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PHYSICOCHEMICAL PROPERTIES OF REAGINIC ANTIBODY

V. Correlation of Reaginic Activity with γ E-Globulin Antibody¹

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In the previous studies of this series (1-3), it was found that reaginic antibody in atopic patients' sera was not precipitated with any of the antibodies specific for γ G-, γ A-, γ M- and γ D-globulins but was coprecipitated with γ G-globulin-anti-L chain antibody complexes. It was also found that the antibody was precipitated with the rabbit antiserum which did not contain any of the anti- γ , anti- α , anti- μ , anti- κ and anti- λ chain antibodies (4). The rabbit antiserum gave a γ_1 precipitin band with a reagin-rich fraction which contained γ A- but no γ G-, γ M- or γ D-globulins, and anti-ragweed antibody was detected in the γ_1 -globulin by radioimmunodiffusion. Since the γ_1 -globulin had a different antigenic structure from the four known immunoglobulins, i.e., γ G, γ A, γ M and γ D, the protein was tentatively designated γ E-globulin. Absorption of γ E globulin from reagin-containing fractions and from two patients' sera was accompanied by the loss of reaginic activity. These findings suggested that the reaginic antibody is γ E globulin. However, the possibility still exists that the antibody is associated with an entirely different protein which has not been detected so far. The work reported here was undertaken to determine whether the precipitation of reaginic antibody in patients' sera with the anti- γ E antibody is consistent, and whether the physicochemical properties of γ E-globulin are similar to those of reaginic antibody. A reaginic serum was fractionated by various methods and the distributions of γ E antibody and skin-sensitizing activity were studied. The light chain(s) of immunoglobulins were detected in γ E-globulin by radioimmuno-electrophoresis.

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MATERIALS AND METHODS

Allergens. Both a crude ragweed allergen extract (Center Laboratories, New York) and purified antigen E from the pollen extract (5) were employed in the experiments. The latter sample was kindly supplied by Dr. M. W. Chase, Rockefeller University, New York.

Patients' sera. All serum specimens used in the experiments were obtained from atopic individuals who gave positive skin reactions against ragweed pollen extract. The specimens from two individuals (Ka and Pr) were kindly supplied by Drs. H. Fudenberg and W. Epstein, University of California, San Francisco. One of these sera (Pr) lacked a detectable concentration of γ A-globulin but had skin-sensitizing activity against ragweed pollen extract. Individual serum specimens from seven atopic patients were kindly supplied by Dr. C. E. Arbesman, Buffalo, New York. A serum sample from another patient (P) was the gift of Drs. L. Perelmutter and L. Goodfriend, Montreal, Canada. Specimen A was obtained from a patient who had been in residence at the Children's Asthma Research Institute and Hospital. The titers of these sera in Prausnitz-Küstner (P-K) reactions are listed in Table I.

Myeloma proteins. The γ G and γ A myeloma proteins, as well as γ M macroglobulin preparations, were the same as those described in a previous article (4). A serum sample from a patient with γ D myeloma was supplied by Dr. H. N. Claman, University of Colorado. γ D-globulin in the serum was purified by gel filtration through a Sephadex G-200 column, followed by chromatography on a diethylaminoethyl (DEAE) cellulose column using the method described by Rowe and Fahey (6). The final preparation contained a trace amount of γ G-globulin and β globulins in addition to γ D-globulin.

Fractionation of reaginic sera

a) γ A-globulin fractions. The γ A globulin fraction of serum K was obtained by the zinc precipitation method described by Vaerman *et al* (7) with slight modifications (8). The γ A-globulin preparation gave a single precipitin band with anti-human serum in immunoelectrophoresis. The preparation was further fractionated into three subfractions by chromatography on a DEAE cellulose (Mann Research Lab., New York) column (3). The preparation was dialyzed against 0.02 M phosphate buffer (pH 8.0) and applied to the column (2×75 cm). Proteins were eluted stepwise with 0.02 M, 0.035 M and 0.08 M phosphate buffers of pH 8.0, and the fractions eluted with each buffer were pooled separately (Frs. γA_1 , γA_2 and γA_3). As reported previously (3), the skin-sensitizing activity of Fr. γA_1 was 10 times higher than the other fractions on a weight basis. This fraction was used to immunize a rabbit for the preparation of anti- γ E globulin antibody.

b) *Chromatography*. The globulin fractions of two patients' sera (Pr. and E. K.) were obtained by precipitation with half saturation of ammonium sulfate followed by chromatography on DEAE cellulose columns (1). Proteins were eluted stepwise with 0.005 M, 0.025 M, 0.035 M, 0.05 M and 0.08 M phosphate buffers of pH 8.0, and the fractions obtained with each buffer were pooled separately (Frs. I-V). As reported previously (1, 2), Fr. II had the highest skin-sensitizing activity. After concentration by negative pressure dialysis, the fraction was applied to Sephadex G-200 (Pharmacia, Uppsala, Sweden) columns. A 5- to 7-ml sample was applied to a column (2.5×100 cm) which was equilibrated with borate buffered saline (pH 8.0) and proteins were eluted with the same buffer.

c) *Sucrose density gradient ultracentrifugation*. The method of Martin and Ames (9) was applied. The γ G-globulin containing anti-A isoagglutinin was used as a marker. The γ G-globulin fraction was obtained from a serum of a type O individual who was immunized with A substance (8). A continuous sucrose density gradient of 5 to 20% in borate buffered saline was made in a total volume of 4.5 ml. A mixture of the reagin-containing fraction and γ G isoagglutinin, in a total volume of 0.2 ml, was layered on top of the

sucrose density gradient. Centrifugation was carried out in a Spinco Model L ultracentrifuge with a SW 39 swinging bucket rotor at 35,000 rpm for 18 hr. Fractions were collected by piercing the bottom of the centrifuge tubes.

d) *Zone electrophoresis*. The reagin-containing fractions from the patients' sera were further fractionated by electrophoresis in agarose gel using the technique described by Ovary *et al.* (10) with modifications. Heated agarose, 1% in barbital buffer, pH 8.2, 0.08 ionic strength, was placed on $1\frac{3}{4} \times 4\frac{1}{4}$ -in. lantern slides and allowed to cool. A trough (2×15 mm) was cut crosswise in the center of the agar approximately 1 in. from the end of the slide. Two troughs were cut at right angles to the center trough (cf., Fig. 7). Of the sample, 0.08 ml was applied to the center trough and, following electrophoresis, 2-mm strips were cut from the center portion of the agarose and each was eluted with 0.3 to 0.5 ml of sterile saline. The agarose strips were macerated, and kept in the cold overnight, followed by centrifugation to remove the agarose. Appropriate rabbit antisera to human serum proteins were added to the longitudinal troughs in order to identify the proteins in the eluted fractions by immunodiffusion.

Rabbit antisera to human immunoglobulins. Rabbit antisera specific for γ G-, γ M-globulins or F_{ab} fragment of γ G-globulin (anti-L) were the same preparations described in a previous article (1). The anti-L chain antibody was prepared by the absorption of anti- γ G-globulin serum with F_c fragment of γ G-globulin. The preparation gave a single precipitin band of identical specificity with γ G-, γ A- and γ M-globulins. The antiserum specific for γ A-globulin was obtained by immunizing rabbits with normal γ A-globulin followed by the absorption of cross-reacting antibodies with γ G-globulin. The supernatant was absorbed with a serum from an agammaglobulinemia patient which was kindly supplied by Dr. H. Fudenberg. The final preparation gave a precipitin band with normal human serum in immunoelectrophoresis. The γ -globulin fractions of the antisera specific for one of the γ G-, γ M-, γ A-globulins or light chains of immunoglobulins were obtained by chromatography on DEAE cellulose columns. The rabbit antiserum specific for γ D-globulin was kindly supplied by Drs. J. Fahey and D. Rowe, National Cancer Institute, Bethesda, Maryland (6).

A rabbit was immunized with Fr. γA_1 from serum Ka to obtain antibody specific for the immunoglobulin with which reaginic activity was associated. The rabbit received 2 mg protein included in complete Freund's adjuvant into the footpads. One week and 3 weeks after the initial injection, the rabbit received intramuscular injections of 1 mg protein in complete Freund's adjuvant. Five weeks after the first injection, 30 ml of blood were obtained from the rabbit. In order to maintain antibody level, the rabbit received 1 mg protein every 3 weeks and bleedings were repeated before each booster injection. Eleven weeks after the initial injection, the rabbit was exsanguinated. The antisera were pooled and absorbed with a γG -globulin fraction which was obtained from a reaginic serum by DEAE cellulose column chromatography. The fraction did not contain any reaginic activity. The rabbit antiserum was incubated with the γG -globulin at the equivalence point and precipitates were removed. The γ -globulin fraction of the supernatant was obtained by precipitation with $\frac{1}{3}$ saturation of ammonium sulfate followed by chromatography on a DEAE cellulose column. The antibody specific for γA globulin in the fraction was precipitated with a type L γA myeloma protein. As described in a previous paper (4), the supernatant still gave a precipitin band with a type K γA myeloma protein but not with three type L myeloma proteins. The antibody against the type K protein was precipitated at the equivalence point. A trace amount of anti- γD antibody, which was detected in the supernatant, was precipitated with γD myeloma protein. The final supernatant (anti- A_1) did not give a precipitin band with any of the γ_{2a} (type K), γ_{2b} (type L), γA , γD myeloma proteins or γM macroglobulin.

Immunologic techniques

Immunodiffusion. Double diffusion in agar was performed by the method of Ouchterlony (11). Immunoelectrophoresis was carried out in agarose on microscope slides (12). The plates were incubated at room temperature for 24 to 48 hr, washed and the precipitin bands stained with Amido-schwartz 10B. The γA -globulin concentration in chromatographic fractions of a reaginic serum was estimated using immunoplates (13) which contained the antiserum specific for γA -globulin (Hyland Laboratories, Los Angeles,

California). As standards, serial dilutions of Fr. γA_2 from a reaginic serum were tested simultaneously. No serum protein other than γA -globulin was detected in the fraction. After incubation at room temperature overnight, the diameters of precipitating areas were measured under magnification and γA -globulin concentration was estimated from a standard curve.

Radioimmuno-electrophoresis was carried out by the method of Yagi *et al.* (14) on agarose slides. The antigen E from ragweed pollen extract was labeled with I^{131} by the method described by Yagi *et al.* (15) using chloramine T. Approximately 0.5 to 1 $\mu g/ml$ of the labeled antigen E was included in appropriate rabbit antisera. In another set of experiments, the γ -globulin fraction of the antiserum specific for light chain of immunoglobulins was labeled with I^{131} by the method of Day *et al.* (16). Immunoelectrophoresis was set up against the anti- γE rabbit antiserum. The slides were washed for 2 days, I^{131} -labeled γ -globulin containing anti-L antibody was applied to the troughs and incubated overnight. The anti-L antibody concentration in the labeled preparation was 5 $\mu gN/ml$.

Radioimmunodiffusion experiments were set up essentially in the same manner as radioimmuno-electrophoresis. The mixture of labeled antigen E and rabbit antisera was applied to Ouchterlony plates. After the precipitin bands were formed, the Ouchterlony plates were washed and radioactive anti-L antibody was applied. The plates were incubated 24 to 48 hr with the radioactive material, washed for 2 to 3 days, dried and wrapped with a plastic film (Saran wrap). Radioautographs were obtained by placing x-ray film (Kodak Industrial x-ray film, type KK) in contact with the plates.

Prausnitz-Küstner reactions. The tests were carried out on individuals who did not give positive skin reactions to the allergens tested. Intracutaneously, 0.05 ml of test samples was injected. Twenty-four hours later the sites received an intracutaneous injection of 0.02 ml of ragweed allergen extract containing 10 PNU or 0.2 μg antigen E. Erythema-wheal reactions, which appeared 15 to 20 min after the injection of allergen, were traced on a transparent plate applied to the area.

RESULTS

A. Detection of light chains in γE -globulin. In a previous article (4), the detection of γE globulin

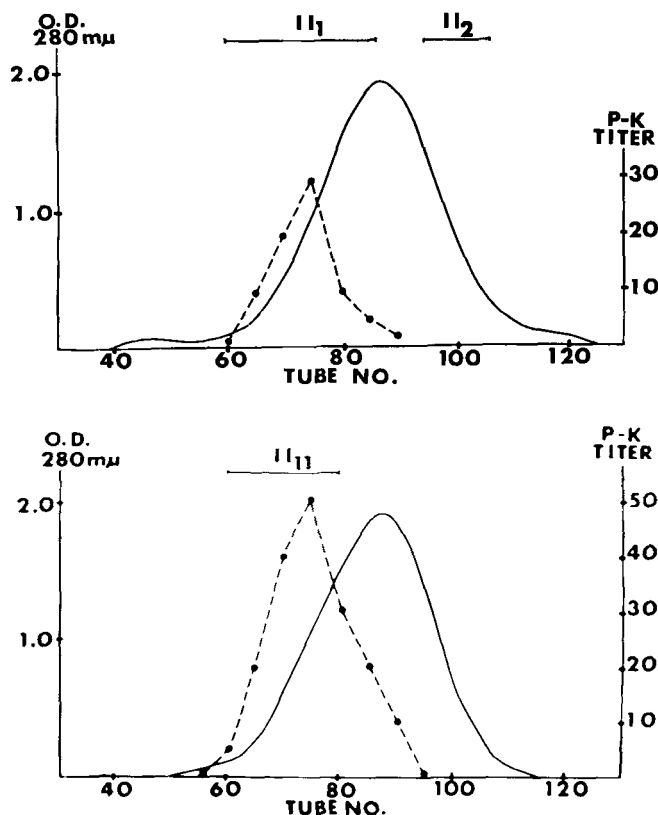


Figure 1. Gel filtration of Fr. II (serum Pr) through a Sephadex G-200 column (upper). Rechromatography of Fr. II₁ above (lower). Solid lines represent absorption at 280 mμ; broken lines represent P-K titers of the fractions.

in γ A globulin fractions from atopic patients' sera was reported. Because the protein is difficult to separate from γ A-globulin by usual fractionation methods, a reagin-rich fraction was prepared from serum Pr which did not contain a detectable amount of γ A-globulin. The globulin fraction of the serum was applied to a DEAE cellulose column, and the proteins were eluted stepwise with phosphate buffers increasing in molarity. Distribution of skin-sensitizing activity in the fractions was determined by P-K reactions using crude ragweed allergen for challenge. As reported previously (1), the first fraction (Fr. I) obtained with 0.005 M buffer did not contain a detectable amount of reagenic activity. On a weight basis, the second fraction (Fr. II) obtained with 0.025 M buffer had the highest activity; 0.2 μ gN of Fr. II and 1 μ gN of Fr. III were sufficient to give positive P-K reactions, whereas as much as 5 μ gN of Frs. IV and V did not. For further

purification, Fr. II was applied to Sephadex G-200 columns, and each 2.5-ml fraction was tested for the presence of reagenic activity by P-K reactions. As reported previously (2), most of the reagenic activity was detected in the ascending portion of a single peak (Fig. 1a). The ascending and descending portions of the peak were pooled separately (Fr. II₁ and II₂), and each fraction was rechromatographed on the Sephadex G-200 column. The fractions from Fr. II₁ and Fr. II₂ were separated into three portions (Fig. 1b). On a weight basis, Fr. II₁₁ had the highest skin-sensitizing activity and the last fraction from Fr. II₂ (Fr. II₂₃) was the least active. Only 0.02 μ gN of Fr. II₁₁ was needed to give positive P-K reactions, whereas as much as 10 μ gN of Fr. II₂₃ did not. In Ouchterlony plates against anti-A₁, Fr. II₂₃ gave a reaction of non-identity with Fr. II₁₁. Although the protein forming the precipitin band in Fr. II₂₃ was not

identified, the anti-A₁ was absorbed with this fraction at the equivalence point. The supernatant (anti- γ E) gave a weak band with Fr. II₁₁ but not with Fr. II₂₃, or with any of the known immunoglobulins when tested by immunodiffusion. It was also found that none of the Frs. I, III, IV or V gave a visible precipitin band with the anti- γ E.

Experiments were undertaken to determine whether the precipitin band formed between Fr. II₁₁ and anti- γ E antiserum is due to a new immunoglobulin. Immunoelectrophoresis was set up between Fr. II₁₁ and anti- γ E in duplicate. After washing for 2 days, the antiserum trough in one slide was filled with normal rabbit γ -globulin and that of another slide was filled with the γ -globulin fraction of anti-light chain antiserum. After incubation for 24 hours at room temperature, the troughs were filled with anti-L chain antibody labeled with I¹³¹. The concentration of radioactive anti-L chain was $\frac{1}{10}$ the concentration of cold anti-L. Both stained slides and radioautograms are shown in Figure 2. It is evident that the precipitin band formed between Fr. II₁₁ and anti- γ E showed radioactivity and that the fixation of radioactive anti-L antibody to the precipitin band was blocked by pretreatment of the slide with cold anti-L antibody. Similar experiments were carried out in Ouchterlony plates. The precipitin band formed between Fr. II₁₁ and anti- γ E fixed radioactive anti-L chain antibody. The other immunoglobulin did not form either a visible or radioactive precipitin band with the anti- γ E preparation (Fig. 3 a, b). It was also found that the visible band on the stained slide was slightly intensified by the application of cold anti-L chain. These findings indicate that a protein having light chain(s) of immunoglobulins was responsible for the formation of the γ E precipitin band and that this protein is not one of the known immunoglobulins, i.e., γ G-, γ A-, γ M- and γ D-globulins.

B. Presence of γ E antibodies in reaginic sera.

Attempts were made to detect γ E anti-ragweed antibody in atopic patients' sera by radioimmunodiffusion. In Ouchterlony plates, peripheral wells were filled with the patients' sera and Fr. II₁₁ from serum Pr, and the center wells were filled with the anti- γ E antibody preparation which had been mixed with I¹³¹-labeled antigen E. In the resulting radioautograms, radioactive precipitin bands were seen between some of the

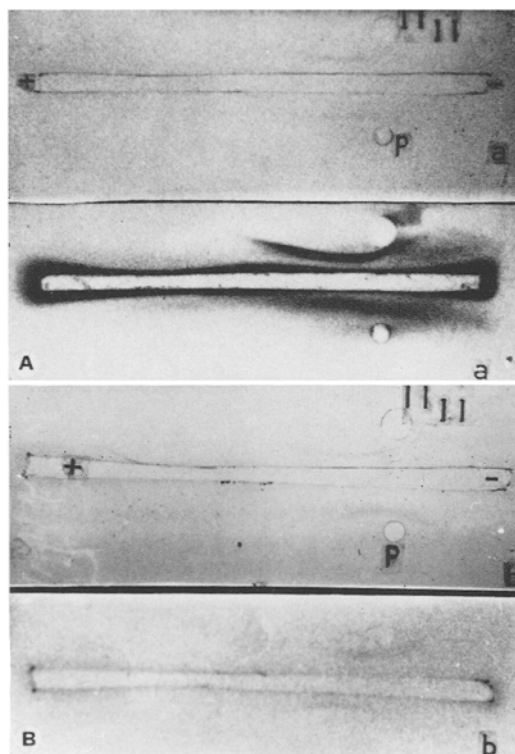


Figure 2. Radioimmuno-electrophoretic analysis of anti- γ E antibody. Both stained slides (upper) and corresponding radioautographs (lower) are shown. Fr. II₁₁ from serum Pr and serum P was placed in wells, and the center troughs were filled with anti- γ E. After washing the slides, radioactive anti-L antibody was placed in the antibody trough in (a); cold anti-L followed by radioactive anti-L was placed in the antibody trough in (b). Radioautograms of the plates showed that γ E band combined radioactive anti-L (a), which was blocked by cold anti-L (b).

patients' sera and the anti- γ E antibody preparation, although a corresponding visible band was not shown in the stained slides. A visible band between Fr. II₁₁ and anti- γ E in the stained slide did not show radioactivity. However, the radioactive bands formed with the patients' sera in the wells adjacent to Fr. II₁₁ terminated at the point where the visible γ E band with Fr. II₁₁ was present (Fig. 4a). Similar results were obtained in radioimmuno-electrophoresis. The patients' sera which showed radioactive bands with anti- γ E in Ouchterlony plates gave a γ ₁ radioactive band in radioimmuno-electrophoresis,

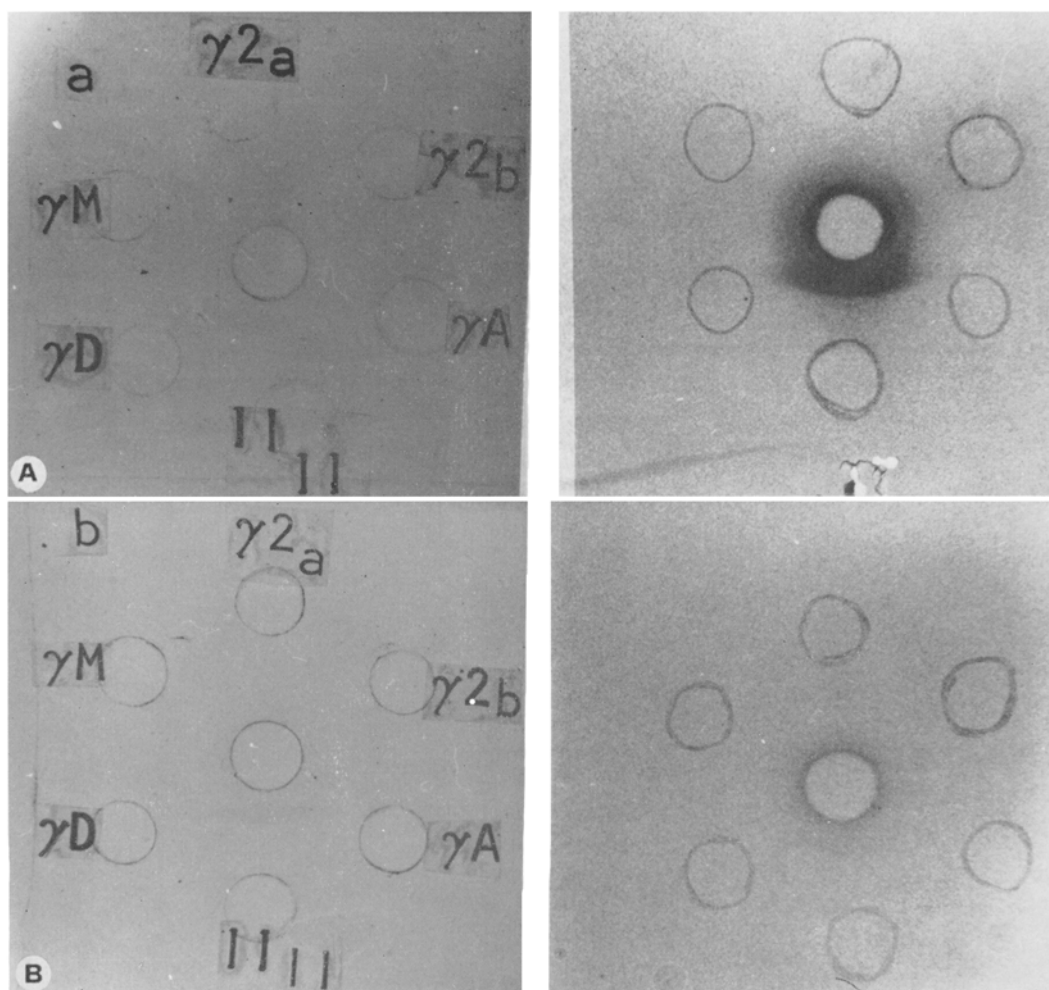


Figure 3. A, B) Radioimmunodiffusion analyses of anti- γ E. Both stained slides (left) and corresponding radioautographs (right) are shown. Anti- γ E was placed in center wells. Peripheral wells were filled with γ_{2a} -(type K), γ_{2b} -(type L), γ A-, γ M-, γ D-globulins and Fr. II₁₁ from serum Pr. After washing the plates, radioactive anti-L chain was applied to the center well of plate A; the center well of B was filled with cold anti-L antibody followed by radioactive anti-L. The γ E band combined radioactive anti-L (a), which was blocked by cold anti-L (b).

but the precipitin band formed between anti- γ E and Fr. II₁₁ did not show radioactivity. Representative results are shown in Figure 5. The intensities of radioactivity of the γ E band in both radioimmunodiffusion and radioimmunoelectrophoresis are listed in Table I. The absence of the radioactive γ E band with Fr. II₁₁ suggests that the fraction does not contain γ E antibody against antigen E. Prausnitz-Küstner reactions with the fraction and the patients' sera were set up using both crude allergen and antigen E. It was found

that the P-K titer of Fr. II₁₁ with the crude allergen was 1:1600, however, a dilution as low as 1:10 of the fraction did not give a positive reaction when antigen E was used for challenging. It is evident that the skin sensitizing antibody in Fr. II₁₁ is specific for an allergen(s) other than antigen E. It was also found that the P-K titers of several other patients' sera with crude allergen and antigen E were significantly different (Table I).

The presence of γ A and/or γ G anti-ragweed

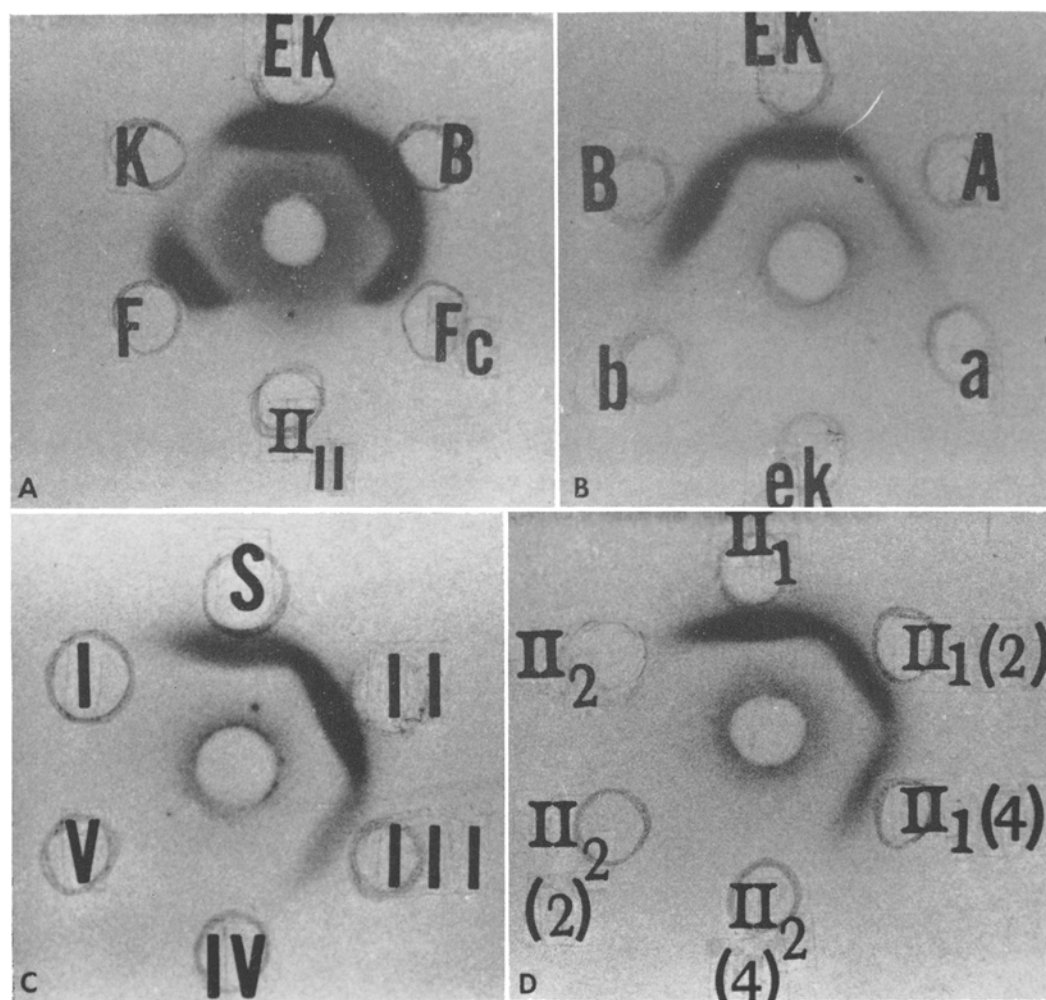


Figure 4. Radioimmunodiffusion analyses of reaginic sera and their fractions. A) The center wells were filled with the mixture of anti- γ E antibody and radioactive antigen E. Peripheral wells were filled with 5 reaginic sera and Fr. II_{11} from serum Pr (upper left). B) Effect of absorption of reaginic sera with anti- γ E antibody (upper right). Peripheral wells of the Ouchterlony plate were filled with the supernatants after the absorption (small letter), and the original sera diluted with saline (capital letter). C) Distribution of γ E antibody in chromatographic fractions from a DEAE cellulose column (lower left). The fractions and the original serum (E. K.) were placed in peripheral wells (cf. Table III). D) Distribution of γ E antibodies in Frs. II_1 and II_2 (serum E. K.) (lower right). The peripheral wells were filled with twofold dilutions (undiluted, $\times 2$, $\times 4$) of the fractions (cf. Table III).

antibodies in patients' sera was also studied by radioimmunodiffusion. Most of the serum specimens contained γ G antibody against antigen E but γ A antibody was detected in only three cases (Table I). Comparison of the intensities of radioactivity in three different immunoglobulin precipitin bands, i.e., γ G, γ A, γ E, and P-K titers

of the specimens with antigen E indicates a parallelism between γ E antibody and P-K titer (Table I).

The effect of absorption of the patients' sera with anti- γ E antiserum on the skin-sensitizing activity was studied. One volume of each serum was incubated with one-half volume of anti- γ E

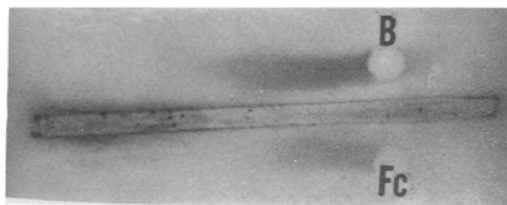


Figure 5. Radioimmuno-electrophoresis of sera B and Fc against anti- γ E. The antiserum was mixed with radioactive antigen E and placed in the center trough.

antibody preparation for 24 hr at 0°C, and a trace amount of precipitate was removed. In one case (Serum A), equal volumes of the serum and the antibody preparation were incubated. As controls, a sample of each serum was mixed with saline instead of anti- γ E. Serial dilutions of the supernatants and controls were injected into normal individuals for passive sensitization and the skin sites were challenged with crude allergen. As shown in Table II, no skin-sensitizing activity was detected in the supernatants. The presence of γ E antibody in the supernatants from three representative sera was tested by radioimmuno-diffusion. In an Ouchterlony plate, a mixture of anti- γ E and radioactive antigen E was placed in the center well and the peripheral wells were filled with the supernatants and controls from the same patients' sera. As shown in Figure 4b, γ E antibody was not detected in the supernatants, whereas the controls gave a radioactive γ E band. It is evident that both skin-sensitizing antibody and γ E antibody were precipitated with anti- γ E. Similar experiments were carried out with Fr. II₁₁ from serum Pr. Three volumes of the anti- γ E antibody were incubated with one volume of the fraction to precipitate γ E-globulin. Serial dilutions of both the supernatant and the control, which was previously diluted 4 times with saline, were injected intracutaneously into normal individuals and the skin sites were challenged with crude allergen. A dilution as high as 1:400 of the control gave P-K reactions, whereas a 1:50 dilution of the supernatant did not. Since antigen E did not give P-K reactions with Fr. II₁₁ and serum K, the reaginic antibody in these patients is specific for some allergen(s) other than antigen E. The results, therefore, indicate that the precipitation of reagin with the anti- γ E antibody preparation is not restricted to the antibody against antigen E.

C. Distribution of γ E antibody and reaginic activity. In order to investigate the correlation between γ E antibody and skin-sensitizing activity, distribution of the antibody in the fractions of a patient's serum was studied. The globulin fraction of serum E. K. was obtained by precipitation with half saturation of ammonium sulfate and was further fractionated by chromatography on a DEAE cellulose column. Proteins were eluted stepwise from the column with five phosphate buffers increasing in molarity and fractions obtained with each buffer were pooled separately. After concentration, the fractions containing 1% protein and the original serum were tested for the presence of γ E antibody by radioimmuno-diffusion (Fig. 4c). The radioactive band was obtained with Frs. II and III but not with the other three fractions. It is apparent that the band with Fr. II showed much higher radioactivity than that of Fr. III. The skin-sensitizing activity of the fractions was determined by P-K reactions using antigen E as a challenging allergen. The results shown in Table III indicate that Fr. II had the highest reaginic activity on a weight basis. The activity of Fr. III was $\frac{1}{8}$ that of Fr. II, on a weight basis, and that of the other fractions was almost negligible when compared with these two fractions. It is evident that the distribution of γ E antibody in the chromatographic fractions correlates with their skin-sensitizing activity. The presence of γ G antibody in the fractions was tested by radioimmuno-electrophoresis. A mixture of the antiserum specific for γ G and radioactive antigen E was placed in the troughs. The γ G precipitin band formed with Fr. I had the highest radioactivity. A definite radioactive γ G band was obtained with Fr. II, and the band with Fr. III had a trace amount of radioactivity. The other two fractions did not give a radioactive γ G band. Although γ A antibody was not detected in the original serum, Fr. III had a detectable amount of the γ A antibody when it was tested by radioimmuno-diffusion. The γ A band formed with Fr. II did not show radioactivity. In order to confirm this finding, the concentration of γ A-globulin in the fractions was measured by immunoplates containing anti- γ A antibody. The relative concentrations of γ A-globulin in the fractions are shown in Table III. It is evident that the skin-sensitizing activity does not have any correlation with the γ A globulin concentration in the fractions.

TABLE I

Sensitizing activity of patients' sera and the antigen-combining activity of γ G, γ A and γ E precipitin bands in radioimmunodiffusion

Serum Samples	Titer of P-K Reactions		Intensity of Radioactivity in Radioimmunodiffusion					
	Crude allergen	Antigen E	γ G		γ A		γ E	
			IEP ^a	ID ^b	IEP	ID	IEP	ID
E. K.	2000	1600	++	++	±	±	++	+++
B	3200	3200	++	++	—	±	+++	+++
Fc	3200	800	+	+	++	++	++	++
F	1600	600	+	+	—	+	++	++
K	300	<100	±	±	—	—	—	±
W	600	600	++	++	+	+	+	++
H	400	400	++	++	±	—	+	+
A	3200	800	++	++	±	±	++	++
P	800	400	+++	+++	—	—	+	+
Pr ^c	1600	<10	—	—	—	—	—	—

^a IEP represents radioimmunoelectrophoresis with I¹³¹ antigen E.

^b ID represents radioimmunodiffusion in Ouchterlony plates with I¹³¹ antigen E. Undiluted samples were applied to Ouchterlony plates to detect γ A and γ E antibodies. For the detection of γ G antibody, 1:10 dilutions of the samples were applied.

^c Fr. II₁₁ from serum Pr.

In order to study the molecular size of γ E antibody, Fr. II was applied to a Sephadex G-200 column. The chromatogram was similar to that in Figure 1, which shows the pattern of gel filtration of the same fraction from serum Pr. Fractions corresponding to the ascending and descending halves of the single peak were pooled separately (Fr. II₁ and II₂) and concentrated to 0.5%. Serial twofold dilutions of the two fractions were placed in the peripheral wells of an Ouchterlony plate, and the center well was filled with a mixture of anti- γ E and radioactive antigen E. No visible precipitin band was formed. However, a radioautogram of the plate showed an intense radioactive band with each dilution of Fr. II₁ (Fig. 4d). In another Ouchterlony plate, even 1:20 dilution of Fr. II₁ gave a definite radioactive band which was comparable to that obtained with undiluted Fr. II₂. It is apparent that almost all γ E antibody is present in the ascending portion of the single protein peak, indicating that γ E-globulin is larger than γ G-globulin. The skin sensitizing activity of the fractions was studied by P-K reactions using antigen E. It was found that the minimum dose of Fr. II₁ required to give a positive skin reaction was about one-twentieth the dose of Fr. II₂ (Table III). These results indicate a close correlation between the distribution of γ E antibody and of skin-sensitizing activity. The γ G antibody against antigen

E was detected in both fractions by radioimmunoelectrophoresis. The intensity of radioactivity in the γ G band was comparable for these fractions.

The sedimentation velocity of γ E antibody was studied by sucrose density gradient ultracentrifugation. The γ G-globulin fraction of anti-A serum was mixed with Fr. II₁ (serum E. K.) and the mixture was applied to sucrose density gradient. After centrifugation, both anti-A hemagglutinin and skin sensitizing activities in the gradient fractions were measured. The results shown in Figure 6 indicate that the sedimentation velocity of the reaginic antibody was higher than that of γ G isoagglutinin. The presence of γ E antibody in the fractions was studied by radioimmunodiffusion. The fractions were placed in peripheral wells of an Ouchterlony plate against a mixture of anti- γ E with radioactive antigen E. The γ E antibody was detected in two fractions which showed high reaginic activity.

The electrophoretic mobility of γ E antibody and of skin sensitizing antibody was studied in agarose gel electrophoresis. 0.08-ml volumes of Fr. II₁₁ from serum Pr and of Fr. II₁ from serum E. K. were applied to the gel. After electrophoresis, 2-mm segments of the center piece of the gel were extracted with saline and the troughs were filled with mixtures of radioactive antigen

TABLE II
Effect of absorption with anti- γ E on skin-sensitizing activity of patients' sera^a

Patient	Absorption	Prausnitz-Küstner Reactions With Dilutions of					
		× 100	× 200	× 400	× 800	× 1600	× 3200
E. K.	—	13 (48)	12 (40)	11 (38)	9 (36)	8 (26)	
	+	0	0	0	0	0	
B	—			11.5 (30)	9.5 (34)	8.5 (28)	7 (23)
	+		0	0	0	0	0
Fc	—			12 (44)	10 (39)	9 (35)	6 (27)
	+		0	0	0	0	0
F	—		14 (44)	11 (38)	9 (33)	7.5 (25)	
	+	0	0	0	0	0	
K	—	8 (31)	7 (25)	5 (15)			
	+	0	0	0			
W	—		9 (35)	7.5 (32)	6 (21)		
	+	0	0	0	0		
H	—		9.5 (39)	8 (34)	±		
	+	0	0	0	0		
A	—			11 (38)	10.5 (36)	9 (33)	7 (28)
	+			0	0	0	0
P	—	11 (33)	10 (32)	9 (27)	7.5 (24)		
	+	0	0	0	0		
Pr ^b	—		11 (40)	9.5 (40)	9 (33)	8 (24)	
	+		0	0	0	0	

^a Crude allergen was used in Prausnitz-Küstner reactions. Numerals in the table represent the average diameters of a wheal; numerals in parentheses represent the average diameters of erythema in millimeters.

^b Fr. II₁₁ from serum Pr.

TABLE III
Distribution of antibodies associated with different immunoglobulins and of reaginic activity

Fraction	Phosphate Buffer for Elution	Relative Concentration of γ A	Presence of Antibodies Against Antigen E in ^a			Minimum Skin-Sensitizing Dose
			γ G	γ A	γ E ^b	
		%				μ gN
I	0.005	0	++	—	—	>1.6
II	0.025	32	+	—	+++	0.05
III	0.035	61	±	+	+	0.4
IV	0.05	39	—	—	—	>1.6
V	0.08	10	—	—	—	>1.6
II ₁	0.025 ^c	56	+	±	+++	0.02
II ₂	0.025 ^c	7.2	+	—	±	0.4

^a Radioactivity of precipitin bands was compared in radioautographs.

^b Protein concentrations of Frs. I-V and Frs. II₁, II₂ applied in radioimmunodiffusion were 1% and 0.5%, respectively.

^c Fractions II₁ and II₂ were obtained from Fr. II by gel filtration through a Sephadex G-200 column.

E and anti-human serum, anti- γ G or anti- γ E antibody preparations. In stained slides, the anti- γ E antibody gave a visible γ E precipitin band with Fr. II₁₁ from serum Pr but not with Fr. II₁ from serum E. K. Radioautograms of the plates were made by contact with x-ray film. In agreement with the results of radioimmunoelectrophoresis, Fr. II₁ from serum E. K. showed radioactive γ G and γ E bands but Fr. II₁₁ from serum Pr did not. The radioactive γ E band with the former sample was observed in anodic side of the place of application, whereas the radioactive

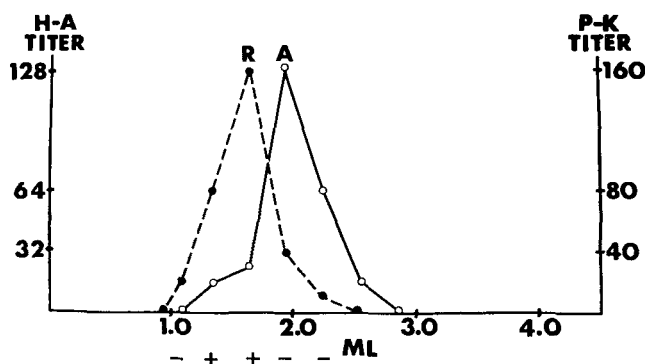


Figure 6. Density gradient ultracentrifugation showing the distribution of γ G isoagglutinin (A) and reagin (R). A broken line represents the P-K titer and a solid line represents hemagglutinin titer of the fractions. The presence of γ E antibody, which was detected by radioimmunodiffusion, in the fractions is shown by + under the figure.

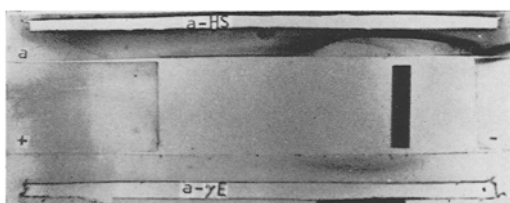


Figure 7. Electrophoretic mobility of reagin and γ E antibodies; Fr II₁₁ of serum Pr was applied to the center well of agarose gel. Reagin activity was detected in the eluates from the segments marked under the figure. Longitudinal troughs were filled with anti-human serum (a-HS) or anti- γ E (a- γ E). The distribution of the reagin activity, shown by (—), corresponded with the γ E precipitin band.

γ G band located in cathodic side. Prausnitz-Küstner reactions were carried out with the extracts from each segment. The skin sites receiving the extract from Fr. II₁ were challenged with antigen E and those receiving the extract from Fr. II₁₁ (serum Pr) were challenged with crude allergen in order to study the distribution of reagin antibody. A stained slide with Fr. II₁₁ of serum Pr is shown in Figure 7. The distribution of reagin antibody in serum Pr was restricted to the position of the visible γ E band. In the case of Fr. II₁ from serum E. K., the reagin activity was detected in a wide range, but the peak of the activity corresponded to the position of the radioactive γ E band. It was also found that the extract from the segments showing the radioactive γ G bands did not have reagin activity.

DISCUSSION

The data presented in this article confirm the previous observations (4) that skin-sensitizing activity in atopic patients' sera was inactivated by the precipitation of serum proteins with the rabbit anti-human globulin serum which did not contain any of the antibodies specific for γ G-, γ M-, γ A- or γ D-globulins and for light chains of immunoglobulins. In all of the 10 sera studied, essentially all skin-sensitizing activity was removed by the precipitation. In immunoelectrophoresis and immunodiffusion experiments, the rabbit antibody preparation gave a γ ₁ precipitin band with a reagin-containing fraction (Fr. II₁₁) of serum Pr but not with any of the four known immunoglobulins. The γ ₁ precipitin band corresponded to the γ E band, which was described in a previous article (4). It was also found that the γ E band formed with Fr. II₁₁ combined with radioactive antibody specific for light chains. Since the combination with the radioactive antibody was specifically blocked by the pre-treatment of the slide with cold anti-L chain antibody, it is evident that γ E globulin has light chain(s). The presence of light chains is the common structural characteristic of immunoglobulins. As none of the antibodies reacting with the known immunoglobulins were detected in the anti- γ E preparation by the same method, the findings indicate the presence of a new immunoglobulin, γ E, in the reagin-rich fraction.

In the previous experiments (4), the γ E band formed with a reagin-rich fraction from patients' sera combined with radioactive antigen E. Un-

fortunately, Fr. II₁₁ from serum Pr did not contain skin-sensitizing antibody against antigen E and the γ E precipitin band did not combine with radioactive antigen E. However, the sera from other atopic patients, which had high skin sensitizing activity against antigen E, showed a radioactive γ_1 band with anti- γ E antibody in radioimmuno-electrophoresis. Although a visible band was not definite in the stained slide, the position of the radioactive band corresponded to the γ E band. Furthermore, in Ouchterlony plates, the radioactive band formed with the atopic patients' sera with anti- γ E antibody terminated at the intersection where the visible but non-radioactive γ E band crosses the radioactive bands (Fig. 4a). This finding indicates that the radioactive band formed with the patients' sera and the visible but nonradioactive γ E band have an identical specificity, and therefore, that the radioactive band is due to γ E-globulin antibody.

The question arose as to whether the reaginic antibody is actually associated with γ E globulin. The present results showed that both the radioactive γ E band and the skin-sensitizing activity were lost by absorption of the patients' sera with the anti- γ E antibody preparation. Furthermore, the distribution of reaginic activity and γ E antibody paralleled each other when a patient's serum (E. K.) was fractionated by various methods. Both reaginic activity and γ E antibody were eluted from a DEAE cellulose column with phosphate buffers of molarity 0.005 to 0.025 M, and the fraction obtained with 0.005 M buffer was inactive in spite of the presence of a large amount of γ G antibody. On a weight basis, Fr. II (0.025 M fraction) was more active than Fr. III (0.035 M fraction) with respect to both skin sensitizing activity and γ E antibody concentration, which was estimated from the intensity of radioactivity in the γ E band. The distribution of γ A antibody in the fractions did not correlate with the skin sensitizing activity. By gel filtration through a Sephadex G-200 column, reaginic activity and γ E antibody came off the column earlier than the γ G-globulin peak. The presence of reaginic activity in the ascending portion of the 7S γ G-globulin peak was in agreement with previous reports (2, 17-19). Furthermore, γ E antibody and reaginic activity paralleled each other when the reagin-rich fraction was applied to sucrose density gradient ultracentrifugation (Fig. 6). It is evident that the sedimentation velocity of γ E antibody is higher than that of

γ G isoagglutinin. Assuming that a partial specific volume is the same for the reaginic antibody and γ G isoagglutinin and that $s_{20, w}$ of γ G-globulin is 6.8S, the sedimentation coefficient of the reaginic antibody was estimated to be 7.7S. This value is in agreement with the observations by Anderson and Vannier (20) and with our previous report (2). In any case the results indicate that γ E antibody and reaginic antibody are comparable with respect to their molecular size. When the reagin-containing fractions were applied to zone electrophoresis in agarose gel, the distribution of reaginic antibody corresponded to the γ E precipitin band but not to the γ G band.

The correlation between γ E antibody and reaginic activity strongly supports the hypothesis that reaginic antibody is γ E-globulin. The question arose as to whether or not all the reaginic activity is associated with this protein. It was found that the skin-sensitizing activity of patients' sera, determined by P-K reactions with antigen E, almost paralleled the intensity of radioactivity in the γ E precipitin band and did not have any relation to radioactivity in either the γ A or γ G band (Table I). It was also found that almost all skin-sensitizing activity disappeared after the precipitation with anti- γ E-globulin. In the case of serum B and E. K. which had high skin sensitizing activity, it is clear that the activity in the supernatant after the precipitation was less than $1/6$ of the original activity. Since rabbit γ -globulin induces nonspecific reactions in normal human skin, lower dilutions of the supernatants were not injected for passive sensitization. As shown in Table I, Fr. II₁₁ from Pr and serum K did not give P-K reactions when the skin sites were challenged with antigen E, indicating that the allergen involved in the skin reactions with crude antigen is different from antigen E. In agreement with the results, radioactive γ E bands were not observed with antigen E in these cases. The precipitation of the skin-sensitizing antibody in these sera by anti- γ E-globulin indicates that the association of the reaginic antibody with γ E-globulin is not restricted to the antibody against antigen E. In addition to the present findings, it was found in previous experiments that the precipitation of γ E-globulin in the reagin-rich fraction from two pooled sera was accompanied by the loss of skin-sensitizing activity (4). Although the number of sera tested is limited, the present as well as the previous findings strongly suggest that almost

all of the reaginic antibody to ragweed pollen allergens is generally associated with γ E-globulin.

The physicochemical properties of human reaginic antibodies have been studied by many investigators. Our studies indicate that the antibody is not associated with any of the γ G-, γ A-, γ M- or γ D-globulins but with γ E-globulin. However, the possibility still remains that the reaginic antibodies are heterogeneous and are associated with two or more classes of immunoglobulins. Terr and Bentz (21) as well as Fireman *et al.* (22) reported the presence of heat stable skin sensitizing antibodies. Reid *et al.* (23) recently suggested that skin-sensitizing antibody may cross the immunoglobulin classification which is based on the antigenic determinants of the heavy chains of the proteins. There is a considerable amount of evidence that the biologic properties of antibodies are different depending on the immunoglobulin classes and that the structures essential for the biologic activity are present in heavy chains (24). However, the possibility remains that heavy chains of different immunoglobulins have a similar structural moiety which is essential for a certain biologic activity. Recent studies of Stechschulte *et al.* (25) on rat antibodies showed two kinds of anaphylactic antibodies; one is involved in histamine release and another is involved in the release of slow-reacting substance. Both of these antibodies give anaphylactic reactions in rat skin under different conditions. This suggests the possibility that human reaginic antibody may well be composed of multiple classes of immunoglobulins. If this is the case, γ E-globulin antibody may represent the classical type of reaginic antibody which is usually found in ragweed sensitive patients.

The present experiments added some information on the physicochemical properties and structure of γ E-globulin. Electrophoretic mobility of this protein in agarose gel is γ_1 . Distribution of γ E-globulin in chromatographic fractions on DEAE cellulose columns is different from that of γ G-, γ M- and γ A-globulins but similar to γ D-globulin. It is also evident that γ E-globulin is eluted from a Sephadex G-200 column earlier than γ G-globulin and sediments faster than γ G-globulin in sucrose density gradient ultracentrifugation. The correlation of γ E antibody and reaginic antibody in sucrose density gradient ultracentrifugation experiments indicates that the sedimentation coefficient of γ E

globulin is around 8S. With respect to the structure, the present results showed the presence of light chain(s) in the γ E molecules. The presence of two L chain types in the immunoglobulin is not known yet. The properties of this protein, especially the behavior on Sephadex G-200 columns and the characteristic antigenic structure, suggest that the molecules contain characteristic heavy chains. Since γ E-globulin has not been isolated, the concentration of this protein in human serum is unknown. However, lack of a visible precipitin band between anti- γ E antibody and patients' sera and the position of a radioactive γ E band which is very close to the antigen wells suggest that the concentration of γ E-globulin in most of the sera might be less than that of γ D-globulin.

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SUMMARY

The skin-sensitizing activity in the sera of nine atopic patients, who are sensitive to ragweed pollen, and in a reagin-rich fraction of another patient's serum was precipitated by anti- γ E-globulin antiserum which did not contain any detectable antibody against γ G-, γ A-, γ M- or γ D-globulins. Absorption of the skin-sensitizing antibody was accompanied by the loss of γ E antibody in the supernatants. The skin-sensitizing activity in the patients' sera, determined by P-K reactions with antigen E, paralleled radioactivity of the γ E band which was observed by radioimmunodiffusion with the radioactively-labeled antigen, whereas no correlation was observed between the skin-sensitizing activity and either γ G or γ A antibodies. When one of the patients' sera was fractionated by chromatography on a DEAE cellulose column, gel filtration, sucrose density gradient ultracentrifugation and by zone electrophoresis on agarose gel, distributions of reaginic activity paralleled those of γ E antibody. These findings strongly support the hypothesis that reaginic antibodies in ragweed sensitive patients' sera are associated with γ E-globulin. Light chains of immunoglobulins were detected in γ E-globulin, indicating that γ E-globulin is a new immunoglobulin.

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