

## ALLERGEN-BINDING ACTIVITY OF $\gamma$ E, $\gamma$ G AND $\gamma$ A ANTIBODIES IN SERA FROM ATOPIC PATIENTS

### *In Vitro* Measurements of Reaginic Antibody<sup>1</sup>

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Our previous studies on human reaginic antibody (1-5) indicated that the antibody was not associated with any of the  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- or  $\gamma$ D-globulins, but with a new immunoglobulin which we tentatively designated  $\gamma$ E. The skin-sensitizing activity of 10 atopic sera was lost by absorption of the sera with antibody specific for  $\gamma$ E-globulin. When one of the sera was fractionated by chromatography on a diethylaminoethyl (DEAE)-cellulose column, and examined by gel filtration, by density gradient ultracentrifugation and by zone electrophoresis in agarose gel, the distribution of reaginic antibody correlated with that of  $\gamma$ E-globulin antibody, but not with that of either  $\gamma$ G or  $\gamma$ A antibodies. It was also found that skin-sensitizing activity of the serum specimens, determined by Prausnitz-Küstner (P-K) reactions, paralleled the intensity of radioactivity of the  $\gamma$ E band formed in radioimmunodiffusion. These findings strongly support the idea that reaginic antibodies are associated with  $\gamma$ E-globulin, and suggest the possibility that quantitation of skin-sensitizing antibody may be estimated *in vitro*, by measuring the allergen-combining activity of  $\gamma$ E antibody in the serum samples. The present investigation was undertaken to study the allergen-binding activity of the antibodies associated with different immunoglobulin classes. Purified allergen from ragweed pollen extract, i.e., the antigen E of King *et al.* (6), was labeled with  $I^{131}$ , and antibodies associated with  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- and  $\gamma$ E-globulins were titrated by radioimmunodiffusion. Attempts were also made to measure the allergen-binding activity of each immunoglobulin antibody. A large excess of radioactive allergen was incubated with the patients' sera, and allergen-antibody complexes

were precipitated with rabbit antibodies specific for one of the immunoglobulins. The results indicate that the allergen-binding activity of  $\gamma$ E antibody in the serum specimens correlates with the skin-sensitizing activity of the samples. The minimum amount of the antibody required for P-K reactions is estimated.

#### MATERIALS METHODS

*Purified ragweed allergen and rabbit antibody.* Purified ragweed allergen (antigen E, Fr. IVc prepared according to King *et al.* (6) for the Committee on Standardization of Allergen) and rabbit antiserum specific for this antigen were kindly supplied by Dr. M. W. Chase, Rockefeller University. The antibody concentration in the rabbit antiserum, which was measured by Dr. King, was 300  $\mu$ gN/ml.

Antigen E was labeled with  $I^{131}$  using the method described by Yagi *et al.* (7) with chloramine T. Two hundred micrograms of the antigen were labeled with 6 mc of  $I^{131}$ , purchased from Abbott Laboratories, North Chicago, Illinois. The protein concentration in the labeled preparation was measured by the Folin-Ciocalteu method (8) using unlabeled allergen as a standard. Gel diffusion precipitin analysis revealed that the radioactive allergen formed a precipitin band with the rabbit antiserum against antigen E. The effective antigen concentration in the labeled preparation was measured by coprecipitation. Eight tenths of a microgram of the radioactive antigen was mixed with 3.2  $\mu$ g of unlabeled antigen and precipitated with the rabbit antiserum in antibody excess. The precipitate was washed three times with cold saline, and radioactivity was determined. It was found that 83% of radioactivity was precipitated by the antibody.

*Sera from atopic patients.* The serum specimens from individual patients are the same as those described in a previous article (5). Six serum

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samples were supplied by Dr. C. E. Arbesman, Buffalo, N. Y. Serum P was supplied by Drs. L. Perelmutter and L. Goodfriend, Montreal, Canada, and serum A was obtained from a patient who was in residence at the Children's Asthma Research Institute, Denver, Colorado. Serum Pr, which contained reaginic activity but no detectable  $\gamma$ A-globulin, was supplied by Dr. H. Fudenberg, University of California, San Francisco, California. A serum pool from patients who had been treated with antigen E (serum Tr) was supplied by Dr. L. M. Lichtenstein, The Johns Hopkins University. Another serum specimen, I, was obtained from a normal individual who received P-K tests every month for 2 years. This subject did not give a positive direct skin reaction with either antigen E or crude ragweed allergen.

Fractions of serum EK are the same preparations used in a previous study (5). The globulin fraction of the serum was applied to a DEAE-cellulose (Mann Research Laboratories, Inc., New York) column, and proteins were eluted stepwise with phosphate buffers of pH 8.0 and of increasing molarity. The fractions obtained with 0.005 M, 0.025 M and 0.035 M buffers were pooled separately (Fr. I, II, III). No serum protein other than  $\gamma$ G globulin was detected in Fr. I. Both Frs. II and III contained  $\gamma$ G- and  $\gamma$ A-globulins. On a weight basis Fr. II had the highest skin-sensitizing activity. This fraction was then applied to a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (2.5 by 100 cm), and the ascending and descending halves of a single peak were pooled separately (Frs. II<sub>1</sub> and II<sub>2</sub>). For further purification, each fraction was rechromatographed on the Sephadex G-200 column, and the ascending half of Fr. II<sub>1</sub> (Fr. II<sub>11</sub>) and the descending half of Fr. II<sub>2</sub> (Fr. II<sub>22</sub>) were obtained. As reported previously (5), Fr. II<sub>11</sub> had high skin-sensitizing activity, whereas Fr. II<sub>22</sub> did not. A reagin-rich fraction from serum Pr which contained no detectable amount of  $\gamma$ A-globulin was also used in the present experiment. This fraction was obtained by chromatography on a DEAE-cellulose column and gel filtration in exactly the same manner as that used to obtain Fr. II<sub>11</sub> from serum EK.

The  $\gamma$ A-globulin fraction of serum Tr was obtained by the zinc precipitation method described by Vaerman *et al.* (9) with slight modifications (10). The preparation, which gave a single precipitin band with anti-human serum on immuno-

electrophoresis, was then subfractionated on a DEAE-cellulose column (3). Protein fractions were eluted stepwise with 0.02 M and 0.035 M buffers at pH 8.0, and the fractions eluted with each buffer were pooled separately (Fr.  $\gamma$ A<sub>1</sub> and  $\gamma$ A<sub>2</sub>). As reported previously (3), the skin-sensitizing activity in the  $\gamma$ A-globulin preparation was concentrated in Fr.  $\gamma$ A<sub>1</sub>. The  $\gamma$ A-globulin fraction of serum Tr was prepared from the zinc precipitate by chromatography on DEAE-cellulose. The precipitate was dissolved by the addition of ethylenediamine tetraacetate (EDTA), dialyzed against 0.005 M phosphate buffer pH 8.0, and applied to a DEAE-cellulose column equilibrated with the same buffer. The protein eluted under these conditions was concentrated.

*Antisera specific for immunoglobulins.* Rabbit antisera specific for human  $\gamma$ G-globulin and for the light chains of immunoglobulins were prepared by absorption of an antiserum against  $\gamma$ G-globulin, with F<sub>ab</sub> or F<sub>c</sub> fragment of the protein. The antisera against  $\gamma$ A- and  $\gamma$ M-globulins were prepared by direct immunization of rabbits with normal  $\gamma$ A-globulin, and with the  $\gamma$ M-macroglobulin from a patient with Waldenstrom's macroglobulinemia, respectively. The antigens for immunization were included in complete Freund's adjuvant (Difco Lab., Detroit, Michigan) and injected *via* the footpads. The antisera were rendered specific by absorption with normal  $\gamma$ G-globulin. The  $\gamma$ -globulin fraction of the supernatant fluids was obtained by precipitation with ammonium sulfate at  $\frac{1}{2}$  saturation at pH 7.8. The specificity of the antibody preparations was tested by immunodiffusion. The anti- $\gamma$ G, anti- $\gamma$ A and anti- $\gamma$ M preparations gave a precipitin band with the homologous immunoglobulin, but not with the other two immunoglobulins. The anti-light chain antibody preparation gave a single precipitin band of identical specificity with  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ M-globulins. The concentration of specific antibody in the final preparations was measured by quantitative precipitin reactions (11) using purified  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ M-globulins from normal serum. Precipitating antibody in the anti- $\gamma$ G, anti- $\gamma$ A, anti- $\gamma$ M and anti-light chain preparations was 350  $\mu$ gN/ml, 420  $\mu$ gN/ml, 510  $\mu$ gN/ml, and 630  $\mu$ gN/ml, respectively.

The anti- $\gamma$ E antibody employed in the present experiment was the same preparation as that used in a previous study (5). A rabbit was immunized with a reagin-rich fraction of an atopic patient's

serum included in complete Freund's adjuvant, and the antiserum obtained was absorbed with normal  $\gamma$ G-globulin, with two preparations of  $\gamma$ A myeloma and  $\gamma$ D myeloma proteins. The supernatant fluid gave a single precipitin band with Fr. II<sub>11</sub> from serum Pr, which did not contain  $\gamma$ A-globulin, but not with any of the  $\gamma$ G,  $\gamma$ A,  $\gamma$ M and  $\gamma$ D myeloma proteins. Presence of the antibody specific for  $\gamma$ E and absence of antibodies reacting with the other four immunoglobulins in the anti- $\gamma$ E preparation was confirmed by radioimmunodiffusion using radioactively labeled anti-light chain (5).

A goat antiserum against rabbit  $\gamma$ G-globulin (RGG) was purchased from Antibody Inc., Davis, California. The antibody concentration in the preparation, measured by quantitative precipitin reactions (11) with RGG, was 1.0 mgN/ml.

*Radioimmunodiffusion.* Immunodiffusion was carried out by the method of Ouchterlony (12). Peripheral wells were filled with serial dilutions of serum specimens or of the fractions of patients' sera. Dilutions were made in either saline or normal serum which was obtained from a person who gave a negative skin reaction with ragweed pollen extract. Center wells were filled with a mixture of the antiserum specific for one of the human immunoglobulins and radioactive antigen E. The concentration of radioactive allergen in the mixture was 1  $\mu$ g/ml. Antibody in the rabbit antiserum specific for antigen E was titrated in the same manner using goat anti-RGG. The rabbit antiserum was diluted in a solution of RGG (50  $\mu$ gN/ml) to maintain the concentration of protein. The dilutions were placed in peripheral wells of Ouchterlony plates and the mixture of anti-RGG and radioactive antigen E in the center wells. The plates were incubated for 48 hr at room temperature and washed for 2 days. The slides were dried and radioautographs were taken by contact with x-ray film (Kodak Industrial, type KK). The exposure time was selected so that the product of time of exposure and the specific activity of I<sup>131</sup> antigen E at the time of exposure was the same for all slides.

*Measurement of antigen-binding activity.* The principle of the method is based on that of Skom and Talmage (13). Radioactive allergen was incubated with serum samples, and each immunoglobulin in the mixture was precipitated in antibody excess with its specific antiserum. All tests were run in duplicate. Dilutions of radioactive

allergen were made in a 1:5 dilution of normal rabbit serum. For the measurement of allergen-binding activity of  $\gamma$ G,  $\gamma$ A and total antibodies, serum samples were diluted in saline. The sera were diluted 10 times for the measurements of  $\gamma$ G and total antibodies, and 2.5 times for  $\gamma$ A antibody measurement. One-tenth milliliter of appropriate dilutions was pipetted into test tubes and incubated for 24 hr at 3°C with an equal volume of radioactive allergen. Serum K, in which no antibody against antigen E was detected by radioimmunodiffusion, was used for a control series. Serial dilutions of radioactive antigen E were incubated with 0.1 ml of either 2.5-fold or 10-fold dilution of the serum. The anti- $\gamma$ G, anti- $\gamma$ A or anti-light chain antibodies were then added to the mixtures to precipitate  $\gamma$ G-,  $\gamma$ A- or whole immunoglobulins. The amount of the antibodies added to precipitate the immunoglobulin was about twice the equivalence amount. The tubes were incubated again for 24 hr at 3°C, and centrifuged for 30 min at 0°C. The precipitates were washed three times with cold saline, resuspended in cold saline and transferred to new tubes which were centrifuged to obtain final precipitates. The radioactivity of the precipitates were measured using a well-shaped scintillation counter. Counts of control tubes, which were considered to be due to nonspecific absorption of radioactive allergen, increased with the concentration of allergen incubated. The amount of specifically bound allergen in each tube was calculated by subtracting the count of control tubes which were incubated with the same concentration of allergen and serum K, from the count of precipitates formed with other specimens. In each test, tubes containing 0.1 ml of serial dilutions of radioactive antigen E served as standards to convert the radioactivity to dose of allergen.

Since the concentration of  $\gamma$ E-globulin in serum specimens was low, the possibility was considered that a part of  $\gamma$ E-anti- $\gamma$ E precipitates might be lost by washing. In order to avoid this, the  $\gamma$ E-anti- $\gamma$ E precipitates were formed in 0.5% agarose. One-tenth milliliter of undiluted serum samples was incubated with an equal volume of appropriate dilutions of radioactive allergen for 24 hr at 3°C. Equal volumes of anti- $\gamma$ E preparation and 2% agarose were mixed at 45°C and 0.2 ml of the mixture was added to each tube. A control series consisting of varying dilutions of radioactive allergen, normal rabbit serum and agarose gel

containing anti- $\gamma$ E was also used. The tubes were incubated at 37°C for 1 hr and then at 3°C for 48 hr. The agarose gel was removed from the bottom of the test tubes by gentle shaking, and washed in 10 ml saline for 7 days at 3°C, with two changes of saline per day. The gel was transferred to new tubes for radioactivity measurements. Standards for the tests were the same as those used in the control series but the gels were not washed. It was found that the presence of gels reduced the count by 5%. Since nonspecific binding of radioactive allergen differed according to the concentration of allergen incubated, the amount of allergen specifically combined was calculated by subtracting the count of the control gel formed in the presence of the same concentration of radioactive allergen.

The allergen-combining activity of the rabbit antiserum specific for antigen E was measured by two methods. In one method, both radioactive allergen and the antiserum were diluted in a 50  $\mu$ gN/ml concentration of RGG and the precipitates were formed by the addition of 0.1 ml of anti-RGG containing 100  $\mu$ g AbN. In the other method, the precipitates were formed in agarose gel. The reagents were diluted in 1  $\mu$ gN/ml of RGG and mixed. After incubation, 0.2 ml of a mixture of 1:5 dilution of anti-RGG serum and 2% agarose was added to each tube for precipitating RGG in agarose gel.

*Prausnitz-Küstner reactions.* The tests were carried out on three nonallergic individuals who gave a negative skin reaction to ragweed-allergen extract. One-twentieth milliliter of test samples was injected intracutaneously into the back. Twenty four hours later, the sites received 0.2  $\mu$ g of antigen E in 0.02 ml. Erythema-and-wheal reactions appearing 15 to 20 min after the injection of allergen were traced on a transparent plate which was applied to the area. In order to compare the sensitivity of the reactions in the three recipients, twofold dilutions of the same patient's serum were injected dorsally followed by an allergen injection. The maximum dilution of the serum required for giving a positive skin reaction was the same in the three recipients, indicating that the test subjects had a similar range of reactivity.

#### EXPERIMENTAL RESULTS

##### A. Detection of antibodies associated with $\gamma$ G-, $\gamma$ A-, $\gamma$ M- and $\gamma$ E-Globulins

The skin-sensitizing activity of the patients' sera and fractions from sera EK and Tr were

determined by P-K reactions. Appropriate dilutions of the samples were injected intracutaneously into two normal individuals for the titration of each sample. Although the size of positive reactions was different depending on the recipients, the maximal dilutions of the same samples giving definite positive reactions were the same in both recipients. The titer of each specimen in P-K reactions conducted at 24 hr is shown in Table I. Among the serum specimens tested, serum K and Fr. II<sub>11</sub> from serum Pr gave negative results when the skin sites were challenged with antigen E (5).

As reported in a previous paper (5), the skin-sensitizing activity of the serum specimens was lost by incubating the specimens with anti- $\gamma$ E antibody. Serum specimens which gave positive skin reactions with antigen E were mixed with one-half volume of the anti- $\gamma$ E preparation. After incubation for 24 hr at 3°C, a trace amount of precipitate was removed by centrifugation. Appropriate dilutions of the supernatant fluids and unabsorbed preparations were injected intracutaneously into normal individuals for passive sensitization. In all cases, the highest concentration of the supernatant fluids, which corresponded to  $\frac{1}{8}$  to  $\frac{1}{16}$  of the P-K titer of the original sera, gave a negative reaction when the skin sites were challenged with antigen E.

Radioimmunoassay in Ouchterlony plates was applied to detect the antibodies against antigen E. Serial dilutions of the patients' sera in saline were placed in peripheral wells of Ouchterlony plates and were run against a mixture of radioactive antigen E and one of the antibodies specific for  $\gamma$ G-,  $\gamma$ A- or  $\gamma$ M-globulins. For the titration of  $\gamma$ E antibody in human sera, a normal human serum which contained no detectable amount of  $\gamma$ E antibody was used as a diluent, and a mixture of anti- $\gamma$ E with radioactive antigen E was placed in center wells. As control, the rabbit antiserum against antigen E was titrated in the similar manner, with the antiserum diluted 1:30 in saline, or diluted further in normal RGG (50  $\mu$ gN/ml); dilutions were placed in peripheral wells and tested against a goat anti-RGG serum which was mixed with radioactive antigen E. The concentration of the radioactive antigen E in the center wells was the same for all experiments. The agar plates were incubated at room temperature for 48 hr, washed, and radioautographs taken. Among the serum specimens tested, sera Pr and K did not give a radioactive band irrespective of

TABLE I  
Correlation of  $\gamma G$ ,  $\gamma A$ ,  $\gamma M$  and  $\gamma E$  antibody and titers of Prausnitz-Küstner reactions

| Serum          | Fraction         | Concentrations of Immunoglobulins |            |            | Maximum Dilutions Giving Radioactive Band |            |                |            | P-K Titer |
|----------------|------------------|-----------------------------------|------------|------------|-------------------------------------------|------------|----------------|------------|-----------|
|                |                  | $\gamma G$                        | $\gamma A$ | $\gamma M$ | $\gamma G$                                | $\gamma A$ | $\gamma M$     | $\gamma E$ |           |
|                |                  | mgN/ml                            | mgN/ml     | mgN/ml     |                                           |            |                |            |           |
| EK             |                  | 3.4                               | 0.55       | 0.19       | 80                                        | $\pm$      | — <sup>a</sup> | 16         | 1600      |
| B              |                  | 2.6                               | 0.44       | 0.15       | 40                                        | $\pm$      | —              | 32         | 3200      |
| F <sub>c</sub> |                  | 2.6                               | 0.20       | 0.19       | 20                                        | 10         | —              | 8-16       | 800       |
| F              |                  | 2.1                               | 0.24       | 0.40       | 20                                        | —          | —              | 8          | 600       |
| A              |                  | 3.5                               | 0.31       | 0.15       | 40                                        | $\pm$      | 1              | 8-16       | 800       |
| W              |                  | 2.1                               | 0.26       | 0.31       | 80                                        | 5          | —              | 8-16       | 600       |
| P              |                  | 1.1                               | 0.30       | 0.19       | 160                                       | —          | —              | 4-8        | 400       |
| K              |                  | 2.3                               | 0.17       | 0.19       | —                                         | —          | —              | —          | <100      |
| I              |                  | 1.5                               | 0.22       | 0.08       | 160                                       | —          | —              | —          | 0         |
| Pr             | II <sub>11</sub> | 0.66                              | 0          | 0          | —                                         | —          | —              | —          | <10       |
|                | I                | 2.89                              | 0          | 0          | 80                                        | $\pm$      | —              | —          | <50       |
| EK             | II <sub>11</sub> | 0.19                              | 0.38       | 0          | 5                                         | —          | —              | 64         | 3200      |
|                | II <sub>22</sub> | 0.3                               | 0          | 0          | 5                                         | —          | —              | —          | <10       |
|                | III              | 0.6                               | 1.22       | 0          | 10                                        | 2          | —              | 2          | 400       |
| Tr             | $\gamma G$       | 8.6                               | 0          | 0          | 320                                       | —          | —              | —          | 20        |
|                | $\gamma A_1$     | 0                                 | 0.23       | 0          | —                                         | 1          | —              | $\pm$      | 50        |
|                | $\gamma A_2$     | 0                                 | 0.26       | 0          | —                                         | 5          | —              | —          | 10        |

<sup>a</sup> Minus signs indicate no radioactive band.

the rabbit antiserum used, indicating that these samples did not contain any detectable antibody against antigen E. A  $\gamma E$  precipitin band was observed visually on a stained slide only in the case of serum A, however, a  $\gamma E$  radioactive band was detected by radioautographs in all cases other than Pr and K.  $\gamma M$  antibody was detected in only one specimen, while two out of nine samples contained  $\gamma A$  antibody (Table I). Under the same conditions, as low as 0.0016  $\mu gN/ml$  of rabbit antibody against antigen E was sufficient to give a radioactive precipitin band. The intensity of radioactivity in precipitin bands formed with patients' sera was compared with those formed with the rabbit antibody. The maximal dilution of each sample required to give a radioactive  $\gamma G$  or  $\gamma A$  band, which is comparable in radioactivity (darkness) to that formed with 0.0016  $\mu gN/ml$  of rabbit antibody is shown in Table I. The radioactive  $\gamma E$  bands are generally diffuse and very close to peripheral wells of Ouchterlony plates (Fig. 1a, b). The appearance of the  $\gamma E$  bands makes the reading of end point of the titration difficult. Therefore, darkness of  $\gamma E$  bands in radioautographs was compared with that of the

precipitin band formed with 0.0062  $\mu gN/ml$  of rabbit anti-antigen E antibody (Fig. 1c). The dilution of each serum sample giving the radioactive  $\gamma E$  band which is comparable to the radioactive band secured with the rabbit antibody is shown in Table I. The results of the titration of  $\gamma E$  antibody were confirmed by using Fr. II<sub>11</sub> of serum Pr as diluent. Serum EK and A were diluted with Fr. II<sub>11</sub> of serum Pr containing 66  $\mu gN/ml$  and the dilutions were placed in peripheral wells of Ouchterlony plates. The position of radioactive  $\gamma E$  bands was intermediate between an antibody well and antigen well, and the radioactivity of the bands was easily compared with that formed with rabbit antibody (Fig. 1d). Again, 1:16 dilution of serum EK and 1:8 dilution of serum A gave radioactive  $\gamma E$  bands which are comparable in radioactivity with that formed with 0.0062  $\mu gN/ml$  of rabbit antibody. It is clear from Table I that the  $\gamma E$  antibody titer correlates with the skin-sensitizing activity of the serum specimens which were determined by P-K reactions, whereas none of the  $\gamma G$ ,  $\gamma A$  or  $\gamma M$  antibody titers did.

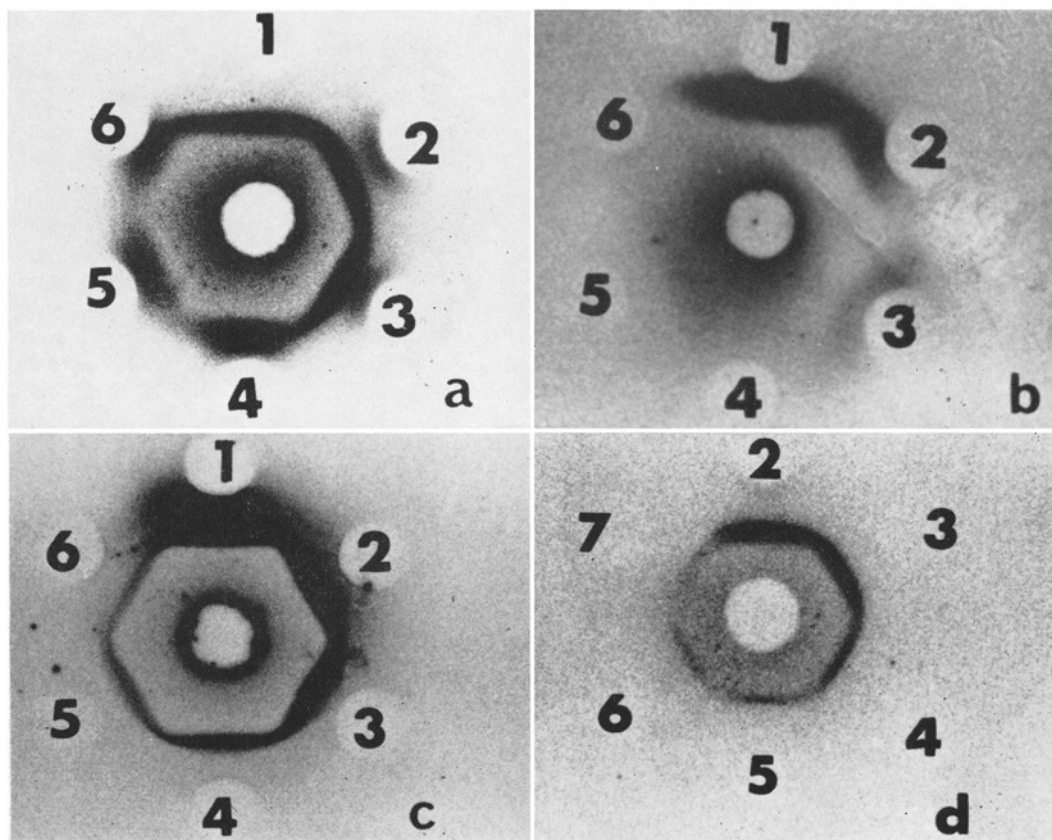


Figure 1. Titration of  $\gamma$ E antibody by radioimmunodiffusion. Twofold dilutions (tested from undiluted serum up to 1:32) of serum A (Fig. 1a) and Fr. III from EK (Fig. 1b), diluted in a normal human serum, were placed in peripheral wells (1 to 6); a mixture of anti- $\gamma$ E with radioactive antigen E was placed in the center wells. In Figure 1c twofold dilutions of rabbit antiserum against antigen E in rabbit  $\gamma$ -globulin (50  $\mu$ gN/ml) were placed in peripheral wells, the antibody concentration in well 1 being 0.1  $\mu$ gN/ml. The center well was filled with the mixture of anti-rabbit  $\gamma$ -globulin with radioactive allergen. In Figure 1d materials placed in the wells were the same as in (a) except that serum A was diluted in Fr. II<sub>11</sub> from serum Pr. Twofold to 64-fold dilutions were placed in wells 2 to 7. Note that the radioactive  $\gamma$ E bands formed in the middle zone of agar between antibody and antigen wells.

Similar experiments were performed with the fractions of serum EK and of serum Tr.  $\gamma$ E antibody was detected only in the fractions having high skin-sensitizing activity, and the titer of the  $\gamma$ E antibody paralleled the P-K titer of the fractions (Table I).

#### B. Quantitative measurements of allergen-binding activity

As a preliminary experiment, the allergen-combining activity of rabbit antiserum against antigen E was measured. The antiserum was diluted in a solution of normal RGG (50  $\mu$ gN/ml)

to prepare dilutions containing 0.03 and 0.05  $\mu$ g AbN/ml. One tenth milliliter of these antibody solutions and of controls which contained 50  $\mu$ gN/ml of normal RGG were incubated with twofold dilutions of radioactive antigen E, after which RGG was precipitated by a goat antiserum raised against rabbit  $\gamma$ G-globulin. After washing the precipitate, radioactivity remaining in the precipitate was measured. The experimental error in duplicate tubes was within 5%. The relations of the specifically bound allergen and the dose of allergen incubated are shown in Figure 2. Although the specific binding of allergen increased

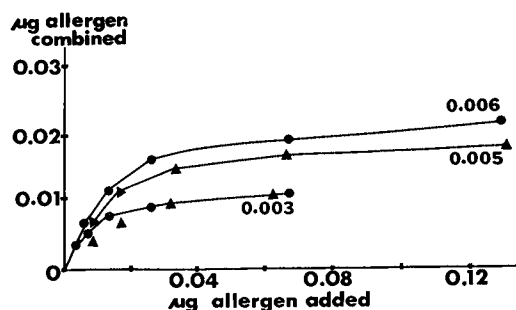


Figure 2. Quantitative measurements of allergen-binding activity of rabbit antiserum against antigen E. Allergen-antibody complexes were precipitated with anti-rabbit  $\gamma$ -globulin, either in 0.5% agarose gel (●—●) or without agarose gel (▲—▲). Numerals in the figure represent micrograms of antibody N incubated.

with the concentration of allergen incubated, the amount of bound allergen was nearly maximal when the amount of added allergen was 5 to 10 times greater. The bound allergen with 0.003 and 0.005  $\mu$ g N of rabbit antibody under the conditions employed was 0.0108 and 0.0179  $\mu$ g, respectively, indicating that the dose of bound allergen is proportional to the amount of antibody with which it is incubated.

The allergen-binding activity of the antibody was measured by another method in which antigen-antibody precipitates were formed in 0.5% agarose gel. Although nonspecific adsorption of radioactive allergen to the gel was slightly higher than that to specific precipitate, the specific binding of allergen measured by this method was in agreement within experimental error with the results obtained by the precipitation method (Fig. 2). When the bound allergen was 10 to 20% of the dose of allergen incubated, the dose of specifically bound allergen with 0.003 and 0.006  $\mu$ g N of rabbit antibody was 0.110 and 0.0213  $\mu$ g, respectively. It is apparent that the allergen-binding activity of the antibody can be quantitatively measured by both methods, provided that the dose of allergen incubated with the antibody is in sufficient excess.

The allergen-binding activity of sera from atopic patients and of the fractions of sera EK and Tr were measured. The concentration of  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ M-globulins in the samples were estimated by agar plates containing one of the antisera specific for these proteins (Hyland Laboratories, Los Angeles, California) (14) and total

immunoglobulin was calculated by adding the concentration of the individual immunoglobulins. The results are shown in Table I. Radioactive antigen E in twofold dilutions was mixed with equal volumes of serum specimens diluted 1:10 or with appropriate dilutions of the fractions. Whole immunoglobulins in the mixture were then precipitated by adding an excess of the rabbit antiserum specific for light chains. The absence of immunoglobulins in the supernatant fluids was confirmed by interfacial ring tests made *vs.* the antilight chain antibody. Serum K and Pr were used as controls since they contained no antibody against antigen E detectable by radioimmuno-diffusion. The relationship between specifically bound allergen and the dose of allergen added is shown in Figure 3. The amount of bound allergen approaches a maximum when 10 to 20% of the added allergen is combined.

For measurement of the allergen bound to antibodies found in each class of immunoglobulins, the concentration of allergen was selected so that 10 to 20% of the allergen added would be bound by all antibodies in the specimens. The concentration of  $\gamma$ G-globulin being much higher than that of  $\gamma$ A-globulin, the serum specimens were used in 1:10 and 1:2.5 dilutions for measurement of  $\gamma$ G and  $\gamma$ A antibodies, respectively. The  $\gamma$ M antibody was not studied because it was detected in only one case by radioimmunodiffusion and its titer was low. An appropriate concentration of radioactive antigen E was incubated with the dilutions of the serum specimens and  $\gamma$ G- and  $\gamma$ A-globulins in the mixtures were precipitated by addition of excess rabbit antibodies specific for

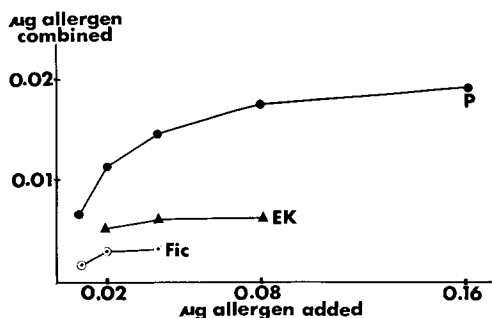


Figure 3. Allergen-binding activity of the sera of ragweed-sensitive patients EK, Fc and P. Allergen-antibody complexes were precipitated with the rabbit antibody specific for light chains of human immunoglobulins.

either  $\gamma$ G or  $\gamma$ A. As controls, serial dilutions of the radioactive allergen were added to serum K and the immunoglobulins were precipitated similarly. The allergen-binding activity of  $\gamma$ G and  $\gamma$ A antibodies in each serum was then calculated from activities bound in the specific precipitates, multiplying by the dilution factor (Table II). The allergen-binding activities of  $\gamma$ G and  $\gamma$ A antibodies in the fractions of serum EK and serum Tr were also measured. In the original serum EK, the activity of the  $\gamma$ A antibody was negligible, yet, Fr. III of EK, containing a high concentra-

tion of  $\gamma$ A-globulin, gave a significant binding of allergen to  $\gamma$ A-globulin (Table III).

The  $\gamma$ E-anti- $\gamma$ E precipitates were formed in agarose gel for the measurement of  $\gamma$ E antibody. One-tenth milliliter of undiluted serum samples was incubated with an equal volume of an appropriate dilution of radioactive antigen E. Serum K, which served as a control, was incubated with varying concentrations of radioactive allergen. Another set of controls consisted of a different concentration of allergen and 0.1 ml rabbit serum. The anti- $\gamma$ E antibody in melted agarose was added to all tubes. It was found that the difference in radioactivity of the two controls (one with serum K and the other with normal serum) was  $\pm 5\%$ , indicating that serum K possesses no detectable  $\gamma$ E antibody. The dose of allergen specifically bound to  $\gamma$ E antibody in the specimens is shown in Tables II and III. It is apparent that the allergen-binding activity of the  $\gamma$ E antibody measured by this method bears no correlation with those of either  $\gamma$ G or  $\gamma$ A antibodies. It will be noted in the tables that the sum of the allergen bound by  $\gamma$ E,  $\gamma$ G and  $\gamma$ A antibodies in the same serum samples is in agreement with the dose of allergen bound with all immunoglobulins measured independently. The results also showed that the allergen-binding activity of  $\gamma$ E antibody correlates with P-K titer of the specimens.

#### DISCUSSION

Measurements of antibodies in sera from atopic individuals have been attempted by many investigators. Lidd and Farr (15) as well as Minden

TABLE II  
*Allergen-binding activity of antibodies  
in patients' sera<sup>a</sup>*

| Serum            | Allergen-Binding Activity |            |            |                 |                     | P-K<br>Titer |
|------------------|---------------------------|------------|------------|-----------------|---------------------|--------------|
|                  | $\gamma$ G                | $\gamma$ A | $\gamma$ E | IG <sup>b</sup> | IG cal <sup>c</sup> |              |
| EK               | 0.40                      | 0          | 0.23       | 0.62            | 0.63                | 1600         |
| B                | 0.26                      | 0          | 0.38       | 0.66            | 0.64                | 3200         |
| Fic <sup>d</sup> | 0.16                      | 0.082      | 0.14       | 0.33            | 0.38                | 800          |
| F <sup>d</sup>   | 0.14                      | 0.016      | 0.097      | 0.25            | 0.27                | 600          |
| A1               | 0.23                      | 0          | 0.10       | 0.34            | 0.33                | 800          |
| W                | 0.61                      | 0.033      | 0.099      | 0.79            | 0.74                | 600          |
| P                | 1.8                       | 0          | 0.07       | 1.9             | 1.9                 | 400          |
| I                | 1.9                       | 0          | 0          | 1.9             | 1.9                 | 0            |

<sup>a</sup> Numerals in the table indicate micrograms of antigen E bound by immunoglobulins in 1 ml of serum.

<sup>b</sup> Measured by precipitation of all immunoglobulins with anti-light chain antibodies.

<sup>c</sup> Allergen-binding activities of  $\gamma$ G,  $\gamma$ A and  $\gamma$ E antibodies were added.

<sup>d</sup> Untreated patients, all other patients were treated.

TABLE III  
*Allergen-binding activity of antibodies in fractions of patients' sera<sup>a</sup>*

| Serum | Fraction                | Protein<br>Concentration<br><br>mg.N/ml | Allergen-Binding Activity |            |            |       |       | P-K Titer |
|-------|-------------------------|-----------------------------------------|---------------------------|------------|------------|-------|-------|-----------|
|       |                         |                                         | $\gamma$ G                | $\gamma$ A | $\gamma$ E | IG    | IGcal |           |
| EK    | I                       | 2.89                                    | 0.59                      | 0          | 0          | 0.61  | 0.59  | <50       |
|       | II <sub>11</sub>        | 0.55                                    | 0                         | 0          | 0.34       | 0.37  | 0.34  | 3200      |
|       | II <sub>22</sub>        | 0.36                                    | ND <sup>b</sup>           | 0          | 0.01       | 0.044 |       | <10       |
|       | III                     | 1.83                                    | <0.05                     | 0.039      | 0.08       | 0.13  | 0.12  | 400       |
| Tr    | $\gamma$ G              | 8.6                                     | 4.0                       | 0          | 0          | 4.4   | 4.0   | 20        |
|       | $\gamma$ A <sub>1</sub> | 0.23                                    | 0                         | 0.015      | 0.01       | 0.026 | 0.025 | 50        |
|       | $\gamma$ A <sub>2</sub> | 0.26                                    | 0                         | 0.040      | 0          | ND    | 0.040 | 10        |

<sup>a</sup> See legend for Table II.

<sup>b</sup> Not done.

*et al.* (16) measured antigen-binding activity of the sera from atopic patients by the precipitation of  $I^{131}$ -labeled antigen-antibody complexes with 50% saturated ammonium sulfate. Pruzansky *et al.* (17, 18) reported the measurement of antibodies in the sera of ragweed-sensitive patients by the precipitation of antigen-antibody complexes with anti-human globulin. Lichtenstein and Osler (19) have determined antigen-neutralizing capacity of allergic sera by the inhibition of antigenically induced release of histamine from sensitive human leukocytes. In none of these studies did the antigen-binding capacity of the sera show correlation with skin-sensitizing activity. In view of the present knowledge that the antibodies are associated with immunoglobulins of various classes and that skin-sensitizing antibody is a part of the antibodies in the patients' sera, lack of correlation may be expected between the allergen-binding capacity and skin-sensitizing activity of the serum specimens. The antibodies associated with  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ M-globulins in atopic patients' sera were detected by Yagi *et al.* (7) by radioimmuno-electrophoresis, and Reisman *et al.* (20) reported that the presence of  $\gamma$ A antibody in the sera did not correlate with the skin-sensitizing activity. Our previous studies (1-5) on the antigenic structure of human reaginic antibodies indicated that the antibody is associated with  $\gamma$ E globulin rather than the four known immunoglobulins, i.e.,  $\gamma$ G,  $\gamma$ M,  $\gamma$ A and  $\gamma$ D.  $\gamma$ E antibody was detected in ragweed-sensitive patients' sera by radioimmunodiffusion (4, 5). In view of these results, the  $\gamma$ E antibody against antigen E was titrated by radioimmunodiffusion in the present experiments. As shown in Table I, the titer of  $\gamma$ E antibody paralleled the skin-sensitizing activity as determined by P-K reactions. Lack of correlation between the skin-sensitizing activity and the antibodies associated with other immunoglobulins was confirmed.

Titration of antibodies by radioimmunodiffusion incurs limitations for quantitative measurement of antibody. The intensity of radioactivity in precipitin bands is determined by 1) the concentration of immunoglobulin tested, 2) concentration of the anti-immunoglobulin used and 3) the relative concentration of antibody in the immunoglobulin precipitated. If the concentration of immunoglobulin in patients' sera falls within a certain range, and if one uses the same concentration of antibody specific for an immuno-

globulin, one can expect a parallelism between the radioactivity and the concentration of the antibody associated with the immunoglobulin. Nevertheless, comparisons among the antibodies of different immunoglobulin classes are impossible by this method. In order to obtain more quantitative data, attempts were made to measure the allergen-binding activity of antibodies associated with each immunoglobulin. The technique was developed in our laboratory by Newcomb and Ishizaka (21) and applied to measure  $\gamma$ G and  $\gamma$ A antibodies against diphtheria toxoid. One of the important precautions to be observed in using this technique is that the concentration of radioactive antigen for incubation should be in great excess compared with the antigen-binding activity of total antibodies. Otherwise, antibodies associated with two or more immunoglobulin classes may form a single antigen-antibody complex which may be precipitated with the rabbit antibodies specific for either of these immunoglobulins. In the present experiments, allergen-binding activity of total antibodies was estimated by the precipitation of all allergen-antibody complexes with the rabbit antibodies specific for light chains. The amount of radioactive allergen combined with all antibodies increases as the concentration of allergen increased. Theoretically, a maximum binding value should be obtained when an infinite concentration of allergen is added. However, nonspecific adsorption of the allergen to the specific precipitate increased with antigen concentration. Since the specific binding of allergen reaches its maximum when the allergen added was about 5 to 10 times as much as the allergen combined, the concentration of allergen for incubation with each specimen was selected, so that 10 to 20% of the allergen would be combined with the antibodies in the specimens. Another problem in this measurement was that  $\gamma$ E-globulin concentration in serum is very low. When anti- $\gamma$ E antibody was added to serum specimens, visible but very small amounts of precipitates were formed. In order to minimize loss from these precipitates occasioned by washing, the precipitates were formed in 0.5% agarose. This technique is based on the fact that a radioactive  $\gamma$ E band remains in 0.5% gel in radioimmuno-electrophoresis. The experiments with rabbit antibody specific for antigen E showed comparable specific binding values when antigen E-rabbit antibody complexes were precipitated with or without agarose.

It was also found that a quantitative relationship existed between the allergen-binding value and antibody concentration.<sup>2</sup> When this method was applied to human serum specimens, it was found that the allergen-binding values of different immunoglobulins were quite independent. In the serum specimen which did not have skin-sensitizing activity,  $\gamma$ E antibody was not detected in spite of the high allergen-binding value of  $\gamma$ G-globulin. Furthermore, the sum of allergen-binding values of  $\gamma$ G,  $\gamma$ A and  $\gamma$ E antibodies were in agreement with that of total antibody, which was measured by the precipitation with anti-light chain antibodies. These findings indicate that the allergen-binding value of one immunoglobulin was not significantly affected by the presence of antibodies belonging to other immunoglobulins.

The presented data showed that essentially all skin-sensitizing activity in the serum specimens was removed by the precipitation with anti- $\gamma$ E antibody, and that their skin-sensitizing activity correlated with antigen-binding values of  $\gamma$ E but not with those of other immunoglobulins. These findings support the idea that the skin-sensitizing antibody is associated with  $\gamma$ E-globulin. With respect to the relationship between the skin-sensitizing antibody and  $\gamma$ E-globulin, the possibility still exists that the antibody may be associated with a subclass of  $\gamma$ E-globulin. It is also possible that the human immunoglobulin family is more complicated than expected, and "unknown immunoglobulins" as well as  $\gamma$ E-globulin may be precipitated by the "anti- $\gamma$ E" preparation. Although these possibilities cannot be

<sup>2</sup> The maximal amount of allergen bound by a given dose of rabbit antibody may be estimated by assuming that the molecular configuration of the immune complex in extreme antigen excess is  $Ag_2Ab$ , and that the molecular weight of the rabbit antibody is 160,000. Thus, antigen bound = (antibody)  $\times \frac{2 \times (\text{molecular weight of antigen})}{(\text{molecular weight of antibody})}$ . If the molecular weight of antigen E is 37,000 (6), the actual dose of antigen bound by the rabbit antibody in the present experiment was higher than the calculated value. The discrepancy cannot be explained by the present experiment; however, it is possible that a part of antigen may be polymerized during iodination which was carried out at pH 8.9. If the dose of allergen (0.0108  $\mu$ g) bound with 0.003  $\mu$ gN of rabbit antibody corresponds to the maximal dose, the average molecular weight of the radioactive antigen, calculated from the present data, is 47,000.

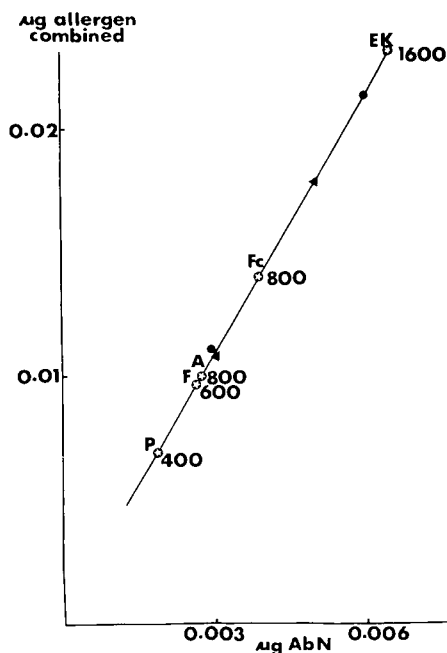


Figure 4. Relation between antibody concentration and allergen-binding activity of rabbit antibody ((●, ▲), *c.f.*, Fig. 2). The allergen-binding values of  $\gamma$ E antibodies in serum EK, Fc, A, F, P were interpolated. The numbers in the figure represent the Prausnitz-Küstner titer of the serum specimens.

eliminated, the parallelism between the P-K titer and allergen-binding activity of  $\gamma$ E antibody suggests strongly that the skin-sensitizing antibody is responsible for the major part of the antibodies precipitated by the anti- $\gamma$ E antibody preparation.

The allergen-binding value of  $\gamma$ E antibody in some specimens was comparable to that of the rabbit antibody preparation containing 0.03 to 0.06  $\mu$ g AbN/ml. This enables us to estimate the concentration of  $\gamma$ E antibody in the specimens. The relation between antibody concentration and allergen-binding values of rabbit antibody is shown in Figure 4. The allergen-binding values of  $\gamma$ E antibodies in some specimens were interpolated to estimate the concentration of the rabbit antibody which has the same allergen-binding value as  $\gamma$ E antibody in the specimens. Since the antigen-binding activity of antibodies is dependent on their avidity and valency, factors unknown for  $\gamma$ E antibody, and the sedimentation coefficient of  $\gamma$ E-globulin is 7.8 to 8 S (2, 5) which

TABLE IV

*Estimation of the minimum sensitizing dose of  $\gamma$ E antibody in Prausnitz-Küstner reactions*

| Serum Specimen | Allergen-Binding Value of $\gamma$ E Antibody | Rabbit Antibody Concentration <sup>a</sup> | Rabbit Antibody Corresponding to Minimum P-K dose |
|----------------|-----------------------------------------------|--------------------------------------------|---------------------------------------------------|
|                | $\mu\text{g/ml}$                              | $\mu\text{gN/ml}$                          | $\times 10^{-6} \mu\text{gN}$                     |
| EK             | 0.23                                          | 0.065                                      | 2.0                                               |
| Fc             | 0.14                                          | 0.038                                      | 2.4                                               |
| F              | 0.097                                         | 0.026                                      | 2.1                                               |
| A              | 0.10                                          | 0.028                                      | 1.8                                               |
| W              | 0.099                                         | 0.027                                      | 2.2                                               |
| P              | 0.07                                          | 0.019                                      | 2.4                                               |

<sup>a</sup> The concentration of rabbit antibody which is comparable to  $\gamma$ E antibody with respect to allergen-binding activity.

is higher than that of  $\gamma$ G-globulin, the concentration of rabbit antibody (Table IV) does not represent the actual concentration of  $\gamma$ E antibody, but gives some idea of the range of  $\gamma$ E antibody concentration in the patients' sera. The dose of rabbit antibody which corresponds to that of  $\gamma$ E antibody in the minimum sensitizing dose of the sera with respect to allergen-binding activity was also calculated. It will be noted in Table IV that the calculated doses in five different specimens are very close to one another, in the range of  $2 \times 10^{-6} \mu\text{gN}$ . If one assumes that the avidity of  $\gamma$ E antibody is comparable to that of rabbit antibody, and if the skin-sensitizing antibody is actually associated with  $\gamma$ E-globulin, the minimum dose of skin-sensitizing antibody required for positive P-K reactions would be in the order of  $10^{-6}$  to  $10^{-5} \mu\text{gN}$  which is about  $\frac{1}{1000}$  of the minimum dose of guinea pig  $\gamma_1$  and rabbit  $\gamma$ G antibodies for the induction of passive cutaneous anaphylaxis in the guinea pig (22, 23).

Another important finding in the present experiments is the ratio of antibody concentrations within the different immunoglobulins. It is clear in Table II that  $\gamma$ E and  $\gamma$ G antibody concentrations in four serum samples, e.g., EK, B, Fc, F, are of the same order of magnitude. The  $\gamma$ E-globulin concentration in the serum specimens is unknown; however, the position of  $\gamma$ E precipitin band in immunodiffusion and the amount of specific  $\gamma$ E-anti- $\gamma$ E precipitates obtained from sera (4) suggest that the concentration of  $\gamma$ E-globulin in the sera may be 10 to 20  $\mu\text{g/ml}$  or less. The  $\gamma$ G-globulin concentration in the serum is

probably 1000 to 2000 times higher than  $\gamma$ E-globulin. Comparisons of allergen-binding activity to the concentration of those immunoglobulins with which antibody is associated, suggests that, in these patients, a high percentage of  $\gamma$ E-globulin has antibody activity. The high concentration of  $\gamma$ E antibody in the sera might be due to the nature of antigen and/or the route of immunization. Another possibility is that  $\gamma$ E antibody synthesis may be higher in atopic individuals than in normal subjects. The high concentration of  $\gamma$ G antibody and the lack of other antibodies in normal subjects who received repeated intracutaneous injections of allergen suggests that the nature of antigen is not the only factor responsible for high  $\gamma$ E antibody levels. In any event, the measurement of allergen-binding activity in antibodies of different immunoglobulin classes may provide a scientific basis for the effect of hyposensitization treatment. It is well known that hyposensitization treatment results in an increase of blocking antibody, a major part of which may be  $\gamma$ G antibody. In fact, the  $\gamma$ G antibody level in some treated patients was very high (Table II). Connell and Sherman (24) reported that skin-sensitizing activity in some patients decreased after a long-term treatment. The possibility may be suggested that the effect of the treatment is due to a decrease of  $\gamma$ E antibody rather than to an increase of  $\gamma$ G antibodies. Studies on antibody concentrations in different immunoglobulins before and after the treatment are planned.

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#### SUMMARY

The antibodies associated with  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- and  $\gamma$ E-globulins in the sera of ragweed sensitive patients were titrated by radioimmunodiffusion using  $I^{131}$ -labeled antigen E and rabbit antibodies specific for each of these immunoglobulins. Quantitative measurements of the allergen-binding activities of the  $\gamma$ G,  $\gamma$ A and  $\gamma$ E antibodies in the specimens were made by precipitation of the radioactive allergen-antibody complexes with rabbit antibodies. The titer of the antibodies in the radioimmunodiffusion method paralleled their allergen-binding activities. The skin-sensitizing

activity of the patients' sera, which was determined by Prausnitz-Küstner reactions with antigen E, correlated with the allergen-binding activity of  $\gamma$ E antibody but not with that of  $\gamma$ G or  $\gamma$ A antibodies.  $\gamma$ M antibody was not detected in most of the samples containing high reaginic activity. Precipitation of  $\gamma$ E-globulin removed the skin-sensitizing activity of the sera. These findings support the hypothesis that the reaginic antibody is associated with  $\gamma$ E-globulin.

The minimum dose of the skin-sensitizing antibody required for giving positive Prausnitz-Küstner reactions was estimated. Comparisons were made between the  $\gamma$ E antibody in patients' sera and rabbit antibody specific for antigen E, with respect to the allergen-binding activity. The binding activity of  $\gamma$ E antibody present in the minimum dose of five patients' sera required for giving positive reactions was comparable to that of  $2 \times 10^{-6}$   $\mu$ gN of the rabbit antibody.

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