

## ANTIGENIC STRUCTURE OF $\gamma$ E-GLOBULIN AND REAGINIC ANTIBODY<sup>1</sup>

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Previous studies of human reagenic antibody indicated that the antibody was not associated with  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ D-globulins but rather with a new immunoglobulin (1-4). The protein was detected in ragweed-sensitive patients' sera with an antiserum which was obtained by immunization of a rabbit with a reagin-rich fraction of human serum. This antiserum after being absorbed with known immunoglobulins contained no antibodies specific for  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- or  $\gamma$ D-globulin or for  $\kappa$ - or  $\lambda$ -chains of immunoglobulins, but it precipitated essentially all of the reagenic activity in patients' sera and gave a  $\gamma_1$  precipitin band with a reagin-rich fraction in immunoelectrophoresis (4, 5). The  $\gamma_1$  precipitin band combined radioactively-labeled allergen, indicating that the protein had antibody activity, and thus, it was tentatively designated  $\gamma$ E-globulin (4). Further studies on the antibody associated with  $\gamma$ E-globulin by radioimmunodiffusion analyses showed that the distribution of reagenic activity paralleled that of  $\gamma$ E-antibody when a patient's serum was fractionated by chromatography on a diethylaminoethyl (DEAE)-cellulose column and examined by gel filtration, by sucrose density gradient ultracentrifugation and by zone electrophoresis. The sedimentation coefficient of  $\gamma$ E-antibody was estimated to be around 8 S by sucrose density gradient ultracentrifugation (5). These findings strongly suggest that  $\gamma$ E-globulin belongs to a unique immunoglobulin class. The possibility still exists, however, that the protein may be a subclass of known immunoglobulins. Since  $\gamma$ E-globulin was detected using a rabbit antiserum from which antibodies specific for  $\gamma$ A-,  $\gamma$ G- and

$\gamma$ D-globulins were precipitated with myeloma proteins, it is possible that the antiserum contained antibodies directed toward minor antigenic determinants in the known immunoglobulins and that " $\gamma$ E"-globulin was detected by one of these antibodies. Subclasses of  $\gamma$ G-globulins were described by Grey and Kunkel (6) as well as Terry and Fahey (7) and those of  $\gamma$ A-globulin have recently been reported by Kunkel and Prendergast (8), Vaerman and Heremans (9)<sup>2</sup> as well as by Terry and Robert (10). The present experiments were therefore undertaken to investigate the relationship between  $\gamma$ E-globulin and the known subclasses of  $\gamma$ G- and  $\gamma$ A-globulins with respect to antigenic structure. Some experiments are also made to study the presence of two light chain types in  $\gamma$ E-globulin.

### MATERIALS AND METHODS

*Reagenic sera and their fractions.* Four serum specimens from ragweed-sensitive individuals were employed in the present experiments. Serum EK was kindly supplied by Dr. C. E. Arbesman, Buffalo, New York, and serum Pr was supplied by Drs. H. Fudenberg and W. Epstein, University of California, San Francisco, California. The latter serum did not contain a detectable amount of  $\gamma$ A-globulin. A pooled serum (U) from untreated patients was supplied by Dr. L. M. Lichtenstein, The Johns Hopkins University, Baltimore, Maryland. Serum A was obtained at Children's Asthma Research Institute and Hospital, Denver, Colorado. Sera EK, A and U contained skin-sensitizing antibody against antigen E (11). Serum Pr gave Prausnitz-Küstner (P-K) reactions with crude ragweed allergen but not with antigen E (5).

<sup>2</sup> Feinstein and Franklin (9a) also reported two subclasses of  $\gamma$ A and indicated that the subclasses described in (8) and (9) and those in their work are identical.

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The reaginic sera were fractionated by chromatography on a DEAE-cellulose (Mann Research Lab., New York, N. Y.) column and by gel filtration through a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column in the same manner as those previously described (3, 5). The globulin fraction of the sera was obtained by precipitation with ammonium sulfate at 1/2 saturation at pH 7.0. The fraction was dialyzed against 0.005 M phosphate buffer, pH 8.0, and applied to a DEAE-cellulose column, equilibrated with the buffer. Proteins were eluted stepwise with phosphate buffers of increasing molarity. A reagin-rich fraction obtained with 0.025 M phosphate buffer (Fr. II) was applied to a Sephadex G 200 column and the ascending and descending halves of a single peak were pooled separately (Frs. II<sub>1</sub> and II<sub>2</sub>). Since reaginic activity was present in Fr. II<sub>1</sub>, this fraction from sera Pr and EK was concentrated and rechromatographed on the Sephadex column to obtain a more active fraction (Fr. II<sub>11</sub>). The fraction from serum Pr contained  $\gamma$ G-,  $\gamma$ D- and  $\gamma$ E-globulins and gave positive P-K reactions with 1:1600 dilution which was eight times as high as the titer of the original serum (5). Further purification was achieved by recycling gel filtration through a Sephadex G 200 column (12). A sample of Fr. II<sub>11</sub> (serum Pr) was applied to a Sephadex column (3.2 by 90 cm) fitted with adjustable plungers (LKB Instruments Inc., Rockville, Md.) and filtered by an upward flow of borate buffered saline. By this procedure, the bulk of  $\gamma$ G-globulin was separated from the fraction containing reaginic activity. The final reagin-rich fractions were pooled and concentrated (Fr. II<sub>12</sub>).

The  $\gamma$ A-globulin fraction was obtained from serum U by the method of Vaerman *et al.* (13) with slight modifications (14). The preparation was subfractionated by chromatography on a DEAE-cellulose column (3). Protein fractions were eluted stepwise with 0.02 M and 0.035 M phosphate buffers of 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>.

*Purified ragweed pollen allergen.* Purified allergen (antigen E, Fr. IV<sub>0</sub> prepared according to King (11)) was kindly supplied for Dr. M. W. Chase, Rockefeller University. The antigen was labeled with I<sup>131</sup> using the method described by Yagi *et al.* (15) with chloramine T.

*Myeloma proteins.* Myeloma  $\gamma$ G-globulins, i.e.,

$\gamma$ G<sub>1</sub>-,  $\gamma$ G<sub>2</sub>-,  $\gamma$ G<sub>3</sub>- and  $\gamma$ G<sub>4</sub>- and myeloma  $\gamma$ A-globulin, i.e., Fu, Ma, He and Jo, were purified from sera of myeloma patients by electrophoresis on Pevikon (Fosfatbolaget, Sweden) block and chromatography on DEAE-cellulose columns. Three  $\gamma$ G<sub>1</sub>-, 3  $\gamma$ G<sub>2</sub>-, 2  $\gamma$ G<sub>3</sub>- and 1  $\gamma$ G<sub>4</sub>-globulins were used in the experiments. Proteins Fu and Ma are the same preparations as those described in (10) and represent different  $\gamma$ A-subclasses (Fu type, Ma type). Serum He and Jo were kindly supplied by Dr. H. Fudenberg. The  $\gamma$ A myeloma protein from serum He was comprised of polymer and monomer forms which were separated by gel filtration through a Sephadex G 200 column. The  $\gamma$ D myeloma serum was supplied by Dr. H. N. Claman, University of Colorado, Denver, Colorado. The myeloma protein was purified by DEAE-cellulose column chromatography followed by gel filtration as described by Rowe and Fahey (16, *cf.* 5). The  $\gamma$ M-macroglobulin was isolated from serum of a patient with Waldenström macroglobulinemia by the combination of gel filtration and block electrophoresis.

*Antisera specific for immunoglobulins.* Rabbit specific for  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M-,  $\gamma$ D- and  $\gamma$ E-globulins used in the experiments are the same preparations described in a previous article (5). The anti- $\gamma$ E-serum contained antibodies against  $\alpha$ -globulins; however, no antibody to known immunoglobulins was detected in the preparation.

Antisera specific for  $\kappa$  or  $\lambda$  antigenic determinants were obtained from rabbits injected intramuscularly with 5 to 10 mg of  $\kappa$ - or  $\lambda$ -type Bence Jones proteins emulsified in complete Freund's adjuvant (CFA). The rabbits were similarly reinjected every 6 to 8 weeks until useful precipitating antisera were obtained. The antisera were absorbed with  $\gamma$ G myeloma proteins of the opposite light chain type.

Antisera with specificities for heavy chain antigenic subclasses were prepared in monkeys and rabbits. A rhesus monkey was immunized intramuscularly with 10 mg of pooled normal human IgG emulsified in CFA, similarly reinjected 4 weeks later and then bled weekly thereafter. The antiserum was rendered specific for  $\gamma$ G<sub>2</sub> and  $\gamma$ G<sub>3</sub> by absorption with a  $\gamma$ G<sub>1</sub> myeloma protein of the  $\kappa$ -type. Anti- $\gamma$ G<sub>4</sub>-serum was obtained from rhesus monkeys similarly immunized with an isolated  $\gamma$ G<sub>4</sub> myeloma protein. The antiserum was absorbed with hypogammaglobulinemic serum and  $\gamma$ G<sub>1</sub>-,  $\gamma$ G<sub>2</sub>- and  $\gamma$ G<sub>3</sub>- myeloma proteins. An antiserum specific for  $\gamma$ G<sub>3</sub> was prepared by intra-

muscularly immunizing a rabbit with 5 mg of a  $\gamma$ G<sub>3</sub> myeloma protein emulsified in CFA, and reinjecting the same amount of protein 6 weeks later. The animal was bled weekly thereafter and the serum absorbed with hypogammaglobulinemic serum and a  $\gamma$ G<sub>2</sub> myeloma protein until it was specific for the  $\gamma$ G<sub>3</sub>-subclass.

**Immunodiffusion and radioimmunodiffusion.** Immunodiffusion was carried out by the method of Ouchterlony (17). Radioactive antigen E was mixed with the antiserum specific for one of the immunoglobulins and the mixtures were diffused against the test samples. The concentration of radioactive allergen in the mixture was 1  $\mu$ g/ml. The plates were incubated for 48 hr at room temperature and washed for 2 days. Plates were dried and radioautographs were taken by contact with x-ray film (Kodak Industrial, X-ray film, type KK). Immunoelectrophoresis was carried out using agarose gel (18). In order to study whether  $\gamma$ E-globulin has both K and L types, radioactively-labeled anti- $\kappa$ - and anti- $\lambda$ -chain antibodies were used. The method was described in detail in a previous article (5). Briefly, the  $\gamma$ E precipitin band was formed between a reagin-rich fraction and anti- $\gamma$ E-antiserum. The slides were washed and radioactively-labeled anti- $\kappa$ - or anti- $\lambda$ -chains antibody was applied to the antibody troughs. The slides were incubated overnight, washed and radioautographs were taken.

**Prausnitz-Küstner reactions.** The tests were carried out on nonallergic individuals who gave a negative skin reaction to ragweed-allergen extract. Test samples of 0.05 ml were injected intracutaneously into these individuals. Twenty-four hours later, the sites received 0.2  $\mu$ g of antigen E or 10 protein nitrogen units (PNU) of crude ragweed-allergen extract (Center Lab., New York, N. Y.) in 0.02 ml saline. Erythema wheal reactions appearing 15 to 20 min after the injection of allergen were traced on a transparent plate which was applied to the area.

#### EXPERIMENTAL RESULTS

**A. Presence of K and L types in  $\gamma$ E-globulin.** In a previous article (5), the presence of light chain(s) in  $\gamma$ E-globulin was suggested by the combination of radioactively-labeled anti-F<sub>ab</sub>-antibody to the  $\gamma$ E precipitin band. Although the antibody used in the experiments gave a single precipitin band of identical specificity with  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ M-globulins, presence of antibody against the F<sub>d</sub> portion of  $\gamma$ -chain could not be

excluded. Similar experiments were therefore carried out using anti- $\kappa$ - and anti- $\lambda$ -chain antibodies. The  $\gamma$ E precipitin band was formed between Fr. II<sub>11</sub> from serum Pr and anti- $\gamma$ E-antiserum in immunoelectrophoresis. After washing for 2 days, three sets of two slides were treated with unlabeled anti- $\kappa$ , anti- $\lambda$  or normal rabbit  $\gamma$ -globulin, which was applied to an antibody trough. After incubation for 24 hr, it was found that  $\gamma$ E precipitin bands were intensified by either anti- $\kappa$ - or anti- $\lambda$ -chain antibody. Radioactively-labeled anti- $\kappa$ - or anti- $\lambda$ -antibody was then added to antibody troughs of a set of three slides which had been treated with anti- $\kappa$ , anti- $\lambda$  or normal  $\gamma$ -globulin. The concentration of radioactive antibodies was  $\frac{1}{10}$  that of unlabeled antibodies used for pretreatment of the slides. The slides were incubated for 24 hr followed by washing for 2 days. Radioautographs of the slides showed that

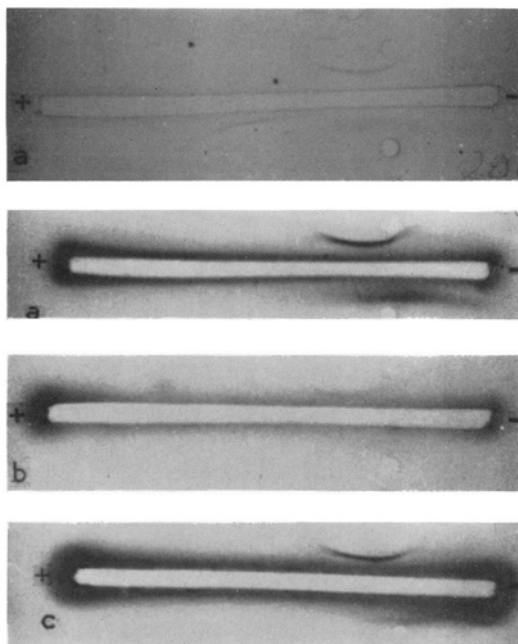


Figure 1. Radioimmunoelectrophoresis of Fr. II<sub>11</sub> (serum Pr) and the original serum. Both stained slides (top) and radioautographs (a, b, c) are shown. Slides (a), (b) and (c) were treated with normal rabbit  $\gamma$ -globulin, unlabeled anti- $\kappa$ - and anti- $\lambda$ -chain antibody, respectively before radioactive anti- $\kappa$ -chain was applied. The radioactive antibody combined with the  $\gamma$ E precipitin band (a, c) and the combination was specifically blocked by unlabeled anti- $\kappa$ -chain antibody (b).

the  $\gamma$ E precipitin band bound radioactive anti- $\kappa$ - and anti- $\lambda$ -chain antibodies; and the combination of the labeled antibody was specifically blocked by pretreatment of the slide with unlabeled antibody of the same specificity but not with antibody of the other specificity. Representative results with radioactive anti- $\kappa$ -chain antibody are shown in Figure 1. It is clear that intensity of radioactivity of the  $\gamma$ E precipitin band greatly diminished by pretreatment with unlabeled anti- $\kappa$ -antibody (Fig. 1b) but not with anti- $\lambda$ -antibody (Fig. 1c). Similar experiments were carried out by radioimmunodiffusion. The center wells of Ouchterlony plates were filled with anti- $\gamma$ E-globulin and peripheral wells were filled with Fr. II<sub>11</sub> of serum Pr,  $\gamma$ G<sub>2</sub>,  $\gamma$ G<sub>3</sub>,  $\gamma$ G<sub>4</sub>,  $\gamma$ A myeloma proteins and  $\gamma$ M-macroglobulin. The concentration of the myeloma proteins was 100 to 200  $\mu$ g N/ml. Only a faint band was formed between anti- $\gamma$ E and Fr. II<sub>11</sub>. After washing, the center well was filled with either unlabeled anti- $\kappa$ , anti- $\lambda$  or normal rabbit  $\gamma$ -globulin followed by radioactive antibody. Again the  $\gamma$ E precipitin band combined labeled anti- $\kappa$ - and anti- $\lambda$ -antibody and the combination

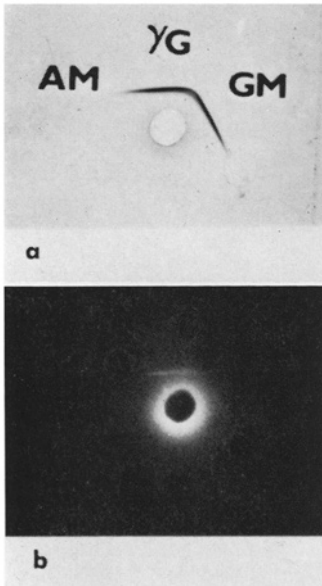


Figure 2. Interruption of a radioactive band by a precipitin band of identical specificity. Both the stained slide (a) and radioautograph (b) are shown. A center well of the Ouchterlony plate was filled with anti- $\gamma$ G mixed with radioactive antigen E. Peripheral wells were filled with  $\gamma$ G-globulin fraction of a patient's serum ( $\gamma$ G), myeloma  $\gamma$ A (AM) and myeloma  $\gamma$ G (GM) proteins.

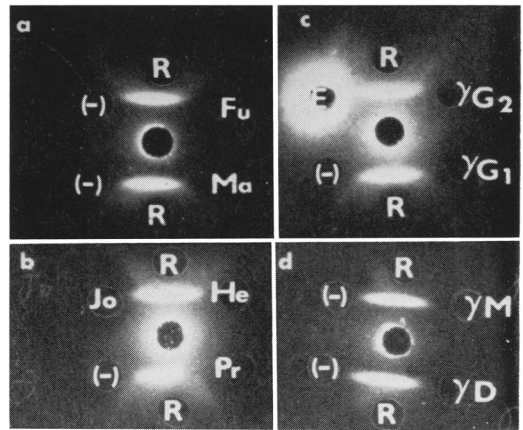


Figure 3. Effect of myeloma proteins on the shape of the radioactive  $\gamma$ E-band. Center wells were filled with anti- $\gamma$ E mixed with radioactive antigen E. Peripheral wells were filled with a reagin-rich fraction (R),  $\gamma$ A,  $\gamma$ G,  $\gamma$ D myeloma proteins,  $\gamma$ M-macroglobulin, Fr. II<sub>11</sub> from serum Pr or antigen E (E). Wells marked (-) were empty. The shape of the  $\gamma$ E-band between the reagin-rich fraction (R) and anti- $\gamma$ E was changed by placing Fr. II<sub>11</sub> (Pr) in a well adjacent to the well (R), but not by  $\gamma$ A (Fu, Ma, He, Jo),  $\gamma$ G<sub>1</sub>,  $\gamma$ G<sub>2</sub>,  $\gamma$ D myeloma proteins or  $\gamma$ M-macroglobulin.

was blocked by unlabeled homologous antibody. No radioactive band was observed between anti- $\gamma$ E-antiserum and myeloma proteins, indicating that the anti- $\gamma$ E-antibody preparation did not contain any of the antibodies against heavy chains of the myeloma proteins. These results indicate that  $\gamma$ E-globulin is composed of molecules containing  $\kappa$  and  $\lambda$  light chains.

*B. Different antigenic determinant in  $\gamma$ E-globulin from subclasses of  $\gamma$ G- and  $\gamma$ A-globulins.* In a previous article (5), a radioactive  $\gamma$ E-band was formed in Ouchterlony plates between sera from ragweed-sensitive individuals and anti- $\gamma$ E-serum mixed with radioactive antigen E. The shape of the radioactive  $\gamma$ E-band changed when the well adjacent to a patient's serum was filled with Fr. II<sub>11</sub> from serum Pr which did not contain any antibody against antigen E. The radioactive  $\gamma$ E-band was interrupted at the intersection of the band with a visible  $\gamma$ E precipitin band formed between the fraction and the anti- $\gamma$ E (cf. Fig. 3b). These results indicate that the radioactive band and the visible  $\gamma$ E-band have the same specificity. The phenomenon was confirmed in a  $\gamma$ G-antibody system. Radioimmunodiffusion was set up be-

tween the  $\gamma$ G-globulin fraction from an atopic patient's serum and anti- $\gamma$ G-antibody mixed with radioactive antigen E. The wells adjacent to  $\gamma$ G-fraction was filled with either  $\gamma$ G or  $\gamma$ A myeloma protein. The radioactive  $\gamma$ G-band was interrupted by a  $\gamma$ G precipitin band formed with myeloma  $\gamma$ G-globulin, whereas the  $\gamma$ A-globulin did not change the shape of the radioactive  $\gamma$ G-band (Fig. 2*a*, *b*). In view of these findings, the effect of  $\gamma$ A myeloma proteins on the shape of the radioactive  $\gamma$ E-band was studied. Radioimmuno-diffusion was set up with anti- $\gamma$ E-serum mixed with radioactive antigen E in center wells. Two peripheral wells were filled with a reagin-rich fraction of serum U and others were filled with  $\gamma$ A myeloma proteins and Fr. II<sub>11</sub> from serum Pr (Fig. 3*a*, *b*). The concentration of the myeloma proteins was 100  $\mu$ g N/ml and that of Fr. II<sub>11</sub> was 40  $\mu$ g N/ml. It is apparent that Fr. II<sub>11</sub> interrupted the radioactive  $\gamma$ E-band whereas none of the  $\gamma$ A myeloma proteins, i.e., Fu, Ma, He and Jo, did. The shape of the radioactive band was the same on the side adjacent to the well containing  $\gamma$ A-globulin and on the side where the well was empty. The  $\gamma$ A myeloma proteins were sub-classified with the antiserum supplied by Dr. Vaerman. Both Fu and Ma were Le subclass and He and Jo were molecules with He subclass specificity (9). As Fu and Ma represent different subclasses described by Terry and Robert (10),  $\gamma$ A myeloma proteins tested include all known subclasses (9*a*, footnote 2). Effect of  $\gamma$ G myeloma proteins,  $\gamma$ G<sub>1</sub>,  $\gamma$ G<sub>2</sub>,  $\gamma$ G<sub>3</sub>,  $\gamma$ G<sub>4</sub>,  $\gamma$ D- and  $\gamma$ M-globulins on the shape of the radioactive  $\gamma$ E-band was also studied by the same method. None of the myeloma proteins changed the shape of the radioactive  $\gamma$ E-band when they were placed in wells adjacent to a reagin-rich fraction. Representative data on  $\gamma$ G<sub>1</sub>,  $\gamma$ G<sub>2</sub>,  $\gamma$ D- and  $\gamma$ M-globulins are shown in Figures 3*c* and 3*d*. The experiments were duplicated with serum EK as a source of  $\gamma$ E-antibody with comparable results. These findings indicate that the antigenic determinant responsible for the formation of  $\gamma$ E-band is not shared by any of the immunoglobulins of the known classes and subclasses.

A problem arose as to whether  $\gamma$ E-globulin may be precipitated with antibodies specific for known immunoglobulins. Radioimmuno-diffusion experiments were set up to study this possibility. Center wells of Ouchterlony plates were filled with anti- $\gamma$ E, two peripheral wells with a reagin-rich fraction from serum U and two other wells with

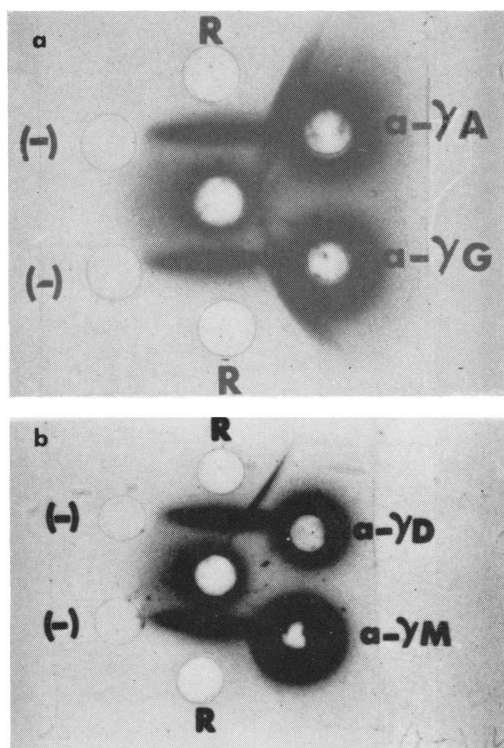


Figure 4. Radioautographs were superimposed on corresponding stained slides in the figure, to show the relationship between the radioactive  $\gamma$ E-band on radioautographs and precipitin bands on stained slides. Center wells of Ouchterlony plates were filled with anti- $\gamma$ E-serum mixed with radioactive antigen E. Peripheral wells (R) were filled with a reagin-rich fraction. Antisera specific for  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- and  $\gamma$ D-globulins (*a-gammaG*, *a-gammaA*, *a-gammaM*, *a-gammaD*) were placed in wells adjacent to well (R). The  $\gamma$ G- and  $\gamma$ E-bands showed radioactivity, whereas the  $\gamma$ A- and the  $\gamma$ D-bands did not.

antiserum specific for  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- or  $\gamma$ D-globulins (Fig. 4). The reagin-rich fraction formed  $\gamma$ G,  $\gamma$ A and  $\gamma$ D precipitin bands. After washing the plates, antibody wells were filled with radioactive antigen E. Radioautographs of the plates showed that  $\gamma$ G- and  $\gamma$ E-globulins had antibody activity, whereas neither the  $\gamma$ A- nor  $\gamma$ D-band showed radioactivity. The radioautographs were superimposed to corresponding stained slides to show the relationship between  $\gamma$ E and other immunoglobulins. It is clear in Figure 4 that the radioactive  $\gamma$ E-band penetrated the  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ D-bands. The findings indicate that the  $\gamma$ E-globulin did not react with any of the antibodies specific for the four known immunoglobulins.

TABLE I  
Distribution of reaginic activity,  $\gamma G_2$ - and  $\gamma G_3$ -globulins

| Fraction                    | Buffer for Elution | Minimum Concentration for Detecting |              |                   |
|-----------------------------|--------------------|-------------------------------------|--------------|-------------------|
|                             |                    | $\gamma G_2$                        | $\gamma G_3$ | Reaginic Activity |
|                             | M/H                | $\mu g$ N/ml                        | $\mu g$ N/ml | $\mu g$ N/ml      |
| A                           | 0.005              | 16                                  | 150          | >20               |
| B                           | 0.01               | 16-32                               | 300          | 8                 |
| C <sub>1</sub> <sup>a</sup> | 0.02               | 75-150                              | 300          | 1                 |
| C <sub>2</sub> <sup>a</sup> | 0.02               | 75-150                              | 300          | 8                 |
| D                           | 0.035              | 300                                 | 1000         | 8                 |
| E                           | 0.05               | >1000                               | >2000        | >20               |
| F                           | 0.08               | >1300                               | >1300        | >20               |
| $\gamma A_1$                | 0.02               | >290                                | >290         | 1                 |

<sup>a</sup> Frs. C<sub>1</sub> and C<sub>2</sub> were obtained by gel filtration through a Sephadex G 200 column (see text).

C. *Reaginic antibody and subclasses of  $\gamma G$ -,  $\gamma A$ - and  $\gamma M$ -globulins.* When reaginic sera were fractionated by DEAE-cellulose column chromatography and by gel filtration, the reaginic activity in the fractions did not parallel the concentration of either  $\gamma G$ - or  $\gamma A$ -globulin (1, 3). In order to study the relationship between the reaginic activity and minor subclasses of  $\gamma G$ -globulin, the globulin fraction of serum U was fractionated by chromatography on a DEAE-cellulose column. Proteins were eluted stepwise with five phosphate

buffers (pH 8.0) of increasing molarity (Table I) and the most active fraction (Fr. C) was applied to a Sephadex G 200 column (3). A single peak was obtained and most reaginic activity was detected in the ascending portion of the peak. The ascending and descending halves of the peak, therefore, were pooled separately (Frs. C<sub>1</sub> and C<sub>2</sub> in Table I). Twofold dilutions of each fraction were placed in peripheral wells of Ouchterlony plates against anti- $\gamma G_2$ , anti- $\gamma G_3$  or anti- $\gamma G_4$  in center wells. Since anti- $\gamma G_2$ -serum contained both anti- $\gamma G_2$ - and anti- $\gamma G_3$ -antibodies,  $\gamma G_2$ -globulin was placed in two of the six peripheral wells to identify the  $\gamma G_2$ -band. The minimum protein concentration of the fractions required for giving definite precipitin band against each antiserum is shown in Table I. None of these fractions gave a positive precipitin band against anti- $\gamma G_4$ -globulin serum. The skin-sensitizing activity of the fractions was determined by P-K reactions, and the minimum concentrations required for positive skin reactions are listed in Table I. It is evident that the skin-sensitizing activity did not parallel either  $\gamma G_2$ - or  $\gamma G_3$ -globulin concentration.  $\gamma A$ -Globulin fraction was obtained from the same reaginic serum and subfractionated by DEAE-cellulose column chromatography. As reported previously (3), the skin-sensitizing activity was concentrated in one of the fractions,  $\gamma A_1$ , which was eluted by 0.02 M phosphate buffer. The

TABLE II  
Effect of absorption on skin-sensitizing activity<sup>a</sup>

| Serum | Reaginic Fraction | Absorption with                 | Dilutions of the Supernatants Injected for P-K <sup>b</sup> Reaction |          |          |          |
|-------|-------------------|---------------------------------|----------------------------------------------------------------------|----------|----------|----------|
|       |                   |                                 | ×50                                                                  | ×100     | ×200     | ×400     |
| A     | II <sub>1</sub>   | Anti- $\gamma G_3$ <sup>c</sup> | 9.5 (36)                                                             | 8.5 (34) | 8.5 (22) | 7 (27)   |
|       |                   | Anti- $\gamma E$                | 0 (0)                                                                | 0 (0)    |          |          |
|       |                   | None                            | 9.5 (39)                                                             | 8.5 (31) | 8 (34)   | 7 (23)   |
| A     | II <sub>1</sub>   | Anti- $\gamma M$ <sup>c</sup>   | 11 (39)                                                              | 10 (27)  | 8.5 (22) | 7 (18)   |
|       |                   | None                            | 11 (38)                                                              | 11 (29)  | 9 (24)   | 7 (20)   |
| EK    | II <sub>11</sub>  | Anti- $\gamma G$ <sup>d</sup>   | 9 (39)                                                               | 8.5 (38) | 8 (34)   | 6.5 (21) |
|       |                   | None                            | 9 (39)                                                               | 8.5 (34) | 7.5 (28) | 6 (20)   |

<sup>a</sup> Numerals in the table indicate average diameters of wheal and those in parentheses represent diameters of erythema in millimeters.

<sup>b</sup> P-K, Prausnitz-Küstner.

<sup>c</sup> Absorption of  $\gamma G_3$ - and  $\gamma M$ -globulins were carried out in the presence of carrier proteins; 0.2 ml of anti-serum was added to mixtures containing 0.1 ml of reaginic fraction and 0.1 ml of carrier protein (20  $\mu g$  N/ml  $\gamma G_3$ , 40  $\mu g$  N/ml  $\gamma M$ -globulin). The unabsorbed fraction was diluted four times with saline.

<sup>d</sup> Both  $\gamma G_2$  (0.05 ml) and  $\gamma G_4$  (0.05 ml) myeloma proteins were added to 0.1 ml of EK II<sub>11</sub>, and all  $\gamma G$ -globulin in the mixture was precipitated with anti- $\gamma G$ -antiserum (0.6 ml); the unabsorbed fraction was diluted eight times with saline.

fraction was tested for the presence of  $\gamma$ G<sub>2</sub>-,  $\gamma$ G<sub>3</sub>- and  $\gamma$ G<sub>4</sub>-globulin by immunodiffusion. None of the antisera specific for these proteins gave a precipitin band with Fr.  $\gamma$ A<sub>1</sub>, which had a higher skin-sensitizing activity than any of the chromatographic fractions from the same serum (Table I).

The  $\gamma$ G<sub>3</sub>-globulin in a reagin-rich fraction (Fr. II<sub>1</sub>) of serum A was precipitated with anti- $\gamma$ G<sub>3</sub>-globulin antibody. Since the concentration of  $\gamma$ G<sub>3</sub>-globulin in the fraction was low,  $\gamma$ G<sub>3</sub> myeloma protein was added to the fraction and the mixture was incubated with anti- $\gamma$ G<sub>3</sub>-globulin for 24 hr at 0°C followed by centrifugation to remove precipitates. Anti- $\gamma$ G<sub>3</sub>-antibody was detected in the supernatant. A separate sample of the serum fraction was absorbed with anti- $\gamma$ E-antiserum. Equal volumes of the fraction and of anti- $\gamma$ E-serum were incubated and a trace amount of precipitate removed. The skin-sensitizing activity of the supernatants and the unabsorbed fraction was determined by P-K reactions. Serial dilutions of the preparations were injected into normal individuals and the skin sites were challenged with ragweed allergen. The supernatant obtained after the absorption of  $\gamma$ G<sub>3</sub>-globulin and the unabsorbed fraction gave the same P-K titer, whereas the supernatant after absorption of  $\gamma$ E-globulin did not give positive P-K reactions (Table II).

The relationship between reaginic antibody and  $\gamma$ G<sub>2</sub>- and  $\gamma$ G<sub>4</sub>-globulins was studied. A reagin-rich fraction (Fr. II<sub>1</sub>) of serum EK was mixed with the myeloma proteins. The concentration of the proteins in the mixture was 40  $\mu$ g N/ml. The mixture was then incubated with an excess amount of anti- $\gamma$ G-antibody and the precipitate removed. The supernatant contained anti- $\gamma$ G-antibody which gave a precipitin band of identical specificity with  $\gamma$ G<sub>1</sub>-,  $\gamma$ G<sub>2</sub>-,  $\gamma$ G<sub>3</sub>- and  $\gamma$ G<sub>4</sub>-globulins in immunodiffusion but did not give any reaction with either anti- $\gamma$ G<sub>2</sub>- or anti- $\gamma$ G<sub>4</sub>-antiserum by interfacial ring tests. Appropriate dilutions of the supernatant and the original fraction were injected intracutaneously into normal individuals for passive sensitization and the skin sites were challenged with ragweed allergen. Results indicated no detectable decrease of skin-sensitizing activity after the absorption of all  $\gamma$ G-globulins (Table II).

Rothfeld *et al.* (19) reported 7 S globulin having  $\gamma$ M antigenic determinant. Although  $\gamma$ M-globulin was not detected in the reagin-containing fractions of the patients' sera and the sedimentation coefficient of the reaginic antibody was

TABLE III  
*Effect of myeloma proteins on passive sensitization with reaginic antibody*

| Proteins Injected with Reaginic Antibody | Wheal | Erythema |
|------------------------------------------|-------|----------|
| $\mu$ g N                                | mm    | mm       |
| $\gamma$ G <sup>a</sup>                  |       |          |
| $\gamma$ G <sub>1</sub>                  | 2.0   | 30 × 32  |
| $\gamma$ G <sub>2</sub>                  | 2.0   | 35 × 42  |
| $\gamma$ G <sub>3</sub>                  | 2.0   | 30 × 43  |
| $\gamma$ G <sub>4</sub>                  | 2.0   | 30 × 49  |
| $\gamma$ A                               |       |          |
| Fu                                       | 2.0   | 30 × 41  |
| Ma                                       | 2.0   | 34 × 37  |
| He                                       | 2.0   | 31 × 45  |
| $\gamma$ D                               | 1.6   | 31 × 34  |
| $\gamma$ M                               | 2.0   | 34 × 36  |
| Fr. II <sub>11</sub> (Pr) <sup>b</sup>   | 0.1   | 0        |
|                                          | 0.025 | 22 × 26  |
| Fr. II <sub>R</sub> (Pr) <sup>b</sup>    | 0.06  | 0        |
|                                          | 0.03  | 20 × 22  |
|                                          | 0.015 | 21 × 25  |
| Saline                                   | 0     | 36 × 42  |

<sup>a</sup> Two other samples of  $\gamma$ G<sub>1</sub> and of  $\gamma$ G<sub>2</sub>, and one other sample of  $\gamma$ G<sub>3</sub> protein gave similar results.

<sup>b</sup> Fr. II<sub>11</sub> contained  $\gamma$ G-,  $\gamma$ D- and  $\gamma$ E-globulins and Fr. II<sub>R</sub> was composed of  $\gamma$ G- and  $\gamma$ E-globulins. No other serum protein was detected in Fr. II<sub>R</sub>.

around 8 S (2), the possibility still remained that the antibody was associated with a minor component having  $\gamma$ M antigenic determinant. In order to study this possibility, a reagin-rich fraction (Fr. II<sub>1</sub>) from serum A was mixed with  $\gamma$ M-globulin followed by precipitation of the protein with anti- $\gamma$ M-antibody in antibody excess. The supernatant contained excess anti- $\gamma$ M-antibody which was demonstrated by interfacial ring test. The supernatant and the original fraction gave the same P-K titer, indicating that essentially all reaginic activity remained in the supernatant (Table II).

In a previous article (3) it was found that Fr.  $\gamma$ A<sub>1</sub> from ragweed-sensitive individuals blocked passive sensitization of normal human skin with reaginic antibody of a heterologous system, whereas  $\gamma$ A myeloma proteins,  $\gamma$ G- and  $\gamma$ M-globulins did not. The results strongly suggested that the blocking of passive sensitization was due

to the same protein with which the reagin was associated. In view of these findings, the blocking effect of  $\gamma$ G and  $\gamma$ A as well as  $\gamma$ M and  $\gamma$ D was studied. Three preparations each of  $\gamma$ G<sub>1</sub> and of  $\gamma$ G<sub>2</sub>, two preparations of  $\gamma$ G<sub>3</sub> and one each of  $\gamma$ G<sub>4</sub> and  $\gamma$ D myeloma proteins were tested. A reaginic serum (EK), which had a P-K titer of 1:1600 was diluted 1:250, and 1 volume of the dilution was mixed with an equal volume of the myeloma proteins which were at concentrations of 80  $\mu$ g N/ml.  $\gamma$ M-Macroglobulin and serial dilutions of Fr. II<sub>11</sub> from serum Pr were also mixed with a 1:250 dilution of the reaginic serum. A volume of 0.05 ml of each of the mixtures was injected into normal human skin for passive sensitization and the skin sites were challenged with antigen E 24 hr later. Representative results are shown in Table III. None of the subclasses of  $\gamma$ G- and  $\gamma$ A-globulins,  $\gamma$ D- or  $\gamma$ M-globulins blocked passive sensitization at the levels tested whereas even 0.1  $\mu$ g N of Fr. II<sub>11</sub> completely blocked the sensitization. In order to confirm that  $\gamma$ E-globulin was responsible for the blocking activity, experiments were repeated with Fr. II<sub>R</sub> from the same serum which was obtained by recycling after gel filtration. It was found by immunodiffusion that Fr. II<sub>R</sub> contained  $\gamma$ G- and  $\gamma$ E-globulins but no other immunoglobulin (Fig. 5). Immunoelectrophoresis of the preparation against goat anti-human serum and anti- $\gamma$ E-serum revealed that no serum protein other than the two immunoglobulins was detectable in it. A relative concentration of  $\gamma$ G-globulin, measured by immunoplates containing

anti- $\gamma$ G-antibody (Hyland Laboratories, Los Angeles, Calif.) (20), was 75% of total protein in the fraction. As shown in Table III, only 0.06  $\mu$ g N of the fraction was sufficient to block passive sensitization of human skin with reaginic antibody.

#### DISCUSSION

Data presented in this paper clearly show that the  $\gamma$ E precipitin band in immunoelectrophoresis specifically combined anti- $\kappa$ - and anti- $\lambda$ -chain antibodies and indicate that  $\gamma$ E-globulin possesses both  $\kappa$  and  $\lambda$  light chains. It was also found by radioimmunodiffusion that the antigenic determinant(s) responsible for the formation of the  $\gamma$ E-band was not shared by any of the  $\gamma$ G,  $\gamma$ A,  $\gamma$ M and  $\gamma$ D myeloma proteins tested. The shape of the radioactive  $\gamma$ E-band formed between reaginic serum and anti- $\gamma$ E-serum was not changed by placing the myeloma proteins in a well adjacent to that of the reaginic serum. Only Fr. II<sub>11</sub> from serum Pr which contained  $\gamma$ E-globulin changed the shape of the radioactive  $\gamma$ E-band (Fig. 3). As demonstrated in the  $\gamma$ G-antibody system,  $\gamma$ G myeloma protein, but not  $\gamma$ A myeloma protein, changed the shape of the radioactive  $\gamma$ G-band in radioimmunodiffusion (Fig. 2). The radioactive  $\gamma$ G-band was terminated at the point where the band fused to that formed with a  $\gamma$ G myeloma protein. The results indicate that a radioactive band is interrupted when a precipitin band of the same specificity is formed by a protein placed in an adjacent well. Failure of myeloma proteins of known classes and subclasses to interrupt the radioactive  $\gamma$ E-band indicates that the proteins tested lacked antigenic determinant(s) responsible for the formation of  $\gamma$ E-band.

If  $\gamma$ E-globulin is a subclass of a known immunoglobulin, the protein should possess a major antigenic determinant characteristic of heavy chains of the immunoglobulin. It has been shown in a previous paper (4) that the  $\gamma$ E-band formed with a subfraction of a  $\gamma$ A-globulin preparation from a ragweed-sensitive serum combined radioactive antigen E but that the  $\gamma$ A-band formed with the same preparation did not, and that the preparation did not contain a detectable amount of  $\gamma$ G-,  $\gamma$ D-, or  $\gamma$ M-globulin. It was also found that  $\gamma$ E-antibody against purified ragweed antigen was not precipitated by either anti- $\gamma$ G- or anti- $\gamma$ A-antiserum (21). Furthermore, radioimmunodiffusion experiments showed that the shape of a radioactive  $\gamma$ E-band formed between a

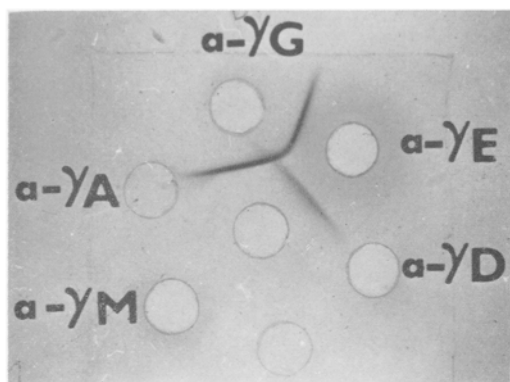


Figure 5. Ouchterlony plate analysis of Fr. II<sub>R</sub> from serum Pr. The fraction (142  $\mu$ g N/ml) was placed in a center well against antisera specific for one of the immunoglobulins. Anti- $\gamma$ G- ( $\alpha$ - $\gamma$ G) and anti- $\gamma$ E- ( $\alpha$ - $\gamma$ E) sera gave precipitin bands.

reagin-rich fraction and anti- $\gamma$ E-serum did not change when antisera specific for other immunoglobulins were placed in adjacent wells (Fig. 4). The radioactive  $\gamma$ E-band penetrated the  $\gamma$ A-,  $\gamma$ G- and  $\gamma$ D-bands. It is apparent that  $\gamma$ E-globulin does not have any of the major antigenic determinants characteristic of  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M- and  $\gamma$ D-globulins but has determinants which are not shared by any of the known immunoglobulins. These findings as well as the presence of  $\kappa$ - and  $\lambda$ -chains in the molecules and the association of antibody with the protein indicate that  $\gamma$ E-globulin represents a distinct immunoglobulin class which may be called IgE.

It has been shown in previous articles (1, 2, 4, 5) that reaginic antibodies are not removed by antibodies specific for  $\gamma$ G-,  $\gamma$ A- and  $\gamma$ D-globulins but that it is precipitated by anti- $\gamma$ E-globulin. The present experiments extended the previous findings through the demonstration that chromatographic behavior of reaginic antibodies is different from that of  $\gamma$ G<sub>2</sub>- or  $\gamma$ G<sub>3</sub>-globulins and that the antibodies are not coprecipitated either with the complexes containing  $\gamma$ G<sub>2</sub>-,  $\gamma$ G<sub>3</sub>- and  $\gamma$ G<sub>4</sub>-globulins or with  $\gamma$ M-anti- $\gamma$ M complexes. It is apparent that reaginic antibody in the ragweed patients studied does not have any of the major antigenic determinants of the four known immunoglobulins.

The hypothesis that reaginic antibodies are associated with  $\gamma$ E-globulin but not with other immunoglobulins was supported by the fact that none of the myeloma proteins of  $\gamma$ G-subclasses,  $\gamma$ A-subclasses, the  $\gamma$ D myeloma protein or  $\gamma$ M-macroglobulin from a serum of Waldenström macroglobulinemia blocked passive sensitization with reagin, whereas Fr II<sub>R</sub> of serum Pr containing  $\gamma$ E-globulin did. Only 0.06  $\mu$ g N of the fraction was sufficient to block the passive sensitization. As the fraction was composed of  $\gamma$ G- and  $\gamma$ E-globulins but did not contain any antibody against purified ragweed allergen,  $\gamma$ E-globulin in the fraction seems to be responsible for the blocking effect. Although the concentration of  $\gamma$ E-globulin in the fraction is unknown, it would be 25% or less of total protein, as  $\gamma$ G-globulin comprised 75%. It seems that the minimum dose of  $\gamma$ E-globulin for complete blocking of passive sensitization is in the order of 0.01 to 0.02  $\mu$ g N. It was shown in our recent studies (22) that the blocking effect of the fraction was lost upon heating at 56°C. This finding is in agreement with the fact that skin-sensitizing activity of

patients' sera is heat-labile and suggests that inactivation of skin-sensitizing antibody on heating is due to loss of affinity of  $\gamma$ E-globulin for skin tissues. Previous and present experiments have shown that 1) physicochemical properties of reaginic antibody are similar to those of  $\gamma$ E-antibody to the same antigen (5); 2) reaginic activity in 10 patients' sera tested was precipitated by anti- $\gamma$ E-antibody (5) and that 3)  $\gamma$ E-globulin but no other immunoglobulin blocks passive sensitization with reagin. In our more recent studies which will be published separately, a reagin-rich preparation was obtained from a patient's serum. By radioimmunodiffusion using I<sup>131</sup>-labeled antigen E, only  $\gamma$ E-antibody, but none of the antibodies associated with four other immunoglobulins was detected in the preparation. Furthermore, precipitation of  $\gamma$ E-globulin in the preparation by anti- $\gamma$ E-serum was accompanied by loss of reaginic activity. These findings indicate that  $\gamma$ E-antibodies have skin-sensitizing activity. Our findings, however, do not necessarily exclude a possible heterogeneity of reaginic antibodies, and skin-sensitizing antibodies in some sera might be associated with immunoglobulins other than  $\gamma$ E-globulin.

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#### SUMMARY

Both  $\kappa$  and  $\lambda$ -chain antigenic determinants were detected in  $\gamma$ E-globulin by radioimmunoelectrophoresis. In addition,  $\gamma$ E-globulin has antigenic determinants which are lacking in other immunoglobulins of the four known classes, i.e.,  $\gamma$ G,  $\gamma$ A,  $\gamma$ M and  $\gamma$ D as well as in  $\gamma$ A- and  $\gamma$ G-subclasses. The protein did not contain the major antigenic determinants of the four known classes of immunoglobulins. The results indicate that  $\gamma$ E-globulin represents a distinct class of immunoglobulin.

Reaginic activity in atopic patients' sera was precipitated by anti- $\gamma$ E-antibody but not by either  $\gamma$ M-anti- $\gamma$ M-antibody complexes or  $\gamma$ G-antibody complexes containing  $\gamma$ G<sub>2</sub>-,  $\gamma$ G<sub>3</sub>- and  $\gamma$ G<sub>4</sub>-globulins. The distribution of reaginic activity in chromatographic fractions did not

parallel that of  $\gamma G_2$ - or  $\gamma G_3$ -globulin. Passive sensitization of human skin with reaginic antibody was not blocked by any of the myeloma proteins of known classes or subclasses, whereas a fraction containing  $\gamma E$ -globulin had blocking activity. The results indicate that reaginic antibody activity is associated with  $\gamma E$ -globulin.

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