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Research Article

MULTIPLE SERUM PROTEIN DEFICIENCIES IN CONGENITAL AND ACQUIRED AGAMMAGLOBULINEMIA [1]

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The absence of serum γ-globulins in patients afflicted with severe recurring bacterial infections has been reported (1, 2). Three forms of this metabolic disturbance, agammaglobulinemia, have been described (3): 1) a physiologic or transient form occurring in infants, 2) a congenital form which is inherited as a sex-linked recessive characteristic occurring in males, and 3) an acquired form, occurring in both sexes, with the onset of infections in adolescence or adulthood. The congenital and acquired forms of the disease are due to an absence of plasma cells in the tissues of these patients (3–5) with a consequent deficiency of γ-globulin and failure of antibody synthesis (4); the plasma cells appear to be the site of antibody synthesis (6). Unlike the transient form, the failure of γ-globulin synthesis in the congenital and acquired groups, once established, seems to be permanent.

Using rabbit and horse antisera against human γ-globulin in the quantitative precipitin reaction, it had been demonstrated that the failure of γ-globulin synthesis in these patients was usually not complete and that small amounts of γ-globulin were often present in their sera, less than 25 mg. per cent in the congenital form and less than 100 mg. per cent in the acquired form (3). In this report, it will be shown that at least two other plasma proteins in addition to γ-globulin are either absent or markedly deficient in these two forms of the disease.

METHODS

Human sera studied: Sera from 8 children with congenital agammaglobulinemia, from 3 adults with the acquired disease, and from 6 normal children and adults were selected for study. The γ-globulin concentrations in the agammaglobulinemic patients ranged from 0 to 35 mg. per cent as determined immunochemically. All of the sera studied were obtained prior to instituting γ-globulin therapy.

Unadsorbed and adsorbed antisera: 1) Rabbit antiserum against human γ-globulin was prepared as described elsewhere (7). 2) Horse antiserum against human γ-globulin was prepared according to the method described for horse anti-human albumin (8), using as the antigen a purified γ-globulin preparation obtained by low temperature ethanol-water fractionation (9). The horse antiserum gave the diphtheria antitoxin type of precipitation curve in its interaction with human γ-globulin (10). As will be shown, these antisera were *not* entirely specific for γ-globulin alone, since small amounts of antibodies to trace plasma protein components in the γ-globulin fraction used as the antigen were also found. It was the latter "contaminating" antibodies that proved so useful for this study. 3) Horse antiserum prepared by the injection of whole human plasma was kindly supplied by Dr. Robert Berg, of the Massachusetts General Hospital.

Aliquots of 20 ml. of each of the antisera were adsorbed with 3 ml. of a patient's serum by mixing, incubating at 37° C for 1 hour and then at 4° C overnight, and finally centrifuging at 0° C at 3,000 RPM to remove any resulting precipitate. In this manner, aliquots of antisera were adsorbed with sera from 3 children with congenital agammaglobulinemia and additional aliquots adsorbed with sera from 2 normal adults. Adsorption of any given aliquot of antiserum was performed only with the serum from a single individual.

Thus, each of the three antisera was used in 3 forms: 1) unaltered or unadsorbed, 2) adsorbed with agammaglobulinemic serum, and 3) adsorbed with normal serum. There were 3 separate aliquots in the second group corresponding to the three separate agammaglobulinemic sera used for adsorption and similarly there were 2 separate aliquots in the third group. *Each* variation of *each* antiserum was tested against *all* of the normal and agammaglobulinemic sera studied.

Antigen and antibody analysis: A modification of the immunoelectrophoresis method of Grabar and Williams (11) was used for antibody analysis of the antisera and for antigenic analysis of the patients' sera. In this method, the patient's serum was electrophoresed in an agar plate and the plate "developed" by permitting antisera to diffuse horizontally into the agar phase. Interaction of antigen and antibody was indicated by the appearance of a band of specific precipitate in the agar.

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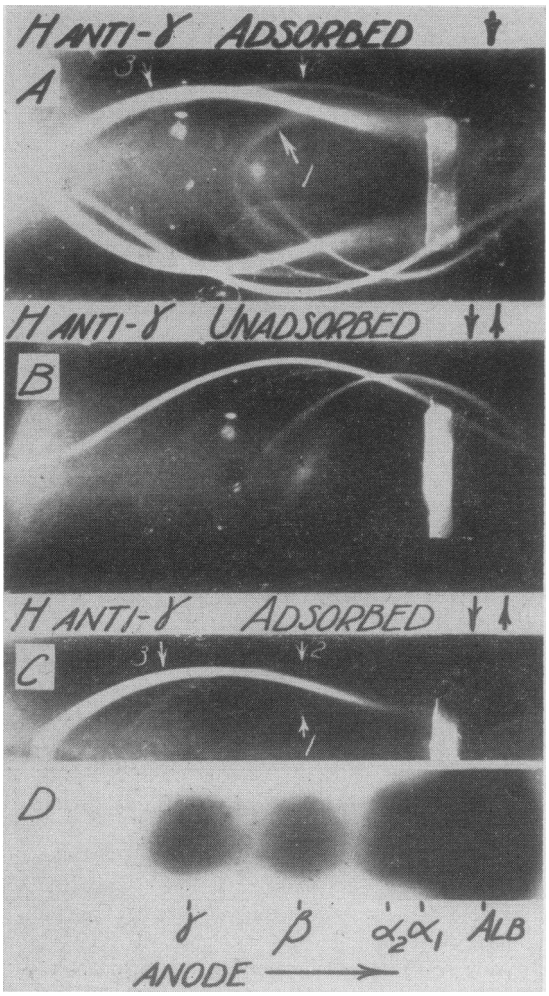

FIG. 1. ELECTROPHORESIS OF NORMAL AND AGAMMAGLOBULINEMIC SERA IN AGAR; DEVELOPED WITH HORSE ANTISERUM AGAINST HUMAN γ-GLOBULIN (H ANTI-γ)

This figure and all subsequent figures represent sections taken from immunoelectrophoresis plates. The horizontal edges indicate the edges of the wells containing antiserum and the vertical white bars on the right half of the figures delineate the transverse wells for the patient's serum-agar solution. The anode was always at the right. Between the agar sections are noted, by arrows, the direction of diffusion of antisera used for development.

A. Electrophoresis of normal serum. Upper portion developed with H anti-γ adsorbed with agammaglobulinemic serum, showing three precipitation bands (white arrows). Lower portion developed with unadsorbed H anti-γ, showing 7 precipitation bands; the eighth band had migrated into the antiserum well by the time this photograph was taken.

B. Electrophoresis of serum from a patient with congenital agammaglobulinemia. Upper portion developed with unadsorbed H anti-γ, showing only 4 of the 7 bands seen in A. Lower part developed with H anti-γ adsorbed with agammaglobulinemic serum; no bands are seen.

C. Electrophoresis of another normal serum developed with the same adsorbed H anti-γ as in 1A and 1B.

D. Electrophoresis in agar of same normal serum as in A but stained with bromphenol blue.

For electrophoresis, pieces of plate glass (¼″ × 8″ × 10″ or ⅛″ × 6″ × 8″) were coated with a thin layer of 1 gm. per cent agar and dried at 100° C. The plates were then edged with plasteline to a height of about one cm.; 3 to 5 bars of lucite, ¼″ × ¾″ × 6″, were arranged in parallel on the plate, dividing its surface into equal longitudinal areas, the bars being placed on the ¼″ edge. Two gm. per cent agar in 0.1 M borate buffer, pH 8.6, was poured over the plate to a depth of 3 to 5 mm. and permitted to gel. The lucite bars and plasteline were removed and narrow linear wells, about 2–3 mm. × 12–20 mm., were cut into the agar transversely to the long dimension of the plate. The patients' sera were dialyzed overnight against 0.2 M pH 8.6 borate buffer and clarified by centrifugation at 24,000 × gravity for 30 minutes; the sera were warmed to 50° C and were then mixed with an equal volume of melted 4 gm. per cent agar, in distilled water, cooled to 50° C. The agar-serum was then placed in the transverse wells and the plate cooled. Electrophoresis was performed at 4° C using 8 to 10 volts per cm. of agar for 16 to 17 hours, using 3 thicknesses of buffer saturated Whatman 3 MM filter paper to make electrical contact between agar plate and buffer reservoirs. After electrophoresis, unadsorbed or adsorbed antisera were placed in the longitudinal wells resulting from removal of the lucite bars and the plates were kept at about 22° C in moist chambers for 1 to 2 weeks; the borate buffer suppressed bacterial and fungal growth during this period of time. Any given antiserum thus diffused laterally into 2 agar-serum sections at once; zones of antigen-antibody interaction were indicated by curvilinear bands of specific precipitate. After the plates were so developed, in some instances, circular wells were cut out of the agar close to a particular band of precipitate and the wells were then filled with 1 gm. per cent γ-globulin or other plasma protein fractions; similarity or identity between the antigen in the patient's serum and that in the circular well was indicated by a shift in the position of the precipitation band. All plasma protein fractions used in this study were isolated by low temperature ethanol-water fractionation (9, 12).

In some instances, the agar plates were stained with bromphenol blue, instead of being developed with antisera, to determine the extent of migration of the plasma proteins under the electrophoretic conditions employed.

It should be noted that separation of the α-globulins in borate buffer is less satisfactory than with barbiturate buffer, but that separation between the γ and β-globulins is relatively greater than that obtained with barbiturate buffer (Figure 1D).

RESULTS

Analysis with horse antiserum vs. human γ-globulin

The unadsorbed horse antiserum against human γ-globulin (H anti-γ) resulted in the appearance of 8 precipitation bands when reacted with normal human sera (Figure 1A). When adsorbed with any of three sera from children with agammaglobulinemia and then again reacted with normal human serum, only 3 bands were obtained (Figures 1A and 1C). Thus, agammaglobulinemic sera failed to remove 3 antibodies against normal serum proteins from H anti-γ. In each instance, one of these 3 remaining precipitation bands (arrow 3) could be demonstrated to be due to the interaction of γ-globulin with its homologous antibody (Figure 4); the other two bands (arrows 1 and 2) were attributable to the interaction of two β-globulins with their respective antibodies. It should be noted that in no instance did the *adsorbed* antisera give any visible reaction against any of the sera from patients with congenital (Figure 1B) or acquired agammaglobulinemia. In view of this evidence of completeness of adsorption of antibodies against *agammaglobulinemic sera,* the 3 antibodies remaining against normal serum proteins indicated the absence of 3 corresponding serum proteins from agammaglobulinemic sera. Adsorption of the antiserum with normal human serum removed all precipitating antibodies for both agammaglobulinemic and normal sera.

When reacted with agammaglobulinemic sera, unadsorbed H anti-γ gave 5 bands in the agar plate (Figure 1B). Thus, there were 3 fewer bands in each instance than when the same antiserum reacted with normal serum. The missing 3 bands corresponded to the 3 bands found when reacting agammaglobulinemia-adsorbed antiserum with normal sera.

Analysis with rabbit antiserum vs. human γ-globulin

The unadsorbed rabbit antiserum against human γ-globulin (R anti-γ) showed 5 bands when reacted with normal human sera (Figure 2A). By far the most dense band was that due to γ-globulin reacting with its homologous antibody. The remaining bands of specific precipitate were exceedingly faint and difficult to detect. When reacted with agammaglobulinemic sera, it was noted that, but for one case, R anti-γ still gave 5 bands. In these instances, however, the γ-globulin band was much fainter and broader (arrows, Figure 2B)

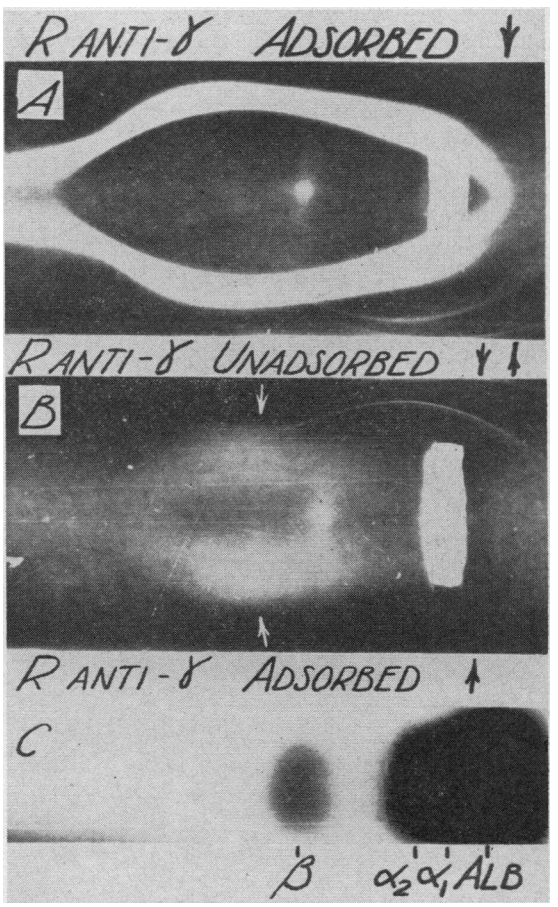

FIG. 2. AGAR PLATES DEVELOPED WITH RABBIT ANTISERUM AGAINST HUMAN γ-GLOBULIN (R ANTI-γ)

A. Electrophoresis of a normal serum. Upper part, developed with R anti-γ adsorbed with the agammaglobulinemic serum used in 2B, shows only the single band due to interaction of human γ-globulin with specific antibodies. Lower part developed with unadsorbed R anti-γ; 4 bands can be discerned in this photograph.

B. Electrophoresis of serum from a patient with congenital agammaglobulinemia. Upper part, developed with unadsorbed R anti-γ and lower part developed with the same adsorbed R anti-γ used in 2A. Diffuse bands (white arrows) were due to presence of small amount of γ-globulin in this patient's serum. Although this serum was used for adsorbing R anti-γ, the amount of γ-globulin present was insufficient to remove all anti-γ globulin antibodies.

C. Electrophoresis of same serum as in 2B, but stained with bromphenol blue; no γ-globulin can be discerned in the serum with this method.

and its position toward the center of the longitudinal axis of the agar plate clearly indicated a markedly reduced concentration of γ-globulin. In the exception noted, no γ-globulin band could be detected with R anti-γ; this serum came from a patient with acquired agammaglobulinemia and contained no γ-globulin by the quantitative precipitin method.

When rabbit antiserum was adsorbed with agammaglobulinemic serum, only the γ-globulin antibody remained in the antiserum (Figure 2A). Although γ-globulin was detected in all but one of the agammaglobulinemic sera tested, the amount was insufficient to remove much of the rabbit antibodies against γ-globulin when these sera were used for adsorption. Adsorption with normal serum removed all detectable antibodies from R anti-γ.

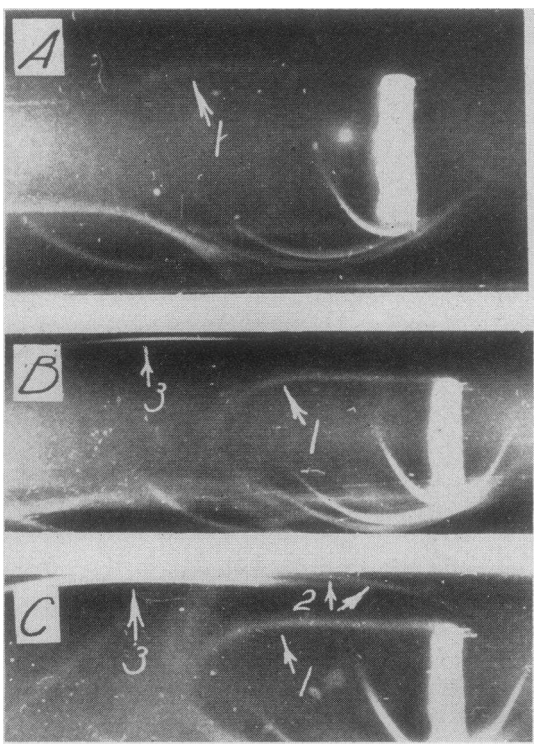

FIG. 3. ELECTROPHORESIS OF A NORMAL SERUM IN AGAR

Only the upper portions of each section will be referred to.

A. Developed with horse antiserum against whole human plasma adsorbed with serum from a patient with congenital agammaglobulinemia. After 5 days only the band indicated by arrow 1 could be seen.

B. The antiserum was then replaced with similarly adsorbed H anti-γ. A day later, band 3 was noted.

C. Three days later band 2 was apparent.

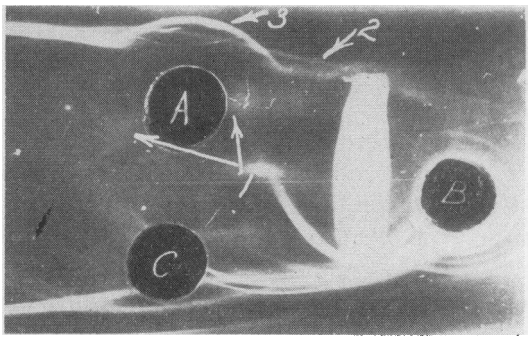

FIG. 4. ELECTROPHORESIS OF A NORMAL SERUM IN AGAR, DEVELOPED WITH UNADSORBED H ANTI-γ (LOWER PORTION) AND AGAMMAGLOBULINEMIA-ADSORBED H ANTI-γ (UPPER PORTION)

The circular wells A, B and C were then cut into the agar and the wells filled with various plasma protein fractions:

A. β-isohemagglutinin
B. Impure α-lipoprotein
C. Iron-binding globulin

The resolution and reprecipitation of the γ-globulin band to form a semicircle at arrow 3 indicated the presence of γ-globulin in the β-isohemagglutinin fraction used. The 2 β-globulin precipitation bands remained unaffected by the isohemagglutinin.

Analysis with horse antiserum vs. whole human plasma

With horse anti-whole human plasma, 8 bands were observed when reacted with normal sera and 6 bands with agammaglobulinemic sera. The human γ-globulin precipitation band, when present, was exceedingly faint in this instance. Adsorption with agammaglobulinemic sera resulted in the elimination of all but one band; this proved to be the same as one of the β-globulin bands observed with H anti-γ (Figure 3, arrow 1). Since the agammaglobulinemic sera used for adsorption contained some γ-globulin and since the concentration of antibodies against γ-globulin in this antiserum was very low, adsorption completely removed these antibodies. The β-globulin band, however, could be better visualized with this antiserum than with H anti-γ. In one instance, there was a question as to whether some of the antigen causing this band was also present in diminished amount in a single patient with congenital agam-

maglobulinemia. By developing a plate first with agammaglobulinemia-adsorbed horse anti-whole plasma for about a week and following this with agammaglobulinemia-adsorbed H anti-γ, all three of the bands due to the antigens missing or diminished in agammaglobulinemic sera could be visualized (Figures 3B and 3C, arrows). Adsorption with normal sera eliminated all precipitation bands.

Attempts at identification of the β-globulins

It is known that isohemagglutinins are absent or deficient in the two forms of agammaglobulinemia studied here (2, 3). Neither of the two β-globulins in question, however, appeared to be isohemagglutinins. Adsorption of the horse antisera with isohemagglutinins isolated from normal plasma did not remove the antibodies for these β-globulins. In addition, diffusion of isohemagglutinins into the agar plates already developed with antisera failed to shift the precipitation bands associated with the unknown β-globulins (Figure 4). Although other plasma protein fractions, such as α_1- and α_2-glycoproteins, α- and β-lipoproteins, and iron-binding globulin, were similarly studied, the β-globulins could not be identified. From the results, it is clear, however, that they have no antigenic relationship with γ-globulin.

DISCUSSION

It would appear from the results presented that at least two plasma proteins besides γ-globulin are absent or markedly diminished in congenital and acquired agammaglobulinemia. These proteins migrate as β-globulins and have no apparent antigenic relationship to γ-globulin. Since the individual components of γ-globulin are immunochemically related and cross-react with antisera against γ-globulin, the data clearly indicate that these two β-globulins cannot be considered as fast-moving components of γ-globulin. The failure of synthesis of γ-globulin in these patients is presumably due to the absence of plasma cells; it is interesting to speculate whether these β-globulins are also products of the plasma cell. However, further study is necessary to establish the identity and function of these proteins. It would appear, from the intensity of the reaction and the position of the precipitation bands in the electrophoresis plates, that the concentration of at least one of these proteins, indicated by arrow 1 in Figures 1 and 3, even in normal plasma is not very great. This does *not,* of course, preclude its possible physiological importance. It must be emphasized that there may be *additional* plasma proteins absent or deficient in this syndrome; the number of proteins which could be studied in this investigation depended entirely upon the number of antibodies present in the antisera used.

It has been shown that the deficiency of γ-globulin in 10 of the sera of the 11 patients with agammaglobulinemia studied was not complete, as has already been pointed out (2, 3); in one instance, no precipitation band for γ-globulin could be obtained even with the rabbit antiserum. The reasons for the apparently greater sensitivity of rabbit antiserum than horse antiserum in the detection of human γ-globulin lies in the differences in the precipitation characteristics of the two types of sera, the horse antiserum requiring a greater amount of γ-globulin for visible precipitation than does the rabbit antiserum under similar conditions (10).

SUMMARY

1. Antigenic analysis of sera from 8 patients with congenital agammaglobulinemia, 3 patients with the acquired form of the disease and 6 normal adults was performed, using rabbit and horse antisera. The rabbit and horse antisera against human γ-globulin contained antibodies against other plasma antigens besides γ-globulin.

2. Through use of agar diffusion and electrophoretic methods, it has been demonstrated that at least 2 plasma proteins besides γ-globulin are absent or deficient in agammaglobulinemia; these proteins migrate as β-globulins in agar.

3. It is confirmed that the deficiency of γ-globulins in agammaglobulinemia is often *not* complete.

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