THE ANALYSIS OF STREPTOCOCCAL INFECTIONS

VI. Immunoelectrophoretic Observations on Extracellular Antigens Detectable with Human Antibodies*

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A complete understanding of any human infection requires knowledge of the toxins or antigens that the organisms release *in vivo* during the disease process. Until recently, discovery of these substances has tended to be on a chance basis. Demonstration of the resolution and sensitivity of antigen-antibody reactions in agar gels (*e.g.* 1, 2) has opened the way to a rational analysis of the total number of antibody responses occurring after a given infection, and thereby the total number of antigens or toxins released by the organisms during its residence in the tissues. These immunological tools also furnish the means for following the purification of each of the antigens thus shown to be released *in vivo* when human convalescent serum, or its equivalent, is used as the source of detecting antibodies.

Studies of this sort with human streptococcal infections have been carried out during recent years in this laboratory (3–8). It has been found that sera from patients with rheumatic fever are very rich in precipitating antibodies to *extracellular* streptococcal products, but contain few if any to *cellular* extracts prepared from the same strain. These observations have since been confirmed (9–11, 27, 28). It appears from these data that non–type-specific cellular antigens often do not reach antibody forming sites in significant amounts during human disease, while extracellular ones do so in great numbers. It was found that a high proportion of sera from non-rheumatic patients without recent histories of upper respiratory infections also contained antibodies to some of the streptococcal extracellular products, although many fewer than were seen with sera from rheumatic fever subjects. For this reason, normal pooled human gamma globulin, such as is used for the prophylaxis of measles or poliomyelitis, was also tested against streptococcal products. A number of different globulin samples were shown to be extraordinarily rich in antibodies against the extracellular secretions and to be poor in antibodies against cellular components.

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On Ouchterlony plates, several normal human gamma globulin specimens revealed at least 8 or 9 distinct antistreptococcal antibodies. When large scale purifications of crude culture supernates containing these antigens were carried out by means of continuous flow electrophoresis combined with column chromatography on calcium phosphate gels, it was then found that the systems were even more complex (5, 7). At least sixteen extracellular antigens were detected for which humans had spontaneously developed antibodies.

Because of the possibility that this number still failed to account for all of the extracellular antigens which the streptococcus might secrete *in vivo* during human infections, immunoelectrophoretic observations have now been made. It was also the aim of this study to explore the potential usefulness of this technique for the analysis of individual sera from rheumatic fever patients during the course of infection.

Materials and Methods

Most of these present observations have been made with culture supernate concentrates or fractions derived from the C203S strain of Group A hemolytic streptococci. The preparation of the crude culture concentrates and the various assay procedures have been described previously (7). The concentrate of the Group C streptococcal filtrate is similar to that used in reference 5. The immunoelectrophoretic technique of Grabar and Williams (12) as modified by Scheidegger (13) was used principally. Early difficulties had been encountered in these studies because of the need to use highly concentrated gamma globulin (16 per cent) solutions which were precipitated by the low ionic strength buffer used in the agar. These difficulties have been adequately overcome by the following procedure. Bacto-agar (Difco) in 0.8 per cent solution was dissolved in veronal-acetate buffer pH 8.2 μ0.05 (14) containing 1:10,000 merthiolate, 4.0 ml. of agar being poured per slide (1 × 4 inches). The antigen wells were usually 2.0 mm. in diameter, and the serum trench was 4.0 mm. wide. The distance between the edges of the two was 6.4 mm. in most instances. A potential difference of 6 to 7 volts/cm. was employed, usually for 2½ hours when the small slides were used. When longer migration paths were required, 1 × 6, or 2 × 10 inch slides were prepared with a proportionate volume of agar and increase in the well dimensions, as well as longer exposure to the electrical field. The electrophoreses were carried out at 4°C. without a cover, but precipitin development was allowed to take place at room temperature. This helped alleviate nonspecific precipitation of the gamma globulin due to the low ionic strength of the system.

Comparative tests were carried out with four horse antisera. These had been prepared by hyperimmunization with different Group A streptococcal filtrates. The Richards strain of streptococcus was the source of the antigens for the lyophilized serum from the Wellcome Laboratories, Beckenham, England. It was reconstituted to contain 2,000 Todd antistreptolysin units/ml. The NY 5 strain of streptococcus, and a mixture of filtrates from 8 different streptococcal types respectively, furnished the antigens for two other horse sera kindly supplied by Dr. L. Hanson[1] of Göteborg, Sweden. The gamma globulin fractions of the latter two had been separated and concentrated roughly tenfold. The fourth horse antiserum was obtained from Lederle Laboratories, New York, as therapeutic scarlet fever antitoxin, lot 1570. It was prepared by injection of filtrates of the NY 5 strain plus filtrates of two other strains. The globulin fraction was concentrated about sixfold.

[1] The authors are very grateful to Dr. Proom and Dr. Hanson for these horse serum specimens, and to Dr. H. Wood for the sera from rheumatic patients.

In addition, samples of sera from rheumatic fever patients were obtained from the Irvington House Hospital, Irvington, New York, and similarly tested. These represented samples obtained at varying times during an acute episode of the illness. Control sera from 22 nonrheumatic patients were obtained from the diagnostic laboratory of the Presbyterian Hospital, New York. The *Haemophilus influenzae* cell extract was obtained by sodium desoxycholate lysis of type b cells as described elsewhere (8).

RESULTS

When tested at a concentration of 47 or 74 mg. protein/ml. against pooled normal human gamma globulin, the crude concentrate used as a standard reference system (H461-A3) revealed a total of at least twenty distinct antigen-antibody systems. These were often best visualized when longer migration paths were used, but the reproducibility of the patterns was excellent. Composite results of numerous tests are diagrammatically represented in Fig. 1,

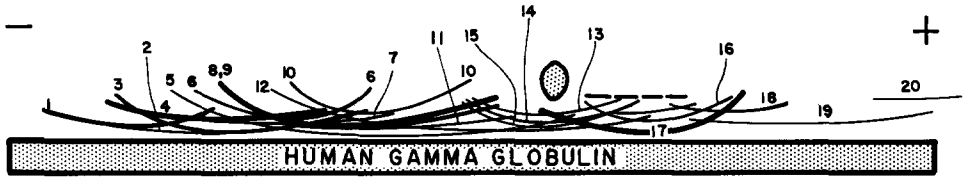

FIG. 1. Diagrammatic representation of Group A streptococcal extracellular antigens detectable with naturally occurring human antibodies in normal pooled gamma globulin, as seen by immunoelectrophoresis. The crude streptococcal concentrate (H461A3) was prepared from the C203S strain of streptococcus, and showed these results at 47 or 74 mg. protein/ml. The human gamma globulin was a 16 per cent solution, as used for the prophylaxis of measles or poliomyelitis.

in which the electrophoretically separated streptococcal antigens are revealed with human antibodies. The complexity of the systems, and the possibility that even larger numbers exist was suggested by the prolonged development of some of the slides for 4 to 7 days. Under these circumstances, either several new bands appeared, *or* artifactual splitting occurred of some of the arcs. These possible new systems usually developed in the region of arcs 4, 6, and 10. In addition, fuzzy precipitates at the level of the antigen well were frequently seen on either side of it. The latter may represent still other systems in which the relative concentrations of the antigens were too low to produce clear precipitin lines.

Other crude concentrates prepared from the same strain of organisms showed surprising variability in relation to the reference system. Several batches revealed significantly fewer precipitin arcs at equivalent protein concentrations, while one showed two heavy arcs where only one was present in the reference system. The differences found between the reference system and this latter crude concentrate is shown in Fig. 2 *a*. It may be seen that H414-6 showed two

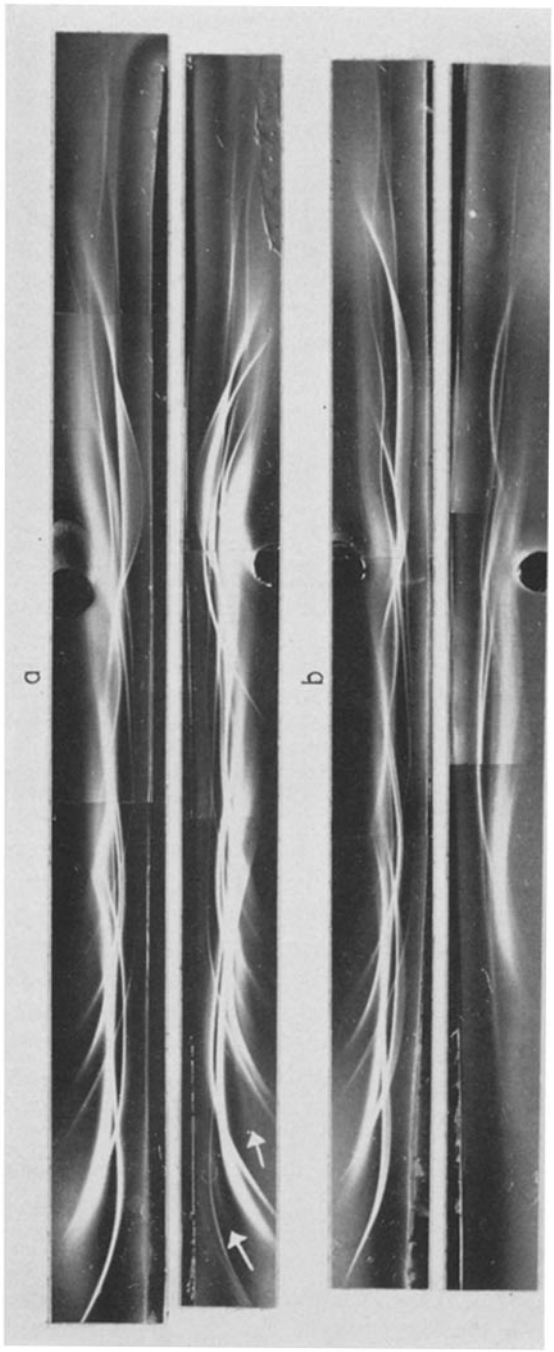

FIG. 2. Streptococcal extracellular antigens detectable with naturally occurring human antibodies in normal pooled human gamma globulin. Slides 2 × 10 inches were used, and 6 volts/cm. were applied for 4½ hours. Photographed after 3 days of development at room temperature. Gamma globulin, Squibb, lot 321-1, was placed in the trenches. Anode on the right.

(*a*) Upper antigen well: standard reference crude concentrate (H461A3) of group A strain C203S, 74 mg./ml.
Lower antigen well: crude concentrate H414-6 of the same strain, 56 mg./ml.
(*b*) Upper antigen well: standard reference crude concentrate H461A3, 74 mg./ml.
Lower antigen well: crude concentrate of a group C streptococcal strain, 40 mg./ml.

large heavy arcs in the region of No. 17 of the standard system, in contrast to the latter. It is possible that this represents an increased concentration of the components forming arcs 13 or 15. In addition, the crude H414-6 reveals a suggestion of at least one other component not seen in the standard. This is indicated by the arrow below arc 4. The No. 2 arc is only occasionally seen in the reference system, but may be barely detected in the photograph with crude H414-6, indicated by the arrow below arc 1. Although other systems may be present, these were not visualized often enough for certainty. Those which are noted in Fig. 1 possessed sufficiently different mobilities from each other to almost certainly represent distinct components. It may be pointed out that the large scale cultures (25 liters) yielding these supernate concentrates were all grown in the same dialyzable medium under presumably identical conditions. The inoculum for each batch was taken from a fresh vial of lyophilyzed organisms drawn from a large supply prepared at one time and stored *in vacuo* at 4°C. It therefore seems that intensity of production of some of these antigens depends on presently intangible factors. In Fig. 2 *b* the reproducibility of the reference pattern may be noted, as well as the occurrence of 8 or 9 systems with a Group C streptococcal culture filtrate concentrate.

When crude concentrates differing in their apparent immunoelectrophoretic composition were placed in adjacent wells on Ouchterlony plates, no instances were observed of "non-identity" reactions. This at first suggested that the differences between the C203S crude concentrates were quantitative, not qualitative. However, recent absorption studies with the standard reference system (H461A3) and other crude preparations indicate that the crude H414-6 may actually contain a component not present in the other concentrates (see inner arrow, Fig. 2 *a*).

In previous studies (5, 7), a number of components had been separated in varying states of immunological purity. Attempts have now been made to identify the arcs seen with the reference crude concentrate and gamma globulin. Examples of these tests are shown in Fig. 3, with proteinase precursor, desoxyribonuclease B (DNase B), streptolysin O, the "far left" antigen, and the "antigen excess" components. The arcs were tentatively identified on the basis of the correlation of the electrophoretic mobilities in the crude concentrates and the separated fractions, as well as by the appearance of the precipitin lines. The data thus far obtained are summarized in Table I. In all of these instances electrophoretic mobility coincided quite well with arcs seen in the crude concentrate, except in the case of DNase B. The arc of this purified fraction was often displaced toward the anode (see Fig. 3 *f*). Identification of these arcs has also been made by a method recently described, using a second trench as antigen source (15–17), and the results confirm the above data. Examples of the latter tests with proteinase precursor and streptolysin O are shown in Figs. 3 *g* and 3 *h*.

Four antistreptococcal horse sera were similarly examined using the refer-

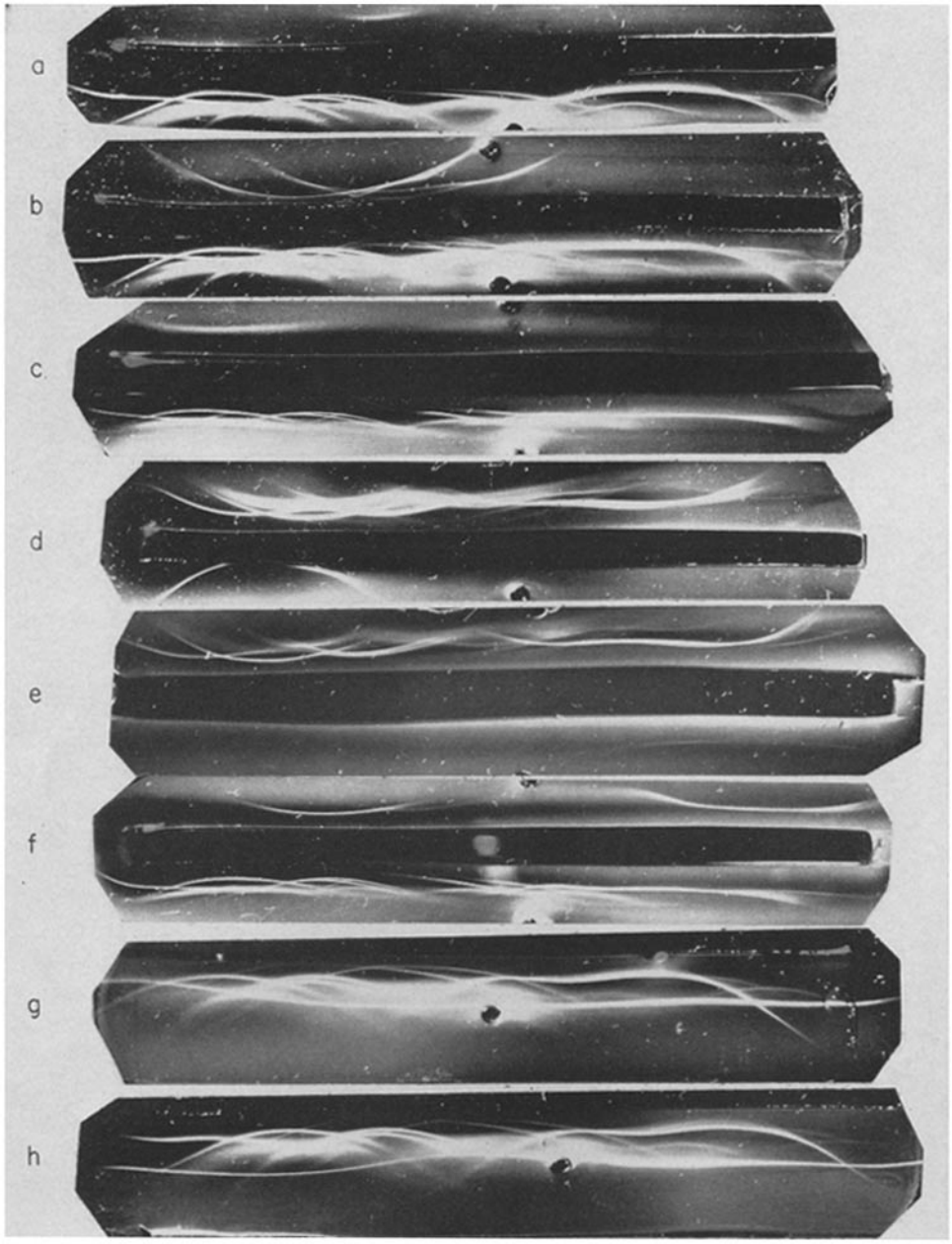

FIG. 3

ence crude concentrate H461A3 and concentrate H414-6. The type of results obtained are shown in Fig. 4. It is worth noting that the serum from England (Fig. 4 *c*) tended to be richer in antibodies to the antigens migrating to the anode than were the other horse sera or gamma globulin.

The horse sera from Sweden were also compared to human gamma globulin for their antibody content against crude concentrate in adjacent wells by the Ouchterlony technique. In spite of the complexity of the precipitin bands which merged for the most part, at least one system was found with human gamma globulin which was not represented with the horse antibodies, and *vice versa*. Previous similar comparative tests with the Wellcome Laboratories horse

TABLE I

Tentative Identification of Group A Streptococcal Extracellular Antigens Involved in Precipitin Arcs Seen with the Reference Crude Concentrate H461A3 and Normal Pooled Gamma Globulin

Arc	Tentative identification
1	Proteinase precursor
4	C carbohydrate-protein (complex ?)
5, 12	"Antigen excess" components, No. 5 possibly related to erythrogenic toxin (see reference 7)
10	Streptolysin O
17	Desoxyribonuclease B
19	Far left component (see reference 7)

antiserum (5) had also shown merging of most of the bands. However, it had been shown that this latter horse antiserum failed to reveal detectable antibodies against proteinase precursor, or C carbohydrate, in contrast to the

FIG. 3. Tests of previously isolated streptococcal extracellular fractions by immunoelectrophoresis with normal pooled human gamma globulin as antibody source, on 1- × 4-inch slides. In all instances, the anode was at the right. In *a*, *b*, *c*, and *f*, the reference crude concentrate (H461A3) was in the lower well, while in *d* and *e* it was in the upper. All of the fractions were dissolved from the lyophilized state at 1 mg./ml. in veronal acetate buffer. In *c* and *f*, the development period was short, somewhat less than 1 day, while the others were developed for 2 to 3 days. In *g* and *h*, the reference crude (H461A3) was separated by electrophoresis, and gamma globulin was placed in the upper trench. The isolated antigen was placed in the lower thin trench. The separated components tested were:—

(*a*) Streptolysin O (peak VII, Text-fig. 5 of reference 7).
(*b*) "Antigen excess" components (peak II, Text-fig. 5 of reference 7).
(*c*) C-carbohydrate-protein (complex?) (peak I, Text-fig. 4 of reference 7).
(*d*) Crystalline proteinase precursor, rechromatographed and crystallized 3 times (peak I, Text-fig. 6, reference 7).
(*e*) "Far left" antigen (peak V, Text-fig. 2, reference 7).
(*f*) Desoxyribonuclease B, contaminated with "antigen excess" components. (similar to peak III, Text-fig. 5, reference 7).
(*g*) Proteinase precursor, as in *d*.
(*h*) Streptolysin O, as in *a*.

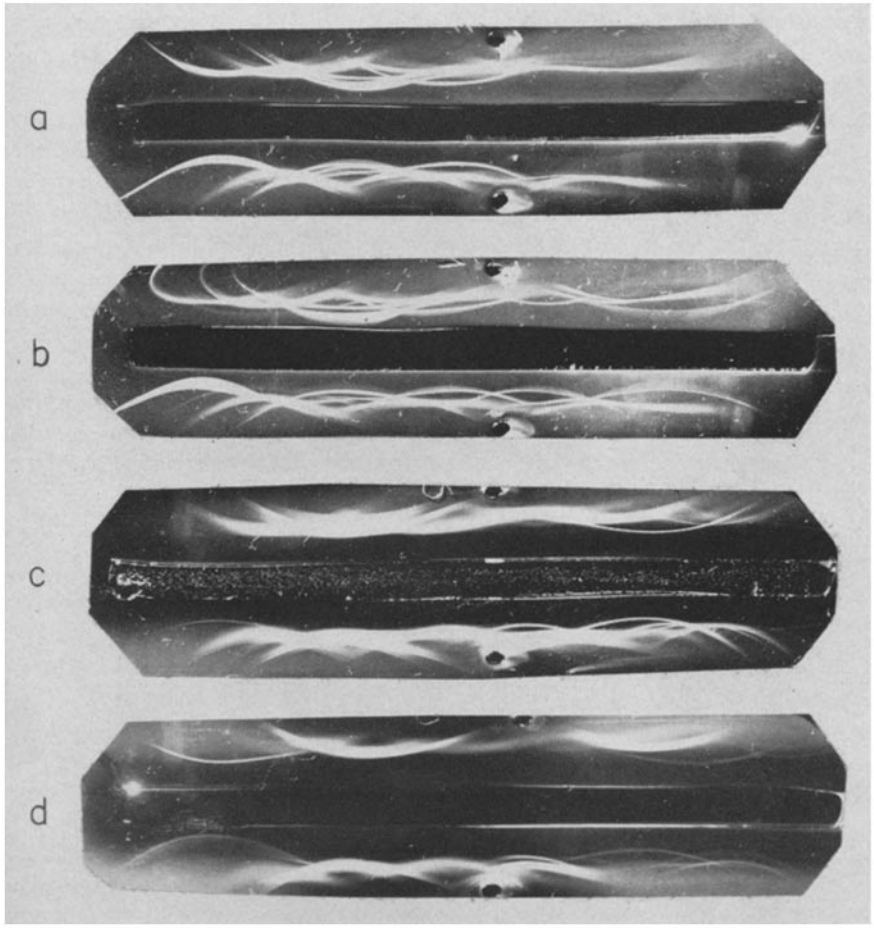

FIG. 4. Immunoelectrophoretic analysis on 1- × 4-inch slides of Group A extracellular antigens as revealed by antibodies from 4 different hyperimmunized horses. In each instance, the upper well contained the standard crude concentrate H461A3 at 74 mg. protein/ml., while the lower well contained crude concentrate H414-6 at 57 mg. protein/ml. The anode was placed on the right.

(*a*) Horse antibody concentrate HO, Sweden.
(*b*) Horse antibody concentrate HO-S, Sweden.
(*c*) Horse antibody concentrate, Wellcome, England.
(*d*) Horse antibody concentrate, Lederle, United States.

human gamma globulin. The data suggest that normal pooled human gamma globulin is at least as rich in anti-streptococcal antibodies as are these several hyperimmunized horse antibody concentrates.

In addition, six individual *whole* sera from rheumatic fever patients were

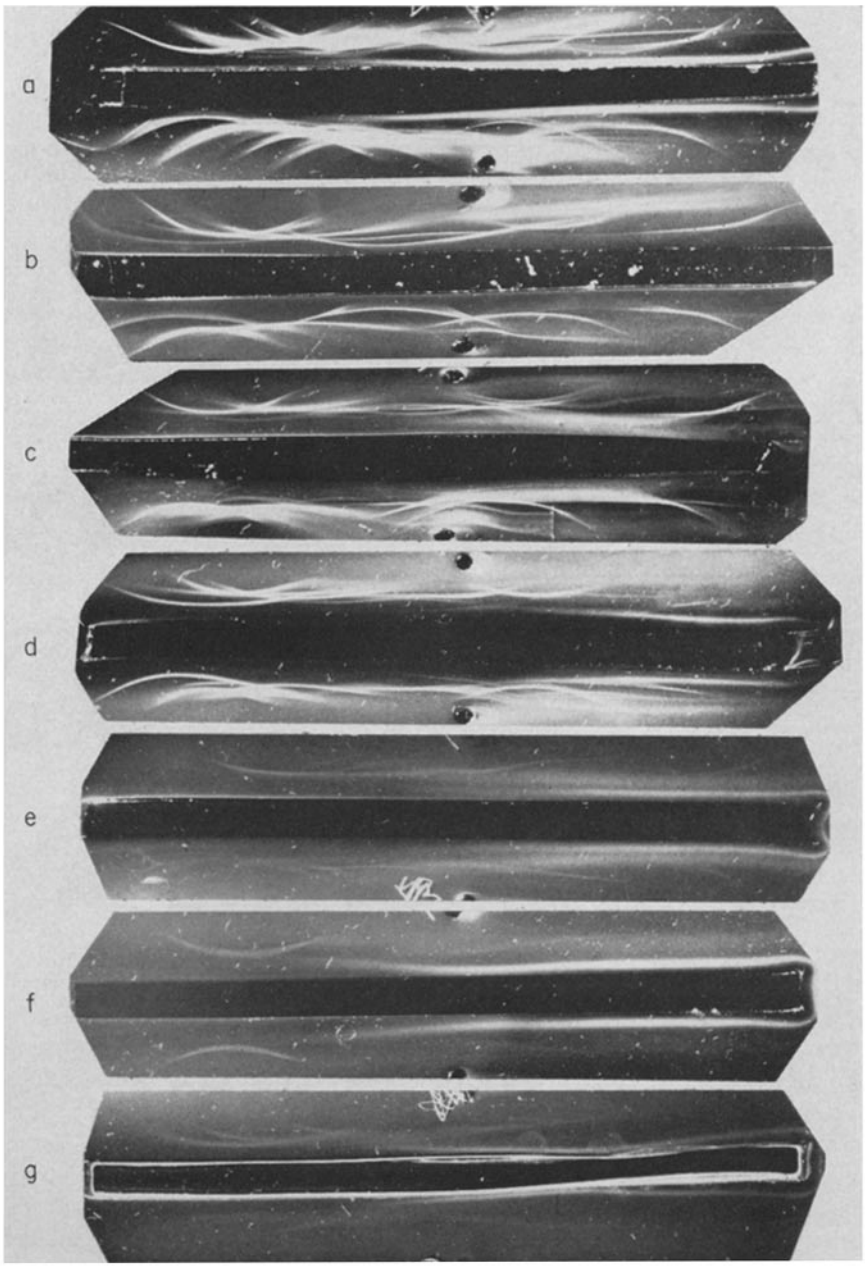

FIG. 5. Immunoelectrophoretic analysis of streptococcal extracellular antigens as revealed by antibodies in *whole* sera from four rheumatic (a, b, c, d) and three control patients, (e, f, g) on 1- × 4-inch slides. In all instances, the upper wells contained the reference crude concentrate (H461A3) at 74 mg./ml., while the lower contained crude concentrate (H414-6) at 57 mg./ml. The anode was placed on the right. Development time 2 days, except *d* which was 24 hours.

tested as antibody source, using the reference crude streptococcal concentrate as antigen. Examples of the types of results with the four strongest sera are shown in Fig. 5. It may be seen that they were remarkably potent, and the one tested in Fig. 5 *a* revealed at least 14 distinct precipitin systems. The results in Fig. 5 *d* were recorded at 24 hours, a development time which experience had shown to be inadequate for detecting faint systems. In spite of this, *at least* 12 arcs could be readily seen on the original photograph. The two other sera tested, but not shown in Fig. 5, showed fewer and less intense streptococcal precipitin systems, 9–10 in one case, and 6–7 in the other. In contrast to these findings, *whole* sera from 22 control non-rheumatic patients

TABLE II

No. of Precipitin Arcs Seen with Sera from Non-Rheumatic Patients with a Variety of Illnesses

The single patient showing 8 systems was suffering from an acute attack of streptococcal pharyngitis and pneumonia, and had an antistreptolysin titer of 1250 units/ml.

No. of arcs	No. of sera
0	1
1	1
2	7
3	3
4	4
5	2
6	1
7	2
8	1

were found to contain considerably fewer and fainter precipitin arcs. Examples of these results are seen in Fig. 4 *e*, *f*, and *g*. On the original slides, these showed 7, 2, and 4 faint arcs respectively. The number of systems found with the control sera varied from 0 to 8, as can be seen in Table II. The patients from whom the control sera were obtained suffered from a variety of disturbances such as ulcerative colitis, goiter, hypertensive heart disease, malignancy, etc., and were mostly in the age group from 30 to 60. The only patient whose serum revealed 8 precipitin arcs was an 18 year old boy suffering from an acute streptococcal pharyngitis and bronchopneumonia. His antistreptolysin titer was 1250 units/ml., but no history or evidence of rheumatic fever was found. Three other patients of this control group were found to have had a mild upper respiratory infection just prior to the blood sampling. The differences between the observed precipitins in the control and rheumatic group is similar in general to that found in previous studies using the more poorly resolving Ouchterlony technic (4).

Because of the possibility that the rheumatic patients possessed antibodies against streptococcal products that were not present in the normal gamma globulin, a few tests were carried out with such sera and gamma globulin being placed in adjacent wells in Ouchterlony plates, and tested against a crude streptococcal concentrate. In no instances were any antigen-antibody systems found with the sera from rheumatic patients, which were not represented

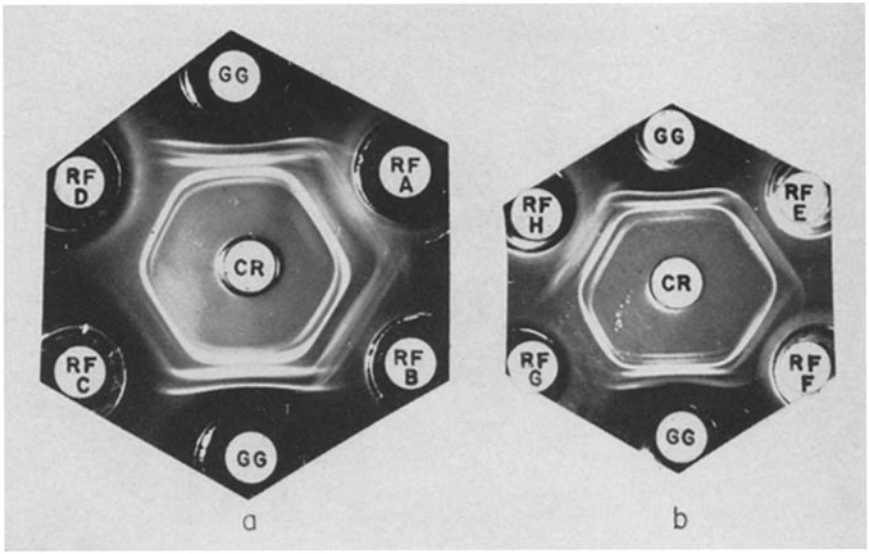

FIG. 6. Comparative tests of human normal pooled gamma globulin and whole sera from rheumatic fever patients. The sera in *a* were also examined by immunoelectrophoresis, but those in *b* were not.

CR —crude concentrate H461A3 at 10 mg./ml.
GG —gamma globulin
RF-A, RF-B, etc.—different rheumatic fever sera

with normal gamma globulin. The relative concentrations of these antibodies might be somewhat different in the individual sera, however, as evidenced by the position and intensity of several of the bands. Examples of these data are shown in Fig. 6.

As a check on the validity of the observations made with pooled human gamma globulin, a few sera from rheumatic patients were tested against several of the isolated streptococcal components described earlier (5, 7). The purpose of these tests was to see if individual patients' whole sera contained antibodies not present in the normal gamma globulin. Such was not the case, and when a single band was found between a given fraction and the gamma globulin, not

more than one band was seen with an individual rheumatic serum. Typical results are shown in Fig. 7 with one serum sample. In this case, desoxyribonuclease B, diphosphopyridinenucleotidase, and streptolysin O showed single bands such as were found earlier with gamma globulin. The "antigen excess" fraction showed two components also similar to the reactions seen with gamma globulin, as did the proteinase precursor containing 105II. The *Haemophilus influenzae* extract failed to show any precipitin bands with this serum while gamma globulin revealed 2 to 3 systems.

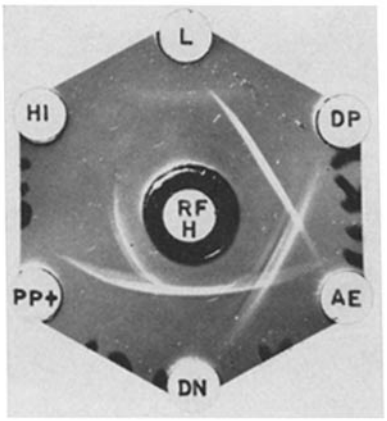

FIG. 7. Reactions of whole serum from a rheumatic fever patient with several isolated streptococcal antigens. The serum is in the central well.

L —streptolysin O, Group C, 0.4 mg./ml.
DP —diphosphopyridine nucleotidase, 0.1 mg./ml.
AE —"antigen excess" components, 0.1 mg./ml.
DN —desoxyribonuclease B, 0.1 mg./ml.
PP+—proteinase precursor containing fraction 105 II
HI —*Haemophilus influenzae* bacterial cell extract

DISCUSSION

The data presented amply confirm the complexity of the immune response to streptococcal extracellular products in humans. It is clear that at least 20 such substances are secreted or released by the organisms in the tissues during the course of human infections. Suggestions were obtained that perhaps even more are involved. It may be stressed that almost all of the observations made here were with materials derived from a single Group A hemolytic streptococcal strain (C203S) isolated from a patient with scarlet fever about 25 years ago (18). It is possible that a detailed examination of different streptococcal strains may reveal still other substances not secreted by the C203S strain. However, it may be advisable to grow the organisms in a completely defined medium

(*e.g.* reference 19) when comparing different strains, in view of the variability shown above with a number of crude concentrates produced by the C203S organisms under presumably identical conditions in a dialysate medium.

Should this variability of antigen production be resolved, it would be of importance to examine the potential capacity of streptococcal strains isolated from patients with distinct clinical syndromes, with respect to the synthesis of these extracellular antigens. Different streptococcal types and groups could also be examined in this way. The clarity and complexity of the reactions found above with individual rheumatic fever serums, indicate that a re-investigation of the "total" antibody responses in patients with various post-streptococcal states might also be most informative. Such a study is planned.

Although emphasis has been placed on *non-type-specific* components in this series of studies, the possibility that *type-specific* antigens may also be detected and explored with such methods is worthy of consideration. It would only be necessary to employ serum from individual convalescent patients and to use the streptococcal strain causing their illness as antigen source. In searching for *cellular type-specific* antigens, sonic-vibrated cell extracts could be used as antigen, and absorption of the sera could be carried out with other streptococcal strains in order to remove *non-type-specific cellular* antibodies. In view of the relative paucity of the latter seen in our laboratory, it seems likely that this problem would be considerably less difficult than in the case of the extracellular antigens.

Although substantial progress has already been made in identifying the arcs of precipitate in the reference system, it is clear that much more effort will be required to complete the identification. The method of using a second trench as antigen source (15, 17) as shown in Figs. 3 *g* and 3 *h* will be of much help in this respect. It should be possible to sort out the components in the numerous and often rather complex chromatographic fractions isolated previously (5, 7).

It has long been known that patients with rheumatic fever tend to develop significantly higher average antibody responses to a number of streptococcal extracellular antigens, than do patients with uncomplicated streptococcal infections. For example, this has been shown to be true for antistreptolysin "O" (*e.g.* reference 21), anti-streptokinase (22), anti-hyaluronidase (23), anti-streptococcal proteinase (24) and anti-desoxyribonuclease (25). Total gamma globulin levels in the sera of rheumatic patients also tend to be higher than those found in patients with uncomplicated streptococcal infections (26). It is therefore worth stressing the anti-streptococcal potency of human gamma globulin, and individual rheumatic sera found here, in comparison to antibody concentrates prepared from hyperimmunized horses. The latter had received numerous injections of other streptococcal filtrates over long periods of time. The abundance of the human antibodies suggests that the antigenic stimuli

with these substances in man must be rather intense, and probably frequently repetitive. This finding tends to support the working hypothesis developed earlier implicating antigen-antibody complexes of streptolysin "O" in the pathogenesis of rheumatic fever (20).

It is also important to point out that the horses were immunized with filtrates of a number of streptococcal strains other than the C203S strain. In spite of this, they responded with antibodies of apparently the same specificities as those seen in human gamma globulin which were reactive with C203S products. These findings imply that the other streptococcal strains secrete many similar or identical extracellular substances.

In some instances, prolonged development of the slides resulted in possible splitting of a few of the arcs of precipitate. Some of these cases might have been artifact, since the site and appearance of the extra arcs seemed to coincide. In a few instances, however, the extra arcs were somewhat different in appearance from the arc which appeared first. It is possible that the arrangement used here produces a relative excess of some antibody concentrations, unlike the numerous tests carried out with these systems in Ouchterlony plates. In the latter instances, no clear cut suggestion of antibody excess artifacts were encountered, although antigen excess effects, such as precipitin band abolition, or splitting, were seen. However, the large amount of accumulated data indicate that the *early* developing precipitin bands or arcs undoubtedly represent distinct streptococcal antigen-antibody systems. In addition, the twenty precipitin arcs seen in immunoelectrophoresis almost certainly are valid because of the different electrophoretic mobilities represented by the arcs.

Finally, it may be pointed out that the distribution of antigens seen here by immunoelectrophoresis appeared to be slightly different from that found by paper-supported continuous flow electrophoresis of crude concentrates (see reference 5). In those separations, however, acetate buffer at pH 6.0 was used as compared to veronal acetate at pH 8.2 in the present experiments. In addition, differences in adsorption properties of the two supporting media could contribute to the observed differences in the patterns.

SUMMARY

It has been found by immunoelectrophoresis, that Group A streptococci release at least 20 distinct extracellular antigens in human tissues, as judged by naturally occurring antibodies present in normal pooled human gamma globulin.

Several of these precipitin arcs have been identified with streptococcal antigens previously purified by electrophoresis and chromatography.

Human gamma globulin, as well as several rheumatic fever sera, were shown to be remarkably potent in antistreptococcal antibodies, when compared to four horse antibody concentrates obtained by hyperimmunization with several streptococcal filtrates.

A Group C streptococcal culture concentrate revealed 8 or 9 antigens for which corresponding antibodies were present in human gamma globulin.

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