STUDIES IN THE RELATION OF THE HEMOLYTIC STREPTOCOCCUS TO RHEUMATIC FEVER

III. Complement Fixation versus Streptococcal Nucleoproteins with the Sera of Patients with Rheumatic Fever and Others*

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In the previous paper of this series (1) the isolation of two fractions of streptococcal nucleoproteins was described: the cytoplasmic particles (CP) and the S fraction, of smaller molecular size. It was pointed out that so far neither fraction had been prepared chemically free of the other. However, the immunologic reactions of each were so distinct and characteristic that the two fractions could be employed in serologic tests in their present state of purity. Although the S fraction was shown by electrophoresis and ultracentrifugation to contain two molecular species, presumably protein, the use of this preparation in exploratory serologic work is indicated by the facts that this fraction has not been described before, and that the relative concentrations of the two components of S are of the same order (33 and 67 per cent of their total, respectively).

Since antibodies to these fractions had never been measured, it was necessary to determine their titer in the normal population. Such tests were performed on the sera of newborn infants, older infants, children, and young adults. For evaluation in streptococcal infections tests were done on a group of patients with scarlet fever, serial specimens being taken at the acute and convalescent stages and several months thereafter. Finally, titrations were done on the sera of patients with active and quiescent rheumatic fever, and longitudinal studies were done during single or repeated rheumatic episodes. For general correlation with the earlier work on measurement of streptococcal antibodies, antistreptolysin tests were done on each serum tested.

Methods and Materials

The Preparation of Sera and Antigens.—The sera of newborn infants were prepared from blood collected from the umbilical cords in the Delivery Rooms of the Philadelphia General

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Hospital. The sera of older infants were collected at the Well-Baby Clinic of The Children's Hospital of Philadelphia. These infants ranged in age from 6 months to 2 years. The sera representing the antibody levels in normal children were those collected in the group of patients with scarlet fever mentioned below for the study of the serologic response in acute streptococcal infection. Only sera collected within the first 3 days of the disease were taken to represent normal antibody levels. The sera of young adults were collected at the Student Health Service of the University of Pennsylvania.

For the study of antibody response to these antigens in acute streptococcal disease, blood specimens were collected from children with scarlet fever at the Philadelphia Hospital for Contagious Diseases during the first half-week and during the 3rd week of the disease. In a number of these cases, an additional specimen was drawn 4 months later.

In the case of rheumatic patients the blood specimens were drawn at weekly, biweekly, or monthly intervals, depending on the severity of the rheumatic process, in correlation with the clinical studies to be described below.

Blood specimens were collected with a minimum of sodium citrate as anticoagulant, in order to allow for the determination of the erythrocyte sedimentation rate, white blood count, and concentration of hemoglobin. The plasma was drawn off each blood specimen and frozen as soon as the tests above had been completed. When a specimen was to be tested, it was thawed, and cleared by centrifugation of the fibrinogen which had precipitated on freezing. A single dilution of 1:8 or 1:16 was made of each serum, in sufficient amount to allow for the two complement fixation tests, the detection of anticomplementary sera, the antistreptolysin titer, and the antihyaluronidase titer (2). It was then heated to 56°C. for 15 minutes to inactivate complement. These dilutions were used within a few days, and were kept frozen between tests.

The S antigen was maintained in the refrigerator in the lyophilized state and was dissolved when needed. In the case of CP desiccation was not feasible, because of poor resolution of the large particles. The CP was maintained in suspension in the refrigerator with merthiolate added to a final concentration of 1:10,000. There were no troublesome anticomplementary effects during many months of such storage.

The Serologic Tests.—Complement fixation tests were performed by incubating 0.4 cc. of various dilutions of serum with 0.1 cc. of the antigen (at twice its minimal optimal concentration) and 0.1 cc. of complement, diluted to contain 1.3 to 1.5 units under the conditions of the test. After 45 minutes of incubation at 37°C., 0.1 cc. of a 4 per cent suspension of sheep erythrocytes was added to each tube, together with 0.1 cc. of rabbit anti-sheep–erythrocyte serum, adjusted to contain 2 units of hemolysin in that volume. The antigen-complement and the sensitized erythrocytes were each added as a mixture, using an automatic pipet. At the end of half an hour of further incubation at 37°C. the degree of hemolysis was read as 0, weak, strong, or complete. As a control on temperature effects in incubating, and because of the time required for the pipetting of reagents, at least one standard serum was included in each rack of every test. Each test with each antigen involved about 450 sera, including the standard sera. The usual precautions of complement titrations were taken, and several experimental sera of each test were repeated in the following one. Finally, for more precise comparison of titers in a given patient at different stages of activity of the rheumatic process, all sera of a given patient were included within one set to be tested.[1]

The antistreptolysin tests were done exactly as suggested by Todd (3), since that is the technic followed in almost all of the reported studies of antistreptolysin titers. Serum dilutions were made in volumes of 0.4 rather than 0.5 cc., in conformity with the other tests in this study.

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[1] The complement used in this study was the lyovac complement of Sharp and Dohme, Inc., to whom the author is indebted for generous gifts of this material.

The Study of the Patients and the Criteria of Activity of the Rheumatic Process.—The rheumatic patients were studied in acute, convalescent, and quiescent stages of the disease. They were usually seen first in the wards of The Children's Hospital of Philadelphia, or the Philadelphia General Hospital. Many of them were studied in the wards of the Children's Seashore House at Atlantic City during the chronic, active and the convalescent stages of the illness. They were then followed at the Rheumatic Fever Clinics of the first named institutions in the quiescent stage. In some cases, of course, the entire cycle was repeated.

The patients were examined at least every 2 weeks while in the acute or convalescent wards and at each visit to the Clinics. These examinations included the following: symptoms—anorexia, headache, precordial, abdominal, or arthritic pain, cough, dyspnea, and epistaxis; physical signs—cardiac rate and rhythm, distance of apex beat from the midline, murmurs, with distance of transmission of each, and other adventitious sounds, palpable thrills and friction rubs, hepatic enlargement and edema, rashes, subcutaneous nodules, and chorea. Laboratory examinations included the erythrocyte sedimentation rate, white blood cell count, and hemoglobin concentration. The vital capacity was usually determined at biweekly intervals, and electrocardiograms were taken as required. The erythrocyte sedimentation rate was done by a method described elsewhere (4). It involved a series of readings of the erythrocyte level at 5 minute intervals, in order to determine the rate of free fall of the corpuscles, and a correction for the relative volume of erythrocytes.

The patients selected for serologic study were, of course, only those in whom the diagnosis of active rheumatic fever was beyond doubt. Very nearly all of these children had active rheumatic carditis.

The recording of clinical evidence of rheumatic activity in correlation with the serologic titers presented a problem. A considerable part of the clinical data was kept in graphic form in the records of this investigation. However, an attempt to present all such data in graphic form in this paper was abandoned because of the necessary complexity of such graphs. On the other hand, the manifestations of active rheumatic disease are so protean that no one or two clinical signs can measure the activity in all cases. The erythrocyte sedimentation rate is probably the most sensitive single indicator of activity of the rheumatic process, but it has been shown that this test may be unreliable in adolescent and overweight children (4), in right-sided heart failure (5, 6), and in prolonged, full dosage of salicylates (7–9). Finally, there are some patients in every series of sufficient size whose erythrocyte sedimentation rate is within normal limits, although there is unquestionable clinical evidence of an active rheumatic process.

For the recording of the clinical evidence of the degree of rheumatic fever, therefore, it was decided to show the erythrocyte sedimentation rate, temperature, resting pulse rate, and such episodes as polyarthritis, carditis, and cardiac failure. The criteria of quiescence of the rheumatic process included the fall to a plateau level of the erythrocyte sedimentation rate, of the size and rate of the heart, and of murmurs and their extent of transmission; a stable pulse rate, absence of significant symptoms and signs, and general well being.

RESULTS

The Optimal Antigen Concentration of the Fractions.—In order to use the two nucleoprotein fractions in large scale serologic tests it was necessary to ascertain that the optimal range of concentration of each fraction as antigen in complement fixation tests was the same, regardless of the titer of the antiserum. Table I shows that such is the case for high and low titered sera of rheumatic patients. The same optimal concentrations were found to obtain also in tests using rabbit antistreptococcal sera of high and low titer.

The Antibody Levels to CP and S in Normal Subjects of Various Age Groups.— Since nothing was known of the occurrence of these antibodies in human sera, determinations were carried out of the complement fixation titers to CP and S in sera of 35 neonatal infants, of 35 older infants ranging from 6 months to 2 years, of 70 children ranging from 4 to 15 years, and of 40 medical students. Comparative tests of antihemolysin were done in parallel. The results of these tests are shown in the form of a percentage frequency chart in Fig. 1.

Fig. 1 shows that antibodies to all three streptococcal antigens under investigation are found in the sera of normal subjects. Although the range of titers

TABLE I

The Constancy of Optimal Antigen Titers of CP and S in Complement Fixation Tests versus Rheumatic Sera of High and Low Titer

	Serum 1315 (active rheumatic)							Serum 1910 (quiescent rheumatic)			
	Dilution 1:							Dilution 1:			
	32	64	128	256	512	1024	2048	8	16	32	64
CP											
0.01 per cent solution	0	0	0	0	0	tr	s	0	w	s	c
0.005	0	0	0	0	0	tr	s	0	w	s	c
0.0025	0	0	0	0	0	0	s	0	w	c	c
0.0013	w	w	w	w	s	s	s	w	s	c	c
S											
0.2 per cent solution	0	0	w	s	c	c	c	0	s	c	c
0.1	0	0	0	s	c	c	c	0	w	c	c
0.05	0	0	tr	s	c	c	c	0	w	c	c
0.025	0	tr	s	c	c	c	c	w	s	c	c
0.013	w	s	c	c	c	c	c	ac	c	c	c

The degree of hemolysis is recorded: O, none; tr, a trace; w, weak; s, strong; ac, almost complete; c, complete.

differs among the three tests, certain broad similarities are seen. In each test, a considerable percentage of relatively high titers is found in neonatal infants. After several months, or a year, the higher titers are no longer found except in a very few cases. In childhood higher titers appear in all three tests, and among young adults the higher titers occur with slightly greater frequency than among children.

The Antibody Response in Acute Streptococcal Disease.—

Since no epidemic of streptococcal infection occurred in the Philadelphia area during the course of these studies, the patients chosen for examining antibody levels in streptococcal infection were children with scarlet fever. These children were admitted to the Philadelphia Hospital for Contagious Diseases within the first day or two of the disease. The diagnosis was verified at the hospital, and the children were not treated with sulfonamides. Sera drawn at

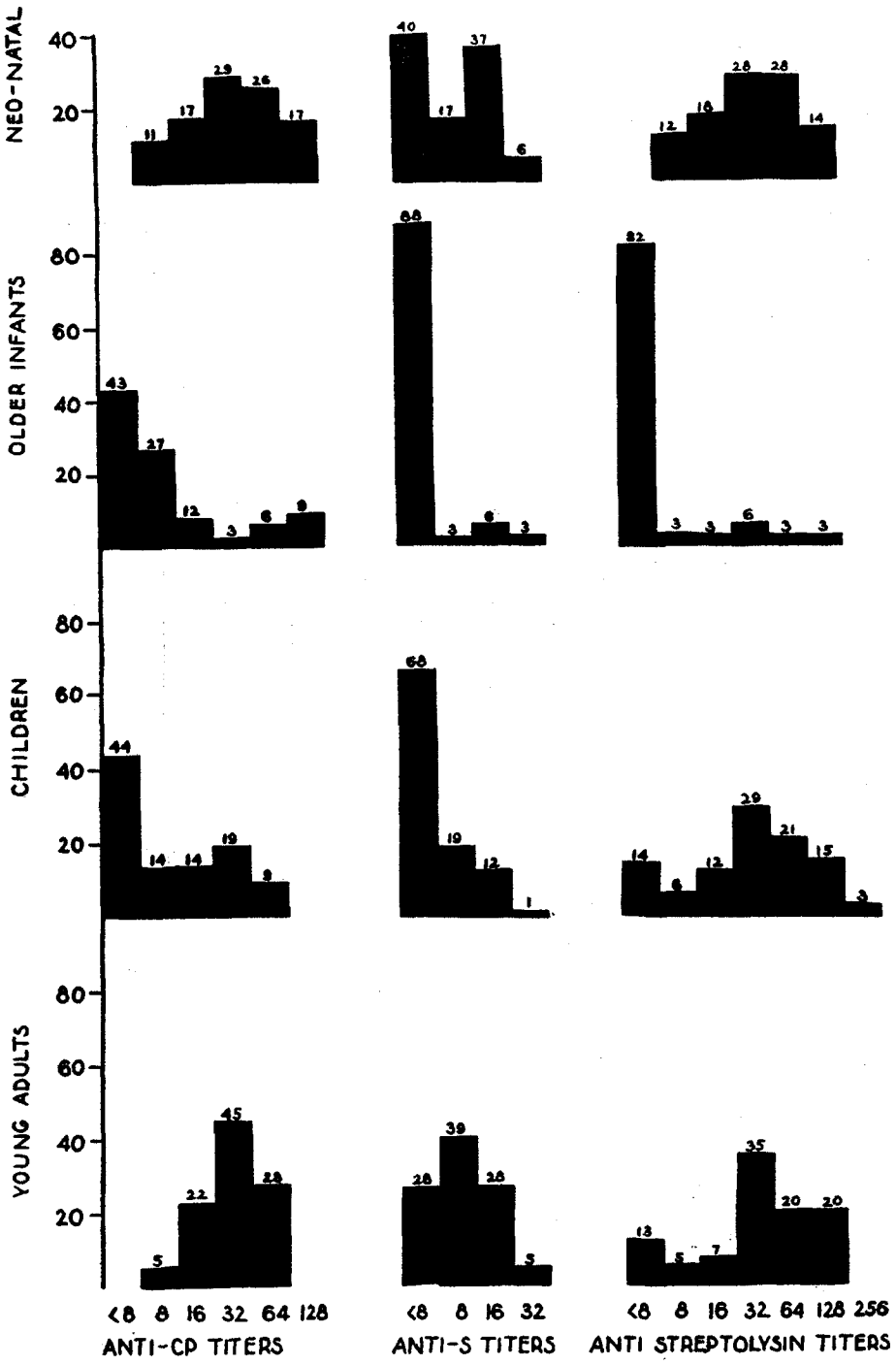

FIG. 1. The frequency distribution, in percentage, of complement fixation titers to CP and S, and of antistreptolysin titers, in sera of normal subjects of various age groups.

the beginning and at the end of the 3 week hospitalization were compared to determine increases in antibodies in the course of the infection. As can be seen by the distribution of normal titers in Fig. 1, the titers at the first bleeding varied widely. Accordingly, the effect of the disease on the antibody level is recorded, in Table II, in terms of individual increases, rather than of average titers.

Table II shows that in all the tests 80 per cent or more of the patients showed at least a twofold rise in titer, so that an increase of antibody level in any of these three tests may be considered characteristic of this acute streptococcal infection. There was poor correlation among the changes of antibody titers to the respective antigens. Although a few sera showed very little or no rise in the titer of all three antibodies, there were examples of sera in which one of the antibodies showed a greater increase in titer than in the case of the other two. This could be

TABLE II

Percentage Frequency of Increases in Antibody Titers to CP, S, and Hemolysin in the First 3 Weeks of a Group of 70 Cases of Scarlet Fever

Increase in titer	CP	S	ASL
None or too small to be significant	20	15	17
2-fold	39	66	37
4-fold	22	17	11
8-fold	14	2	8
16-fold	4	0	7
32-fold or higher	1	0	20

observed for any of the three antibodies, especially in the case of anti-CP and antihemolysin, in which the range of increase tended to be higher.

In order to study the persistence of these antibodies after recovery from the streptococcal infection, sera were obtained from these patients 4 months after they were discharged from the hospital. Twenty such specimens were obtained. Comparison of the titers of these sera with those of the specimens drawn at 3 weeks showed that the majority of titers to each of the antigens remained elevated for at least that period of time. In the case of the CP, 70 per cent of the pairs of sera showed no significant change in titer. In 10 per cent, there was a twofold fall in titer, and in 20 per cent, there was a twofold rise. Anti-S antibodies showed as high a titer after 4 months in 80 per cent, a fall in 10 per cent, and a rise in 10 per cent. The antistreptolysin titers were unchanged in 65 per cent of the pairs tested. The 4-month specimens showed higher titers in 10 per cent of the pairs, and slightly lower titers in the remaining 25 per cent. In general the titer of all three antibodies was in the same range after 4 months of health as during the preceding episode of scarlet fever.

Antibody Titers in Acute and Quiescent Rheumatic Fever.—The sera of 100

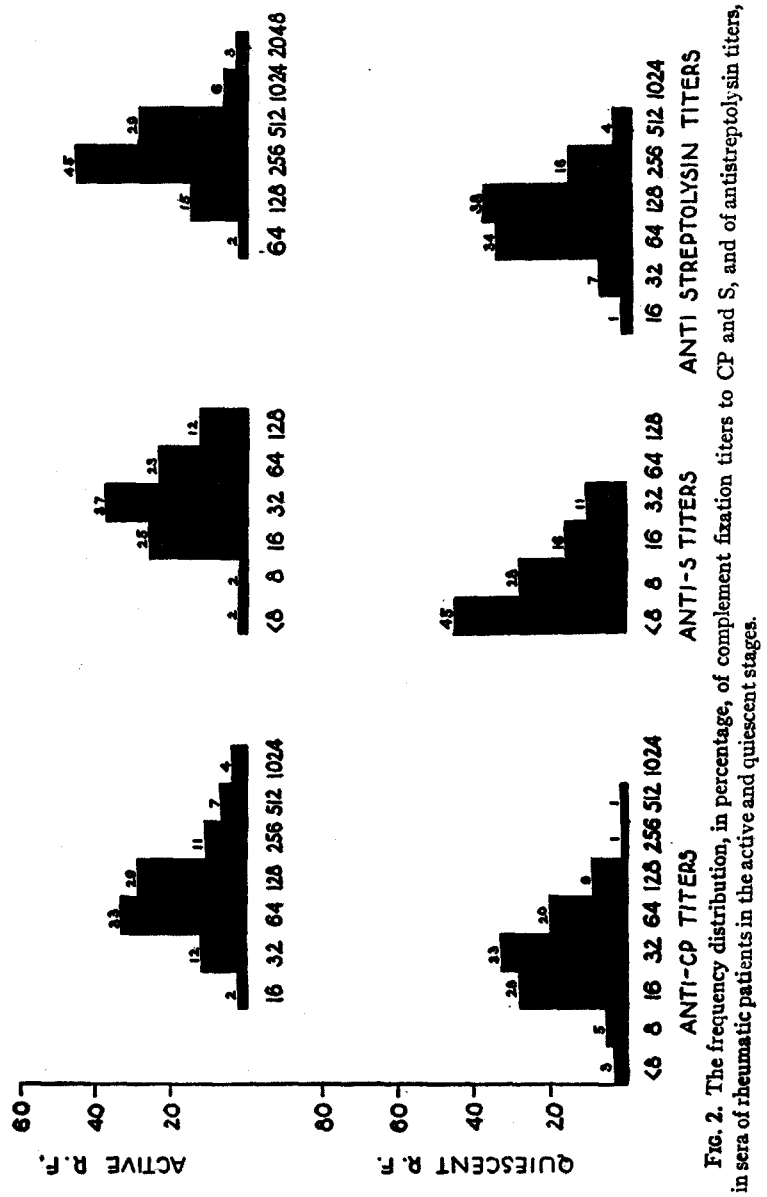

FIG. 2. The frequency distribution, in percentage, of complement fixation titers to CP and S, and of antistreptolysin titers, in sera of rheumatic patients in the active and quiescent stages.

children with active rheumatic fever were examined for antibodies to these three streptococcal antigens, as well as the sera of 81 rheumatic patients in the quiescent stage of the disease. The frequency with which various titers were found is shown in percentages in Fig. 2.

Fig. 2 shows that the antibodies in all three tests were elevated in the great majority of active cases, in comparison with those of normal children shown in Fig. 1. In the case of quiescent rheumatic patients fewer elevated titers are found, but there are more of these than among the normal individuals.

A comparison of the distribution of titers in active rheumatic patients with those of normal controls of the same age group suggests a titer of 32 as a dividing line between the anti-CP titers of normal children and active rheumatic patients, 8 for the anti-S titers, and 128 as the analogous antistreptolysin level. The significance of these levels and of the difference between titers of normal and acute rheumatic children is brought out in Table III.

TABLE III

Titers of Normal, Active Rheumatic, and Quiescent Rheumatic Children against CP, S, and Hemolysin, in Percentages

	CP		S		ASL	
	Up to 32	64 and above	Up to 8	16 and above	Up to 128	256 and above
Normal children	91	9	87	13	97	3
Active rheumatic fever	13	87	4	96	17	83
Quiescent rheumatic fever	69	31	73	27	80	20

Table III shows that a range of anti-CP titers exists which includes so high a percentage of normal children that, for streptococcal work, it can be considered the upper normal limit, and that the great majority of active rheumatic subjects have titers above that level. The same is true of anti-S titers. The analogous results with antistreptolysin titers confirm those recorded in the literature. The sera of quiescent rheumatic patients show a much smaller number of titers above the upper normal limit, but more than in the case of normal individuals. The average intervals between the foregoing acute rheumatic episode and the time of collection of serum from these quiescent rheumatic patients were 17 months.

Antibody Titers during Fluctuations in the Severity of the Rheumatic Process.— The great majority of the patients with acute rheumatic fever were followed into the stage of quiescence. A few had continuous rheumatic activity either of fulminating severity ending in death, or of a low grade. A number, however, ran a classical polycyclic course with clearly defined recurrences or changes in clinical estimate of rheumatic activity. In the single rheumatic episodes

studied the titers of all three antibodies did not decline as the clinical activity of the disease subsided, but remained elevated for months after complete quiescence of the rheumatic process, in the absence of any clinical streptococcal infection. The patients with a polycyclic course afforded an opportunity to correlate antibody titers with variations in degree of rheumatic activity. The charts of three such patients, shown in Fig. 3, reveal that, although the titers vary during the course of the rheumatic infection, there is no correlation with the clinical changes used to evaluate the severity of rheumatic fever. In none of the cases shown had sufficient time elapsed between recurrences for the titers to fall to normal levels.

DISCUSSION

The presence of the antibodies measured in these studies in normal sera, and their concentrations at various age groups, are a consequence of the prevalence of the hemolytic streptococcus in the human throat and of the breadth of specificity of the antigens. After several months or a year the infant has lost the streptococcal antibodies derived from the maternal blood; the titers are quite low at that age. In childhood, however, as opportunity for contact with the ubiquitous streptococcus increases, higher titers appear and these are again slightly higher in young adults. Among the antistreptolysin values, the comparison of titers between neonatal life, later infancy, and childhood is in good agreement with that reported for that test by Gordon and Janney (10).

The prevalence of the hemolytic streptococcus and the broad specificity of these antigens are also the basis for the prolonged maintenance of elevated titers after acute streptococcal or rheumatic disease. In this case it is probable that minute amounts of antigens, produced by streptococci too few in number to excite an inflammatory reaction, suffice to act as "boosters" to maintain or raise a titer already elevated by disease. In the case of both streptococcal and rheumatic infection, Mote and Jones (11) have shown that antistreptolysin titers may remain elevated for as long a period as 1 year, without clinical infection by the streptococcus. Solomon (12) has shown the same effect in streptococcal infection, and Yannet and Leibovitz (13) had similar results with antifibrinolysin in streptococcal disease. In the studies reported here anti-CP and anti-S titers were followed for 4 months after streptococcal infection. At the end of that time the average titers were the same as at the height of the disease. Following acute rheumatic infections there was a better opportunity to observe the rate of fall of the antibodies to CP and S. These antibody titers could remain elevated for many months after the evidence of rheumatic activity had subsided. It is very likely that the greater percentage of elevated titers in the quiescent rheumatic group than among normal controls was due to the same phenomenon as that which keeps these titers above normal after streptococcal infection, and not to any characteristic reaction of the quiescent rheumatic patient to these antigens.

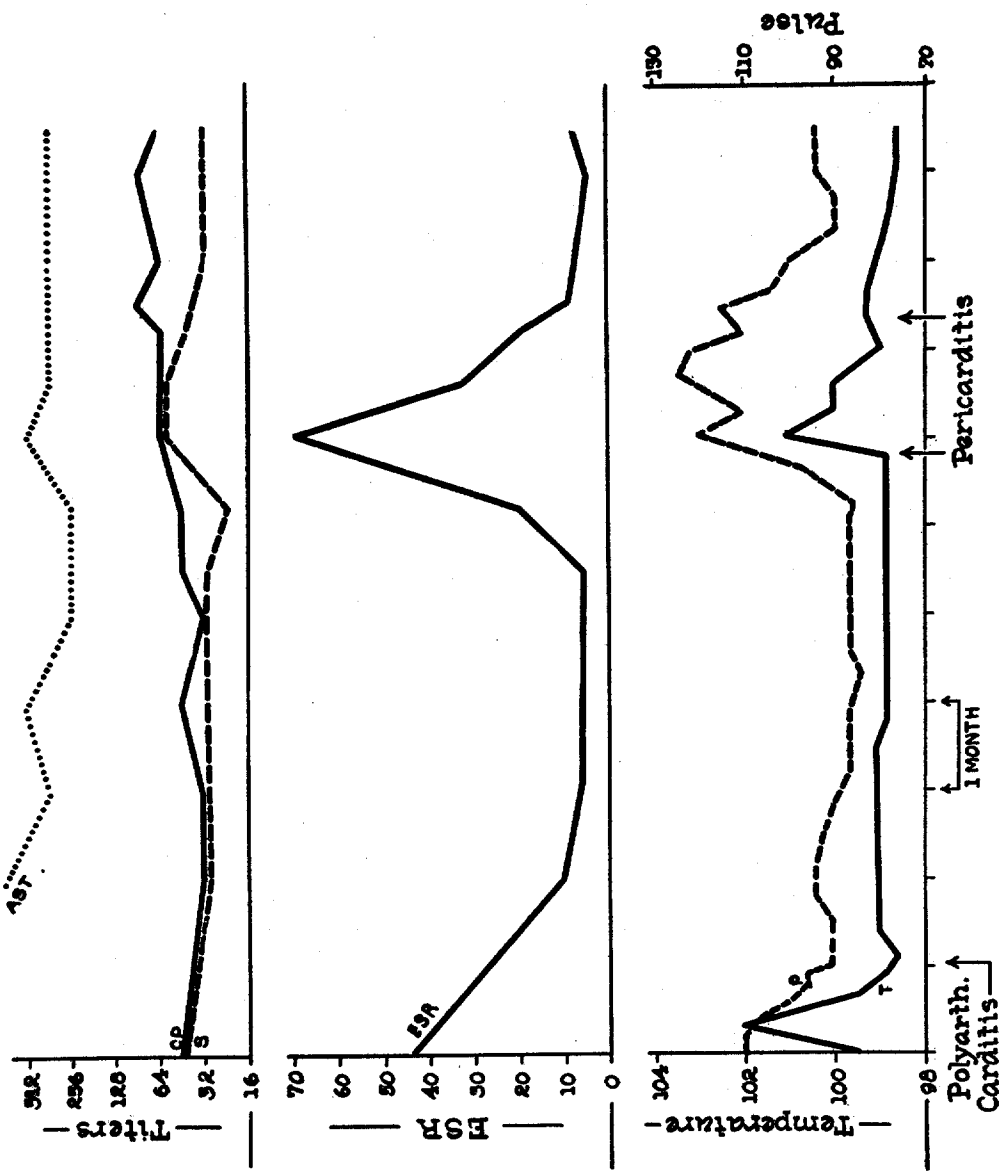

FIG. 3 *a*

FIG. 3, *a*, *b*, and *c*. Correlation of antibody titers to CP, S, and hemolysin with the degree of activity of the rheumatic process.

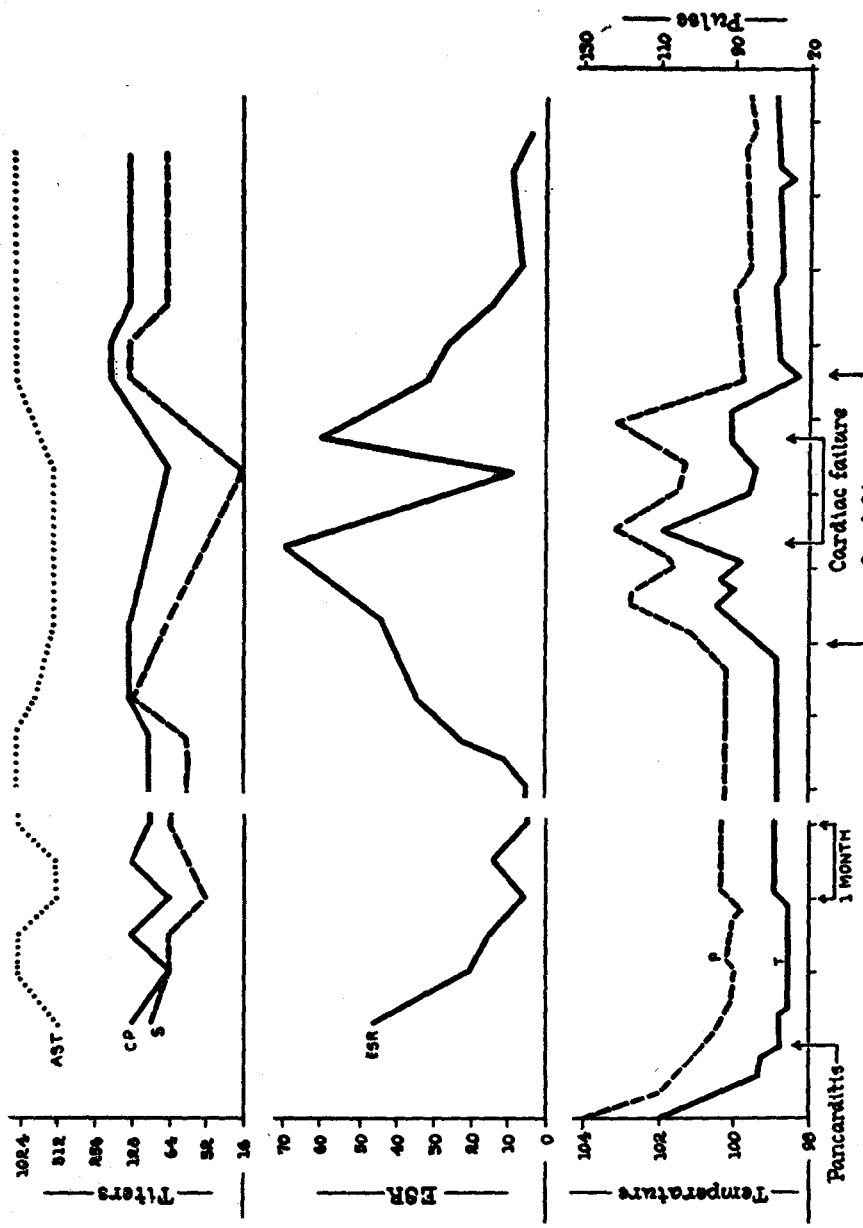

FIG. 3 *b*

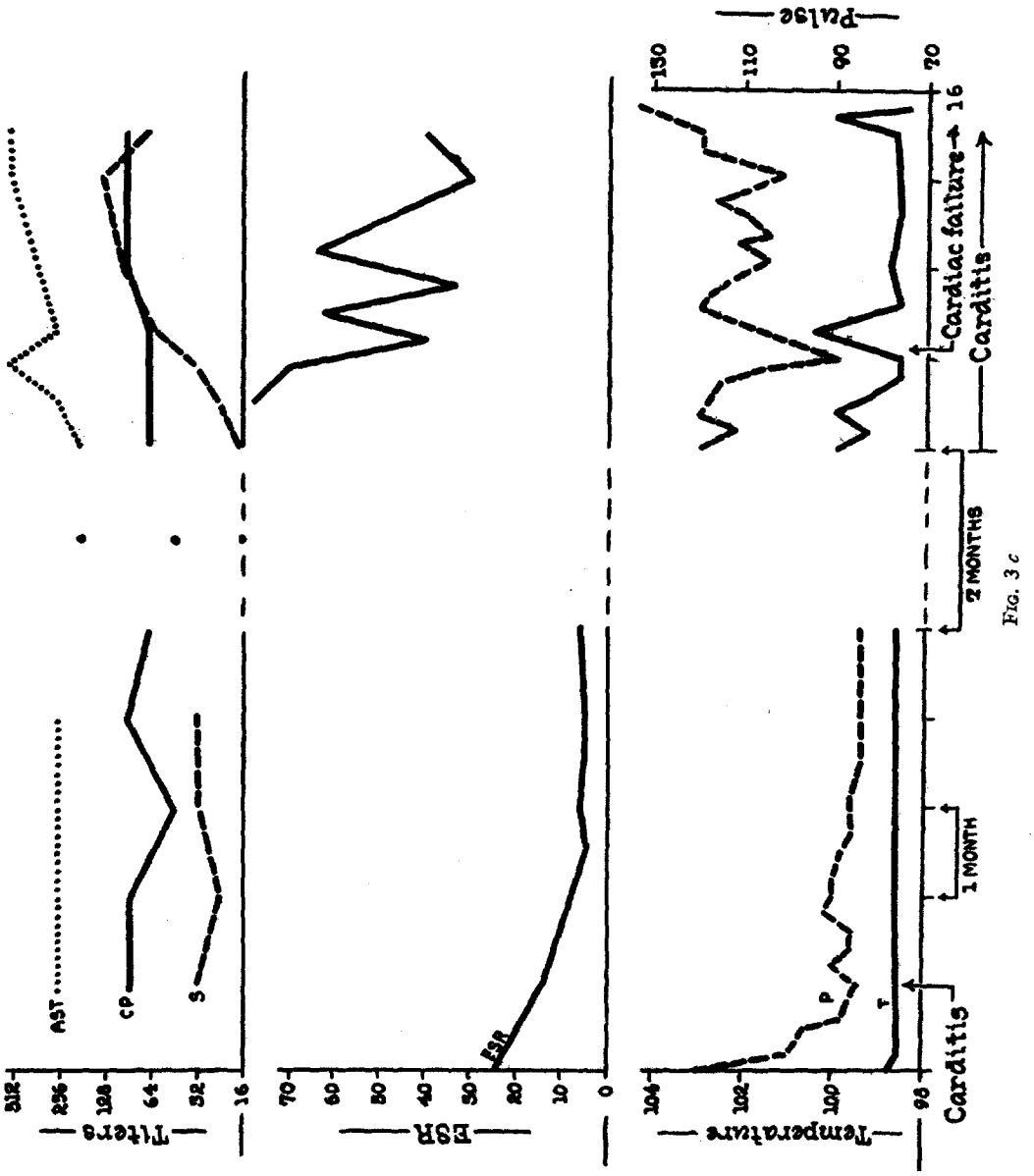

FIG. 3 c

The factors which account for the prevalence and persistence of these antibodies also make it necessary to assume an upper limit of normal titers which does not actually include the highest titers among normal subjects. As striking as is the contrast in distribution of titers between active rheumatics and controls, it would be impossible to demonstrate it if one stipulated that the upper limit accepted for the normal population include all the titers found in that group. The epidemiology of the streptococcus makes it necessary to set the upper limit of normal at a titer which includes only 85 or 90 per cent of normal sera tested. Even without accepting the levels indicated in Table III as upper limits of normal for the respective antibody titers, the difference in distribution of titers between normal subjects and active rheumatics is striking, and statistically significant. The serologic tests with CP and S confirm the suggestion derived from antifibrinolysin and antistreptolysin tests that the active rheumatic patient is one in whom streptococci are present in numbers comparable to those in an acute streptococcal infection.

It is not possible to be certain at present whether either of the new tests is preferable to the antistreptolysin test for general use. The anti-S titers show the greatest difference between the acute and quiescent phase groups. Whether this difference is sufficiently great to indicate the use of anti-S complement fixation for semiroutine work will be decided as additional serologic tests are done.

The question inevitably arises as to the rôle of CP and S in the pathogenesis of rheumatic fever. It should be pointed out that if the pathogenesis of rheumatic fever involves sensitization, it is possible that the sensitizing agent will not be detected by serologic tests, because there is no necessary quantitative correlation between the degree of sensitization and the titer of humoral antibodies to a given substance. Especially in the case of streptococcal immunology, the disturbing factor of prevalence of subclinical contacts with the organism would make the serologic identification of a streptococcal sensitizing agent very unlikely, should such an agent exist in rheumatic fever. The work presented here should not be regarded, therefore, as an examination of the CP and S as possible pathogenetic agents in rheumatic fever, but rather as a serologic exploration of sera of normal, streptococcal, and rheumatic subjects by these new antigens.

SUMMARY

Complement-fixing antibodies to the cytoplasmic particles (CP) and to the S fraction of streptococcal nucleoproteins are present in normal human sera, the range of concentrations varying among the age groups.

The titer of these antibodies rises between the first half-week and the 3rd week of scarlet fever, in more than 80 per cent of the cases. The titers then remain elevated for at least 4 months.

In children, 91 per cent of the normal sera examined showed anti-CP titers up to 32; 87 per cent of sera in active rheumatic disease had titers above this level. Corresponding data with S fell in the same range of percentage distribution.

Anti-CP and anti-S titers remained elevated long after the rheumatic process had reached quiescence. No correlation of serologic titer with the degree of clinical activity was found in the case of either antibody.

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