Differentiation of 19S and 7S Complement Fixing Antibodies in Primary *Versus* Recrudescent Typhus by Either Ethanethiol or Heat.* (30161)

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(Introduced by R. S. Chang)
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The 19S and 7S antibodies can be differentiated by any one of a number of methods. Among the more commonly used procedures are immunoelectrophoresis, ultracentrifugation, zone electrophoresis, column chromatography and disulfide-bond reducing agents(1). By means of such tests it has now become well established that 19S antibodies are generally associated with primary responses, and 7S antibodies with late primary or secondary immune responses(2). The vast majority, however, of the studies establishing this phenomenon have been carried out with sera which were relatively easily obtained in sufficient volume from experimentally-produced immune responses in man and animals(3).

During the past decade we have been studying naturally-occurring antibody patterns in typhus fever, an infectious disease of man which appears to offer an unusually favorable example for studying 19S and 7S antibody patterns. Epidemiologic and serologic studies have clearly established that primary epidemic typhus is a primary infection, and that Brill-Zinsser disease (recrudescent typhus) is a secondary attack coming years later and caused by the same organism which has lain dormant in the host during the interim(4).

In preliminary studies with typhus sera in which we employed immunoelectrophoresis, it was demonstrated, as might be predicted, that early primary epidemic typhus antibodies produced precipitin arcs characteristic of 19S gamma globulins, while precipitin arcs produced by both early and late Brill-Zinsser disease antibodies were characteristically 7S(5). Similar results were obtained when the same sera were tested in an agglutination test with the disulfide-bond reducing agent 2-mercaptoethanol. Agglutination tests, however, proved extremely expensive, difficult to standardize, and yielded equivocal results in low dilutions. Moreover, low-titered sera would not yield precipitin arcs in routine immunoelectrophoresis. Other conventional methods for differentiating 19S and 7S gamma globulins, such as column chromatography and ultracentrifugation were equally inconvenient or impossible to adapt to the study of antibodies of very low titer in the very small volumes of sera (often fractions of milliliters) which were available as serial serum specimens from acutely ill patients.

These difficulties encouraged us to determine whether the complement fixation (CF) test could be so modified as to permit the assay of 19S *versus* 7S antibodies.

The present report describes a simple and rapid complement fixation test by means of which the sequence and development of 19S and 7S antibodies in the primary and secondary forms of typhus fever can be demonstrated.

Materials and methods. At the outset 3 findings from previous serologic studies in these laboratories proved indispensable in designing a CF test to compare 19S and 7S antibodies. In the first place, during 10 years of testing all types of typhus sera by complement fixation it had gradually become evident that early phase primary epidemic typhus antibodies required 8 times more antigen than Brill-Zinsser disease antibodies in order for maximum fixation of complement to occur(6). It was necessary, therefore, to employ 8 standard units of antigen in the

* This work was sponsored by the Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board and was supported by a contract from the office of the Surgeon General (DA-49-007-55) Dept. of the Army, Washington, D.C.

new CF test designed to compare primary and secondary immune response antibodies. Secondly, during the same studies it had been observed that primary epidemic typhus CF antibodies were reduced in titer when sera were inactivated at 60°C for 30 minutes(6). Brill-Zinsser disease antibodies were not affected by such temperatures. Protocols of the new CF test therefore included both 60°C and 56°C inactivation of sera. Finally, a sulfhydryl compound, ethanethiol† (ET-SH) was found which had a boiling point of 34.5° to 35.5°C. The low boiling point of ET-SH made it possible to mix this sulfhydryl compound with sera in the CF test and, after incubation of the serum-sulfhydryl mixture, remove the major part of the chemical during routine 56°C inactivation of sera. This eliminated the tedious and inconvenient dialysis procedure which is the conventional method for removing sulfhydryl compounds from mixtures.

Selection of sera. Paired sera were obtained from 26 patients diagnosed as suffering from typhus fever in Bosnia, Yugoslavia, between 1960 and 1963. These 52 sera were subjected to 4 differential tests: (a) proteus OX_{19} agglutination, (b) CF test for presence or absence of cross-reacting murine antibody, (c) CF test for high or low antigen requirement of antibodies, and, (d) 56° and 60°C inactivation of sera. By means of differential criteria previously described(6) these 4 tests clearly separated the sera into 2 groups: 13 pairs gave a serologic pattern typical for primary epidemic typhus and 13 pairs, typical for the secondary Brill-Zinsser disease.

CF test technic. A standard CF test technic was used(7) with several modifications. (a) Sera were pretreated and inactivated in the following manner with 3 parallel tests being performed on each serum. First, all 3 specimens were diluted to 1/5 in the veronal buffer (8) (V.B.) used throughout the test. Immediately following this, specimens 1 and 2 were diluted to 1/10 in V.B. and specimen 3 was mixed with an equal part of a 0.06 M solution of ethanethiol (ET-SH). All 3 specimens then were allowed to stand at 26°C for 3 hours. At this point specimens 1 and 3 were inactivated at 56°C for 30 minutes and specimen 2 was inactivated at 60°C for 30 minutes. (b) Following inactivation, the standard CF technic was carried out with the exceptions that 8 rather than 2 units of antigen were employed and fixation was carried out for one hour in a 37°C water bath instead of overnight at 4°C.

The antigen used in the CF test was an epidemic typhus soluble antigen produced in this laboratory by a standard method(9). Titrations of the antigen to determine the dilution giving 8 units were carried out with the secondary type antibodies in Brill-Zinsser disease sera. To minimize the objectionable stench of concentrated ET-SH, a large volume of 0.06 M ET-SH was made up under a hood. Ten to 20 ml quantities of this 0.06 M ET-SH, which has only a mild odor, were then sealed in glass ampules and stored at 4°C to be opened as needed. The diluted chemical can be stored for at least 4 months without loss in effective molarity.

Immunoelectrophoresis. Tests were carried out with a slight modification of Scheidegger's method(10). The antigen used was a sonic vibrated emulsion of concentrated and purified epidemic typhus richettsiae. A detailed description of the test is published elsewhere(5).

Results. The results of the 3 parallel CF tests on each of the 26 primary epidemic typhus sera are recorded in Table I. Data from 25 of the 26 sera recorded in Table I are represented graphically in Fig. 1. Data from the one-day serum which had no demonstrable antibodies were not included in the figure.

In Fig. 1 it can be seen that when the 25 sera were tested in a standard CF test (56°C serum inactivation and no treatment with ET-SH), geometric mean titers ranged from 1/127 at 0-5 days to a maximum of 1/1141 at 6-10 days following onset; thereafter titers declined irregularly. The same sera treated with ET-SH and 56°C inactivation showed markedly lower titers with differences being most pronounced in the early acute phase sera. When the same sera were inactivated at 60°C with no ET-SH treatment, there

† Eastman Organic Chemicals, Rochester, N. Y.

TABLE I. Complement Fixation Test Data for Paired Sera from 13 Primary Epidemic Typhus Patients. A comparison of parallel tests employing ethanethiol and 60° serum inactivation against a standard control.

Patient No.	Age	Day of disease	Complement fixation tests* 56°C Inactivation (standard control)	56°C Inactivation and ET-SH	60°C Inactivation	Immunoelectrophoresis pattern
1	7	10	640	80	160	19S
		41	80	80	80	
2	16	11	5120	40	1280	19S
		22	2560	80	640	
3	8	9	2560	40	320	19S
		20	1280	40	160	
4	7	4	320	$\leq$20†	40	19S
		21	320	40	160	19S weak
5	16	1	$\leq$10	$\leq$10	$\leq$10	
		32	1280	160	640	19S weak strong 7S
6	10	9	320	40	80	
		16	160	20	40	19S
7	12	17	320	$\leq$20	$\leq$20	19S
		30	640	40	40	
8	4	6	1280	80	320	19S
		18	640	160	160	
9	7	4	40	$\leq$ 5	10	
		10	640	20	40	19S
10	13	4	160	10	20	
		10	5120	20	320	19S
11	12	14	640	80	320	19S weak
		20	320	160	320	
12	14	20	640	160	320	
		28	160	80	160	19S weak
13	11	11	320	$\leq$10	80	19S
		13	1280	$\leq$10	320	

* Figures represent denominators of CF antibody titers.

† $\leq$20 considered as 20 in geometric mean calculations (see Fig. 1 & 2).

was considerable lowering of antibody titers but to a degree somewhat less than that produced by ET-SH treatment of the sera.

At least one serum from each pair belonging to a primary epidemic typhus patient was subjected to agar immunoelectrophoresis. All showed precipitin arcs characteristic of 19S antibody (see last column Table I). One of the late (32 day) sera also produced a 7S precipitin arc. Of interest is the fact that this serum had a relatively high titer ($\frac{1}{160}$) of sulfhydryl-resistant antibody.

In Table II the geometric mean antibody titers shown in Fig. 1 are converted into percentages of antibody inhibited by treatment with ET-SH or 60°C serum inactivation. Ninety-four to 98% of the antibody contained in the 13 sera obtained during the first 15 days of primary epidemic typhus infection was shown to be sulfhydryl sensitive. As noted above this antibody was also demonstrated to be consistent with 19S antibody by immunoelectrophoresis. The real percentage of early acute phase primary epidemic typhus antibody which is 19S may be even greater. For it is possible that some recombination(1) of the reduced 19S macroglobulins may have occurred during the 2 to 3 hours required to carry out the CF test after the ET-SH had been boiled off. In addition 3 of these sera treated with ET-SH were not carried out to their lowest CF end points.

Subsequent to the 15th day after onset the percentage of antibody which is either sensitive to ET-SH or lowered by 60°C

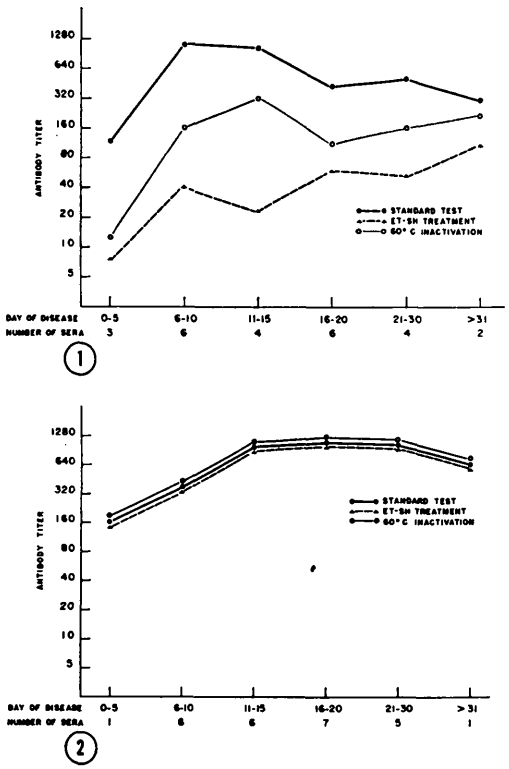

FIG. 1. Primary epidemic typhus CF titers. Geometric mean titers of 25 sera arranged according to day of disease. For each serum, 3 parallel tests: a) with ET-SH treatment and 56°C inactivation, b) with no ET-SH treatment but 60°C inactivation, and c) with no ET-SH treatment but 56°C inactivation—a standard control.

FIG. 2. Brill-Zinsser disease CF titers. Geometric mean titers of 26 sera arranged according to day of disease. For each serum, 3 parallel tests: a) with ET-SH treatment and 56°C inactivation, b) with no ET-SH treatment but 60°C inactivation, and c) with no ET-SH treatment but 56°C inactivation—a standard control.

serum inactivation declines irregularly. Conversely, the antibody resistant to ET-SH treatment or 60°C serum inactivation increases as the acute phase merges into convalescence.

Fig. 2 portrays the results of the 3 parallel CF tests performed on each of the 26 Brill-Zinsser disease sera. It is obvious that Brill-Zinsser disease antibodies are resistant to the action of a final concentration of 0.03 M ET-SH. Furthermore, these antibodies are not lowered by 60°C inactivation. At least one serum from each pair belonging to a Brill-Zinsser disease patient was subjected to agar immunoelectrophoresis. All produced precipitin arcs characteristic of 7S antibody.

Fig. 3 presents typical precipitin arcs obtained with immunoelectrophoresis of a primary epidemic typhus and a Brill-Zinsser disease serum.

Discussion. It is assumed in these studies that antibodies sensitive to a disulfide-bond reducing agent are 19S antibodies. Other investigators have made a similar assumption and found no inconsistencies when they cross-checked the sulfhydryl compound method with sucrose-gradient ultracentrifugation, starch-block electrophoresis, immunoelectrophoresis or ion exchange chromatography (11). However, some reports have appeared recently suggesting that certain 7S or closely related antibodies may lose immunological activity after mercaptan treatment. Rockey and Kunkel(12) reported on an antibody having an intermediate sedimentation constant which was inactivated by 2-mercaptoethanol. Wiedermann *et al*(13) and Schur and Christian(14) have described loss of complement fixing activity, without corresponding loss in precipitating ability after treatment of certain 7S antibodies with 0.01 M to 0.1 M mercaptoethanol and subsequent alkylation. In all of these studies considerably more physical and chemical manipulation of antibodies was employed over a longer period of time than in our CF tests in which no alkylating agent, no dialysis, and no centrifugation was employed and in which not more than 6½ hours elapsed from the time when ET-SH was first mixed with the sera until the final readings of the titers were made.

While variations in the animal species as well as the nature of the antigen including

TABLE II. Comparison of Percentages of Antibody in Primary Epidemic Typhus Sera Which Are Inhibited by ET-SH and 60°C Inactivation.

Day of disease	No. of sera tested	% of antibody inhibited by ET-SH treatment (19S)	% of antibody inhibited by 60°C Inactivation
0– 5	3	94	90
6–10	6	97	86
11–15	4	98	70
16–20	6	86	75
21–30	4	89	70
>30	2	64	20

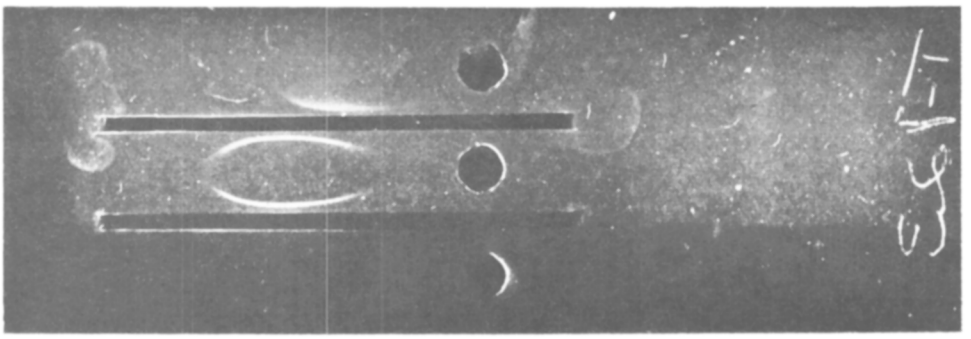

FIG. 3. Typical precipitin arcs obtained with immunoelectrophoresis of primary epidemic typhus and Brill-Zinsser disease sera; the former serum was electrophoresed from the upper well, the latter from the middle well. No serum was placed in lower well. Epidemic typhus soluble antigen (see text) was placed in both troughs.

the dose and route of injection might explain differences reported by various investigators, no clear explanation of differences is obvious. It can be emphasized, however, that in our studies, wherever a patient's serum was demonstrated to contain antibodies sensitive to ET-SH, immunoelectrophoretic arcs were consistent with 19S macroglobulins being the major antibody present. Conversely, where ET-SH was shown to have no effect on antibody titers, characteristic 7S gamma 2 precipitin arcs were produced in immunoelectrophoresis. Moreover, epidemiologic and other serologic data relating to each sera were in agreement with the results obtained by sulfhydryl compound treatment and immunoelectrophoresis(6).

The final concentration of the disulfide-bond reducing agent in the serum-ET-SH mixture is, of course, a very important factor in the test. When titrating the effect on antibody titers of various molarities of ET-SH, it was observed that slight loss of complement fixing ability of some 7S antibodies did occur when the final concentration of ET-SH in the serum was brought to 0.10-0.20 M. Conversely, we noted that there was slightly less inhibition of 19S antibody titers if the final molarity of the serum-ET-SH mixture was decreased below 0.03 M. Consequently, in all treatment procedures we used a final concentration of 0.03 M ET-SH. This latter concentration allows a moderate amount of leeway in the test for it was observed that optimal inhibition of the 19S antibody titers and no change of 7S antibody titers occurred as long as the final concentration of ET-SH employed was kept within the limits of 0.03 to 0.06 M.

One of the interesting developments in our previous studies was the unexpected demonstration of a clear cut differential effect of 60°C inactivation of the sera of primary and secondary forms of typhus(6). Our present studies have confirmed this phenomenon. The 7S antibodies uniformly gave identical CF titers following either 56° or 60°C serum inactivation. Conversely, wherever 50% or more of the antibody was shown to be 19S by its sensitivity to ET-SH, antibody titers obtained following 60°C serum inactivation were considerably lower than titers following 56°C inactivation.

The exact effect of 60°C on 19S molecular structure is not immediately obvious. It is not likely that 60°C inactivation of sera lowers 19S antibody titers by breaking disulfide bonds. The action is more likely due to some other denaturation process(15). Further studies are necessary to elucidate this point. In any case, the regular and sharp differential effect of heat on 19S and 7S antibodies within the very narrow temperature range of 59.5 to 60.5°C offers a possible tool for studies of the immune globulins.

It is well known that the breaking of disulfide bonds by a sulfhydryl compound with consequent loss of antibody activity may be a reversible phenomenon(1). Following removal of the sulfhydryl compound, recombination usually occurs at a rate related to pH, temperature, and concentration of the

reduced 19S gamma globulins. Under the conditions of pH, time, and temperature prevailing in the specially designed CF test employed in these studies, there appeared to be little recombination of reduced early phase 19S antibodies, for they remained 94-98% reduced at the end of the CF test. In fact, in order to reduce recombination, we employed a "same-day" CF test with fixation at 37°C for 1 hour rather than fixation overnight at 4°C. Studies are in progress to measure and possibly control with an alkylating agent any recombination which may occur.

It might be asked why we have not employed one of the more refined CF tests such as one using 50% end-point readings. We have used a conventional CF test intentionally. Our primary objective was to find a simple test to differentiate 19S and 7S antibodies—a test which would be adaptable to the routine study of antibodies in serial serum specimens of small volume from patients suffering from an infectious disease. The majority of studies of 19S and 7S antibodies up to the present have been reported on experimentally-produced immune responses, hence data relating to the actual 19S and 7S antibody patterns produced in man by natural infections remain fragmentary.

However, if the simple CF test described here proves to be adaptable to studying antibodies of other infectious diseases, general diagnostic laboratories will be able to include information on 19S and 7S antibody characteristics of a patient's serum along with routine antibody titer determinations. Such information, in addition to its theoretical interest, should have direct clinical and epidemiological value; for a laboratory report indicating that the antibodies of a patient under study are primary rather than residual or secondary could not only be of aid to the clinician in his differential diagnosis but could also assist the epidemiologist in tracing the chain of infection where there is suspected man-to-man or animal-to-man transmission of disease. Studies are in progress to determine whether the ethanethiol-CF test used in these studies can be adapted to the differentiation of 19S and 7S antibodies produced in other infectious diseases.

Summary. The CF test can be modified so as to permit the differentiation of 19S and 7S antibodies in primary and secondary typhus infections. The 19S CF antibodies are markedly inhibited by the sulfhydryl compound ethanethiol and to a lesser degree by 60°C inactivation of the sera. The 7S CF antibodies are not inhibited by either of these procedures. The technic described may have application to the clinical and epidemiological investigation of other infectious diseases and may prove useful as a tool in studies of the immune globulins.

The authors are indebted to Dr. John C. Snyder, Dr. Robert S. Chang, Dr. John H. Peters and David Coleman for valuable advice and continuous interest in these studies.

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Received February 23, 1965. P.S.E.B.M., 1965, v119.