

THE SEROLOGICAL DIAGNOSIS OF RELAPSING FEVER*

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Diagnosis in relapsing fever is often difficult because of the inadequacy of available methods. Clinical identification of the disease may be unreliable since the physical signs vary and are frequently atypical. Moreover, the symptomatology may resemble any one of several acute infectious diseases, particularly those having an intermittent febrile course. Outstanding among the infections which may be confused clinically with relapsing fever are malaria, spotted fever, typhus, Weil's disease, and certain of the enteric diseases particularly in their early stages. According to Magee (1) the occurrence of pulmonary involvement in relapsing fever may cause difficulty in making a differential diagnosis from certain acute respiratory infections. In some instances rat bite fever may also be confused with relapsing fever.

The laboratory diagnosis of relapsing fever is more dependable. Although frequently direct microscopic demonstration of the spirochetes in the blood of patients is possible, this method often meets with failure. In that event blood from suspected cases may be injected into suitable experimental animals. Here again, however, unless conditions are optimal, demonstration of the characteristic spirochetes in the blood of experimental animals may be unsuccessful.

Various explanations have been advanced to account for difficulties in detecting the etiological agent. Patients' blood obtained during the "decline" phase of the paroxysm often shows no spirochetes and may rapidly lose infectivity. An inter-relapse specimen may be repeatedly negative both by direct examination and animal inoculation. Inability to induce experimental infection may be attributed in certain instances to the absence of spirochetes from the inoculum, and in others to loss of viability, even though the organisms are present. Failure to infect may also be due to variations in virulence of the spirochetes as well as to differences in host susceptibilities.

In the present report methods are described which it is believed will assist in the differential diagnosis of relapsing fever. Data are presented which suggest that diagnosis can be accomplished by the use of serum from man or animals infected with one or other of the recognized "species" of relapsing fever spirochetes. In order to make this possible, saponin-treated antigens have been prepared which possess the dual advantages of stability and broad specificity.

The immunological picture in relapsing fever is complex. The multiplicity of strains and the ready evolution of relapse variants from any given strain, may give rise to the

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production in man or animals of antibodies which are specific for each strain or variant thereof. In addition, it is possible that antigenic variation may occur on changing from one host to another, as for example by passage through a rodent or an arthropod vector. Because of the many antigenically distinct strains and variants, immunological studies have been difficult to interpret.

A survey of the literature reveals that most of the immunological investigations have followed one of two directions, namely, attempts to elucidate the mechanisms of the relapse phenomenon or efforts to classify the many so called species of relapsing fever spirochetes. These studies made use of a variety of techniques in which spirochete-containing blood or suspensions of spirochetes constituted the antigen.

Because of the antigenic instability of the organisms and difficulties in controlling certain physical factors it is extremely difficult to duplicate many of the investigations which have been carried out previously.

In 1896 Gabritschewsky (2) demonstrated the immobilizing and spirochetolytic action of specific antiserum. Ten years later Novy and Knapp (3) observed that immune serum prepared in experimental animals agglutinated the homologous strain of spirochetes. These workers as well as Manteufel (4) and others, subsequently showed that animals which had recovered from infection with one strain could be infected with another. This led to the now questionable segregation of relapsing fever spirochetes into separate "species." To determine the mechanisms of the relapse phenomenon and to classify the so called "species," both cross-immunity tests and various serological techniques were employed. Levaditi and Roché (5) applied Gabritschewsky's criteria of immobilization and spirochetolysis. Toyoda (6) and Yano (7) adapted the complement fixation reaction to their studies. Slide agglutination tests using darkfield preparations were described by Meleney (8) and Cunningham, Theodore, and Fraser (9). Brussin (10), Adler and Ashbel (11), and Schuhardt (12) relied on the adhesion phenomenon of Rieckenberg (13) to investigate the relapse phenomenon.

Schuhardt (12) has presented an evaluation of the various tests. He has pointed out the merits and limitations of each, expressing the opinion that conflicting reports result from the use of spirochetes having dissimilar antigenic constitutions. He has postulated that these variations in antigenic composition are due to the diverse environments to which the spirochetes may have been subjected. Schuhardt has emphasized, therefore, the need of conducting comparative studies with uniform antigenic strains of relapsing fever spirochetes.

Wenyon (14) and Hindle (15) have asserted that antigenic differences between variants of a single strain may be as marked as those between strains. Kudicke, Feldt, and Collier (16) and Sagel (17) have stated that passage of a given strain through a different animal species alters the antigenic constitution of relapsing fever spirochetes.

On technical grounds, Coleman (18) has criticized the use of infected blood as an antigen for agglutination tests. He has indicated the possibility of drawing erroneous conclusions as a result of spontaneous agglutination of spirochetes in infected blood. He has also pointed out that non-specific agglutination due to excessive incubation or drying may invalidate experimental results.

It is apparent from this review that reliance cannot be placed on available immunological tests since relapsing fever spirochetes possess marked physical and antigenic instability.

Cultivation of the spirochetes *in vitro* would probably assist in resolving many of the problems in relapsing fever. However, reliable cultural techniques have not yet been developed. Consequently, infected blood, despite its known disadvantages, must necessarily serve as a source of spirochetes, since it alone provides the organisms in copious numbers. With this in mind it seemed possible that proper treatment of heavily infected blood might yield an antigen of value in the serological diagnosis of relapsing fever.

EXPERIMENTAL

Separation of Spirochetes from Infected Blood Following Hemolysis with Saponin.—In 1913 Mohler, Eichhorn, and Buck (19), working with dourine, reported the separation of trypanosomes from the blood of infected dogs. The blood was laked with saponin and the trypanosomes separated by centrifugation followed by repeated washings with physiological saline. In modified form, application of these procedures to the present investigation proved to be satisfactory.

Certain precautions are necessary to insure uniformly good results. It is desirable to carry out the separation procedures with blood from animals showing a severe infection (4+ spirochetemia).¹ However the blood should be drawn during the period when multiplication is still rapid, since later, degraded and agglutinated spirochetes may be present, together with specific agglutinins, spirocheticidins, and spirochetolysins. Immediately after cardiac puncture, to avoid non-specific agglutination of the spirochetes, it is necessary to mix the infected blood and the saponin solution thoroughly.

The method of separation follows in detail:—

Four volumes of blood from heavily infected rats or mice were aspirated by cardiac puncture into a syringe containing one volume of a 2 per cent solution of sodium citrate in physiological saline. The mixture was immediately discharged into 15 cc. centrifuge tubes containing a solution of 10 per cent saponin in isotonic saline. The ratio of saponin to citrated blood was maintained at 3:5. The centrifuge tubes were well shaken and allowed to remain at room temperature for from 30 minutes to 1 hour. At the end of this period, although considerable hemolysis had occurred, the mixtures were turbid. This turbidity was largely due to the great numbers of spirochetes in suspension. Examination of the saponinized blood by darkfield microscope revealed many ghost erythrocytes in addition to the numerous thinned and shadowy spirochetes.

The mixture was then centrifuged at 2500 R.P.M. for 45 minutes. This resulted in a reddish-white sediment and a slightly cloudy supernatant. Darkfield inspection

¹ In this report the following symbols represent the degree of infection in experimental animals: < 1+, from 1 to 10 spirochetes per oil immersion field; 1+, from 11 to 20 per field; 2+, from 21 to 50 per field; 3+, from 51 to 80 per field; 4+, more than 80 per field. Darkfield examination was used for these determinations. The virulence of the strains employed was maintained by frequent passage in rats and mice in which overwhelming blood stream infections could be induced. These infections when allowed to follow their natural course often resulted in death.

showed a few spirochetes and many ghost red blood cells in the supernatant, whereas the sediment contained many spirochetes but few red cells. The supernatant was discarded and the sediment resuspended in 5 cc. of physiological saline. Physiological saline containing merthiolate in 1–15,000 concentration was used throughout for washing the spirochetes.

The suspension was centrifuged at 2500 R.P.M. for 30 minutes. The supernatant was now slightly cloudy and contained few spirochetes whereas the white sediment showed very many organisms and some red cell stroma. As washing proceeds the stroma is gradually eliminated with the discarded supernatants. The supernatant was discarded and the sediment resuspended in 10 cc. of saline.

A total of four washings was carried out by the same technique. After the final washing the spirochetal suspensions were pooled and centrifuged at 1300 R.P.M. for 30 seconds to remove the large particles. The sediment was discarded. The extremely cloudy supernatant was then diluted with saline to a turbidity matching that of the No. 5 tube of the MacFarland turbidimetric standards.² Darkfield examination of such a suspension always revealed more than 80 organisms per oil immersion field (4+).

For subsequent serological studies “antigens” of this concentration were employed, although it was determined that spirochetal suspensions of one-half or one-third this concentration could be satisfactorily substituted in the complement fixation tests.

*Spirochetal Suspensions.*³—Suspensions of four different strains or “species” of relapsing fever spirochetes were prepared by the technique outlined above. Suspensions of *Borrelia obermeieri*⁴ were prepared from infected mouse and rat blood; suspensions of spirochetes of which the tick *Ornithodoros hermsi* (Colorado) is the vector, were prepared from the blood of infected mice as well as from rats. The latter strain is referred to as *Borrelia hermsi*, following the suggestions of Brumpt (20) and Davis (21) on the nomenclature of the spirochetes of relapsing fever.

*Sera.*⁵—Sera to be examined for antibodies by agglutination and complement fixation reactions were divided into groups. These included sera from rats, mice, and guinea pigs experimentally infected with various strains of relapsing fever spirochetes; sera from rats and rabbits hyperimmunized with an “antigen” prepared as described from the blood of mice infected with a strain of *B. obermeieri*; hyperimmune serum prepared in rabbits against normal mouse blood; sera from man and animals either

² The No. 5 MacFarland tube represents a turbidity which equals that of a suspension containing 1,500,000,000 bacteria per cc. However, as the spirochetes of relapsing fever are many times the size of ordinary bacteria, exact estimations of spirochetes per cubic centimeter based on the MacFarland series are invalid. Hence, the comparison is one of turbidity only.

³ The strains of relapsing fever spirochetes were generously supplied by Dr. M. S. Soule, Dr. G. E. Davis, Dr. H. W. Moursund, Dr. H. G. Johnstone, and Dr. V. T. Schuhardt.

⁴ Wherever appropriate the generic name for relapsing fever spirochetes is herein given as *Borrelia*.

⁵ Some of the sera were kindly furnished by Dr. J. G. M. Bullowa, Dr. I. A. Bengston, Dr. H. C. Evans, Dr. L. Foshay, Miss N. Styron, Dr. A. Packchianian, Dr. A. M. Pappenheimer, and Dr. E. Hazen. To all the writer expresses his gratitude.

hyperimmunized against or convalescent from various bacterial or rickettsial diseases; sera from three cases of relapsing fever, one infection of spontaneous origin, the others being induced therapeutically in neurosyphilis,⁶ and sera from normal human beings and animals.

Complement Fixation Reactions.—0.1 cc. of spirochetal suspension, 0.1 cc. of varying dilutions of the test sera, and 0.1 cc. (2 units) of complement were pipetted into tubes, mixed, and incubated in a water bath at 37°C. for 30 minutes. 0.2 cc. of the suspension of sensitized sheep erythrocytes was added, making a total volume of 0.5 cc. The tubes were then kept at 37°C. for an additional 10 minutes and the reactions read. Complete fixation of complement is expressed as 4+, absence of fixation as 0. 1+, 2+, and 3+ indicate intermediate degrees of fixation. The highest dilution of a given serum showing 1+ or better fixation is taken as the titre.

Anticomplementary and hemolytic control tests were carried out with each of the different sera and with the various antigens. The volume was made to 0.5 cc. in all instances by the addition of physiological saline.

Complement.—Fresh or lyophilized guinea pig serum served as the source of complement. It was titrated against sensitized sheep erythrocytes. The standard unit (0.05 cc. of the proper dilution) represented the smallest amount of complement which completely hemolyzed 0.1 cc. of a 5 per cent suspension of sensitized sheep red blood cells after incubation in a 37°C. water bath for 15 minutes. Two units (0.1 cc.) of complement were used in the tests.

Hemolytic System.—Washed sheep erythrocytes and amboceptor, prepared by immunizing rabbits with sheep red cells, constituted the hemolytic system. A unit of amboceptor was considered the smallest amount, which in the presence of 2 units of complement (0.1 cc.) and 0.1 cc. of a 5 per cent suspension of sheep erythrocytes, would completely hemolyze the cells after incubation at 37°C. for 15 minutes. Accordingly, the amboceptor was standardized so that 0.05 cc. contained one unit. To prepare a suspension of sensitized sheep red blood cells, one volume of a 5 per cent suspension of erythrocytes was mixed with an equal volume of amboceptor containing 2 units in 0.1 cc. The mixture was allowed to stand at room temperature for 15 minutes, and 0.2 cc. of this suspension of sensitized erythrocytes was used in the diagnostic tests. Sera were inactivated by heating in a water bath at 56°C. for 30 minutes.

Agglutination Reactions.—These tests were carried out by mixing 0.2 cc. volumes of various serum dilutions and 0.2 cc. of spirochetal suspensions. Readings were made after 1 to 2 hours at 37° and again after overnight refrigeration. The titre of a given serum indicates the highest dilution giving 1+ or better agglutination.

The character of agglutination obtained with the saponin-treated spirochetes differs considerably from that observed with ordinary bacterial suspensions. It is soft and cumulous, forming a relatively loose, irregular sediment. The supernatant is clear. On moderate agitation the sediment is broken up and resuspended as extremely minute, uniformly distributed particles. Agglutination and sedimentation take place slowly.

⁶ Patients J.L. and A.B. suffered from syphilis of the central nervous system. The spinal fluid of each showed a 4+ Wassermann reaction. Therapeutic inoculation with both tertian and quartan malaria had been given with only minor improvement, and for this reason therapy with relapsing fever was resorted to.

In contradistinction, the control tubes show little sedimentation, since the spirochetes remain suspended for a comparatively long time.

The Use of Spirochetal Suspensions for the Serological Diagnosis of Relapsing Fever

Agglutination and Complement Fixation Tests with Experimental Relapsing Fever Sera.—Suspensions of spirochetes were prepared by treating infected blood with saponin and repeated washing as described above. Samples of serum were

TABLE I
Agglutination and Complement Fixation Reactions with Serum from Animals Experimentally Infected with Spirochetes of Relapsing Fever

Sera Infecting strain of spirochetes	Animal source	Agglutination with saponinized antigens			Complement fixation with saponinized antigens		
		<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from rats	<i>B. hermsi</i> from mice	<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from rats	<i>B. hermsi</i> from mice
<i>B. duttonii</i>	Rat	1-100 (4+)*	1-100 (1+)		1-1000 (2+)	1-1000 (1+)	
<i>B. kochii</i>	"	1-100 (3+)	1-100 (1+)		1-100 (2+)	1-100 (1+)	
<i>B. novyi</i>	"	1-100 (4+)	1-10 (3+)		1-1000 (2+)	1-100 (3+)	
<i>B. obermeieri</i>	"	1-100 (4+)	1-100 (1+)		1-1000 (1+)	1-100 (3+)	
<i>B. hermsi</i>	"	1-100 (1+)	1-100 (1+)		1-100 (2+)	1-100 (3+)	
<i>B. turicata</i>	"	1-100 (4+)	1-100 (4+)				1-100 (4+)
<i>B. obermeieri</i> †.....	Mouse			1-10 (1+)	1-100 (1+)		1-10 (1+)
<i>B. obermeieri</i> ‡.....	"	1-100 (4+)	1-100 (1+)		1-1000 (1+)		
<i>B. hermsi</i> †.....	"			1-100 (2+)	1-100 (1+)		1-100 (1+)
<i>B. hermsi</i> ‡.....	"	1-10 (1+)	1-100 (1+)		1-100 (1+)	1-100 (1+)	
<i>B. hermsi</i> ‡ from case 2, J.L.....	"			1-10 (4+)	1-100 (1+)		1-100 (1+)
<i>B. turicata</i> †.....	"			1-10 (4+)	1-10 (2+)		1-10 (1+)
<i>B. hermsi</i>	Guinea pig	1-100 (2+)	1-100 (1+)		1-100 (1+)	1-100 (2+)	

* Highest dilution of serum giving a 1+ or better reaction; initial dilution, 1-10. Symbols 1+ to 4+ indicate varying degrees of agglutination or complement fixation.

† Serum obtained from mouse 4 days after infection.

‡ Serum obtained from mouse 11 days after infection.

obtained from various species of animals experimentally infected with one or other strain of spirochetes. These sera were tested in serial dilutions with the spirochetal suspensions for specific antibodies by agglutination and complement fixation reactions. The results are summarized in Table I.

From Table I it can be seen that serum obtained from rats, mice, or guinea pigs which had been infected with one or other of 6 strains or "species" of relapsing fever spirochetes contained antibodies which reacted with each of the three test antigens. Differences in titre occur, but these are not sufficiently great to invalidate the suggestion that the "antigens" possess relatively broad specificity.

The possibility may exist that under similar conditions constant passage of originally distinct strains tends to make them assume antigenic resemblance.

B. duttonii, *B. kochii*, *B. novyi*, and *B. obermeieri* are laboratory strains long maintained in white rats. These were the only strains employed in this study which, to a degree, might fit into the above category. The different tick strains of spirochetes, however, had been recently recovered from nature and possessed a varied host history. One of the antisera tested was obtained from mice which had been infected with the *B. hermsi* strain. Earlier in the course of the present studies this strain of organisms had been resident in ticks. These ticks were allowed to feed on mice, thus establishing the strain in a mammalian species. The spirochetes from mice effectively induced relapsing fever in a patient (case 2, J.L.). Finally, from the human host, the organisms were reestablished in mice. Thus, it becomes plain that this strain had been subjected to a variety of environmental influences.

TABLE II
Complement Fixation Reactions with Serum of Hyperimmunized Animals

Antisera		Complement fixation with saponized antigens	
Antigen	Species immunized	<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from mice
<i>B. obermeieri</i> from mice.....	Rat	1-1000 (1+)*	
<i>B. obermeieri</i> from mice.....	Rabbit	1-1000 (1+)	
Normal mouse blood.....	Rabbit	1-10 (1+)	1-10 (1+)

* Highest dilution of serum giving a 1+ or better reaction; initial dilution, 1-10.

It is of interest to note that specific antibodies were present in mouse sera 4 days after initiation of infection.

Complement Fixation Tests with Serum of Hyperimmunized Animals.—This experiment was carried out to determine whether there was sufficient rat protein in spirochetal suspensions (“antigens”) prepared by saponin treatment to interfere with the interpretation of the complement fixation tests.

Hyperimmune sera were prepared in rats and rabbits by multiple injections of saponinized spirochetal suspensions. Similarly, rabbits were immunized to normal mouse blood. These sera and the saponinized antigens were used to carry out complement fixation tests as shown in Table II.

The data presented in Table II indicate that suspensions of antigen contained insufficient amounts of rat or mouse protein to evoke anything but a minor immune response even upon repeated injection. The slight degree of fixation observed in the mouse protein-antiprotein system (1+ in a 1-10 dilution of serum) is insufficient to affect appreciably the interpretation of the results observed with the spirochete-antispochete system (1+ in a dilution of 1-1000).

The Absence of Relapsing Fever Antibodies in Heterologous Immune Sera.—

Agglutination and complement fixation reactions were carried out with serum from animals which had been immunized by the repeated injection of various

TABLE III
Agglutination and Complement Fixation Reactions with Heterologous Sera

Antisera Immunizing agent	Source	Agglutination with saponinized antigens		Complement fixation using saponinized antigens		
		<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from rats	<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from rats	<i>B. hermsi</i> from mice
<i>Salmonella paratyphi</i> A.....	Rabbit	0*		0		
<i>Salmonella paratyphi</i> B.....	Rabbit	0		0		
<i>Salmonella enteritidis</i>	Rabbit	0		0		
<i>Salmonella typhi murium</i> ...	Rabbit	0		0		
<i>Shigella dysenteriae</i> (Flexneri).	Rabbit	0		0		
<i>Shigella dysenteriae</i> (Sonnei)..	Rabbit	0		0		
<i>Eberthella typhosa</i>	Human	0	0			1-10 (0)‡
<i>Bacillus proteus</i> OX 19.....	Rabbit	0		0		
<i>Brucella abortus</i>	Rabbit	0		0		
<i>Brucella abortus</i>	Human	0	0			
Pneumococcus, Type I.....	Horse	0		0		
Pneumococcus, Types I, III, VII, VIII, pooled.....	Rabbit	0	0	0	0	
β hemolytic streptococcus, group A.....	Rabbit	0		0		
<i>Pasteurella tularensis</i>	Goat	1-10 (1+)	0	1-10 (1+)	0	0
<i>Streptobacillus moniliformis</i> ...	Human					0
<i>Plasmodium falciparum</i>	Human					0
<i>Rickettsia rickettsi</i>	Human	0	0		0	1-10 (0)‡
<i>Rickettsia rickettsi</i>	Guinea pig	0		0	0	
<i>Rickettsia prowazeki</i>	Human	0	0	1-10 (0)§	0	1-10 (0)§
<i>Rickettsia prowazeki</i>	Guinea pig			0	0	
<i>Leptospira icterohemorrhagiae</i> ..	Human	0	0	0		
<i>Treponema pallidum</i>	Human	0	0	0		

* Initial serum concentration, undiluted or diluted 1-10; 0 signifies absence of agglutination or complement fixation.

‡ Undiluted serum was anticomplementary.

§ With undiluted human serum from a typhus fever convalescent, reaction was slightly positive; however, when this serum was diluted 1-10, no reaction occurred.

infectious agents. Serum from man and animals convalescent from infections other than relapsing fever were also examined. Again, suspensions of spirochetes separated from infected mouse and rat blood constituted the antigens. The results of these tests are shown in Table III.

From the data in Table III it can be seen that with the exception of a convalescent human typhus serum (1+ in undiluted serum) and of a goat antitularse serum (1+ in a 1-10 dilution) no reactions occurred. It is of interest that serum from patients infected with species of two other spirochetal genera, *Leptospira icterohemorrhagiae* and *Treponema pallidum* did not contain demonstrable antibodies against *Borrelia*.

*Agglutination and Complement Fixation Tests with Sera from Cases of Relapsing Fever.*⁷—Agglutination and complement fixation tests were carried out with serum from three human beings who had suffered from relapsing fever.

Case 1 (A.P.).—This individual contracted relapsing fever in 1906 while carrying out experimental studies of the disease in the laboratory. The infecting strain is now known as *B. novyi*. Blood for the present studies was obtained in 1942, 36 years later.

Case 2 (J.L.).—White male; age 45 years. The patient was a neurosyphilitic who had been infected therapeutically with tertian malaria 1 year previously with only minor improvement in neurological symptoms. 2 months before the present inoculation with *B. hermsi*, he had been given quartan malaria, but because of the development of jaundice, malaria was terminated after the second chill. The Wassermann reaction of the spinal fluid remained strongly positive. Because of the failure of malarial therapy, the patient was inoculated with the tick strain of *B. hermsi*. Skin test with normal mouse serum, diluted 1-100 with normal saline, showed no evidence of sensitivity to mouse protein. 1 cc. of whole citrated blood from heavily infected mice was inoculated intramuscularly. The 1st paroxysm of fever occurred 12 hours after inoculation, and during the succeeding 17 days he experienced 5 additional paroxysms. Following the 6th paroxysm, relapsing fever subsided spontaneously. At no time during the course of the disease were spirochetes demonstrable in the patient's blood by direct microscopic examination. Spirochetes were recovered, however, from mice inoculated with blood obtained from the patient 48 hours after the original inoculation. Blood for serological studies was obtained before infection and at intervals thereafter for 59 days.

Case 3 (A.B.).—Negro male; age 37 years. The patient was a neurosyphilitic who had been given a therapeutic inoculation of tertian malaria 2 years previously without the development of infection. 9 months before infection with relapsing fever spirochetes, he had been inoculated with quartan malaria, and although 9 paroxysms occurred, his neurological symptoms were only slightly improved and the Wassermann reaction of the spinal fluid remained strongly positive. Because of the failure to improve following malaria, therapeutic inoculation with *B. hermsi* was carried out. The strain employed was recovered from mice which had been infected with the blood of case 2 (J.L.). The inoculum consisted of 1.0 cc. of whole citrated blood obtained from heavily infected mice, and was injected intramuscularly. Skin test with diluted

⁷ Numerous attempts were made to procure sera from naturally infected cases of relapsing fever in order to extend the present investigations. However, these efforts proved unsuccessful. Since the writer entered the United States Army during the course of the study, further work with human relapsing fever was interrupted.

normal mouse serum, carried out before the injection of whole mouse blood, showed no evidence of sensitivity. The 1st paroxysm occurred 24 hours following injection, and 4 additional paroxysms were experienced over the succeeding 12 days, whereupon the infection subsided spontaneously. The patient's blood was infective for mice during the 4th paroxysm, although repeated microscopic examination of his blood during the course of the disease failed to reveal spirochetes. Blood for serological studies was obtained from the patient before infection with *B. hermsi*, and at intervals thereafter for 26 days.

TABLE IV
Agglutination and Complement Fixation Reactions with Sera from Cases of Relapsing Fever

Case No.	Sera		Agglutination with saponinized antigens	Complement fixation with saponinized antigens	
	Infecting strain	Interval between infection and bleeding for serum	<i>B. hermsi</i> from mice	<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from mice
1	<i>B. novyi</i>	36 yrs.	Undiluted (3+)*	1-10 (1+)	1-10 (1+)
2	<i>B. hermsi</i> in mouse blood	Preinfection	Undiluted (0)	1-10 (0)‡	1-10 (0)‡
		17 days	1-500 (1+)	1-10 (2+)	1-500 (1+)
		38 days	1-100 (2+)	1-25 (1+)	1-50 (1+)
		45 days	1-100 (1+)	1-50 (1+)	1-50 (1+)
		59 days	1-10 (1+)	1-10 (1+)	1-100 (1+)
3	<i>B. hermsi</i> in mouse blood	Preinfection	1-10 (0)‡	Undiluted (0)	1-10 (0)‡
		6 days	1-10 (3+)	1-10 (1+)	1-50 (1+)
		20 days	1-100 (2+)	1-100 (1+)	1-100 (1+)
		26 days	1-10 (4+)	1-10 (2+)	1-10 (3+)

* Tests included undiluted as well as serially diluted sera; 0 signifies absence of agglutination or complement fixation; symbols 1+ to 4+ indicate varying degrees of agglutination or complement fixation.

‡ Reactions with undiluted sera were slightly positive. However, after these sera were diluted 1-10, no reactions occurred.

Two different suspensions of saponinized spirochetes constituted the antigens. One was prepared from the blood of rats infected with the *B. obermeieri* strain; the other was prepared from mice infected with the *B. hermsi* strain. The results of the agglutination and complement fixation reactions are shown in Table IV.

The data recorded in Table IV suggest that serum from an individual long recovered from relapsing fever (36 years) still contained specific circulating antibodies, although in low concentration. Results of tests with sera from the two recent relapsing fever infections (case 2 and case 3) indicate the presence of specific antibodies in both acute phase and convalescent sera. These sera fixed complement in the presence of the two different saponinized antigens, one prepared from rats infected with *B. obermeieri*, the other from mice infected with

B. hermsi. The titres of the sera differed somewhat with the two spirochetal suspensions, but not to an extent which would invalidate the suggestion that the antigens possessed broad specificity.

The weak reactions observed with the undiluted serum obtained from cases 2 and 3 before infection, disappeared when the serum was diluted 1-10.

All postinfection sera from the relapsing fever patients agglutinated saponin-separated spirochetes of the *B. hermsi* strain, the only antigen used in the tests.

Absence of Antibodies to Mouse Proteins in Serum from Case 2.—Case 2, J.L., had been inoculated with infected mouse blood. In consequence, the coexistence in his blood of antibodies specific for mouse proteins as well as for spirochetes seemed a possibility. Moreover, although the spirochetal antigens had

TABLE V
Antibodies to Mouse Proteins and Spirochetes in Serum of Case 2 (J.L.)

Antigens	Dilutions of human relapsing fever serum									
	Precipitin reaction		Complement fixation				Agglutination reaction			
	1-10	1-100	1-10	1-100	1-250	1-500	1-10	1-100	1-250	1-500
Normal mouse serum										
1-10	0	0	0	0						
1-100	0	0	0	0						
1-1000	0	0	0	0						
1-10,000	0	0	0	0						
<i>B. hermsi</i> *			4+	4+	2+	1+	4+	4+	3+	1+

* Spirochetes separated from infected mouse blood following treatment with saponin.

been separated from infected mouse or rat blood, some blood elements might conceivably still be present in the spirochetal suspensions. It seemed necessary, therefore, to determine whether or not a mouse protein-antiprotein system had been inadvertently established, and if so, to what extent it affected the results with the spirochete-antispochete systems. To this end, precipitin and complement fixation tests were carried out. Normal mouse serum constituted the antigen, while the 17 day serum from case 2 was used as the human relapsing fever antiserum. For purposes of comparison, agglutination and complement fixation tests, employing a saponinized spirochetal antigen and the patient's serum, were also performed.

The results of the precipitin, complement fixation, and agglutination tests are recorded in Table V. The first two reactions indicate that by the methods employed the patient's serum contained no detectable antibodies against mouse proteins. In contradistinction the same serum, in a 1-500 dilution, agglutinated the spirochetal antigen and also fixed complement in its presence.

Agglutination and Complement Fixation Tests with Normal Sera.—Normal sera were obtained from rats, mice, guinea pigs, rabbits, and human beings. Antigens consisted of four different saponin-separated suspensions of spirochetes. Two were obtained from the blood of rats and mice infected with the *B. obermeieri* strain, and two from rats and mice infected with *B. hermsi* strain. The various normal sera were tested for their agglutinating and complement-fixing capacities.

The results presented in Table VI show that normal sera of man and animals contained no antibodies to the spirochetes of relapsing fever as determined by either agglutination or complement fixation reactions.

TABLE VI
Agglutination and Complement Fixation Reactions with Normal Sera

Sera		Agglutination with saponinized antigens			Complement fixation using saponinized antigens		
Source	No. tested	<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from rats	<i>B. obermeieri</i> from mice	<i>B. obermeieri</i> from rats	<i>B. hermsi</i> from rats	<i>B. hermsi</i> from mice
Rats.....	8	0*	0	0	0	0	
Mice.....	9	0		0	0		
Guinea pigs.....	6	0		0	0		
Rabbits.....	8	0	0	0	0	0	
Human beings.....	6	0	0	0	0		0

* Initial serum concentration undiluted or diluted 1-10; 0 represents absence of agglutination or complement fixation.

DISCUSSION

Solution of certain of the perplexities associated with relapsing fever awaits the application of exact methods. One of the chief difficulties has been the fact that the source of spirochetes has been infected blood almost exclusively, a medium which precludes precise bacteriological and immunological analysis.

Although a suitable medium for *in vitro* cultivation was not available, a method of separating the spirochetes from infected blood and preserving them in antigenically stable form seemed worth procuring. By laking spirochete-containing blood with saponin and washing the separated spirochetes with physiological saline such an antigen was obtained. This antigen, when tested with sera of animals experimentally infected with various strains or "species" of relapsing fever spirochetes, reacted with all. This would indicate that antigens prepared in this manner have broad immunological specificity. These observations suggested its applicability to the serological diagnosis of relapsing fever.

The antigen possesses a feature of distinct merit, namely its stability. After being stored in the refrigerator for as long as 4 months, no apparent change in its

reactivity could be detected. From the viewpoint of practicability, the quantity of antigen prepared from several heavily infected rats is sufficient to carry out a considerable number of diagnostic tests. Furthermore, during the course of the present studies it was determined that even after the standard (4+) suspension of antigen was diluted threefold or more, its ability to bind complement was unaffected.

The mechanism by which saponin appears to alter the antigenic constitution of the spirochetes from one of extremely narrow specificity to one which is relatively broad has not been investigated.

Of the two techniques employed to detect circulating antibodies in relapsing fever serum the macroscopic agglutination test possesses the advantage of technical simplicity. However, the appearance of the agglutinated spirochetes is different from that usually observed with bacteria. Agglutination of the saponinized spirochetes occurs in the form of loose, cumulous, irregularly shaped particles whose sedimentation rate is low. In positive reactions the supernatant is clear while the sediment is fluffy. On agitation this sediment breaks up into minute, uniformly suspended particles. The complement fixation reaction, on the other hand, although technically more complicated, in addition to being somewhat more sensitive than agglutination, can be read without difficulty. For this reason, the complement fixation reaction was found to be more desirable in the present study.

For reasons already indicated the present investigations could not be extended. Obviously it would be desirable to study additional human relapsing fever sera obtained during various stages of active disease and convalescence. Moreover, serological determinations using antigens prepared from additional strains of spirochetes should be carried out. It may be suggested that serodiagnostic efficiency might be enhanced by attempting complement fixation tests with a "polyvalent antigen," *i.e.*, a mixture of saponin-separated antigens from two or more different strains of relapsing fever spirochetes.

SUMMARY AND CONCLUSIONS

1. Spirochetes of relapsing fever have been separated from the blood of heavily infected mice and rats by hemolysing with saponin, followed by repeated washing of the spirochetal suspension with physiological saline.

2. Spirochetes obtained in this manner appear to have broad antigenic specificity. Antigens of this type fixed complement in the presence of serum obtained from man or animals infected with one or other of the recognized strains or "species" of relapsing fever spirochetes. Macroscopic agglutination of the antigens likewise was observed with sera from the same sources.

3. Positive serological reactions were not observed with convalescent sera obtained following infection with other diseases, for example, typhus fever, malaria, Rocky Mountain spotted fever, Weil's disease, syphilis, and typhoid

fever. Hyperimmune sera prepared against other pathogens also failed to react with the relapsing fever antigens.

4. No apparent change in the antigen occurred following storage in the ice box for as long as 4 months.

5. The results indicate that treatment of the spirochetes of relapsing fever with saponin yields a relatively stable antigenic preparation which may prove useful in the serological diagnosis of this disease.

BIBLIOGRAPHY

1. Magee, G. R., in Relapsing fever in the Americas, Washington, American Association for the Advancement of Science, (F. R. Moulton, editor), 1942, No. 18, 106.
2. Gabritschewsky, G., *Ann. Inst. Pasteur*, 1896, **10**, 629.
3. Novy, F. G., and Knapp, R. E., *J. Infect. Dis.*, 1906, **3**, 291.
4. Manteufel, *Arb. k. Gsndhtsamte*, 1908, **29**, 355.
5. Levaditi, C., and Roché, J., *Compt. rend. Soc. biol.*, 1907, **62**, 815.
6. Toyoda, H., *Kiiasato Arch. exp. med.*, 1920, **4**, 40.
7. Yano, M., *Acta. dermat.*, 1937, **29**, 85.
8. Meleney, H. E., *J. Exp. Med.*, 1928, **48**, 65.
9. Cunningham, J., Theodore, J. H., and Fraser, A. G. L., *Indian J. Med. Research*, 1934, **22**, 105.
10. Brussin, A. M., *Z. Immunitätsforsch.*, 1925, **44**, 328.
11. Adler, S., and Ashbel, R., *Ann. Trop. Med. and Parasitol.*, 1937, **31**, 89.
12. Schuhardt, V. T., in Relapsing fever in the Americas, Washington, American Association for the Advancement of Science, (F. R. Moulton, editor), 1942, No. 18, 58.
13. Rieckenberg, H., *Z. Immunitätsforsch.*, 1917, **26**, 53.
14. Wenyon, C. M., *Protozoology*, London, Baillière, Tindall & Cox, 1926, 1244.
15. Hindle, E., *A system of bacteriology in relation to medicine*, Great Britain Medical Research Council, London, His Majesty's Stationery Office, 1931, **8**, 101.
16. Kudicke, R., Feldt, A., and Collier, W. A., *Z. Hyg. u. Infektionskrankh.*, 1924, **102**, 135.
17. Sagel, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1930, **34**, 429.
18. Coleman, G. E., *J. Infect. Dis.*, 1934, **54**, 1.
19. Mohler, J. R., Eichhorn, A., and Buck, J. M., *J. Agric. Research*, 1913, **1**, 99.
20. Brumpt, E., *Compt. rend. Soc. biol.*, 1933, **113**, 1366, 1369.
21. Davis, G. E., quoted by Johnstone, H. G., in Relapsing fever in the Americas, Washington, American Association for the Advancement of Science, (F. R. Moulton, editor), 1942, No. 18, 35.