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STUDIES IN BORRELIAE (*)

II. SOME IMMUNOLOGIC, BIOCHEMICAL AND PHYSICAL PROPERTIES OF THE ANTIGENIC COMPONENTS OF *BORRELIA TURICATAE*

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The immunologic activities of the antigenic components of borreliae causing relapsing fever have not been studied as yet, though some work on the chemical composition of borreliae has been reported. The amnio acids present in whole organisms and the nucleoproteins in crude extracts were listed by Geiman (1). Calabi (2) studied the influence of the plasma inhibitors in infections with *B. novyi* and found that they are related to beta-globulins. The sera and the organisms used in agglutination, adhesin, complement fixation, borreliocidin, immobilizing and cross-protection tests were not studied beyond their basic thermal sensitivity and complement requirements (3. Levaditi *et al.* (4), however, established that *Borrelia* "immobilizines" have a low molecular weight, but did not study their composition.

Relapsing fever in man and animals is characterized by a primary attack (hereafter called "attack") which may or may not be followed by relapses. The

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disease is either self-limited or it is a fatal illness, with death usually occurring during one of the clinically manifest acute phases. One of the remarkable features of relapsing fever is that the borreliae undergo antigenic variations during the attack as well as during relapses. This phenomenon has been demonstrated by the study of the immobilizing and borreliolytic activity of immune sera as well as by animal cross-protection tests. The immunologic response to variant or mutant borreliae appearing during the course of the disease consists of the formation of antibodies different from those observed during the attack (for a review see (3)). The role of the physically and/or chemically separable antigenic fractions in these variations has not been investigated as yet. Perhaps the reasons for the lack of information on *Borrelia* antibodies and antigens are that these organisms have to be grown in the presence of living cells, that they are not numerous in the blood of infected animals or in developing chick embryos, and that the antigenic composition may vary from strain to strain.

B. novyi has been employed in the United States as an experimental model for *Borrelia* studies. This organism has been maintained in rats for several decades and has become well adapted to those animals. *B. novyi* has to be transferred from animal to animal by syringe and not by a natural vector. Since the aim of the present experiments was to reproduce natural conditions as closely as possible, one of the most frequent relapsing fever borreliae of the United States, *B. turicatae*, was selected for the initial studies.

In working with this tick-borne strain, the choice of a feasible experimental animal was limited to young rats and monkeys. In rats, only one or two borreliae were found in the blood smears per high power field. The number of the organisms obtained after cardiac puncture was small. The amount of blood which could be obtained from a 3-week-old rat was 5 to 7 ml. This volume seldom harbored more than 1×10^7 borreliae. Since approximately 2.5×10^9 borreliae/ml of 0.15 M NaCl solution are necessary to make a suspension that will match the third tube of the McFarland nephelometric scale, it is evident that large numbers of animals had to be sacrificed to obtain sufficient material for merely fundamental studies. The results of these investigations, carried out over a period of 2 years, are given in this report.

PROCEDURES

The strain selected for the present studies was *Borrelia turicatae* (BT) Brumpt 1933 maintained in *Ornithodorus turicata* Dugès 1876. The ticks were received from Dr. W. Burgdorfer from the United States Public Health Sevrice Laboratory in Hamilton, Montana. They were fed at 3-month intervals on 18- to 22-day-old rats. In the interims, the ticks were kept in a simple tickorium consisting of a gauze covered Coplin jar with short glass tubes loosely fitted into it. Moist absorbent cotton was kept at the bottom. The ticks lived in the short glass tubes, which had cotton stoppers and strips of filter paper in them.

Nearly all (93 to 96%) *O. turicatae* fed on infected animals took up this

Borrelia and were able to transmit it by bite when fed on young (20- to 23-day-old) white rats of an undetermined laboratory strain. The rats developed one attack and about one half (44 to 52%) of them had one relapse. Second and third relapses were not observed under these experimental conditions. The animals did not die as a result of the infection.

B. parkeri var. *california* (BP) from the George W. Hooper Foundation in San Francisco, another North American *Borrelia*, was used as control. It was maintained in *O. parkeri* var. *california* Rafyi under identical conditions as *O. turicata*. It caused one attack in young (20- to 23-day-old) rats, but no relapse or death. The organisms were scarce in the peripheral circulation. Their count never exceeded 2×10^6/ml blood.

All animal bleedings were performed under anesthesia. The borreliae were collected from the rats by heart puncture during the attack or the relapse. Nine parts of blood were allowed to flow into 1 part of 0.15 M NaCl with 0.12 M sodium citrate. After centrifuging at 2500 × G for 30 min, then at 2000 × G for 15 min, and repeated washing of the sediment with 0.15 M NaCl until the supernatant did not precipitate with rabbit antirat serum, the organisms were suspended in distilled water and broken up by repeated freezing at —40°C and thawing. Ultrasonic vibration under cooling with the Sonifer apparatus (Branson Instrument Company), however, gave better yields. The latter method was adopted, therefore, for the preparation of homogenates which were considered the crude antigen (CR) of borreliae. The abbreviations BTA and BTR will be used for crude antigens from *B. turicatae* collected during the attack and in the relapse, respectively, while BPA will be used for antigens from *B. parkeri*.

Immune sera. The CR antigen, as well as fractions thereof *(vide infra)*, were standardized to contain 2 to 5 mg protein/ml and injected into rabbits together with Freund's incomplete adjuvant. The rabbits received 0.5 and, 3 days later, 1.0 ml s.c. in divided doses in several areas. Then 0.1, 0.25, 0.5 and 1.0 ml of the antigen solution without adjuvant were given i.v. at 3- to 4-day intervals, the last dose being repeated 6 times. The rabbits were bled 5 to 8 days after the last injection. Rabbit anti-rat sera for immuno-electrophoresis studies were prepared in the same manner but without Freund's adjuvant and omitting the s.c. injections.

Blood for serologic studies was collected from infected rats 3 to 4 days after the attack or the relapse. Sera from uninfected rats of the same age were used for comparison.

The sera were kept at —40°C without a preservative.

Chemical procedures. The crude antigens were subjected to chemical and physical treatment. The ninhydrin test (5) was used for protein, and nesslerization (6) for N determinations. The biuret reaction (7) was utilized as a rapid test for eluates after electrophoresis.

Amino acids were tested by paper chromatography after hydrolysis in 3 N HCl and subsequent neutralization to pH 7.0. Amino acids which may be destroyed by harsh hydrolysis, such as tryptophan, serine, threonine, cystein and cystine, were estimated according to the method of Young (8).

The lipopolysaccharides and nucleic acids were separated by the method of O'Neill and Todd (9). Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were differentiated according to the method of Brown *et al.* (10) and checked by the procedure of Hotchkiss (11).

Immunologically active lipopolysaccharides were extracted from CR by the method of Fife and Kent (12) and studied by silica gel chromatography (13).

Electrophoretic and chromatographic separation. Zone electrophoresis was performed with the E-C electrophoretic apparatus and with the Buchler standard model. As a rule, Whatman No. 3MM paper was used for paper electrophoresis. Slides with 1% special agar (Difco) in 0.025 M barbiturate buffer at pH 8.6 and 4 V/cm for 45 min were employed for immunoelectrophoresis. For better separation of the beta-globulin in studies related to this protein, the modification of serum protein fractionation of Lataste-Dorolle *et al.* (14) was used. The Buchler apparatus was employed for starch gel vertical plate electrophoresis with 15% special starch (Connaught Laboratory) in 0.03 M borate and 0.012 M NaOH buffer at pH 8.6 and 5 V/cm for 16 to 18 hr.

The velocity of movement in the electric field was determined by using dextran and bromphenol blue as an indicator (15).

The stains employed were amido black 10B, Schiff's reagent and Oil Red O saturated in alcohol, as well as pyronin Y, following the recommendations of Smith (15).

Paper chromatograms for carbohydrates were set up according to Baar and Bull (16). Thinlayer chromatography of lipid materials was performed in 30% silica gel in comparison with standard lecithin and cholesterol solutions (13). The two-dimensional paper chromatography of amino acids was done on Whatman No. 1 paper, using Cassidy's procedure (17) with standard amino acid solutions as controls.

Antigenic fractions. The main means for studying the components of the CR antigenic material were paper and starch gel electrophoresis. Parallel strips were run in the former procedure. After completed paper electrophoresis one strip was stained with amido black 10B. The eluates of representative segments were used in the agar gel diffusion technique for precipitation experiments. The protein content of these eluates was determined by the biuret reaction. Starch plates were cut horizontally. The upper layer was tinted with the appropriate stain. Blocks were cut from the underlaying parts and were homogenized in phosphate or in 0.08 M tris buffer at pH 7.4 and centrifuged at 2500 × G at 0°C for 30 min. The supernatant was decanted, the sediment homogenized again and centrifuged, and the supernatants dialyzed against distilled water in Viking tubings and kept at —40°C.

Serum fractioning. gamma- and beta-globulins were fractioned by the method of Heremans *et al.* (18) and purified by starch gel electrophoresis. In some experiments it was more convenient to simply separate the gamma-globulins as described by Heremans *et al.* (18) and to use the remaining serum as a "—gamma serum." Parallel serologic tests were run with the "—gamma serum" and with

the isolated gamma fraction as well as with the original serum as recommended by Cohen and McGregor (19).

Proof of activity. Agar gel diffusion experiments served as *in vitro* tests for the activity of the sera and antigens, as well as of their fractions. It was understood that this precipitation did not necessarily indicate other antigenic activity as, e.g., that of adhesin, immobilizine or borreliolysin.

Because of the scarcity of the materials, the Preer method (20) was used to a great extent. It was performed in tubes of approximately 1.7 to 2.0 mm diameter. Three-tenths per cent special agar (Difco) in 0.15 M NaCl and 0.02 M phosphate buffer at pH 7.4 with 1×10^{-4} merthiolate was employed. For slide and plate diffusion tests, the same medium but with 0.8% agar was used . In the latter, wells 2 mm in diameter, placed 3 mm apart, gave clear-cut results.

In agar gel electrophoresis utilizing slides with a trough, alternate experiments were set up for more detailed studies of antigen-antibody relations by first subjecting the antiserum in the well to electrophoresis and then filling the trough with the CR or one of its fractions, then putting CR in the well and, after electrophoresis, filling the trough with the antiserum or its components.

Tests in which precipitated antibody N had to be determined were carried out in 0.15 M NaCl and 0.02 M phosphate buffer at pH 7.4 for 2 hr at 37°C followed by repeated centrifuging at 2500 × G, decantation and washing for 30 min. The equivalence zones were determined by varying the amount of antigen against standard portions of the antiserum.

Establishing the composition of antigenic fractions. The analytic methods used for the separation of nucleoproteins, lipids and polysaccharides from the CR were not applied to antigenic fractions separated with the aid of electrophoresis because of the shortage of antigenic material. The fractions were exposed, however, to the following physical and chemical agents to determine their basic characteristics:

Heating in the boiling water bath for 30 min in 0.25 M HCl—KCL buffer at pH 2; in 0.5 M phosphate buffer at pH 7.0; and in M ammonia-ammonium chloride buffer at pH 11.0, respectively.

Pepsin (Armour Company), 1 mg/ml in 0.2 M HCl and 0.2 M KCl at pH 2.0 for 30 min at 37°C.

Chymotrypsin (Sterile, Nutritional Biologicals Laboratory), 0.2 mg/ml in 0.05 M phosphate buffer at pH 7.4 for 18 hr at 37°C.

Papain (Crystalline, N.B.L.), 0.1 mg/ml in 0.03 M phosphate buffer at pH 7.0 for 18 hr at 37°C.

Lipase (448, N.B.L.), 5 mg/ml in M sodium bicarbonate-sodium carbonate buffer with 0.005 M CaCl2 at pH 9.2 for 18 hr at 37°C.

Lysozyme (Crystalline, Armour & Company), 0.5 mg/ml in 0.5 M phosphate buffer at pH 7.4 for 18 hr at 37°C.

Ribonuclease (Crystalline, N.B.L.), 10 micro g/ml in Krebs buffer at pH 7.4 for 30 min at 37°C.

Deoxyribonuclease (Crystalline, N.B.L.), 50 Dornase units/ml in Krebs buffer with 0.04 M MgSO4 for 30 min at 37°C.

Urea. 4 M aqueous in distilled water for 18 hr at 37°C.

Periodic acid, 0.2 M aqueous for 15 min at room temperature followed by neutralization with 0.1 M NaOH.

Ultracentrifugal analysis. The sedimentation coefficient of the material in each purified fraction was determined with a Spinco model E ultracentrifuge. The samples were centrifuged at 52,640 rpm in an aluminum centerpiece at 25°C in 0.1 M phosphate buffer. Most of the samples had a protein nitrogen concentration of 0.5 to 1%, so that the sedimenting boundary could be observed as a distinct peak with the Schlieren optics. The sedimentation coefficient was calculated and corrected to water at 20°C for the $s_{20,w}$ values.

An estimate of the sedimentation coefficient of the antigenic material in each purified fraction was also made by a method described by Yphantis and Waugh (21). This method makes use of a special cell which is divided into two compartments. The fraction of active material remaining in the upper compartment after centrifugation for a given time is used to calculate the sedimentation coefficient. Samples were centrifuged for various times at 52,640 rpm at 25°C, the rotor was stopped and the antigenic titer in the upper chamber was determined for these calculations.

Immobilizing and lytic antibodies. These tests were performed in 0.15 M NaCl and 0.02 M phosphate buffer at pH 7.2. Suspensions of 5000 + 380 borreliae/ml of buffer were used. The sera were employed in 1.5-fold dilutions, from 1:4 to 1:1180. Two-tenths milliliter of the *Borrelia* suspensions were added to each 0.8-ml portion of the serum dilutions. The tubes were incubated at 37°C for 2 hr, centrifuged at 1500 × G for 10 min and the borreliae observed under the microscope. Appropriate controls were used in all instances.

The absorption of undiluted sera was carried out according to Sonneborn (22). Absorbed sera were tested for their immobilizing and lytic activities in the same manner as the unabsorbed sera.

The procedure of Mayer *et al.* (23) and Heidelberger (24) was followed for the determination of hemolysin and the amount of fixed complement. Sera were prepared for the test by heating at 56°C for 30 min. All sera were absorbed with sheep RBC's at 0° for 30 min before testing. The CF test was carried out in 4.5×10^{-4} M CaCl2 and 5×10^{-4} M MgCl2 in phosphate-saline buffer at pH 7.3. Parallel tests were run omitting antisera to measure the degradation of the complement due to the incubation at 37°C.

CF tests for diagnostic purposes were carried out according to the method of Fife and Kent (12).

Passive protection tests were performed on groups of 6 young (20- to 24-day-old) rats. The immune sera were administered in 0.1-ml amounts/100 g body weight, 5 to 15 min before infection.

All tests were performed in triplicate. The means and the ranges of the results are shown in the tabulations.

RESULTS

Chemical constitution of CR. The crude antigens of the examined borreliae did not show significant variations in their gross biochemical composition. When *B. turicatae* from attack (BTA) and from relapse (BTR) as well as *B. parkeri* from attack (BPA) were analyzed, their total protein content was 34.2 (32.7 to 39.2%).

All crude antigens contained alanine, aspartic acid, cystine, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine, but arginine, cysteine, histidine and methionine could not be demonstrated.

The nucleoproteins had almost no mobility in starch gel electrophoresis at pH 8.6. The DNA: RNA ratio was 1:1.27 (1.22 to 1.31). The nucleoprotein fraction showed only weak precipitation with homologous anti-CR sera and was not studied further.

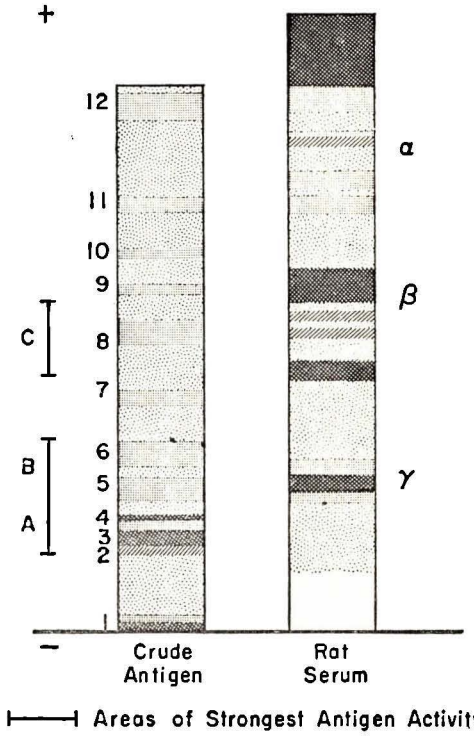

Figure 1. Schematic starch electrophoretic pattern of *Borrelia turicatae.*

The total lipids constituted 12.2 (11.1 to 14.1) % of the CR.

The lipopolysaccharide materials were 2.3 (2.1 to 2.6) % of the total weight, with 2.2 (1.9 to 2.4) % N and 0.9 (0.7 to 1.1) % P. The lipopolysaccharide moved at pH 8.6 at -2×10^{-5}/cm^2/V/sec in the electric field. The lipid part constituted 34.2 (32.1 to 36.4) % of the lipopolysaccharide and gave lines identical with cholesterol and lecithin, as well as 4 other unidentified zones, by silica gel chromatography. Lipopolysaccharides were used in the CF test. After hydrolysis with 2 N HCl, 46.1 (45.3 to 48) % reducing sugars were found, of which glucose was identified.

Antigenic fractions. Crude BTA, BTR and BPA antigens gave similar results by electrophoretic analysis. Several protein-like constituents were separated according to their mobility and zone formation after starch gel electrophoresis and subsequent staining as well as the biuret reaction of the eluates from segments of the starch gel. Figure 1 demonstrates the results of the starch gel electrophoresis of BTA CR, showing the 12 lines and bands usually observed when the CR of the borreliae used in this experiment were subjected to starch gel electrophoresis.

Of the 12 lines or bands observed in starch gel electrophoresis, 1, 3 and 4 were strongly marked (Fig. 1). Line 1 was diffuse. Lines 3 and 4 coalesced in several instances, presenting a broad band. The staining intensity of Line 2 varied. Figure 1 also shows the starch gel electrophoretic pattern of the serum of a young uninfected rat. Bands 5 and 6 of the BTA design were opposite that of rat gamma-globulin, while Bands 7 to 10 covered an area occupied in the rat serum pattern by beta-globulins. The alpha-globulins did not give strong tracing in these rats. The rat serum albumins formed a diffuse tract. The anodic end of the CR pattern tapered off.

The eluates from the areas of the starch gel from Line 2 to and including Line 4, the area of Bands 5 and 6, and the area between Bands 7 and 9 showed strong precipitating activity against homologous CR antisera. The eluates from these segments were considered antigenic fractions or factors.

The crude antigens were tested also with the aid of the Ouchterlony technique. Figure 2 is a schematic representation of the results. There seemed to be one line common to BT and BP. It did not appear when the CR antiserum was absorbed with Fraction B eluted from the starch gel plate but it was visible after the antiserum was absorbed with Fractions A, C or A + C. It was considered, therefore, that Fraction B represented part or all of a nonspecific antigen. The line nearest to the wells containing the antigen was not apparent when then CR antiserum was absorbed with the homologous Fraction C. A precipitation line, often composed of several fine lines, was found to be caused by an AB-AG reaction involving Fraction A in tests with absorbed antisera. Factor A appeared to be specific for BTA, BTR and BPA. Since the presence of "lines in the Ouchterlony test is a function of quantities of antigens present, further studies are indicated in this direction.

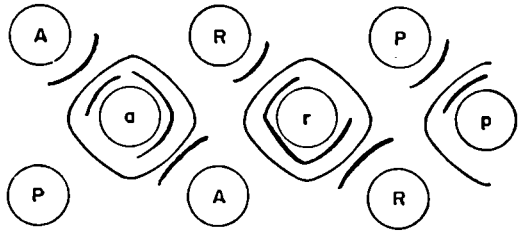

Figure 2. Cross-reaction between borreliae in the Ouchterlony test. *A*, *R*, *P*, antigens from *Borrellia turicatae* from attack and relapse, and from *B. parkeri*; *a*, *r*, *p*, corresponding antisera.

The over-all relationship of the antigenic components was confirmed by testing rabbit immune sera prepared against the individual antigenic factors against CR and its components. Immune sera were subjected to agar gel electrophoresis, and the precipitation lines corresponding to the individual serum globulins were established with the aid of rabbit antirat sera. CR and antigenic Fractions A, B and C when permitted to act against electrophorized rat sera showed that all three antigenic fractions precipitated with gamma-globulin but only A and B with beta-globulin including beta 2M. This was confirmed by employing isolated rat immune globulin and "—gamma" sera and the isolated antigenic factors.

In agar gel electrophoresis of CR antigens, 4 lines were observed when the troughs were filled with homologous anti-CR sera. Homologous A, B and C anti-

sera caused precipitation characterized by a single line which had a different location according to the individual fraction. The fourth line, which remained close to the well and appeared diffuse, perhaps represented a non-specific component.

There was no cross-reaction between the A and C antisera and antigens of BT and BP when tested by agar gel electrophoresis. The anti-B fraction did not display a specific strain affinity.

It was necessary to test the chemical composition of rat sera since the animals which were used in these experiments were at the age where all protein fractions except the gamma-globulin are increasing. Quantitative protein determinations, however, revealed no statistically significant deviations from the values found in non infected rats. The A/G ratio was approximately 3:2. The gamma-globulin constituted 10.6 (10.2 to 11.1) % of the total serum proteins; the beta-globulin, 14.3 (13.8 to 14.7) % in both groups of rats at the age of 25 to 30 days. Nor did immunization with borreliae affect significantly the per cent of beta-and gamma-globulin in rabbit sera.

The lack of such changes may be due to the very minor pathogenicity of the *Borrelia* strains used in these experiments.

Physical and chemical properties of the antigenic fractions. Table I shows some of the differential characteristics of the antigenic fractions obtained by starch gel electrophoresis.

While all fractions contained alanine, aspartic acid, cystine, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, serine, tryptophan and valine, Fraction A did not harbor tyrosine and Fraction C was free of proline and threonine. The significance of these differences is unknown. They were observed in BTA, and BTR as well as in BPA antigens.

The total per cent N of Fractions A and B was that expected in antigen proteins, but that of C was low.

The per cent of precipitable protein in the homologous anti-CR sera was partly precipitated by the individual fractions. Fraction C precipitated the smallest proportion of this N, while A and B precipitated about twice as much N. The three fractions together removed about 85% of the total precipitable protein from the anti-CR sera.

When "—gamma" immune serum was used, the precipitable protein was removed from this serum to the greatest extent by Fraction A and, to a lesser degree, by Fraction C. Since these two fractions reacted with beta-globulins and component B did not, this experiment confirmed the relationship of these antigens to the beta-globulins.

Dialysis against distilled water for 24 hr showed that components A and B were not dialyzable, while 8% (7 to 11%) of Fraction C was dialyzed.

The mean electrophoretic mobility of the fractions increased from A to C, in accordance with the molecular weight of the fractions.

The concentration of the material in each fraction was high enough to permit direct observation of the sedimenting boundary and calculation of the sedimentation coefficient. This coefficient was corrected for viscosity and temper-

ature, and the $s\,20{,}w$ values are given in Table I. The sedimentation coefficient was also estimated by the Yphantis and Waugh (21) technique. The sedimentation coefficients calculated from these antigenic measurements are given in Table I. These values are based on three or more centrifuge experiments, but the sedimentation coefficient calculated in this way should be considered only an estimate because of the uncertainties involved in determinations of antigenic titers. However, the average sedimentation coefficient of the antigenic components corresponded to the observed peaks. Thus the main sedimenting component contained the antigenic activity.

TABLE I

Differential characteristics of antigenic fractions of Borrelia turicatae (BT)

	Fraction		
	A	B	C
Amino acids			
Proline	+[a]	+	0
Threonine	+	+	0
Tyrosine	0	+	+
Total N(%)	12.7 (11.5-13.8)	10.8 (9.3-12.3)	6.3 (5.9-7.0)
Precipitable N fraction removed (%) from anti-CR (crude antigen) serum	37.8 (35.3-41.5)	32.1 (30.1-33.6)	16.4 (15.5-16.9)
Precipitable N fraction removed (%) from "$-\gamma$" anti-CR serum	70.2 (61.9-71.8)	<5	13.1 (11.5-15.0)
Dialysis against distilled water for 21 hr	Not dialyzed	Not dialyzed	8% dialyzed (7-11)
Mean electrophoretic mobility on paper -1×10^{-5}/cm²/V	2.2	3.4	6.1
S_{20w}	7.5	6.4	4.6
S calculated from antigenic activity	8 (5-11)	7 (5-9)	5 (1-9)
Molecular weight (estimated)	100,000	80,000	50,000
Precipitating activity inactivated by			
Heating at 100°C for 30 min			
at pH 2.0	+	+	+
at pH 7.0	+	+	0
at pH 11.0	+	+	+
Pepsin	+	+	0
Chymotrypsin	+	+	0
Papain	+	+	+
Lipase	+	+	0
Urea	+	+	0
Periodic acid	+	+	0
Lysozyme	0	0	0

[a] Symbols used are +, reactivated; 0, not reactivated.

The C antigenic fraction showed a 10% fraction that was dialyzable and

therefore assumed to be of low molecular weight. Such a minor component may be present, but it was not possible to detect it by the centrifuge methods used. The centrifuge studies were not complete enough to distinguish between antigens formed during attack and relapse.

The observed sedimentation coefficients are in the range 4 to 8*s* which corresponds to a molecular weight of an idealized spherical protein in the range 50,000 to 100,000. This is also the range for beta- and gamma-globulins with which these antigenic fractions show comparable electrophoretic mobility. The resistance to heat of Fraction C was different from that of the others. It did not lose its precipitating activity when heated for 30 min at 100°C at pH 7.0. Neither was the precipitating capability of component C lost after the action of pepsin, chymotrypsin, lipase, urea, periodic acid and lysozyme. It was decomposed, however, by papain. The inactivation of Fractions A and B by periodic acid may be due to the splitting of a polysaccharide component, but proteins with a thiol, phenol or indol entity also may be inactivated by this reagent. The partial dialysability, relative heat stability and low N content of component C are compatible although they are not proof of a glycoproteinic glycopolypeptidic or other complex. If this assumption is correct, the lack of inactivation by periodic acid would indicate that the carbohydrate moiety is not involved in the precipitation reaction. Fraction C contained at least 6 carbohydrates of which only dextrose has been identified to date.

TABLE II

Precipitable rabbit antibody N precipitated by crude and fractioned antigens

Antigen	Antiserum against crude antigen (CR)		
	BTA[a]	BTR	BPA
	%	%	%
BTA CR	100	52 (46-58)	26 (22-31)
A	38 (35-41)	16 (14-19)	<5
B	32 (29-34)	28 (25-32)	16 (11-20)
C	16 (15-17)	<5	<5
BTR CR	71 (64-76)	100	29 (25-33)
A	28 (24-31)	35 (31-39)	5 (2-8)
B	30 (24-35)	33 (30-37)	18 (15-20)
C	8 (5-11)	15 (13-17)	<5
BPA CR	32 (28-35)	30 (27-34)	100
A	7 (5-9)	8 (4-13)	36 (34-40)
B	17 (13-21)	12 (9-14)	32 (27-36)
C	<5	<5	16 (15-18)

[a] Abbreviations used are BTA, *Borrelia turicatae* from attack; BTR, *B. turicatae* from relapse; BPA, *B. parkeri* from attack.

RNAse and DNAse had no influence on the A, B and C fractions under these experimental conditions.

The gross chemical and physical investigations of BTA, BTR and BPA gave closely similar results in spite of the different immunologic reactions of the antigenic components.

Precipitation tests. In the study of the N precipitable by antigens and by their fractions from immune sera the reference curve was determined only for CR, with standard

amounts of antiserum and variable doses of antigens. The equivalence zone and the optimal precipitation established by this procedure for each CR and each serum were also applied in the testing of the antigenic components against the same sera. This abbreviated procedure was necessary because of the lack of sufficient antigenic material. Table II shows the results.

Antigenic fractions A, B and C together precipitated more than 80% of the precipitable N from the homologous CR antiserum. The proportions were 86% with BTA, 83% with BTR and 84% with BPA. The antigenic fraction A of BTA precipitated about twice as much N from BTA as from BTR and very little or no N from BPA, while only slightly less N was precipitated by the A fraction of BTR from BTA antiserum than from the homologous anti-BTR serum. There was little or no cross-reaction between R antigens and antisera from BT and BP. Antigenic component B from BTA and BPA precipitated approximately the same per cent of N from BTA as from BTR antisera. The proportion of the N precipitated by antigenic fraction B of BT from BP antiserum was about one half of that precipitated from BT antisera and *vice versa*. Component B was considered. therefore, not a stirctly strain specific factor.

The C fractions precipitated less N than Factors A and B from the anti-CR sera.

Component C from BTR precipitated a significantly higher proportion of N from BTA antiserum than Fraction C from BTA from the BTR antiserum, showing a one-sided relationship between BTA and BTR, as was observed also with antigenic fraction A.

TABLE III

Immobilizing activity of crude and absorbed rat sera

Serum	Absorbed with	Organisms Immobilized	
		BTA[a]	BTR
		%	%
None	0	3 (2-4)	2 (0-4)
Uninfected rats	0	2 (1-3)	5 (2-8)
Post attack (BTA)	0	92 (89-96)	17 (14-20)
	BTA CR	12 (8-16)	12 (10-15)
	A	31 (28-36)	12 (10-15)
	B	78 (69-86)	14 (12-15)
	C	45 (41-50)	13 (12-15)
	A + C	22 (19-25)	14 (12-16)
Post relapse (BTR)	0	80 (72-86)	87 (81-93)
	BTR CR	15 (12-16)	12 (9-14)
	A	24 (20-29)	26 (21-30)
	B	72 (64-79)	76 (71-82)
	C	56 (52-59)	44 (41-48)
	A + C	24 (21-28)	20 (16-22)

[a] Abbreviations used are BTA, *Borrelia turicatae* from attack; BTR, *B. turicatae* from relapse.

Immobilizing activity. Sera of uninfected rats showed no immobilizing activity. Table III demonstrates that the difference between the number of organisms

TABLE IV

Lytic activity of crude and absorbed rat sera

Serum	Absorbed with Antigen	Organisms Lysed	
		BTA[a]	BTR
		%	%
None	0	2 (0-5)	3 (1-5)
Uninfected	0	8 (6-9)	6 (5-9)
Post attack (BTA)	0	73 (68-77)	9 (7-10)
	BTA CR	10 (8-12)	7 (5-10)
	A	28 (25-32)	6 (5-8)
	B	66 (61-73)	7 (4-11)
	C	48 (40-54)	8 (6-9)
	A + C	13 (10-17)	8 (6-11)
Post relapse (BTR)	0	68 (60-76)	74 (67-79)
	BTR CR	5 (2-8)	8 (6-11)
	A	33 (28-37)	37 (34-40)
	B	60 (55-67)	63 (58-70)
	C	44 (39-49)	48 (45-51)
	A + C	16 (13-19)	18 (16-21)

[a] Abbreviations used are: BTA, *Borrelia turicatae* from attack; BTR, *B. turicatae* from relapse.

immobilized by unabsorbed and with CR-absorbed homologous sera was 90% or more. Sera collected after the attack immobilized borreliae from the attack 92 (89 to 96) % but to only a limited degree, 17 (14 to 20) %, the borreliae from the relapse, while post-relapse serum immobilized both variants in approximately the same proportions, 80 and 87 (91 to 93) %, respectively. The absorption of the rat relapse sera with BTA CR or its fractions did not change significantly its limited activity on BTR organisms. Absorption of BTA serum with CR from either BTA or BTR and their A and C components reduced the immobilizing power of this serum. Antigen BTA was perhaps more effective in absorbing BTA than BTR antiserum. The same phenomenon was observed when BTR antiserum was absorbed with BTR antigenic fractions. BTR CR and its components absorbed part of the immobilizing activity both from BTR and BTA antisera to approximately the same degree. This, again, may be construed as a demonstration of the one-sided immunologic relationship between attack and relapse strains. The main agents absorbing the immunizing ability were antigenic fractions A and C, indicating that antigenic components A and C play a significant role in immobilization.

Isolated gamma-globulin and "—gamma" sera from infected rats did not reveal immobilizing or lytic activity against BTA or BTR.

Immunization of rabbits with individual antigenic fractions did not produce antisera with significant immobilizing or lytic titers.

Lysin. The lactic activity of crude and absorbed immune sera against BTA and BTR followed the pattern observed in the study of immobilization (Table

IV). Absorption with BTR CR reduced significantly this activity of BTR as well as BTA antisera, while BTA sera did not lyse a significant number of BTR organisms. Antigenic fractions A and C were effective in absorbing this activity of the antisera. Antigen B did not show such a property. Combinations of A and C fractions caused a significant reduction of the lytic capability of the antisera.

Since it has been reported (3) that heated antisera do not lyse borreliae, the influence of the complement on the immobilizing and lytic activities of such sera was investigated.

Fixation of C'. Preliminary tests showed that complement was fixed in BTA antiserum—BTA CR precipitation tests. When the proportion of antigen tests. When the proportion of antigen N and antibody N was 1:10, and 50 C'H50 units of guinea pig complement were added, 8.2 (6.8 to 10) C'H50 units were fixed in the precipitation test. When the immobilizing and lysin tests were performed with 100 C'H50 complement added to the inactivated rat sera, 10.1 (7.9 to 13.7) C'H50 units were fixed if immobilization took place. When inactivated sera were used, 30.2 (27.3 to 34.9) C'H50 units were necessary for the production of a 50% lysis of the borreliae. Active (fresh) sera mixed with their homologous organisms bound 5 (2 to 7) C'H50 units in the lytic reaction. These C'H50 values were calculated with consideration for the deterioration of the complement during the 2-hr incubation of the antiserum- *Borrelia* mixtures. The loss of C'H50 averaged 18 (16.8 to 22.1) % units under these experimental conditions.

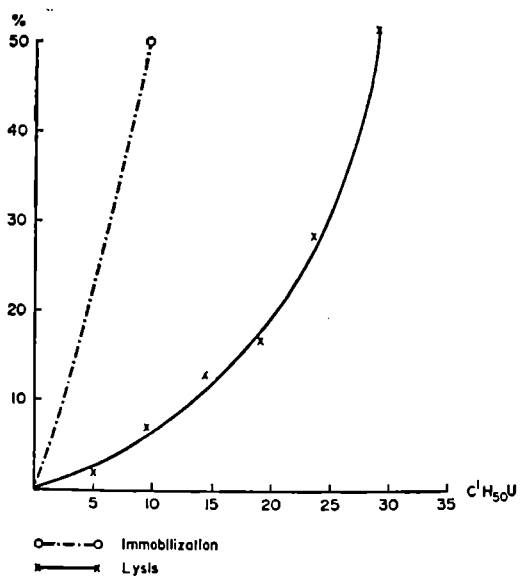

Figure 3. Fixation of complement in immobilizing and lytic antibody tests with *Borrelia turicatae* and rat immune sera.

The fixation of complement in immobilizine and lysin titration, using inactivated BTA rat serum against BTA organisms, is shown in Figure 3. Thus significant amounts of complement were fixed in lysis but not in immobilization tests. Antisera which had no immobilizing or lysing ability, fixed 8.5 (7.1 to 10.9). C'H50 units. The same amount was fixed in precipitation tests.

CF tests. CF tests with rat and rabbit immune sera showed (Table V) that cross-reactions exist between BTA and BTR but that BP could be differentiated from BT by this method with ease. While the method used in this study (12) has not been employed previously in *Borrelia* infections, and the number of strains used in this study was limited, one seems to be justified in recommending a further

TABLE V

Reciprocal complement fixation titers of rabbit and rat sera

Serum	Antigen from		
	BTA[a]	BTR	BPA
Rat			
Not Infected	<5	<5	<5
BTA	40 (20-40)	20 (20-40)	<5
BTR	20 (20-40)	40 (20-40)	<5
BPA	<5	<5	20 (20-40)
Rabbit			
Not Immunized	<5	<5	<5
BTA	20 (20)	20 (10-20)	<5
BTR	20 (10-20)	40 (20-40)	<5
BPA	5 (5-10)	<5	40 (20-40)

[a] Abbreviations used are: BTA, *Borrelia turicatae* from attack; BTR, *B. turicatae* from relapse BPA, *B. parkeri* from attack.

exploration of this technique in relapsing fever since the CF test has not been favored in borreliosis by many investigators (3).

Passive protection tests. Rabbits were immunized with CR and their sera used in attempts to prevent disease in rats. Rat anti-BTA and BTR sera were collected after the respective phases of the infection and employed as a source of protective antibodies. Infected ticks were used to convey the borreliae through their bite for a challenge.

Rabbit serum against BTA did not protect rats against BT infections when absorbed with either BTA or BTR, while the anti-BPR sera protected these animals against BTA and BTR organisms. The activity of the BTR antiserum in preventing BTR infection was not reduced by absorption with BTA, while absorption with BTR deprived it of this activity. The same relationships were found when immune rat sera were employed for passive protection (Table VI).

Anti-BP serum did not protect rats against BT infections and *vice versa*. As in the *in vitro* experiments, in passive protection tests the ability of relapse sera to prevent infection with organisms isolated from attack and relapse was proven.

The brevity of the time interval during which our rat strains were susceptible to infections with the borreliae employed in these experiments did not permit experimentation with active immunization.

DISCUSSION

Since this is the first attempt to analyse the antigenic composition of a *Borrelia*, and the previous literature is meager even on the basic immunologic features of relapsing fever, comparison cannot be made with results of a study of other borreliae. The immunochemistry of *B. tuircatae*, as described in this communication, however, resembles that of leptospirae and trypanosomata (25-29). Leptospirae are closely related to borreliae, both being Treponemataceae, and some borreliae modify *Trypanosoma* infections in experimental animals (3). *B. turicatae* had a gross chemical composition not very different from *T. rhodesiense*

TABLE VI

Passive protection of rats with crude and absorbed sera

Serum			Infection with *Borrelia*		
Animal	Against	Absorbed with	BTA[a]	BTR	BPA
Rabbit	BTA CR	0	0/6	4/6	6/6
		BTA CR	6/6	6/6	5/6
		BTR CR	4/6	5/6	6/6
		BPA CR	0/6	4/6	6/6
	BTR CR	0	0/6	0/6	6/6
		BTA CR	5/6	1/6	5/6
		BTR CR	6/6	6/6	6/6
		BPA CR	0/6	2/6	6/6
	BPA CR	0	6/6	6/6	0/6
		BTA CR	6/6	5/6	0/6
		BTR CR	6/6	5/6	1/6
		BPA	6/6	6/6	6/6
Rat	BTA	0	0/6	5/6	6/6
		BTA CR	5/6	6/6	5/6
		BTR CR	4/6	5/6	6/6
	BTR	0	0/6	6/6	5/6
		BTA CR	5/6	5/6	6/6
		BTR CR	6/6	6/6	6/6

[a] Abbreviations used are: BTA, *Borrelia turicatae* from attack; BTR, *B. turicatae* from relapse; BPA, *B. parkeri* from attack; CR, crude antigen.

as described by Williamson and Brown (30), Cohen and McGregor (19) and Brown (31). *B. turicatae* and *B. parkeri* had three protein or protein-like antigenic components as *T. rhodesiense* while leptospirae have a broader and more variable antigenic spectrum. It is possible, however, that antigenic structures will be observed. The lipopolysaccharide present in leptospirae has a chemical constitution (32) which closely resembles that which we found in *B. turicatae*. The importance of the beta-globulins pointed out in immobilizine studies with leptospirae by Lataste-Dorolle *et al.* (14) and in *B. novyi* relapses by Calabi (2) was evident also in the present experiments. Further investigations are expected to yield results which will shed more light on this problem.

Antigenic components A and B are proteins. The antigenic fraction C is probably a complex between a protein or a polypeptide and some other component which, together with Factor A, is responsible for antigenic specificity. Since A and C isolated from organims collected during different attacks and even from *B. parkeri* had the same basic physical and chemical properties and contained the same amino acids and the same amount of N, one may suspect that the cause for their divergent antigenicity may lie in a different arrangement of some receptor sites. Our findings of amino acids are in general agreement with those of

Geiman (1) who studied *B. novyi*. The implications of the differences in the amino acid composition of the three antigenic components found in these experiments are unknown.

The ability of relapse sera to protect against both relapse and attack strains has been confirmed, and quantitative data have been furnished proving this "asymmetrical immune phenomenon" (3). It is possible that the mutant *Borrelia* which appears during the relapse is already present during the first attack and "outmultiplies" the borreliae prevalent in the initial attack after the latter are suppressed by the antibodies formed during the attack. It is also possible that surviving borreliae which retreat to the internal organs during the crisis at the end of the first attack, are modified during this extracirculatory sojourn and reappear after multiplication as a mutant relapse strain. While most writers are inclined towards the second theory (reviewed in (3)), the present experiments do not permit us to decide between these two theories.

Immobilization of borreliae by antisera could take place without complement, but complement was needed for lysis. This phenomenon may be considered a differential characteristic between the mechanisms of the two reactions. Neither serum deprived of beta-globulin nor serum deprived of gamma-globulin was able to produce lysis in spite of the addition of complement. One may conclude that the lytic antibody is shared by both serum proteins or by an undetermined component which is extracted from the sera when the beta- or the gamma-globulins are separated from it. Since adhesion was not seen with the strains employed in these reactions observed must be considered independent of that phenomenon.

The role of polysaccharides in the immunologic behavior of borreliae is a moot question. The lipopolysaccharides extracted from the crude antigen had complement fixing ability. The scarcity of material did not permit the analysis of polysaccharides in the isolated antigenic fractions. The answer to this question may be of value also for the taxonomic classification of borreliae since bacterial antigens usually contain polysaccharides while the role of such components in protozoa has not been clarified.

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SUMMARY

One strain of *Borrelia turicatae* and one of *B. parkeri* were studied. The organisms had chemical compositions similar to those of leptospirae and of

Trypanosoma brucei. Three antigenic factors were isolated, of which two were proteins and one a protein or polypeptide complex. One antigen, designated B, was shared by *B. turicatae* and *B. parkeri*. It did not differ in strains isolated from the first attack and from relapse. Antigens A and C were starin and relapse specific. These two antigens also played a role in the immobilization and lysis of borreliae. They reacted with antibodies present in the beta- and gamma-globulin fractions of the immune sera. Relapse sera protected young rats against borreliae from both the relapse and the first attack, while sera collected after the first attack did not protect against borreliae isolated from the relapse. The immune sera had a strong immobilizing and lytic activity, the latter dependent on complement. The strains used in this experiment did not show adhesion. Lysis was, therefore, considered independent of the adhesion phenomenon.

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