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Antileptospiral Activity in Lower-Vertebrate Sera

N. W. CHARON,[1] R. C. JOHNSON, AND L. H. MUSCHEL[2]
University of Minnesota, Department of Microbiology, Minneapolis, Minnesota 55455

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Normal serum from the painted turtle (*Chrysemys picta*), the snapping turtle (*Chelydra serpentina*), and the frog (*Rana pipiens*) were found to possess bactericidal activity towards *Leptospira*. Leptospires from both the parasitic and biflexa complexes were killed by these sera at high dilutions. This pattern differs from that of mammalian serum, as generally only the biflexa complex leptospires are killed by normal mammalian serum. The activity in *C. picta* serum was characterized as being complement dependent and not mediated by basic proteins. Because complement-inactivated *C. picta* serum regained leptospiricidal activity after the addition of fresh rabbit serum, antibody is also likely to participate in the killing activity. Further support that *C. picta* serum contained leptospiral antibodies was found by the detection of serotype-specific agglutinins.

Leptospira are spirochetes ubiquitous in nature. Although *L. interrogans* is the only recognized species of *Leptospira*, the genus is divided into two groups or complexes (4, 25). Leptospires of the parasitic complex are associated with infection, parasitism, and disease, whereas leptospires of the biflexa complex are isolated from soil and water and are generally not pathogenic (25, 27). Nutritional and biochemical differences between the two groups exist, and as a result they can be differentiated on the basis of a number of tests such as 8-azaguanine sensitivity and growth at low temperature (11, 17).

The biflexa and parasitic complexes also differ from one another in their sensitivity to normal mammalian serum. Leptospires of the parasitic complex are generally resistant to the killing activity of normal mammalian serum, whereas leptospires of the biflexa complex are readily killed by such serum (7, 15). This ability to survive in normal serum is likely to play a role in the pathogenesis of leptospirosis, as the pathogens are not cleared nearly as rapidly as the biflexa complex leptospires upon injection into the host (15). A similar relationship of serum resistance correlating with pathogenicity has been found for the *Enterobacteriaceae* (18).

In the present study, we addressed ourselves to whether lower-vertebrate sera exhibit a killing pattern to *Leptospira* similar to that which mammalian sera exhibit. In the case of the *Enterobacteriaceae*, the rough strains are more serum sensitive than the smooth strains in both lower-vertebrate and mammalian sera (L. H. Muschel, J. E. Jackson, and H. Gewurz, Fed. Proc. 23:505, 1964; and reference 22). Possibly similar results would be found with *Leptospira*. It was also expected that the results would offer some insight into the relationship of the lower vertebrates with *Leptospira*. We report here the results of these experiments using turtle (*Chelydra serpentina* and *Chrysemys picta*) and frog (*Rana pipiens*) sera. In addition, because the pattern of killing in the lower-vertebrate sera markedly differed from that of mammalian sera, the killing activity in *C. picta* serum was partially characterized.

MATERIALS AND METHODS

Organisms and maintenance. The leptospires were grown and maintained at 30 C in the modified Tween-80 albumin medium (11). The origin of these leptospires was previously reported (13, 17). Serotypes of the biflexa complex include *patoc* Patoc I, *semaranga* Veldrat Semarang 173, *biflexa* CDC, and *sao-paulo* Sao Paulo. Each of the leptospires has been characterized as a member of the biflexa complex by standard procedures such as 8-azaguanine resistance (17), growth at low temperature (11), mammalian serum sensitivity (15), $CuSO_4$ resistance (8), lipase production, and 2,6-diamino purine resistance (12). The serotypes of the parasitic complex used in this study include *canicola* Hond Utrecht IV, *arboreae* Arborea, *ballum* Mus 127, *pomona* Pomona, and *grippotyphosa* Mal 1540. These leptospires, originally isolated from infected animals, were characterized as belonging to the parasitic complex by the criteria mentioned above.

Sera. Separate pools of serum were obtained from

[1] Present address: West Virginia University, Department of Microbiology, Medical Center, Morgantown, W. Va. 26506.

[2] Present address: American Cancer Society, Inc., New York, N.Y. 10017.

the painted turtle (*C. picta*), the snapping turtle (*C. serpentina*), the frog (*R. pipiens*), and the New Zealand rabbit. Some of the *C. serpentina* serum was purchased from Schettle Frog Farm, Houlton, Wis., but most of the sera were obtained from animals caught in the Minnesota-Wisconsin area, including the Carlos Avery Game Reserve. All animals were adults and no leptospires could be isolated from them (R. Henry, Ph.D. thesis, Univ. of Minnesota, Minneapolis, 1972). Turtles were exsanguinated by severing neck blood vessels and rabbits were bled by cardiac puncture. The sera were harvested and pooled from blood held for 1 h at 23 C and for 18 h at 4 C. The pH of the sera was adjusted to 7.35 to 7.40; the sera were sterilized by filtration and stored at −50 C.

Leptospiricidal assays. A modification of the method of Johnson and Muschel was used to assay killing of *Leptospira* (15). Leptospires from a 24-h culture were adjusted to a concentration of 3×10^7 leptospires per ml with a diluent containing 0.5% NaCl and 0.5% bovine serum albumin. A 0.1-ml amount of this cell suspension was incubated with the diluted test serum in a final volume of 1 ml for 1 h at 37 C. At the end of the reaction period, percentage of immobilization was determined under dark-field illumination. To determine growth inhibition, 6 ml of growth medium was added to the reaction tubes. Growth was measured turbidimetrically using a photonephelometer (Coleman model 9). Readings on the photonephelometer were correlated with the number of organisms per milliliter using the Petroff-Hausser counting chamber (T. Auran, M.S. thesis, Univ. of Minnesota, Minneapolis, 1968). Percentage of survival was determined by dividing the number of leptospires per milliliter in experimental reaction tubes by the number per milliliter in the control preparation without serum and multiplying by 100. This was done when the leptospires in the control preparation grew to a density of approximately 3×10^8 leptospires per ml.

Release of labeled nucleic acids. Serum-induced release of leptospiral nucleic acid material was determined by a method similar to that of Johnson and Muschel for *Leptospira* (15) and Spitznagel for gram-negative bacteria (24). Labeled leptospires were prepared by culturing the organisms in growth medium containing [8-^{14}C]adenine (specific activity, 0.008 μCi/μg, Volk Radiochemical Co.). This compound is almost exclusively incorporated into leptospiral nucleic acids (16). The volume of the test reaction was 10 ml at a concentration of 3×10^8 leptospires per ml. The test involved incubating serum at a final dilution of 1:10 with washed, labeled leptospires for 1 h at 37 C. After determining percentage of immobilization, the cells were removed from the reaction mixture by centrifugation and the supernatant fluid was assayed for radioactivity. This was done using a model 181 B scaler and a model D47 gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) at 1,150 V with a gas mixture of 98.7% helium and 1.3% butane. Samples were counted on glass planchets.

Assay and absorption of wall lytic activity. A procedure similar to that of Crombie and Muschel was used to detect lysozyme-like factors present in *C. picta* and rabbit serum (5). Lysozyme-like cell wall lytic activity was assayed by incubating a specified amount of serum with 2 ml of ultraviolet light-killed *Micrococcus lysodeikticus* (Worthington) in 0.5% NaCl. A dense suspension (optical density 1.7 at 420 nm) was required to give a linear rate of change in optical density in the presence of serum or egg-white lysozyme. As little as 0.05 μg of lysozyme per ml of reaction mixture could be detected by this assay. Absorption of serum basic proteins, including lysozyme, was achieved by treatment with Montmorillonite clay (bentonite, American Colloid Corp., Chicago, Ill.). The bentonite used was prepared as by Crombie and Muschel (5) and was the kind gift of L. B. Crombie.

EDTA treatment of serum. To determine whether divalent cations were required for killing *Leptospira*, equal volumes of 0.8 mM ethylenediaminetetraacetic acid (EDTA) and *C. picta* serum diluted 1:25 were incubated for 1 h with 3×10^6 leptospires. Percentage of immobilization was determined and either 1 ml of diluent or 1 ml of 0.8 mM $CaCl_2$ was added to the reaction tubes. After further incubation for 1 h, percentage of immobilization was determined.

Serum bactericidal activity to Salmonella and hemolytic complement. Standard procedures were used to assay for the serum-mediated killing of *Salmonella typhi* 0901 (19) and for titration of hemolytic complement (20). The results of the bactericidal tests are expressed as the estimated volume of serum needed to inhibit growth 50%. Hemolytic activity is expressed at the estimated amount of serum needed to hemolyze 50% of the unsensitized sheep red blood cells.

Leptospiral agglutination. To determine whether specific antibodies participate in the agglutination of *Leptospira*, *C. picta* serum was absorbed with various serotypes of *Leptospira*. Leptospires from a 24-h culture were sedimented by centrifugation, washed with albumin-sodium chloride diluent, and recentrifuged. The sedimented leptospires (approximately 10^{10}) constituted the absorbing antigen. A 4.5-ml amount of a 1:3 dilution of heat-inactivated (10 min, 56 C) serum was incubated with the absorbing antigen for 2 h at 37 C. The leptospires were sedimented by centrifugation and the serum was absorbed a second time with fresh antigen. After the second absorption, the serum was collected by centrifugation and clarified by filtration through an 0.8-μm membrane filter (Millipore Corp.). Agglutination of leptospires was determined by adding 2×10^7 leptospires in 0.2 ml to 0.2 ml of the serum dilution. The percentage of the leptospires agglutinated was estimated under dark-field illumination after 1 h at 30 C.

RESULTS

Killing of serotype canicola by C. serpentina serum. *C. serpentina* normal serum was found to differ from normal mammalian serum in that it killed leptospires of the parasitic complex at high serum dilutions. Three criteria were used to assay this killing activity. Evi-

dence was found that *C. serpentina* serum immobilized and inhibited the growth of serotype *canicola* (Table 1). Serum dilutions of 1:1,000 or greater immobilized less than 10% of the leptospires, and growth in these tubes equaled or exceeded growth in the control without serum. At serum dilutions of 1:250 or less, greater than 90% of the leptospires were immobilized, and growth was less than 4% of the control. *C. serpentina* serum also induced cellular leakage. [^{14}C]adenine-labeled serotype *canicola* released 37% of its nucleic acid material after 1 h of serum treatment (Table 2). This amounted to almost three times that of the control, and, as with growth inhibition, immobilization showed a high correlation with cellular leakage. In each of the three methods used to assay for killing, heated serum (56 C, 30 min) had no inhibitory effect on the leptospires (Tables 1 and 2).

Pattern of killing of C. serpentina serum. Nine serotypes were tested using immobilization and growth inhibition as an assay for cell death. In all experiments, immobilization data agreed with growth inhibition. The results are expressed as that serum dilution which immobilized and growth inhibited greater than 90% of the leptospires. The results of *C. serpentina* serum on these serotypes are presented in Table 3. Most of the serotypes tested were killed (>90%) at serum dilutions of 1:250. Serotypes *semaranga, pomona,* and *grippotyphosa* were more resistant than the other serotypes, as they had end points of 1:50, 1:25, and 1:25, respectively. In contrast to *C. serpentina* serum, rabbit serum only killed leptospires of the biflexa complex (Table 3, column 3). Leptospires of the parasitic complex were resistant to rabbit serum concentrations of 1:3. These results are similar to those reported by Johnson and Muschel (15) and by Faine and Carter for mammalian sera (7). The results indicate the *C. serpentina* serum differs from mammalian sera in its ability to kill serotypes of the parasitic complex at higher serum dilutions than rabbit serum.

TABLE 1. *Growth inhibition and immobilization of serotype canicola by C. serpentina serum*

Final dilution of turtle serum	Immobilization (%)	Leptospires ($\times 10^7$/ml)	Percent survival compared with control[a]
1:2,000	<10	26	100
1:1,000	<10	35	135
1:250	>90	<1	<4
1:125	>90	<1	<4
1:50	>90	<1	<4
1:25	>90	<1	<4
No serum (control)	<10	26	100
Heated serum[b]	<10	23	88

[a] Based on growth.
[b] Heated at 56 C for 30 min.

TABLE 3. *Antileptospiral activity of C. serpentina serum against serotypes of the biflexa and parasitic complexes*

Serotype	>90% end point[a]	Rabbit serum 1:3[b]
Biflexa complex		
patoc	1:250	S
semaranga	1:50	S
Sao Paulo	1:250	S
biflexa CDC	1:250	S
Parasitic complex		
arboreae	1:250	R
canicola	1:250	R
ballum	1:250	R
pomona	1:25	R
grippotyphosa	1:25	R

[a] Dilution in which >90% are immobilized and growth inhibited. Serum dilution schedule as in Table 1.
[b] S designates >90% killed; R designates no growth inhibition or immobilization in test reaction.

TABLE 2. *Release of nucleic acid material of serotype canicola by C. serpentina serum*

Determination	Total counts/min in sample	Corrected for control (counts/min)	Label released (%)	Immobilization (%)
Untreated leptospires suspended in diluent	50,406			
Supernatant fluid of untreated leptospires (control)	9,443	0	0	<10
Supernatant fluid of leptospires treated with heated serum[a]	10,579	1,136	2	<10
Supernatant fluid of leptospires treated with fresh serum	27,679	18,236	37	>90

[a] Heated at 56 C for 30 min.

Pattern of killing in C. picta and R. pipiens sera. Serum from *C. picta* and *R. pipiens* were tested for their ability to kill three serotypes. As can be seen from the results in Table 4, sera from *C. picta* and *R. pipiens* were similar to that from *C. serpentina* in that each immobilized the parasites in dilutions of 1:12 or greater. In addition, in each of the three sera tested, serotype *grippotyphosa* was most resistant.

Lysozyme and basic protein involvement of C. picta serum in the killing of Leptospira. The marked killing activity in the three sera led us to examine the nature of the killing activity. One approach taken was to examine if a lysozyme-like activity or basic protein was involved in the killing. Using the *M. lysodeikticus* assay for lysozyme, both *C. picta* and rabbit sera were found to possess wall lytic activity (Table 5). These sera were absorbed with bentonite to remove basic proteins including lysozyme and tested in the leptospiricidal assay at a 1:10 serum dilution. The killing activity remained, but wall lytic activity was essentially removed (less than 0.2 μg of lysozyme material per ml). These results suggest that basic proteins are not involved in the killing of *Leptospira* by *C. picta* and rabbit sera.

Complement involvement in the killing of Leptospira. A number of criteria suggest that complement participates in the killing of *Leptospira* by *C. picta* and *C. serpentina* serum. First, the killing activity in *C. picta* serum demonstrated a divalent cation requirement, as EDTA-treated serum failed to immobilize serotype *patoc*. The addition of Ca^{2+} reactivated the activity. We note that, although EDTA-Ca^{2+} treatment per se did not result in immediate (1 to 3 h) immobilization of the leptospires, it did result in killing of the leptospires as assayed by growth inhibition. Second, heating the serum at 56 C for 30 min destroyed the killing activity (Tables 1, 2, and 4). A 10-min heating at 56 C was found to be sufficient to destroy the activity in all three lower vertebrate sera. The same heat treatment of *C. serpentina* sera (56 C, 10 min) also destroyed killing activity against *Salmonella typhi* 0901 (0.025 serum units preheating) and hemolytic activity against sheep red blood cells (0.07 serum units preheating). Finally, serum from *C. picta* heated at 56 C for 10 min regained leptospiricidal activity to serotype *canicola* after the addition of fresh but not heated rabbit serum (Table 6). Fresh rabbit serum or heated *C. picta* serum alone was inactive in the assay. When incubated together, greater than 90% of the leptospires were killed. Thus, the divalent cation requirement, the heat lability, and the reactivation of the killing activity of heated serum by the addition of fresh rabbit serum indicate the participation of complement.

Antibodies in C. picta serum to Leptospira. Attempts were made to determine if specific antibody participated in the killing of *Leptospira*. EDTA-treated serum was absorbed at 4 C with one serotype and tested for killing

TABLE 4. *Antileptospiral activity of C. picta and R. pipiens serum*

Serotype	>90% end point[a]			
	C. picta serum		*R. pipiens* serum	
	Unheated	Heated 1:12[b]	Unheated	Heated 1:12[b]
patoc	>1:100	R	>1:100	R
canicola	>1:100	R	1:25	R
grippotyphosa	1:12	R	1:12	R

[a] Serum dilutions of 1:100, 1:50, 1:25, and 1:12.
[b] Serum heated at 56 C for 30 min. R designates no growth inhibition or immobilization.

TABLE 5. *Effect of bentonite treatment on the leptospiracidal activity of C. picta serum and rabbit serum*[a]

Sample	Lytic activity[b]	Serum (diluted 1:10)
Normal *C. picta* serum	4	S
Bentonite-treated *C. picta* serum	<0.2	S
Normal rabbit serum	5	S
Bentonite-treated rabbit serum	<0.2	S

[a] Test organism *patoc* Patoc I. S designates greater than 90% killed.
[b] Expressed as the concentration of egg white lysozyme (μg/ml) to bring about an equivalent rate of lysis of *M. lysodeikticus*.

TABLE 6. *Reactivation of heated C. picta serum with fresh rabbit serum*[a]

Final dilution			Leptospires killed (%)
Heated *C. picta* serum[b]	Fresh rabbit serum	Heated rabbit serum[b]	
1:10	1:5		>90
1:10			<10
	1:5		<10
1:10		1:5	<10

[a] Serotype *canicola*.
[b] Heated at 56 C for 10 min.

activity after Ca^{2+} reactivation on the homologous or heterologous serotype. In some experiments the absorbed serum lost activity towards the homologous but not the heterologous serotype tested. The results, however, were variable, as total killing activity was often lost after absorption. On the other hand, evidence for antibody being directed to *Leptospira* was demonstrated by agglutination. Serotype-specific agglutinins were found to *Leptospira* in low serum dilutions (1:16 or less). Sera adsorbed with *biflexa* CDC, *patoc*, or *canicola* resulted in removal of agglutinating activity to the homologous serotype, but not to the heterologous serotypes tested (Table 7).

DISCUSSION

The results reported in this communication indicate that sera from *C. serpentina*, *C. picta*, and *R. pipiens* possess bactericidal activity against *Leptospira*. This leptospiricidal activity was demonstrated using immobilization, growth inhibition, and, in the case of *C. serpentina* serum, altered permeability. These three methods have been previously employed to assay for cell death of *Leptospira* by mammalian sera (15).

The pattern of killing in lower-vertebrate sera differed from that of mammalian sera. Both the parasitic and biflexa complex leptospires were readily killed by lower-vertebrate sera. On the other hand, as demonstrated here and by others (7, 15), the parasitic leptospires were resistant to the killing activity of mammalian serum. Because this difference could reflect alternate mechanisms of killing *Leptospira* by serum, the killing activity directed to the parasitic leptospires was partially characterized.

The results indicate that the killing of both groups of leptospires by lower-vertebrate sera involved complement. Similar results have been found with mammalian sera (7, 15). The activity in *C. picta* serum required divalent cations and was heat labile (56 C, 10 min). Both a divalent cation requirement and heat lability are attributes of lower-vertebrate complement (21). Because the killing activities directed to both groups of leptospires in *R. pipiens* and *C. serpentina* sera were also heat labile, complement involvement in the leptospiricidal activity in these sera is strongly suggested. In addition, as with mammalian sera (14), the killing activity in *C. picta* serum did not require basic proteins.

The leptospiricidal activity directed to the parasitic leptospires in lower-vertebrate sera involved the participation of antibodies. Thus, heated *C. picta* serum regained its leptospiricidal activity to serotype *canicola* by the addition of fresh rabbit serum. Specific agglutinins were also found to serotype *canicola* in *C. picta* serum. Agglutinins to parasitic leptospires in lower vertebrate sera have been reported by others (2, 9, 23, 26). However, these agglutinins may not be related to antibody and may not be epidemiologically related. For example, the adult turtle *Clemmys caspica* possesses in its serum a 4.8*S* gamma globulin fraction which nonspecifically agglutinates four serotypes with high titers (500 to 128,000) (23, 26). In addition, the appearance of these agglutinins is unrelated to exposure to *Leptospira* (23, 26). Because the agglutinins reported in this study were relatively low in titer (1:16) and were specific, they apparently differ from those of *C. caspica*. If mammals are immunized to parasitic leptospires, agglutinins and leptospiricidal activity to the immunizing serotype appear in the sera (15). Accordingly, the lower-vertebrate sera resembles immune mammalian sera with respect to these two activities.

The most likely explanation for the different pattern of killing of *Leptospira* in lower-vertebrate sera is either an immunization to *Leptospira* themselves or an immunization to cross-reacting antigens. The animals used in this study were likely to come in contact with *Leptospira*, as leptospires of the biflexa complex were readily isolated from soil and water from the vicinity they were caught (R. Henry, Ph.D. thesis). Because the leptospires of the biflexa complex grow considerably faster than the parasitic leptospires, it was difficult to ascertain the presence of the latter group in the environment without animal inoculations. However, parasitic leptospires have been isolated from turtles and frogs by others (3, 6, 9). In addition, turtles have also been shown to be experimentally infected with *Leptospira*, as renal shedding infec-

TABLE 7. *Specific agglutination of various serotypes of Leptospira by C. picta serum*[a]

Absorbing serotype	Specific agglutination[b]		
	Test serotype		
	patoc	*biflexa* CDC	*canicola*
None	++++	++++	++
patoc Patoc I	0	++++	+
biflexa CDC	++++	0	++
canicola Hond Utrecht IV	++++	++++	0

[a] Final serum dilution, 1:6.

[b] Symbols: 0, no agglutination; +, 25% of cells agglutinated; ++, 50% of cells agglutinated; +++, 75% of cells agglutinated; ++++, 100% of cells agglutinated.

tions have been established in the laboratory (1). On the other hand, the origin of the antibodies may be related to an immunization to antigens which cross-react to *Leptospira*. Although there is no evidence to our knowledge that this occurs with *Leptospira*, evidence for this phenomenon has been found in other systems such as *Haemophilus* and *Escherichia coli* (10).

The pattern of killing in the lower vertebrate sera directed to *Leptospira* contrasts to the pattern found to the *Enterobacteriaceae*. Carp serum has a similar pattern as mammalian sera (L. H. Muschel, J. E. Jackson, and H. Gewurz, Fed. Proc. 23:505, 1964). Thus the smooth strains are generally more resistant than the rough strains to both mammalian and carp sera. Data for this general pattern are also found in toad and lizard sera (22). However, lizard serum readily killed a smooth strain of *E. coli*, and a smooth strain of *S. paratyphi* B was found to be as sensitive as a rough strain in both toad and lizard sera (22). Schwab and Reeves attribute the ability of these sera to kill the smooth strains to the presence of specific antibody (22). A similar proposal is presented here with respect to turtle and frog sera and parasitic leptospires.

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