Presence of Hemolysin in Cultures of Pathogenic Leptospires. (22214)

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Hemolytic activity of a pathogenic leptospiral culture was observed in blood agar medium containing sheep erythrocytes when this culture was examined for bacterial contamination. The hemolytic potency of a "non-pathogenic" culture of water leptospires for various mammalian red blood cells had previously been reported by Sugimota(1). Except for Jungherr's casual reference to this phenomenon in reporting an outbreak of canine leptospirosis(2), no observations similar to Sugimota's has been reported for the pathogenic leptospires. Since toxic and hemolytic manifestations are frequently observed in leptospiral infections, further investigation of this chance observation was initiated by the authors—a study which demonstrated that a soluble hemolysin can be found in cultures of specific "serotype" strains of leptospires. Some characteristics of this soluble hemolysin will be described below.

Materials and methods. Preparation of hemolysin. Leptospires were cultivated in a 10% solution of inactivated normal rabbit serum (56°C for 1 hour) in isotonic phosphate buffered salt solution (pH 7.4) consisting of 0.667 g Na_2HPO_4, 0.087 g KH_2PO_4, 8.213 g NaCl, 0.190 g $MgCl_2 \cdot 6H_2O$ and 0.132 g asparagine dissolved in one liter of distilled water. Inoculated cultures were incubated 10-13 days at 30°C. Cultures were then centrifuged at approximately 4000 g, and the clear supernatant fluid removed and filtered through a Seitz E-K pad. Part of the supernatant fluid was stored in the refrigerator (5°C) and the remainder was frozen and maintained at –60°C. The sedimented leptospires were washed twice in phosphate buffer, resuspended with isotonic phosphate solution to 20% of the volume of the original culture and stored at about –60°C. For certain procedures, sedimented washed leptospires were disrupted by alternate freezing and thawing in dry-ice-alcohol and 37°C water baths, respectively. Cells were also disrupted when 20 ml aliquots of a 10-fold concentration of washed leptospires were subjected to sonic vibration at frequency of 10 kc and amplitude of 1.25 amps in a water-cooled magnetostriction oscillator. *Preparation of red blood cell (RBC) suspensions.* Sheep blood, collected by sterile venipuncture, was preserved in modified Alsever's solution(3). On the day of test, preserved cells were filtered through gauze, centrifuged at 1000 g for 10 minutes, washed 3 times with isotonic phosphate base and resuspended in a sufficient volume of base to produce approximately a 20% suspension. The concentration of cell suspensions was adjusted so that the complete lysis of a 1:40 dilution of RBC suspension had an optical density of 0.570 at 550 mμ. Erythrocyte suspensions from other animals were similarly prepared for one experiment. *Hemolysin test.* Two procedures were employed. In Procedure I, one part of a 20% sheep RBC suspension was mixed thoroughly with 9 parts of test substance in test tube and incubated in water bath adjusted to the desired temperature. After suitable intervals of time, aliquots were removed, rapidly centrifuged and diluted 1:4 with isotonic buffer. The percent hemolysis was estimated spectrophometrically at 550 mμ. In later studies this procedure was discarded and a more sensitive indicator system (Procedure II) was substituted. In this procedure, 1 ml of test fluid was mixed with an equal volume of a 1% RBC suspension in a 10 x 65 mm cuvette. After appropriate intervals of incubation, cuvettes were centrifuged and direct readings of the optical density were taken. A hemolytic unit was defined as the reciprocal of the dilution of test fluid in a 1 ml dose which results in lysis of 50% of the red cells of 1 ml of a 1% sheep

TABLE I. Comparative Hemolytic Potency of Supernatant and Cellular Fractions of *L. hemolyticus* Cultures.

Culture	Fraction*	Milieu*	% hemolysis of dilutions: Undil.	1:2	1:3	1:4	1:5	1:6	1:8	1:10	Units of hemolysin§
A	S	b + r	90	94	93	66	62	38	17	6	5.5
(16-day-old)	W (FT)†	b			93	71	48	17	8	6	.5
	W (FT)†	b + r	0	0	0	0	0	0	0	0	0
B	S	b + r	86	85	88	80	71	40	7	7	5.6
(21-day-old)	W (FT)†	b		29	0	0	0	0	0	0	<.2
	W (SV)‡	b		75	49	13	0	0	0	0	.3

* S = Supernatant; W = Washed cells; b = Base medium; r = Rabbit serum.
† 10-fold concentration of cells disrupted by successive freezing and thawing.
‡ " " " " " " sonic vibration.
§ Calculated/ml of original culture volume.

RBC suspension. The final volume of this test mixture is 2 ml. To determine 100% hemolysis and "normal" hemolysis of culture medium and diluent, suitable controls were set up with each test. *Preparation of antiserum.* A 2 kg rabbit was inoculated intravenously with successive doses of 5.0 ml, 5.0 ml, and 10.0 ml of filtered supernatant fluid at 5 to 6-day intervals. Seven days following the last inoculation, the rabbit was exsanguinated, the serum was collected, and stored at −20°C.

Results. Hemolytic studies were conducted on a strain of leptospires isolated from a human patient in Malaya. Upon antigenic analysis, the isolate was found to be a hitherto unrecognized serotype member of the hebdomadis group, and the strain was subsequently designated as *Leptospira hemolyticus* in this laboratory(4). Initial studies established the presence of a soluble hemolysin in cultures of *L. hemolyticus*. Treatment of a 2% suspension of sheep RBC with 12-day-old culture as well as with this culture's supernatant fluid, resulted in visible lysis of erythrocytes after a remarkably long incubation period of one to 2 hours at 37°C. On the other hand, a 10-fold concentration of washed leptospires prepared from the same culture and resuspended in uninoculated media demonstrated slight hemolytic activity after 6 hours of incubation. More definitive studies to determine the relative concentration of hemolysin in the culture supernatant fluid and in disrupted leptospires were conducted with two separate cultures of *L. hemolyticus*. In this procedure the more sensitive hemolysin test Procedure II was employed. The results of this study, shown in Table I, indicate that nearly all activity of *L. hemolyticus* cultures can be attributed to the soluble hemolysin present in the culture milieu. In addition, the presence of a RBC lytic inhibitor in rabbit serum is made evident by the lack of hemolytic activity of a suspension of disrupted cells in culture medium (buffered base + 10% rabbit serum). Even in the presence of RBC lytic inhibitors in cell-free liquor, the hemolytic potency of this fraction was 10- to 30-fold greater than comparable amounts of cells contained in the same volume of culture. Therefore, most of the subsequent work was conducted on the supernatant fluid.

The effect of 0°, 30°, 37°, 56°C temperatures on the hemolytic activity of the supernatant fluid, prepared from a 13-day-old culture of *L. hemolyticus*, was determined. In this study a 2% sheep RBC suspension was employed. After test tubes containing supernatant fluid were equilibrated in water baths of the desired temperatures for 10 minutes, sheep RBC were introduced and readings were taken at chosen intervals. The results shown in Fig. 1 indicate that the rate of hemolysis is considerably affected by temperature. At 0°C no hemolytic activity was manifested. The preliminary period before the onset of hemolysis, designated as the "induction period" by Bernheimer(5), was markedly reduced when the temperature was increased to 37°C. Hemolysin was completely inactivated at 56°C within 10 minutes.

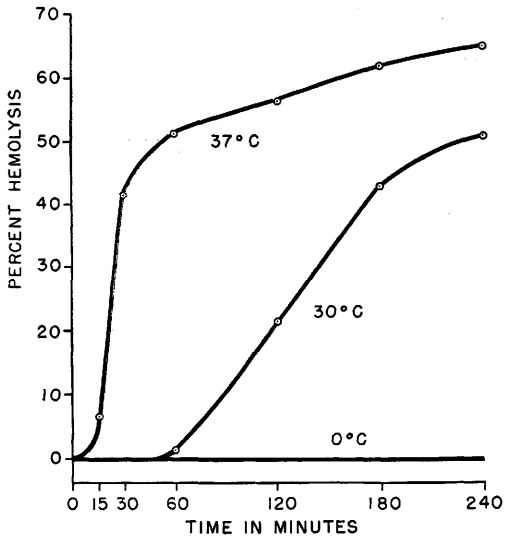

FIG. 1. Effect of temperature on course of hemolysis.

Additional studies of the culture supernatant fluid indicated that the hemolytic activity of the preparation was not reduced by continuous dialysis for 7 days against cold running tap water. No marked diminution of hemolytic activity was noted in the supernatant fraction, after this cell-free fluid was stored at 5°C (in the presence of air) or at −60°C during four months' observation. The hemolysin was not destroyed by lyophilization.

The hemolytic activity of the supernatant fraction of a culture of *L. hemolyticus* was tested against the RBC of the following species: sheep, horse, cow, goat, pig, dog, cat, chimpanzee, guinea pig, white rat, hamster, rabbit, human and chicken. Hemolysin test Procedure I was employed. Preparations were incubated at 37°C and readings were taken at the end of 2 and 4-hour intervals. Hemolytic activity was observed with sheep, cow and goat RBC only.

Forty-three different leptospiral strains representing multiple "serotypes" were screened to determine if the production of hemolysin was a generic or serotype-specific characteristic. Cell-free culture liquids were obtained from 13- to 14-day-old cultures of leptospires according to the methods previously outlined. When the spirochetes were harvested, the concentration of leptospires in each culture was determined spectrophotometrically on centrifuge-packed cells resuspended to effect a 10-fold concentrate of the culture's original volume. Supernatant preparations were treated with sheep RBC (Procedure I), incubated at 37°C, and the percent hemolysis was recorded at the 4th and 6th hour of incubation. The results of this study are shown in Table II. The minimum O. D. of any culture in which hemolysin was detected was 0.102. This figure was therefore chosen as the criterion for determining the amount of growth necessary to produce hemolysin. The results obtained with seven cultures showing lesser concentrations of leptospires were not considered to be significant. The production of hemolysin, demonstrated by the test procedures employed was restricted to specific serotype strains.

A close examination of the data in Table II revealed no apparent correlation between either the optical density or the length of the *in vitro* cultivation and the production of hemolysin by serotype strains. All strains employed were recognized "type" strains or strains identified in this laboratory through agglutinin-absorption procedures, with the following exceptions: Coke, Asbahadur, Campbell, Moulton, Perret, Williams, and Buxton. The disparity between the hemolytic reactions of various strains belonging to the same "serotype," such as, canicola, bangkinang, and hemolyticus, may reflect differences in the antigenic composition of these various strains, particularly in view of the fact that the serotype designations of some of these strains were based upon similarities in cross-agglutination lysis reaction patterns and not upon the more definitive agglutinin-absorption studies.

The results observed in various experiments indicated that the differences in hemolytic potential of *L. hemolyticus* cultures could be attributed to the age of the culture. Attempts were, therefore, made to determine the incubation period necessary to produce optimum hemolysin. A freshly seeded culture was distributed in 10 ml amounts in a

TABLE II. Hemolysis of Sheep RBC by Cell-Free Culture Liquids of Various Serotype Strains of Leptospires.

Serogroup	Serotype	Strain	Year isolated	Culture optical density	% hemolysis 4-hr	% hemolysis 6-hr
icterohemorrhagiae	ictero. AB	Wijnberg	1926	.153	—	—
	new serotype A	DeGray	54	.148	—	—
javanica	javanica	Veldrat, Bat. 46	38	.115	—	—
schüffneri	schüffneri	ML-2	51	.110	—	—
	"	Bishop	54	.105	—	—
	"	Cake*	"	.132	—	—
	"	Asbahadur*	"	.115	—	—
canicola	canicola	Ruebush	48	.168	—	—
	"	Campbell*	54	.148	16	46
	"	Moulton*	52	.125	74	85
	new serotype	Jones	54	.152	—	—
benjamin	benjamin	Benjamin	37	.250	—	47
ballum	ballum	Pasteur	–	.129	—	—
pyrogenes	pyrogenes	Salinem	24	.183	—	—
	new serotype	Biggs	54	.136	—	—
cynopteri	cynopteri	3522	38	.102	7	78
sentot	sentot	Sentot	37	.225	—	—
autumnalis	autumnalis AB	Akiyami A	25	.165	—	—
	bangkinang	Perret*	54	.108	—	—
	"	Mason	"	.148	—	55
	new serotype	Williams	"	.180	—	—
djasiman	djasiman	Djasiman	37	.249	—	—
australis A	australis A	Ballico	"	.175	73	74
pomona	pomona	LC 73*	53	.214	48	74
	"	LC 78*	54	.145	62	76
	"	V-42*	52	.118	47	74
hebdomadis	medanensis	HC	29	.192	—	—
	Wolffi A	ML-34	51	.149	—	—
	hemolyticus	Marsh	54	.270	65	78
	"	Buxton*	"	.164	—	—
bataviae	bataviae	Van Tienen	32	.127	—	85
	djatzi	HS-26	51	.169	75	85
	paidjan	Quigley	54	.135	—	45
semaranga	semaranga	RS 173	37	.248	—	—
andaman	andaman	CH 11	31	.309	—	—
biflexa		CDC	–	.315	—	—

* Serotype designation was based on cross agglutination-lysis reactions.

series of 17 x 150 mm screw cap, chemically cleaned tubes. These tubes were incubated at 30°C. At specified intervals of time, the contents of 5 culture tubes were pooled, the supernatant fraction was then harvested, and the optical density of a 10-fold concentration of cells was determined as previously described. The supernatant fluid of harvested cultures was stored at –60°C. Tests, employing hemolysin Procedure II at 37°C, were conducted on the samples. When necessary, serial dilutions of supernatant samples were tested. The hemolytic potency of each sample was expressed in units of hemolysin. The results shown in Table III indicate that the hemolysin content of actively growing cultures increases with the increased concentration of leptospires and reaches maximum values at 1-3 days following optimal growth. Continued incubation of cultures reduces the hemolytic potency to approximately one-half the maximal value by the 50th day. This decrease in hemolytic activity may in large measure be due to thermal inactivation.

Studies on the effect of concentration of hemolysin on the kinetics of sheep RBC lysis were conducted with various dilutions of a supernatant preparation that was concentrated 2-fold by pervaporation and dialysis. Hemolysin Procedure I was employed and

TABLE III. Hemolytic Activity as a Function of Culture Growth.

Age of culture (days)	Culture optical density	Units of hemolysin/cc
0	.029	<1
1	.053	<1
2	.072	<1
3	.121	<1
4	.183	1– 4
5	.203	1– 4
6	.200	8–10
7	.209	10
8	.210	10
9	.214	12–16
10	.197	16–20
12	.192	16–20
14	.157	10
16	.161	10
18	.150	10
20	.133	8–10
24	.117	8–10
48	.109	8
50	.090	8

tests were conducted at 30°C. The plotting of percent hemolysis of various dilutions of the supernatant fraction as a function of time (Fig. 2) resulted in a series of curves which did not conform to the pattern generally observed with known hemolytic systems(5).

Attempts were made to determine the presence of specific hemolysin inhibitors in rabbit antiserum prepared against supernatant fractions (agglutination-lysis titer, 1:1600). The hemolysin employed was a 20-fold concentration of washed cells, disrupted by alternate freezing and thawing. The results summarized in Table IV indicated that normal rabbit serum inhibited lysis of sheep blood cells to the same degree as antiserum.

Discussion. The clinical symptomatology of the leptospiroses has been attributed to the toxigenic manifestations of these spirochetes (6,7). Gsell(7) maintains that leptospires produce a toxin with hemolytic properties and that the severity of disease reflects the intensity of intoxication. The hemolytic manifestations of leptospiral infections in man and animals have been frequently reported (6-9). The toxic effects in guinea pigs inoculated with preparations obtained from *L. icterohemorrhagiae* cultures have been demonstrated by Fukishima and Hosoya(10), and Higuchi(11), respectively. These observations, coupled with the fact that most, if not all of the known bacterial toxin products, other than the classic exotoxins, are hemolytic(12) strongly suggest that the hemolytic activity of leptospires is an attribute of a leptospiral toxin.

An appraisal of the experimental data in the light of current concepts of leptospirosis affords an opportunity to consider several factors that may operate in differential pathogenesis of various serotype strains for particular hosts. The various serotype strains show marked over-all differences in severity of disease produced in a particular host. Thus, in man, for example, mild, frequently subclinical, infections are generally produced by *L. pomona, L. mitis, L. grippotyphosa* and *L. hebdomadis* while the more severe icteric and hemorrhagic manifestations are more frequently observed in *L. icterohemorrhagiae, L. australis A* and *L. bataviae* infections(7). In addition, particular strains show discriminate pathogenicity for various mammalian hosts as exemplified by *L. pomona* infection in rats (inapparent), hamsters (lethal), guinea pigs (inapparent), humans (rarely icteric), swine (nonicteric), and cattle (frequently icteric) (6,7,9,13). Close examination of the data in which the hemolytic potency of 36 serotype strains, including 14 very recent isolates, was tested against sheep RBC (Table II) discloses differences among serotypes with respect to this characteristic. In some instances, *e.g., L. icterohemorrhagiae,* Wijnberg, the absence of hemolysin in strains with

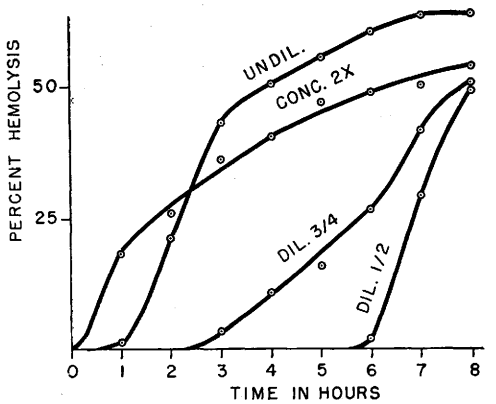

FIG. 2. Course of hemolysis of various concentrations of the supernatant fraction of a *L. hemolyticus* culture at 30°C.

TABLE IV. Comparative Titration of RBC Lytic Inhibitors in Specific Rabbit Antiserum and Normal Rabbit Serum.

Serum dilution	——Undiluted——			1:5	1:50	1:500	1:5000	—	—
" , ml	1	.6	.2	1	1	1	1	—	—
Buffer, ml	—	.4	.8	—	—	—	—	—	2
Hemolysin, ml	1	1	1	1	1	1	1	2	—
	Incubated test mixtures 30 min., 30°C								
1% sheep RBC, ml	2	2	2	2	2	2	2	2	2
	% hemolysis (1 hr 37°C)								
Antiserum	0	0	0	0	38	83	87	100	0
Normal serum	0	0	0	0	33	71	73		

a past history of "hemolytic" manifestations may perhaps be due to loss of this characteristic through continued *in vitro* subculturing over extended periods of time. In this respect, however, it is interesting to note that hemolysin was observed in 4 strains that were maintained in cultures for 17 to 23 years. The possible loss or maintenance of hemolytic properties after continued *in vitro* passage may perhaps reflect the varying degrees of adaptability of different strains to culture mediums, just as strains vary in their ability to maintain their virulence after repeated transfer *in vitro* medium. In our laboratory, a strain of *L. pomona* (S-91) was no longer lethal for hamsters after one year's passage in Fletcher's medium, while *L. djatzi* isolated from a human in Puerto Rico retained its lethality for hamsters after repeated transfers in this medium for 3 years. Van Riel observed an increase in pathogenicity for guinea pigs of an isolate after 8 months' cultivation in medium(14).

In addition to differences in the frequency of hemolysin-producing strains among heterologous serotypes, strain variation in the production of hemolysis is apparent within serotypes (Table II). Analogous observations of the differences in the pathogenicity of "homologous" serotype strains isolated from different sections of the world have been observed. The severity of *L. grippotyphosa* (bovis) infections in Israel and *L. bataviae* infection in Indonesia, are in marked contrast to the benign symptomatology produced by the respective European strains(7). On the basis of extensive studies of canine leptospirosis, Meyer *et al.*(15) concluded that—"The observations in California amply confirm the findings of Walch-Sorgdrager and Schüffner(16) that the icterogenic tendencies of *L. canicola* are a fixed property of certain strains." Stavitsky's(17) failure to substantiate the previously mentioned work of Fukishima, Hosoya and Higuchi, may be attributable to the toxigenic differences in the *L. icterohemorrhagiae* strains employed. In view of these observations, further consideration of the differences in pathogenicity of "homologous" strains may possibly be correlated in part with the hemolytic potential of these spirochetes.

The few species' RBC that are susceptible to the hemolysin of *L. hemolyticus* is particularly noteworthy. In contrast to our findings, Higuchi showed that "water leptospires" hemolysed rat, rabbit or guinea pig RBC. The data presented in Higuchi's report does not preclude the possibility that pathogenic leptospires were isolated. Whether or not this preferential activity is a function of the particular strain, reflecting perhaps the selective pathogenicity of that strain for various mammalian hosts, remains to be determined.

In large measure the activity of the hemolysin was masked by the presence of a relatively high concentration of rabbit serum (10%), necessary for the cultivation of leptospires. The inhibitory activity of normal rabbit serum on the lysis of mammalian RBC has been recognized for other hemolytic systems(18). The interfering activity of rabbit serum is demonstrated by data presented in Tables I and IV and from kinetic studies on the course of hemolysis of different concentrations of supernatant fluid (Fig. 2). The plotting of percent hemolysin as a function of time, employing different dilutions of the known bacterial hemolytic systems, elicits a typical consecutive series of sigmoid curves

with decreased slopes and increased "induction periods" as the concentration of hemolysin decreases(5). The atypical series of curves shown in Fig. 2 is attributed to the differences in the kinetics of the hemolysin and inhibitor.

Superficially, the hemolytic potency of mature cultures of *L. hemolyticus* (Table III) is low when compared to the activity of cultures of beta streptococci(5). However, the hemolytic potential of leptospires is indeed considerable when one compares the extremely low yield of approximately 25-75 mg (dry weight) of leptospires obtained per liter of culture(19) with the several grams of streptococci obtained in an equivalent volume of medium(12). In addition, the hemolytic activity of leptospires is manifested in the presence of high lytic inhibitor (10% rabbit serum) concentrations.

Although it is interesting to consider our experimental data in terms of the current knowledge of the leptospiroses, definitive interpretation of the role of leptospiral hemolysin in the virulence of leptospiral strains and host reactions to infections must be approached with caution at this time. Further study of the nature and activity of leptospiral hemolysin and its mode of formation may provide clues to a better understanding of the factors involved in the pathogenesis of leptospiral infections.

Summary. A chance observation of the hemolysis of sheep red blood cells by a culture of leptospires was investigated. Studies were conducted on a newly disclosed serotype strain, isolated in Malaya and designated as *Leptospira hemolyticus*. The presence of a soluble non-dialysable, thermolabile, oxygen-stable hemolysin in the supernatant fluid of cultures was demonstrated. The optimum temperature for hemolytic activity was 37°C. At lower temperatures the activity of hemolysin progressively decreased. The hemolytic activity against sheep RBC occurred after a prolonged induction period. This phenomenon as well as the atypical kinetic activity of varying concentrations of hemolysin preparations were attributed to the inhibitory activity of the rabbit serum present in culture supernatants. The production of hemolysin was not a generic characteristic but was restricted to specific serotype strains. Hemolytic activity of *L. hemolyticus* was manifested against sheep, cow, goat RBC, but not against RBC of eleven other species screened. The antigenicity of this hemolysin could not be demonstrated. In actively growing cultures, maximum amounts of hemolysin were produced 1 to 3 days following optimum growth. The presence of a soluble hemolysin in specific serotype strains may figure significantly in explaining the pathogenicity of leptospiral infections.

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