Antileptospiral Activity of Serum
I. Normal and Immune Serum

RUSSELL C. JOHNSON AND LOUIS H. MUSCHEL
Department of Microbiology, University of Minnesota, Minneapolis, Minnesota

Received for publication 9 December 1965

Abstract

JOHNSON, RUSSELL C. (University of Minnesota, Minneapolis), and LOUIS H. MUSCHEL. Antileptospiral activity of serum. I. Normal and immune serum. J. Bacteriol. 91:1403–1409. 1966.—Normal serum was found to exert a leptospiricidal effect, mediated by the complement system, against the nonpathogenic leptospires. Although resistant to normal serum, the pathogenic serotypes were susceptible to antiseraum plus complement. Several variables in these immune leptospiricidal reactions were investigated. A reaction period of 3 hr at 37 C between serum substances and 1-day-old cells provided a maximal leptospiricidal effect. The normal serum of the rabbit, guinea pig, bovine, and human were leptospiricidal against the nonpathogenic serotypes, and, in conjunction with rabbit antiserum, rabbit and bovine complement were leptospiricidal against the pathogenic serotypes. Studies with C14 labeled leptospires indicated that the immune leptospiricidal reaction was associated with a loss of permeability control. Thus, like the gram-negative bacteria, the treponemes, erythrocytes, and nucleated mammalian cells, the leptospires may be included as cell types susceptible to the antibody-complement system.

In a previous communication (10), the presence of antileptospiral activity in normal serum mediated by the complement system was reported. Further studies have been concerned with the standardization of an assay for this activity and the determination of the serum susceptibility of a larger number of pathogenic and nonpathogenic leptospires. These studies have indicated that pathogenic leptospires may be differentiated from the nonpathogenic forms by the greater resistance of the former to normal serum. These results nicely supplement another method of discrimination involving the purine analogue 8-azaguanine, which, in contrast to serum, has greater inhibitory action against the pathogenic leptospires (12).

The role of complement in the leptospire-antibody system was studied previously by Lawrence (14) and Dymowska and Babudieri (2). These authors indicated that complex did not influence this system in any significant manner. Since we had noted that “normal” antibodies in conjunction with complement would exert a leptospiricidal action, the role of complement in the leptospire-antibody system was re-evaluated. The results of this investigation are presented in this report.

Materials and Methods

Nine strains of nonpathogenic leptospires and eight strains of pathogenic serotypes of leptospires were used in this investigation. Leptospira pomona Wickard was obtained from J. B. Wilson, Department of Bacteriology, University of Wisconsin, and L. biflexa Lt695 from Mildred M. Galton, Communicable Disease Center, Atlanta, Ga. The remaining cultures were provided by A. D. Alexander, Walter Reed Army Institute of Research.

Cultures of leptospires were maintained in a growth medium containing 10% heat-inactivated (56 C, 30 min) pooled rabbit serum (Pel-Freez Biologicals, Inc., Rogers, Ark.), 0.02 m Na2HPO4-KH2PO4, pH 7.4 (13), 10−3 m NH4Cl, 5 μg/ml of thiamine (9), and 100 μg/ml of 5-fluorouracil (11). For use in the test procedures, the cultures were obtained from semisolid stock medium (growth medium containing 0.2% agar) and transferred at least three times in the liquid growth medium after incubation at 30 C for 3 to 5 days.

The pooled normal rabbit serum used in the antileptospiral assay of normal serum, and as a complement source in the experiments with immune sera, was obtained from 15 adult New Zealand rabbits. Human serum was obtained from a single donor, and bovine and guinea pig sera were purchased from Grand Island Biological Co., Inc., Grand Island, N.Y. All sera were adjusted to pH 7.3 with 1 N HCl.
sterilized by filtration through a Seitz filter at 5 C, and stored at -50 C. The diluent used in the anti-leptospiral assay was 0.85% NaCl containing 0.5% bovine albumin (Cohn fraction V).

The assay for antileptospiral activity of normal rabbit serum was conducted in the following manner. A 0.2-ml amount of this cell suspension plus test serum and diluent were combined in a final volume of 2 ml and incubated at 37 C for 1 to 3 hr with members of the L. biflexa group, and for 16 to 18 hr with the pathogenic serotypes. At the end of the reaction period, immobilization of the leptospires by the test serum was determined by dark-field microscopy. In addition, 5 ml of growth medium was added to each tube for the assay of surviving organisms. The tube contents were mixed and incubated at 30 C. Growth was measured daily with a Coleman (model 7) photonephelometer. Periodic microscopic counts with the Petroff-Hausser counting chamber were performed to verify the relationship between cell count and nephelometer reading. The experiments were terminated when growth in the control tubes (cells plus heated serum) reached approximately 4 x 10^8 cells per milliliter. The growth in the assay tubes was compared with the growth in the control, and the percentage of growth inhibition was calculated. The percentage of growth was, as determined by direct microscopic examination, corresponded with percentage of growth inhibition.

5-Fluorouracil (100 μg/ml) was present in the growth medium added to the assay tubes to prevent the growth of contaminants. To determine whether this pyrimidine analogue influenced the results of the antileptospiral assay, an experiment was conducted employing growth medium with and without 5-fluorouracil. No difference was observed with these two media.

For the production of immune sera, adult New Zealand rabbits were injected three times with 1, 2, and 4 ml of living 5-day-old cultures of leptospires (2 x 10^9 leptospires per milliliter) at weekly intervals. The first injection of 1 ml consisted of the culture plus an equal volume of Freund's complete adjuvant administered subcutaneously. The second injection of 2 ml and the third injection of 4 ml were given intravenously without Freund's adjuvant. One week after the final injection, the rabbits were bled, and sera were harvested, heated at 56 C for 30 min, and stored at -20 C.

The microscopic agglutination test with the use of immune serum was performed as described by Babudieri (1), and was also modified to determine the effect of complement on the reaction. The immune leptosporical test was performed with test tubes (18 by 150 mm) containing 0.5 ml of antiserum dilution, 0.2 ml of fresh rabbit serum (source of complement), 0.2 ml of the leptospires (3 x 10^8 cells per milliliter), and diluent to a final volume of 2 ml. After mixing, the tubes were incubated at 37 C for 1 to 3 hr. The test material was then examined microscopically and assayed for growth in the same manner as described for the antileptospiral assay of normal serum.

C^4-labeled L. pomona cells were used to study the possible leakage of cellular substances as a result of serum action. The cells were prepared in the following manner. A 5-ml amount of a 3-day-old culture was added to an equal volume of growth medium containing 5 μc of uniformly labeled C^14-palmitate. The culture was incubated for 18 hr at 30 C, and cells were harvested by centrifugation, washed once with the diluent, and adjusted to a concentration of 10^8 to 5 x 10^9 cells per milliliter. These cells were used in the immune leptosporical test with the total volume of the reaction mixture increased to 10 ml. After the reaction period of 1 hr at 37 C, the reaction mixture was examined for leptospiral motility and centrifuged for 1 hr at 12,000 x g, and the supernatant fluid was examined for the presence of leptospires. In the absence of leptospires, the supernatant fluid was assayed for radioactivity with a model 181 B scaler and a model D47 gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) at 1,450 v with a gas mixture of 98.7% helium and 1.3% butane. Samples were plated in infinite thinness on glass planchets.

The in vivo survival of L. pomona Wickard and L. biflexa Patoc I was studied in the following manner. Four adult female New Zealand rabbits were bled, and their sera were tested against L. pomona and L. biflexa to determine whether their antileptospiral activity was within normal limits. Two of the rabbits were injected with approximately 10^9 cells of L. biflexa. In one rabbit, the cells were injected intracardially, and 5-ml blood samples were withdrawn from the heart at 15, 30, 60, and 90 min thereafter. In the second rabbit, the same number of leptospires were injected intravenously in the left marginal ear vein, and 0.5-ml blood samples were collected from the right marginal ear vein at 1, 2, 3, 4, 5, 30, and 60 min after the injection. All blood samples were diluted 1:20 and 1:200 in the growth medium, and immediate microscopic examinations were made. The samples were also incubated at 30 C for 14 days and examined daily for the presence of leptospires. These procedures were then repeated with L. pomona.

RESULTS

Several variables that might influence the results of the antileptospiral assay of normal serum were investigated. In these studies, pooled normal rabbit serum was tested against L. biflexa Patoc I. The number of leptospires used in the assay had a marked influence on antileptospiral titer of the test serum: the sensitivity of the assay was inversely proportional to the number of cells used. Normal rabbit serum (1:50 dilution) was leptosporical to 95, 90, 75, 50, and 5% of the cells when the reaction mixture contained 2.5, 5, 10, 20, and 40 x 10^4 leptospires per milliliter, respectively. The use of between 10^4 and 5 x 10^5 cells per milliliter in subsequent assays resulted in a sensitive test which allowed the growth assay to be completed within a reasonable time period.

The reaction mixture was incubated at 37 C.
for different time intervals to determine the effect of different reaction periods on the serum antileptospiral titer (Table 1). The activity of the serum increased as the incubation time increased. On the basis of these results, the incubation period for the *L. biflexa* group was between 1 and 3 hr. Normal rabbit serum did not manifest antileptospiral activity against the pathogenic serotypes, even when the incubation period was extended to 18 hr. It is interesting to note that at zero-time an inhibitory effect was observed, although the 5 ml of growth medium was added to the reaction mixture before the test serum. This observation indicated that the 1:3.5 dilution of the serum-leptospiral mixture did not result in the complete inhibition of serum action, and, further, that the growth medium was not significantly anticomplementary.

The temperature at which the assay mixture was incubated was varied from 0 to 40 C for a period of 1 hr (Table 2). Very little antileptospiral activity was observed at 0 C. As the temperature was raised from 0 to 27 C, the degree of growth inhibition increased and reached its maximum at 37 or 40 C.

The effect of cell age on the sensitivity of cul-
tures to normal serum was investigated next. A 5-ml amount of a 3-day culture was added to 5 ml of growth medium and incubated for 16 to 24 hr. These 1-day-old cultures showed a slight increase in nephelometer reading during this incubation period and were considered to be comprised of cells in the late lag phase and early log phase of growth. The serum sensitivity of these cells was compared with 6-day-old cells which had just terminated their log phase of growth (Table 3). The 1-day-old cells were markedly more sensitive to the action of normal serum than were the 6-day-old cells, and, subsequently, cells of this age were used in the assay system.

With the assay method developed, members of the nonpathogenic *L. biflexa* group and certain pathogenic serotypes of leptospires were tested for their sensitivity to the antileptospiral activity of normal rabbit serum (Table 4). The growth of all nine members of the *L. biflexa* group tested was strongly inhibited by a 1:33 dilution of rabbit serum, with the exception of *L. biflexa* Lt 430 which survived a serum concentration of 1:5. Rabbit serum at dilutions of 1:50 and 1:100 had a variable effect, and 1:200 dilution had little or no antileptospiral activity on these leptospires. In contrast to the nonpathogenic *L. biflexa* group, the eight pathogenic serotypes were almost completely refractory to the action of normal serum. Little or no inhibition of the growth of these pathogenic serotypes occurred, even at the 1:3 dilution of normal rabbit serum. Thus, a marked difference in the sensitivity to the antileptospiral action of normal rabbit serum exists between the *L. biflexa* group of leptospires and the pathogenic serotypes.

Rabbit serum was compared with bovine, guinea pig, and human sera against the pathogen *L. pomona* and the nonpathogen *L. biflexa* Patoc I (Table 5). The sera of the four species were leptospiricidal to *L. biflexa* at a dilution of 1:40. Rabbit, bovine, and human sera had little or no antileptospiral activity against *L. pomona*. In contrast to these sera, guinea pig serum possessed

### Table 1. Effect of incubation time on the reaction of normal rabbit serum against *Leptospira biflexa* Patoc I

<table>
<thead>
<tr>
<th>Length of incubation before the addition of growth medium</th>
<th>Per cent survival* compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>1:50</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>30</td>
<td>&lt;1</td>
</tr>
<tr>
<td>60</td>
<td>&lt;1</td>
</tr>
<tr>
<td>180</td>
<td>&lt;1</td>
</tr>
<tr>
<td>300</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Survival based on growth.
† Serum dilution.

### Table 2. Effect of incubation temperature on the reaction of normal rabbit serum against *Leptospira biflexa* Patoc I

<table>
<thead>
<tr>
<th>Incubation temp</th>
<th>Per cent survival* compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:66†</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td>27</td>
<td>45</td>
</tr>
<tr>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Survival based on growth.
† Serum dilution (v/v).

### Table 3. Effect of cell age of *Leptospira biflexa* Patoc I on serum sensitivity

<table>
<thead>
<tr>
<th>Culture age</th>
<th>Per cent survival compared with control*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:30†</td>
</tr>
<tr>
<td>days</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

* Survival based on growth.
† Dilution of normal rabbit serum.
marked leptospiricidal activity against this organism.

Each of the pathogenic serotypes, i.e., *L. pomona*, *L. canicola*, *L. icterohaemorrhagiae*, and *L. wolfii*, which were resistant to normal serum, was then tested against specific antiserum in conjunction with normal rabbit serum, diluted 1:10, as a complement source. Different amounts of the antisera were tested against the homologous organism in the presence and absence of complement. All the dilutions of antiserum which caused agglutination of the majority of cells were leptospiricidal in the presence of complement (Table 6). In the absence of complement, growth occurred in all dilutions of antisera. Agglutination of the cells by antiserum before the addition of

complement did not protect them from leptospiricidal activity. Bovine serum also functioned as a satisfactory source of complement at a 1:10 dilution. As previously observed with the interaction of *L. biflexa* Patoc I and normal serum, the number of cells and the age or physiological state of the cells have a significant effect on the sensitivity of the immune leptospirosis test.

The question whether leptospires were lysed by the antibody-complement system was investigated with the use of C14-labeled *L. pomona* cells. Two identical experiments were performed with *L. pomona* antiserum diluted 1:100 and fresh rabbit serum as the source of complement against C14-labeled leptospires. In the presence of anti-serum and complement, the cells were killed and about 50% of the label was released from these cells (Table 7). Antiserum without added complement (heated fresh rabbit serum) did not kill the cells, and only about 4% of the label was released. These results demonstrated a marked loss of permeability control in the presence of specific antiserum and complement, and indicated that significant leakage of intracellular material does not occur in the absence of complement.

The capacity of the comparatively serum-resistant and -sensitive leptospires to survive in vivo was investigated with the use of adult rabbits. Two rabbits were injected with the nonpathogen *L. biflexa* Patoc I. In one rabbit the cells (10⁹) were injected intracardially and, in the other, intravenously. Only blood samples obtained at
Table 6. Effect of complement on the leptospiricidal activity of immune serum

<table>
<thead>
<tr>
<th>Antiserum against</th>
<th>Without complement</th>
<th>With complement*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:10</td>
<td>1:100</td>
</tr>
<tr>
<td>Leptospira pomona Wickard</td>
<td>±† ±</td>
<td>+ ++</td>
</tr>
<tr>
<td>L. canicola Hond Utrecht IV</td>
<td>0 ±</td>
<td>+ + +</td>
</tr>
<tr>
<td>L. icterohaemorrhagiae M20</td>
<td>± ±</td>
<td>+ +</td>
</tr>
<tr>
<td>L. wolffi 3705</td>
<td>± ±</td>
<td>+ +</td>
</tr>
<tr>
<td>L. biflexa Patoc I</td>
<td>± +</td>
<td>++</td>
</tr>
</tbody>
</table>

* Source of complement was a 1:10 dilution of fresh rabbit serum for the pathogenic serotypes and 1:100 dilution for L. biflexa. In both instances, this amount of normal serum alone was without effect.
† Symbols: 0 = no growth, no viable leptospires seen; + = partly in clumps, largely as single leptospires; ++ = growth equivalent to control; ± = growth occurred in large clumps, could not be measured.

Table 7. Release of C4-labeled material from Leptospira pomona by antiserum and complement

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Expt I</th>
<th>Expt II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Corrected</td>
</tr>
<tr>
<td>Untreated cells suspended in albumin-</td>
<td>5,450</td>
<td>0</td>
</tr>
<tr>
<td>saline diluent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant fluid of untreated cells</td>
<td>625</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant fluid of cells plus heated rabbit serum</td>
<td>700</td>
<td>75</td>
</tr>
<tr>
<td>Supernatant fluid of cells plus complement (unheated rabbit serum)</td>
<td>850</td>
<td>225</td>
</tr>
<tr>
<td>Supernatant fluid of cells plus antiserum heated rabbit serum</td>
<td>950</td>
<td>250</td>
</tr>
<tr>
<td>Supernatant fluid of cells plus antiserum complement</td>
<td>3,200</td>
<td>2,450</td>
</tr>
</tbody>
</table>

* Total number of cells in sample was $4 \times 10^7$.

1, 2, and 3 min contained even a few L. biflexa cells. No leptospires were seen microscopically in subsequent samples taken, and no growth was observed in any of the blood samples. Two other rabbits were injected in a similar manner with the pathogen L. pomona. In contrast to L. biflexa, L. pomona was seen microscopically and was cultured from all the blood samples obtained from these rabbits. Decreased numbers of L. pomona in the blood samples were not observed until 90 min.

Discussion

In conjunction with appropriate antisera, it is well established that complement may be involved in the destruction of a wide variety of cells. This includes the killing of many gram-negative bacteria, the immobilization and killing of treponemes, the hemolysis of erythrocytes, a cytotoxic effect on nucleated cells, and others (18). To these, the immobilization and killing of the leptospires may now also be added.

In a previous report, it was found that normal sera of various mammalian species possessed antileptospiral activity mediated by the complement system (10). The presence of normal antibodies in such sera may be inferred from the work of Linscott (15), which showed that such substances functioned in the immune adherence of leptospires. These normal antibodies provide the required immune complex for the activation of complement. Moreover, the results of these studies have indicated that the nonpathogenic leptospires are extremely sensitive to normal serum. This is in marked contrast to the comparatively high serum resistance of the pathogenic serotypes tested. However, when antiserum was used against these forms, with normal serum as a source of complement, an easily demonstrable leptospiricidal effect was observed. These results
are in accord with the findings among the gram-
negative enteric bacteria which suggested an
association between serum susceptibility and
virulence (16).

Various biological characteristics have been
associated with the nonpathogenic leptospires,
and these have been useful in differentiating them
from the pathogenic serotypes. Some of these
characteristics are the ability of the nonpathogenic
organisms, but not the pathogenic organisms, to
grow in the feces medium (7, 20) in the presence
of 10 ppm of copper sulfate (4), in the absence of
CO₂ (21), and in the presence of high concentra-
tions of the purine analogue 8-azaguanine
(12). The nonpathogens also have greater oxidase
activity than do the pathogenic serotypes (5).

The marked difference in rabbit serum suscepti-
bility between the nonpathogens and the patho-
gens is another parameter which can be used for
the differentiation of these two groups of lepto-
spires. This method of differentiation is as simple
and as rapid as the 8-azaguanine test. With the
serum antileptospiral assay, normal rabbit serum
concentrations of up to and including the 1:3 dilu-
tion should be used (Table 4). The antileptospiral
assay used in conjunction with the 8-azaguanine
test has given unequivocal results in differentiating
these leptospires.

Many investigators in the field of leptospirosis
do not routinely heat-inactivate the rabbit serum
used in their growth medium. This is probably of
no consequence when pathogenic serotypes or
large inocula of the nonpathogenic leptospires
(10% v/v) are used, since unheated sera will
exert little or no significant antileptospiral ac-
tivity under these circumstances. However, when
isolating these leptospires from natural sources,
where they usually occur in small numbers, the
heat inactivation of the rabbit serum used in the
growth medium is imperative. The use of an
albumin medium would similarly be helpful in
this regard (3).

The relationship demonstrated in vitro with the
serum was found to hold true in the blood stream of
the rabbit. When nonpathogenic leptospires
were injected into rabbits, they were removed or
destroyed, probably as a result of the action of
normal antibody and complement, far more
rapidly than the pathogenic serotype. Although
these results are of a preliminary nature, they
suggest a meaningful relationship between serum
resistance and the pathogenicity of different lepto-
spires.

The anatomical or physiological basis for the
resistance or susceptibility of leptospires to nor-
mal serum was not investigated. However, by
analogy with the gram-negative enteric bacteria,
capsular antigens associated with the pathogenic
forms may be suspected to confer serum resistance
(16). Normal antibodies are probably either ab-
sent or present in too low a concentration for
significant activity against such antigens.

The incubation time and temperature found to
be suitable for serum antileptospiral activity,
the heat lability of this activity, and its inhibition
by treatment of serum with an immune complex
(10) are compatible with a complement require-
ment for this activity. Normal rabbit serum pos-
sesses little or no leptospiridal activity against
the pathogenic serotypes of leptospires. Thus, it
serves as an excellent source of complement for
the immune leptospiridal assay of antisera.

The finding that cells in a 1-day-old culture
were more susceptible than those of a 6-day-old
culture to the leptospiridal activity, but not to
the agglutinating activity, of antisera is also of
interest and is analogous to results obtained with
the immune bactericidal reaction against gram-
negative bacteria (16).

The effect of antiserum upon leptospires has
been confused largely because of the term ag-
glutination—"lysis," which is used frequently to
describe leptospiral agglutination when live
antigens are used. This so-called lysis was unique,
because it did not require the participation of
complement (19). However, Lawrence (14) and
Dymowska and Babudieri (2) demonstrated very
clearly that antiserum alone does not kill or
lyse leptospires, and that the "lysis balls" that
result from the action of antiserum upon lepto-
spires contain intact and viable leptospires. In
addition, both of these investigations showed
that antiserum, even in the presence of comple-
ment, did not manifest a leptospiridal or lytic
action. Insufficient complement or unsuitable
conditions are probably responsible for these
observations. In this investigation, C4-labeled
leptospires were used to determine whether these
organisms were lysed by antiserum and comple-
ment. As noted previously, a leptospiridal ef-
fact was observed, and microscopically the killed
leptospires appeared to be partially disintegrated.
Such cells were found to release about 50% of
their C4 label. When complement was not in-
cluded in the reaction mixture, only an insignifi-
cant amount of label was released, and lepto-
spiridal action was not observed. Thus, the
action of the complement system upon leptospires
resulted in an irreversible loss of permeability
control and partial disintegration of the cell.
This is similar to the action of complement upon
other bacteria (17) and mammalian cells (6).
Moreover, with mammalian erythrocytes, func-
tional holes in the cell membrane have been
demonstrated by electron microscopy (8).
ACKNOWLEDGMENT

The investigation was supported by Public Health Service grants AI-06589 and AI-05454 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED