Natural Antibody in Mammalian Serum Reacting with an Antigen in Some Leptospires

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Serum from normal mammals agglutinated and immobilized nonpathogenic Leptospira biflexa and agglutinated avirulent lines of pathogenic serotypes L. icterohaemorrhagiae and L. zanoni. Virulent lines of L. icterohaemorrhagiae and L. zanoni were not affected, nor were any of three strains of L. pomona, one of which was avirulent. The active principle in serum was a β-macroglobulin which was heat-labile and reduced by 2-mercaptoethanol, and acted in conjunction with complement and lysozyme; it was absorbable from serum by Formalin-treated susceptible leptospires. The Formalin-stable receptor antigen, named "Z antigen," is associated with virulence rather than pathogenicity, but may not be a determinant of virulence.

Johnson and Muschel (10, 11) described an antileptospiral activity of serum which selectively affected the nonpathogenic Leptospira biflexa, but not the pathogenic L. pomona, in vivo as well as in vitro. They regarded this selective action of serum as a determinant of pathogenicity. We have studied the nature and mode of action of the antileptospiral substance, and its possible significance as a mediator of pathogenicity or of virulence. "Pathogenicity" and "virulence" are used as defined by Miles (13) and in previous studies of leptospiral infection (3). Briefly, pathogenicity is a genotypic characteristic which may be measured quantitatively as virulence. Among the leptospires, pathogenicity is associated with serological type; within a pathogenic serotype, there may be variations from highly virulent to avirulent without any accompanying serological change which can be identified by currently available methods. Nonpathogenic serotypes (e.g., L. biflexa) are free-living, do not cause infection, and, thus, are never virulent.

Materials and Methods

Leptospiral cultures. All leptospiral cultures were grown at 30°C in modified Korthof medium containing 10% rabbit serum and 10 μg of added thiamine HCl per ml. L. biflexa (Patoc), a nonpathogenic leptospira, was obtained from A. D. Alexander, WHO Reference Laboratory, Walter Reed Army Medical Center, Washington, D.C. L. icterohaemorrhagiae (22), avirulent for guinea-pigs and chick embryos, was obtained in 1958 from L. Kirschner, New Zealand. Leptospira Reference Laboratory, Dunedin, New Zealand. L. icterohaemorrhagiae (GP) was the avirulent straight form of L. icterohaemorrhagiae (GP-B), hooked, and virulent for guinea-pigs (4) and 10-day chick embryos, freshly resolated in culture after its second guinea pig passage. L. zanoni (E), virulent (carrier state for mice; 3), was freshly resolated from mice after its fifth mouse passage; its avirulent cultured line was L. zanoni. L. pomona (EP; 2) and L. pomona (B), isolated from a patient in Queensland in 1966 and obtained from D. J. W. Smith, Laboratory of Microbiology, Brisbane, Australia, were virulent for chick embryos, and the latter was presumably virulent for man. L. pomona (STM), obtained from J. W. Wannan, School of Public Health and Tropical Medicine, University of Sydney, was avirulent for chick embryos. "Virulent" for chick embryos indicates typical lesions within 5 days of inoculation of approximately 5 × 10^7 leptospires from a 6-day culture onto the dropped chloroallantoic membrane. "Avirulent" for chick embryos indicates no lesions or leptospires detected after similar inoculation.

Animals. Normal guinea pigs, rabbits, rats and mice were obtained from University of Sydney stocks. The animal stocks were not known to have leptospiral infections. Sheep were obtained from local sources. All sera were separated from clots, pooled, and either used fresh or frozen at −20°C in small portions.

Immunization procedures. Antisera against leptospires were prepared by injecting rabbits intravenously with 1 to 5 ml of suspensions of leptospires three times weekly for 3 weeks, and bleeding after an interval of 3 days after the last injection.

Titration of sera. Dilutions of sera, usually 1:2 to 1:128 were made in a titrating tray with Veronal-buffered Ca-Mg saline, pH 7.3, as a diluent. Equal volumes of 2- to 6-day leptospiral cultures were added, and the tray was incubated at 37°C for 90 min. Occasionally, a little autoagglutination occurring in the cultures was cleared by centrifuging at approximately 800 × g for 5 min just before use.
Gel filtration. Sephadex G-200, in a buffer of 0.1 M tris(hydroxymethyl)aminomethane-chloride (pH 8.0) in 0.2 M NaCl, was packed in a column approximately 30 x 1.5 cm. A sample volume of up to 0.75 ml was layered on the gel surface and washed through with the same buffer at a hydrostatic pressure of 1.5 to 2 meters. Fractions of 3 ml were collected, dialyzed against 0.015 M NaCl for 4 hr, and concentrated about one-tenth volume by pervaporation or evaporation under reduced pressure.

Immunoelectrophoresis. Electrophoresis was carried out on microscope slides coated with 1% agar by use of a Veronal buffer (ionic strength, 0.1; pH 8.6) at 7 V/cm for 90 min. After addition of antiserum, slides were kept at 4 C for 48 hr to develop precipitin lines.

Elution of absorbed globulins. Globulins absorbed onto leptospiras were eluted by incubation of the absorbing suspension with 0.1 ml of a glycine buffer, pH 3.0, at 4 C overnight. The sediment was separated by centrifugation, and the supernatant fluid was neutralized and kept.

RESULTS

Immobilizing and agglutinating effects of normal serum. Normal guinea pig serum (GPS) was diluted in 0.85% NaCl to 1, 10, 20, 30, 40, and 50%. One half of each sample was heated at 56 C for 30 min. Each serum dilution was incubated at 37 C for 2 hr with an equal volume of a culture of either L. biflexa (Patoc) or L. pomona (EP-F). On microscopic examination, it was found that unheated serum immobilized L. biflexa (Patoc) but not L. pomona (EP-F), in concentrations of 30, 40, or 50%. Heated serum did not immobilize either of the serotypes. These results confirm the findings of Johnson and Muschel (10) in similar experiments. The same serum concentrations that immobilized L. biflexa (Patoc) also agglutinated it. L. pomona (EP-F) was not agglutinated. L. biflexa (Patoc) and GPS were mixed and observed at intervals of 5 min. An estimated 50 to 75% of the leptospiras were agglutinated after 10 min and 100% were agglutinated by 35 min. The first sign of immobilization was sluggish motility at 10 min, followed by very slow movement at 15 min; 90% of the leptospira were nonmotile after 20 min and 100% after 50 min; controls were motile and unagglutinated after 50 min. No differences were observed between the use of Veronal-buffered saline (pH 7.3) and phosphate-buffered saline (pH 7.2). Pooled sera from normal man, sheep, rat, rabbit, mouse, and guinea pig agglutinated L. biflexa (Patoc) and L. icterohaemorrhagiae (22; titers, 1:4 to 1:128), and all except rabbit sera immobilized L. biflexa (Patoc; titers, 1:4 to 1:16). Human serum agglutinated L. biflexa (Patoc) within 20 sec, then immobilized it, and finally lysed it within 5 min in a 1:2 dilution at 18 C.

Evidence of the participation of complement and lysozyme. The agglutinating and immobilizing power of GPS, lost on heating at 56 C for 30 min, could be restored to heated GPS by adding a final 1:80 concentration of unheated GPS, which itself had no agglutinating or immobilizing effect. A final concentration of 0.15% ethylenediaminetetraacetic acid (EDTA) in GPS was kept at 4 C for 45 min. Dilutions of EDTA-treated GPS in Veronal buffer were incubated with equal volumes of 6-day cultures of L. biflexa (Patoc) or of L. icterohaemorrhagiae (22) at 37 C for 1.5 hr. EDTA alone had no effect. GPS agglutinated L. biflexa (Patoc) and L. icterohaemorrhagiae (22), and in addition immobilized L. biflexa (Patoc). GPS treated with EDTA had no effect on either serotype of leptospira, but activity similar to that of GPS alone was restored by adding a 1:80 concentration of GPS or by a final 100 μg of Ca++ or Mg++. A sample of GPS was absorbed twice with bentonite to remove lysozyme (7). A control untreated sample agglutinated L. icterohaemorrhagiae (22) to a titer of 1:64 when incubated at 37 C for 1.5 hr. After bentonite absorption, the corresponding titer was reduced to 1:16, and was restored to the control level by 8 μg or more of crystalline lysozyme per ml (but not by 6 μg/ml or less), or by prolonging the incubation period to 4 hr. Adding excess lysozyme (up to a final 200 μg/ml) to GPS did not increase its titer on incubation for 45 or 90 min.

Susceptibility of various leptospiral strains. Table 1 shows that of the strains tested only L. biflexa (Patoc) was immobilized by GPS. Only avirulent cultures of the pathogenic serotypes L. icterohaemorrhagiae and L. zanoni were agglutinated; virulent cultures freshly passaged through animals were unaffected. None of the cultures of L. pomona was affected, even though one (STM) was avirulent.

Nonspecificity of antileptospiral activity. Agglutinating and immobilizing ability were absorbed from GPS by packed susceptible leptospiras, without treatment or after treatment with 0.5% Formalin. Sera absorbed with Formalin-treated leptospiras were titrated, with the results shown in Table 2. Susceptible strains cross-absorbed agglutinating activity and immobilizing activity, even though the strains were not themselves immobilized. Insusceptible strains did not absorb either agglutinating or immobilizing activity.

Antibody nature of the active component in GPS. Undiluted GPS (titer, 1:64) was heated at 56 C for 6 hr with intermittent agitation. Every 10 min for the first 90 min, and after 6 hr, a sample was removed and its agglutinating titer against L. icterohaemorrhagiae (22) was measured with the addition of 1:80 GPS as a source of complement.
The first five of the protein-containing fractions were active. These fractions were pooled, concentrated, and again run down a similar column. The fractions were dialyzed and concen-

The titer fell by one-half each 10 min until 30 min, and then remained at 1:2 for 60 min to 6 hr.

In a similar experiment, GPS diluted 1:2 in Veronal buffer was heated at 45, 50, 56, 60, 65, and 75 C for 2 hr; samples were removed at 10-min intervals, chilled, and later titrated. Activity was completely destroyed at 65 to 75 C within 20 min. A residual titer of 1:2 remained after 50 to 60 min at 56 to 60 C. From the resulting graph of time versus titer at each temperature, it was apparent that half the original titer of 1:64 remained after 5 min at 65 to 75 C, 22 min at 60 C, 38 min at 56 C, 90 min at 50 C, and >120 min at 45 C.

**TABLE 1. Agglutinating and immobilizing effect of normal guinea pig serum on various leptospiral strains, at 37 C for 1.5 hr**

<table>
<thead>
<tr>
<th>Strain of Leptospira</th>
<th>Dilution of guinea pig serum&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em> (22)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em> (GP)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em> (GP-B)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td><em>L. zanoni</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td><em>L. zanoni</em> (-E)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td><em>L. pomona</em> (STM)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td><em>L. pomona</em> (EP-F)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td><em>L. pomona</em> (B)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td><em>L. biflexa</em> (Patoc)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4X</td>
</tr>
</tbody>
</table>

<sup>a</sup> Symbols: 0 = no effect; 1-4 = degrees of agglutination; 1 = approximately 25% agglutinated; 4 = approximately all agglutinated; X = immobilized. No effect was observed with diluent alone or a dilution 1:128.

<sup>b</sup> Avirulent.

<sup>c</sup> Virulent (see Materials and Methods).

**TABLE 2. Agglutinating and immobilizing titers of guinea-pig serum absorbed with Formalin-treated packed leptospires**

<table>
<thead>
<tr>
<th>Guinea pig serum absorbed with</th>
<th>Titer (reciprocal serum dilution) against test organism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agglutination</td>
</tr>
<tr>
<td><em>Leptospira biflexa</em> (Patoc)</td>
<td>0</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em> (22)</td>
<td>0</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em> (GP)</td>
<td>0</td>
</tr>
<tr>
<td><em>L. icterohaemorrhagiae</em> (GP-B)</td>
<td>32</td>
</tr>
<tr>
<td><em>L. pomona</em> (EP-F)</td>
<td>16</td>
</tr>
<tr>
<td>Unabsorbed control</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not immobilized.

**TABLE 3. Effect of treatment with 2-mercaptoethanol and of heating of guinea-pig serum on its agglutinating and immobilizing activity against Leptospira biflexa (Patoc)**

<table>
<thead>
<tr>
<th>Type of treatment of serum (40% dilution)</th>
<th>Dilution of GPS added as a source of complement&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, diluent only</td>
<td>4X 2X 1</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>0 0 0</td>
</tr>
<tr>
<td>56 C for 30 min</td>
<td>4X 2X 1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Symbols: 0 = no effect; 1-4 = degrees of agglutination (see Table 1); X = immobilized. No reaction was observed without added GPS.

A sample of GPS was treated with 2-mercaptoethanol (ME; 1) and a similar sample was heated at 56 C for 30 min. Both samples were made up to a 40% dilution of GPS in phosphate-buffered saline and titrated against *L. biflexa* (Patoc) with dilutions of 1:80, 1:120 or 1:160 GPS as sources of complement. The results in Table 3 show that GPS treated with ME was unable to agglutinate or immobilize leptospires, and that the effects of treatment with ME were not reversible by the addition of complement, in contrast to the effects of heating at 56 C for 30 min.

Identification of active fraction of GPS. GPS was gel-filtered on a column of Sephadex G-200. The fractions containing protein were identified and tested for agglutinating activity against *L. biflexa* (Patoc). The first five of the protein-containing fractions were active. These fractions were pooled, concentrated, and again run down a similar column. The fractions were dialyzed and concen-
treated, and a sample of each fraction was treated with ME. Titers ranging from 1:4 to 1:32 were found in 7 of 20 fractions (no. 6 to 13; peak titer, no. 8 to 10), and proteins were distributed in fractions 6 to 17. Treatment with ME abolished all activity. The treated and the untreated fractions were immuno-electrophoresed, with the use of rabbit anti-GPS to develop the precipitin lines. All fractions showed precipitin lines corresponding to $\alpha$ and $\gamma$ migration. Fractions with agglutinating activity showed, in addition, a $\beta$-migrating component, approximately proportional in intensity to the agglutinating titer, and absent after treatment with ME (Fig. 1).

Formalin-treated cultures of *L. biflexa* (Patoc), *L. icterohaemorrhagiae* (22; susceptible to GPS), and *L. pomona* (EP-F; insoluble to GPS) were each used to absorb samples of 20 ml of GPS at 4 C for 48 hr. Each Formalin-treated suspension was then washed three times in phosphate-buffered saline, pH 7.4, and resuspended to make 10 ml. One rabbit was immunized with each suspension as described in Materials and Methods, and its serum was used as an antisera against the proteins absorbed from GPS by the corresponding leptospires. Samples of GPS and of GPS treated with ME were immuno-electrophoresed with the use of these antisera to develop precipitin lines. All antisera developed $\alpha$- and $\gamma$-globulin lines in the GPS, but an additional $\beta$ ME-sensitive line was developed with antisera against the two susceptible strains of leptospires (Fig. 2).

A heavy suspension of each of *L. biflexa* (Patoc), *L. icterohaemorrhagiae* (GP and GP-B), and *L. pomona* (EP-F), each from a 2-liter culture, was used to absorb 10 ml of GPS at 4 C for 1 hr. After treatment with glycine as described in Materials and Methods, the eluates were neutralized and dialyzed against saline; volumes of 0.1 ml were tested for leptospiral agglutinating and immobilizing activity by adding 0.1 ml of complement (1:80 GPS) and 0.1 ml of a culture of *L. biflexa* (Patoc) at 37 C for 1.5 hr. Eluates from the susceptible leptospires, *L. biflexa* (Patoc) and *L. icterohaemorrhagiae* (GP), agglutinated and lysed similarly to GPS. The eluates from the other strains were inactive. Each eluate was concentrated to half its original volume and immuno-electrophoresed, with the use of rabbit anti-GPS to develop the precipitin lines. In addition to $\alpha$ and $\gamma$ bands, a $\beta$-migrating band, similar to those shown in Fig. 1 and 2, was developed in eluates from the two susceptible leptospires, but not from the insensitive strains.

In a complementary experiment, a sample of GPS was absorbed with *L. icterohaemorrhagiae* (22) and immuno-electrophoresed. When the immuno-electrophoretic pattern of this sample was compared with that of unabsorbed serum, it was found that $\alpha$, $\beta$, and $\gamma$-migrating proteins were absorbed, and that the $\beta$-migrating band corresponded to the ME-sensitive proteins absorbed by, and capable of being eluted from, susceptible leptospires, and to that obtained from active gel-filtration column fractions (Fig. 3). After absorption of GPS by susceptible leptospires, an additional $\beta_{1A}$ line appeared (Fig. 3), corresponding to a $\beta_{1A}$ line observed to appear in human serum
after absorption with immune precipitates (14). Consequently, GPS was absorbed with an immune precipitate of group A streptococcal carbohydrate and rabbit antibody; after immunoelectrophoresis, an additional \( \beta_{1}A \) line was observed, compared with unabsorbed controls, exactly similar in appearance to the \( \beta_{1}A \) line described above.

**DISCUSSION**

*Natural antibody in serum.* The occurrence of antileptospiral activity in normal mammalian sera (10, 11) has been confirmed. However, in the experiments reported here, *L. biflexa* (Patoc) was agglutinated as well as immobilized, and avirulent strains of *L. icterohaemorrhagiae* and *L. zanoni* were agglutinated; virulent strains of these serotypes and *L. pomona* were not affected. The factor in GPS responsible for agglutination and immobilization of leptospires may be regarded as a natural antibody, for it was shown to be a heat-labile, ME-sensitive, \( \beta \)-migrating globulin of large molecular size on gel filtration, requiring complement and lysozyme, and capable of absorption onto and elution from susceptible organisms. It is similar in these properties to the macroglobulin natural antibodies against gram-negative enteric bacteria. It is less clear whether it is similar to specific immune antileptospiral antibody with regard either to immunoglobulin class or to specificity of action.

There is evidence that immune leptospiral agglutinating antibodies are mainly IgM in sera from human patients and from immunized animals (5, 6, 12, 15) even after extensive hyperimmunization (*unpublished data*). It is doubtful that they represent the same IgM as the natural antibody, because the immune antibodies are characteristic for the serotype-specific antigens of the immobilizing leptospires. The natural antibody IgM is, by contrast, absorbed onto the antigens of susceptible leptospires, cutting across the conventional serotype specificity decided for taxonomic purposes by cross-absorption of hyperimmune rabbit sera. Although the antigen is unknown, it is preserved by Formalin and is lost or masked in the *L. pomona* and the virulent *L. icterohaemorrhagiae* and *L. zanoni* used in these studies; it is common to all susceptible leptospires, as shown by cross-absorption, and is manifested by immobilization or agglutination. It is proposed that this antigen be provisionally designated "Z" antigen. Lack of Z antigen may not be related to pathogenicity, but rather to virulence, as seen in the *L. icterohaemorrhagiae* and *L. zanoni* serotypes. Change from virulence to avirulence includes acquisition or unmasking of Z antigen. This does not necessarily mean that this antigen is a determinant of virulence.

Johnson and Harris (8) showed that virulent leptospires were less susceptible than avirulent leptospires to the lethal action of serotype-specific immune antibody and complement, although the immune antibody cross-agglutinated completely (4, 8). Thus, there is no obvious serological difference between virulent and avirulent leptospires. It may be assumed that serotype-specific immune antibody reacts on more superficial antigens, allowing access of the macromolecular natural antibody to deeper Z antigen. This is not inconsistent with the high titers of activity for immune serum reported by Johnson and Harris (8), for their titration system contained one-tenth volume of normal rabbit serum. This assumption preserves the simple concept that a single antigen-complement-antibody system is responsible for the lethal activity of serum, rather than requiring the existence of two systems having the same end result, one of which is serotype-specific and the other not. Confirmation of this hypothesis requires further research into the identity and location of the Z antigen.

The observation that freshly isolated virulent strains are resistant to the antibody-complement system was explained by "virulence factor" (8). However, an alternative is that growth under usual laboratory conditions of cultivation, such as temperature and composition of medium, favors the development or unmasking of Z antigen and thus, eventually, susceptibility to serum. This view fits more closely the observations that 8-azaguanine added to cultures may confer susceptibility without loss of virulence (8), and that saprophytic *L. biflexa* freshly isolated from surface waters may be resistant to serum (9). The results with *L. pomona* (STM) in this study remain discrepant, and are not easily explicable unless a further assumption is made, that it is incapable to producing any, or enough, Z antigen.

The role of Z antigen as a determinant of virulence, or its significance as a common antigen of *Leptospira* cannot be explained until it has been characterized and localized.

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**LITERATURE CITED**