OBSERVATIONS ON THE AGGLUTINATION OF POLYSACCHARIDE-TREATED ERYTHROCYTES BY TULAREMIA ANTISERA

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Antibody reactive with the somatic polysaccharide of Pasteurella tularensis can be measured in the sera of vaccinated or convalescent persons by means of the quantitative precipitin method (1). Although direct proof of the protective effectiveness of this antibody has not been obtained, it seems probable that it plays a role in human resistance to infection (1, 2). The concentration and reactivity of the antibody in the serum as measured by the precipitin method appear to be correlated with the degree of immunity of the individual; the agglutination titer shows no such correlation (2). Determination of antibody to the polysaccharide thus appears to be the best serological method for measuring acquired immunity to tularemia, and hence for assessing the effectiveness of prophylactic vaccines. Use of the quantitative precipitin method for this purpose is rendered difficult, however, by the technical complexity of the procedure and the relatively large volume of serum required.

Keogh et al. (3, 4) have reported that erythrocytes treated with certain bacterial polysaccharides become specifically agglutinable by antibody to the polysaccharide. Middlebrook and Dubos (5) have shown that polysaccharides from M. tuberculosis also have this property. We have found that the polysaccharide from Past. tularensis combines with erythrocytes, rendering them agglutinable by specific antisera, and have studied the application of this reaction to the measurement of antibody reactive with the polysaccharide.

Materials and Methods

The polysaccharide was prepared by phenol extraction of Past. tularensis (6), and had been used previously for the measurement of antibody by the quantitative precipitin technique (1). Although the chemical nature of this material has not been investigated in detail, it appears to have the properties of a polysaccharide and will be referred to as such.

Human sera were obtained from persons who have been vaccinated with Foshay's gelatin-hydrolysate tularemia vaccine (7) or from persons who had recovered from tularemia. The authors are indebted to Dr. T. W. Green for providing many of these sera. Fresh sera were tested on the day the blood was drawn; others were stored under various conditions as described. Hyperimmune goat serum was obtained from animals immunized with virulent...
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living organisms; we are indebted to Dr. Lee Forshay for this material. Hyperimmune horse serum was provided by Sharp and Dohme, Glenolden, Pennsylvania. A standard formalin-treated suspension of Past. tularensis was used as antigen in the bacterial agglutination titrations.

Preparation of Sensitized Erythrocyte Suspension.—Preliminary experiments indicated that, after treatment with polysaccharide, human type O erythrocytes were agglutinated to a higher titer by immune goat serum than human types A or B or chicken erythrocytes, and they have been used exclusively in the experiments to be reported. Erythrocytes from several type O individuals gave identical results. Rabbit and guinea pig erythrocytes proved unsatisfactory, since they were agglutinable by the sera even before treatment with tularemia polysaccharide. The polysaccharide-treated suspension was prepared in the following manner: Blood was taken into citrate and the cells washed three times with ten volumes of 0.85 per cent sodium chloride solution, hereafter referred to as saline. The cells were diluted in saline to a concentration of 5 per cent and mixed with two parts of 0.02 per cent polysaccharide solution in saline. This mixture was allowed to stand at room temperature for 2 hours and at 4°C overnight. The cells were then washed twice with fifteen volumes of saline and diluted to 0.5 per cent. The polysaccharide treatment produced no agglutination of the cells, and the suspension could be kept in the refrigerator for about 48 hours without hemolysis or appreciable change in agglutinability.

Hemagglutination Titration.—The test was carried out in 10 x 75 mm. agglutination tubes. Serial twofold dilutions of the sera contained in 0.5 ml. were prepared in saline, and 0.5 ml. of the sensitized cell suspension was added to each tube. The tubes were well shaken and allowed to stand at room temperature. Agglutination was determined by examining the pattern of settling of the erythrocytes in the bottom of the tube as in virus hemagglutination. In tubes containing a high concentration of immune serum a positive agglutination pattern appeared within 45 minutes. The cells then gradually settled to a compact pattern which appeared almost identical with the negative control, except that it has a somewhat granular appearance and a slightly ragged edge. Such cells gave macroscopic evidence of agglutination when resuspended, as did the cells from typically positive patterns in some cases. Similar effects are observed in virus hemagglutination-inhibition studies (8). Readings were therefore made after 45 minutes and also after 2 hours. The titer is expressed as the final dilution of serum in the last tube which showed complete or nearly complete agglutination. Where agglutination occurred but was not complete in any tube the result was considered questionable. Known positive and negative sera and other appropriate controls were included. Parallel titrations, using untreated erythrocytes, were carried out in a considerable number of cases and showed no agglutination.

EXPERIMENTAL RESULTS

Hemagglutination by Various Antisera

The results of hemagglutination titrations with various tularemia antisera are given in Table I. The figures from one to four refer to the degree of agglutination as read by the pattern of settling, and represent, respectively, slight, 50 per cent, 75 per cent, and complete agglutination. The first five human sera were examined while fresh, and the next three sera had been stored under refrigeration for 1 to 4 weeks. The goat serum had been stored for a considerable time under refrigeration before the tests were carried out, and the horse serum had been lyophilized and reconstituted. It will be observed that considerable variation occurred in the behavior of the sera from vaccinated persons, and that titers of 1–5,000 or more were encountered in convalescent
sera. With the higher titered sera definite prozones appeared. Normal human, normal goat, and normal horse sera produced no agglutination of the treated cells when tested under identical conditions.

**TABLE I**

*Agglutination of Polysaccharide-Treated Erythrocytes by Various Tularemia Antisera*

<table>
<thead>
<tr>
<th>Source of immune serum</th>
<th>Final dilution of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-10</td>
</tr>
<tr>
<td>Convalescent person; 6 wks. after onset of tularemia</td>
<td>—</td>
</tr>
<tr>
<td>Recovered person; tularemia 4 yrs. previously</td>
<td>—</td>
</tr>
<tr>
<td>Vaccinated person; 5 injections vaccine, last 4 mos. previously</td>
<td>2</td>
</tr>
<tr>
<td>Vaccinated person; 6 injections vaccine, last 5 mos. previously</td>
<td>3</td>
</tr>
<tr>
<td>Vaccinated person; 9 injections vaccine, last 1 yr. previously</td>
<td>3</td>
</tr>
<tr>
<td>Vaccinated person; 4 injections vaccine, last 4 yrs. previously; 1 day after onset of tularemia</td>
<td>1</td>
</tr>
<tr>
<td>Same person 14 days later, 15 days after onset of tularemia</td>
<td>1</td>
</tr>
<tr>
<td>Same person 8 days later, 23 days after onset of tularemia</td>
<td>3</td>
</tr>
<tr>
<td>Hyperimmune goat</td>
<td>4</td>
</tr>
<tr>
<td>Hyperimmune horse</td>
<td>—</td>
</tr>
</tbody>
</table>

**Factors Influencing the Hemagglutination Titer**

The titers of a number of human immune sera were determined after various intervals of storage at 4°C. under aseptic conditions. A gradual decrease in titer was observed, apparently somewhat greater in low titered sera from vac-
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cinated persons than in sera taken after recovery from tularemia. Storage up to 1 week, however, never caused more than a one tube decrease in titer. Storage in the frozen state at $-25^\circ\text{C.}$ caused less decrease in titer than storage at $4^\circ\text{C.}$, and would appear to be the preferred method when preservation of serum is necessary. Inactivation of the fresh serum for 30 minutes at $56^\circ\text{C.}$ usually caused only slight variation in the serum titer, sometimes increasing and sometimes decreasing it. Absorption of complement on a forming specific precipitate (9) also produced no significant change in the hemagglutination titer. These observations indicate that the gradual diminution of the titer of sera stored under refrigeration is not due to loss of complement.

### TABLE II

**Comparison of Agglutination and Hemagglutination Titers of Human Sera**

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Agglutination titer</th>
<th>Hemagglutination titer</th>
<th>Hemagglutination titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered</td>
<td>1-80</td>
<td>1-640</td>
<td>8</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-320</td>
<td>1-1280</td>
<td>4</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-160</td>
<td>1-2560</td>
<td>16</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-160</td>
<td>1-1280</td>
<td>8</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-320</td>
<td>1-2560</td>
<td>8</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>1-20</td>
<td>1-320</td>
<td>8</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-80</td>
<td>1-640</td>
<td>8</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-80</td>
<td>1-20</td>
<td>0.25</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-20</td>
<td>1-80</td>
<td>4</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-20</td>
<td>1-80</td>
<td>4</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-160</td>
<td>1-320</td>
<td>2</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-160</td>
<td>1-40</td>
<td>0.25</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-20</td>
<td>1-80</td>
<td>4</td>
</tr>
<tr>
<td>&quot;</td>
<td>1-160</td>
<td>1-160</td>
<td>1</td>
</tr>
</tbody>
</table>

In the course of the work repeated titrations were carried out on fresh samples of serum from several vaccinated individuals. Titers were usually constant, although occasionally a one tube variation was encountered. These results, together with the constancy of the titers obtained with frozen serum, are evidence of the reproducibility of the technique, and suggest that different preparations of treated erythrocytes do not vary appreciably in agglutinability.

**Relationship of Hemagglutination Titer to Agglutination Titer**

Hemagglutination titers and agglutination titers with bacterial cells were determined on a number of human sera, some from vaccinated persons and some from persons recovered from tularemia. The results are given in Table II.

It will be observed that the ratios of the hemagglutination to the agglutination titer varied from 0.25 to 16. This 64-fold variation is much greater than...
the experimental error of the determinations, and must indicate that the two
titrations measure different activities of the sera. The ratio appeared to be
somewhat higher in recovered than in vaccinated individuals, suggesting that
infection produces a larger proportion of antibody reactive with the polysac-
charide than does immunization with the present vaccine.

Preliminary experiments have been carried out on sera obtained after success-
ive courses of vaccine in the same individual. In general the hemagglutination
titer appeared to be maintained or to increase on continued immunization,
whereas the agglutination titer, in agreement with previous reports (2, 10),
remained constant or decreased. Two sera from cases of brucellosis which
showed bacterial agglutination titers for tularemia were examined for hemag-

TABLE III
Correlation of Quantitative Precipitin and Hemagglutination Determinations on Various
Human Sera

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Antibody N/ml</th>
<th>Hemagglutination titer</th>
<th>Mag. antibody N</th>
<th>Hemagglutination titer × 10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered person</td>
<td>0.043</td>
<td>1-2560</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.062</td>
<td>1-2560</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.026</td>
<td>1-640</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.023</td>
<td>1-1280</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.076</td>
<td>1-2560</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.084</td>
<td>1-2560</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Vaccinated person</td>
<td>0.0097</td>
<td>1-320</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>0.0065</td>
<td>1-640</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

glutination activity with negative results, suggesting that the hemagglutination
method is more specific than the bacterial agglutination titration.

Relationship of Hemagglutination Titer to Precipitable Antibody

Hemagglutination titrations were carried out on a number of human immune
sera on which antibody precipitable with polysaccharide had been determined
previously (1). The sera had been stored in the refrigerator under aseptic
conditions between the two measurements. Results are given in Table III.
The ratio of antibody nitrogen to hemagglutination titer shows only a moderate
variation in the group of sera, suggesting that the two determinations are
correlated.

DISCUSSION

The experimental results indicate that the hemagglutination titer with
polysaccharide-treated erythrocytes is a measure of the amount of antipolysac-
charide antibody in a serum. The method is sensitive and convenient, and re-
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quires only simple serological apparatus and technique. Only a small amount of
serum is required for each determination. While the titration is not capable of
the same precision as the quantitative precipitin method, and indicates only
the quantitative variations of antibody in different sera, the accuracy would
appear to be adequate for studying the duration and intensity of the antibody
response to immunization procedures if the quantity of antibody produced is
the criterion of immunity.

The fact that sera from patients with brucellosis which agglutinated Past.
tularensis gave negative results in the hemagglutination reaction in evidence of
the greater specificity of the hemagglutination technique. Middlebrook and
Dubos (5) have mentioned the high degree of specificity of a similar hemag-
glutination reaction in tuberculosis. It seems possible that in other infectious
diseases the substitution of erythrocytes treated with a specific polysaccharide
antigen for the usual bacterial suspension may produce simple diagnostic
hemagglutination titrations of improved specificity and sensitivity.

SUMMARY

Erythrocytes treated with polysaccharide from Past. tularensis are specifically
agglutinated by the sera of persons recovered from tularemia or vaccinated
against it, and by the sera of animals immunized with living cultures. The
serum hemagglutination titer is correlated with the amount of antibody as
measured by the quantitative precipitin method with polysaccharide, but not
with the bacterial agglutination titer. The meaning of the results is discussed.

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