Cumulative Hemagglutination by *Mycoplasma pneumoniae* and Other Agglutinins

T. JACOB JOHN,† MARLENE STAHL, AND VINCENT A. FULGINITI

Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado

Received for publication 27 June 1966

**ABSTRACT**

JOHN, T. JACOB (University of Colorado School of Medicine, Denver), MARLENE STAHL, AND VINCENT A. FULGINITI. Cumulative hemagglutination by *Mycoplasma pneumoniae* and other agglutinins. J. Bacteriol. 92:1002-1004. 1966.—The phenomenon of cumulative hemagglutination, or hemagglutination by two agglutinins, each in subagglutinating concentration, was demonstrated by use of four different systems, namely, horse serum and *Mycoplasma pneumoniae*, horse serum and measles antigen, *M. pneumoniae* and measles antigen, and parainfluenza 2 virus and *M. pneumoniae*. Cumulative hemagglutination appears to be the mechanism by which a horse-serum diluent causes high hemagglutination titers of *M. pneumoniae*, since both contain hemagglutinins against vervet erythrocytes. It was also shown that antibodies against either one of the two antigens may cause inhibition of such hemagglutination.

An unusual hemagglutination (HA) reaction by *Mycoplasma pneumoniae* was recently reported by Feldman and Suhs (1). They used 30% horse serum for suspension of the organisms, and demonstrated the agglutination of 0.1% vervet red cells. *M. pneumoniae* alone, or suspended in horse serum devoid of heteroagglutinins against vervet red cells, did not show this property. For want of a ready explanation for this phenomenon, the authors suggested that the heteroagglutinins in the horse serum sensitized the red cells and, either by altering the red cell surface or by forming bridges, made it possible for *M. pneumoniae* to agglutinate them.

This paper reports an investigation of this phenomenon.

**MATERIALS AND METHODS**

*M. pneumoniae* was kindly supplied by R. M. Chanock. It was propagated in our laboratory in a previously described biphasic medium (2) and stored at −70 C. A concentrated suspension of organisms was made (John et al., unpublished data). Samples of horse sera, obtained commercially, were collected from different laboratories in our institution. They were designated as lots 1 to 5, and were used after heat inactivation at 56 C for 30 min.

Diluents used were 0.85% saline (NS), phosphate-buffered saline (PBS, pH 7.2), and PBS with 0.2% gelatin (Difco; PBSG).

† Present address: Christian Medical College, Vellore, India.

Vervet erythrocytes were obtained from Cercopithecus aethiops monkeys in our animal quarters. They were collected in Alsever’s solution and stored at 4 C. Prior to use they were washed three times in NS or PBS and made into a 10% suspension. Guinea pig erythrocytes were handled similarly.

Measles hemagglutinin was obtained commercially from Microbiological Associates, Inc., Bethesda, Md. Tween-ether treatment of measles antigen was performed according to the method of Norrby (3). Parainfluenza 2 virus (strain ATCC 92) was obtained from the American Type Culture Collection, and passed four times in primary rhesus monkey kidney cell culture.

Horse-serum antibodies were prepared in two adult rabbits by injecting each one intramuscularly with 3 ml of horse serum lot no. 4, and bleeding them 3 weeks later. These rabbit sera were stored at −70 C. Pre-inoculation sera were collected for control purposes and stored similarly. Before use they were inactivated at 56 C for 30 min.

HA studies were done in disposable glass Kahn tubes. To 0.4 ml of serial twofold dilutions of the antigen, equal volumes of red-cell suspensions were added, mixed well, and incubated at room temperature for 2 to 3 hr. HA was read by pattern as 2+ for complete, 1+ for nearly complete, ± for partial, and 0 for no agglutination. The last dilution showing 2+ or 1+ agglutination was read as the HA titer, or 1 HA unit in 0.4 ml.

Hemagglutination inhibition (HAI) studies were done by adding 0.2 ml of antigen containing 4 HA units to equal volumes of serial twofold dilutions of the rabbit sera. After incubation for 1 hr at room temperature, 0.4 ml of red-cell suspension was added,
mixed well, and incubated for 2 to 3 hr. The last serum dilution showing ± or 0 HA was read as the HAI titer.

**RESULTS**

*Direct HA by M. pneumoniae.* With 0.1 or 0.2% vervet or guinea pig erythrocytes in NS, PBS, or PBSG, *M. pneumoniae* grown in diphasic medium never gave HA titers above 1:8. However, concentrated suspensions consistently gave a titer of 1:64 against 0.2% guinea pig erythrocytes and 1:32 against the same concentration of vervet red cells.

**HA titers of horse sera.** The HA titers of five lots of horse sera diluted in PBSG and tested with vervet red cells in PBSG are shown in Table 1. The HA titers varied widely. Closer ranges of dilution were tried for lots 1 and 3 to find the lowest dilution showing no HA. The results were 1:50 for lot 1 and 1:38 for lot 3, provided 0.1% cells were used. A 30% dilution of serum, lot 5 gave no agglutination of 0.2% cells.

**HA by *M. pneumoniae* and horse serum.** A suspension of *M. pneumoniae*, with HA titer of 1:8 when diluted in PBSG and tested with 0.1% vervet cells, yielded a titer of 1:64 when diluted in PBSG containing 1:50 horse serum no. 1. The concentrated suspension of organisms with HA titer of 1:32 in PBSG against 0.2% vervet cells gave a titer of 1:256 when diluted in 30% horse serum no. 5 in PBSG. Thus, in both cases, sub-agglutinating concentrations of horse serum caused an eightfold rise in *M. pneumoniae* HA titer.

**HA by *M. pneumoniae* and measles antigen.** Measles antigen treated with Tween-ether was diluted in NS and tested with 0.5% vervet red cells. The HA titer was 1:256, but there was partial agglutination at 1:512. After further studies, a 1:528 dilution was found to be the lowest dilution showing no HA. When a concentrated suspension of *M. pneumoniae* was titrated under the same conditions, an HA titer of 1:4 was obtained. The same suspension, when diluted in NS containing 1:528 measles antigen, gave an HA titer of 1:32. Here, again, a subagglutinating level of measles hemagglutinin was found to enhance *M. pneumoniae* HA titer.

**HA by parainfluenza 2 virus and *M. pneumoniae*.* Parainfluenza 2 virus was titrated in PBSG against 0.2% guinea pig erythrocytes, and the lowest dilution with negative HA was found to be 1:26. When the same dilution was used as the diluent for *M. pneumoniae* titration, the HA titer was enhanced from 1:8 to 1:64.

**HA by horse serum and measles antigen.** The measles antigen used in this experiment was an old stock with an HA titer of 1:16 in PBSG against 0.1% vervet cells. When PBSG containing 1:50 horse serum (no. 1) was used as the diluent, the titer was 1:128. Horse-serum dilutions of 1:53 and 1:56 gave measles HA titers of 1:64 and 1:16, respectively.

**Role of horse-serum antibodies in HAI tests.** Sera from rabbits before and after inoculation with horse serum were used in HAI studies in which measles and *M. pneumoniae* antigens diluted in PBSG containing horse serum were used. The results are shown in Table 2. Horse-serum antibodies had an inhibitory effect in both systems.

**DISCUSSION**

In four separate systems, namely, horse serum and *M. pneumoniae*, horse serum and measles antigen, *M. pneumoniae* and measles antigen, and parainfluenza 2 virus and *M. pneumoniae*, hemagglutination was demonstrated by a combination of two antigens, each in subagglutinating concentrations. The term cumulative hemagglutination has been applied for this effect, which may be universal for any two hemagglutinins.

Feldman and Suhs (1) found that the presence of vervet red-cell agglutinins in the horse serum was essential for high HA titers of *M. pneumoniae*. Since cumulative hemagglutination requires that both components be agglutinins, it is crucial to demonstrate direct HA by *M. pneu-

<table>
<thead>
<tr>
<th>Horse serum lot no.</th>
<th>Reciprocal of HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1% cells</td>
</tr>
<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>256</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

**Table 2. HAI titers of the sera of two rabbits before and after inoculation with horse serum**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Reciprocal of HAI titers against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measles in horse-</td>
</tr>
<tr>
<td></td>
<td>serum diluent</td>
</tr>
<tr>
<td>Preinoculation 1</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Preinoculation 2</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Postinoculation 1</td>
<td>64</td>
</tr>
<tr>
<td>Postinoculation 2</td>
<td>64</td>
</tr>
</tbody>
</table>
moniae. This has been done recently (John et al., unpublished data), and the above experiments have confirmed it.

If a mixed antigen is used for HA, it seems likely that antibodies against either component would be able to cause HAI. Thus, Feldman and Suhs (1) demonstrated HAI by antibodies against M. pneumoniae, and we have shown HAI by antibodies against horse serum. If cumulative hemagglutination was not taken into consideration, the two rabbit sera would have been mistaken to contain measles and M. pneumoniae HAI antibodies when, in fact, they contained only horse-serum antibodies.

The above fact poses a possible disadvantage in using such a test as an epidemiological tool. Individuals who have had horse-serum injection(s) in the past may show HAI activity in their sera, which may be mistaken for antibodies against M. pneumoniae. This may be an insignificant factor if the incidence of individuals with anti-horse serum antibodies is low in any given population studies.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service contract no. 43-62-477 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

