Passive Hemagglutination Test for Measles Immunity and Serodiagnosis

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A passive hemagglutination (PHA) test for measles was evaluated in comparison with hemagglutination inhibition (HI) and neutralization (NT) tests. The PHA test determines exclusively the level of antibody directed to the hemagglutinin protein of measles virus. The ratio of PHA to HI titer was 1 to 32 (geometric mean, 6.5) for the first 5 weeks of infection but declined to near unity thereafter. It gradually increased again to 4 to 32 (geometric mean, 11.7) over several years. The initial high PHA titer relative to the HI titer was most likely due to the presence of the immunoglobulin M antibody known to be efficient in agglutination, because 2-mercaptoethanol (2ME) treatment of sera reduced the PHA titer to a level similar to that of the HI titer. The PHA titer in sera obtained after the convalescent phase was insensitive to 2ME, and the relative increase in the PHA over the HI titer was presumably a result of increased antibody avidity. In some individuals, the HI titer fell to below detectable levels several years after either natural infection or vaccination, but the PHA as well as the NT titer remained positive. The PHA titer was therefore a more reliable and more sensitive indicator of immune status against measles than the HI titer. The decrease in PHA titer by 2ME treatment provided evidence of a current or very recent infection. PHA was found to be useful both for assessing immunity status and for serodiagnosis.

The hemagglutination inhibition (HI) test has been and is extensively used for the determination of antibody to measles virus. The test is relatively simple, reproducible, and reliable for the serodiagnosis of measles infection and evaluation of measles vaccines. However, it is not sensitive enough to assess immunity status against measles long after infection or vaccination (10, 17). In one study, the HI antibody titer was below 1:8 in 15 and 36% of subjects 16 years after natural infection and successful vaccination, respectively (9), while in other studies HI antibody declined to an undetectable level in more than 50% of vaccinees in 16 years (14) and was no longer detected in some adults who had undoubtedly been infected (17). The neutralization (NT) test is more sensitive than the HI test (9, 13) but too cumbersome and time-consuming for a routine serological test. The agglutinin assay and enzyme-linked immunosorbent assay were reported to be as sensitive as the NT test and reliable indicators of immunity status (10, 17), while in another report it was concluded that enzyme immunoassay is more sensitive than the HI test, but since the former may detect antibody irrelevant for protection, it is not necessarily suitable for evaluation of immunity status (2). The passive hemagglutination (PHA) test with monkey erythrocytes sensitized with measles virus hemagglutinin (HA) and fixed with glutaraldehyde was reported to be sensitive and specific in the detection of measles antibody (6). We report here that the PHA test with sheep erythrocytes developed recently is not only as sensitive as the NT test but also useful for the diagnosis of recent measles infection when combined with the treatment of sera with 2-mercaptoethanol (2ME).

MATERIALS AND METHODS

Serum specimens. The following serum specimens were used: sera from 19 unvaccinated children, 14 to 36 months of age, with no history of measles; 28 acute- and early-convalescent-phase sera from 13 measles patients; 11 postvaccination sera; sera from 19 healthy subjects of various ages; and 9 sera taken sequentially from a monkey up to 31 weeks after experimental inoculation with 6 \times 10^6 PFU of the YMT (wild-type) strain of measles virus. Patient sera were placed on a time scale on the assumption that the onset of illness occurs 2 weeks after infection.

Antisera. Polyclonal antisera directed to the HA protein, fusion (F) protein, nucleoprotein (NP), phosphoprotein or polymerase (P), and matrix (M) protein of measles virus were taken from rabbits immunized with the respective proteins separated by and eluted from sodium dodecyl sulfate-polyacrylamide gels after electrophoresis of purified virions of the Toyoshima strain of measles virus. The antisera were provided by T. Kohama. The monoclonal antibodies have been described elsewhere (15).

PHA test. Reagents for the PHA test were manufactured by Denka Seiken Co. (Tokyo, Japan). Briefly, sheep erythrocytes were fixed with glutaraldehyde, treated with an equal volume of 10-mg/dl tannic acid at 37°C for 30 min, and sensitized with a suitable concentration of Tween 80-ether-treated measles antigen at 37°C for 60 min. The test was performed as specified by the manufacturer. In brief, a doubling dilution series of test serum starting from 1:8 was made in duplicate in phosphate-buffered saline containing 0.2% normal rabbit serum in V-bottomed microtiter plates. An equal volume of a 0.5% suspension of sensitized erythrocytes was added to each well of one dilution series and left for 2 h. Agglutination was read by the pattern method. The highest dilution of a test serum which gave partial agglutination was taken as the endpoint. Antibody titer was expressed as the reciprocal of the serum dilution for this and all other tests. The specificity of the PHA test was verified by at least a fourfold reduction in agglutination titer in the second dilution series to which measles virus antigen was added as a competitor before sensitized erythrocytes. A PHA titer of 16 or higher was taken as positive and lower than 8 as negative. The titer of 8 was equivocal because its specificity could not be verified by competition. Product-to-product
variability appeared to be minimal, as an identical PHA titer was given by a standard positive serum with three different lots of sensitized erythrocytes. Nevertheless, a reference positive serum was included in each test. Sensitized erythrocytes had a shelf life of at least 6 months when stored at 4°C.

NT test. The NT antibody titer was expressed as the highest dilution of a test serum which reduced the number of plaques by 50%. A fourfold serial dilution of a test serum was combined with an equal volume of appropriately diluted large-plaque variant of the Toyoshima strain of measles virus. The serum-virus mixture was kept sequentially for 30 min at 37°C, 30 min at room temperature, and overnight at 4°C, and 0.1 ml was inoculated in triplicate to Vero cell monolayers grown in Costar 12-well cluster plates (well diameter, 2.2 cm). All other procedures for plaque assay have been described previously (4), except that plaques were counted on day 4 after monolayers were fixed with 7% Formalin and stained with 0.01% crystal violet because the number of plaques does not increase after day 4 with this variant strain.

HI test. A test serum was heat-inactivated, treated with Kaolin, and absorbed with African green monkey erythrocytes. The HI test was done by the standard microrititer method (5). An HI titer of 8 or higher was taken as positive.

2ME treatment. A test serum diluted 1:8 either without treatment or after Kaolin and African green monkey erythrocytes treatment was mixed with an equal volume of 0.2 M 2ME in phosphate-buffered saline (pH 7.4) and incubated at 37°C for 1 h (8). Thus, the starting dilution of 2ME-treated serum was 1:16.

RESULTS

We tried to identify the virus protein(s) present on the surface of sensitized erythrocytes that was responsible for antibody-mediated agglutination. The antibody directed to the HA protein, whether polyclonal or monoclonal, efficiently agglutinated sensitized erythrocytes, but antibodies directed to the NP, P, F, and M proteins failed to cause agglutination (Table 1). It was concluded that the PHA test, like the HI test, exclusively detects the antibody reacting with the HA protein of measles virus.

Sera from various donors were tested by both the PHA and HI tests. Sera from 19 children with no history of either measles or measles vaccination were negative (titer less than 8) by both tests (not shown). The relationship between PHA and HI titers is shown in Fig. 1. The PHA relative to the HI titer varied by the time of bleeding. While the antibody titers given by the two tests were closely similar to those for the sera obtained 6 to 8 weeks after either natural infection or vaccination, the PHA titer was 1 to 32 (geometric mean, 6.5)-fold higher than the HI titer with the sera from those in the first 5 weeks of infection and 4 to 32 (geometric mean, 11.7)-fold higher in healthy subjects presumed to have had measles or measles vaccination years before. Within each group, the antibody titers given by the two tests were nearly proportional. The chronological change of the two antibody titers was more evident when the PHA-to-HI titer ratio was plotted against the time after infection (Fig. 2A). The ratio was between 4 and 32 shortly after infection but declined to close to unity (0.5 to 2) in the course of several weeks. It was back to 4 to 32 after an unspecified time.

It was suspected that the initial high ratio of PHA to HI titers is due to the presence of immunoglobulin M (IgM) antibody, which is known to be highly efficient in agglutination because of its multiple valency. The effect of 2ME on the antibody titers was then examined. The change of antibody titers for individual sera in various groups is shown in Fig. 3. In early-phase sera, the drop in antibody titer was greater for PHA (4- to 128-fold) than for HI (up to 8-fold), indicating the greater dependence on IgM antibody of the former than the latter activity. With some sera, the HI titer did not change in spite of a significant drop in the PHA titer, suggesting that IgM antibody was a minor population of HI antibody in these sera. With sera taken from later stages of infection, neither titer was affected by 2ME treatment (Fig.

### TABLE 1. PHA test with polyclonal antisera and monoclonal antibodies specific to individual proteins of measles virus

<table>
<thead>
<tr>
<th>Antibody (designation)</th>
<th>PHA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal</td>
<td></td>
</tr>
<tr>
<td>Anti-HA (B5)</td>
<td>2.048</td>
</tr>
<tr>
<td>Anti-HA (A26)</td>
<td>2.048</td>
</tr>
<tr>
<td>Anti-F (C527)</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Anti-NP (B1)</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Anti-P (C6)</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Anti-M (A27)</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Polyclonal</td>
<td></td>
</tr>
<tr>
<td>Anti-HA</td>
<td>1.024</td>
</tr>
<tr>
<td>Anti-F</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Anti-NP</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Anti-P</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Anti-M</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

FIG. 1. Correlation between PHA and HI titers. Symbols: □, ■, ○ sera from measles cases and vaccinees within 5 weeks (□) and 6 to 8 weeks (■) after infection (for measles cases, the onset of illness was set at 2 weeks after infection); ○, sera from healthy subjects; ○, sera from young children with no history of measles infection or vaccination (the number inside the symbol is the number of serum specimens).

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The resulting change in the profile of the PHA-to-HI titer ratio is shown in Fig. 2B. The right half of the profile remained unchanged by 2ME treatment (Fig. 2B). The high PHA relative to HI activity for the sera from healthy subjects probably reflected an efficient agglutinating activity of antibody with increased avidity.

The above findings were obtained with sera taken from different individuals at different times after infection. In order to follow the antibody response in one individual host throughout the course, a monkey that had been experimentally infected with wild-type measles virus was bled sequentially for up to 31 weeks. Sera were tested for PHA and HI titers before and after 2ME treatment (Fig. 4). The antibody response in the monkey was similar in many respects to the one in humans described above except that the response appeared to be terminated somewhat prematurely in the monkey. In the first week, the PHA titer markedly exceeded the HI titer, but the difference diminished over the following 2 weeks by decline of the former and increase of the latter and was no longer seen by the third week. The HI titer reached its peak 3 weeks after infection and started to decline by 15 weeks. Earlier waning of HI antibody in monkeys than in humans has often been observed (unpublished observation). On the other hand, the PHA titer continued to increase until 15 weeks, and the level was sustained for at least the following 15 weeks. As a result, the PHA-to-HI titer ratio steadily increased from the third week.
on. 2ME caused a reduction in antibody titer for up to 2 weeks for HI and up to 3 weeks for PHA.

Sera from 19 healthy subjects, a majority of them being school-age children, were examined by the HI, PHA, and NT tests (Table 2). Nine of them had received measles vaccination years before. The remaining 10 without a history of vaccination had probably had natural measles. Some of the latter have been included in Fig. 1, 2, and 3. They were all positive by PHA and NT, but 2 of 19 sera were negative by HI (titer less than 8), and 4 were marginally positive (HI titer of 8). The PHA and NT titers were thus shown to persist longer than the HI titer whether after natural infection or vaccination. The PHA titer was about threefold higher than the NT titer.

**DISCUSSION**

The PHA test is similar in principle to the HI test, as both tests determine the level of antibody directed to the HA protein of measles virus. The reason that other viral proteins, e.g., NP, are not involved in PHA is not known. As a constituent of virions, NP is as abundant as HA protein, and antibody to NP dominates over antibody to other components in most sera (12). Whether NP does not attach to the erythrocytes or does attach but attains insufficient density over the erythrocyte surface to render the erythrocytes agglutinable by the corresponding antibody remains to be determined. F protein, antibody to which is believed to play a role in immunity to measles (1), might have been rendered unreactive or poorly reactive antigenically by Tween 80-ether treatment, because the treatment is known to destroy the hemolytic activity and antigenicity of measles virus hemolysin (11). The HI and PHA tests, both detecting the same species of antibody, were nevertheless different in the following two respects.

The first was a much longer persistence of antibody determined by the PHA than the HI test. In most cases of measles virus infection, as in other viral infections, the antibody concentration starts to decline when virus is eliminated from the body and antigenic stimulation ceases. On the other hand, the affinity of antibody to an antigenic determinant progressively increases with time by the phenomenon termed affinity maturation (3; reviewed in reference 11). The increase in antibody avidity has also been described for the human immune response to a number of viruses (7, 8). Because holding erythrocytes together requires very firm binding of antibody to the antigen on the erythrocytes surface, PHA is more dependent on antibody avidity than HI, and an increase in antibody avidity makes up for the decline of antibody concentration to a greater extent in PHA than in HI, resulting in the increasing PHA-to-HI titer ratio with time and the longer persistence of the former than the latter (8). The temporal change in NT titer has not been studied as intensively as that in PHA titer, but its long persistence compared with the HI titer suggests that antibody avidity also plays a role in the efficiency of the NT reaction. As a consequence, the PHA titer was found to be better correlated with the NT titer in late-phase sera than the HI titer and therefore is a more reliable and more sensitive indicator of long-lasting immunity characteristic of measles than the HI titer.

The second is the greater efficiency of IgM antibody in PHA than other classes of antibody, including IgG, at the early phase of antibody response. A high PHA titer at this phase reflects a high efficiency of IgM antibody in agglutination due to its multiple valency and is not necessarily accompanied by a high NT titer (unpublished observation). The drop in PHA titer after 2ME treatment is evidence of a current or very recent measles infection and serves as a convenient means of serodiagnosis with a single serum specimen. An additional advantage of PHA over HI for serodiagnosis is that the rise in PHA titer precedes that in HI titer, and therefore, sera taken within 1 week of the onset of illness are often positive by the PHA but still negative by the HI test.

It is noteworthy that both of these aspects distinguishing PHA from HI are based on the unique feature of the former, which requires very firm binding of antibody to the antigen on the erythrocyte surface. The firm binding is effected by increased avidity for IgG antibody and by multiple valency for IgM antibody.

The relationship between PHA titer after 2ME treatment and HI titer has been reported to be useful in estimating the time of past rubella infection (8). A PHA-to-HI titer ratio of less than 1 was a sign of a recent infection, while a ratio of more than 1 was suggestive of an infection in the remote past. The change was also brought about by the increase in PHA titer with antibody avidity. Rubella PHA, however, differed from measles PHA in the relative inefficiency of IgM in the former (8). The mechanism underlying the difference remains to be elucidated.

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**LITERATURE CITED**


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