Studies on the Persistent Infection with Measles Virus in HeLa Cells

III. Immunolysis of Cells in Carrier State by Anti-Measles Sera

Tomonori MINAGAWA and Morihide YAMADA

Department of Bacteriology, Hokkaido University School of Medicine, Sapporo

(Received for publication, March 29, 1971)

ABSTRACT

Almost all cells in HeLa/MV culture contained measles antigen which was mainly detected on the cell surface by immunofluorescent and hemadsorption technique. Cytotoxic effect on HeLa/MV cells was demonstrated by anti-measles sera and complement (immunolysis), indicating the existence of viral surface antigen. Anti-measles serum absorbed with purified measles particles completely lost the ability of immunolysis. Though the cytolysis by anti-measles sera was due to antigen-antibody reaction on the cell surface, the mechanism of lysis seemed to be different from that of the cytolysis by anti-HeLa cell serum. It was suggested that some unknown serum factors other than components of complement are necessary for immunolysis by anti-measles serum.

In immunolysis, antibody and complement act not only on histocompatibility antigen and tumor specific transplantation antigen [2], but also on newly synthesized antigen on the surface of cells infected with certain viruses. Oncogenic viruses usually produce a surface antigen distinct from virion antigens [1, 9]. Some non-oncogenic viruses such as poxvirus [16] and herpes simplex virus [20] can also produce such a surface antigen. As to myxovirus and paramyxovirus, it is not certain whether a surface antigen is produced, because they mature at the surface of cells [14].

It is of interest to investigate the immunological behavior of cells carrying non-cytocidal myxovirus infection. Carrier cultures of rabies virus were lysed completely with anti-rabies serum and complement [24]. Cells infected with mumps virus were lysed by contact with immune lymphocytes but this reaction was inhibited by anti-mumps virus serum [23].

In our measles virus culture (HeLa/MV), immunolysis was shown by anti-measles sera and complement. Studies in this report were aimed at determining (a) the location in the cell of the antigen responsible for the immunolysis by anti-measles sera, and (b) the difference between the reaction of the cell with anti-measles serum and with anti-HeLa cell serum.

MATERIALS AND METHODS

Purification of virus. The standard measles virus (Toyoshima strain) was pre-
pared by the method described in the previous report [15]. Virus was purified partially by sucrose density gradient centrifugation and used for immunization of rabbits. Purified measles particles for the absorption of anti-measles serum were prepared from culture fluids of infected Vero cells. The infectious materials were subjected to sucrose density gradient (5–45%) centrifugation, and 3 peaks of hemagglutinin (HA) were usually observed. The fractions of the middle peaks were pooled, concentrated with polyethylene glycol #6000 (Nakarai Chemical Co.), and fractionated again by the sucrose density gradient centrifugation. The resulting heavy HA-peak fractions were pooled and used for absorption of serum.

Cell cultures. HeLa and HeLa/MV cells were maintained as described previously [15].

Procedure of immunization. Purified measles virus mixed with an equal volume of Freund’s complete adjuvant (Iatron) was injected intramuscularly into rabbits 5 times at weekly intervals. HeLa and HeLa/MV cells were dispersed with 0.02% EDTA, washed 3 times with Hanks’ BSS, and 10⁸ cells were injected intramuscularly into rabbits 5 times at weekly intervals. The animals were bled 7 days after the last injection.

Antisera. The sera used in this study were 1) rabbit antiserum against purified measles virus (A-MV) with a hemagglutinin-inhibiting antibody titer of 1:4096 and a neutralizing antibody titer of 1:8192 against the standard measles virus; 2) rabbit antiserum against HeLa/MV cells (A-H/M) with an HA-inhibiting titer of 1:8192; 3) human serum with measles virus infection (A-Inomata) with an HA-inhibiting titer of 1:1024; 4) rabbit antiserum against HeLa cells (A-H). All sera were heated at 56°C for 30 min.

Antibody absorption with HeLa cells. HeLa cells were dispersed with 0.02% EDTA and washed 3 times with Hanks’ BSS by centrifugation. About 0.5 ml of packed cells were added to 2 ml of each antiserum used in the experiment. The mixture was incubated at 37°C for 1 hr with frequent shaking and centrifuged. After 3 cycles of absorption, the supernatants were stored at −20°C. The absorbed antisera showed no changes in measles HA-inhibiting titer.

Antibody absorption with purified measles virus. Measles virus purified by the method described above was sedimented by high-speed centrifugation, and the pellet containing 75,200 HA units was incubated with diluted anti-measles sera at 37°C for 1 hr. These absorbed sera showed no HA-inhibiting activity.

Complement. Lyophilized commercial guinea pig sera were used in this experiment. The cytolytic activity of complement was expressed as the amount of complement required to obtain a cytotoxic index of 50% (CL₅₀).

Cytotoxic test. The test was performed by the technique of Gorer and O’Gorman [8]. HeLa/MV cells were dispersed with 0.02% EDTA, washed with Hanks’ BSS and suspended in Eagle’s MEM in a concentration of 10⁶/ml. A mixture of 0.1 ml of the cell suspension and 0.1 ml of diluted antiserum was incubated at 37°C for 15 min at room temperature. The mixture then received 0.1 ml of complement and was incubated at 37°C for 45 min with frequent shaking. The percent of viable cells was determined microscopically after the addition of 2 drops of isotonic trypan blue solution. The cytotoxic index (CTI) was calculated as follows: 

$$\text{CTI} = \frac{100 - C - (100 - E)}{100 - C}$$
where C was percent of stained cells in the control, and E that of stained cells in the experiment.

Absorption with bentonite. Ten mg of bentonite (Wako Pure Chemicals Co.) was added to 1.0 ml of guinea pig sera, and the mixture was incubated at 4°C for 10 min and then centrifuged. The absorption procedure was repeated twice [11].

Incorporation of 14C-amino acids. Monolayer cultures of HeLa/MV were washed once with Hanks' BSS and then antibody and complement were added. Immediately after antibody and complement were discarded at the indicated time, the monolayer sheets were washed twice with Hanks' BSS. Then the cultures were incubated at 37°C for 20 min with 2 ml of 14C-amino acid mixture in Hanks' BSS (0.125 μCi/ml). At the end of the labeling period the cultures were chilled to 0°C, and the labeling medium was discarded. Cellular protein was extracted as follow: To each bottle was added 5 ml of 1% SDS, 0.1 M NaCl, 0.01 M EDTA and 0.01 M Tris-HCl buffer (pH 7.4), and further 1 ml of 50% TCA. The TCA-insoluble precipitates were washed 3 times with 5% TCA by centrifugation and then collected on Millipore filters. The filters were dried and placed in 10 ml of toluene containing PPO 6 g/liter and POPOP 200 mg/liter. Retained radioactivity was measured in a Horiba LS-551 liquid scintillation spectrometer.

RESULTS

Cytotoxic Effect on HeLa and HeLa/MV Cells of Anti-HeLa Cell Serum and Anti-HeLa/MV Cell Serum

Both A-H and A-H/M sera induced cytolysis in HeLa and HeLa/MV cells to the same degree by the addition of one half diluted guinea pig sera used as a source of complement (Fig. 1).

Cytotoxic Effect on HeLa and HeLa/MV Cells of Anti-Measles Sera

The A-H serum absorbed with HeLa

---

**Fig. 1.** Cytotoxic tests on HeLa and HeLa/MV cells with A-H (a) and A-H/M (b). Procedures of these experiments were described in Materials and Methods. Guinea pig sera as the source of complement were diluted twofold with Eagle's MEM. —, cells of HeLa/MV culture; ----, cells of uninfected HeLa culture.
cells (A-H-H) did not react with HeLa and HeLa/MV cells, while the A-H/M serum absorbed with HeLa cells (A-H/M-H) reacted with HeLa/MV cells but did not react with HeLa cells (Fig. 2).

This reaction between A-H/M-H and HeLa/MV cells appeared to have resulted from a reaction between antibody and virus-specific antigen on the cell surface of HeLa/MV. Then anti-measles sera from various sources were absorbed with HeLa cells and examined for their reactivity with HeLa/MV cells (Fig. 3a). These sera reacted to the same degree with HeLa/MV cells. The maximum of CTI was low from 0.4 to 0.7 as compared with 1.0 for the A-H or A-H/M sera. These anti-measles sera

![Graph of Cytotoxic Test](image1)

**Fig. 2.** Cytotoxic test on HeLa and HeLa/MV cells with A-H/M-H. A-H/M-H was the anti-HeLa/MV cell serum absorbed with HeLa cells. •-•, cells of HeLa/MV culture; ○—○, cells of HeLa culture.

![Graph of Cytotoxic and HA-inhibiting Test](image2)

**Fig. 3.** a. Cytotoxic tests on HeLa/MV cells with A-MV-H, A-H/M-H and A-Inomata-H. These measles sera were sufficiently absorbed with HeLa cells. In this experiment, guinea pig sera were diluted fourfold with Eagle’s MEM. •—•, cytolysis by A-MV-H; ▲—▲, cytolysis by A-H/M-H; ○—○, cytolysis by A-Inomata-H. b. Cytotoxic effect and HA-inhibiting effect of A-MV-H on HeLa/MV cells. After the cells and serially twofold diluted antisera were mixed and incubated for 30 min at 37°C, each mixture was divided into 2 groups. One was tested for cytolysis using twofold diluted guinea pig sera. The other was used to show the inhibition of hemadsorption by antibody. The cells were mixed with 2% green monkey red blood cells and incubated for 1 hr and then washed with Hanks’ BSS. HA-positive cells were observed under microscopy. HA-inhibiting index (HII) was calculated by the same method as the calculation of cytotoxic index (CTI). •—•, cytotolytic effect of A-MV-H; ○—○, HA-inhibiting effect of A-MV-H.
also inhibited specifically the adsorption of green monkey red blood cells on the surface of cells infected with measles virus [12]. Hemadsorption-inhibiting index (HII) which was calculated by the same method as CTI roughly paralleled CTI as shown in Fig. 3b, supporting our working hypothesis that the immunolysis resulted from the interaction of measles antibody with the measles antigen on the cell surface of HeLa/MV.

Cytotoxic Effect on HeLa/MV Cells of Anti-Measles Sera Absorbed with Measles Particles

It was of interest whether the antigen on the cell surface of HeLa/MV was a virion antigen or a new antigen. The A-MV and A-H/M-H sera lost the ability to produce cytolysis by absorption with measles virus particles (Table 1). This indicates that the antigen on the cell surface responsible for the cytolysis is the antigen of measles virion.

Time Course of Cytotoxic Reaction on HeLa/MV Cells with Anti-Measles Sera or Anti-HeLa Cell Serum

The maximum titer of cytolysis by A-MV serum was so low that cytotoxic titer of A-MV serum could not be determined. The time course of the cytolysis by both A-MV and A-H sera were recorded to determine the time necessary for the maximum cytolysis (Fig. 4a, b). The cytolysis by A-MV gradually increased even after 90 min, while that by A-H increased rapidly and reached a plateau. The cytolysis by A-H serum was similar to the one-hit reaction of immune hemolysis. On the other hand, the cytolysis by A-MV serum seemed to be dependent not only on antibody and complement but also on some unknown factors in the serum.

The Effect of Anti-Measles Serum and Anti-HeLa Cell Serum on Protein Synthesis of HeLa/MV Cells

As shown in Fig. 5, normal rabbit serum did not affect the incorporation of $^{14}$C-amino acids into HeLa/MV culture, while the A-MV and A-H sera gradually decreased the incorporation of $^{14}$C-amino acids. Particularly, the A-H serum and complement stopped the protein synthesis of HeLa/MV cells in 15 min after the treatment. These results agreed with the results of the above experiments.

<table>
<thead>
<tr>
<th>Table 1. Cytotoxic test on HeLa/MV cells with anti-measles sera absorbed with measles particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisera (1:8)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>A–H/M</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>A–MV</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control (C’ only)</td>
</tr>
<tr>
<td>Control (A–MV only)</td>
</tr>
<tr>
<td>Control (Medium only)</td>
</tr>
</tbody>
</table>

Abbreviations of A–H/M and A–MV are illustrated in text.

$^{a}$ Titers of hemagglutinin-inhibiting units/ml.

$^{b}$ Standard deviation.

$^{c}$ Not tested.
Fig. 4. Time courses of cytotoxic test on HeLa/MV cells with A-H (a), A-MV and A-H/M-H (b). A-H was diluted serially two-fold with Eagle's MEM, while A-MV and A-H/M-H were diluted eightfold to show the maximum cytolysis. At indicated times after the addition of guinea pig sera, percents of cytolysis of cells of HeLa/MV were determined. a. ○, cytolysis by A-H (1:12); △, cytolysis by A-H (1:24); ▲, cytolysis by A-H (1:48); □, cytolysis by A-H (1:96); ●, cytolysis by C only (control). b. ●, cytolysis by A-MV (1:8); ○, cytolysis by A-H/M-H (1:8).

Requirement for Complement in the Cytolysis with Anti-HeLa Cell Serum and Anti-Measles Sera

The concentration of complement required to produce 50% cytolysis (CL50) was determined (Fig. 6). Activity of complement to show CL50 of HeLa/MV cells by A-H was 270 CL50/ml. On the other hand, in the cytolysis by anti-measles sera, the higher the concentration of complement, the more marked was the cytolysis. The CL50 titer for HeLa/MV cells with anti-measles sera could not be accurately expressed.

Relationship between Consumption and Cytotoxic Activity of Complement

It was of interest to determine whether or not the complement required for the cytolysis by A-MV was effectively consumed. The consumption of complement...
Fig. 6. Requirement for complement in cytolysis by A-H (a) and anti-measles sera (b). These antisera were diluted eightfold to show maximum cytolysis. a. ○, cytolysis by A-MV; ▲, cytolysis by A-Inomata-H; ○, cytolysis by A-H/M-H.

Fig. 7. Relationship between consumption and cytotoxic effect of complement. Fourfold diluted guinea pig sera which showed the activity of 290 C₅₇H₅₀/ml were used in these cytotoxic reactions. After the cytotoxic reactions, each mixture was diluted with 0.7 ml of Eagle's MEM and centrifuged. 0.5 ml of the supernatant was diluted with GVB++ and then 10⁷ sheep red blood cells sensitized with hemolysin were added to each dilution. Thus the activity of the residual complement was determined. At the same time, the cytotoxic index (CTI) was determined using the cell pellets.

in the cytolysis by A-H was closely related to CTI, but that in the cytolysis by A-MV was little and did not coincide with CTI (Fig. 7). Then it was not yet determined whether or not the cytolysis by A-MV essentially needed complement.
Decrease of Cytotoxic Activity of Complement absorbed with Bentonite

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude C'</td>
<td>200</td>
<td>100</td>
<td>63.8</td>
</tr>
<tr>
<td>Absorbed with bentonite</td>
<td>200</td>
<td>100</td>
<td>40.0</td>
</tr>
<tr>
<td>Absorbed twice with bentonite</td>
<td>200</td>
<td>100</td>
<td>28.0</td>
</tr>
<tr>
<td>Heated at 56°C for 30 min</td>
<td>25</td>
<td>27.4</td>
<td>19.5</td>
</tr>
<tr>
<td>Diluted with 0.02% EDTA</td>
<td>NT</td>
<td>29.0</td>
<td>17.4</td>
</tr>
<tr>
<td>Control (C' only)</td>
<td>NT</td>
<td>26.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Control (A-MV-H only)</td>
<td>NT</td>
<td>26.3</td>
<td>17.8</td>
</tr>
<tr>
<td>Control (Medium only)</td>
<td>NT</td>
<td>23.9</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Abbreviations of A-H, A-MV-H and NT indicate anti-HeLa cell serum, anti-measles serum absorbed with HeLa cells and not tested, respectively.

a) One half diluted guinea pig sera.
b) The highest dilution to show the maximum hemolysis.
c) Percentage of trypan blue-stained cells of HeLa/MV.

DISCUSSION

The viruses of the myxovirus group form their progeny virions under the surface of cells by budding. Viral antigen at the surface of infected cells was detected by the fluorescent antibody, hemadsorption [14], or ferritin-conjugated antibody technique [5, 10]. Though it was not known whether or not the cells infected with measles virus had non-virion antigen on the cell surface, microvilli-modification of the infected cell membrane has been observed [17]. The appearance of a new antigen in the membrane fraction had been shown in the cells infected with parainfluenza virus [13]. Nevertheless a major antigen occupying the surface of infected cells was a viral antigen which would be constituted by an envelope antigen.

Lysis of nucleated cells by the action of complement and antibody against cell membrane antigens such as Forssman antigen, histocompatibility antigens and tumor specific antigens induced by oncogenic viruses had been reported [4, 8, 18, 19, 22]. Viral antigens on the surface of cells infected with non-oncogenic viruses were also damaged with anti-viral antibody and complement [24].

Cells persistently infected with measles virus were destroyed by a combined action of anti-measles sera and normal guinea pig sera. Anti-HeLa/MV serum absorbed with sufficient HeLa cells retained the ability of the cytolysis for HeLa/MV cells but lost that for HeLa cells, and further absorption with purified measles particles abolished the ability of the cytolysis for HeLa/MV cells. In addition, anti-measles serum absorbed with purified measles particles did not show any cytolytic activity. Therefore, the cytolysis by anti-measles serum appears to be due to a reaction between measles antibody and viral antigen on the surface of infected cells. The purification of measles particles seems insufficient and further purification with DEAE-cellulose column is needed, but we failed to purify the virus by this method because of a poor
recovery of viral hemagglutinin. Though the fractions used for absorption largely consisted of measles particles of heavy density, contamination by other virus-specific antigens cannot be excluded.

A prozone phenomenon in cytolysis by higher concentrations of anti-measles sera was noticed. This prozone could not be effectively eliminated by washing cells before the addition of complement. Anti-complementary effect of anti-measles sera was not observed. The mechanism of the prozone phenomenon is unknown [6, 7].

The effects of complement in the cytolysis by anti-HeLa cell serum resembled those in the immune hemolysis [21]. The time course of the cytolysis by anti-HeLa cell serum showed that it follows a one-hit reaction which proceeds rapidly until reaching a plateau.

Activity of complement for 50% cytolysis was able to be calculated with the value of Y/I-Y as reported in the case of immune hemolysis. Consumption of some components of complement (C3 and C4) in the cytolysis by various concentrations of anti-HeLa cell serum was closely correlated with the cytotoxic index. These results suggest that the cytolysis by anti-HeLa cell serum needs essentially all components of complement.

On the other hand, the cytolysis by anti-measles sera showed that it follows a multiple hit reaction which proceeds slowly with time. The higher the concentration of guinea pig serum used was, the more marked was the cytolysis. However, the consumption of complement was negligible and did not parallel the extent of cytolysis. If complement contributed to the cytolysis by anti-measles sera, the antigenic sites on the surface of HeLa/MV cells would be very spare and/or antibody combined with surface antigen would have a poor capacity to combine with complement. However, the following findings support that complement is involved in the cytolysis by anti-measles sera. In the cytolysis by anti-measles sera, antigen–antibody complexes are formed. By heating at 56°C for 30 min or dilution with 0.02% EDTA normal guinea pig serum lost the activity of cytolysis by antibodies as well as that of hemolysis.

Non-specific cytotoxin in these sera, if any, may not be inactivated by the above treatment [3]. Absorption with bentonite decreased the activity of guinea pig serum for cytolysis by anti-measles sera without any change for immune hemolysis, indicating that the cytolysis by anti-measles sera is dependent upon some factors present in guinea pig serum. Although the effect of lysozyme as observed in the immune bacteriolysis [11] was examined in the immunolysis of nucleated cells, lysozyme exerted no effect on the immunolysis. Further studies about the serum factors in the immunolysis in our system are now in progress.

ACKNOWLEDGEMENT

The authors wish to thank Prof. H. Iida, Department of Bacteriology, Dr. T. Shirai, Cancer Institute, Hokkaido University School of Medicine, for their kind advices and stimulating discussions. This investigation was supported in part by a grant from Scientific Research Fund of the Ministry of Education of Japan.

REFERENCES


