Mechanism of Polykaryocytosis Associated with Noncytopathic Infection by Measles Virus

JOHN G. ATHERTON,1 SOTIROS D. CHAPARAS,2 MARTHA CREMER, and IRVING GORDON

Department of Microbiology, University of Southern California School of Medicine, and Los Angeles County General Hospital, Los Angeles, California

Received for publication 8 February 1965

Abstract

Atherton, John G. (University of Southern California, Los Angeles), Sotiros G. Chaparas, Martha Cremer, and Irving Gordon. Mechanism of polykaryocytosis associated with noncytopathic infection by measles virus. J. Bacteriol. 90:213-219. 1965.—Infection with a measles virus variant resulted not only in formation of polykaryocytes (PK) but also in formation of multicellular immunofluorescent foci (IFF) in which no cytopathic effect could be detected. The ratio of IFF to PK changed from 27 to 4 during the first passage and remained 4 after a second passage. PK were plaques. Plaque assay was linear in the presence of IFF. To investigate the mechanism of PK formation, radioautography was done on cells pulse-labeled with tritiated thymidine before virus multiplication began. The results showed that PK were formed by fusion; there were no PK whose nuclei contained no label, and the proportion of labeled nuclei (32%) and distribution of grain counts was the same in PK as in uninfected cells, ruling out nuclear replication without concomitant cytoplasmic membrane formation as the mechanism of formation of these PK. Early in PK development, neutral red uptake was markedly increased ("red" plaques). As PK matured, hyperchromicity disappeared ("white" plaques). This sequence provided an index of rate of evolution of PK. Rate of PK maturation was more rapid at 37 than at 32 C.

The large multinucleated syncitia or polykaryocytes that appear during infection of a cell sheet with measles virus (Enders and Peebles, 1954) are thought to be the result of damage to cytoplasmic membranes and subsequent fusion of infected cells with neighboring cells. The evidence is largely based on sequential microscopic observations (Bech and von Magnus, 1958; McCarthy, 1959; Thomison, 1962). Noninfectious measles virus suspensions prepared by ultraviolet irradiation (Toyoshima, Hata, and Takashi, 1960; Cascardo and Karzon, 1964) and noninfectious fractions separated by gradient-density ultracentrifugation (Schluenderberg, 1962) can induce similar cell fusion in vitro. Roizman (1962a, b) suggested that polykaryocytes induced by measles and other viruses are formed in two steps, and called attention to the discrepancies between rate of recruitment of cells into polykaryocytes during herpesvirus infection (Hoggan, Roizman, and Turner, 1960) and the absolute concentration of infectious virus (Hoggan and Roizman, 1959).

This paper reports quantitative studies of polykaryocyte formation during multiplication of measles virus. Grain counts done after autoradiography of infected cell sheets pulse-labeled with tritiated thymidine verified the microscopic observations that polykaryocytes consist of fused cells. Rate of polykaryocyte maturation was estimated by following conversion from an early to a late stage, recognized by characteristic change in reaction to vital stain with neutral red. This index was employed to compare rate of polykaryocyte evolution at two temperatures of incubation known to result in different output of infectious virus. During these investigations we found that the virus variant employed, selected in this laboratory by Seligman and Rapp (1959) from the Edmonston strain, contained infectious noncytopathic measles virus detectable by immunofluorescence. Since plaque formation was linear in the presence of the associated noncytopathic virus, ratios between noncytopathic and

1 Present address: Department of Bacteriology, University of Queensland Medical School, Brisbane, Australia. On leave 1959-1960 as Fulbright Scholar.

2 Present address: Division of Biologics Standards, National Institutes of Health, Bethesda, Md.
cytopathic virus found in different passages and under different plating conditions were compared.

**Materials and Methods**

**Tissue cultures.** HEp-2 cells were derived from the C-3 clone, which has been described (Rapp, 1969). FL human amnion cells were obtained from Microbiological Associates, Inc., Bethesda, Md. Both cell lines were maintained in bottles containing 10% calf serum plus 90% Eagle’s (1959) basal medium (BSE) and 100 units of penicillin plus 100 μg of streptomycin per ml. The pH was adjusted to 7.5 to 7.6 with NaHCO₃. For growing virus, serum content was reduced to 2%. Both uninfected and infected cells were incubated at 37 C for routine passages.

**Virus.** The “giant cell” (GC) variant selected from the Edmonston strain by Seligman and Rapp (1959) was employed for all experiments. It had undergone 10 additional passages in HEp-2 cells and three to five additional transfers in FL cells. Stock virus was harvested after disruption of infected cells by three cycles of freezing at −65 C and thawing at room temperature, and the virus was stored in sealed ampoules at −65 C in a mechanical freezer or at −190 C in liquid nitrogen. Adsorption of virus to cells was carried out at 37 C for 1 hr.

Between 90 and 99% of the virus was adsorbed to HEp-2 or FL monolayers in 1 hr at 37 C, as determined by replicate plaque counts of cell monolayers washed with balanced salt solution (BSS) and overlaid with agar at intervals after inoculation. This is more rapid than the rate of adsorption with some strains (Underwood, 1959). No fusiform or spindle-shaped cells developed at any passage level. Polykaryocyte formation was virtually the only cytopathic effect detectable microscopically; inclusions were rare, and, at some of the passage levels used, they were absent.

**Plaque assay.** Plaque assays were done in 60-mm glass or plastic (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) petri dishes. Monolayers of HEp-2 or FL cells were initiated with 4 × 10⁴ to 1 × 10⁵ cells in 10% calf serum plus 90% BSE. Growth was confluent in 1 to 2 days of incubation at 37 C in 5% CO₂. Virus assays were done in triplicate. At 1 hr after inoculation with 0.1 ml of virus suspension, cells were overlaid with 2% agar containing double-strength BSE plus 2% calf serum and were incubated at 37 C in 5% CO₂. A second overlay, consisting of 4 ml of the same agar plus neutral red (1:10,000), was added to 6 days after inoculation. Plaques were counted on the day after the second overlay was added.

**Hemadsorption.** African green monkey erythrocytes (1% in 0.85% NaCl) were added to the cell sheet under test. After 15 min at room temperature, the cells were washed three times with an excess of BSS. Results were read both grossly and microscopically.

**Technique for immunofluorescent studies.** This procedure, reported in detail by Rapp et al. (1959), requires methylcellulose instead of agar in the overlay. Either a Leitz microscope and HBO-200 light source or a Zeiss microscope and AH-6 water-cooled mercury vapor are employed.

**Technique for autoradiographic studies.** Nuclei of cells grown on cover slips were labeled with tritiated thymidine. For autoradiography, cover slips were rinsed in isotonic saline, fixed (20 min) in four changes of acetate acid-alcohol (1:3), washed, rinsed in 95% alcohol, dried, and mounted on slides, with the cell monolayer uppermost. The next day, they were dipped into Eastman Kodak nuclear track emulsion (NTB2) at 42 to 44 C, wiped, dried, and held for 1 week in the dark at room temperature. They were developed for 2 min (Eastman Kodak D19), washed, fixed for 4 min (Kodak acid fixer), rinsed, stained in 0.25% toluidine blue (pH 6) for 1 min, rinsed in 95% alcohol, and air-dried.

**Table 1.** Formation of plaques, polykaryocytes, and immunofluorescent foci by different virus stocks

<table>
<thead>
<tr>
<th>Stock</th>
<th>Dilution</th>
<th>Virus</th>
<th>Infectious units/ml estimated from</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PFU/ml *</td>
<td>PKFU/ml †</td>
</tr>
<tr>
<td>GC 1A</td>
<td>Undiluted</td>
<td></td>
<td>365</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td></td>
<td>325</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td></td>
<td>260</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td></td>
<td>320</td>
<td>200</td>
</tr>
<tr>
<td>GC 1C</td>
<td>Undiluted</td>
<td></td>
<td>479</td>
<td>489</td>
</tr>
<tr>
<td>GC 1D</td>
<td>Undiluted</td>
<td></td>
<td>332</td>
<td>180</td>
</tr>
<tr>
<td>GC 2</td>
<td>Undiluted</td>
<td></td>
<td>170</td>
<td>170</td>
</tr>
<tr>
<td>GC 3</td>
<td>Undiluted</td>
<td></td>
<td>879</td>
<td>901</td>
</tr>
</tbody>
</table>

* PFU/ml = plaque-forming units per milliliter.
† PKFU/ml = polykaryocyte-forming units per milliliter estimated from counts of Giemsa-stained cover slips.
‡ IFFU/ml = immunofluorescent focus-forming units per milliliter estimated from cover-slip counts.
POLYKARYOCYTE FORMATION BY MEASLES VIRUS

Results

Relationship between plaques, polykaryocytes, and immunofluorescent foci. Under the microscope, plaques were identified as large polykaryocytes (PK). Plaque counts were compared with microscopic counts of PK and immunofluorescent foci (IFF), respectively, done on pairs of cover slips incubated under methylcellulose for 2 to 3 days (Rapp et al., 1959). One of each pair was assayed for IFF, the other was stained with Giemsa stain. For a given virus dose there was close correspondence between the number of plaques and the number of PK determined by either method (Table 1). PK contained large quantities of virus antigen, which was usually restricted to the cytoplasm. In addition to PK, however, a large number of multicellular IFF were present. By interchanging phase condensers and objectives with those used for demonstration of immunofluorescence, it was established that specific fluorescence was completely intracellular. Inclusions or other cytopathic effects could not be found in these foci either under phase optics or after cover slips were stained with Giemsa stain. The presence of IFF-forming virus did not affect the linearity of the plaque assay; when the number of plaques was plotted against the concentration of virus plated, the slope of the resultant straight line was approximately one and extrapolated to the origin.

The ratio between the number of immunofluorescent focus-forming units (IFFU) and polykaryocyte-forming units (PKFU) induced by a given virus suspension did not change when cover slips were incubated for an extra 3 or 4 days, during which time both PK and IFF increased in size. Secondary spread of virus under or through the methylcellulose, therefore, could not be involved as a mechanism to explain the excess of IFFU over PKFU. This confirms previous evidence that methylcellulose prevents the spread of measles virus (Rapp et al., 1959).

Variation of IFFU-PKFU ratios of samples of virus stock GC-1, stored frozen in separate vials, was within probable experimental error (Table 1). Stocks GC-2 and GC-3, prepared by further passage, had different IFFU/PKFU ratios than did stock GC-1.

Attempts to establish an IFF clone by selection at terminal dilutions and serial transfer were unsuccessful.

Distribution of tritiated thymidine in infected cell sheets. The proportion and relative amount of tritiated thymidine (H^3Tdr) in the nuclei of polykaryocytes was compared with that in uninvolved cells on the same cover slip. FL amnion cells were infected and washed; H^3Tdr (specific activity, 6.70 c/m mole) was added at a concentration of 0.05 µC/ml. Incorporation of label was stopped after 1 to 3 hr by washing and addition of cold Tdr (0.08 mM). The cells were incubated in BSE plus 2% serum for 48 hr at 37°C in 5% CO<sub>2</sub>, rinsed, and fixed. Uninfected control cells were

Table 2. Reduced silver grain counts in nuclei of polykaryocytes due to measles virus infection, and in uninvolved cells, after a pulse of tritiated thymidine

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of cover slips</th>
<th>Total no. of nuclei examined</th>
<th>Per cent of total nuclei labeled with H^3Tdr</th>
<th>Grain counts per labeled nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polykaryocytes (293)</td>
<td>21</td>
<td>5,696</td>
<td>31.6</td>
<td>48.8</td>
</tr>
<tr>
<td>Uninvolved</td>
<td>21</td>
<td>14,627</td>
<td>32.5</td>
<td>50.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grain counts per labeled nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 to 39</td>
</tr>
<tr>
<td>10 to 19</td>
</tr>
<tr>
<td>5 to 10</td>
</tr>
</tbody>
</table>

Fig. 1. Early polykaryocyte in wet mount after intra vitum staining with neutral red, showing hyperchromicity of cytoplasm in comparison with uninvolved cells, and hemadsorption with African green monkey erythrocytes. N = nucleus; C = cytoplasm containing neutral red; E = erythrocyte. X 300.
similarly labeled, but were rinsed and fixed a few minutes after cold Tdr was added. Autoradiography was done and the number of reduced silver grains was counted.

The percentage of labeled nuclei was no greater in cells exposed to H'Tdr for 3 hr than for 1 hr. It was also the same in the uninfected control cells fixed immediately after labeling as in the infected cells incubated for an additional 48 hr. This indicated that incorporation of H'Tdr into nuclei of infected cells had taken place within 3 hr after infection. This was during the time of virus penetration or early eclipse; infectious virus was not detectable.

Since the different labeling periods employed resulted in the same percentage of labeled nuclei, these data were pooled for analysis (Table 2). A total of 5,696 nuclei in 293 polykaryocytes were examined, the average number of nuclei per PK being 19. All of the 293 polykaryocytes contained the label. H'Tdr uptake, as estimated by reduced silver grains in nuclei, had approximately the same distribution among PK as among 14,627 neighboring cells uninvolved in polykaryocytosis, and approximately one-third of the nuclei in each group were labeled. These results strongly support the conclusion that the 293 PK examined were formed by fusion.

Reaction to neutral red as an index of PK maturation. Involvement of cells in a polykaryocyte was closely correlated with increased intensity of cytoplasmic staining with neutral red. This was true in the presence and absence of overlay. Newly formed PK could thus be identified as "red plaques" (Fig. 1). Hyperchromicity with neutral red continued to develop at the periphery of enlarging PK where new cells were recruited, but the central area lost its red color as the PK enlarged. Nuclei simultaneously migrated toward the center. A "white plaque" ultimately resulted

---

**Fig. 2.** Mature polykaryocyte showing hypochromicity of cytoplasm in comparison with uninvolved cells, and adherence of African green monkey erythrocytes to slide in center where nuclei are detached after washing. (Prepared and labeled as in Fig. 1.) $\times$ 800.
Table 3. Conversion of red to white plaques at 32 and 37 C

<table>
<thead>
<tr>
<th>Temp</th>
<th>Plate no.</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R*</td>
<td>W†</td>
<td>%W</td>
<td>R</td>
<td>W</td>
</tr>
<tr>
<td>32 C</td>
<td>1</td>
<td>30</td>
<td>1</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>45</td>
<td>1</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>27</td>
<td>1</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>39</td>
<td>1</td>
<td>3</td>
<td>35</td>
</tr>
<tr>
<td>37 C</td>
<td>1</td>
<td>43</td>
<td>4</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>2</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50</td>
<td>5</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>41</td>
<td>4</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>40</td>
<td>4</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

* Abbreviation: R = red plaques.
† Abbreviation: W = white plaques.
‡ Abbreviation: %W = per cent of total plaques that were white plaques.

(Fig. 2). Plaques formed by parainfluenza viruses 2 and 3 consistently followed a very similar progression, and those formed by Newcastle disease virus occasionally did so. Sometimes syncytial areas were connected by long cytoplasmic processes as plaques enlarged.

Hemadsorption could be demonstrated with ease in and around both red and white plaques (Fig. 1 and 2). When cell sheets were rinsed with BSE after addition of erythrocytes, the central area of a white plaque often detached. The red blood cells continued to adhere to the glass or plastic of the container. They lysed after several hours.

An attempt was made to isolate a mutant that would produce red plaques, but not white plaques, by picking red plaques from cell sheets under agar and inoculating monolayers with and without agar overlays. Red plaques were also subcultured at end point dilution through serial transfers in tubes. The sequence of red to white plaque conversion took place in all progeny.

Rate of conversion of red to white plaques at different temperatures. In the absence of overlay, red plaques could be seen under the microscope within 24 hr after incubation at 37 C began, but under agar they usually could not be detected until the 3rd day. Rate of conversion of red to white plaques provided an index of rate of recruitment of cells into PK. FL amnion monolayers were infected, overlaid to prevent secondary spread, incubated at 37 and 32 C, respectively, and stained with neutral red on the 3rd day. Plaques were classified and ringed with a diamond stylus under the microscope. Counts were made daily (Table 3). Conversion from red to white plaques was approximately linear at 37 C, and conversion at 37 C preceded conversion at 32 C by approximately 1 day (Fig. 3).

Discussion

The experiments in which nuclei were prelabeled with tritiated thymidine verify the con-
cept that large measles polykaryocytes are formed by fusion. Roizman and Schluederberg (1962) reported that small PK can arise from mononucleated cells in which measles virus damages, but does not destroy, the mitotic apparatus. They found small PK containing metaphase figures arrested by colchicine in infected cell sheets, and concluded that they arose from aberrant cleavage. During time-lapse microcinematography, Thomson (1962) saw small PK incorporate neighboring cells only occasionally. As these investigators point out, nuclear replication in the absence of concomitant cytoplasmic membrane formation is apparently an unusual mechanism of PK formation by measles virus.

Under our experimental conditions, approximately a third of the nuclei of uninvolved cells contained H3Tdr label. Approximately 200 of the 293 PK examined were unlabeled, and hence could have given rise to an unlabeled PK by nuclear division. None did, so that our estimated rate of PK formation by nuclear division is < 5 × 10⁻². Since there was an average of 19 nuclei per PK in our study, the fact that label was distributed similarly in PK and in uninvolved cells is further evidence supporting fusion as the mechanism of PK formation.

In these experiments, the H3Tdr pulse was administered immediately after virus adsorption was completed and before virus multiplication could occur. In the studies reported by Takahashi, Miyamoto, and Kato (1963), which had a different objective, a 30-min pulse of 2 μc/ml of H3Tdr was administered to FL cells 3 days after infection with measles virus, when cytopathic changes were well advanced. Their finding that H3Tdr incorporation into the nuclei of PK was less than that into uninvolved cells, therefore, does not bear on the problem of nuclear replication as a mechanism of PK formation.

PK formation was virtually the only cytopathic change induced by the GC strain employed in our studies, and the plaques formed under agar or methyl cellulose were demonstrated to be PK both by direct inspection and by the close correspondence between plaque counts and PK counts. The presence of noncytopathic virus, demonstrable only by immunofluorescence, requires explanation. It is not attributable to spread from PK to uninfected areas under or through the methylcellulose overlay. If this spread occurred, and the IFU were capable of becoming PK, the ratio of IFU to PFU or PKFU would have decreased during further incubation of the cover slips at 37 C. This was not the case.

While the ratios differed for different virus stocks, the ratio remained approximately 27 not only for GC stocks 1C and 1D, which were plated undiluted, but also for stock 1A, plated at dilutions of 1:4 and 1:8 (Table 1). This argues against inhibition of virus multiplication by interferon (deMaeyer and Enders, 1961) as an explanation for presence of noncytopathic virus. The three inocula were samples of the same virus stock. An eightfold dilution of the inoculum resulted in an eightfold reduction of both IFFU and plaque count. Interferon present would also have been diluted eightfold from a starting concentration that only partially inhibited virus multiplication. Based on previous experiences with partially inhibitory doses of interferon, the ratio would be expected to decrease. Repeated attempts to select virus that would yield only IFU failed, however, and it is possible that PK formation is affected by cultural conditions, in which interferon formation might play an important part, and which would account for the variation in IFFU/PKFU ratio in different stocks. Interferon formation might also account for the noninfectious virus associated with a carrier culture of measles virus (Rustigian, 1962), since it is present in carrier cultures of other myxoviruses (Henle et al., 1959).

Increased absorption of neutral red is a feature of PK formation induced not only by measles virus (Kohn and Yassyk, 1962; Ruckle-Enders, 1962; Rapp, 1964) but also of closely related myxoviruses such as parainfluenza viruses 2 and 3. It might be due to increased cell membrane permeability. As the PK develop, hypochromicity is lost and plaques change from "red" to "white." The rate of conversion of red to white plaques was used as an index of the rate of maturation of PK. The rate is greater at 37 than at 32 C. In contrast, growth curves indicate that the concentration of both cell-associated and released infectious virus is greater at 32 than at 37 C for the GC variant (unpublished data) and other stocks of the Edmonston strain (Underwood, 1959; Bunyak et al., 1962; Rapp, 1964). The data suggest that concentration of infectious virus per se is not correlated directly with rapidity of PK maturation, and are consonant with the evidence that virus rendered noninfectious by UV can induce PK (Toyoshima et al., 1960; Cascardo and Karzon, 1964). Our data also suggest that, as appears to be the case with herpesvirus (Hoggan et al., 1960), the responsible fusion factor may be more thermostable than infectious virus. Measles virus produces a hemolysin (Perié and Chany, 1960) that has been separated from infectious virus (Schluederberg, 1962). Roizman (1962b) pointed out that recruitment of cells into a polykaryocyte may depend on the rate at which virus emerges from infected
compartments. Our data neither belie nor support this postulated mechanism.

Acknowledgments

We are grateful to Douglas Stevenson, Leslie Eva Hamilton, Shirley Northrop, and Dorothy Emery for excellent assistance.

This investigation was supported by Public Health Service grants from the National Institutes of Health, and was supported in part by the U.S. Army Medical Research and Development Command under the sponsorship of the Commission on Viral Infections of the Armed Forces Epidemiological Board.

Literature Cited


