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# PLAQUE DIFFERENTIATION AND REPLICATION OF VIRULENT AND ATTENUATED STRAINS OF MEASLES VIRUS

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## ABSTRACT

RAPP, FRED (Baylor University College of Medicine, Houston, Tex.). Plaque differentiation and replication of virulent and attenuated strains of measles virus. J. Bacteriol. **88**:1448-1458. 1964.—Plaque formation by strains of measles virus in a stable line of African green monkey kidney cells (BSC-1) is characterized by development of large plaques (>1 mm) within 4 days after inoculation of the cultures with the virulent Edmonston strain or by small plaques (<1 mm) after inoculation with the attenuated Edmonston strain of virus. Plaque formation by measles virus is not influenced by iododeoxyuridine, cytosine arabinoside, isatinthiosemicarbazone, streptonigrin, actinomycin D, or mitomycin C. The predominant cytopathic effect observed with both strains is the formation of large, multinucleated giant cells. Development of the giant cells is correlated with development of virus antigen and synthesis of infectious virus. Synthesis of virus is similar at 34 and at 37 C. Appearance of intracellular virus precedes release, and is later in the attenuated virus-infected cells than in cells infected with the virulent strain. With the virulent strain, equal concentrations of intra- and extracellular virus are found but, with attenuated virus, only a small fraction reaches the extracellular fluids, and more than 95% of the newly synthesized virus remains cell-associated.

The isolation and propagation of measles virus in tissue culture by Enders and Peebles (1954) allowed further characterization of the properties of this virus (Karzon, 1962), and led to the development of both attenuated, live vaccines (Enders, Katz, and Holloway, 1962; Schwarz, 1962), as well as an inactivated vaccine (Warren and Gallian, 1962). The development of a plaque assay for the virulent Edmonston strain of virus by Hsiung, Mannini, and Melnick (1958) and by Underwood (1959) suggested that the differentiation of measles virus variants and the replication of the virus could be undertaken with the help of these assay systems. In practice, this did not occur, presumably because the smallness of the plaques and the length of time (7 to 8 days) required for plaque formation do not readily allow separation of virulent and attenuated variants. In addition, the vaccine virus, attenuated by passage in chicken embryo fibroblast cultures, does not grow readily in the patas monkey and HeLa cells employed.

In the present study, plaquing of virulent and attenuated strains of measles virus in a stable line of monkey cells revealed a marked difference in plaque size by the strains tested. Studies carried out to explain this observation suggest that plaque development in the cells employed may be a consequence of differences in the yield, rate of synthesis, and release of newly formed virus. The plaque assay to be described was also used to measure the effect of antimetabolites and antibiotics on the replication of measles virus.

## MATERIALS AND METHODS

*Virus.* The virulent Edmonston strain of measles virus (kindly furnished by David Karzon, Buffalo, N.Y.) was received after more than 60 passages in primary human amnion cultures. The virus was passed twice in cultures derived from human embryonic lungs, and was then passed an additional five times in a stable line of cells derived from an African green monkey, the BSC-1 cell line (Hopps et al., 1963). This strain, as well as a fresh isolate of measles virus (supplied by M. Benyesh-Melnick), were also tested for plaque characteristics prior to passage in monkey cells.

The attenuated Edmonston strain of measles virus (kindly supplied by Merck Sharp & Dohme Laboratories, West Point, Pa.) was also passed five times in BSC-1 cells prior to the virus growth studies reported below. The virus was tested at all passage levels for cytopathic changes (CPE), plaque characteristics, and virus production. Some tests were also carried out with the direct plaquing of two additional commercial lots of

Edmonston vaccine, one prepared by Merck Sharp & Dohme, and the second by Wyeth Laboratories, Philadelphia, Pa.

*Tissue cultures.* BSC-1 cells were routinely grown in 16-oz (0.473 liter) bottles in a fluid medium composed of 90% Eagle's (1959) basal medium and 10% calf serum. The fluid contained 100 units of penicillin and 100 μg/ml of streptomycin, and was adjusted to a pH of 7.4 to 7.6 with 7.5% $NaHCO_3$. Stocks of virus were prepared in these cultures at 37 C. Virus was harvested 3 to 4 days (virulent strain) or 6 to 7 days (attenuated strain) after inoculation of the cultures by two cycles of rapid freezing of the cells in the supernatant fluid followed by thawing at room temperature. The fluids were clarified by low-speed centrifugation, dispensed in 1-ml volumes into ampoules which were then flame-sealed, quick-frozen in a Dry Ice-alcohol bath, and stored at −65 C.

Cells were also grown in plastic petri dishes (60-mm). Seeding of the dishes with $1.8 \times 10^6$ cells resulted in confluent monolayers in 2 to 3 days. Cover-glass cultures in petri dishes were prepared as previously described for other cells (Rapp, 1962; Rapp, Rasmussen, and Benyesh-Melnick, 1963). All petri dish cultures were incubated at 37 C in 5% $CO_2$.

*Plaque assay.* Titrations were carried out in monolayers of BSC-1 cells growing in plastic petri dishes (60-mm). The techniques used were the same as those previously described for herpes simplex virus in rabbit kidney cells (Rapp, 1963), except that 2% agar was substituted for the methylcellulose. The final concentration of agar was therefore 1%. After washing the cells and withdrawing the fluids, adsorption of the virus took place for 2 hr at room temperature or for 1 hr at 37 C in 0.1 ml. After addition of 5 ml of overlay, cultures were incubated for varying periods at 37 C in 5% $CO_2$; at the desired time, 3 ml of a 1:7,500 solution of neutral red were added to each culture, and plaques were enumerated after an additional overnight incubation. Most plaque assays were read 4 days after inoculation of the cultures. All assays utilized two to four cultures per dilution of virus.

*Inhibitors.* The preparation of the stocks of 5-iodo-2′-deoxyuridine (IUDR), 1-β-D-arabinofuranosylcytosine hydrochloride (cytosine arabinoside), and *N*-methylisatinthiosemicarbazone (NMITC) was described, and is the same as that used in the experiments carried out with herpes simplex and herpes zoster viruses (Rapp, J. Immunol., *in press*).

Streptonigrin was dissolved in acetone (1 mg/ml), and was then diluted 1:10 in sterile distilled water to give a stock solution of 100 μg/ml. Actinomycin D and mitomycin C were dissolved in Eagle's basal medium at 37 C at a concentration of 1 mg/ml. The inhibitors were diluted, and were incorporated into the overlay in the amounts cited below.

*Cytochemical and immunofluorescent studies.* Cover-glass preparations were washed three times with warm tris(hydroxymethyl)aminomethane (tris) saline (pH 7.4). For cytochemical studies, the cultures were fixed in Bouin's solution, and were stained with hematoxylin and eosin as previously described (Rapp et al., 1963). Immunofluorescent procedures were carried out with pooled human serum and antihuman globulin labeled with fluorescein isothiocyanate, by the method of Riggs et al. (1958) as modified by Marshall, Eveland, and Smith (1958). The procedures and the light system employed were described previously (Rapp, 1962; Rapp et al., 1963; Rapp and Vanderslice, 1964).

## RESULTS

*Characteristics of plaque development.* Round plaques regularly developed within 4 days after inoculation of the cultures with either the virulent or attenuated strains of measles virus (Fig. 1). The plaques produced by the virulent strain were larger (1 to 2 mm in diameter) than were those produced by the attenuated strain (approximately 1 mm in diameter). The plaques tended to form comets when viewed 7 days after inoculation of the cultures (Fig. 2), making them difficult to count. The size of the plaques produced by the virulent strain was more heterogeneous than were those produced by the attenuated strain. Genetic

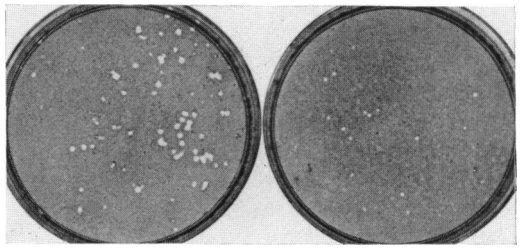

FIG. 1. *Plaques produced in BSC-1 cultures by virulent (left) and attenuated (right) strain of measles virus 4 days after inoculation of the cells.*

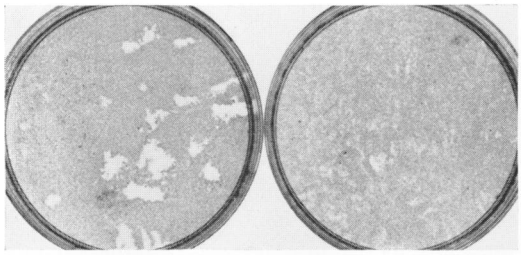

FIG. 2. *Plaques produced in BSC-1 cultures by virulent (left) and attenuated (right) strains of measles virus 7 days after inoculation of the cells.*

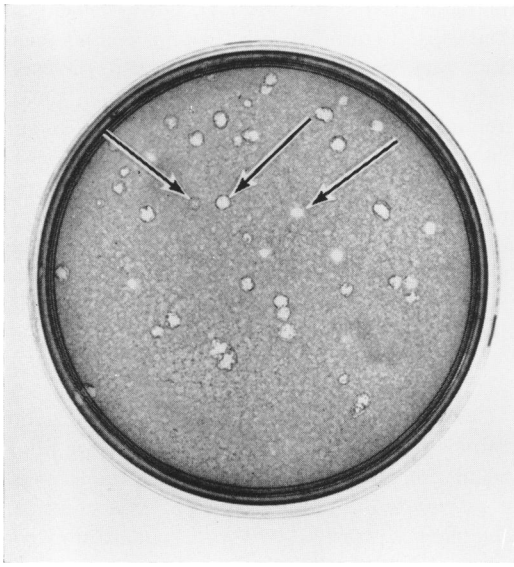

FIG. 3. *Plaques produced in BSC-1 cultures by virulent strain of measles virus 4 days after inoculation of the cells. Arrows point to a clear, a red, and a red-border plaque.*

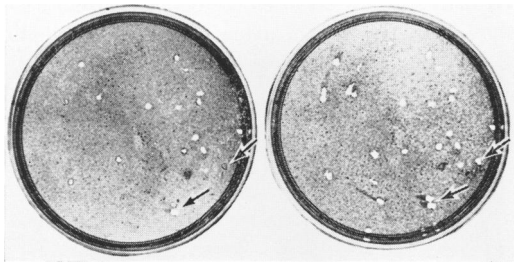

FIG. 4. *Plaque characteristics of measles virus in BSC-1 cells after various times of exposure to neutral red. Left plate: 2-hr exposure; right plate, 18 hr exposure.*

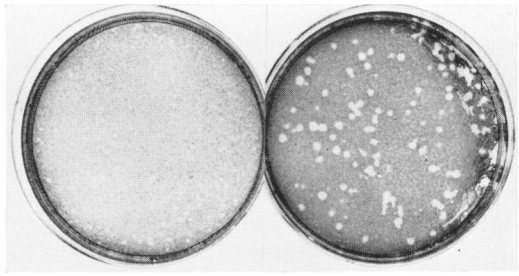

FIG. 5. *Plaque obtained 4 days after inoculation of primary green monkey kidney cells (left) and BSC-1 (right) cells with a dilution of 1:600 and 1:1,200, respectively, of the virulent strain of measles virus.*

purification of the virulent virus by three serial passages of plaque progeny reduced this tendency to heterogeneity, and populations of both large and small plaque-forming virus variants could be established.

The tendency of the virulent strain of measles virus to produce clear, red, and red-border plaques after application of neutral red can be seen in Fig. 3. Such plaques were also observed by Chaparas, Atherton, and Gordon (*personal communication*) in Hep-2 cells. These plaques do not represent genotypic heterogeneity (Fig. 4). The arrows on the left plate point to a red plaque and to a red and clear plaque. These plaques were visualized 2 hr after addition of the neutral red. Overnight incubation in the dark caused a clearing of the plaques (as seen by the arrows pointing to the same plaques in the plate on the right). Other plaques on these plates show a similar effect. Some of the plaques on the plate at the right are beginning to "tail." In addition, populations of virus plaque-purified three times also yielded the three types of plaques described above.

Attempts to utilize primary cultures of kidney cells from African green monkeys were successful, but the plaques were smaller than those obtained in the BSC-1 cultures (Fig. 5). A similar finding was made with Hep-2 cells. Numerous observations supported the concept that a direct linear relationship exists between the concentration of virus plated and the number of plaques produced in BSC-1 cells. A representative experiment is plotted in Fig. 6.

A fresh isolate of measles virus from a fatal case and tested without passage in BSC-1 cells

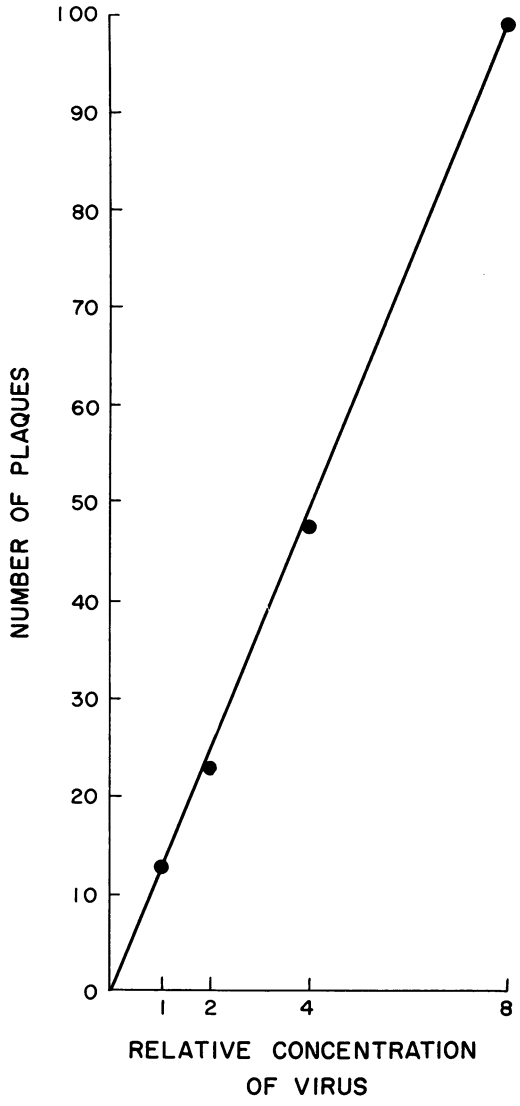


FIG. 6. *Dose-response relationship between number of plaques produced and concentration of measles virus (virulent) plated on monolayers of BSC-1 cells.*

yielded virulent-type plaques. Additional tests were also carried out by direct plaquing of commercial and experimental lots of the Edmonston attenuated strain of virus. The results of plaque assays in BSC-1 cells are compared with those obtained in tube titrations carried out by laboratories supplying the virus (Table 1). The tests compare favorably, although it was found necessary to read plaque assays 7 days after inoculation of the cultures; plaques obtained after 4 days were either too small to count or did not represent maximal titers.

*Inhibition of plaque development.* The identity of the virulent strains as measles virus was carried out in a plaque-reduction test. Convalescent human and immune monkey sera inhibited plaque formation in the absence of inhibition by either the acute human or preinoculation monkey serum (Table 2).

Compounds known to inhibit deoxyribonucleic acid (DNA)-containing virus were then incorporated into the overlay medium. Neither IUDR nor cytosine arabinoside inhibited the development of measles virus in the concentrations of inhibitor tested (Table 3). These compounds inhibited plaque formation by herpes simplex and herpes zoster viruses; the results with these viruses in tests carried out at the same time as those with measles virus are reported elsewhere (Rapp, 1964). NMITC did not significantly inhibit plaque formation by measles virus (Table 3); this compound also did not inhibit the herpesviruses (Rapp, *in press*), but does inhibit plaque formation of vaccinia virus (Rapp, *in press*) by apparently inhibiting maturation of the virus (Easterbrook, 1962). Various antibiotics incorporated into the overlay yielded similar results (Table 4). Significant inhibition was not observed with either the virulent or attenuated strain tested against various antibiotics at different concentrations. Tenfold increases in the

TABLE 1. *Comparison of plaque and tube titrations of attenuated measles virus strains*

| Virus* | $TCD_{50}$ per ml† | PFU per ml‡ |
|---|---|---|
| Merck Sharp & Dohme | $1.3 \times 10^4$ | $8.5 \times 10^3$ |
| Wyeth, P | $2.0 \times 10^3$ | $3.0 \times 10^3$ |
| Wyeth, S | $2.5 \times 10^3$ | $1.1 \times 10^3$ |
| Wyeth, F | $4.0 \times 10^3$ | $5.4 \times 10^3$ |

* Titer supplied by Merck Sharp & Dohme Laboratories represents average of three titrations on a single vial of reconstituted vaccine. P, S, and F viruses from Wyeth Laboratories represent virus populations obtained after further attenuation of Edmonston attenuated strain of measles virus.

† TCD = tissue culture dose.

‡ PFU = plaque-forming units; read 7 days after inoculation.

TABLE 2. *Identification of measles virus* with human and monkey serum by plaque reduction*

| Serum | Dilution | Avg no. of plaques |
|---|---|---|
| Human acute† | 1:5 | 60 |
| | 1:10 | 54 |
| | 1:20 | 56 |
| | 1:40 | 52 |
| Human convalescent† | 1:5 | 0 |
| | 1:10 | 0 |
| | 1:20 | 0 |
| | 1:40 | 0 |
| None | — | 52 |
| Monkey, preinoculation‡ | 1:5 | 35 |
| | 1:10 | 34 |
| Monkey, postinoculation‡ | 1:5 | 0 |
| | 1:10 | 0 |
| | 1:20 | 0 |
| | 1:40 | 0 |
| None | — | 31 |

* Virulent strain.

† Kindly furnished by Robert Huebner, National Institutes of Health.

‡ Kindly furnished by Joseph L. Melnick, Baylor University College of Medicine.

TABLE 3. *Effect of inhibitors of DNA-containing viruses on plaque formation by measles virus**

| Compound | Concn | Titer (× $10^5$) | Inhibition |
|---|---|---|---|
| | *mg/ml* | *PFU/ml* | % |
| IUDR | 1 | 4.7 | 0 |
| | 10 | 4.7 | 0 |
| CA | 1 | 4.6 | 0 |
| | 10 | 4.5 | 0 |
| None | — | 4.0 | — |
| NMITC | 0.01 | 1.7 | 23 |
| | 0.1 | 2.7 | 0 |
| | 1 | 2.3 | 0 |
| None | — | 2.2 | — |

* Abbreviations: IUDR, 5-iodo-2′-deoxyuridine; PFU, plaque-forming units; CA, cytosine arabinoside; NMITC, isatinthiosemicarbazone.

highest concentration of the antibiotics used were toxic to the cells, and thus rendered the plaque tests invalid at these higher concentrations.

*Replication of measles virus.* When BSC-1 cells were under fluid medium, the predominant CPE produced by both the virulent and the attenuated strain of measles virus was the formation of multinucleated giant cells. Small giant cells were seen within 24 hr after inoculation of the cultures (Fig. 7 and 8). The giant cells produced in response to the virulent strain of virus were often larger (Fig. 7) than those produced by the attenuated strain (Fig. 8). The giant cells enlarged, although the cells produced in response to the attenuated strain continued to remain smaller (Fig. 9 and 10). The giant cells vacuolated, the nuclei became pyknotic, and disintegration of the cytoplasm in the area often followed (Fig. 11 and 12). It was not uncommon for a culture to convert to syncytia of few very large giant cells after inoculation with the virulent strain; giant cells formed in response to the attenuated strain remained more focal and localized.

Immunofluorescent detection of measles virus antigen yielded negative results for 18 hr after inoculation. Particulate antigen was observed in small giant cells 6 hr later in cultures infected with the virulent strain (Fig. 13). Generally, cultures infected with the attenuated strain did not yield positive results until 32 hr postinoculation. Giant cells, as they formed, contained large quantities of antigen (Fig. 14). The antigen was restricted to the cytoplasm (Fig. 13), although

TABLE 4. *Effect of antibiotics on plaque formation by measles virus*

| Antibiotic | Concn | Titer | Inhibition |
|---|---|---|---|
| | *μg/ml* | *PFU/ml** | % |
| Streptonigrin† | 0.001 | 5.2 × $10^5$ | 0 |
| | 0.010 | 4.1 × $10^5$ | 0 |
| None | — | 4.0 × $10^5$ | — |
| Actinomycin D† | 0.1 | 4.6 × $10^5$ | 8 |
| | 1 | 4.1 × $10^5$ | 18 |
| None | — | 5.0 × $10^5$ | — |
| Actinomycin D‡ | 0.1 | 3.4 × $10^4$ | 0 |
| | 1 | 6.9 × $10^4$ | 0 |
| None | — | 3.4 × $10^4$ | — |
| Mitomycin C† | 0.1 | 4.5 × $10^5$ | 8 |
| | 1 | 6.0 × $10^5$ | 0 |
| | 10 | 5.9 × $10^5$ | 0 |
| None | — | 5.0 × $10^5$ | — |
| Mitomycin C‡ | 0.1 | 3.2 × $10^4$ | 35 |
| | 1 | 3.5 × $10^4$ | 28 |
| None | — | 4.9 × $10^4$ | — |

* PFU = plaque-forming units.

† Tests carried out with the virulent strain.

‡ Tests carried out with the attenuated strain.

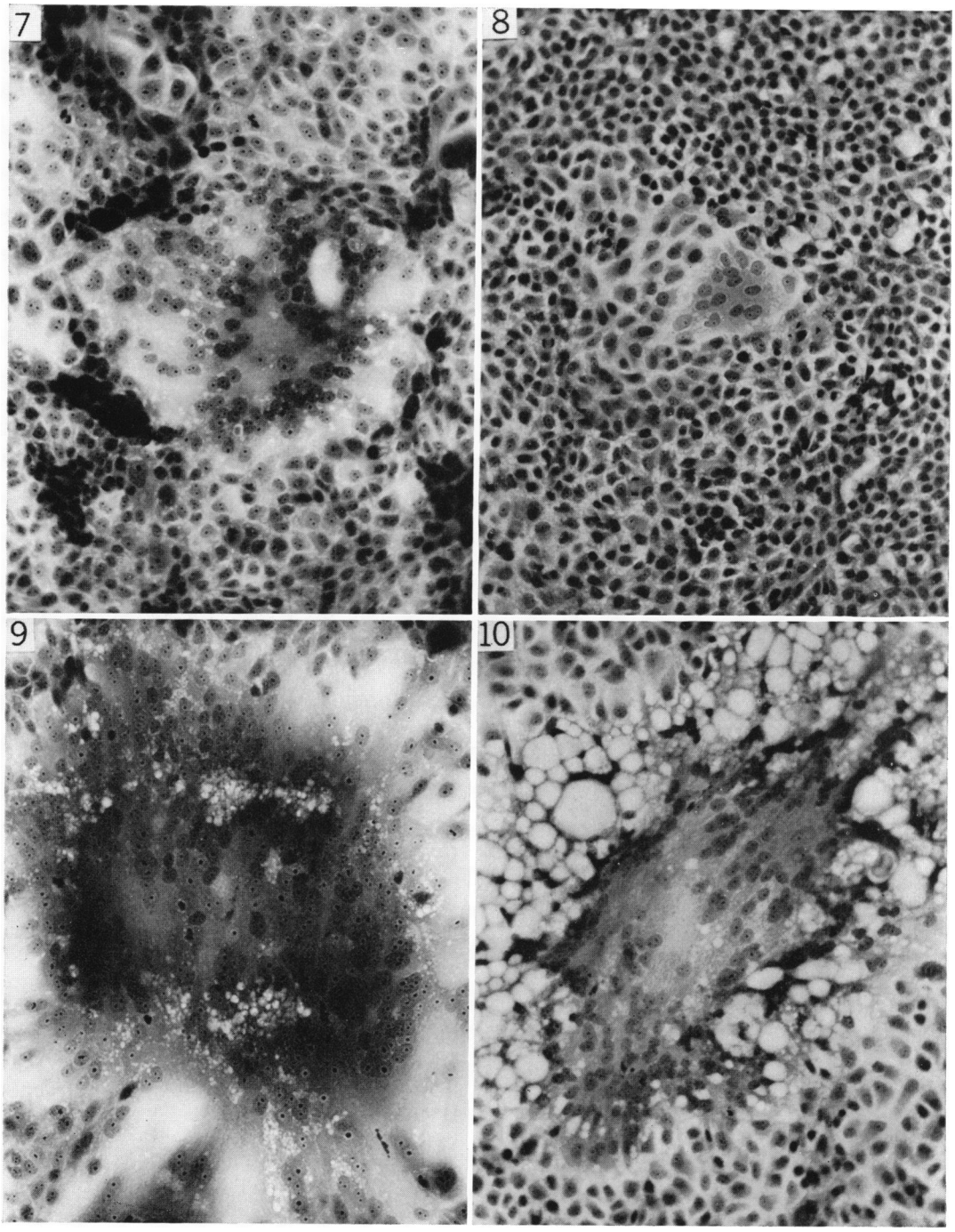


FIG. 7. *Multinucleated giant cell 24 hr after inoculation of BSC-1 cultures with the virulent strain of measles virus. Stained with hematoxylin and eosin.* × *200.*

FIG. 8. *Small, multinucleated giant cell 24 hr after inoculation of BSC-1 cultures with the attenuated strain of measles virus. Stained with hematoxylin and eosin.* × *200.*

FIG. 9. *Multinucleated giant cell 48 hr after inoculation of BSC-1 cultures with the virulent strain of measles virus. Stained with hematoxylin and eosin.* × *200.*

FIG. 10. *Multinucleated giant cell 48 hr after inoculation of BSC-1 cultures with the attenuated strain of measles virus. Stained with hematoxylin and eosin.* × *200.*

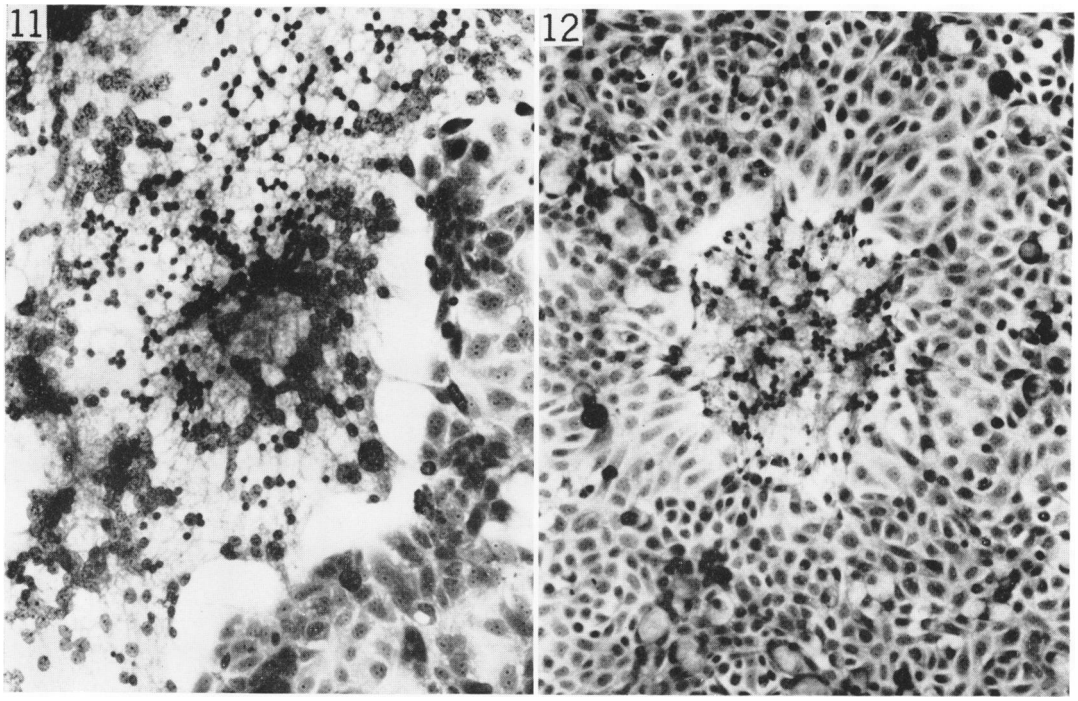

FIG. 11. *Multinucleated giant cell 48 hr after inoculation of BSC-1 cultures with the virulent strain of measles virus. Stained with hematoxylin and eosin.* × *200.*

FIG. 12. *Multinucleated giant cell 48 hr after inoculation of BSC-1 cultures with the attenuated strain of measles virus. Stained with hematoxylin and eosin.* × *200.*

occasional nuclei in the giant cells contained virus antigen.

Concurrent experiments designed to measure both the extracellular and intracellular yields of measles virus growing at 34 and 37 C in BSC-1 cells were carried out. Virus in the extracellular fluid was harvested at various intervals after inoculation of cells in bottle cultures. After washing the cells three times with tris saline, intracellular virus was harvested by two cycles of freezing and thawing of the cells. Experiments were carried out in duplicate, and the results of the plaque assays are plotted in Fig. 15 and 16. It is obvious that virus yields were similar at both temperatures tested, although somewhat higher yields were achieved at 34 C. The virulent strain required 18 to 24 hr before new virus was synthesized (Fig. 15). Virus began to be liberated into the extracellular fluids between 24 and 34 hr after inoculation of the cultures (Fig. 15). Peak titers were obtained 48 hr postinoculation; at this time, approximately 50% of the virus was cell-associated and 50% was extracellular (Fig. 15).

The attenuated virus had a somewhat longer latent period; 32 hr were required for the detection of new intracellular virus, and 34 to 48 hr elapsed before virus was detected in the extracellular fluid (Fig. 16). Maximal levels of intracellular virus were obtained 72 hr postinoculation (Fig. 16). The intracellular curves obtained with the virulent and attenuated strains were therefore similar, except for the slower growth cycle of the attenuated strain. The attenuated virus released into the extracellular fluids did not comprise more than 5% of the total virus harvest (Fig. 16). This differed from the virulent strain for which extracellular virus always represented approximately 50% of the total virus detected (Fig. 15).

## DISCUSSION

The ability of strains of measles virus to cause plaque formation in BSC-1 cells, and the added finding that virulent and attenuated strains yield plaques differing in size, suggest that this cell is well suited for the study of the properties of this virus. This is especially true because the BSC-1 cells are a stable line, and the fluctuation

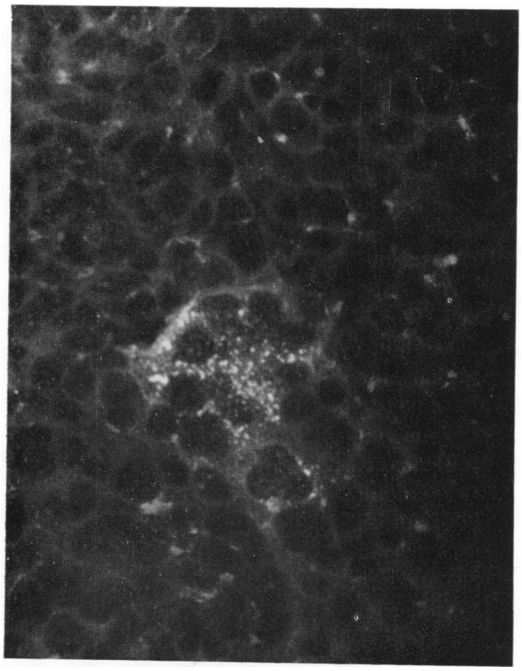

FIG. 13. *Immunofluorescent photomicrograph of BSC-1 cells inoculated 24 hr previously with the virulent strain of measles virus. Note particulate antigen in the cytoplasm.* × *400.*

in cell susceptibility encountered in the use of primary cells may be minimized. The rapidity of plaque formation (4 days) and sharpness of the plaques offer hope that genetic studies with the virus can be undertaken in this system. A previous study with primary grivet kidney cells (Buynak et al., 1962) yielded plaques with the attenuated but not virulent Edmonston strain of measles virus, although Hsiung et al. (1958) had no problem in developing a plaque assay for virulent measles virus by employing patas monkey cells. The methods and fluids used were substantially different from those used in this study; however, "comet" plaques, similar to those described in the present report, were observed in cultures inoculated with the attenuated virus (Buynak et al., 1962). Plaques of this type were also seen with other myxoviruses (Hotchin, Deibel, and Benson, 1960; Grossberg, 1964).

The formation of giant cells in response to measles virus has been attributed to absence of glutamine in the growth fluid (Reissig, Black, and Melnick, 1956) and to the genome of the virus (Seligman and Rapp, 1959; Oddo, Flaccomia, and Sinatra, 1961). Because both strains of virus used in this study are known to yield monocellular CPE in other cells in addition to syncytial formation (i.e., "strand-formation" in Hep-2 cells), it would appear that the BSC-1 cells also play a role in the effect produced by measles virus. Though this does not reduce the importance of the virus genome in regulating the effect on the host cell, it stresses the need for evaluating virus-cell interaction from all aspects. The rapidity of giant cell development supports Thomison's (1962) suggestion that the spread of virus from cell to cell is accompanied by incorporation of adjacent cells into the syncytia.

The antigens of measles virus were detected in the nuclei of Hep-2 cells (Rapp, Gordon, and Baker, 1960; Roizman and Schluederberg, 1961), human amnion cells (Rapp et al., 1960), and rhesus kidney cells (Cohen et al., 1955). Intranuclear crystallites were also observed in human amnion cells after inoculation of cultures with measles virus (Baker, Gordon, and Rapp, 1960). Failure to detect virus antigens in more than an occasional nucleus of BSC-1 cells suggests that the nucleus is not the place where viral antigens

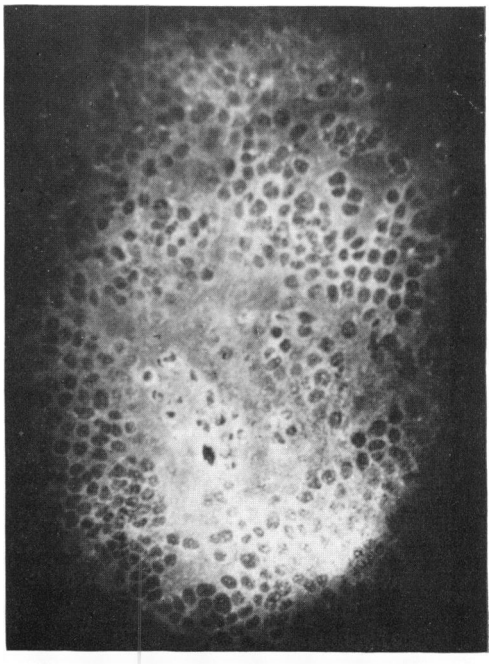

FIG. 14. *Immunofluorescent photomicrograph of BSC-1 cells inoculated 72 hr previously with the virulent strain of measles virus. Giant cell in center of field contains large quantities of virus antigen.* × *160.*

develop in the cell. However, this does not rule out the formation of viral nucleic acid or other components in the host nucleus.

Evidence presented here supports the hypothesis that measles virus is a ribonucleic acid virus. The use by other investigators of halogenated deoxyuridines to support this assumption was also recently described (Sultanian and Gordon, 1963; Lam and Atherton, 1963; Levine and Olson, 1963; St. Geme, 1964). Failure of the other antibiotics tested to inhibit measles virus is therefore not surprising, because all compounds tested interfere with normal DNA or DNA-dependent synthesis.

The growth cycle of measles virus in BSC-1 cells is similar to that reported for other cell systems. The observation that equal amounts of the virulent strain of the virus can be detected intra- and extracellularly is therefore not surprising. The finding that very little detectable infectious virus is released after infection of BSC-1 cells with the attenuated strain is, however, unusual. De Maeyer and Enders (1961) described an interferon in cell cultures infected with measles

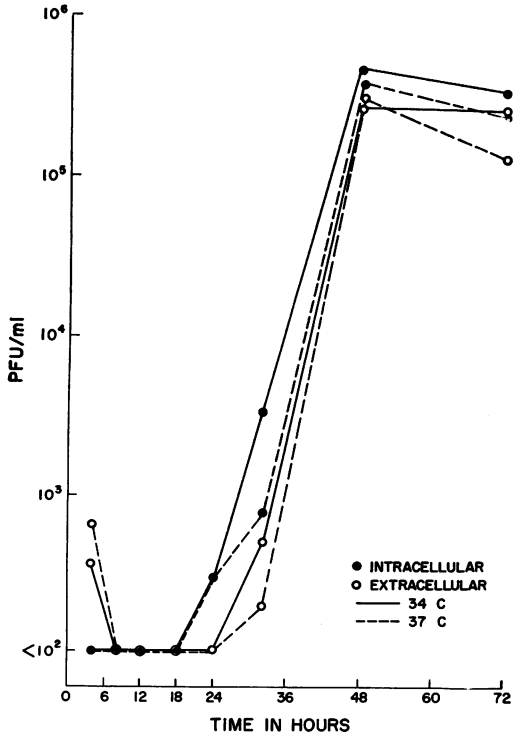


FIG. 15. *Synthesis of virulent strain of measles virus in BSC-1 cells growing at 34 and 37 C.*

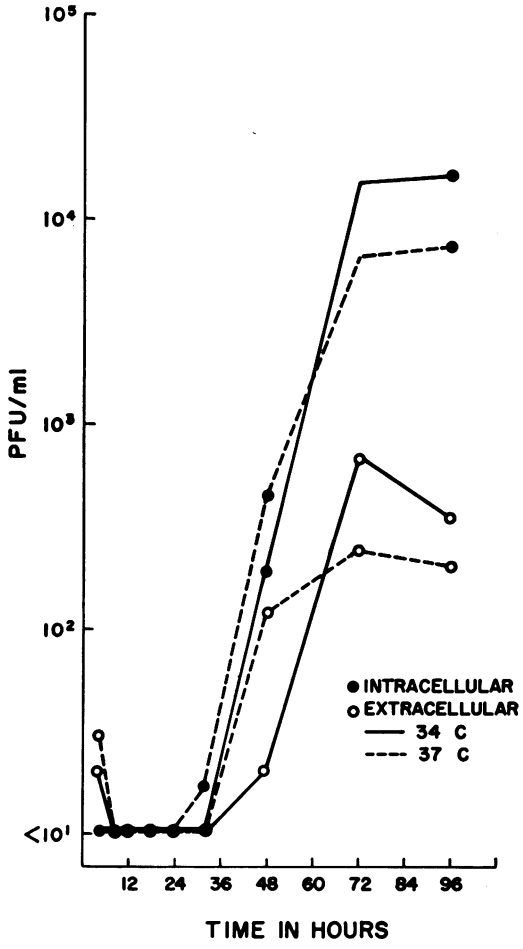


FIG. 16. *Synthesis of attenuated strain of measles virus in BSC-1 cells growing at 34 and 37 C.*

virus, and Enders (1962) suggested that production of this substance may be related to the attenuation of the virus. The production of interferon was not measured in the present study, but it would appear unlikely that interferon would depress extracellular virus yields without equal depression of intracellular virus production. If an analogous situation exists in vivo, failure of children inoculated with attenuated virus to infect susceptible contacts (Katz et al., 1960) despite active infection may be due to release of little or no virus into the respiratory tract.

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