Observations of Measles Virus Infection of Cultured Human Cells

I. A Study of Development and Spread of Virus Antigen by Means of Immunofluorescence*, §, ‡

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PLATES 17 TO 21

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ABSTRACT

The development of measles virus in cultures of both primary human amnion cells and H.Ep.-2 cells has been followed by means of the indirect fluorescent antibody technic and concurrent light and electron microscope observations. The immunofluorescence studies revealed that there is a latent period for development of demonstrable measles virus antigen. In amnion cells the latent period lasted for at least 3 days. In contrast, virus antigen could be detected in H.Ep.-2 cells as early as 12 hours following inoculation.

In each cell system virus antigen was seen in either nucleus or cytoplasm of infected cells, or both. Early localization tended to be perinuclear. Intranuclear fluorescence was generally less bright and less widespread than cytoplasmic fluorescence. Giant cells and long cytoplasmic spindle-shaped processes appeared regularly in infected cultures. Infectious virus was liberated into the nutrient fluid but when extracellular virus was inhibited by antibody, spread of infection from cell to cell in the monolayer still continued.

Results obtained in concurrent electron microscope studies will be presented separately. Correlation of the results of the immunofluorescence and electron microscope studies suggests the possibility that much of the immunofluorescence observed might be due to antigen in virus precursors or components.

INTRODUCTION

In 1954 Enders and Peebles (1) discovered that measles virus would cause consistent abnormalities in cultures of infected human or monkey kidney cells, and that sufficient virus antigen was produced so that the culture fluid would fix complement in the presence of antibody. This was soon confirmed (2); it was shown that monkey kidney tissue cultures inoculated with blood or respiratory secretions from measles patients produced antigen that would react with measles antibody not only in complement fixation tests but also in indirect fluorescent antibody tests. The sequence of appearance of antigen in different parts of the infected monkey kidney cells, however, was not followed at that time. Furthermore, there are disadvantages in the use of monkey kidney cells for demonstration of measles virus infection.

The present study was therefore undertaken to investigate the sequence of spread of intracellular measles antigen, and the mode of development of multicellular infected foci, in human amnion and human epidermoid carcinoma cells. It was found

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§ Preliminary observations were presented at annual meetings of the Society of American Bacteriologists, 1957 and 1958.

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that antigen first appeared either in the perinuclear area or in the nucleus, or both. It spread peripherally and finally occurred in the long fusiform or spindle-shaped processes that are a constant feature (3) of measles virus infection in these and other cell types.

A concurrent electron microscope study of the fine structure of infected human amnion and human epidermoid carcinoma cells was guided by these results. The electron microscope observations will be presented separately (4, 5), where they will be correlated with those reported here. The correlated findings raise the possibility that some, if not all, of the immunofluorescence seen during early stages of cellular infection is due to antigen in virus precursors or components, and not necessarily to fully mature virus.

Materials and Methods

The Edmonston strain of measles virus (1)[1] was employed. This strain had undergone 26 passages in primary human kidney cells and 8 passages in primary human amnion cells. The virus used in the studies of infected human epidermoid carcinoma No. 2 (H.Ep.-2) cells had been further passaged a variable number of times in H. Ep.-2 cells.

The amnion cell cultures were prepared by the method of Weinstein *et al.* (6), with slight modifications. Human placentas were aseptically collected in the delivery or operating rooms of the Los Angeles County General Hospital; the amniotic membrane was trypsinized, and the sedimented cells were diluted 75-fold with fluid consisting of 78 per cent medium 199, 20 per cent horse serum, and 2 per cent beef embryo ultrafiltrate. Penicillin (100 units/ml.), streptomycin (100 μg./ml.) and nystatin (mycostatin, E. R. Squibb & Sons, New York, 100 units/ml.) had been added to the fluid. The pH was then adjusted to 7.5 with $NaHCO_3$. From 0.5 to 1.0 ml. of the resulting suspension was used to initiate growth of cell sheets in screw-capped tubes. For fluorescence microscopy, the cells were dislodged from the wall of the tube with a rubber scraper, sedimented, and placed on glass slides.

H.Ep.-2 (7) cells were grown on coverslips in Leighton tubes. The coverslips had been attached to the bottom of the well with 1 per cent agar. The cells were grown in 80 per cent Eagle's basal medium[2] plus 20 per cent human or horse serum. Antibiotics were added as mentioned above.

Monolayers of both cell types were usually obtained in 3 to 5 days. At this time, the fluids were replaced with 0.8 ml. of a maintenance solution composed of 95 per cent Eagle's basal medium and 5 per cent horse serum. For virus assays and growth curves, the cell sheets were inoculated with 0.2 ml. of appropriate virus dilutions. The cells were lysed by 2 cycles of freezing and thawing for assay of infectious virus in cells.

The fluorescent antibody technic that was employed has been described (8). Controls consisted of (1) uninfected cells stained with convalescent-phase measles serum and labeled antihuman globulin, and (2) infected cells reacted with acute-phase measles serum and the labeled antiglobulin. Photomicrographs were taken with a camera coupled to a Leitz-Micro-Ibso attachment. Anscochrome film was exposed for 8 minutes and processed as recommended by the manufacturer, although it was found advantageous to develop at ASA 125. Black and white reproductions were then prepared from the color slides.

RESULTS

Human Amnion Cells.—The development of measles virus antigen in infected human amnion cells was followed by the fluorescent antibody technic. Virus antigen was not observed for 3 days following inoculation. On the 4th day specific fluorescence could be detected in either nucleus or cytoplasm, or both. Nuclear fluorescence was localized in a few small round homogeneous bodies, while cytoplasmic fluorescence was entirely perinuclear. Perinuclear fluorescence tended to be brighter than nuclear fluorescence.

Between 6 and 8 days following inoculation, generalized fluorescence developed; in many of the cells, measles antigen was distributed throughout the cytoplasm, and many nuclei contained large fluorescent inclusions (Fig. 1). While virus antigen appeared to be localized in areas of the cytoplasm of some cells (Fig. 2), other cells contained intranuclear inclusions with little antigen in the cytoplasm (Fig. 3). It is assumed that cells showing small fluorescent bodies in the nucleus or cytoplasmic virus antigen restricted to the perinuclear area (Fig. 4), are representative of earlier stages of the infection.

Correlated light and phase microscopy of living preparations showed that cytopathic changes occurred 5 to 8 days after inoculation. The changes consisted of spindle-cell and syncytial cell formation identical with those described by previous workers (9, 10) and closely resembled cytopathic manifestations of the infection in H.Ep.-2 cells (Figs. 5 to 8).

Human Epidermoid Carcinoma No. 2 Cells.—

[1] Dr. John Enders of Children's Hospital, Boston, kindly gave us the Edmonston strain of measles virus.

[2] Microbiological Associates, Bethesda, Maryland.

TABLE I

Comparison of the Spread of Measles Virus Antigen with Development of Morphologic Changes in H.Ep.-2 Cells

Time post-inoculation*	Virus titer‡		Fluorescence of cells§	Morphologic details
	Cells	Nutrient fluid		
hrs.				
0			−	Gray-blue autofluorescence.
9	1:10	1:10	−	Gray-blue autofluorescence.
12	1:10	1:10	<+	Stippled, granular yellow-green fluorescence in single cells or small groups of cells. Mostly in cytoplasm but a few nuclei infected.
18	1:10	1:10	<+	As at 12 hours. Stippled, granular. Nuclear bodies larger. Some cells beginning to "strand."
24	1:100	1:10	+ to ++	Fluorescence more diffuse. Local areas of destruction marked by stranding of cells. Fluorescence in strands. Some cells exhibit concentrations of antigen in nuclear area.
36	1:10	1:100	++	Stranding of cells prominent. Fluorescence concentrated on outer edge of strands and cells. Giant cells present exhibit round fluorescent bodies in cytoplasm and in some nuclei.
48	1:100	1:100	++ to +++	As at 36 hours but effects more widespread.
72	1:1000	1:1000	+++ to ++++	Marked destruction. Extensive flowing of cells. Giant cell nuclei contain large fluorescent bodies.

* 100 to 1000 ID_{50} of measles virus inoculated.

‡ Highest dilution of virus at which cytopathic changes could be recognized in 50 per cent of the tubes.

§ Yellow-green fluorescence. + Approximately 25 per cent of cells contain virus antigen. ++ Approximately 50 per cent of cells contain virus antigen. +++ Approximately 75 per cent of cells contain virus antigen. ++++ Approximately 100 per cent of cells contain virus antigen.

Formation of measles virus antigen in infected H.Ep.-2 cells was much more rapid than in human amnion cells; while antigen was not demonstrated in H.Ep.-2 cells prepared 9 hours after inoculation (Table I), specific fluorescence was found in widely spaced single or small groups of cells only 3 hours later (Fig. 9). The fluorescent bodies were generally coccoid and often were less than 1 μ in diameter. As in amnion cells, concentration of antigen around the nucleus was frequently observed. A few cells contained small nuclear inclusions but these were pale in comparison with cytoplasmic fluorescence.

Eighteen hours postinoculation, localization of antigen was still the same as it was 12 hours following inoculation, but nuclear inclusions were larger (Fig. 10), and the foci of fluorescence involved more cells than before. Cells whose nuclei did not exhibit inclusions usually contained the coccoid bodies seen at 12 hours (Fig. 11). At this time, a few cells were beginning to develop long cytoplasmic spindle-shaped processes or "strands," and many of the strands contained virus antigen (Fig. 12).

At the end of 24 hours, involvement of the cell sheet had increased to such an extent that 30 to 50 per cent of the cells contained virus antigen in the cytoplasm, and many cells contained intranuclear inclusions. Spindle-cell formation was prominent, and small areas of the sheet had degenerated (Fig. 13). Such areas contained large amounts of measles virus antigen although neighboring cells appeared uninfected. In some instances, single cells appeared "positive" for measles antigen. This somewhat resembles the status at 12 hours. Often the virus antigen appeared to be concentrated again in the perinuclear area (Fig.

TABLE II

Spread of Measles Virus in H. Ep.-2 Cells in Presence of Antimeasles Serum

Time after inoculation*	Antibody‡ in nutrient fluid	Virus		
		In nutrient fluid (dilution titer, tubes)		In cells (immunofluorescence)
		Exp. 1	Exp. 2	
Days				
1	Present	0	0	One or two cells, separate areas.
	Absent	Undiluted	0	As above.
2	Present	0	0	Spread obvious; "stranding" of cells.
	Absent	Undiluted	0	As above, more extensive.
3	Present	0	0	Progressive spread, larger area involved.
	Absent	>1:100	1:10	Larger, localized areas positive. Single or small groups of cells also positive (secondary spread?).
4	Present	0	0	Large groups of cells involved, giant cells prominent.
	Absent	>1:100	1:100	Larger areas beginning to merge.
5	Present	0	0	Large areas positive; some cells still without virus antigen.
	Absent	>1:100	1:1000	As above; somewhat more widespread.

* 10 ID_{50} of measles virus inoculated at 0 time.

‡ "Present" = contained 20 per cent human serum. Serum exhibited endpoint neutralization when diluted 1:16–1:32 in the presence of 100 ID_{50} of measles virus.

14). Conceivably this was a result of secondary virus spread. Infectivity titrations indicated that mature virus was present in lysed cells, although extracellular fluid contained relatively small amounts of infectious virus at this time (Table I).

At 36 hours postinoculation, the strands still contained virus antigen that was apparently concentrated at the outer edges of the strands and of the cells themselves (Fig. 15). For the first time in the sequence, some of the long processes appeared to be devoid of virus antigen. Syncytial cells had formed, however, and such multinucleated giant cells contained virus antigen in their cytoplasm as well as in some of their nuclei (Fig. 16).

Twelve hours later (48 hours postinoculation), many of the strands exhibited round protuberances with virus antigen lined up on the outer edge (Fig. 17), and many cells showed a similar concentration of virus antigen near their outer walls. Degeneration of the monolayer had become marked. Nuclear inclusions were not prominent although small intranuclear bodies were sometimes seen. Infectious virus could now be recovered from the extracellular fluids in moderate amounts (Table I).

The architecture of the cell sheet was lost within the following 24 hours (72 hours postinoculation); there was widespread destruction, with prominent stranding (Fig. 18). Localization of antigen was similar to that observed 48 hours following inoculation. Giant cells were present. They still contained virus antigen in their cytoplasm as well as in some of their nuclei (Fig. 18).

During the course of these observations, it was repeatedly noted that in involved nuclei, specific fluorescence appeared to be associated with the nucleoli. In no instance were blue-white antigen-free nucleoli seen in a nucleus containing virus inclusions that were green-yellow due to specific fluorescence. This suggests that intranuclear virus antigen is possibly associated with the nucleoli, and that the number of virus inclusions seen in the nucleus might be governed by the number of nucleoli present.

Spread of Virus Antigen in the Presence of Antimeasles Antibody.—To study further the conditions under which measles virus infection can spread from cell to cell, the effect of specific antibody was investigated. H.Ep.-2 cells on coverslips were inoculated with 10 ID_{50} of measles virus. One set

of coverslips was maintained under routine fluids. The second set was maintained under fluids containing sufficient antibody against measles virus to neutralize 600 to 1200 endpoint infective doses of virus. There was, hence, excess antibody present with respect to the amounts of extracellular virus that were liberated during virus multiplication (Table II). Inhibition by antibody of spread of the virus via extracellular fluid, however, did not prevent spread of the virus in the cell sheet; immunofluorescence microscopy revealed a sequence of widening areas of cellular involvement (Figs. 19 and 20).

The studies reported here suggested that specific immunofluorescence develops in infected cells sooner than detectable cytopathic changes occur. This observation has been repeated many times in this laboratory during this study and in other experiments with measles virus (8).

DISCUSSION

The behavior of viruses within host cells can be deduced in part by locating and identifying virus components during various stages of their developmental cycle. An early study of immunofluorescence by some of us (2) had been done in monkey kidney cells infected with measles virus. This study had shown that presumably uninfected monkey kidney cultures degenerated, and therefore, the fluorescent antibody technic was used to aid in the recognition of infected cultures. Enders (11) also found that immunofluorescence could serve this purpose. Monkey kidney cells, however, are unsuitable for investigations of the type reported here; Peebles *et al.* (12) and Ruckle (13, 14) showed that monkeys, and cell cultures derived from them, are often infected with an agent serologically indistinguishable from human measles virus, which causes cytopathic changes in monkey kidney cell cultures almost identical with those caused by human measles virus. The Edmonston strain of measles virus (1) was isolated and passaged in human cells, and is a human strain.

The fluorescent antibody procedure and electron microscopy are both especially useful for the study of the sequence of intracellular spread of viruses. Concurrent use of both technics can decrease sampling problems inherent in electron microscopy (4, 15); by means of the fluorescent antibody method it is easy to determine when generalized involvement of the host tissue has occurred. A correlation of the immunofluorescence and electron microscope observations will be presented in a subsequent report (4).

The studies reported here demonstrate that following a period of less than 12 hours virus antigen is detectable in the infected H.Ep.-2 cell. It appears probable that less than 18 to 24 hours are required for a cycle of virus multiplication. This is in accord with conclusions reached by Black (16) from the results of infectivity titrations. In our studies, the developmental cycle of measles virus in H. Ep.-2 cells was appreciably shorter than in human amnion cells and the former, therefore, are more convenient than the latter for investigations of this sort. This may be a reflection, however, of adaptation and selection during serial transfer in the H.Ep.-2 studies; the virus strain had been passaged through 20 more transfers in H.Ep.-2 cells than in amnion cells.

The apparent asynchrony of development of early virus antigen in different cells may reflect the asynchrony of the tissue culture system with respect to cell division, differences in cell susceptibility, and differences in cell metabolism. At the present time, a number of clones of H.Ep.-2 cells growing synchronously are being investigated (17).

The observation that intranuclear virus antigen appears to be associated with the region of the nucleoli deserves further study, particularly in the light of the associated electron microscope findings (4, 5). It may be that this material represents virus nucleoprotein similar to that described by Breitenfeld and Schäfer (18, 19) for fowl plague virus. At the present time, however, it is not known whether measles virus contains ribonucleic acid or deoxyribonucleic acid. Work on purified virus preparations will be necessary.

The release of infective virus into the fluid was consonant with that found by Black (16) and resembles that of some other animal viruses. In the case of measles virus, fluid titers closely followed the titer of infective virus within the cell. It seems likely that measles virus spreads from cell to cell both via nutrient fluid and directly from cell to adjacent cell. It is tempting to postulate that the latter mechanism was mediated in part by the intercellular processes, but direct confirmation of this hypothesis is lacking.

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Explanation of Plates

Plate 17

Figs. 1 to 4. Immunofluorescent micrographs of human amnion cells from a culture infected 8 days previously with measles virus. The extracellular, white areas did not exhibit specific yellow-green fluorescence; with respect to fluorescence, they are artifacts thought to be due to cellular debris. In Fig. 1 note the intranuclear inclusions and widespread fluorescence in the cytoplasm. Fig. 2 demonstrates sharp localization of antigen in cytoplasm. Note the perinuclear location of one of the two cytoplasmic concentrations of virus antigen. Fig. 3 also shows intranuclear inclusions but there is little specific fluorescence in the cytoplasm. Fig. 4 was selected to show that even in cells in which virus antigen is diffusely spread throughout the cytoplasm, the concentration of antigen, as measured by intensity of fluorescence, is greatest in the perinuclear region. Note the small intranuclear inclusions. × 380.

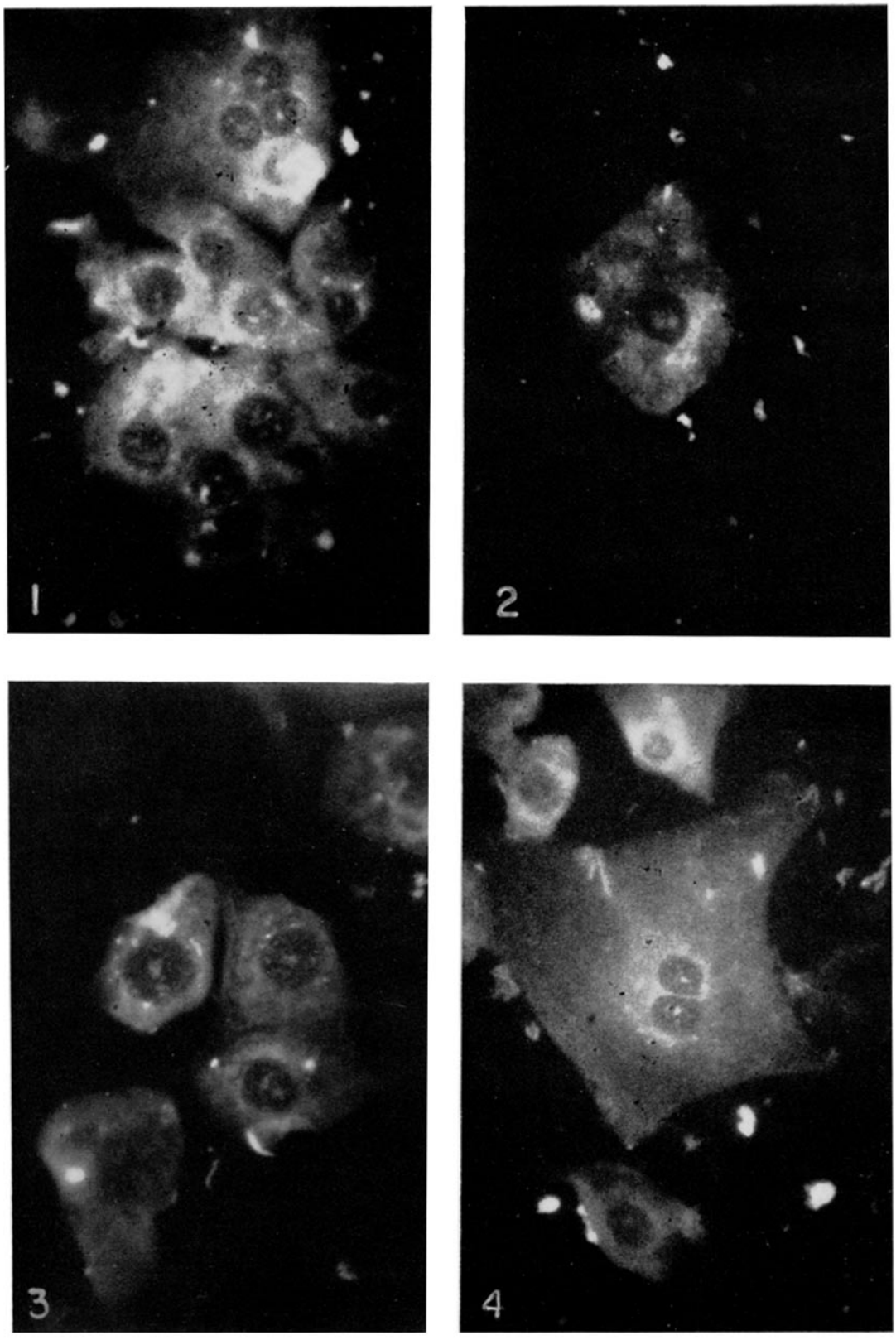

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PLATE 18

FIG. 5. Phase contrast photomicrograph of uninfected human amnion cells. × 184.

FIG. 6. Phase contrast photomicrograph of human amnion cells infected 6 days previously with measles virus. Note spindle cells and the multinucleated giant cell. × 92.

FIG. 7. Phase contrast photomicrograph of uninfected H.Ep.-2 cells. × 92.

FIG. 8. Phase contrast photomicrograph of H.Ep.-2 cells infected 48 hours previously with measles virus. Note spindle cells and the multinucleated giant cell. × 92.

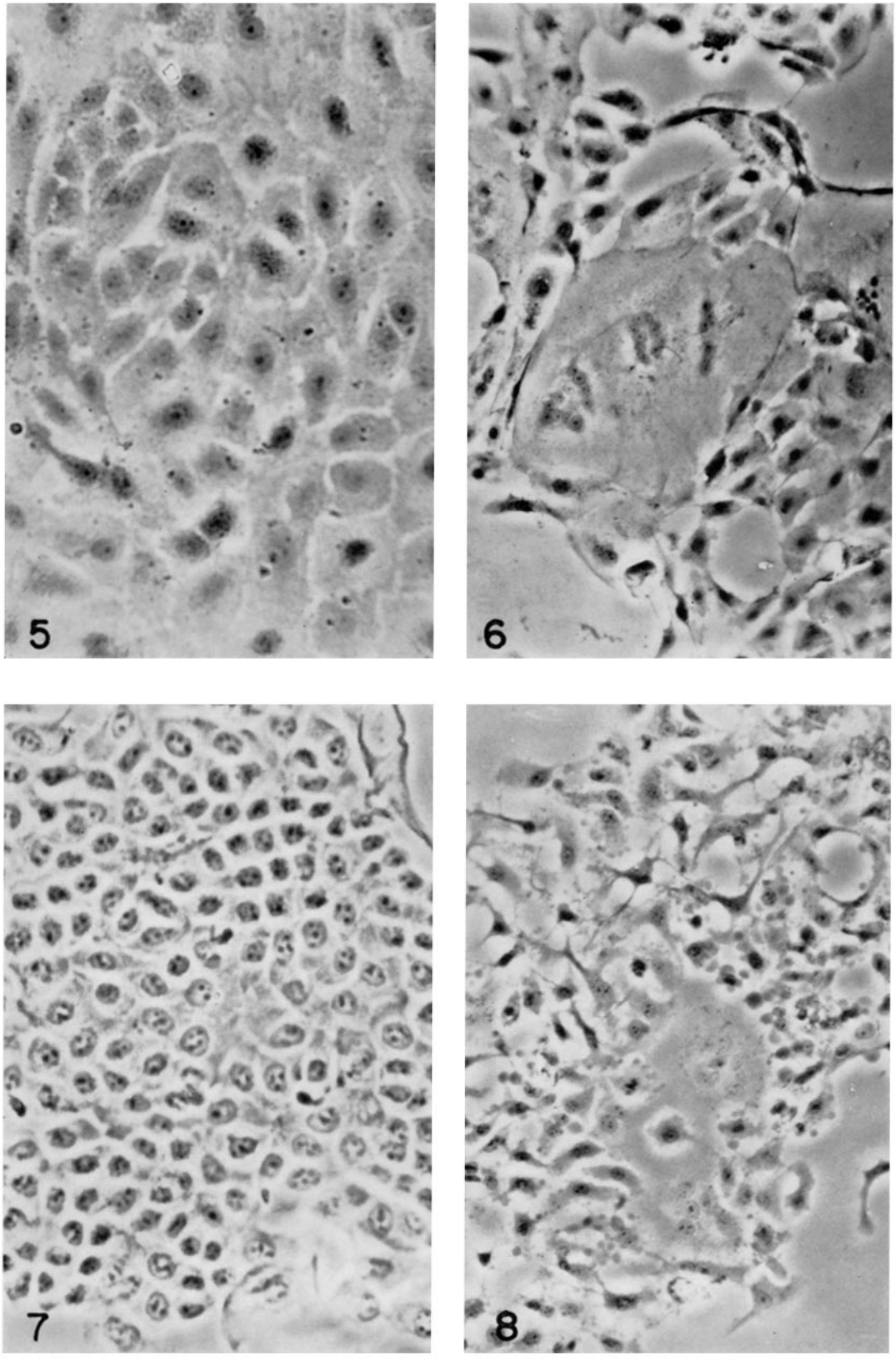

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PLATE 19

FIG. 9. Immunofluorescent micrograph of H.Ep.-2 cells infected 12 hours previously with measles virus. Virus antigen is concentrated in round bodies in the cytoplasm. Note a few small inclusions in the nuclei. × 360.

FIGS. 10 to 12. Immunofluorescent micrographs of H. Ep.-2 cells infected 18 hours previously with measles virus. In Fig. 10, large intranuclear inclusions can be seen. Cytoplasmic fluorescence is widespread although round bodies can still be observed. Fig. 11 demonstrates that antigen is concentrated in round bodies in the perinuclear areas. Note that the dark central nuclei show no specific fluorescence. Cells bordering the three infected cells appear to be uninvolved; they are visible because of the soft gray-blue autofluorescence uniformly excited in these and other cells by the band of ultraviolet radiation employed. In Fig. 12, early "stranding," presumably caused by measles virus, can be seen. Note the many long processes originating from the infected cell. This cell contains virus antigen in both nucleus and cytoplasm. × 360.

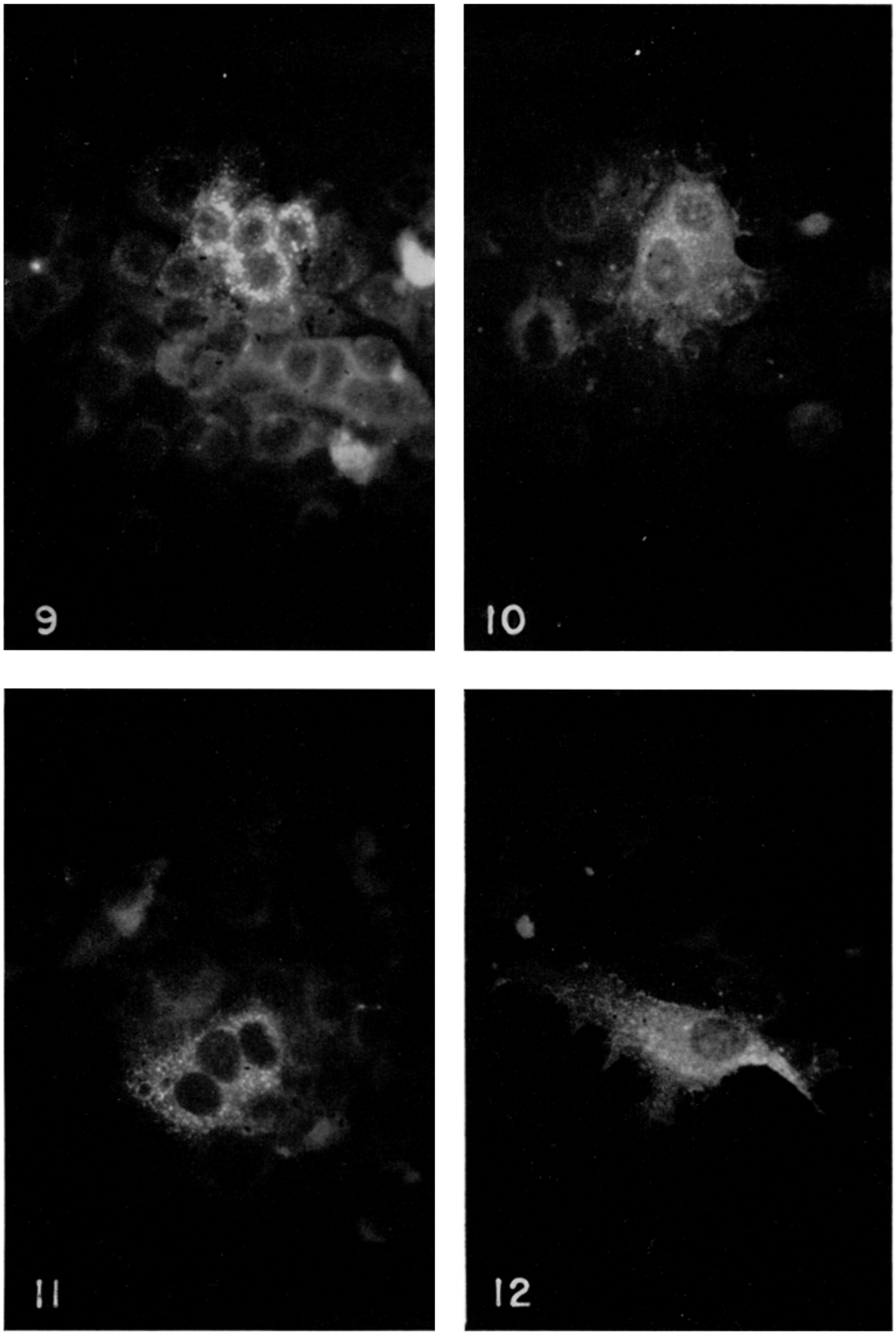

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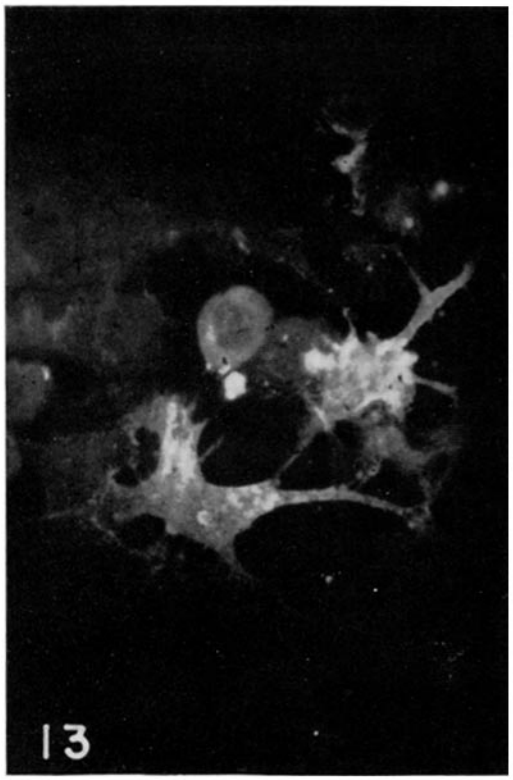
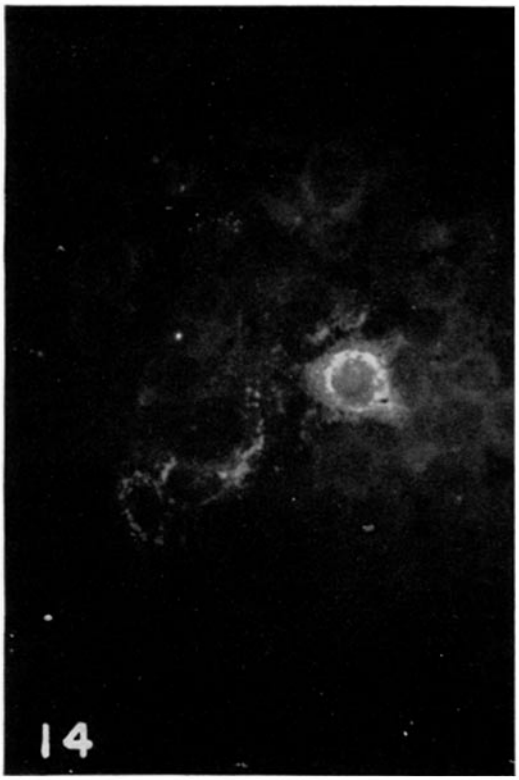
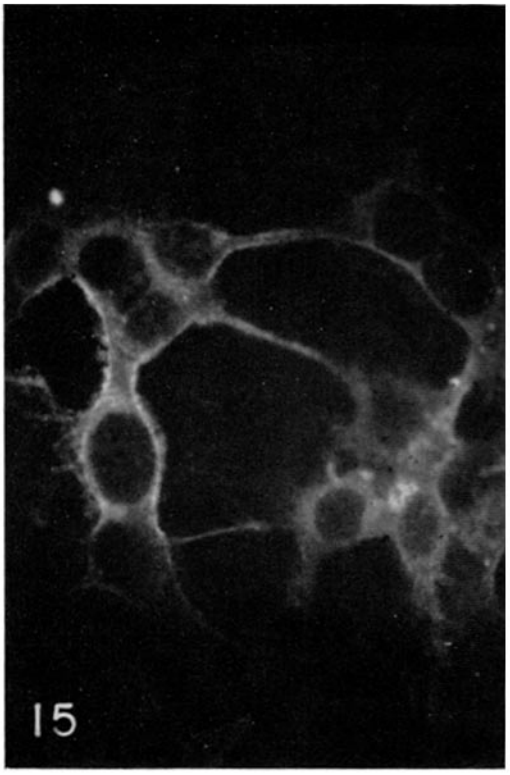
PLATE 20

FIGS. 13 and 14. Immunofluorescent micrographs of H.Ep.-2 cells infected 24 hours previously with measles virus. Fig. 13 is included to show local necrosis accompanied by spindle-cell formation. Note that the intercellular processes contain large amounts of virus antigen. In Fig. 14 perinuclear fluorescence is evident. × 360.

FIGS. 15 and 16. Immunofluorescent micrographs of H.Ep.-2 cells infected 36 hours previously with measles virus. In Fig. 15, long intercellular processes are seen to be lined with virus antigen. Fig. 16 shows a multinucleated giant cell. Cytoplasmic foci of antigen and a few intranuclear inclusions containing antigen can be seen. × 360.







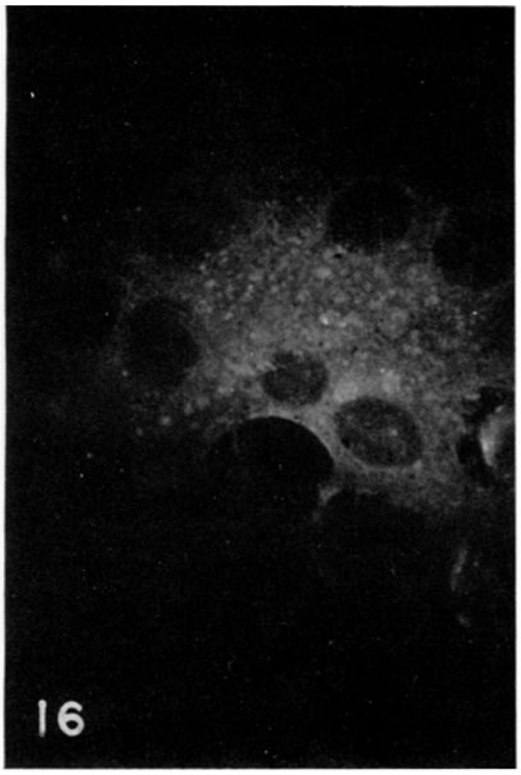


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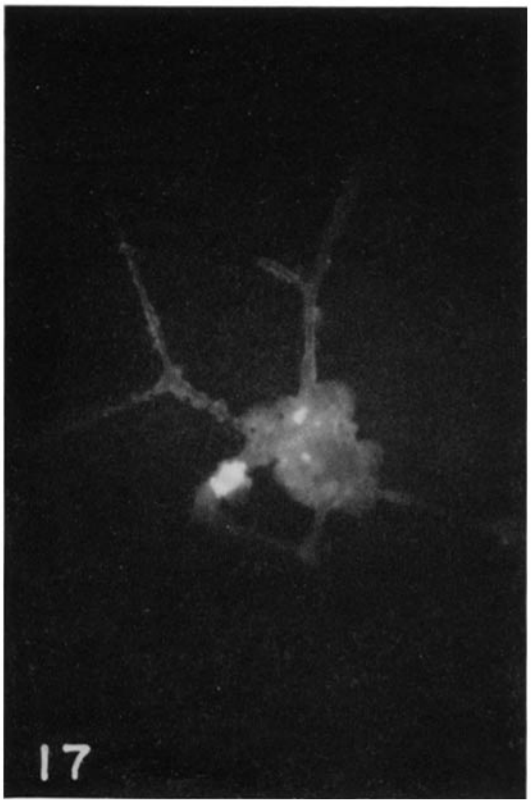
PLATE 21

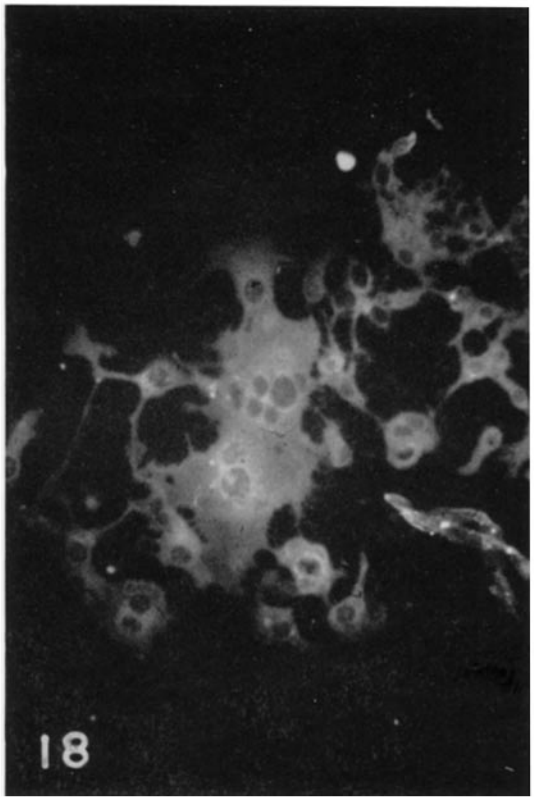
FIG. 17. Immunofluorescent micrograph of H.Ep.-2 cells infected 48 hours previously with measles virus. Intercellular processes exhibit round protuberances containing antigen. × 360.

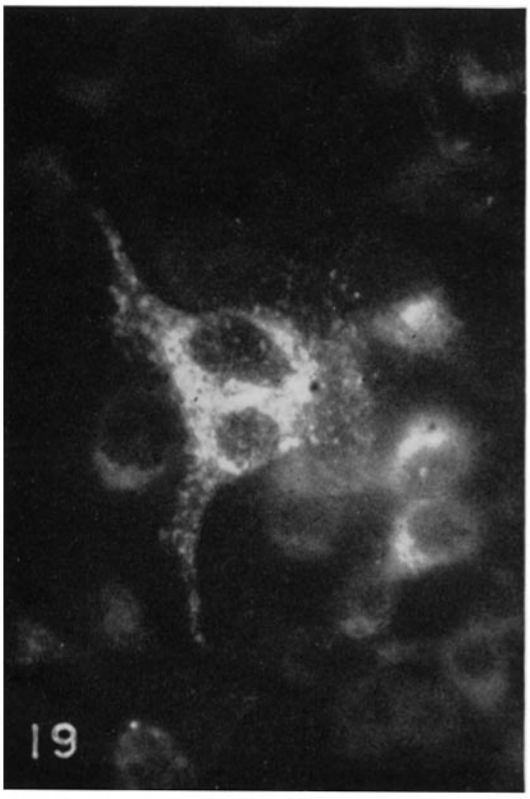
FIG. 18. Immunofluorescent micrograph of H.Ep.-2 cells infected 72 hours previously with measles virus. Note the extensive necrosis and loss of architecture of the cell monolayer. There is a giant cell in the center of the field. × 90.

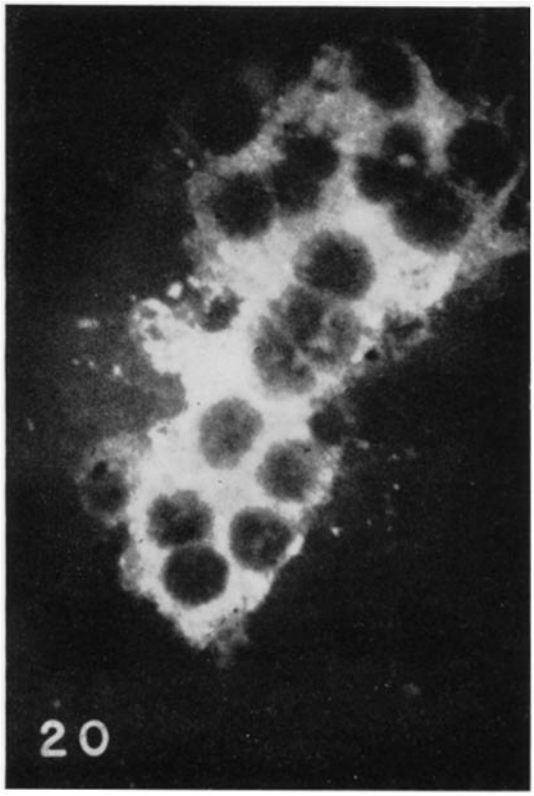
FIG. 19. Immunofluorescent micrograph of H.Ep.-2 cells infected 24 hours previously with measles virus. There was antimeasles antibody in the nutrient fluid. Note antigen in the cellular processes. × 360.

FIG. 20. Immunofluorescent micrograph of H.Ep.-2 cells infected 72 hours previously with measles virus. Localized area of involvement can be seen. Contrast the brightness of cytoplasmic fluorescence with the relatively weak intranuclear fluorescent inclusions. × 360.









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