Studies on Measles Virus in Tissue Culture

(I) Morphological Changes and Development of Virus Antigen Examined by Fluorescent Antibody Technique.

Noboru MATSUMOTO
Department of Pathology,
(Director: Prof. S. Hosokawa)
Yamaguchi University School of Medicine, Ube, Japan.
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Among the studies on the etiologic agent of measles, Goldberger and Anderson[1] made the significant contribution to demonstrate that macaque monkeys were susceptible to measles infection. In 1936, Rake and Shaffer[2] succeeded in serial propagation of the agent in chick embryos. The more important contribution to the studies of measles was done by Enders and Peebles[3]. In 1954, they isolated measles virus from the blood and throat washing of a child during the acute phase of the disease and they discovered that measles virus caused consistent morphological changes in cultures of human and monkey kidney cells. Furthermore, they succeeded in serial passage of measles virus in human kidney tissue cultures and they designated this virus as Edmonston strain, which has been employed for various studies. Since then, this agent has been introduced into cultures of certain other cells and essentially the same cytopathogenic effects have been observed[4-10].

Only recently, measles virus has been classified with myxovirus group. Nature of the virus has been gradually clarified by chemical and morphological analyses in a recent few years. However, there still remain many problems as to exact nature of inclusion body which is one of outstanding cytopathogenic effects of this virus. This study was chiefly concerned with morphological changes of the infected cells and with the nature of inclusion bodies. Correlation between morphological changes and virus multiplication was also pursued.

MATERIAL AND METHODS

1) Tissue Culture Cells

A human amnion cell line (FL cell) and green monkey cell line (VERO cell) were used. Slide cell cultures were prepared for morphological observasions and tube for virus titration. The trypsinized cells were suspended in the culture medium in a concentration of approximately 10×10^4 cells per ml.

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2) Virus

Enders' Edmonston strain of measles virus[3] was employed. The virus used in this study had been further passed at least ten times in FL and VERO cells respectively to adapt it to these cultures before it was used for morphological studies. Virus material from infected cells was concentrated by centrifugation at 5000 r.p.m. for 30 minutes.

3) Medium

For growth medium of FL cells, Earle's balanced salt solution containing 0.5 per cent lactalbumin hydrolysate (LE) with 10 per cent calf serum was used. For growth medium of VERO cells, Hanks' balanced salt solution containing 0.5 per cent lactalbumin hydrolysate (LH) with 5 per cent calf serum was employed. For maintenance medium, LE with 2 per cent calf serum was used in both cell lines. To each solution penicillin and streptomycin were added to give final concentration of 100 units and 100 micrograms respectively.

4) Infection of Tissue Culture Cells

Monolayers of both cell types were obtained in two or four days. When the monolayer was established, the fluids were replaced with 2 ml of maintenance solution containing 0.1 ml of virus suspension. This added virus had a titer of approximately 10^4TCID50/0.2 ml.

Titrations were made on ten fold serial dilution. Two tubes of cells were innoculated respectively with 0.2 ml of each diluent and 1.8 ml of maintenance medium was added to each tube. Maintenance medium war changed two or three times during the observation period. After two weeks' observation, 50 % tissue culture infectious dosis was calculated by the method of Reed and Muench.

5) Fixation and Staining

Cold ethanol and Carnoy's solution were used for the various staining procedures. For hematoxylin-eosin staining, the specimen was fixed in cold ethanol at -25°C for as least ten minutes. Feulgen reaction, methylgreen pyronine staining and acridine orange staining were performed after the slides were fixed in cold Carnoy's solution.

For acridine orange staining, 0.01 per cent acridine orange in McIlvaine's citric acid disodium phosphate buffer at pH 4.0 was employed and this buffered solution was used for washing off the excess dye. The preparations stained with acridine orange were examined with a Leitz photomicroscope equipped with an ultraviolet source.

Some specimens were examined by a phase contrast microscope without any previous fixation or staining.

6) Fluorescent Antibody Technique

Antisera were made in rabbits by repeated subcutaneous injection of 5 ml of antigenic solution. The injection was repeated three times after an interval of one

week. Two weeks after the third injection, the rabbits were bled and antisera were collected. Immune globulins prepared by repeated ammonium sulphate precipitation were conjugated to fluorescein isothiocyanate by the method of Riggs as described by Marshall et al.[11]. This conjugate was further purified by gel-filtration through a Sephadex column and by fractionation through DEAE cellulose column. The purified, conjugated immune globulin was absorbed repeatedly with VERO cells.

Slide cell cultures were washed with phosphate buffered saline and fixed in Cold acetone at $-20°C$ for at least 30 minutes. Then they were air dried and directly stained with fluorescein-labeled antibody for 12 hours at 4°C or for one hour at 37°C. Labeled antibody solution was removed and cover slips were washed with phosphate buffered saline (pH 7.2) and they were mounted on a glass slide in Elvanol mounting medium. The stained and mounted preparations were examined and photomicrographed with a Leitz photomicroscope with a dark field condenser, fitted with an ultraviolet-light source. No specific fluorescence was seen in uninfected cultures likewise stained with the same conjugated immune globulin.

RESULTS

1) General Morphological Changes of Measles Virus Infected FL and VERO cells in Routinely Stained Preparation.

Both uninfected FL and VERO cells had the epitherial character, showing closely packed pavement appearance. Most of these cells had abundant cytoplasm and almost round nuclei with one or two prominent nucleoli (Fig. 1, 2).

The morphological changes due to virus infection were essentially the same in both cell lines. The first recognizable changes occured 24 hours after the infection. The pavement like sheets were partially disrupted and the formation of characteristic syncytia or multinuclear giant cells was observed (Fig. 3). At this time, the nucleoli seemed to be somewhat enlarged.

Two or three days after the infection, eosinophilic inclusion bodies appeared both in the nucleus and cytoplasm especially in the syncytial lesion. Intranuclear inclusion bodies were almost round and homogeneous with apparent hallow around them (Fig. 4). Cytoplasmic inclusion bodies were, on the contrary, markedly irregular in size and shape, and more eosinophilic than intranuclear inclusions (Fig. 5). As the infection proceeded, the number and the size of cytoplasmic inclusion bodies gradually increased. Intranuclear inclusion bodies were also increased in their size and number, occasionally filling the whole nuclear region (Fig. 6).

The third type of cytopathogenic effect consisted in spindle cell tsansformation. Uninfected FL and VERO cells were both polygonal in shape and never showed spindle-like appearance. However, at relatively late stage of infection. some of the cells became fusiform of stellate somewhat resembling fibroblasts (Fig. 7). In

more advanced stage, much of the cell population became involved with increased cytoplasmic eosinophilia (Fig. 8). About two weeks after infection, these changes terminated in complete cellular destruction.

Although inclusion bodies in the nucleus and cytoplasm were negative for Feulgen reaction, with Unna-Pappenheim's stain pyroninophilic materials were occasionally found in the cytoplasm, which might be identical to cytoplasmic inclusions. In more advanced stage of infection, the cytoplasm showed increased pyroninophilia, especially in the spindle shaped cells.

2) Acridine Orange Staining

Both FL and VERO cells showed almost the same changes in acridine orange stained preparations. In control cells, the cytoplasm exhibited flame-red or orange fluorescence, while the nucleus, which contains deoxyribonucleic acid, revealed brilliant green-yellow fluorescence (Fig. 9).

In the early stage of infection, the nucleoli were slightly enlarged exhibiting brilliant orange color. In the cytoplasm, there was an increase in red to orange fluorescence especially in the syncytial lesion (Fig. 10).

In more advanced stage, intranuclear inclusion bodies became prominent, which were recognized as decreased fluorescent areas in yellowish green nuclear matrix (Fig. 11, 12). In these nuclei, brilliant yellow stained clumping of chromatin was revealed near the nuclear membrane. Cytoplasmic inclusions, on the contrary, were hardly visible because of increased fluorecence of the whole cytoplasm. However, irregularly shaped, deep orange fluorescent areas were occasionally found in the cytoplasm (Fig. 13). After removing acridine orange with 40 per cent ethanol, the preparation was restained with hematoxylin and eosin. Decreased sites of green fluorescence in the nucleus and deep orange fluorescent areas in the cytoplasm were identified as intranuclear and cytoplasmic inclusion bodies respectively.

In the later stage of infection, intranuclear inclusion bodies became more prominent with margination of chromatin, but cytoplasmic ones were hardly distinguishable. In most cell, the cytoplasm developed more brilliant reddish orange fluorescence sometimes showing reticular appearance. In some cells, characteristic findings were obtained. The cellular membrane revealed markedly strong orange fluorescence and a number of small cytoplasmic processes with deep reddish orange fluorescence were recognized at the cell membrane (Fig. 14). This finding might be closely related to viral maturation and release at the cellular surface.

3) Phase Contrast Microscopy

Infected VERO cells observed by phase contrast microscope revealed enlargement of the nucleoli at early stage of infection (Fig. 15). In more advanced stage, formation of syncytia with increased granularity of the cytoplasm (Fig. 16) and appearance of spindle shaped cells became prominent, Although cytoplasmic inclusion bodies could not be detected because of abundant granules and small va-

cuoles in the cytoplasm, intranuclear ones were clearly visible which appeared as homogeneous dark arears with distinct clear zone around them (Fig. 17). In some cells, the nucleoli were remarkably enlarged and deformed, suggesting the participation of the nucleoli to the formation of intranuclear inclusion (Fig. 18).

4) Fluorescent Antibody Staining

The development of measles virus antigen in infected VERO cells was followed by means of direct fluorescent antibody technique. As early as 12 hours after innoculation, the cytoplasm of a new cells began to fluoresce faintly and 24 hours later cytoplasmic fluorescence tended to be accumulated around the nucleus (Fig. 19). Most of the nucleoli revealed faint fluorescence but the nuclear matrix did not develope specific fluorescence.

Between 3 and 5 days following innoculation, measles antigen was distributed throughout the cytoplasm of most cells (Fig. 20). At this time fluorescent inclusions were occasionally recognized in the cytoplasm. Multinucleated giant cells also contained virus antigen in the perinuclear region (Fig. 21). In spindle shaped cells, the cytoplasmic processes developed strong fluorescence and some processes exhibited round protuberances with abundant virus antigen (Fig. 22). At the advanced stage of infection, most of the cells contained measles virus antigen in their cytoplasm and distinct fluorescent inclusions were frequently visible in the cytoplasm (Fig. 23). Cytoplasmic fluorescence showed the tendency to be concentrated at the outer surface of the cell membrane (Flg. 24).

During the course of these observations, it was noted that no specific fluorescence which corresponded to the intranuclear inclusion bodies was recognized, though most of the nucleoli exhibited faint fluorescence. Uninfected control cultures, which were likewise stained, revealed no specific fluorescence.

DISCUSSION

Since Enders and Peebles[3)] first succeeded in isolation of measles virus in tissue cultures of human and monkey renal cells from the blood and throat washings of the patients in the acute stage of the disease, several reports as to the isolation of the agent of measles have appeared[12-15)]. In Japan, Mutai isolated four strains of measles virus in monkey kidney cells in 1959[16)]. Toyoshima and his associates also succeeded in isolation of measles virus from throat washings of the patients using FL cells, and they found that there was no immunological and cytopathogenical differences between Enders' Edmonston strain and Toyoshima strain[17)]. Although it has been possible for these ten years to grow measles virus not only in tissue cultures but also in experimental animals[18-21)], information as to the nature of measles virus particles has been scanty. Cooper[22)] provisionally classified the virus with DNA viruse measuring about 120-150 millimicrone in diameter. Toyoshima et al., on the contrary, suggested that measles virus might be RNA virus from

the result of histochemical studies of virus infected cells[23]. Waterson et al.[24] studied the fine structure of measles virus particle by electron microscope and suggested that it might be related to the large myxoviruses. Nowadays, measles virus is involved with large myxovirus group (Newcastle disease virus group) on the basis of chemical and structural properties of the particle.[25,26]

Morphological changes of measles virus infected cells in vitro were first described by Enders and Peebles[3] and they pointed out that cytopathogenic effect consisted of the formation of syncytial giant cells with accumulation of an acidophilic substance in the nuclei. A few years later, Enders and his associates[27] summarized the infection with measles virus in vitro and reported that the formation of syncytia, appearance of inclusion bodies both in the nucleus and cytoplasm and development of spindle-shaped cells resembling fibroblast were the major morphological changes. Thereafter, similar cytopathogenic effects have been reported in various tissue culture systems[4-10]. In this experiment, almost the same morphological changes were obtained in both FL and VERO cells. Enlargement of the nucleoli and slightly increased granularity of the cytoplasm were the first detectable changes. In a few days following infection, formation of multinuclear giant cells with accompanying cleft of the cell sheet was observed. Such giant cells have been observed in the epithelium of respiratory system and in the lymphoid tissue in autopsy and experimental cases of measles[28], and these giant cells observed in vivo has been commonly designated as Warthin-Finkeldey's cell. As to confirm whether giant cells are formed by the amitotic division or by the fusion of the infected cells, Aoyama[29] observed the process of the formation of the giant cells using special culture chamber and he concluded that the giant cells were formed by fusion of the infected cell. Though the author did not tried time-lapse observation, frequent clefts at the vicinity of newly formed giant cells might be morphological evidence which suggests that these cells are formed by fusion of the individual cell and not by the amitotic division.

Inclusion bodies do not always mean the aggregate of the viral particles in the cytoplasm or in the nucleus. It only designates the small bodies, either acidophilic or basophilic, which appear in the cytoplasm or in the nucleus, sometimes in both of them, of the virus infected cells. There are many reports concerning the inclusion bodies of measles virus infected cells, but the exact nature of them are not identical. With H-E stained preparation, eosinophilic inclusion bodies were easily detectable both in the nucleus and cytoplasm at advanced stage of infection. Someone describes that the inclusions appear in the cytoplasm at first and a while later intranuclear ones become apparent[16,23,29]. But Milovanovic et al.[7] reported that the inclusion bodies were found almost at the same time both in the nucleus and cytoplasm. In this experiment, small eosinophilic inclusions were recognized both in the nucleus and cytoplasm about two days after innoculation. As was

reported by Aoyama[29], cytoplasmic inclusions showed the tendency to appear in the perinuclear zone at first and to gather into large ones. Intranuclear inclusions were prominent, especially in the syncytial lesion. Gradually these inclusions increased in their size and number. Though intranuclear inclusion was rather homogeneously stained, cytoplasmic ones, on the contrary, showed fine reticular network in the inclusion matrix.

The results of histochemical staining of inclusion bodies were almost similar to those reported by several authors[23,29,30]. Inclusion bodies both in the nucleus and cytoplasm showed negative Feulgen reaction, but they revealed slight pyroninophilia with Unna-Pappenheim's staining. With acridine orange staining, more characteristic findings were obtained. The fluorochrome, acridine orange, is of great importance nowadays and is commonly employed as a sensitive cytochemical indicator for the detection and identification of nucleic acid. When suitably fixed tissue or virus preparation are stained with acridine orange and viewed by blue-violet light, the components containing deoxyribonucleic acid fluoresce a brilliant yellow-green while those containing ribonucleic acid exhibit a flame-red fluorescence[31]. When syncytial giant cells became prominent, intranuclear inclusion bodies were recognized as decreased fluorescent area in the yellow green nuclear matrix. This would indicate that intranuclear inclusion does not contain ribonucleic acid. The cytoplasm of the infected cells fluoresced more brilliant reddish orange than was recognized in the control cells, which would be indicative of increased ribonucleic acid probably due to virus multiplication. Cytoplasmic inclusions were not so distinct because of increased cytoplasmic fluorescence, but they were occasionally visible as irregularly shaped brilliant red masses characteristic of ribonucleic acid.

To confirm whether the inclusion bodies are composed of viral particles or they are only secondary degenerative changes caused by virus infection, the fluorescent antibody technique is very helpful. In the early stage, cytoplasmic fluorescence was entirely perinuclear and as infection proceeded, the whole cytoplasm gradually developed specific fluorescence. However, no specific fluorescence was recognized in the nucleus even at the advanced stage of infection when intranuclear inclusion bodies were easily detectable in H–E stained preparation. Therefore, it will be assumed that intranuclear inclusions do not contain any viral antigen and viral antigen is only localized in the cytoplasm. Fluorescent inclusions, which might be identical with cytoplasmic inclusions observed in H–E staining, were recognized at the advanced stage of infection. Rapp et al.[32] studied the development of viral antigen by means of immunofluorescence and they described that early localization of viral antigen tended to be perinuclear and later specific fluorescence could be detectable in either the nucleus or the cytoplasm, or in both of them. Cohen et al.[12] and Toyoshima and his associates[23] also reported that

viral antigen was noted in the nucleus as well as in the cytoplasm. However, they could not find out any relation between intranuclear inclusion bodies and virus antigen and they also pointed out that large cytoplasmic inclusion was not the site of accumulation of viral antigen. Recent knowledge about myxovirus reveals that virus synthesis occurs in the cytoplasm and virus particles mature at the cell surface[26,33,34]. Therefore, it seems likely that neither intranuclear nor cytoplasmic inclusion bodies contain mature virus particles and that these inclusion might be an aggregate of immature virus particles or viral nucleic acid. As to clarify the nature of inclusion bodies more precisely, electron microscopic observation will be presented in a subsequent report[35].

When measles virus was first isolated in cultured cells, cytopathogenic effects consisted in the formation of syncytia and appearance of inclusion bodies. A few years later, Enders et al.[7,27] found that after prolonged cultivation in vitro cytopathogenic properties of measles virus became altered. As passage of the virus in successive cultures of amnion cells was continued, transformation to spindle-shaped cells tended to predominate over formation of syncytia. By neutralization and complement fixation tests, they proved that spindle cell transformation represented another expression of cytopathogenicity of measles virus.

Seligman and Rapp[36] also reported the same phenomenon in human amnion cultures and succeeded in isolation of "giant cell" strain and "spindle cell" strain. In this experiment, after at least ten passages in VERO cells a number of fusiform or stellate cells were noted in addition to presence of syncytial giant cells. In spindle shaped cells intranuclear inclusion was often found and with acridine orange staining, cytoplasmic processes exhibited brilliant reddish-orange fluorescence. Specific fluorescence was also revealed in the cytoplasmic processes by fluorescens antibody technique. There is no doubt to assume that the spindle-shaped cells also participate in replication of measles virus.

There are several reports concerning the growth characteristics of measles virus[8,37,38], but the data on the relation between viral multiplication and morphological changes have been relatively incomplete. Enlargement of the nucleolus at the early stage of infection may be more significant than has been estimated before. Brilliant flame red fluorescence of the nucleolus stained with acridine orange suggests increased nucleolar RNA activity and possible participation of the nucleoli in the synthesis of viral nucleic acid. Toyoshima et al. pointed out that the enlarged nucleoli seem to be correlated with virus reproduction[23]. By fluorescent antibody technique, faint fluorescence was recognized in most of the nucleoli throughout the course of infection just as Rapp et al.[32] suggested the possible association of intranuclear viral antigen with the nucleoli. To clarify the exact role of the nucleolus in virus multiplication, further chemical and morphological studies will be necessary.

The nucleocapsid of subgroup II of myxovirus is regarded to be synthesized in the cytoplasm. But mature virus particles have never been found in the cytoplasm as they mature only at the cell membrane acquring an envelop which is derived from the cell membrane. Finally they are released from the host cell by a direct process of "budding". These morphological changes of the host cell membrane are easily visible by electron microscopic observation[35]. Even in acridine orange stained preparations, characteristic findings which suggest viral maturation at the cell membrane was obtained. In advanced stage of infection, an increared intensity in RNA fluorescence was noted at the cell periphery and characteristic cytoplasmic processes with strong RNA fluorescence was readily visible, which might be indicative of maturation and release of measles virus particles.

SUMMARY

The cytopathogenic effects of measles virus (Edomonston strain) were examined using FL and VERO cells by standard light microscope, phase contrast microscope, acridine orange staining and fluorescent antibody technique. The morphological changes of the infected cells were essentially similar to those observed by several authors in various cell cultures.

Enlargement of the nucleoli and formation of syncytial giant cells were outstanding changes at the early stage of infection. Enlarged nucleoli developed brilliant flame red fluorescence in acridine orange stained preparation, suggesting increased RNA activity and possible participation of them in the synthesis of viral nucleic acid.

Early localization of virus antigen tended to be perinuclear and specific fluorescence was gradually distributed throughout the cytoplasm as infection progressed.

In more advanced stage, inclusion bodies appeared either in the nuclei or in the cytoplasm, or in both of them. Intranuclear inclusion bodies were almost round and eosinophilic with relatively homogeneous inclusion matrix. As they showed negative RNA or DNA staining and did not develope any specific fluorescence by direct fluorescent antibody technique, it would be difficult to assume their participation in viral reproduction. Cytoplasmic inclusion bodies, on the contrary, were remarkably large and irregularly shaped and revealed positive RNA staining, and some of them contained measles virus antigen. So, they are not the secondary degenerative changes due to infection but may have close relation to viral reproduction.

At the late stage of infection, small cytoplasmic processes with strong reddish orange fluorescence, characteristic of ribonucleic acid, was observed with acridine orange staining. Virus antigen showed the tendency to be concentrated at the outer surface of the infected cells.

From these findings, it may be assumed that viral nucleic acid (RNA) or virus

precursor are synthesized chiefly in the cytoplasm and maturation and release of virus particle take place at the cell membrane.

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REFERENCES

1) J. Goldberger and J. F. Anderson: An experimental demonstration of the presence of the virus of measles in the mixed buccal and nasal secretions. *J. A. M. A.* **57**: 476, 1911.
2) G. Rake and M. F. Shaffer: Propagation of the agent of measles in fertile hens' egg. *Nature,* **144**: 672, 1936
3) J. F. Enders and J. C. Peebles: Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc. Soc. Exper. Biol. and Med.,* **86**: 277, 1954
4) F. Dekking and K. McCarthy: Propagation of measles virus in human carcinoma cells. *Proc. Soc. Exper. Biol. and Med.,* **93**: 1, 1956
5) F. L. Black, M. Reissig and J. L. Melnick: Propagation of measles virus in a strain of human epidermoid cancer cell (HEp-2). *Proc. Soc. Exper. Biol. and. Med.,* **93**: 107, 1956
6) A. J. Girardi et al.: Growth and CF antigenicity of measles virus in cells derived from human heart. *Proc. Soc. Exper. Biol.* and *Med.,* **98**: 18, 1958
7) M. V. Milovanovic, J. F. Enders and A. Mitus: Cultivation of measles virus in human amnion cells and developing chick embryo. *Proc. Soc. Exper. Biol. and Med.,* **95**: 120, 1957
8) G. E. Underwood: Studies on measles virus in tissue culture. I) Growth rate in various cells and development of plaque assay. *J. Immunol.,* **83**: 198, 1959
9) K. Toyoshima et al.: Virological studies on measles virus. II) Growth of Toyoshima strain in four established cell lines. *Biken's J,* **2**: 313, 1959
10) R. B. Berg and M. S. Rosenthal: Propagation of measles virus in suspensions of human and monkey leukocytes. *Proc. Soc. Exper. Biol. and Med.,* **106**: 581, 1961
11) J. D. Marshall, W. C. Eveland and C. W. Smith: Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technique with a modification of its application. *Proc. Soc. Exper. Biol. and Med.,* **98**: 898, 1958
12) S. M. Cohen et al.: Fluorescent antibody and complement-fixation test of agents isolated in tissue culture from measles patients. *Proc. Soc. Exper. Biol. and Med.,* **90**: 118, 1955
13) G. Ruckle: Studies with measles virus. I) Propagation in different culture systems. *J. Immunol.,* **78**: 330, 1957
14) G. Ruckle: Studies with measles virus. II) Isolation of virus and immunologic studies in persons who have had the natural disease. *J. Immunol.,* **78**: 341, 1957
15) V. Bech and P. von Magnus: Studies on measles virus in monkey kidney tissue cultures. I) Isolation of virus from 5 patients with measles. *Acta Path. Microbiol. Scand.,* **42**: 75, 1958
16) M. Mutai: Isolation and identification of measles virus. *Japan. J. Exper. Med.,* **29**: 283, 1959
17) K. Toyoshima et al.: Virological studies on measles virus. I) Isolation of measles virus using FL cells and immunological properties of the isolated agents. *Biken's J.,* **2**: 305, 1959
18) J. Brunstein, J. H. Jensen and B. H. Waksman: The development of a neurotropic strain of measles virus in hamster and mice. *J. Inf. Dis.,* **114**: 265, 1964

19) H. Gordon and H. J. Knighton: Experimental measles.: The lymphoid tissue of animals innoculated with the virus of human measles. *Am. J. Path.*, **17**: 165, 1941
20) T. Taniguchi et al.: Animal experiments of measles on monkeys. *Med. J. Osaka Univ.*, **4**: 381, 1954
21) T. Taniguchi et al.: Pathology of monkeys experimentally infected with measles virus. *Med. J. Osaka Univ.*, **5**: 367, 1954
22) P. D. Cooper: A chemical basis for the classification of animal viruses. *Nature*, **190**: 302, 1961
23) K. Toyoshima et al.: Virological studies on measles virus. III) Morphological changes and virus growth in FL cells. *Biken's J.*, **3**: 241, 1960
24) A. P. Waterson et al.: The nature of measles virus. *Virology*, **15**: 379, 1961
25) M. Green: Chemistry and structure of animal virus particles. *Am. J. Med.*, **38**: 651, 1965
26) J. G. Cruickshank: The structure of myxovirus and its biological significance. In: *Cellular Biology of Myxovirus Infections*, pp. 5-21. Edited by G. E. W. Wolstenholme and J. Knight, Ciba Foundation Symposium. 1964. J. & A. Churchill Ltd.
27) J. F. Enders et al.: Measles virus; A summary of experiments concerned with isolation, properties and behavior. *Am. J. Pub. Health*, **47**: 275, 1957
28) F. E. Sherman and G. Ruckle: In vivo and in vitro cellular changes specific for measles. *A. M. A. Arch. Path.*, **65**: 587, 1958
29) Y. Aoyama: Changes of cultured cells infected with measles virus. *Japan. J. Exper. Med.*, **29**: 535, 1959
30) B. Roizman and A. E. Schluederberg: Virus infection of cells in mitosis. III) Cytology of mitotic and amitotic HEp-2 cells infected with measles virus. *J. Nat. Cancer Inst.*, **28**: 35, 1962
31) H. D. Mayer: The nucleic acids of virus as revealed by their reactions with fluorochrome acridine orange. *Int. Rev. Exper. Path.*, **2**: 1, 1963
32) F. Rapp, I. Gordon and R. F. Baker: Observation of measles virus infection of cultured human cells. I) A study of development and spread of virus antigen by means of immunofluorescence. *J. Biophysic. Biochem. Cytol.*, **7**: 43, 1960
33) I. Tamm and H. J. Eggers: Biochemistry of virus reproduction. *Am. J. Med.*, **38**: 678, 1965
34) H. A. Blough: Role of the surface state in the development of myxoviruses. In: *Cellular Biology of Myxovirus Infections*, pp. 120-143. Edited by G. E. W. Wolstenholme and J. Knight, Ciba Foundation Symposium. 1964. J. & A. Churchill Ltd.
35) N. Matsumoto: Studies on measles virus in tissue culture. II) Electron microscopic studies of measles virus infected cells and localization of virus antigen examined by ferritin conjugated antibody technique. *Bull. Yamaguchi Med. School*, **13**: 167, 1966.
36) S. J. Seligman and F. Rapp: A variant measles virus in which giant cell formation appears to be genetically determined. Virology, 9: 143, 1959
37) F. L. Black: Growth and stability of measles virus. *Virology*, **7**: 184, 1959
38) K. Toyoshima et al.: Virological studies on measles virus. IV) The effect of active and inactivated measles virus on cultured cells. *Biken's J.*, **3**: 281, 1960

EXPLANATION OF PLATE 1.

Fig. 1. Control VERO cells. H. E. ×400

Fig. 2. Control FL cells. H. E. ×400

Fig. 3. VERO cells two days after infection. Note the characteristic giant cells with numerous nuclei. H. E. ×100

Fig. 4. VERO cells four days after infection. In the nuclei, homogeneously stained round inclusion bodies with thin clear zone around them are seen. Deeply stained inclusion with distinct hallo is also visible. H. E. ×400

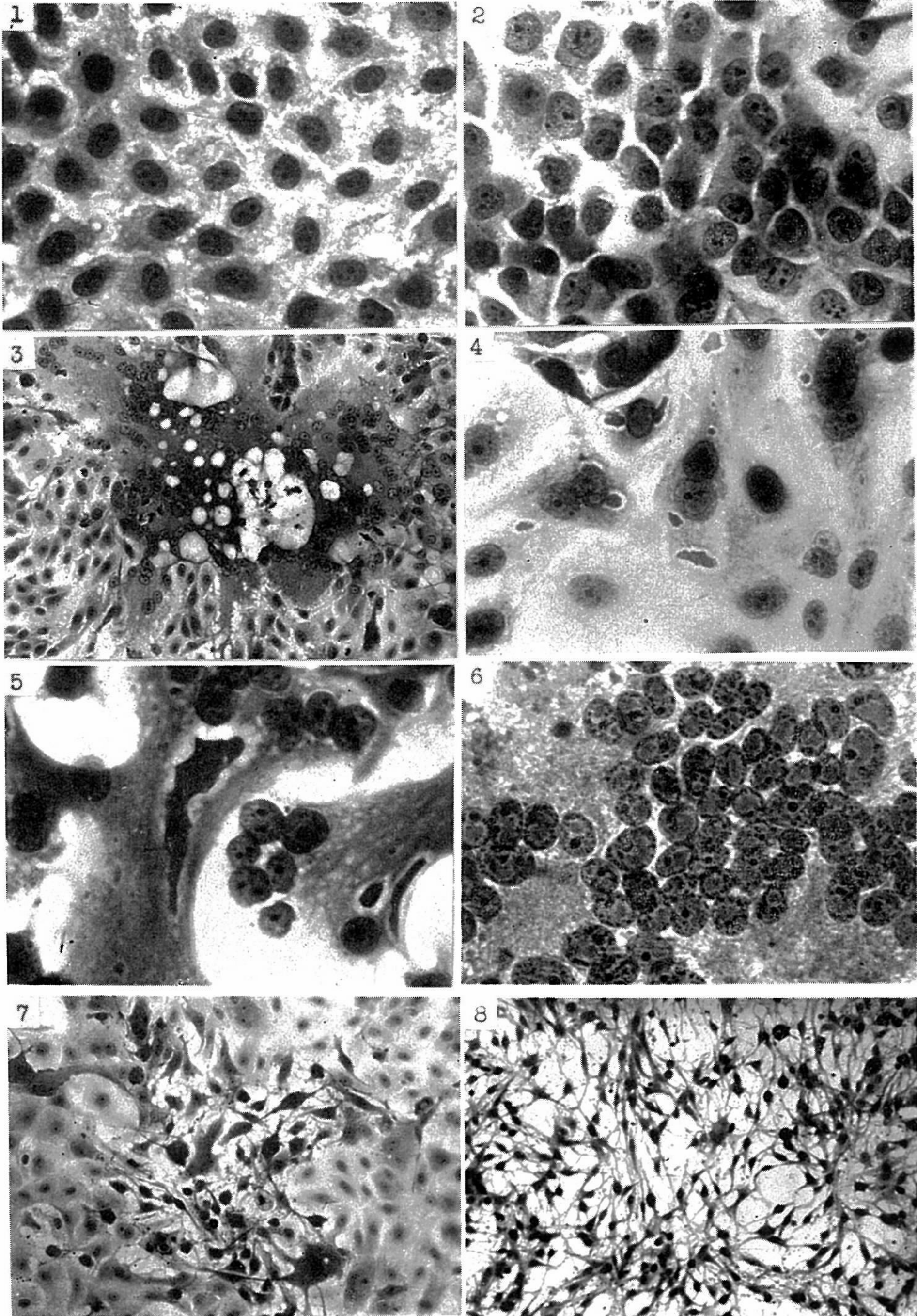
Fig. 5. FL cells 5 days after infection. Note irregularly shaped, deeply eosinophilic cytoplasmic inclusions. H. E. ×400

Fig. 6. VERO cells 7 days after infection. In the syncytial lesion, a lot of large inclusion bodies are clearly visible in most of the nucleus. H. E. ×400

Fig. 7. VERO cells 6 days after infection. Early stage of spindle cell transformation is seen in the center. Note increased cytoplasmic eosinophilia of spindle-shaped cells. H. E. ×100

Fig. 8. VERO cells 8 days after infection. Most cells are transformed to spindle-shaped cells resembling fibroblasts. H. E. ×100

PLATE 1



EXPLANATION OF PLATE 2.

Fig. 9. Control VERO cells. Mitotic figure is visible at the lower left. Acridine orange staining. ×400

Fig. 10. VERO cells 3 days after infection. Syncytial giant cells with somewhat increased cytoplasmic fluorescence. The nucleoli are slightly enlarged and exhibit strong fluorescence. Acridine orange staining. ×400

Fig. 11. VERO cells 4 days after infection. Intranuclear inclusion bodies which appear as decreased fluorescent areas. Some cells show spindle-cell transformation. Acridine orange staining. ×400

Fig. 12. Syncytial VERO cell 5 days after infection. Large intranuclear inclusion bodies which appear as decreased fluorescent areas. Note margination of chromatin and enlargement of the nucleoli. Acridine orange staining. ×400

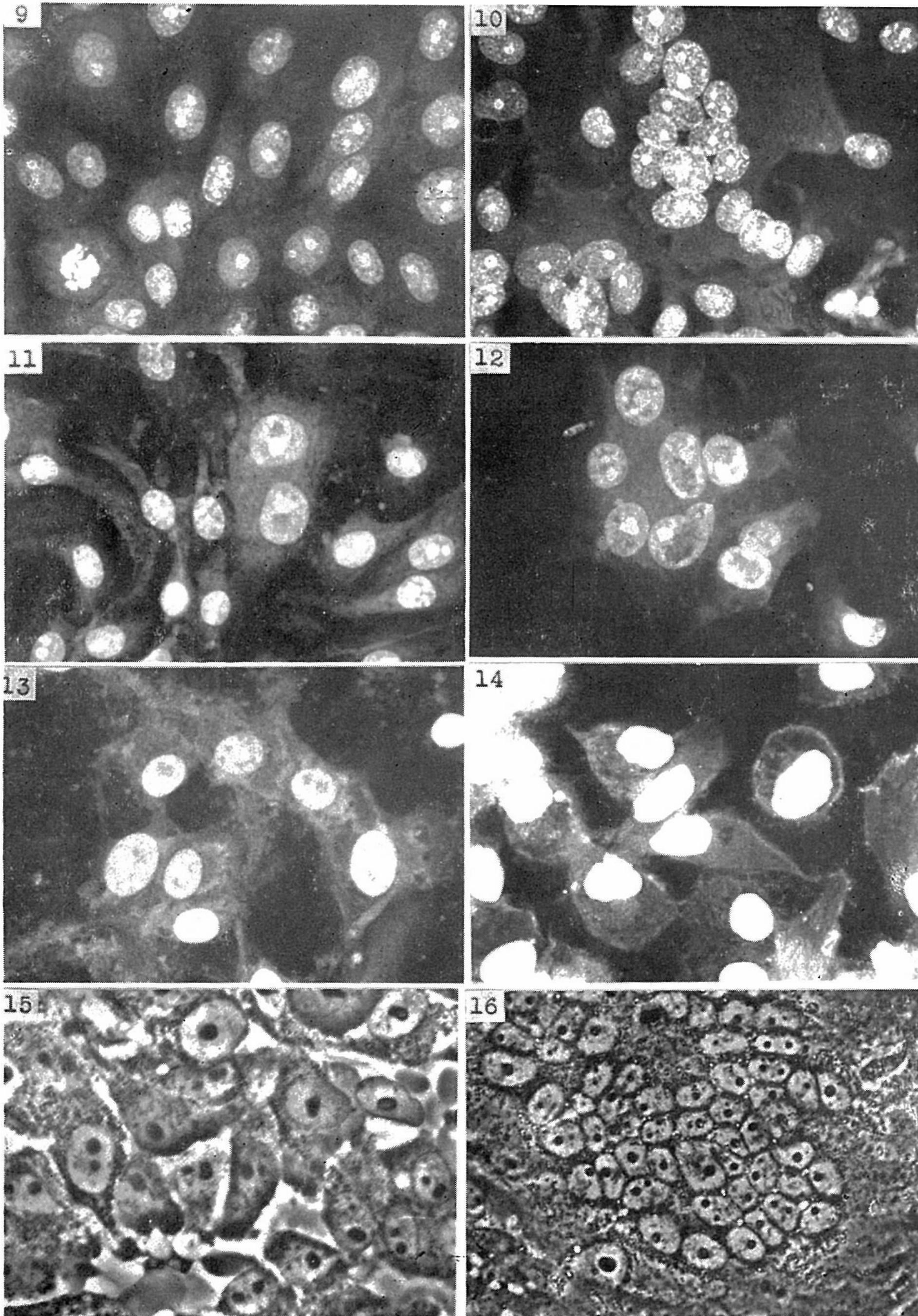
Fig. 13. VERO cells 7 days after infection. The cytoplasm shows increased reddish orange fluorescence and some cells contain irregularly shaped, deep fluorescent areas. Acridine orange staining. ×400

Fig. 14. FL cells 7 days after infection. The cell membrane with characteristic small processes is brilliantly stained for RNA. Acridine orange staining. ×400

Fig. 15. VERO cells 24 hours after infection. Note enlargement of the nucleoli. Phase contrast microscope. ×400

Fig. 16. VERO cells 3 days after infection. Multinuclear giant cell with increased cytoplasmic granules. Phase contrast microscope. ×400

PLATE 2



EXPLANATION OF PLATE 3.

Fig. 17. VERO cells 4 days after infection. Intranuclear inclusion bodies are seen as homogeneously dark areas with clear zone around them. Phase contrast microscope. ×400

Fig. 18. VERO cells 5 days after infection. The nucleoli are remarkably enlarged suggesting their relation to the formation of nuclear inclusion bodies. Phase contrast microscope. ×1000

Fig. 19. VERO cells 2 days after infection. Viral antigen is visible at the perinuclear area. The nucleoli reveals faint fluorescence. Fluorescent antibody technique. ×400

Fig. 20. VERO cells 4 days after infection. The cytoplasm exhibit strong fluorescence. No specific fluorescence is visible in the nuclei except in the nucleoli. Fluorescent antibody technique. ×400

Fig. 21. VERO cells 3 days after infection. Syncytial giant cell also contains viral antigen around the nucleus. Fluorescent antibody technique. ×400

Fig. 22. VERO cells 6 days after infection. Spindle-shaped cell which contains viral antigen in the cytoplasmic processes. Fluorescent antibody technique. ×400

Fig. 23. VERO cells 6 days after infection. The cytoplasm of small syncytial cell exhibits strong fluorecsence and fluorescent inclusion is visible at the lower left. No viral antigen is found in the nuclei. Fluorescent antibody technique. ×400

Fig. 24. VERO cells 7 days after infection. At the late stage, viral antigen is concentrated at the outer surface of the cells. Fluorescent antibody technique. ×400

PLATE 3