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IMMUNOFLUORESCENT STUDIES ON THE LOCALIZATION OF VIRAL STRUCTURAL COMPONENTS AND SURFACE ANTIGEN IN FL CELLS INFECTED WITH CHIKUNGUNYA VIRUS

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SUMMARY The intra-cellular localization of viral structural components of Chikungunya virus (ChV) and the viral specific surface antigens of FL cells infected with ChV were studied with the direct fluorescent antibody technique using rabbit antisera against purified-ChV (anti-V), ChV-hemagglutinin (anti-HA) and the nucleoprotein core of ChV (anti-X) and mouse antiserum against infectious ChV (anti-V-M).

1) The HA antigen of ChV, which contains one of the structural proteins of the virus, is localized exclusively in a certain perinuclear site in the cytoplasm, corresponding to the position of V antigen. 2) The X antigen, the nucleoprotein core of ChV, is diffusely distributed throughout the cytoplasm. 3) Viral specific surface antigen was observed around the cell margin with anti-V, anti-HA and anti-V-M globulin conjugated with fluorescein-isothiocyanate, but not with anti-X globulin conjugate. These fluorescent cells with surface antigen appeared at almost the same rate as cells with HA or V antigen observed after fixation.

INTRODUCTION

Two major structural proteins of purified Chikungunya virus (ChV) were found by polyacrylamide gel electrophoresis. One was associated with the hemagglutinin (HA) and the other with the nucleoprotein core of ChV, formerly named X (Igarashi and Fukai, 1969; Igarashi et al., 1970 a). The serological properties of rabbit anti-sera against these structural components of ChV were also analysed (Igarashi et al., 1970 b).

Chikungunya virus-specific antigens were previously demonstrated by fluorescence (Mantani et al., 1967; Hahon and Hankins, 1970).

In the present experiments rabbit antisera against purified ChV (anti-V), against HA (anti-HA) and against X (anti-X) and mouse antiserum against infectious ChV (anti-V-M) were used to demonstrate the intracellular distributions of the antigens of ChV and ChV-specific surface antigens by the direct immunofluorescence method.

Fluorescent antibodies have already been used to study the antigens in several enveloped RNA viruses, the S and V antigens of influenza

(Liu, 1955) and fowl plague viruses (Breitenfeld and Schäfer, 1957), G or NP and HA antigens of Newcastle disease virus (Reda et al., 1964) and S and V antigens of Sendai virus (Zhdanov et al., 1965).

Virus-specific surface antigens of various viruses have already been demonstrated by immunofluorescence (Tevethia et al., 1965; Irlin, 1967; Miyamoto and Kato, 1968; Ueda et al., 1969; Chen and Purchase, 1970; Klein et al., 1970; Meyer and Birg, 1970; Hahon, 1970) and by immunohemadsorption (Fagraeus and Espmark 1961; Miyamoto and Kato 1968).

But with regard to arboviruses, there is only one report of the surface antigen induced by Venezuelan equine encephalomyelitis virus (Hahon, 1970).

MATERIALS AND METHODS

1. *Virus*

Chikungunya virus (ChV), African strain was obtained from Dr. S. Ahandrik of the Virus Research Institute, Bangkok, Thailand. The stock of seed virus was prepared as described before (Mantani, et al., 1967).

2. *Cells*

The host cell for virus growth was a human amnion cell line (FL). FL cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum.

3. *Infection of cells*

FL cells were grown in Leighton tubes with cover slips. The cells were used after 2 days incubation at 37 C. Virus was allowed to become adsorbed for 1 or 2 hr at 37 C and then maintenance medium (MEM with 2% calf serum) was added.

4. *Assay of infectivity of virus*

Assay was performed by the plaque technique in an established cell line of green monkey kidney cells (BSCl) essentially as described previously (Igarashi and Tuchinda, 1967). BSCl cells was kindly given by Dr. S. Nii of this Institute.

5. *Preparation of anti-serum*

Four kinds of anti-sera were used: 1) rabbit antiserum against purified, formalin-inactivated ChV (anti-V), 2) rabbit antiserum against hemagglutinin of ChV (anti-HA), 3) rabbit antiserum against the nucleoprotein core of ChV after formalin treatment (anti-X) and 4) mouse antiserum against ChV (antiV-M). The procedures for preparation of anti-V, anti-HA and anti-X and of anti-V-M were described previously (Igarashi et al., 1970 a, b; Mantani et al., 1967).

6. *Preparation of fluorescene conjugated antibody*

The procedures used were described previously (Mantani et al., 1967). The staining titers of the anti-V, anti-HA, anti-X and anti-V-M globulin conjugates were 1 : 8, 1 : 8, 1 : 16 and 1 : 8, respectively.

7. *Staining procedures with fluorescent antibody*

The direct fluorescent antibody method was used throughout to demonstrate immuno-fluorescence of V, HA and X antigens and antigen stained with anti-V-M serum (infectious virus antigen) in infected FL cells. Infected cells on coverslips were washed with Hank's solution. Then some of each sample was immediately exposed to each anti-globulin conjugated with fluorescent dye at 37 C for 1 hr, to study the surface antigens of FL cells infected with ChV. The rest of the sample was dried and fixed with acetone at 4 C for 15 min. Then samples were exposed to each anti-globulin conjugated with dye at 37 C for 1 hr. The details of the procedures were described previously (Mantani et al., 1967). After observation of fluorescent cells, the preparations were post-stained with Giemsa solution. More than 500 cells in each sample were examined at random and the percentage of fluorescent cells was calculated.

8. *Block test for surface antigen of cells infected with ChV*

Infected cells were removed from Leighton tubes, and immediately overlayed with one of the four kinds of antiserum (anti-V, anti-HA, anti-X and anti-V-M) as the first antiserum. After 30 min contact at 37 C in a vapor saturated chamber, each sample was divided into four parts, which was overlayed with one of the four kinds of anti-

globulin conjugate, as the second antiserum, and incubated for 30 min at 37 C. The preparations were washed thoroughly with Hank's solution and covered with a coverslip with a drop of mounting medium. For observation, fluorescence was generated by a 250 W high pressure mercury vapor lamp (Nikon Optical Co.). These procedures were described previously (Mantani et al., 1967).

RESULTS

1. *Topography of V, HA and X antigens*

In experiments at high multiplicity (500 moi) of ChV with an adsorption period of 2 hr, V antigens were clearly observed 5 hr after infection in the cytoplasm with anti-V globulin conjugate. All the V antigens were localized exclusively in an area round the nucleus (Fig. 3).

In experiments with anti-HA and anti-V–M globulin conjugates, the antigens were also localized exclusively round the nucleus as in experiments with the anti-V globulin conjugate (Fig. 4 and 5). These findings were consistent with previous observations (Mantani et al., 1967).

On the other hand with the anti-X globulin conjugate, the whole cytoplasm of infected cells became filled with bright fluorescent granules of X antigen, between 5 and 11 hr after infection (Fig. 6 and 7).

The number of these fluorescent cells increased rapidly. The kinetics of the appearance of fluorescent cells are shown in Fig. 1. The curves of V, HA and infections V antigen increased at the same rate till 11 hr after infection while the increase of X antigen seemed to be slightly slower.

For more precise analysis of the delay in the appearance of X antigen, an experiment was made at low-multiplicity with an adsorption period of 1 hr. At the same time the surface antigens were studied with the anti-V globulin conjugate.

As shown in Fig. 2, the percentages of cells with V antigens increased gradually till 21 hr after infection. The percentages of cells with X antigens increased gradually, but the increase appeared 2 or 3 hr later than that of V-antigens.

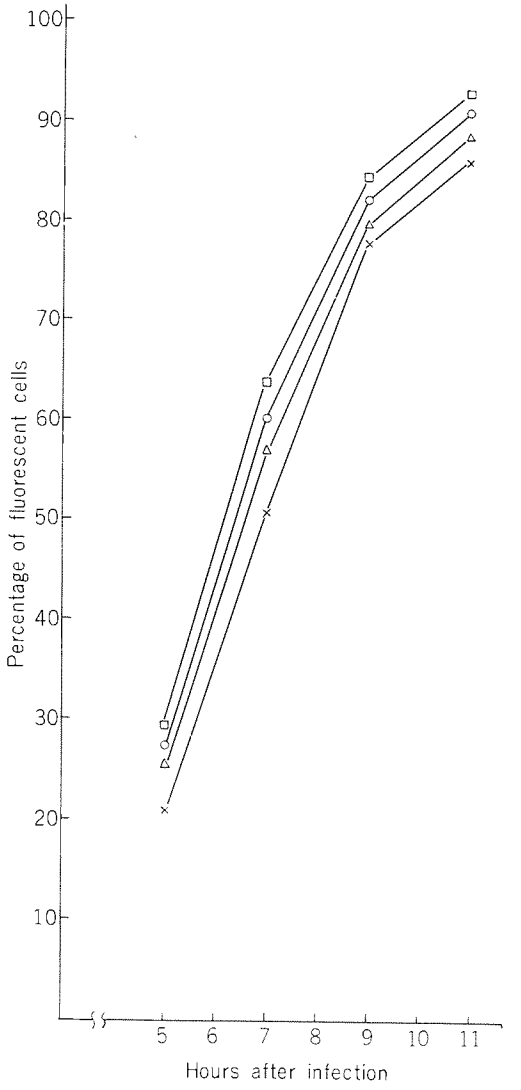


FIGURE 1. *Percentages of fluorescent cells after fixation, stained with various anti-γ globulin conjugates at high multiplicity of infection (500 moi).*
○: *Anti-V*, △: *anti-HA*, ×: *anti-X*, □: *anti-V-M*.
The percentage of fluorescent cells stained with the anti-X globulin conjugate is slightly the lowest.

2. *Surface antigens of FL cells infected with ChV*

The percentages of cells with surface antigens, which was stained with the anti-V globulin conjugate without fixation, increased at the same rate as that of cells with V antigen, as shown in Fig. 2. Twenty-one hr after infection, infected cells were stained with anti-V, anti-HA, anti-X and anti-V–M globulin conjugates without fixation to demonstrate the surface antigen. At the same time block tests were carried out with anti-V, anti-HA, anti-X and anti-V–M sera.

As shown in Table 1, the percentages of fluorescent cells stained with anti-V, anti-HA and anti-V–M globulin conjugates without fixation were almost the same 21 hr after infection. Bright fluorescence was seen around the cell margin, as shown in Fig. 8, 9, and 10. But no fluorescent cells were seen when infected cells were stained with anti-X globulin conjugate without fixation.

Bright fluorescence around the margin of cells stained with anti-V, anti-HA and anti-V–M globulin conjugates was not inhibited by preincubation of the sample with anti-X rabbit serum. But anti-V, anti-HA and anti-V–M sera inhibited the fluorescence of infected cells stained with any of the anti-globulin conjugates without fixation.

3. *Degeneration of FL cells infected with ChV*

Fig. 11 and 12 show results on the same sample of FL cells infected with ChV, 21 hr after infection. The fluorescent cells in Fig. 11 stained with the anti-X globulin conjugate are stained with Giemsa in Fig. 12. There are no inclusion bodies. Several picnotic cells still have bright fluorescence at a late stage of infection. The number of these karyolytic cells increased with time after infection (Fig. 13 and 14).

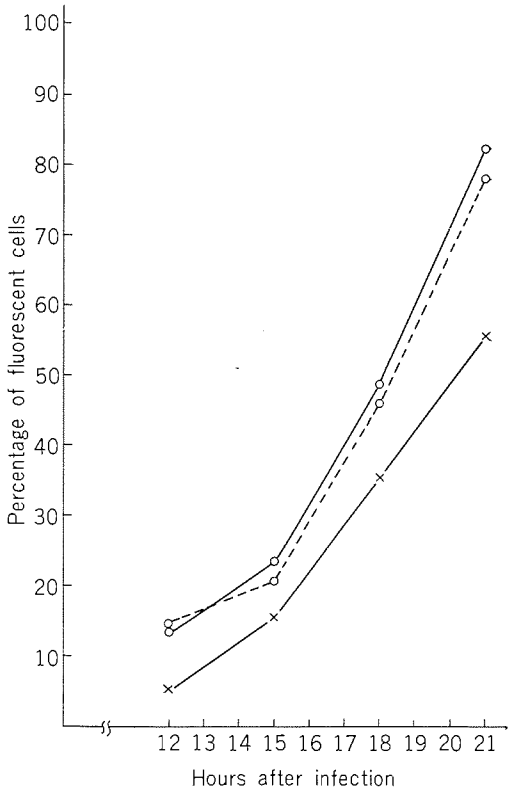


FIGURE 2. *Percentages of fluorescent cells with anti-V and anti-X globulin conjugates with or without fixation at low multiplicity (5 moi).*

○———○: *fixed cells with anti-V*

○- - - -○: *unfixed cells with anti-V*

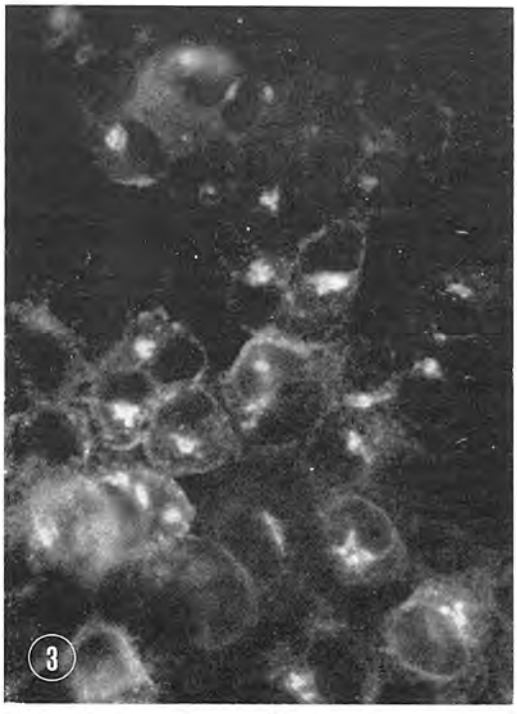
×———×: *fixed cells with anti-X*

No fluorescent cells were seen when infected cells were stained with the anti-X globulin conjugate without fixation. X antigen appeared 2 or 3 hr later than V antigen and surface antigen.

TABLE 1. *Inhibition of surface antigen of FL cells infected with ChV by homologous and heterologous anti-sera*

Second anti-globulin conjugate[a]	Percentage of fluorescent cells	First antiserum[a]			
		anti HA	anti V	anti X	anti V-M
anti HA	++[b] 70%	−[e]	−	+[c]	±[d]
anti V	++ 77.5	−	−	+	±
anti X	− 0	−	−	−	−
anti V-M	++ 81	±	±	+	−

a For details of procedure see text.
b bright fluorescence.
c clear fluorescence.
d obscure.
e no fluorescence.



FIGURES 3, 4, 5, 6 and 7. *Various antigens in fixed FL cells 21 hr after infection with ChV (5 moi).*

FIGURE 3. *Stained with anti-V rabbit globulin conjugate.*

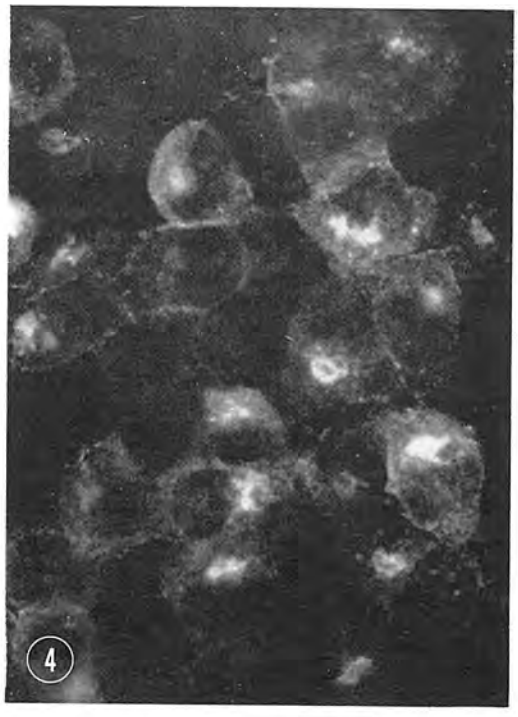


FIGURE 4. *Stained with anti-HA rabbit globulin conjugate.*

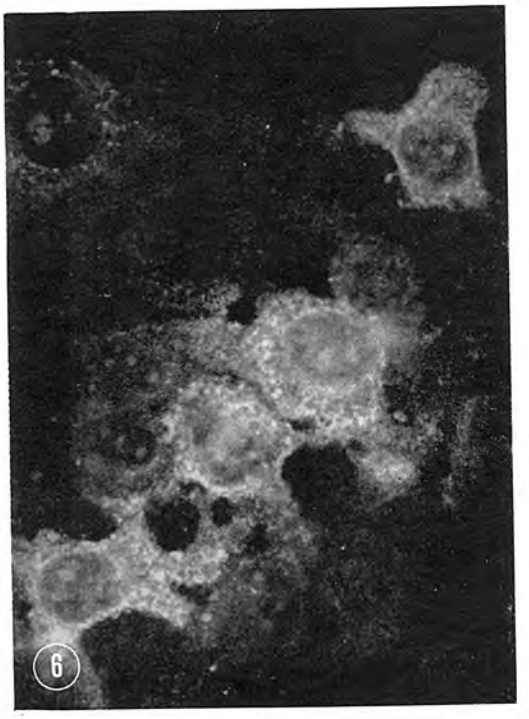
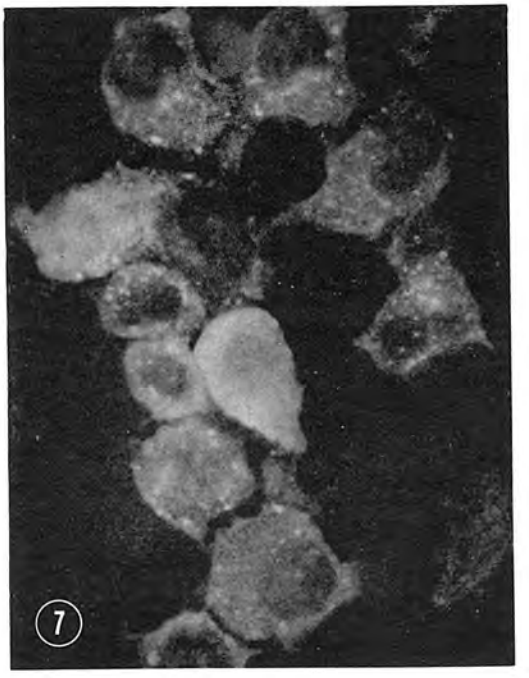


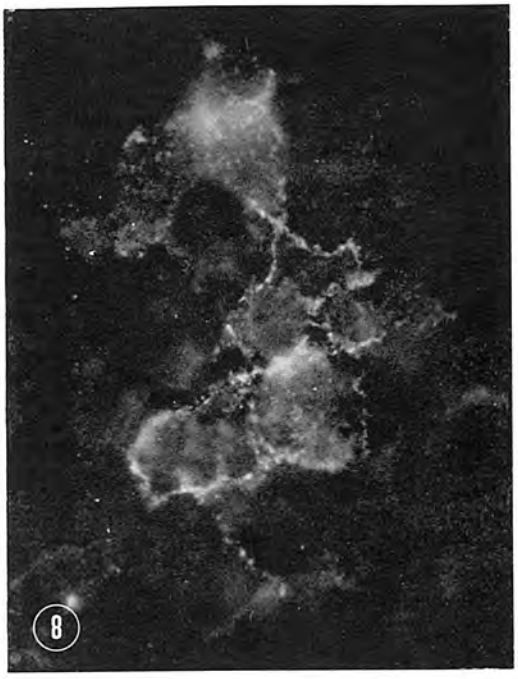
FIGURE 5. *Stained with anti-V-M mouse globulin conjugate.*

FIGURE 6 and 7. *Stained with anti-X rabbit globulin conjugate.*

In Fig. 3, 4, and 5, bright localized areas of fluorescence are seen in the cytoplasm round the nucleus.

In Fig. 6 and 7, bright fluorescent granules are distributed throughout the cytoplasm and no localized areas of fluorescence like those of V, HA and V-M antigens are seen.





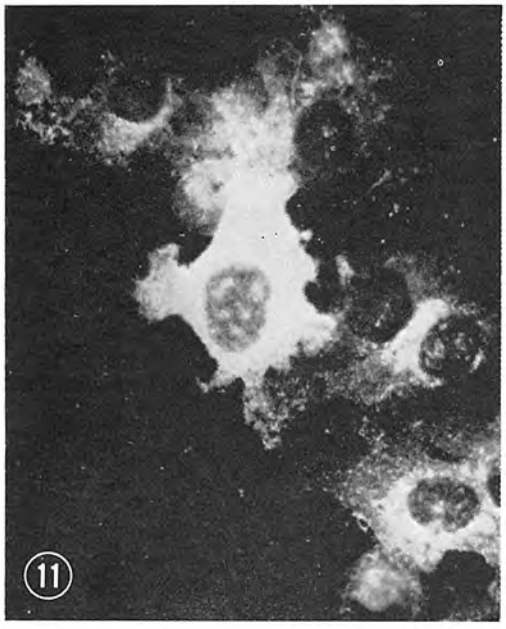
FIGURES 8, 9 and 10. *Surface antigen of unfixed FL cells 21 hr after infection with ChV (5 moi).*

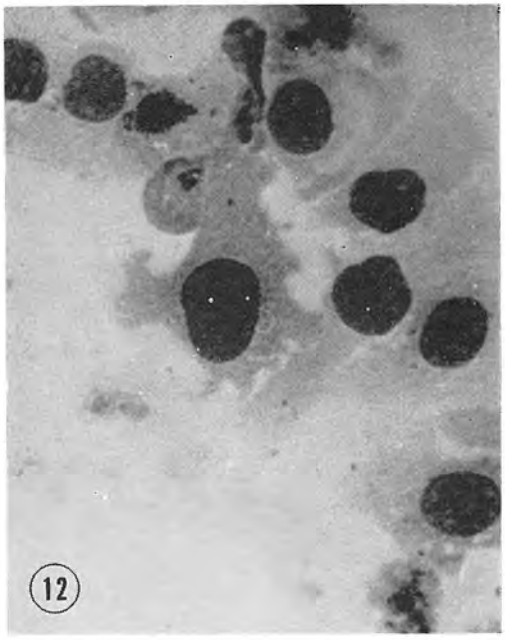
FIGURE 8: *stained with anti-V globulin conjugate.*

FIGURE 9: *stained with anti-HA globulin conjugate.*

FIGURE 10: *stained with anti-V-M glubulin conjugate.*

In Fig. 8, 9 and 10, bright fluorescent areas are seen at the cell margin.





FIGURES 11 and 12. *The fluorescent cells in Fig. 11 are stained with anti-X globulin conjugate 28 hr after infection. The same cells are shown in Fig. 12 stained with Giemsa solution after staining with anti-globulin conjugate.*

The picnotic or karyolytic cells in Fig. 12 have bright fluorescence in Fig. 11. No inclusion bodies are seen in Fig. 12.

FIGURE 13. *Uninfected FL cells stained with Giemsa solution.*

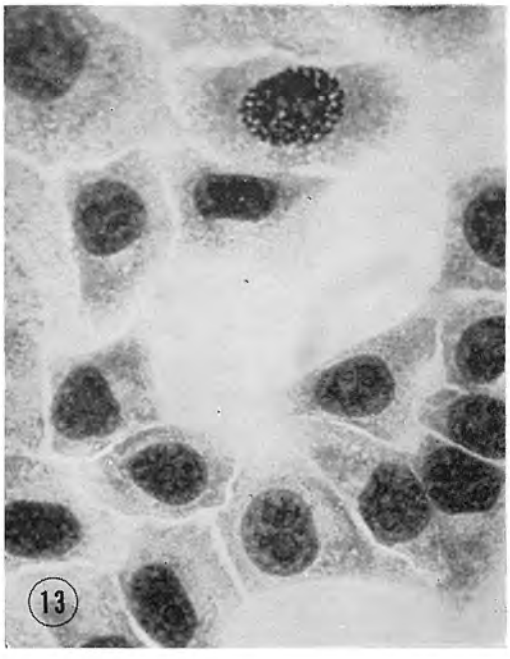


FIGURE 14. *Numerous picnotic and karyolytic cells are seen 28 hr after infection with ChV stained with Giemsa.*

DISCUSSION

The present data demonstrate that the HA antigen of ChV is localized in the same intracellular site as V antigen and infectious virus antigen. This suggests that these three antigens are almost the same. In this discussion the term V antigens refers to HA, and infectious viral antigens. V antigens develop in a certain localized area round the nucleus in the cytoplasm and accumulates there, causing intense concentrated fluorescence in the cytoplasm between 5 and 11 hr after infection at high multiplicity. But at a later stage of infection, the intensely fluorescent area in the cytoplasm became obscure, since infected cells became picnotic and degenerated. On the other hand X antigen develops diffusely throughout the cytoplasm and accumulates there so that small granules of intense fluorescence are seen throughout the cytoplasm of infected cells.

The curve of the percentage of fluorescent cells at low multiplicity suggests that X antigen develops 2 or 3 hr later than V antigen. In the fowl plague virus system, G-antigen (internal component of the virion) was detected earlier than HA-antigen (Breitenfeld and Schäfer, 1957).

In earlier studies on influenza (Liu, 1955), fowl plague viruses (Breitenfeld and Schäfer, 1957) and Sendai virus (Zhdanov et al., 1965) the inner component of the viruses was first observed in the nucleus or nucleoli of infected cells and later seemed to be transported into the cytoplasm. However, more recently Reda et al. (1964) reported that the inner component of Newcastle Disease virus is observed in general exclusively in the cytoplasm, where it is to be found in juxta-nuclear positions. Moreover our findings show that the nucleus is not related to the development of either of the ChV-specific antigens (HA and X).

The viral RNA of ChV is synthesized throughout the cytoplasm like X antigen (Mantani et al., 1967). HA antigens are transported to the cell margin at an early stage of infection to form surface antigen. Then X antigens containing viral RNA are transported to the inside of the cell membrane, where synthesis of ChV is completed, as revealed by electron microscopy (Higashi et al., 1967). When the X antigen moves to the cell membrane, it becomes covered with HA antigen to form a complete virus particle, because surface antigens were not detected by immunofluorescence using the anti-X globulin conjugate.

Hahon (1970) reported a specific surface antigen induced by Venezuelan equine encephalomyelitis virus in infected L-929 cells. However, the relation between this surface antigen and antigens of structural components of the virus was not demonstrated. In this report the anti-V–M conjugate was used to detect cell surface antigen which was not completely

blocked by anti-V or anti-HA sera. But it is still uncertain whether the cell surface antigen is identical with HA antigen, or whether it is a component of HA antigen.

We observed picnotic and karyolytic cells at a late stage of ChV infection. No inclusion bodies were formed on ChV infection, so it is uncertain from microscopic observation after Giemsa staining whether cell degeneration was a result of ChV infection. This problem could be solved using fluorescent antibody or autoradiography. We are now planning autoradiographic studies on these degenerated cells observed on ChV infection.

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