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IMMUNOFLUORESCENCE AND CELLULAR FRACTION STUDIES ON THE MULTIPLICATION OF INFECTIOUS CANINE HEPATITIS VIRUS*¹

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INTRODUCTION

In several electron microscope studies, it has been reported that the virus particles of adenoviruses were located in the nucleus of the infected cell (HARFORD et al., 1956; MORGAN et al., 1957; TAJIMA & MOTOHASHI, 1958; TAJIMA et al., 1961). It should be interesting to know whether the viral proteins of the infectious canine hepatitis virus (ICH virus) are also synthesized in the nucleus or the ribosomes in the cytoplasm as is the synthesis of proteins in the normal cell. For investigational purposes the synthesis of the ICH viral protein and the multiplication of ICH virus in dog kidney cell cultures were undertaken in two experiments described below.

1) Dog kidney cell cultures (DKC) infected with ICH virus were divided into the nuclear and cytoplasmic fractions at various times after infection, the infectivity, complement fixing (CF) and hemagglutinating (HA) titers were measured in each fraction.

2) The multiplication process of the ICH virus in DKC was investigated, using immunofluorescent antibodies prepared against ICH virus particles and against the soluble antigens (CF and HA antigens) which were considered to be the two antigenic components of the virus particle.

MATERIALS AND METHODS

Virus, cell culture and inoculation of the virus The Matsuda strain of ICH virus and DKC were used, as described in detail in the previous report (SUGIMURA & YANAGAWA, 1968).

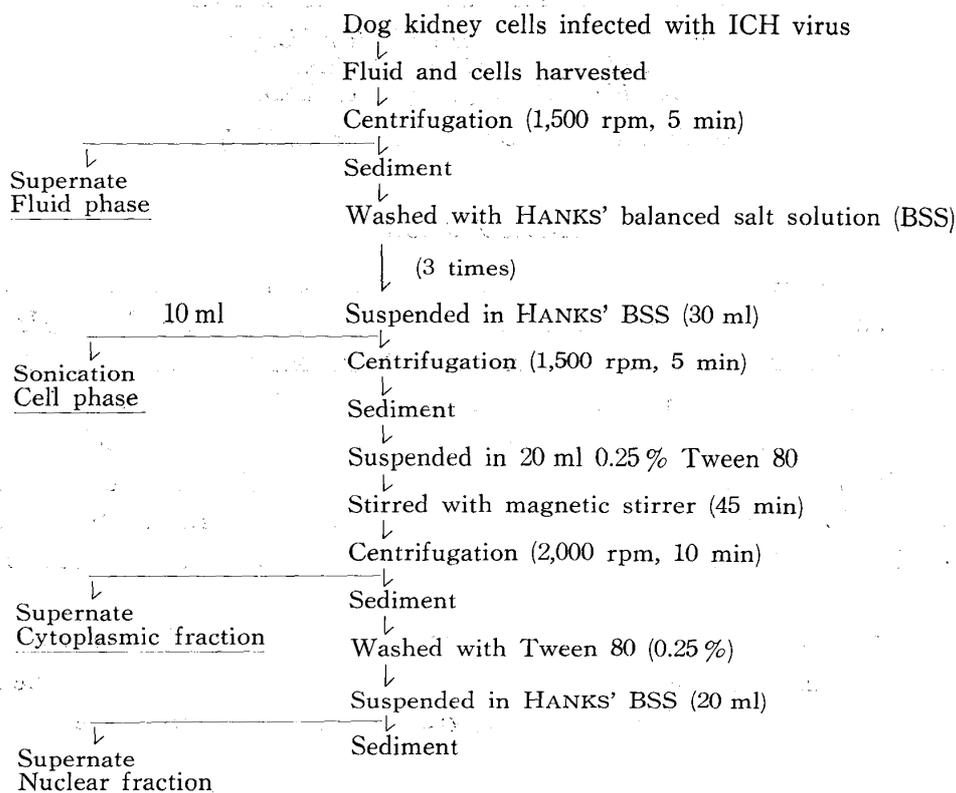
Cellular fraction experiments DKC, in a 4×10×4 cm bottle, infected with ICH

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virus (multiplicity of infection=2), were fractionated at various times by the fraction method using 0.25% Tween 80 as originally described by FISHER & HARRIS (1962). The details of the fractionation procedure are shown in figure 1. Infected materials from 3 bottles were pooled and fractionated. Fifty per cent tissue culture infectious doses (TCID₅₀), CF and HA titers were measured in each fraction as described in the previous report.

FIGURE 1 Procedure of cellular fractionation



Preparation of antiserum

(1) Anti-virus particle sera

Serum D-20 This serum was obtained from a puppy infected with ICH virus. The immunization procedure was described in the previous report (SUGIMURA & YANAGAWA, 1968).

Serum G-1 A guinea pig was inoculated intraperitoneally with 2 ml of the fluid obtained from DKC infected with ICH virus. Two weeks after the first inoculation, the guinea pig was inoculated with the same material 3 times weekly. When the CF and hemagglutination inhibition (HI) titers extended nearly to the maximum after immunization procedures were completed, the animal was bled.

(2) Anti-CF antigen sera

Serum DS-1 The procedure for preparing this serum was described in the previous report.

Serum DS-2 A 2 month old puppy was inoculated intravenously with ICH virus. Two

weeks later the animal was inoculated intravenously with the CF antigen which was purified and concentrated as previously described (SUGIMURA & YANAGAWA, 1968). A week following the last infection, the animal was bled.

(3) Anti-HA antigen sera

Serum G-2 A guinea pig was inoculated intranasally with ICH virus. Two weeks after infection, the guinea pig was inoculated intraperitoneally, twice weekly, with HA antigen. The HA antigen was prepared by fractionation with diethylaminoethyl (DEAE) cellulose column chromatography, and concentrated by dialysis against polyethylene glycol, as described in the previous report. A week following the last inoculation, the animal was bled.

Serum G-6 This serum was obtained from a guinea pig inoculated with HA antigen. The HA antigen used for the immunization was prepared as follows. The soluble antigen obtained from infected DKC was added to guinea pig red cells at 10% of the final concentration, and had been refrigerated overnight. The red cells which adsorbed the HA antigen were collected by centrifugation (1,000 rpm, 5 min) and washed twice with a cooled medium for hemagglutination (SHIMIZU, 1964). A guinea pig was inoculated intramuscularly with the red cells which adsorbed the HA antigen, and were mixed with an adjuvant consisting of Aracel A and Drakeol as described previously (SUGIMURA & YANAGAWA, 1968). After 2 weeks, the same antigen without adjuvant was inoculated intraperitoneally. A week later, the animal was bled.

Titration of infectivity and viral antigens Infectivity was expressed with TCID₅₀ calculated by BEHRENS-KÄRBER's method. CF and HA antigens were titrated as previously described (SUGIMURA & YANAGAWA, 1968).

Fluorescent antibody technique The procedure of labeling γ -globulin with fluorescein isothiocyanate was performed as described by GOLDSTEIN et al. (1961). Before staining, infected and uninfected (control) coverslip cultures were washed in staining buffer saline (pH 7.1), dried, fixed in acetone for 10 min at room temperature and dried for 30 min. The coverslips were then overlaid with the labeled serum diluted to staining titer units (usually 2 units were used) at room temperature for 30 min. The coverslips were then washed in staining buffer saline, mounted in elvanol-glycerol mounting medium, and examined under the fluorescent microscope. The specificity of fluorescence was determined as follows: uninfected cells were not stained, the fluorescence was blocked by homologous anti-serum.

RESULTS

1 Cellular fractionation studies

The infectivity, CF and HA titers were measured in the fluid and the cell phases of infected DKC and also in the nuclear and cytoplasmic fractions.

As shown in figure 2, the infectivity titer began to rise 12 hrs after infection, initiating in the cell and then in the fluid. In the nuclear fraction, the infectivity titers increased 12 hrs after infection, rose to maximum at 24 hrs and remained at the same level until 48 hrs. The infectivity titer of the nuclear fraction was 10 times higher than that of the cytoplasmic fraction. The pattern of increase of infectivity in the nuclear fraction was similar to that in the cell phase.

FIGURE 2 *Infectivity titer in fluid and cell phases, and cytoplasmic and nuclear fractions*

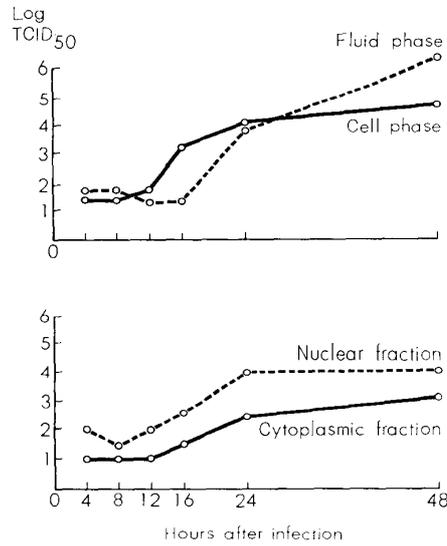


FIGURE 3 *CF titer in fluid and cell phases, and cytoplasmic and nuclear fractions*

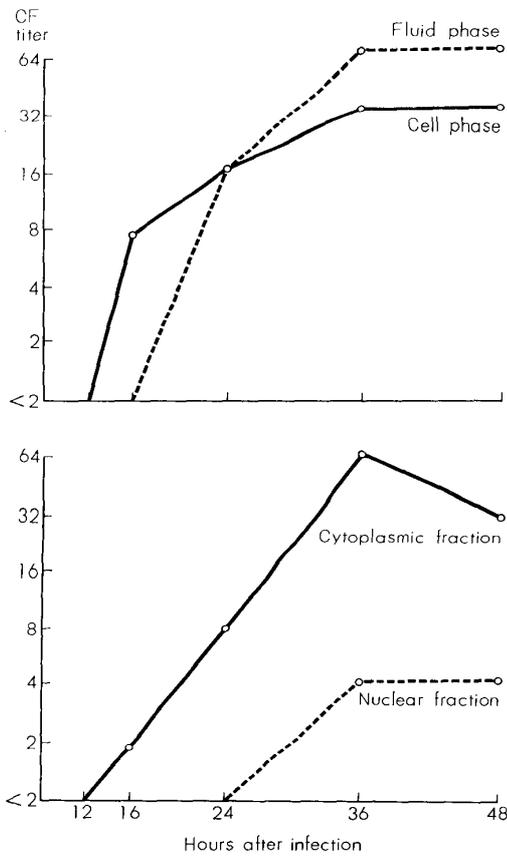
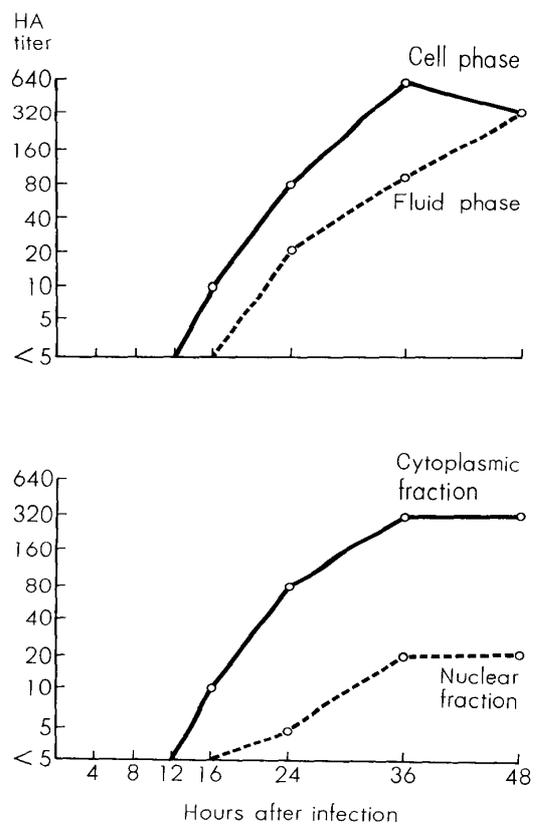


FIGURE 4 *HA titer after infection in fluid and cell phases, and cytoplasmic and nuclear fractions*



The CF titer appeared, as shown in figure 3, at the 16th hr in the cell phase and at the 24th hr in the fluid phase. Little difference was found in the increase of CF titer between the cell and the fluid phases. The CF titer in the nuclear fraction was not detected until the 36th hr. The titer was only 1 : 4 even after 48 hrs. On the contrary, the CF titer in the cytoplasmic fraction appeared at the 16th hr, and rose to 1 : 64 at the 36th hr. The pattern of increase of the CF titer was similar to that of the cell phase.

The increase of HA titer is shown in figure 4. HA titer was detected at the 16th hr in the cell phase and at the 24th hr in the fluid phase. The HA titer in the cytoplasmic fraction was about 16 times higher than that in the nuclear fraction.

The above results reveal that the infectivity titer was high in the nuclear fraction while CF and HA titers were high in the cytoplasmic fraction.

2 Fluorescent antibody study

The CF and HA titer of 6 sera used for fluorescent antibody technique were shown in table 1. D-20 and G-1 were anti-virus particle sera containing antibodies against both CF and HA antigens. DS-1 and DS-2 were anti-CF antigen sera showing high CF titer and low HI titer. G-2 and G-6 were anti-HA antigen sera which showed high HA titer but low CF titer.

TABLE 1 *CF and HI titers of the sera used in the fluorescent antibody study*

SERA	ANIMAL	CF TITER	HA TITER
D -20	dog	128*	1,024*
DS- 1	"	128	4
DS- 2	"	64	8
G - 1	guinea pig	64	128
G - 2	"	4	64
G - 6	"	4	32

* Expressed with reciprocals

1) The findings stained with labeled anti-virus particle sera, D-20 and G-1.

The specific yellow green fluorescence was first detected in the form of granules in the nucleus 6 hrs after infection. This stage is shown in figure 5, and designated stage 1. Nine hrs after infection, small granules with weak fluorescence distributed throughout the nucleus was observed and designated stage 2 (a in fig. 6). Stages 1 and 2 were often observed in the same cell (b in fig. 6). Twelve hrs after infection, a small number of cells showed fibrous fluorescence in the nucleus and was designated stage 3 (a in fig. 7). However, a large number of cells showed fluorescence throughout the nucleus or showed the bright fluorescent ring due to the presence of specific virus antigen along the nuclear membrane (b in figs. 7 & 8). This was designated stage 4. Fifteen hrs after infection, a specific fluorescence spread throughout the cytoplasm (b in fig. 9). After 24 hrs of infection, a rounding of cells occurred, the result of a cytopathic effect. These cells showed a swollen nucleus

and diminished cytoplasm, some cells showed bright fluorescence in the nucleus or along the membrane of nucleus (fig. 10) and were designated stage 7. Finally, fluorescence was absent in but present around the inclusion body (fig. 12). This was designated as stage 8. The inclusion bodies appeared at a stage late in infection and was not stained with the fluorescence. Only a few cells showed fluorescence at the same position in the inclusion body (fig. 11—designated stage 6).

2) The findings using labeled anti-CF antigen sera, DS-1 and DS-2

The first specific fluorescent stain was observed at the 9th hr. In a large number of cells, the fluorescence was weak and in the form of small granules (a in fig. 13). These small granules aggregated in the nucleus in many cells (b in fig. 13). These findings corresponded to stage 2. Twelve hrs after infection, the fibrous fluorescence in the nucleus (stage 3) was observed (a in fig. 14). The fibrous fluorescence appeared more frequently and more clearly than in the case of the stain with the labeled anti-virus particle sera. In addition, the fluorescence was observed throughout the nucleus especially near the nuclear membrane (b & c in fig. 14). Fifteen hrs after infection, the fluorescence was also found in the cytoplasm, and thereafter the findings were the same as those obtained with the labeled anti-virus particle sera.

3) The findings using labeled anti-HA antigen sera, G-2 and G-6

The fluorescence was detected first 9 hrs after infection. The granules with strong fluorescence were identical with stage 1 shown by using the labeled anti-virus particle sera (a in fig. 15). Twelve hrs after infection, these granules increased in number and spread throughout the nucleus (fig. 16). Lack of the fibrous fluorescence and lack of the small granules with weak fluorescence throughout the nucleus was noticeable. These were the important differences between the findings obtained with anti-CF antigen and anti-HA antigen sera. Thenceforth, the findings were similar to those shown by anti-CF antigen and anti-virus particle sera.

DISCUSSION

DENNY et al. (1959) and KONO et al. (1960) fractionated cells infected with adenovirus into nuclear and cytoplasmic fractions. The infectivity and CF titers were measured in each fraction. They stated that the infectivity and the CF titers were high in the cytoplasm but low in the nucleus. In the case of ICH virus, IGARASHI et al. (1961) fractionated infected DKC and titrated CF and infectivity titers. They reported that infectivity titer was high in the nucleus and CF titer was high in the cytoplasm. The reason for the above difference between adenovirus and ICH virus is not clear, but is probably due to the different experimental methods used, adenovirus-HeLa cell and ICH virus-DKC, or fractionation technique. There is a possibility that the virus propagated in the nucleus contaminated the cytoplasmic fraction.

In this report, it was clearly shown that infectivity appeared in the nuclear fraction 12 hrs after infection, and remained high in titer for 48 hrs. Therefore,

it was concluded that most of the infectious particles existed in the nucleus. Electron microscopically, ICH virus particles were found primarily in the nucleus and little in the cytoplasm (TAJIMA et al. 1961), accordingly, ICH virus has been considered to mature in the nucleus. CF and HA titers, however, were detected in this study in the cytoplasmic fraction 16 hrs after infection, the higher titer remained in the cytoplasmic fraction rather than in the nuclear fraction. It could not be determined whether these antigenic materials detected in the cytoplasmic fraction were synthesized in the cytoplasm or synthesized in the nucleus and transferred to the cytoplasm. Supposedly, these antigens were synthesized in the cytoplasm because they were detected earlier and more abundantly in the cytoplasm than in the nucleus.

The multiplication of ICH virus with fluorescent antibody technique was reported by COFFIN et al. (1953) and MOULTON et al. (1961, 1964 & 1965). COFFIN et al. using infected dog tissue concluded that the increase in the viral antigen, demonstrated with fluorescent antibodies, began on or within the nuclear membrane and spread from there to the interior of the nucleus. However, MOULTON et al. using the dog kidney cell culture reported that fluorescent was observed at the nuclear margin only at the late stages of infection, and that fluorescent granules appeared first in the nucleus at 10~12 hrs after infection. During the next stage, the number of granules increased and concentrated on the nuclear membrane. Also they noted loss of viral components in the inclusion body and accumulation of the viral components at the nuclear margin in later stages of infections.

In this study, the multiplication of ICH virus in DKC was studied applying the fluorescent antibody technique using immune sera prepared against the virus particle, CF and HA antigens. The results of the experiment using infected cells stained with immune sera prepared against the virus particle were nearly similar to those reported by MOULTON et al. (1961, 1964 & 1965). Fluorescence along the nuclear membrane was observed during stages 4 and 5 and not at earlier stages. Our findings were, therefore, in accordance with those of MOULTON et al. (1961 & 1965) and not with those of COFFIN et al.

The results obtained with anti-CF antigen and anti-HA antigen sera differed from those obtained with anti-virus particle sera. CF antigen appeared as small granules with weak fluorescence throughout the nucleus, and in the next stage particularly, as the fibrous fluorescence. In contrast, HA antigen appeared as the granules with strong fluorescence and in the next stage they increased in number. It should be stressed that the fibrous fluorescence characteristic of anti-CF antigen sera was never observed. In the advanced stage of infection, CF and HA antigens were detected similarly and were considered to be synthe-

sized in the same location. The immunomicroscopic observation of CF and HA antigens of ICH virus was different from those of S and V antigens of myxovirus (BREITENFELD & SCHÄFER, 1957; ZHDANOV et al., 1965).

Results of the cellular fraction and immunofluorescence studies will be discussed below. The fluorescence was detected greatly in the nucleus and weakly in the cytoplasm, consistent with the report of MOULTON et al. (1965). The fluorescence of the cytoplasm was detected 15 hrs after infection. At this time, the infectious virus was not detected in the cytoplasm, according to the cellular fraction study. Lack of the virus particle in the cytoplasm at this stage of infection was also reported by TAJIMA et al. (1961). Therefore, the fluorescence in the cytoplasm should be the soluble antigens, and not virus particles.

CF and HA antigens were detected immunomicroscopically first in the nucleus and later in the cytoplasm. The fluorescence in the nucleus was stronger than that in the cytoplasm. On the contrary, by fractional study, CF and HA antigens were detected first in the cytoplasm in greater concentration than in the nucleus. This discrepancy was considered as follows. The volume of cytoplasm was larger than that of the nucleus, therefore if the antigens were distributed uniformly in the cytoplasm, they must be diluted and not so easily detected by fluorescent antibody technique. In regard to the infectivity titer, it was always larger in quantity in the nucleus than in the cytoplasm, by both cellular fraction and immunofluorescence studies. The fluorescence in the cytoplasm was uniform and was not particularly strong around the nucleus, therefore it was thought that the viral proteins were synthesized in the cytoplasm. Probably, the HA and CF antigens were synthesized in the cytoplasm and transferred to the nucleus, where virus multiplication is completed. However, no evidence was found to substantiate the transfer of the antigens from the cytoplasm to the nucleus.

SUMMARY

The process of multiplication of ICH virus in the nucleus and cytoplasm of the infected dog kidney cell cultures were studied by cellular fractionation and immunofluorescence techniques.

The infectivity was detected in the nuclear fraction 12 hrs after infection. The virus titer was always higher in the nuclear fraction than in the cytoplasmic fraction throughout the course of infection. CF and HA titers were detected in the cytoplasmic fraction 16 hrs after infection, while in the nuclear fraction both were detected after 24 hrs. The titers of these antigens were always found to be higher in the cytoplasmic fraction than in the nuclear fraction.

Applying the fluorescent antibody technique using anti-CF antigen and anti-HA antigen sera, specific fluorescence was detected 9 hrs after infection. The

CF antigen first appeared in the nucleus as small granules with less intense fluorescence. In the next stage, the fluorescence was found as a fibrous structure. The HA antigen was found throughout the nucleus as granules with bright fluorescence, and never found as a fibrous structure. When the infectious process advanced, no difference was found between the infected cells stained with anti-CF and -HA antigen sera.

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PLATE I

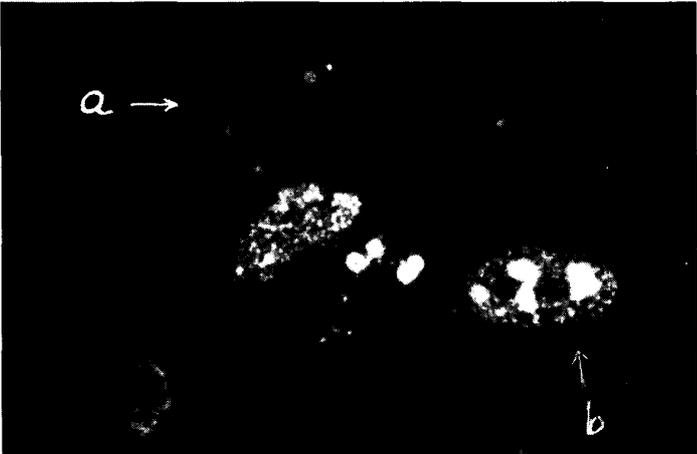
Infected cells stained with labeled anti-virus paricle serum

- Fig. 5 stage 1
- Fig. 6 a. stage 2
b. stages 1 and 2
- Fig. 7 a. stage 3
b. stage 4
- Fig. 8 stage 4
- Fig. 9 a. stage 1
b. stage 5
- Fig. 10 stage 7

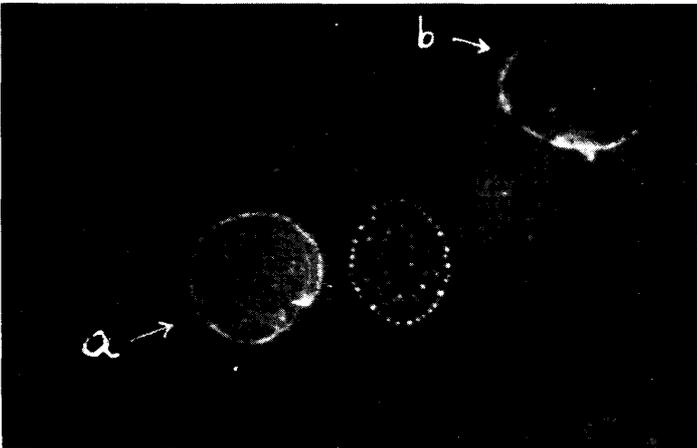
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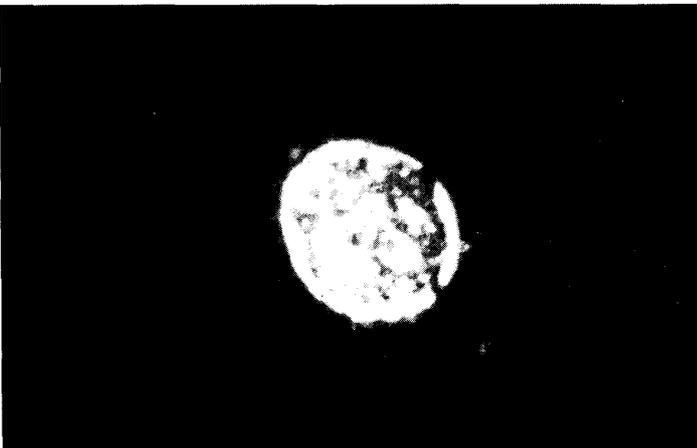
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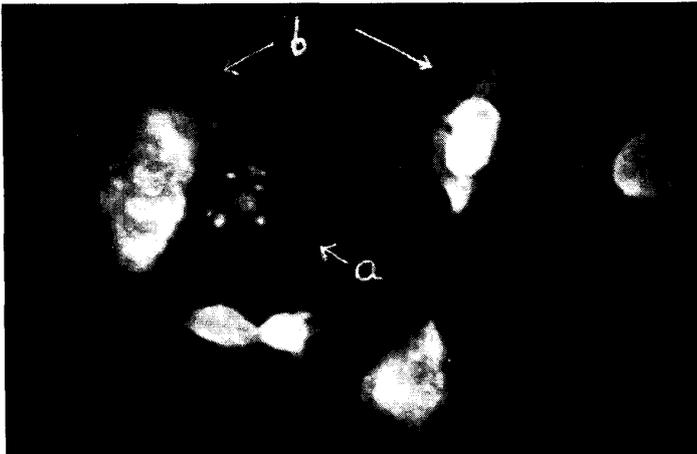
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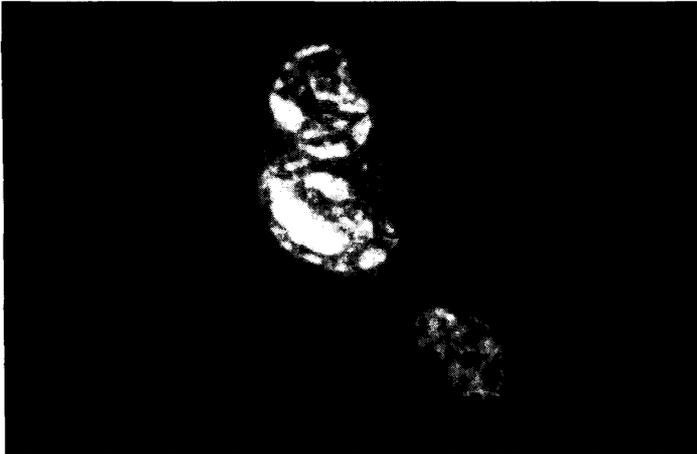
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PLATE II

Infected cells stained with labeled anti-virus particle serum

Fig. 11 stage 6

Fig. 12 stage 8

Infected cells stained with labeled anti-CF antigen serum

Fig. 13 a. stage 2

b. stage 2

Fig. 14 a. stage 3

b. stage 4

c. stage 4

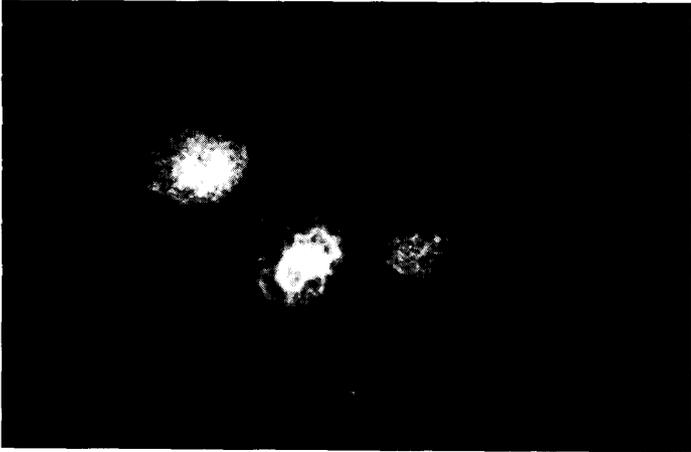
Infected cells stained with labeled anti-HA antigen serum

Fig. 15 a. stage 1

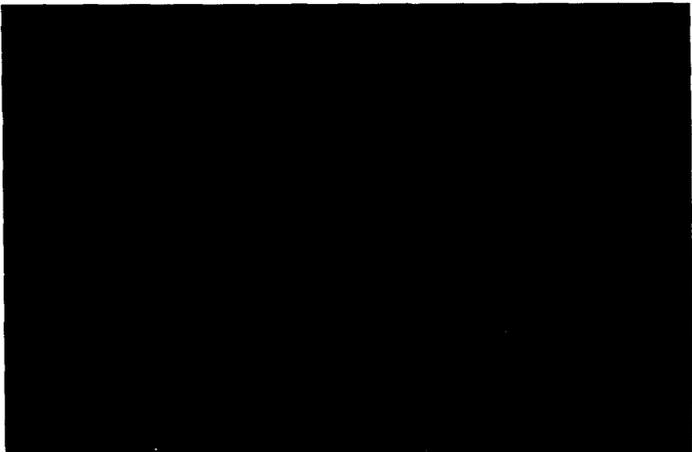
b. stage 4

Fig. 16 stage 1

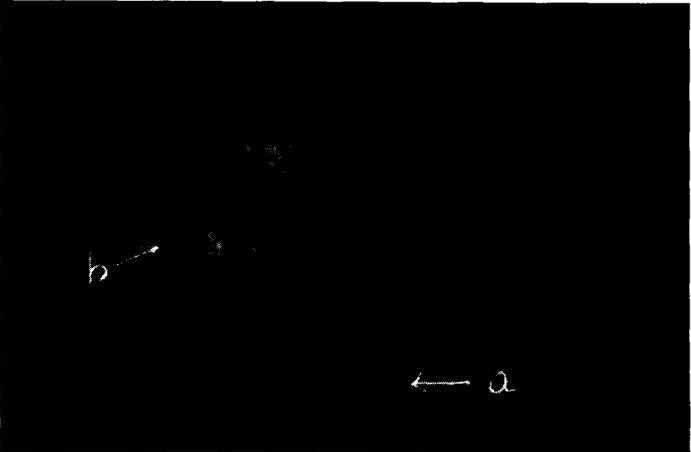
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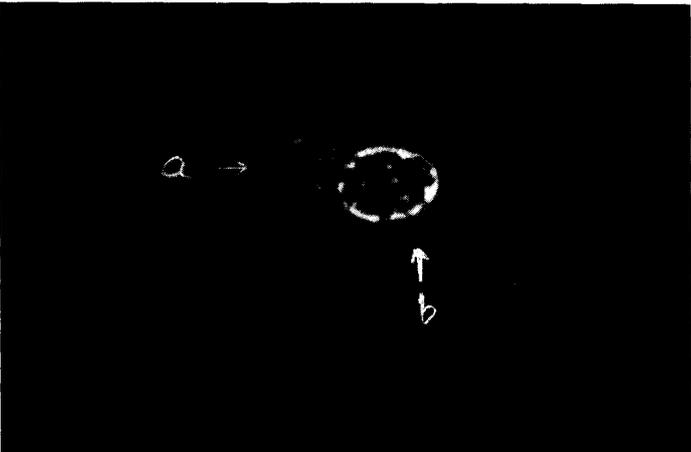
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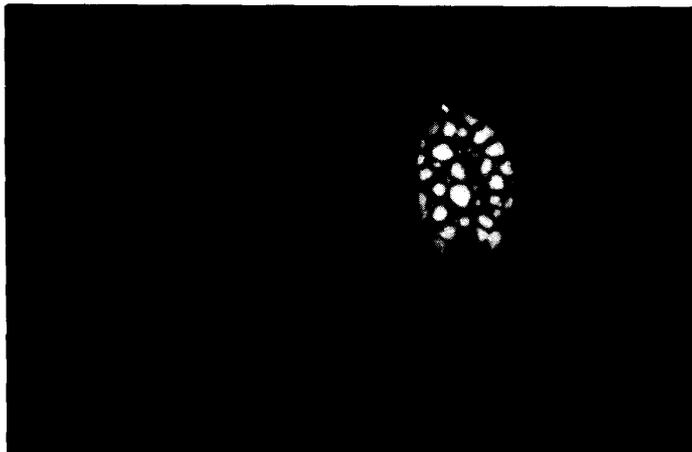
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