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Fluorescent Antibody Studies in Tissue Culture of Parainfluenza 3 Infection.* (27371)

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Fluorescent antibody has been applied by a number of investigators to study the sites of synthesis of the various units of animal viruses in infected cells. Studies on the multiplication of myxoviruses have shown that some, like influenza and fowl plague viruses, have their nucleoproteins and hemagglutinin components synthesized in the nuclei and the cytoplasm respectively(1,2,3); others like mumps, Sendai, and Newcastle disease viruses have not been found by fluorescent antibody to produce specific antigens in the nucleus at any stage of the development cycle(4). Because of recent interests in the newer myxoviruses, an investigation was undertaken to characterize parainfluenza 3 or hemadsorption type I, to study its growth in a continuous line of cells and to make a dynamic approach to the events occurring during viral biosynthesis.

Materials and methods. Conjugation procedure. Rabbit antiserum against parainfluenza 3 was labeled with trimethyl rhodamine isothiocyanate (TMRITC), and its gamma globulin component isolated by the cellulose anion exchange method of Riggs *et al.*(8). The conjugated preparations were successively absorbed with liver powder, from monkey and man, to reduce non-specific staining.

Staining procedures. 1) Fluorescent antibody. Infected cells on glass coverslip were stained with the labeled specific gamma globulin using the direct method(9). Uninfected cells stained with labeled antibody and infected cells treated with unlabeled antibody prior to staining, served as controls. In each instance, the specificity of the labeled serum was indicated, *i.e.,* uninfected cells showed no specific fluorescence and infected cells pretreated with the unlabeled antibody blocked the binding of the labeled one. 2) Acridine orange. The method of Armstrong and Niven(10) was followed for the acridine orange fluorochrome technic.

Microscopy. A standard Zeiss GF425 microscope with an Osram HBO 200 lamp as a light source was used. A UG2 filter and OG4 filter in combination with a GG4 were used as exciter and barrier filters, respectively. All photomicrographs were taken with a 35 mm Zeiss camera and super Anscochrome daylight film (ASA 100).

Tissue culture. The line of continuous monkey kidney (LLC-MK_2) cells used was obtained from Dr. Robert Hull of Eli Lilly research laboratories. As routine, the cultures were grown in synthetic mixture 199(5) with 1% inactivated horse serum. The maintenance medium used throughout during virus multiplication was composed only of the mixture 199.

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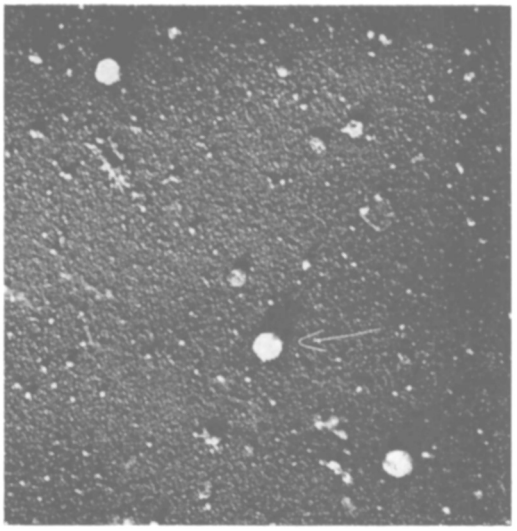

FIG. 1. Electron micrographs of a semipurified preparation of parainfluenza 3 virus, shadowed with palladium (× 26,250).

Virus. The virus HA1 (parainfluenza 3), supplied by Dr. Robert Chanock of the Nat. Inst. of Health, has been passaged 22 times in primary monkey kidney cells and 10 times in the established line of monkey kidney.

Infection procedure. After 5 days of cellular growth, a uniform monolayer was formed, the growth medium was discarded and a heavy virus inoculum was added in order to infect all susceptible cells. The multiplicity of infection was 10 to 1. The virus was allowed 2 hours' adsorption at 37°C and the cultures were washed once with Hanks' balanced salt solution to remove residual and loosely bound particles. At selected intervals duplicate cultures were removed for infectivity titration and staining.

Virus assays. Parainfluenza 3 was titrated in culture tubes of LLC-MK_2 cells by both cytopathology and the hemadsorption technic of Vogel and Shelokov(6) and the 50% end point calculated by the method of Reed and Muench(7).

Results. Size of the virus. Electron micrograph of a semipurified preparation of parainfluenza 3 virus is shown in Fig. 1. The sample was prepared by a freeze-dry method and shadowed with palladium. The particle appears to be between 100-130 mμ in diameter and has a roughly spherical shape. Using negative staining, Waterson *et al.*(11) found that the diameter of the virus varied between 140-250 mμ.

Cytopathology. The cytological changes initiated in the continuous monkey kidney line consisted of rounding, increase in granularity and cytoplasmic basophilia. There was clumping of infected cells with fragmentation and death. At no time was there evidence of syncytium formation. Lepine *et al.* (12) and Diebel and Hotchin(13) reported that the cytopathic action of the virus in HeLa cells and F1, respectively, is very dramatic with syncytial or giant cell formation. However, it was shown by Marston and Vaughan(14) that there is variation in cell responses to infection with parainfluenza 3 of different cell systems.

Growth characteristics. The growth cycle of the virus was obtained from cultures which had been heavily inoculated in order to infect all susceptible cells. At selected intervals a culture was withdrawn, to determine the infectivity titer of the virus in the supernate as well as in the cells following sonication. The data are presented in Fig. 2. It will be noted that in a one-step growth curve, development of the virus proceeded along the following sequence. Intracellular increase in virus titer was detectable between the 2nd and 4th hour after infection. The virus continued to increase and accumulate in the cytoplasm until the 16th hour when no further significant increase in yield was measured. Release of the virus from the cells starts between the 8th and 10th hour after infection. Rate of release is rapid between the 10th and 20th hour, when maximum titer is reached. After the 20th hour the rate of release becomes constant; at this time viral synthesis is no longer evident and visible cellular alterations become apparent. During this study, it was found that some mature virus stays firmly bound at the periphery of infected cells and in order to obtain maximum yield, sonication of cells is essential.

Fluorescent antibodies. Accompanying immuno-fluorescent studies showed good correlation between the growth pattern of the virus as measured by infectivity and the appearance of specific fluorescence in infected

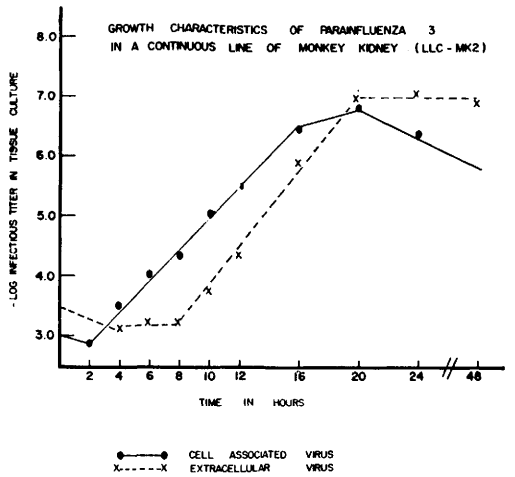

FIG. 2. Growth curve of parainfluenza 3 in a continuous line of monkey kidney cells (LLC-MK_2).

cells. At no time did uninfected cells stained with specific labeled antibody show specific fluorescense. Specific viral antigen was not detected until the 4th hour after infection. At this time a small area of fine granules of fluorescence was seen in the cytoplasm, particularly in the perinuclear region of infected cells (Fig. 3 A). The area of fluorescence increases in size with time and by the 12th hour post-infection specific fluorescence of a fine particulate nature is seen in the cytoplasm toward the periphery of infected cells (Fig. 3 B). Between 16 and 36 hours aggregates of fluorescence arranged in rosette-like structures were seen at the periphery of infected cells (Fig. 3 C).

Examination of infected coverslips up to 72 hours after infection revealed a retention of aggregates of viral antigen at the periphery of cells even when cellular morphology was significantly distorted (Fig. 3 D). At no time during the developmental sequence of the virus was any specific fluorescence observed in the nuclei of infected cells.

Acridine orange. Infected cells when stained with acridine orange revealed characteristic cytochemical changes. The changes in staining quality indicative of an increase in cytoplasmic ribonucleic acid content were observed as early as 3 hours post-infection. At 18 hours after infection when aggregates of specific fluorescence of the viral antigens were observed, replicate cultures of the same period showed similar red-orange aggregates indicative of ribonucleic acid (RNA) in the cytoplasm. Later, when rosette-like structures of specific fluorescence were seen, these structures also stained specifically for RNA. At no time in the developmental cycle of the virus were any DNA-containing structures observed in the cytoplasm.

Discussion. Traver and co-workers(4) have shown, using fluorescent staining technic, that the intracellular site of 3 non-influenza myxoviruses was the cytoplasm. The present findings which describe the developmental sequence of yet another non-influenza myxovirus, substantiates these earlier observations that the site of production of antigen is the cytoplasm. However, it was shown previously(15) that parainfluenza 3 infection of HeLa cells induces increase in the nucleic acids of both nucleus and cytoplasm. While these studies did not reveal any nuclear site of synthesis, it could be that the hyperactivity of the nuclei of infected cells is a reflection that the synthesis of the nucleic acid moiety of the S antigen of a non-influenza myxovirus is in the nucleus and that the complete S antigen is assembled in the cytoplasm. The growth of the virus in LLC-MK_2 cells does not parallel the burst phenomenon seen with bacterial viruses. The results presented here show that the liberation of virus is over a period of many hours and this is in agreement with the work of Marston and Vaughan(14) using a different cell system. The data also indicate clearly that there is a phase of intracellular accumulation of the mature virus before its release. The cytopathic changes seen in this host-system were apparent after maximum infectivity titer is reached. However, generalization cannot be made as Diebel and Hotchin(13) reported that in F1 cells, the cellular changes were evident as early as 6 hours after infection.

Summary. Growth characteristics of parainfluenza 3 virus in LLC-MK_2 line of tissue culture cells have been described using 2 staining technics. The appearance of specific fluorescence was correlated with the appearance of newly formed virus. The site of synthesis of virus was shown to be restricted to the cytoplasm of infected cells. Maximum in-

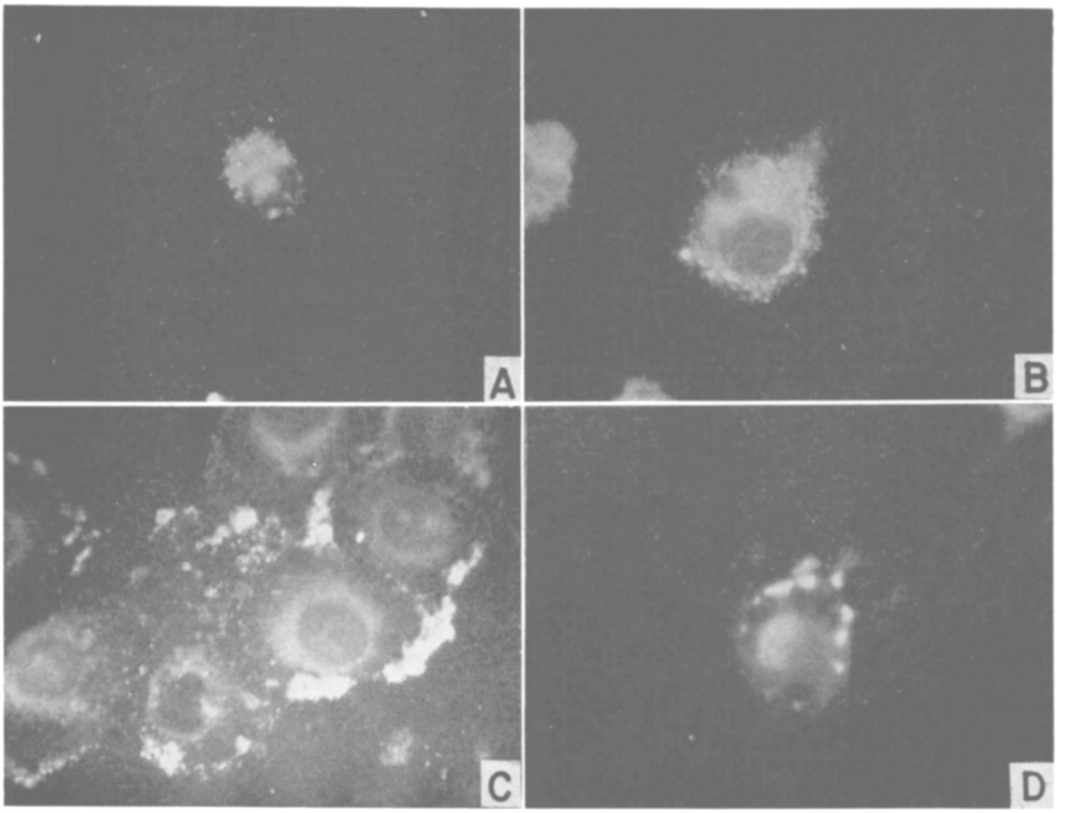

FIG. 3. Photomicrographs of continuous monkey kidney cells (LLC-MK_2) infected with parainfluenza 3 and stained with rhodamine labeled antibody (× 600). (A) 4 hr post-infection: Specific fluorescence is confined to perinuclear region. The extent of the cytoplasm of the cell is not reproduced in the photograph. (B) 12 hr post-infection: Specific fluorescence is more diffuse throughout the cytoplasm and is of a more particulate nature. (C) 36 hr post-infection: The antigen is confined almost exclusively to areas at the periphery of the cell. The grey areas around the nucleus are not viral antigen, but are due to autofluorescence characteristic of the cell line. (D) 72 hr post-infection: Some retention of viral antigen at periphery of the cell, but now appears in more condensed packets. The blue autofluorescent area is present in perinuclear region.

fectivity titer intracellularly is reached around the 16th hour, while viral release is maximal around the 20th hour post-infection.

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