DEMONSTRATION OF THE SEQUENTIAL DEVELOPMENT OF VACCINIAL ANTIGENS AND VIRUS IN INFECTED CELLS: OBSERVATIONS WITH CYTOCHEMICAL AND DIFFERENTIAL FLUORESCENT PROCEDURES*

By PHILIP C. LOH, Ph.D., and JOHN L. RIGGS, Ph.D.

(From the Virus Laboratory, Department of Epidemiology, School of Public Health, The University of Michigan, Ann Arbor)

Plates 20 and 21

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Several antigenic products smaller than the virus particle are found in cells infected with vaccinia virus. These include the immunologically distinct heat-labile-heat-stable LS antigen (1–3), the nucleoprotein NP antigen (3), and the hemagglutinating HA antigen (4). Using immunofluorescent and cytochemical procedures, it has now been possible to chart their development in infected cells or in the same cell. This has permitted relating the time of their appearance and sites of intracellular production to the appearance of the final infective virus. In the present communication such relationships are described.

The LS antigen is a protein with a molecular weight of about 240,000 (5–7). Although considered as a single molecule, it was found to contain a heat-labile (L) and a heat-stable (S) factor, which can be degraded independently by appropriate treatment (5, 7). The NP antigen contains 6 per cent deoxyribonucleic acid (DNA) and constitutes at least 50 per cent of the substance of a virus particle (7). Because their respective antibodies react with whole virus, both the LS and NP antigens are considered surface constituents (7). The HA antigen which accompanies virus multiplication in most tissues is a lipoprotein complex which agglutinates the erythrocytes of certain fowl (4, 10). It has been associated with particles 65 mμ in diameter and with the virus (8, 9). It is, however, considered a non-essential part of the virus since hemagglutination activity and infectivity may be dissociated by centrifugation or adsorption with suitable red cells (10).

Materials and Methods

Cell Culture.—HeLa cells were originally obtained from Difco Laboratories, and routinely grown in Eagle's basal medium (EBM) (11) containing 10 per cent equine serum. For cytological studies, the cells were grown on glass coverslip pieces in 16 × 150 mm screw-capped culture tubes or in Leighton tubes. Approximately 35,000 cells suspended in 1 ml of medium were inoculated into each tube and incubated at 37°C.

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Infection Procedure.—The culture fluids from 4-day-old HeLa cell coverslip preparations were removed and the cells washed twice with 2 ml of Hanks' balanced salt solution (BSS) and HeLa-passaged virus in 1 ml of EBM with 2 per cent equine serum was introduced. The input multiplicity of virus to cell was approximately 7 PFU per cell. The infected cultures were further incubated at 37°C.

Virus and Virus Assay.—To avoid contamination of cultures with avian antigens which might stain with fluorescent antibody to antigens prepared from chorioallantoic membranes (CAM) of chick embryonated eggs, experimental cell cultures were infected with virus that had been passaged four times in HeLa cells. The fluorescent antibody used was prepared against antigens obtained from the CAM-infected with vaccinia virus. The number of infectious units was assayed on the CAM of 12-day-old embryonated chick eggs by the method of Beveridge and Burnet (12), and expressed as the number of pock-forming units (PFU) per ml. The virus passed in HeLa cell cultures yielded a titer of 7.2×10^7 PFU per ml, and the CAM-passaged virus had a titer of 1.5×10^9 PFU per ml.

Preparation of Antigens.—The LS and HA antigens were prepared from homogenates of infected CAM. Chorioallantoic membranes of 12-day-old eggs were each inoculated with 0.2 ml of virus (10^5PFU/ml). After 2 days the membranes were harvested, homogenized, and sonically treated (9 kc for 10 minutes). The LS antigen was prepared from this homogenate by the method of Craigie and Wishart (2). Briefly, the procedure is as follows: virus and cellular debris are removed by centrifugation and LS antigen recovered and concentrated by mild acid precipitation (pH 4.5), and centrifugation. The precipitate was dissolved in 0.01 M citric acid-phosphate buffer (pH 6.3), reprecipitated and then redissolved in the buffer to give the final preparation for immunization. The supernatant after removal of the LS antigen and neutralization (pH 7.0), contains the HA antigen. No virus was detected in preparations of either antigen when tested on CAM.

NP antigen was prepared from partially purified CAM-passaged virus by treatment with dilute alkali (3). Partial purification of the virus was achieved by several cycles of differential centrifugation (13). The antigen preparation was stored in barbital buffer solution (pH 8.6) at 0°C, and showed no infectivity when tested on the CAM.

Preparation of Immune Sera.—For the preparation of hyperimmune sera, 6-week-old rabbits were immunized intravenously with alum-precipitated preparations of each antigen. Each rabbit was inoculated three times a week with increasing concentrations of antigen for 4 weeks. Fourteen days after the last inoculation the rabbits were bled and the sera recovered. The specificity of each serum was tested by the quantitative precipitin method of Cohn (14).

Conjugation Procedure. The immune serum of each antigen was conjugated with fluorescein isothiocyanate (FITC)[1] (15). A portion of the anti-NP serum was conjugated with tetramethylrhodamine isothiocyanate (TMRITC).[2] The labeled gamma globulins were obtained either by ethanol fractionation (16), or by the cellulose ion-exchange method (17). These conjugated globulin fractions were routinely absorbed twice with normal human liver powder to remove non-specific staining.

Staining Procedure.—The direct fluorescent antibody technique of Coons and Kaplan (18) was used for the visualization of viral antigens. Uninfected coverslip preparations served to control the specificity of staining by the labeled sera. An additional test for specificity was the prior treatment of infected cultures with non-labeled immune serum followed by its labeled counterpart. In both tests, the specificity of each immune serum was indicated, *i.e.* uninfected cells when stained showed no specific fluorescence, and prior treatment with unlabeled sera blocked the binding of its labeled immune sera.

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[1] FITC, fluorescein isothiocyanate.

[2] TMRITC, tetramethylrhodamine isothiocyanate, kindly supplied by Baltimore Biological Laboratory, Baltimore.

Cytochemical Stains.—In addition to the fluorescent antibody technique, the following cytochemical stains were used: (*a*) the acridine orange fluorochrome technique (19); (*b*) Pearse's methyl green—pyronin (20); and (*c*) Stowell's Feulgen staining method (21). Coverslip preparations for methyl green–pyronin and Feulgen stains were fixed in Newcomer's fixative (22), and for acridine orange stain with Carnoy's fixative.

Fluorescence Microscopy and Photomicrography.—A standard Zeiss GF 425 microscope was used with a 32 mm diameter and a 3 mm thickness, BG 12 or UG 2 exciter filter. An OG4 filter alone or in combination with a GG4 filter served as the ocular barrier system. An Osram HBO 200 lamp served as the light source. All photomicrographs were taken with a 35 mm Zeiss camera, and super Anscochrome daylight film (ASA 100). Exposure times ranged from 30 to 70 seconds.

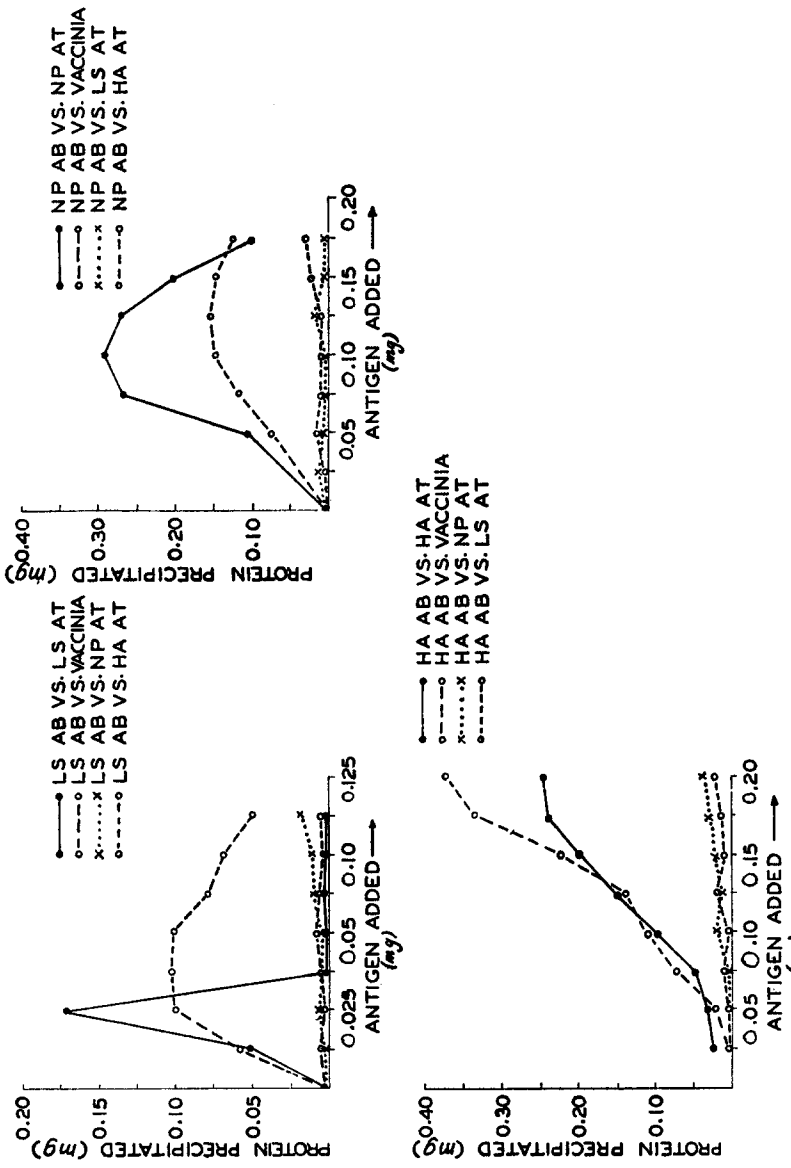
EXPERIMENTAL RESULTS

The Specificity of the Immune Sera.—Quantitative precipitin tests were carried out on the immune sera made against LS, NP, and HA antigens, respectively. Each serum was tested for its specificity against the homologous and the heterologous antigens. Text-fig. 1 shows the results of this experiment. The anti-LS and -NP sera, but not the anti-HA serum, gave typical precipitin curves to their respective homologous antigens. None of the three immune sera reacted with heterologous antigens, and each reacted with whole virus. Although the anti-HA serum reacted only with homologous antigen and whole virus, the precipitin pattern obtained suggested the presence of "contaminating" protein, probably cellular in nature. Subsequent labeling of these immune sera with the fluorescent dye did not alter their specificity.

Virus Multiplication in HeLa cells.—To relate the morphologic changes and appearance of viral antigens in the cell to virus multiplication, the time course of production and liberation of virus was first determined. Text-fig. 2 shows the growth characteristics of the virus when a sufficiently large inoculum of virus is used so as to give a single cycle of multiplication.

Four-day old tube cultures of HeLa cells containing 8 to 10 $\times$ 10^4 cells were inoculated with approximately 7 PFU of virus per cell. Cultures were then incubated at 37°C and samples taken at 4 hour intervals. To obtain extracellular virus the medium from 5 tubes was removed and the cells washed twice with Hanks' BSS. The first washing was combined with the medium for titration. At later times (beyond 10 hours) samples were centrifuged to remove cellular debris. To determine the cell-associated virus (CAV) the cells were frozen and thawed 4 times, disintegrated by sonic oscillation, and then titered on the CAM.

The first detectable increase in the amount of CAV occurred at about 8 hours post infection, and by 24 hours the yield per cell was approximately 70 PFU. The amount of virus in the medium did not increase until about 16 hours post infection and by 24 hours the yield was about 15 PFU per cell. Cytologic changes became apparent only beyond 12 hours post infection, and were marked by the 24th hour. Perhaps, because of the relatively small input

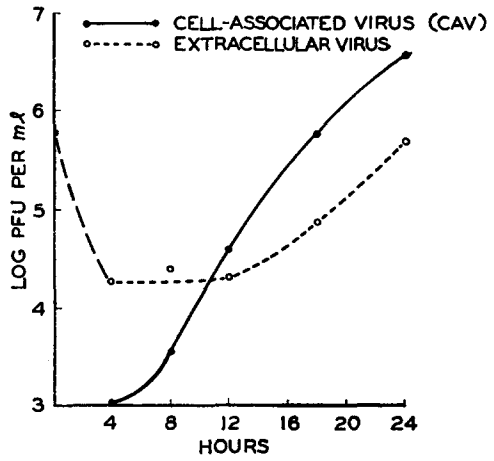


TEXT-FIG. 1. Precipitin reactions of hyperimmune anti-LS, -NP, and -HA sera against homologous and heterologous antigens.

multiplicities used in these experiments, the early "cytotoxic" action seen by others (23, 24) with certain strains of vaccinia virus was not observed.

Cytologic Observations of HeLa Cell Cultures Infected with Vaccinia Virus

Microscopic examination of tissue culture cells infected with vaccinia virus revealed a variety of characteristic cytochemical and cytologic alterations. Coverslip preparations of HeLa cells, infected with an appropriate amount of virus according to the method described above, were examined at 2 hour intervals after infection, and the sequential nature of the cellular changes established.



TEXT-FIG. 2. The growth of HeLa-adapted vaccinia virus in HeLa cell monolayers. The number of infectious units was assayed on the CAM of 12 day embryonated chick eggs, and expressed as the number of pock-forming units (PFU) per milliliter. The input multiplicity was approximately 7 PFU per cell.

1. Cytochemical Studies:

(*a*) *Feulgen and Methyl Green–Pyronin Stains.*—Examination of infected cultures on coverslips stained by either of these two cytochemical techniques, revealed the appearance of typical DNA-containing inclusion bodies in the cytoplasm (Figs. 1 A and 1 B). These Feulgen or methyl green–positive bodies can be seen as early as 5 to 6 hours after infection, and are at this time small and frequently round in shape. Several bodies can be seen within the cytoplasm of some cells, and with time, they may enlarge and coalesce to form a larger and irregular shaped body (about 8 hours post infection). At this time a small increase in the CAV content can be detected (Text-fig. 2). In infected cells stained with methyl green–pyronin, a relative increase in staining with pyronin, indicative of RNA (ribonucleic acid) was observed as early as 2 hours post

infection. Alterations in staining quality became markedly difficult to evaluate at times later that 12 hours post infection, when the cells became rounded.

No significant changes in morphology or staining characteristics of the nuclei were observed, except in the later stages when the cells became rounded and the nuclei pyknotic.

(*b*) *Acridine Orange Stain.*—Once again cytoplasmic DNA-containing elements were observed with the use of this stain. They can be seen as early as 4 hours post infection, as compared to 5 to 6 hours post infection with Feulgen and methyl green–pyronin stains. At this time the inclusions vary in size and number within the cytoplasm (Fig. 2A). As the infection progresses, the individual bodies enlarge and fuse, and the number of cells containing inclusion bodies increases. At 8 to 10 hours post infection, some of the bodies appear to fragment around the edges (Fig. 2B) coinciding with the first detectable increase in CAV.

Supportive evidence for the increase in cytoplasmic RNA of vaccinia virus-infected cells was obtained with coverslip preparations stained with acridine orange. This relative increase in RNA was again seen as early as 2 hours post infection, and before the first detectable increase in CAV. At later stages (8 to 10 hours post infection) an increase was observed in nuclear DNA fluorescence, which was probably due to pyknosis.

2. Fluorescent Antibody Studies:

To determine relationships between the formation of the various viral antigens and the final mature infective product, preparations of fluorescent labeled antibody specific for the respective antigens were used.

(*a*) *Fluorescein Isothiocyanate-Labeled Antibody Staining.*—The first specific subviral unit to appear is the LS antigen. This antigen was detected in the cytoplasm at about 4 hours post infection. At that time staining was limited to a diffuse perinuclear area, but by 6 hours post infection (Fig. 2C) and later, more of the cytoplasm is involved, and the staining characteristics took on a diffuse but more granular distribution. In the cytoplasm of some of the infected cells, pockets of non-fluorescence were seen, which, as will be described below, hold the NP antigen–containing inclusions (Fig. 2D, 8 hours post infection). By 10 to 12 hours post infection, intensive LS fluorescence is seen over the entire cytoplasm of the rounding infected cell.

In contrast to the LS antigen, the NP antigen was first detected in the cytoplasm at about 6 hours post infection (Fig. 2E), and occurred as focal centers of discrete particulate fluorescence (green). Although these centers were most often found in the perinuclear area, they were occasionally more distant from the nucleus. Also, some infected cells showed more than a single center of NP antigen synthesis. As the infection progressed, the centers enlarged and, if multiple, appeared to coalesce (Fig. 2F, 8 hours post infection). In addition at this time marginal disintegration of the centers could be observed in some cells.

The number of cells showing such fragmented "inclusions" increased with time. As with the evidence obtained by use of the acridine orange stain, this phenomenon coincided with the first appearance of CAV. Beyond the 12th hour post infection, specific fluorescence of a discrete and particulate nature for the NP antigen extended throughout most of the cytoplasm.

The "soluble" HA-antigen, which is found in vaccinia-infected cells, and is a by-product of virus-cell interaction, was first detected by the fluorescent labeled antibody at about the 10th hour post infection. It was found initially as a small diffuse fluorescent mass, particularly in the perinuclear region, and beyond 12 hours post infection it had increased in size and intensity of fluorescence, throughout the entire cytoplasmic area (Fig. 2G, 12 hours post infection). In contrast to the LS and NP antigens, it should be emphasized that the HA antigen was not detected before the appearance of infectious virus. At no time during the infectious periods studied was any specific fluorescence observed within the infected cell nucleus for any of the three antigens.

(*b*) *Combined Fluorescein- and Rhodamine-Labeled Antibody Staining.*—It is apparent that despite the differences observed in the morphology, time and sequence of appearance of the LS and NP antigens, the ideal comparison would be made in preparations where the antigens were visualized simultaneously. For this purpose, the anti-NP serum was labeled with an orange-red fluorescing stain, tetramethylrhodamine isothiocyanate (TMRITC), which can be readily distinguished from the green fluorescence of the fluorescein dye used to label the anti-LS serum. For the detection of the two antigens within the same cell, equal volumes of FITC-labeled LS serum and TMRITC-labeled NP serum were mixed, and the mixture used for staining.

Using the double stain, the two antigens were first detected in single cells at essentially the same times as in previous experiments. At 5 to 6 hours post infection, when the orange-red NP antigen appears in cells already containing the green LS antigen, the two antigens are readily distinguished from one another. At this time the compact NP antigen inclusions are discrete masses within the diffuse field of the LS antigen (Fig. 2H, I). Beyond 8 hours post infection, when specific fluorescent antibodies demonstrate a diffuse distribution of the two antigens within the cell, the staining by anti-NP serum labeled with TMRITC and anti-LS serum labeled with FITC is also more widespread and intimately mixed (Fig. 2I). The resultant color at this time and later, when intracellular detail becomes less distinguishable, is yellow-green. Temporally, this blending of antigens, according to labeled antibody reaction, corresponds with appearance of infectious virus. It appears therefore to represent the final assembly of the antigenic components into mature active virus.

DISCUSSION

Although fluorescent antibody studies have been used by a number of investigators (25–27) to study the development of vaccinia virus–infected cells,

all of them have used labeled antibody made against whole virus. Furthermore, only Cairns (27) has made a time study of production of the complete viral antigen, and found that it could be detected in the cytoplasm as early as 3.5 hours post infection. From the present investigation it appears that the initial specific fluorescence seen by Cairns was probably LS antigen. Furthermore, for the first time with this present combination of techniques, the stepwise development of vaccinia virus has been charted to reveal an orderly sequence of events. The earliest antigen formed is the LS protein which appears at about the 4th hour post infection as fine particulates in the perinuclear region. The NP antigen appears at about the 6th hour as coarse but distinct aggregates in the same region. Although both antigens are seen only in the cytoplasm, even when they become more diffusely distributed in the cell, their centers of synthesis are easily differentiated through the use of the double fluorescent antibody method. Both of these antigens appear before the first increase in cell-associated virus is detected. About 8 hours post infection, as the antibody staining of both LS and NP antigens extends throughout the cytoplasm, the orange-red and green stains intermix to produce a yellow-green color. Coincidentally there occurs fragmentation of the DNA-containing bodies in the cytoplasm detectable by the acridine orange stain, and the first increase in cell-associated virus. This combination of events clearly marks the final organization of the various components into mature vaccinia virus. However, it was not possible in the present studies to discern the structural and functional development of the infectious units.

In contrast the HA antigen does not appear to participate in viral formation, since it is not found until 10 or more hours after infection, at which time production of mature virus is well advanced (Text-fig. 2). This is in keeping with conclusions reached from other studies (10, 28), that the HA antigen is not an integral part of the vaccinia virus, but a by-product of infection.

The finding of inclusion bodies containing DNA through cytochemical stains is not new (23, 26, 29–31). These are the bodies within which the mature infective virus is formed (32–34). In the present study, the centers of viral DNA synthesis are easily demonstrable as early as the fourth hour post infection by use of the more sensitive acridine orange stain. The inclusion bodies, however, are stained by the labeled anti-NP sera only at a later stage of development (about 6 hours post infection), which strongly suggests that viral nucleic acid is synthesized before, or at a greater rate, than its accompanying protein. Cairns (27) has demonstrated through the use of tritiated thymidine and the autoradiographic technique, that the newly formed viral DNA, detectable as early as 3.5 hours post infection, is susceptible to deoxyribonuclease. The inclusion body itself was found to be a nucleoprotein complex resistant to the action of the enzyme, unless pretreated with a proteolytic enzyme (18). The staining of these bodies by the labeled anti-NP serum also suggests that they contain mainly NP antigen.

The reactivity of both anti-LS and anti-NP sera with whole vaccinia virus again supports the contention that these are surface antigens (7). Until further correlative immunological studies are made, it is difficult at this time to correlate the antigens studied with the complex electron microscopical structure of the virus as seen by Peters (35) and Epstein (36), or to the single- and double-membrane particles observed by Morgan *et al.* (34) in the infected tissue. Furthermore, the relationship of the formation of LS and NP antigens to the appearance of the mature virus, is not clear. Biochemical studies have shown that an increase in cytoplasmic protein occurs early in the infectious sequence before the appearance of infectious virus (37). A more detailed examination of these relationships by biochemical methods is now in progress.

The accompanying apparent increase of RNA in the cytoplasm of infected cells as demonstrated through the use of specific cytochemical stains, reaffirms the evidence that cellular RNA is involved in the synthesis of a DNA-containing virus such as vaccinia (37–40). As in other studies (27, 37, 41), the techniques used here do not suggest any but an indirect role of the nucleus in the vaccinia-infected cell.

SUMMARY

Virus-induced alterations in vaccinia virus-infected HeLa cells have been followed by immunofluorescent and cytochemical techniques. In a time sequence study, infected cells show an early increase in cytoplasmic RNA content, followed by appearance of centers of viral DNA synthesis in the cytoplasm. The centers of synthesis were detected at 4 hours post infection, with the acridine orange fluorochrome stain as compared to 6 hours with Feulgen and methyl green–pyronin stains. Marginal fragmentation of the inclusion bodies was seen at 8 to 10 hours post infection, and appears to coincide with the first increase in cell-associated virus.

With the immunofluorescent technique, it was found that the LS antigen of the virus can be detected at about 4 hours post infection. This is followed at 5 to 6 hours post infection by the appearance of the NP antigen. Both antigens are found only in the cytoplasm, and precede the appearance of the infective particle. The HA antigen, a by-product of virus-cell interaction, is not seen until about 10 hours post infection; that is, several hours after the appearance of both the LS and NP antigens, and only after the appearance of mature virus.

The successful application of the use of two immune sera, each labeled with a different fluorescent dye for the simultaneous visualization of two antigens within a cell, is reported. Using this technique, the sites of LS and NP antigen synthesis, were easily differentiated. The intimate mixing of the two antigens at a later stage appears to coincide with the fragmentation of the inclusion body and the first detectable increase in cell-associated virus. The evidence

obtained strongly suggests that the typical inclusion body observed in vaccinia-infected cells is composed mainly of the NP antigen.

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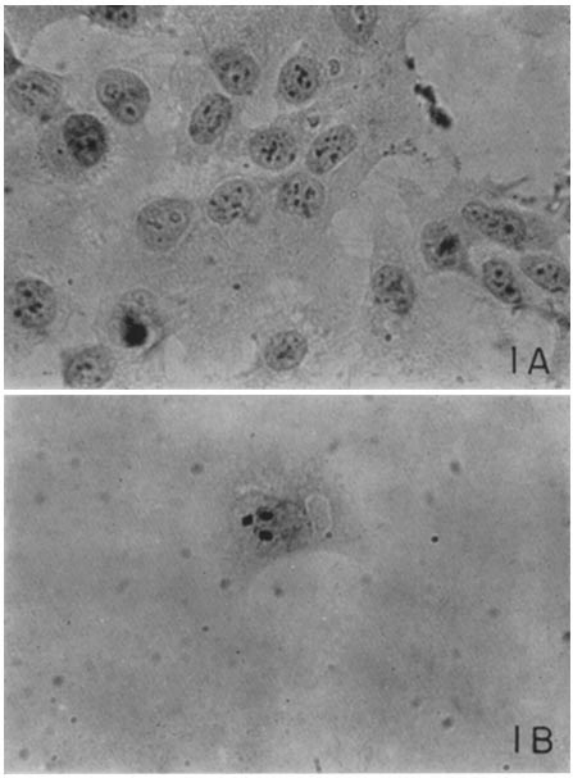
EXPLANATION OF PLATES

PLATE 20

FIG. 1A and 1B. Inclusion body formation in vaccinia virus-infected HeLa cell monolayers.

FIG. 1A. Six hours post infection Feulgen stain. × 320.

FIG. 1B. Eight hours post infection. Methyl green–pyronin stain. × 480.



(Loh and Riggs: Vaccinial antigens and virus in infected cells)

PLATE 21

FIGS. 2A to 2I. Inclusion body formation and the development of subviral antigens in vaccinia virus-infected HeLa cell monolayers.

FIG. 2A. Early appearance of inclusion body four hours post infection. Acridine orange stain. × 1600.

FIG. 2B. Marginal fragmentation of the inclusion body about 10 hours post infection. Acridine orange stain. × 1600.

FIG. 2C. LS-antigen stained with FITC-labeled anti-LS serum 6 hours post infection. × 640.

FIG. 2D. Same as 2C but at about 8 hours post infection. Note pockets of nonfluorescence suggesting sites of NP-antigen synthesis. × 640.

FIG. 2E. NP-antigen stained with FITC-labeled anti-NP serum 6 hours post infection. Note several centers of NP-antigen synthesis. × 960.

FIG. 2F. Same as 2E but at 8 hours post infection. Several centers of NP antigen synthesis have enlarged and fused. × 960.

FIG. 2G. HA-antigen stained with FITC-labeled anti-HA serum. 12 hours post infection. × 640.

FIG. 2H. 6 hours postinfection preparation stained with FITC-labeled anti-LS and TMRITC-labeled anti-NP sera. Note aggregated NP-antigen surrounded by diffuse LS antigen. × 640.

FIG. 2I. Same as 2H but at 8 hours post infection. Note mixing of antigens to give an intermediate color, and also another cell in an earlier state exhibiting NP antigen surrounded by LS antigen as in 2H. × 640.

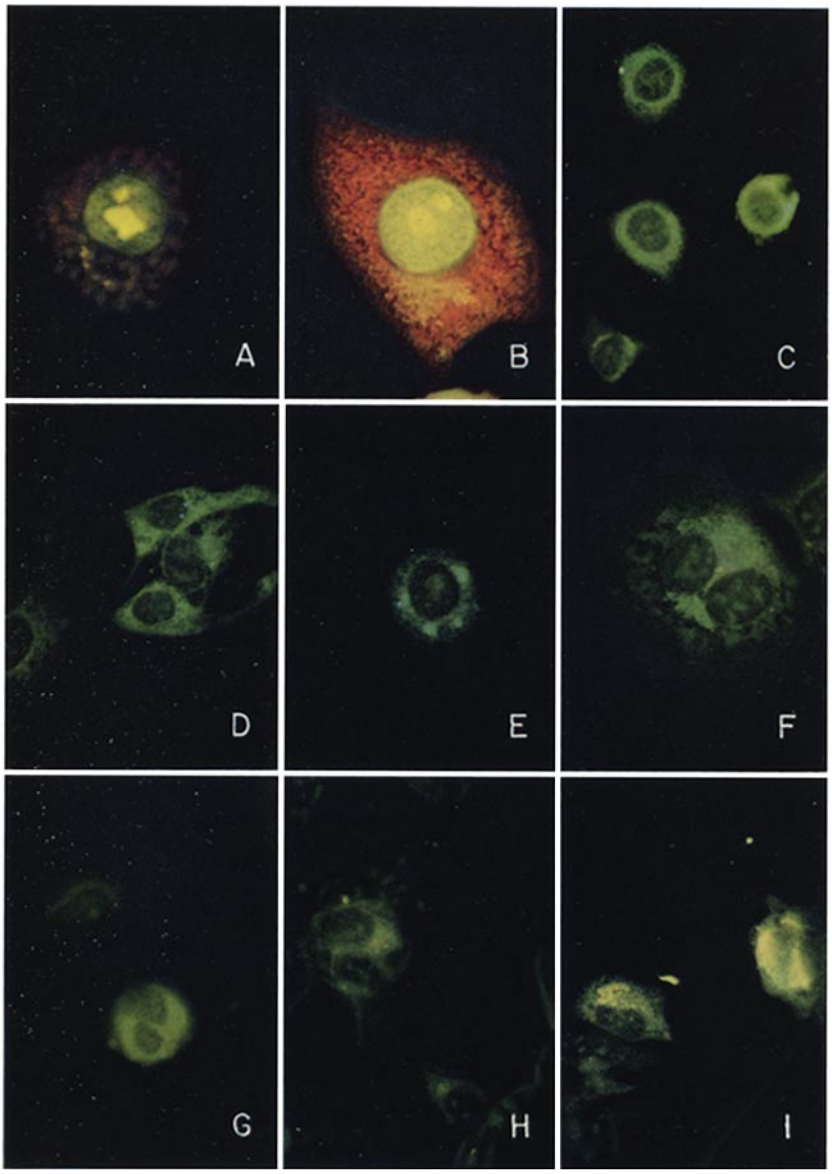


FIG. 2

(Loh and Riggs: Vaccinial antigens and virus in infected cells)