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INTERFERENCE OF ULTRAVIOLET INACTIVATED
PARAINFLUENZA 3 AND ECHO 21 VIRUSES
WITH THE ACTIVE VIRUSES IN TISSUE CULTURE

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William Ernest Steinmetz

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ABSTRACT

Experiments were conducted to investigate the possibility of the production of interference by ultraviolet inactivated (UVI) Parainfluenza 3 (Para 3) and Echo 21 viruses with active Para 3 and Echo 21 viruses in tissue culture (TC).

Four major experiments were conducted: the effect of UVI Para 3 on active Para 3 in rhesus monkey kidney cell (MKC) tissue culture; the effect of UVI Para 3 on active Para 3 in HEp-2 TC; the effect of UVI Para 3 on Echo 21 in MKC TC; and the effect of UVI Echo 21 on Echo 21 in MKC TC. The rates of production of cytopathic effect (CPE) by active viruses at a tissue culture infective dosage of 100% in TC was suppressed to a greater degree by UVI Para 3 and active Echo 21, than when the same virus combinations were used in HEp-2 TC.

INTRODUCTION

An animal or a culture of animal cells, infected by one virus, is sometimes rendered resistant to infection by another virus. This phenomenon is known as "interference." Henle (1950) presented a review of the interference phenomenon. He defined viral interference as "the ability of one virus, active or inactive, or component thereof, to interfere with the production of another virus when inoculated into the same host." Magrassi (1935) described interference by a non-encephalitogenic strain of Herpes virus with the growth of an encephalitogenic strain in rabbits. Hoskins (1935) stated that a neutropic strain of Herpes virus prevented infection with a viscerotropic strain. These authors reported that simultaneous inoculation of two different viruses will induce interference whereby neither will grow. The results reported by Magrassi (1935) and Hoskins (1935) have been disputed as being examples of true interference; protection in these cases might have been due wholly or in part to immunological factors (Isaacs and Burke, 1959). Findlay and MacCallum (1937) found that monkeys were protected by Rift Valley Fever virus from infection with yellow fever virus. It is widely accepted that this was the first report of true viral interference, as yellow fever virus and Rift Valley fever virus are immunologically

unrelated. Cross-immunity seems not to be involved (Isaacs and Burke, 1959).

Henle and Henle (1944) made the first studies dealing with factors influencing the phenomenon of interference between active and inactive influenza viruses. Work by Hollander and Oliphant (1944) using monochromatic ultraviolet radiation on influenza viruses and demonstrations by Henle, et al. (1947) of interference between ultraviolet-irradiated viruses and active viruses in host cells established the groundwork for further use of ultraviolet inactivation in interference studies.

Since the time of the initial work of Henle and Henle (1944) with the demonstration of interference between active and inactive viruses, researchers such as Isaacs (1963), among others, have reported widespread occurrence of the phenomenon and have demonstrated its clinical significance.

The use of tissue culture techniques has enhanced greatly research on interference. Harrison (1907) introduced tissue culture techniques that were modified and improved in succeeding years by Burrows (1911), Carrell (1912), and others. Steinhardt (1913) is credited with first growing viruses in tissue culture. Carrell and Ebeling (1926) and Maitlands (1928) further improved upon the earlier techniques

The work presented in this thesis was undertaken in view of the fact that no reports were found in the literature

concerning interference between ultraviolet inactivated (UVI) Parainfluenza 3 (Para 3) (Andrews, et al., 1959) and UVI Enteric Cytopathic Human Orphan 21 (Echo 21) (Wenner, 1962) with active Echo 21 in tissue culture. Para 3, a ribonucleic acid (RNA) myxovirus that causes infections of the respiratory system, was chosen because its interfering capacity previously has been demonstrated by many researchers (Schlesinger, 1959). Echo 21, a RNA containing enterovirus, immunologically unrelated to Para 3, is the eitological agent of aseptic meningitis, rubelliform rashes, respiratory infections, and diarrhea (Rhodes and van Rooyen, 1962). The active viruses used in the experiments were highly pathogenic and infective (Ciba Foundation, 1960). Two different tissue culture systems were employed for experimental comparison, as Isaacs, et al. (1961) have proposed that the degree of viral interference may be influenced by the choice of host tissue culture employed.

METHODS AND MATERIALS

Collection of Virus and Tissue Cultures

Echo 21 virus was supplied by the State Health Laboratory of Virginia in a host-tissue culture of kidney cells of the rhesus monkey, Macaca mulatta. Para 3 virus was supplied by the City of Richmond Public Health Laboratory in both rhesus monkey kidney and human epithelium tissue cultures.

Tissue cultures, rather than live animals, were used in the interference experiments because immunological factors due to the production of antibodies could be ruled out (Habel, et al., 1958 and Isaacs and Burke, 1959). Continuous cell line human epithelium (HEp-2), originally derived from epidermoid carcinoma of human larynx (Toolan, 1954),was obtained from the Virginia State Health Laboratory, and was maintained by subculturing. Non-continuous, primary cell line rhesus monkey kidney cells (MKC) were obtained from both the Virginia State Health Laboratory and the City of Richmond Public Health Laboratory. The HEp-2 (Fig. 2C) and MKC (Fig. 1A) lines were grown and maintained according to methods given by Becton-Dickinson Laboratories, Inc. (1966). The tissue cultures were tested for contamination with pleuropneumonialike organisms, bacteria, L-forms, yeasts, and filamentous fungi by the use of appropriate standard methods (Merchant, et al., 1964). Contaminated cultures were discarded.

Subculturing of Tissues

Detailed recipes for the various media and solutions used are presented in the Appendix.

Buffered crystalline-potassium Penicillin G (Eli Lilly and Company) and Pfizer Laboratory Combistrep (a mixture of Dihydrostreptomycin Sulfate and Streptomycin Sulfate) were dissolved in single strength (1X) Hanks Balanced Salt Solution (BSS) (Hanks, 1949) and were added to TC media at the rate of 100 units/ml and 100 µg/ml respectively, to minimize contamination. Two-tenths percent phenol red, as a pH indicator, was included in all media, and in other solutions where appropriate.

Tenfold strength (10X) Ca^{++}- and Mg^{++}-free phosphate-buffered saline (CMF-PBS) was prepared according to a recipe given by Merchant, et al. (1964). The 10 X CMF-PBS was diluted tc 1X in preparing the stock solution of 0.25% Difco 1:250 trypsin used for dispersal of cell sheets.

To obtain subcultures of HEp-2 for experiments, growth medium was decanted from TC monolayers grown in 500 ml oval, soft-glass bottles (Duraglass, Owens-Illinois Glass Company). Tissue monolayers were washed twice with 1X BSS. Following the washes, the monolayers were dispersed by trypsinization using modifications of the techniques reported by Scherer (1953), Scherer et al. (1958), and Syverton, et al. (1954). Ten ml of 0.25%

trypsin in CMF-PBS were allowed to remain on each HEp-2 TC for one minute and then decanted. The residual trypsin acted at 35 C until the cell sheet became dispersed. as determined by visual inspection (approximately 20 to 45 minutes). Cell clusters were dispersed to single cells by repeatedly drawing the suspension into a pipette and then ejecting it back into a solution of 1X CMF-PBS. The resulting suspension was then pipetted into sterile 10 ml tubes that were corked and then centrifuged at 250 rpm for five minutes. The supernatant was decanted, the cells were resuspended in 1X CMF-PBS, and centrifuged again at 250 rpm for five minutes. The supernatant was decanted again, and the cells were resuspended in 10 ml of growth medium (GM) consisting of Eagle Minimal Essential Medium (MEM) (Eagle, 1959) plus 10% fetal calf serum (both obtained from Microbiological Associates). Ten ml of dispersed cell suspension were diluted to 100 ml with additional GM to give a concentration of approximately 100,000 cells/ml (Earle and Sanford, 1951). To initiate stock cultures, bottles (500 ml) were seeded with 20 ml of cell suspension. A Cornwall pipette was used to distribute 1.0 ml of cell suspension into soft-glass test tubes (30 ml capacity, from Microbiological Associates). The tubes were then capped tightly with rubber-lined screw caps, and were incubated in a slanted position at 35 C until complete cell monolayers had formed. A sterile, aqueous solution of 1.4%

(wt/vol) sodium bicarbonate was added to adjust changes in pH where necessary.

The MKC were originally planted in Melnick medium with Hanks base (Melnick, 1955). Monkey kidney cells were fed with Melnick medium with Earle base. pH 7.0 ± 0.2 (Earle, 1943).

When a complete HEp-2 or MKC monolayer had formed, the GM was decanted and an equivalent amount of Scherer's maintenance medium (Microbiological Associates) was added (Scherer, 1953). The cultures were incubated on maintenance medium for 24 hours before use.

All bottles and test tubes for tissue cultures were prepared for use by soaking in potassium dichromate-sulfuric acid cleaning solution for one hour, followed by fifteen rinses with tap water and five with glass-distilled water. The glass culture vessels were sterilized by hot air at 360 C for two hours. Other glassware, such as graduated cylinders and medium flasks, were washed in Haemosol, rinsed ten times with tap water and three times with glass-distilled water.

Media, buffer solutions, salt solutions, and serum were sterilized by filtration through either a Seitz or a 0.5 μ Millipore filter. Glass-distilled water, flasks, cylinders, caps for TC tubes, bottles, and acid and base solutions were autoclaved at 15 psi for 15 minutes.

Preliminary Experiments

A number of preliminary experiments were performed to obtain basic information necessary to conduct the major experiments.

A virus when passed through tissue culture may lose its major disease-producing properties and become avirulent. Para 3, however, demonstrated greater degrees of CPE on each passage, as noted by Lennette and Schmidt (1964). Echo 21 and Para 3 viruses produced one or more of the following readily visible cytopathic effects in a TC system in agreement with CPE criteria outlined by Rhodes and van Rooyen (1962): necrosis of cells (Figure 2G and 3B), formation of syncytia (Fig. 1G), sloughing of the cell sheet from the vessel wall (Figures 2F and 3E). changes in pH, formation of giant cells (Figures 1D and 2D). and cell lysis (Fig. 1H).

Because a large volume of virus-containing inoculum was needed, cultures of Echo 21 grown in MKC were frozen at -20 C and were pooled after the third stage cytopathic effect (3+ CPE) in which the TC monolayers were 75% destroyed (Lennette and Schmidt, 1964). The pooled cultures subsequently were stored at -20 C. Para 3 virus was grown in both MKC and HEp-2 TC that were frozen at -20 C after sufficient time had been allowed for the production of abundant giant cells and partial monolayer destruction (Lennette and Schmidt. 1964), and were pooled before use.

The infective strength of viruses (concentration of inocula) was judged by the extent to which they could be diluted before they failed to produce signs of growth in TC. Serial dilutions of a virus were prepared as follows: 0.9 ml BSS was pipetted into each of 8 tubes; 0.1 ml of virus inoculum was added to the first tube and mixed; 0.1 ml of the mixture from the first tube was pipetted into the second, and this process was repeated for the remaining six tubes to obtain dilutions of 10^{-1} through 10^{-8}; 0.1 ml was discarded from the last tube.

The first step in determining tissue culture infectious dosage (TCID) was to set up a control tube containing TC. Next, tubes of TC each containing 0.9 ml of maintenance medium were arranged in eight sets of five each for MKC, and ten each for HEp-2. One set was inoculated with 0.1 ml of active virus concentrate, and into each successive set, 0.1 ml of the appropriate active virus dilution was inoculated; dilutions of 10^{-1} through 10^{-9} resulted. Stages of CPE were observed at intervals of 12, 24, 48, and 72 hours, or until 3+ CPE stage was obtained in 50% of the tubes (Figures 1, 2, and 3). Tissue culture infectious dose 50% ($TCID_{50}$) (Reed and Muench, 1938) and effective dose 50% (ED_{50}) (a more precise estimate) were determined (Finney, 1951).

The $TCID_{50}$ for Echo 21 in MKC occurred at a dilution of 10^{-3} (Table 1A), and the ED_{50} occurred between $10^{-3.5}$ and

$10^{-3.0}$ (Table 1B). By interpolation, ED_{50} was determined to occur at a dilution of $10^{-3.17}$. If the virus particles were actually distributed at random in the original suspension, the proportion of inocula that contained no particles would have been e^{-m}, where e was equal to 2.718 (the base of the natural logarithms) and m was the average number of particles/0.1 ml of inoculum. The average number of infectious particles/0.1 ml of inoculum, d, was determined to be 0.22 for a dilution of $10^{-3.5}$ and 0.92 for a dilution of $10^{-3.0}$ (Table 1C), from the formula used by Finney (1951) and Chang (1958): $d = \log_e(1-p) = 2.302 \log_{10}(1-p)$. The particle density estimate expressed in terms of the single dilution, $10^{-3.0}$, for $10^{-3.5}$ was 0.70, and for $10^{-3.0}$ was 0.92 (Table 1C). In the intermediate dilutions, the estimates of d were proportional to the concentration of the virus. In the original suspension, the average number of infectious particles were approximately 0.81×10^7 particles/0.1 ml of inoculum.

The $TCID_{50}$ for Para 3 in MKC occurred at a dilution of 10^{-5} (Table 2A) and ED_{50} occurred between dilutions of $10^{-4.0}$ and $10^{-5.0}$ (Table 2B). By interpolation, ED_{50} was determined to occur at a dilution of $10^{-4.7}$. The average number of infectious particles/0.1 ml of inoculum was determined to be 0.51 for a dilution of $10^{-5.0}$ and 1.61 for a dilution of $10^{-4.0}$ (Table 2C). The particle density estimate expressed in terms of the single dilution, $10^{-5.0}$, for $10^{-5.0}$ was 0.51;

and for $10^{-4.0}$, was 0.48 (Table 2C). In the original suspension, the average number of infectious particles was determined to be approximately 0.50 X 10^{7} particles/0.1 ml of inoculum.

The $TCID_{50}$ for Para 3 in HEp-2 occurred at a dilution of 10^{-4} (Table 3A), and the ED_{50} occurred between dilutions of $10^{-4.0}$ and $10^{-3.0}$ (Table 3B). By interpolation ED_{50} was determined to occur at a dilution of $10^{-3.68}$. The average numbers of infectious particles/0.1 ml inoculum were determined to be 0.11, 0.51, and 1.20 for dilutions of 10^{-5}, 10^{-4}, and 10^{-3}, respectively (Table 3C). The particle density estimates expressed in terms of the single dilution, 10^{-4}, were 0.35, 0.51, and 0.36 for dilutions of 10^{-5}, 10^{-4}, and 10^{-3} respectively (Table 3C). In the original suspension, the average number of infectious particles was determined to be approximately 0.40 X 10^{7} particles/0.1 ml of inoculum.

An unsuccessful attempt was made to adapt Echo 21 to HEp-2 TC by inoculating three sets of HEp-2 cultures with dilutions of 10^{-1} through 10^{-9} (Table 4)

The titer of active Echo 21 inoculum was determined to be approximately 1:1000 (Table 5) by a modification of the antiserum neutralization procedure as outlined by Lennette and Schmidt (1964). Dilutions of Echo 21 antiserum (Microbiological Associates) in maintenance medium were incubated for one hour with Echo 21 at a concentration that was 100% infective to

tissue cultures ($TCID_{100}$). After an hour's incubation at 35 C, dilutions were inoculated into MKC TC and the rate of CPE was observed (Table 5).

The titer of active pooled Para 3 virus inoculum was determined to be approximately 1:1000 (Table 6), by a modification of the red blood cell (RBC) hemagglutination procedure given by Lennette and Schmidt (1964). The procedure was as follows: 0.1 ml of 2.5% chicken RBC in physiological saline (0.85% NaCl) was distributed to each hemagglutination tube: the degrees of hemagglutination of dilutions of standardized antigen in physiological saline were compared with the degrees of hemagglutination of dilutions of active Para 3 in physiological saline.

Ultraviolet Inactivation of Viruses

Ultraviolet inactivation (UVI) was accomplished in the following manner: 3 ml of each pooled virus were pipetted into the bottom of an open 60 ml Petri dish; each dish was placed 15 cm below a Westinghouse germicidal lamp (2537Å) and was agitated every 40 seconds for two hours (Baluda, 1957); as evaporation occurred, Dulbecco phosphate-buffered saline (Dulbecco and Vogt, 1954), as recommended by Powell and Setlow (1956), was added to maintain a total volume of 3 ml. Dilutions of 10^0 through 10^{-4} of each UVI virus were tested for signs of infectivity in TC. The titer of UVI Para 3 was

approximately 1:1000 as determined by the same method used for active Para 3 in the previous preliminary experiment (Table 6).

Ultraviolet rather than hard radiation was used to inactivate the virus, as Powell and Pollard (1956) found that, occasionally, "nonuniformity of the cyclotron beam left significant residual infectivity titers in the irradiated samples." A Westinghouse germicidal lamp of the wavelength 2537Å was used for ultraviolet inactivation of the viruses because the greatest percentage of absorption of ultraviolet light by nucleic acids normally occurs between 2500 and 2650Å (Giese, 1958).

Photomicrographs

Photomicrographs of unstained tissue cultures, uninfected and infected, were taken with a 35 mm camera using the 100 X lens combination of a compound microscope. The tissues were photographed through the relatively thick walls of the culture vessels, and thus lack the clarity that would result from other types of preparations. The figures in this thesis all are at the same degree of magnification (approximately 345 X).

Experimental Procedure

The following general procedures were used to conduct the major experiments: two sets of dilutions for each UVI virus

inoculum (Tables 14-17) were allowed to remain on TC at the rate of 0.1 ml/culture and were decanted after 24 hours incubation at 35 C. Into these same cultures, dilutions of active virus were inoculated at the rate of 0.1 ml/culture. Simultaneously, to serve as controls, sets of cultures not previously inoculated, but of the same incubation age, were inoculated with corresponding dilutions of active virus. The four major experiments were as follows:

1. The Effect of UVI Para 3 on Active Para 3 in MKC TC
2. The Effect of UVI Para 3 on Active Para 3 in HEp-2 TC
3. The Effect of UVI Para 3 on Active Echo 21 in MKC TC
4. The Effect of UVI Echo 21 on Active Echo 21 in MKC TC

RESULTS

Experiment 1. The Effect of UVI Para 3 on Active Para 3 in MKC TC

The initial culture inoculum of approximately 100,000 monkey kidney cells/test tube increased in number to approximately 1,000,000.

The rate of CPE by active Para 3 virus was suppressed by the previous addition of UVI Para 3 virus as compared to the cytopathic rate of the controls. The addition of UVI Para 3 in relative $TCID_{100}$ concentration completely suppressed production of CPE by active Para 3 $TCID_{50}$ concentration, and altered the rate of the production of CPE by $TCID_{100}$ concentration of active virus (Table 7).

Interference of growth of active Para 3 virus by UVI Para 3 virus for dilutions of 10^{-1}, 10^{-2}, 10^{-3} occurred at relative calculated concentrations of 5.0, 0.5, and 0.05 UVI Para 3 virus particles/MKC.

The photomicrographs in Fig. 1 show the stages in the production of CPE by active HEp-2 virus in MKC TC.

Experiment 2. The Effect of UVI Para 3 on Active Para 3 in HEp-2 TC

The initial culture inoculum of approximately 100,000 HEp-2 cells/test tube increased in number to approximately 1,000,000.

The addition of UVI Para 3 virus to HEp-2 TC before inoculation with active Para 3 in $TCID_{100}$ concentration delayed the production of CPE in HEp-2 TC inoculated with active Para 3 in $TCID_{50}$ concentration. Other concentrations of UVI Para 3 showed no significant alteration of the rate of production of CPE by active virus (Table 8).

Interference of growth of active Para 3 virus by UVI Para 3 virus for a dilution of 10^{-4} occurred at the relative calculated concentration of 4.0 UVI Para 3 virus particles/ HEp-2 cell.

The photomicrographs in Fig. 2 demonstrate the stages in the production of CPE by active Para 3 virus in HEP-2 TC.

Experiment 3. The Effect of UVI Para 3 on Active Echo 21 in MKC TC

The addition of UVI Para 3 to MKC TC before active virus inoculation delayed significantly the rate of CPE by Echo 21 virus in MKC TC (Table 9). A normally rapidly infective $TCID_{50}$ inoculum of Echo 21, as compared to the controls, showed delayed CPE when the TC had first been inoculated with UVI Para 3 virus in $TCID_{100}$ concentration.

Interference of growth of active Echo 21 virus by UVI Para 3 virus for a dilution of 10^{-1} occurred at the relative calculated concentration of 5.0 Para 3 virus particles/MKC.

The photomicrographs in Fig. 3 show the stages in the

production of CPE by active Echo 21 virus in MKC TC.

Experiment 4. The Effect of UVI Echo 21 on Active Echo 21 in MKC TC

Monkey kidney cell tissue cultures previously inoculated with $TCID_{100}$ relative concentration of UVI Echo 21 showed no delay in the production of CPE by $TCID_{100}$ of active Echo 21. The rate of CPE by $TCID_{50}$ active Echo 21 was delayed by previous addition of $TCID_{100}$ relative concentration of UVI Echo 21 in MKC TC (Table 9).

Interference of growth of active Echo 21 virus by UVI Echo 21 virus for a dilution of 10^{-1} occurred at the relative calculated concentration of 8.1 UVI Echo 21 virus particles/ MKC.

DISCUSSION

These experiments were unique in that not only UVI Para 3 but also UVI Echo 21 caused interference of growth of the active viruses in TC. Earlier workers demonstrated numerous examples of interference among strains of a single virus, pairs of immunologically related viruses and unrelated viruses (Schleisinger, 1959). The initial studies dealt mainly with interference between "live" viruses in a number of animals and TC systems. Henle and Henle (1944) and Henle (1950) showed that viruses inactivated by a number of means caused interference where they otherwise would have caused CPE in tissue culture.

Production of CPE

One of the main criteria for the determination of growth of viruses has been the detection of readily visible CPE. The production of bacterial CPE was held at a minimum by the use of antibiotics in TC as introduced by Enders, et al. (1949). Penicillin and streptomycin were the only antibiotics used because others have often been toxic to the TC system.

Cytopathic effect due to changes in pH were ruled out by maintenance of pH 7.0$\pm$ 0.2 by various buffer systems included in media and the addition of dilute acid, base, or buffer to the individual test tubes and bottles.

Glass distilled water was used because it proved to be

relatively free from organic contamination, toxins, and other substances that may cause CPE and are often present in distilled water from other sources.

The production of malignancy in in vitro cells at the glass interphase was lessened by the use of specially cleaned and prepared soft-glass bottles and test tubes (Earle, et al., 1950). The dispersal of cell clumps into individual cells was facilitated by the use of CMF-PBS trypsin solution from which chelating agents, Ca^{++}, and Mg^{++} were omitted (Zeidman, 1947); and for best proliferation 100,000 cells inoculum per test tube was used (Earle, et al., 1951).

Attempt to Adapt Echo 21 Virus to HEp-2 TC

An unsuccessful attempt was made to adapt Echo 21 virus to HEp-2 TC in order to obtain additional data for experimental comparisons and to determine, for Echo 21, the validity of the proposals of Isaacs, et al. that the degree of viral interference may be influenced by the choice of host TC employed. Another purpose of this experiment was to seek a reason for UVI virus interference in HEp-2 TC, where interferon activity has been ruled out (Isaacs, 1961). Echo 21 virus may not have grown in HEp-2 TC because of pH factors (Barron and Karzon, 1957). Rhodes and van Rooyen (1962) noted that Echo 21 grew poorly in HeLa cell TC, a carcinoma cell line similar to HEp-2. Isaacs (1961) suggested that since viruses, including the Echo group, differed in their sensitivity to

oxygen, some grew in cells under less aerobic conditions than others. Cancer cells which did not require aerobic phosphorylation would support the growth of viruses adaptable to this condition. Viruses, when inoculated in sufficient infective quantity, will grow in a tissue culture system surrounded by a nutrient medium, but the tissue and medium both must reproduce closely the conditions in the original host source of infection (Geyer, 1958 and Morgan, 1958). Thus, the process of bringing about virus adaptation to various TC systems becomes a difficult task.

Ultraviolet Inactivation

It was observed that the UVI viruses retained interfering ability, hemadsorption, and hemagglutination qualities in agreement with experiments by Woese and Pollard (1954) and Baluda (1957) that dealt with the effects of ionizing radiation on various properties of Newcastle disease virus, a myxovirus containing RNA.

Two advantages of using inactivated virus rather than active virus for interference experiments were considered: inactive viruses have less influence on metabolic activity in host cells, thus simplifying the search for the point where the interference with virus growth occurs (Henle and Henle, 1944); and inactivated viruses can induce interference at dosages which do not produce obvious cytopathic effects, so that cellular disorganization can be excluded as a cause of

interference by inactive virus (Ziegler and Horsfall, 1944).

Possible explanations of the mechanisms by which ultraviolet light inactivates viruses have been proposed by a number of workers. Powell, et al. (1956) further improved, expanded, and refined previous work in their studies to determine the effects of monochromatic ultraviolet radiation on the interfering property of RNA containing influenza virus. They found that oxygen had no effect on their results and that UVI reduced infectivity without reducing interfering ability. Research by Marmur, et al. (1961) with bio-physio-chemical effects of ultraviolet light on DNA led Setlow and Setlow (1962) to propose that ultraviolet-induced thymine dimers in DNA caused the biological damage.

Production of Interference

The UVI virus particle to cell ratios from the major experiments in this thesis were consistent with those described by previous workers.

Henle (1950) stated that about ten UVI viruses per cell were sufficient to induce interference. Groth and Edney (1952) hypothesized that under certain conditions one interfering virus particle per cell can completely inhibit the production of challenge virus by that cell. This was the case when ultraviolet inactivated Melbourne Influenza (Mel) virus was used as the interfering agent and a neurotropic variant of WS virus (NWS), as the challenge agent (Powell and Pollard, 1956).

A number of researchers have proposed explanations of the mechanisms of virus interference. Baluda (1957) demonstrated interference of the growth of active viruses by UVI Newcastle disease virus of chickens. He proposed that interference came about as a result of the blockage and destruction of specific virus receptors on cells. Henle (1950) described another distinct variety of interference in which UVI influenza virus was found to interfere with the growth of active virus of the same serological group.

Cases in which inhibition of virus growth occurred after host inoculation, and not explained by immunity due to antibodies, resistance, or competition have been attributed a protective substance, interferon, which was purified by Burke (1961), with interference properties. Isaacs and Burke (1959) proposed that interferon was not a single substance but was a group of similar substances; that it was not self-replicating; was serologically distinct from the virus; and had protein properties. Isaacs and Hitchcock (1960) found that lungs of mice infected with influenza virus produced interferon. Enders (1960) found that in some cases attenuated strains of a virus produced more interferon than unmodified strains.

Research conducted by Somer, et al. (1962) lead to an explanation of the mechanism of action of interferon. They stated that interferon action occurred within the cell, after

penetration of virus and before formation of mature virus particles. Their studies indicated that virus DNA or RNA introduced into target cells forces the cell to produce viral messenger RNA that instructs the cell to make proteins for the virus. The process of synthesizing virus protein is hampered by the co-stimulation of the cell to produce another protein, interferon, protective in nature, which blocks the production of viral RNA. Extracted RNA of myxoviruses has been shown to be non-infectious (Rhodes and van Rooyen, 1962). Lockhart (1964) demonstrated the necessity for cellular RNA and protein synthesis for viral inhibition resulting from interferon. Isaacs (1963) and Friedman (1964) conducted further research dealing with viral interference induced by interferon. Research by Ho, et al. (1965) agreed with that of Somer, et al. (1962), Isaacs (1963), and Friedman (1964). Ho and Breinig (1965) found that interferon appeared as early as two hours after absorption of inoculum of interferon inducer and represented new protein synthesis. Large-scale interferon production (Burke and Buchan, 1965) is stimulated in chick embryo cells by ultraviolet inactivated viruses. The rate of interferon production in their studies varied with the virus used. The production of interferon increased with the dosage of ultraviolet-inactivated virus until a plateau was reached. Isaacs, et al. (1961) found that interferon uncoupled oxidative phosphorylation, probably in the nucleus of the cell;

cancer cells and embryonic cells that did not require aerobic phosphorylation for their energy requirements were much less sensitive to interferon than those that required oxygen.

In sum, experiments were conducted to investigate the possibility of the production of interference by UVI Para 3 and Echo 21 viruses with active virus in TC. Since one of the main criteria used for the determination of virus growth was the detection of readily visible CPE, precautions were taken to rule out production of CPE in TC by factors other than virus growth. The four major experiments were: the effect of UVI Para 3 on active Para 3 in MKC TC; the effect of UVI Para 3 on active Para 3 in HEp-2 TC; the effect of UVI Para 3 on active Echo 21 in MKC TC; and the effect of UVI Echo 21 on active Echo 21 in MKC TC. The rates of production of CPE by active viruses at a $TCID_{100}$ were suppressed to a greater degree by UVI Para 3 in MKC when the challenge viruses were active Para 3 and active Echo 21 than when the same virus combinations were used in HEp-2 TC.

These experiments were unique in that not only UVI Para 3 but also UVI Echo 21 virus caused interference of the growth of active virus in TC; that the UVI virus particle to TC cell ratios were within numerical boundaries determined by previous interference research; that ultraviolet inactivation did not destroy the hemadsorption and hemagglutination

qualities of the active viruses: and that the degree of viral interference may be influenced by the choice of TC employed.

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TABLES

TABLE 1

A Determination of Infectivity of Echo 21 Virus in Monkey Kidney Cell Tissue Culture

A. Determination of $TCID_{50}$ for Echo 21 Virus in Monkey Kidney Cell Tissue Culture
B. Determination of ED_{50} for Echo 21 Virus in Monkey Kidney Cell Tissue Culture
C. Particle Density Estimate for Echo 21 in Monkey Kidney Cell Tissue Culture

A.

Dilution of Virus	Logarithm of Dilution	Proportion of Positive Cultures
$10^{-1.0}$	-1.0	5/5 = 1.0
$10^{-2.0}$	-2.0	5/5 = 1.0
$10^{-2.5}$	-2.5	5/5 = 1.0
$10^{-3.0}$	-3.0	3/5 = 0.6
$10^{-3.5}$	-3.5	1/5 = 0.2
$10^{-4.0}$	-4.0	0/5 = 0.0
$10^{-5.0}$	-5.0	0/5 = 0.0

B.

Logarithm of Dilution of Virus	Observed Number of Cultures		Accumulated Number of Cultures (p)		% Positive $100(\frac{p}{n+p})$
	+	-	+	-	
-5.0	0	5	0	16	0.0
-4.0	0	5	0	11	0.0
-3.5	1	4	1	6	15.7
-3.0	3	2	4	2	66.6
-2.5	5	0	9	0	100
-2.0	5	0	14	0	100
-1.0	5	0	19	0	100

C.

Dilution of Virus	Proportion of Positive Cultures (p)	Estimated Number of Particles/Inoculum (d)	Estimated Number of Particles/Inoculum at a $10^{-3.0}$ Dilution
$10^{-4.0}$	0.0		
$10^{-3.5}$	0.20	0.22	0.70
$10^{-3.0}$	0.60	0.92	0.92
$10^{-2.5}$	1.0		

TABLE 2

A Determination of Infectivity of Parainfluenza 3 Virus in Monkey Kidney Cell Tissue Culture

A. Determination of $TCID_{50}$ for Parainfluenza 3 Virus in Monkey Kidney Cell Culture

B. Determination of ED_{50} for Parainfluenza 3 Virus in Monkey Kidney Cell Tissue Culture

C. Particle Density Estimate for Parainfluenza 3 Virus in Monkey Kidney Cell Tissue Culture

A.

Dilution of Virus	Logarithm of Dilution	Proportion of Positive Cultures
$10^{-1.0}$	-1.0	5/5 = 1.0
$10^{-2.0}$	-2.0	5/5 = 1.0
$10^{-3.0}$	-3.0	5/5 = 1.0
$10^{-4.0}$	-4.0	4/5 = 0.8
$10^{-5.0}$	-5.0	2/5 = 0.4
$10^{-6.0}$	-6.0	0/5 = 0.0
$10^{-7.0}$	-7.0	0/5 = 0.0

B.

Logarithm of Dilution of Virus	Observed Number of Cultures		Accumulated Number of Cultures (p)		% Positive $100(\frac{p}{n+p})$
	+	-	+	-	
-7.0	0	5	0	14	0.0
-6.0	0	5	0	9	0.0
-5.0	2	3	2	4	33.3
-4.0	4	1	6	1	85.7
-3.0	5	0	11	0	100
-2.0	5	0	16	0	100
-1.0	5	0	21	0	100

C.

Dilution of Virus	Proportion of Positive Cultures (p)	Estimated Number of Particles/Inoculum (d)	Estimated Number of Particles/Inoculum at a $10^{-5.0}$ Dilution
$10^{-6.0}$	0.0		
$10^{-5.0}$	0.40	0.51	0.51
$10^{-4.0}$	0.80	1.61	0.48
$10^{-3.0}$	1.0		

TABLE 3

A Determination of Infectivity of Parainfluenza 3 Virus in HEp-2 Tissue Culture

A. Determination of $TCID_{50}$ for Parainfluenza 3 Virus in HEp-2 Tissue Culture
B. Determination of ED_{50} for Parainfluenza 3 Virus in HEp-2 Tissue Culture
C. Particle Density Estimate for Parainfluenza 3 Virus in HEp-2 Tissue Culture

A.

Dilution of Virus	Logarithm of Dliution	Proportion of Positive Cultures
$10^{-1.0}$	-1.0	10/10 = 1.0
$10^{-2.0}$	-2.0	10/10 = 1.0
$10^{-3.0}$	-3.0	7/10 = 0.7
$10^{-4.0}$	-4.0	4/10 = 0.4
$10^{-5.0}$	-5.0	1/10 = 0.1
$10^{-6.0}$	-6.0	0/10 = 0.0

B.

Logarithm of Dilution of Virus	Observed Number of Cultures		Accumulated Number of Cultures (p)	(n)	% Positive $100(\frac{p}{n+p})$
	+	-	+	-	
-6.0	0	10	0	28	0.0
-5.0	1	9	1	18	5.2
-4.0	4	6	5	9	35.7
-3.0	7	3	12	3	80.0
-2.0	10	0	22	0	100
-1.0	10	0	32	0	100

C.

Dilution of Virus	Proportion of Positive Cultures (p)	Estimated Number of Particles/Inoculum (d)	Estimated Number of Particles/Inoculum at a $10^{-4.0}$ Dilution
$10^{-6.0}$	0.0		
$10^{-5.0}$	0.1	0.11	0.35
$10^{-4.0}$	0.4	0.51	0.51
$10^{-3.0}$	0.7	1.20	0.36
$10^{-2.0}$	1.0		

TABLE 4

An Attempt to Adapt Echo 21 Virus to HEp-2 Tissue Culture

Virus Concentration	Cytopathic Effect (Hours)			
	24	48	72	96
$10^{-1.0}$	All -	+ - -	+ - -	3+ - -
$10^{-2.0}$	"	+ - -	+ - -	2+ - -
$10^{-3.0}$	"	+ - -	+ - -	+ - -
$10^{-4.0}$	"	- - -	- - -	+ - -
$10^{-5.0}$	"	- - -	- - -	+ - -
$10^{-6.0}$	"	- - -	- - -	+ - -
$10^{-7.0}$	"	+ - -	2+ - -	4+ - -
$10^{-8.0}$	"	+ - -	2+ - -	4+ - -
$10^{-9.0}$	"	+ - -	2+ - -	4+ - -

TABLE 5

A Determination of the Titer of Active Echo 21 Virus by Antiserum Neutralization Test in Monkey Kidney Cell Tissue Culture

Dilution of Antiserum	Dilution of Virus	Cytopathic Effect (Hours) 24	48	72	96
$10^{-1.0}$	10^{-1} (TCID100)	-	-	-	-
$10^{-1.5}$	"	-	-	-	-
$10^{-2.0}$	"	-	-	-	-
$10^{-2.25}$	"	-	-	-	-
$10^{-2.5}$	"	-	-	-	-
$10^{-3.0}$	"	-	+	2+	3+
$10^{-4.0}$	"	+	2+	3+	4+

TABLE 6

A Determination of the Titer of Active and Ultraviolet Inactivated Parainfluenza 3 Virus from Hemagglutination Test

	Hemagglutination		
Dilution	Para 3 Antigen	Active Para 3	Ultraviolet Inactivated Para 3
$10^{-1.0}$	+	+	+
$10^{-2.0}$	+	+	+
$10^{-3.0}$	-	+	+
$10^{-4.0}$	-	-	-
$10^{-5.0}$	-	-	-
$10^{-6.0}$	-	-	-
$10^{-7.0}$	-	-	-
$10^{-8.0}$	-	-	-
$10^{-9.0}$	-	-	-

TABLE 7

Interference of Ultraviolet Inactivated Parainfluenza 3 Virus with Active Parainfluenza 3 Virus in Monkey Kidney Cell Tissue Culture

Dilution of UVI Para 3	Dilution of Active Para 3	Cytopathic Effect (Hours after Inoculation with Active Para 3)										
		24		48		72		96		120		
		1	2	1	2	1	2	1	2	1	2	
$10^{-1.0}$	$10^{-1.0}$	-	-	-	-	+	+	+	+	3+	3+	
$10^{-1.0}$	$10^{-2.0}$	-	-	-	-	+	+	+	+	3+	3+	
$10^{-1.0}$	$10^{-3.0}$	-	-	-	-	+	+	+	+	2+	+	
$10^{-1.0}$	$10^{-4.0}$	-	-	-	-	+	-	+	-	+	-	
$10^{-1.0}$	$10^{-5.0}$	-	-	-	-	-	-	-	-	-	-	
$10^{-1.0}$	$10^{-6.0}$	-	-	-	-	-	-	-	-	-	-	
$10^{-2.0}$	$10^{-1.0}$	-	-	-	-	+	+	+	+	3+	3+	
$10^{-3.0}$	$10^{-1.0}$	-	-	-	-	+	-	+	-	3+	2+	
$10^{-4.0}$	$10^{-1.0}$	-	-	+	+	+	+	2+	2+	3+	3+	
$10^{-5.0}$	$10^{-1.0}$	-	-	+	+	+	+	2+	2+	3+	3+	
$10^{-6.0}$	$10^{-1.0}$	-	-	+	+	+	+	2+	2+	3+	3+	

	Dilutions of Controls	24	48	72	96	120	(3+)
	$10^{-1.0}$	-	+	+	2+	3+	5/5
	$10^{-2.0}$	-	+	+	2+	3+	5/5
	$10^{-3.0}$	-	+	+	2+	3+	5/5
	$10^{-4.0}$	-	-	+	+	3+	4/5
	$10^{-5.0}$	-	-	-	+	3+	2/5
	$10^{-6.0}$	-	-	-	-	-	0/5

TABLE 8

Interference of Ultraviolet Inactivated Parainfluenza 3 Virus with Active Parainfluenza 3 Viruses in HEp-2 Tissue Culture

Dilution of UVI Para 3	Dilution of Active Para 3	Cytopathic Effect (Hours after Inoculation with Active Para 3)									
		24		48		72		96		120	
		1	2	1	2	1	2	1	2	1	2
$10^{-1.0}$	$10^{-1.0}$	-	-	+	+	2+	2+	2+	2+	3+	3+
$10^{-1.0}$	$10^{-2.0}$	-	-	+	-	2+	+	2+	2+	3+	3+
$10^{-1.0}$	$10^{-3.0}$	-	-	-	+	+	+	2+	2+	3+	3+
$10^{-1.0}$	$10^{-4.0}$	-	-	-	-	+	-	+	+	+	+
$10^{-1.0}$	$10^{-5.0}$	-	-	-	-	-	-	-	-	-	-
$10^{-1.0}$	$10^{-6.0}$	-	-	-	-	-	-	-	-	-	-
$10^{-2.0}$	$10^{-1.0}$	-	-	+	+	2+	+	2+	2+	3+	3+
$10^{-3.0}$	$10^{-1.0}$	-	-	+	+	2+	2+	2+	2+	3+	4+
$10^{-4.0}$	$10^{-1.0}$	-	-	+	+	2+	2+	2+	2+	3+	3+
$10^{-5.0}$	$10^{-1.0}$	-	-	+	+	2+	2+	2+	2+	3+	3+
$10^{-6.0}$	$10^{-1.0}$										

	Dilutions of Controls	24	48	72	96	120 (3+)
	$10^{-1.0}$	-	+	2+	2+	3+ 10/10
	$10^{-2.0}$	-	+	2+	2+	3+ 10/10
	$10^{-3.0}$	-	+	2+	2+	3+ 7/10
	$10^{-4.0}$	-	+	2+	2+	3+ 4/10
	$10^{-5.0}$	-	-	-	2+	3+ 1/10
	$10^{-6.0}$	-	-	-	-	- 0/10

TABLE 9

Interference of Ultraviolet Inactivated Parainfluenza 3 Virus with Active Echo 21 Virus in Monkey Kidney Cell Tissue Culture

Dilution of UVI Para3	Dilution of Active Echo 21	Cytopathic Effect (Hours after Inoculation with Active Echo21)									
		24		48		72		96		120	
		1	2	1	2	1	2	1	2	1	2
$10^{-1.0}$	$10^{-1.0}$	+	+	2+	2+	3+	3+	4+	4+		
$10^{-1.0}$	$10^{-2.0}$	+	+	2+	2+	3+	3+	4+	4+		
$10^{-1.0}$	$10^{-2.5}$	-	+	-	2+	+	3+	2+	3+	3+	4+
$10^{-1.0}$	$10^{-3.0}$	-	-	+	-	2+	+	3+	+	3+	2+
$10^{-1.0}$	$10^{-3.5}$	-	-	-	-	+	-	2+	-	2+	+
$10^{-1.0}$	$10^{-4.0}$	-	-	-	-	-	-	-	-	-	-
$10^{-1.0}$	$10^{-5.0}$	-	-	-	-	-	-	-	-	-	-
$10^{-2.0}$	$10^{-1.0}$	+	+	2+	2+	3+	3+	4+	4+		
$10^{-3.0}$	$10^{-1.0}$	+	+	2+	2+	3+	3+	4+	4+		

	Dilutions of Controls	24	48	72	96	120	(3+)
	$10^{-1.0}$	+	2+	3+	4+		5/5
	$10^{-2.0}$	+	2+	3+	4+		5/5
	$10^{-2.5}$	+	2+	3+	4+		5/5
	$10^{-3.0}$	-	+	2+	3+	4+	3/5
	$10^{-3.5}$	-	-	+	3+	4+	1/5
	$10^{-4.0}$	-	-	-	-	-	0/5

TABLE 10

Interference of Ultraviolet Inactivated Echo 21 Virus with Active Echo 21 Virus in Monkey Kidney Cell Tissue Culture

Dilution of UVI Echo 21	Dilution of Active Echo 21	Cytopathic Effect (Hours after Inoculation with Active Echo 21)							
		24		48		72		96	
		1	2	1	2	1	2	1	2
$10^{-1.0}$	$10^{-1.0}$	+	+	2+	2+	3+	3+	4+	4+
$10^{-1.0}$	$10^{-2.0}$	-	+	2+	2+	3+	3+	4+	4+
$10^{-1.0}$	$10^{-3.0}$	-	-	-	-	2+	2+	3+	3+
$10^{-1.0}$	$10^{-4.0}$	-	-	-	-	-	-	-	-
$10^{-2.0}$	$10^{-1.0}$	+	+	2+	+	3+	2+	4+	4+
$10^{-3.0}$	$10^{-1.0}$	+	+	2+	2+	3+	2+	4+	3+
$10^{-4.0}$	$10^{-1.0}$	+	+	2+	2+	3+	3+	4+	4+

	Dilutions of Controls	24	48	72	96	(3+)
	$10^{-1.0}$	+	2+	3+	4+	5/5
	$10^{-2.0}$	+	2+	3+	4+	5/5
	$10^{-2.5}$	-	+	2+	3+	5/5
	$10^{-3.0}$	-	+	2+	3+	3/5
	$10^{-3.5}$	-	-	+	3+	1/5
	$10^{-4.0}$	-	-	-	-	0/5

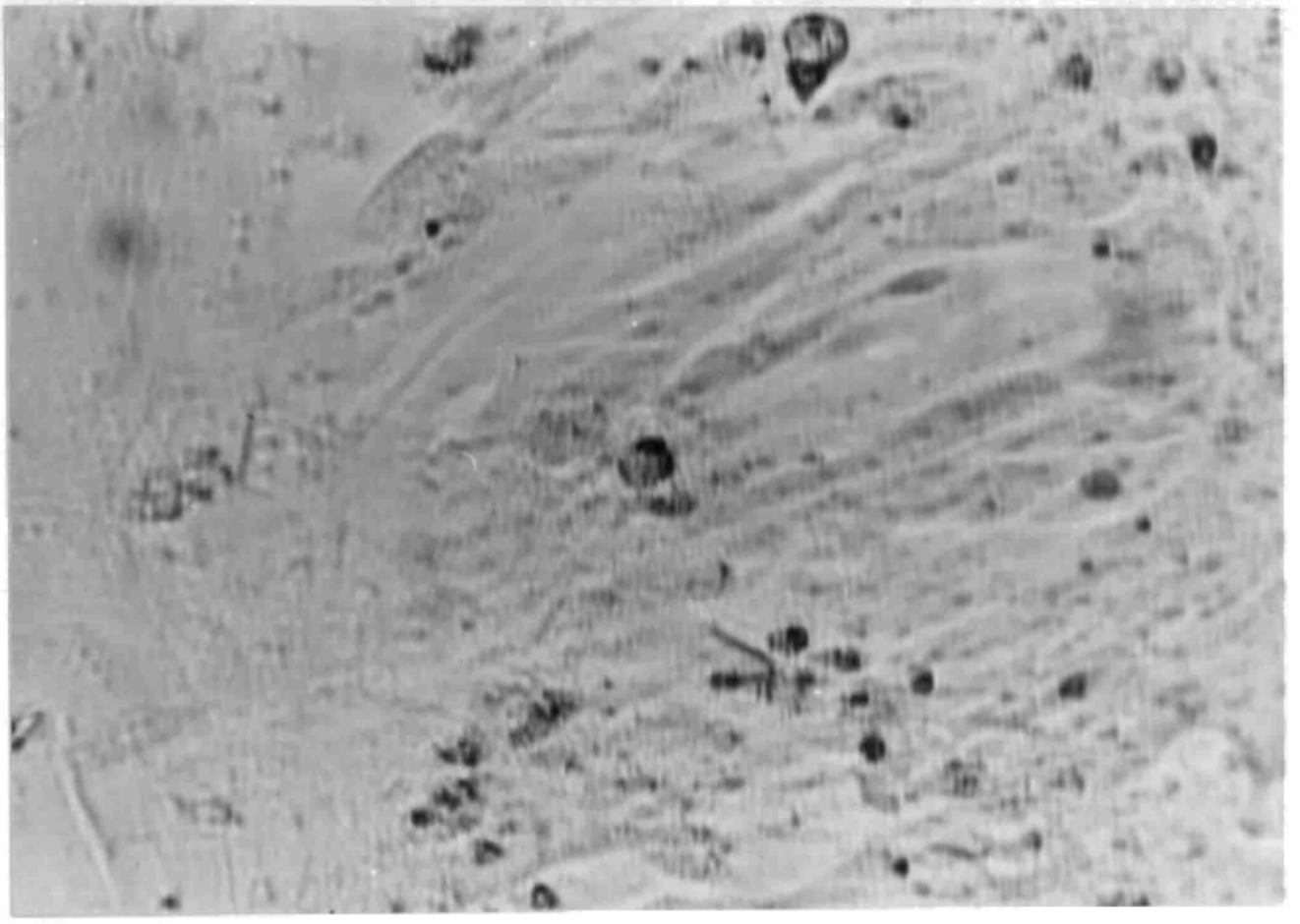
FIGURES

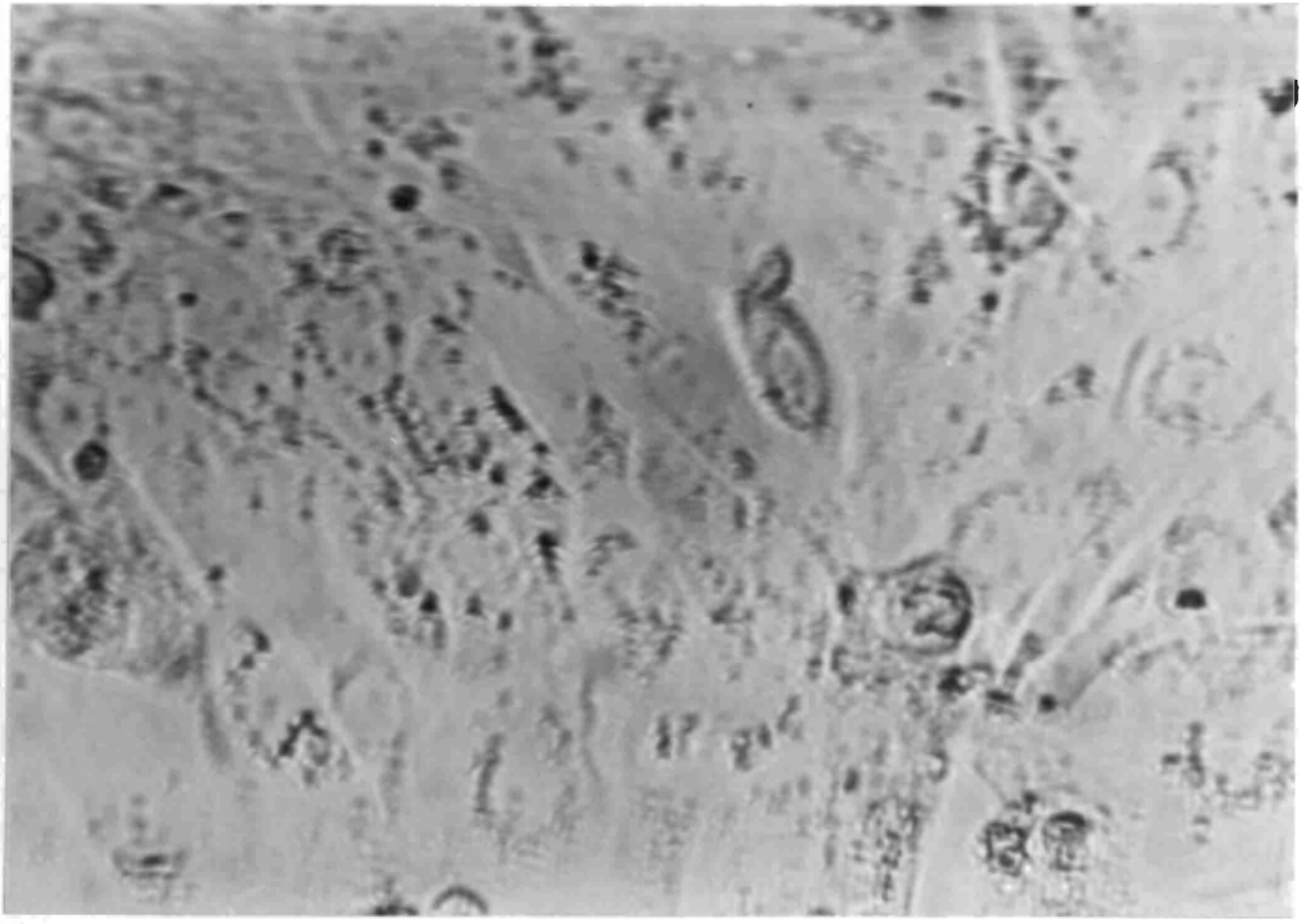
FIGURE 1

Stages in the Production of Cytopathic Effect by Parainfluenza 3 Virus in Rhesus Monkey Kidney Cell Tissue Culture

A. Uninoculated, rhesus monkey kidney cell tissue culture in which the cell monolayer was at the proper stage of growth for virus inoculation

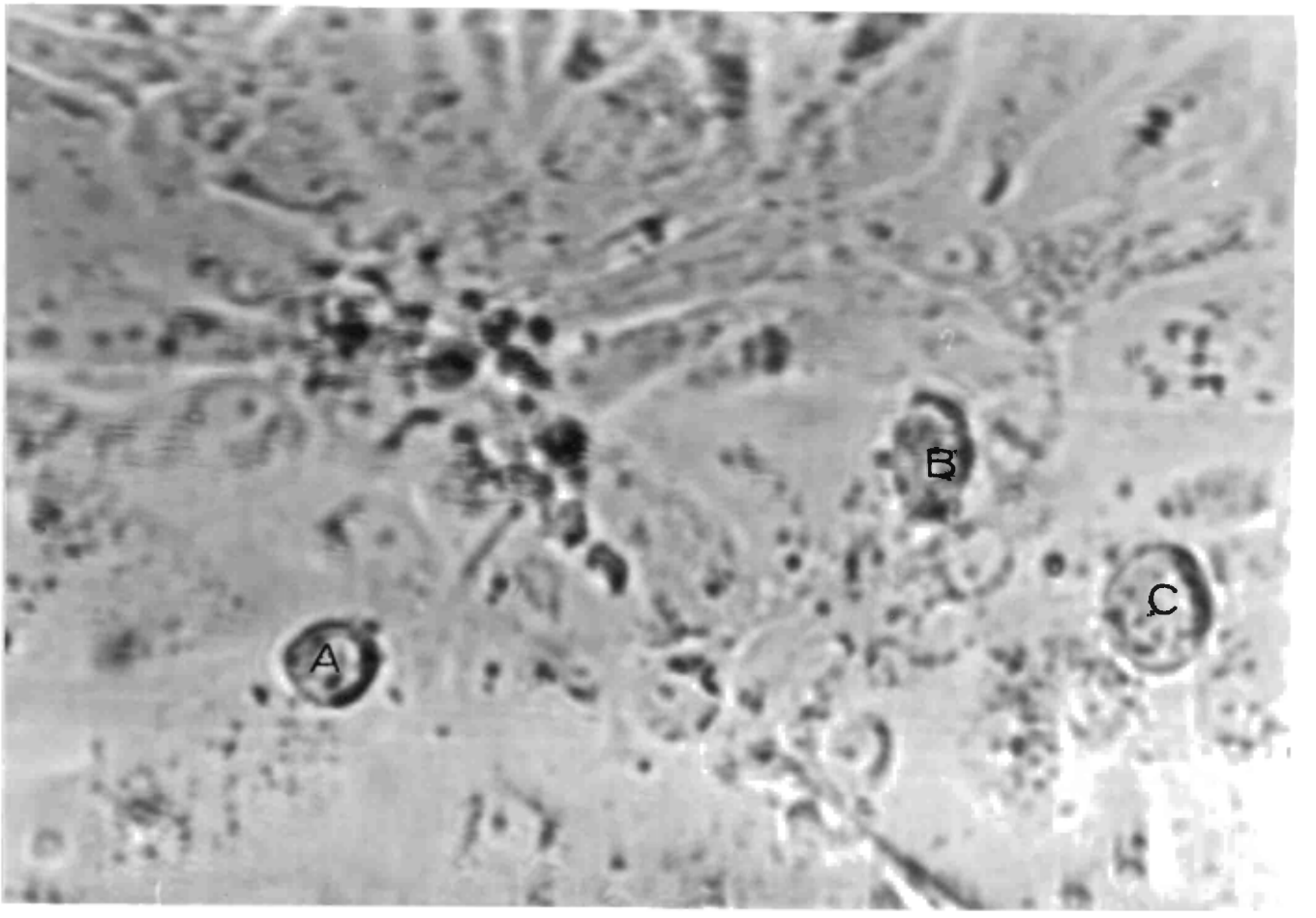
B. Stage one cytopathic effect (1+ CPE) in which the monkey kidney cell monolayer was 25% destroyed by Parainfluenza 3 virus

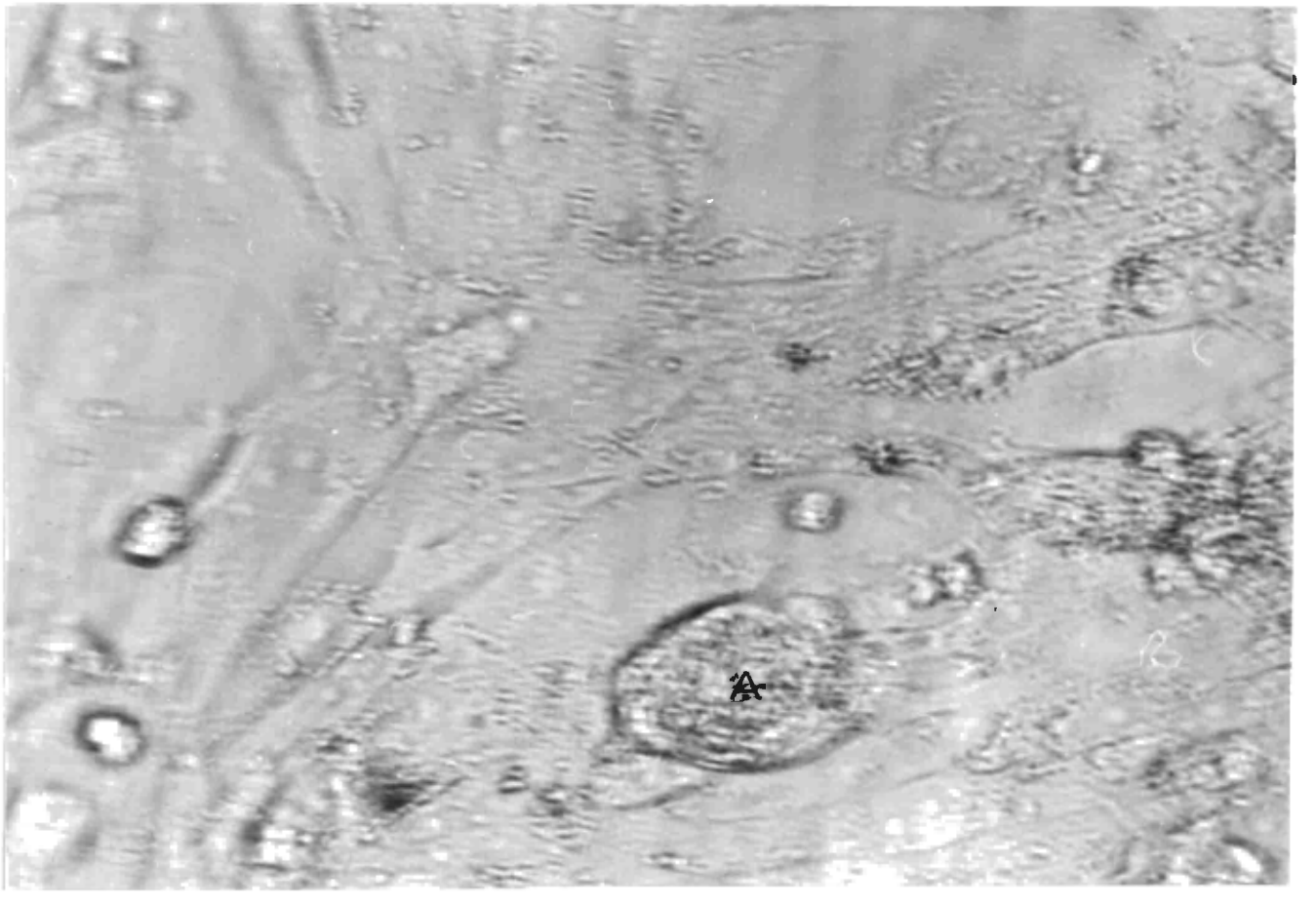




C. Parainfluenza 3 virus produced giant cells (A., B., and C.)

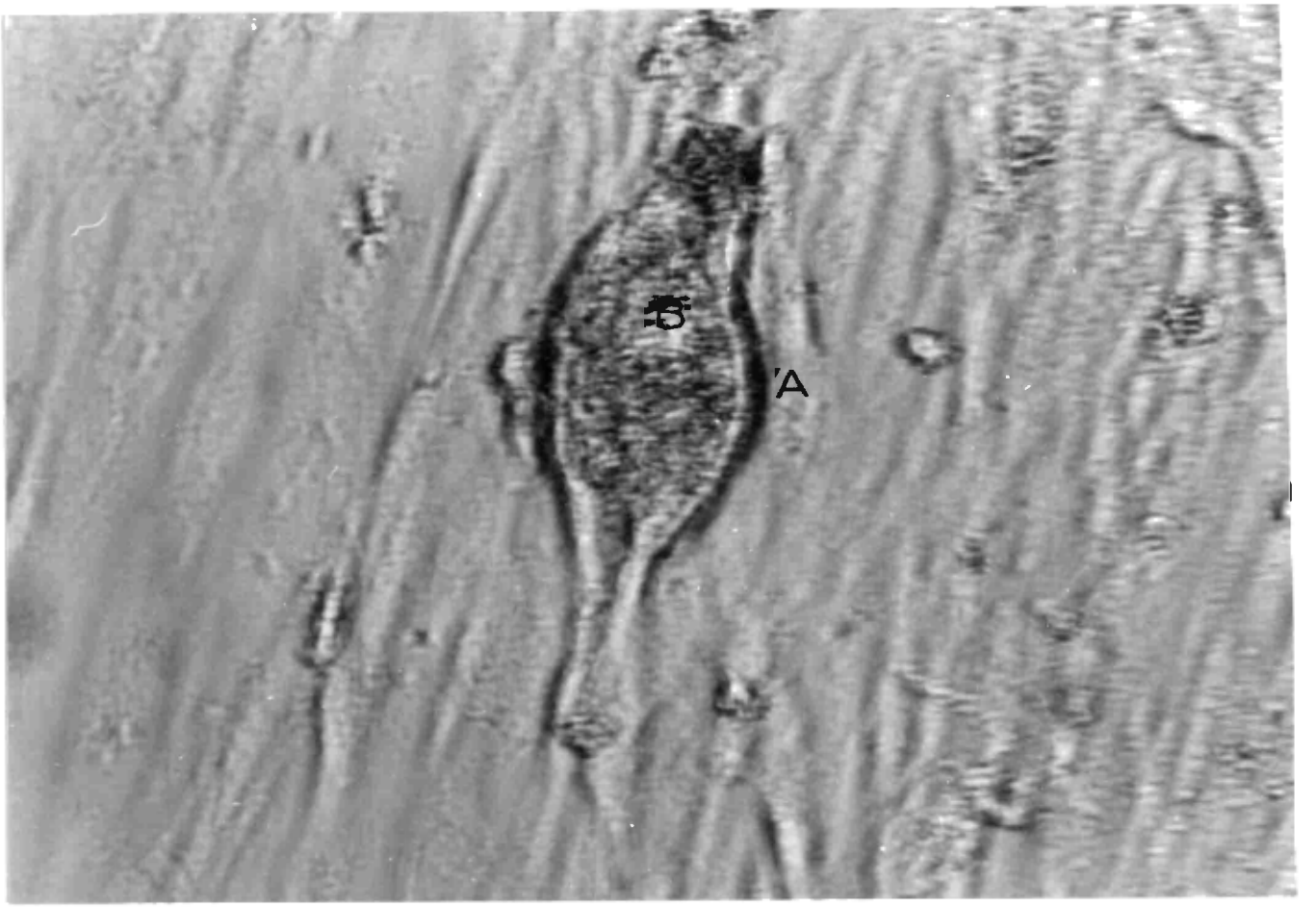
D. A single giant cell (A.) among normal cells in MKC tissue culture inoculated with Parainfluenza 3 virus

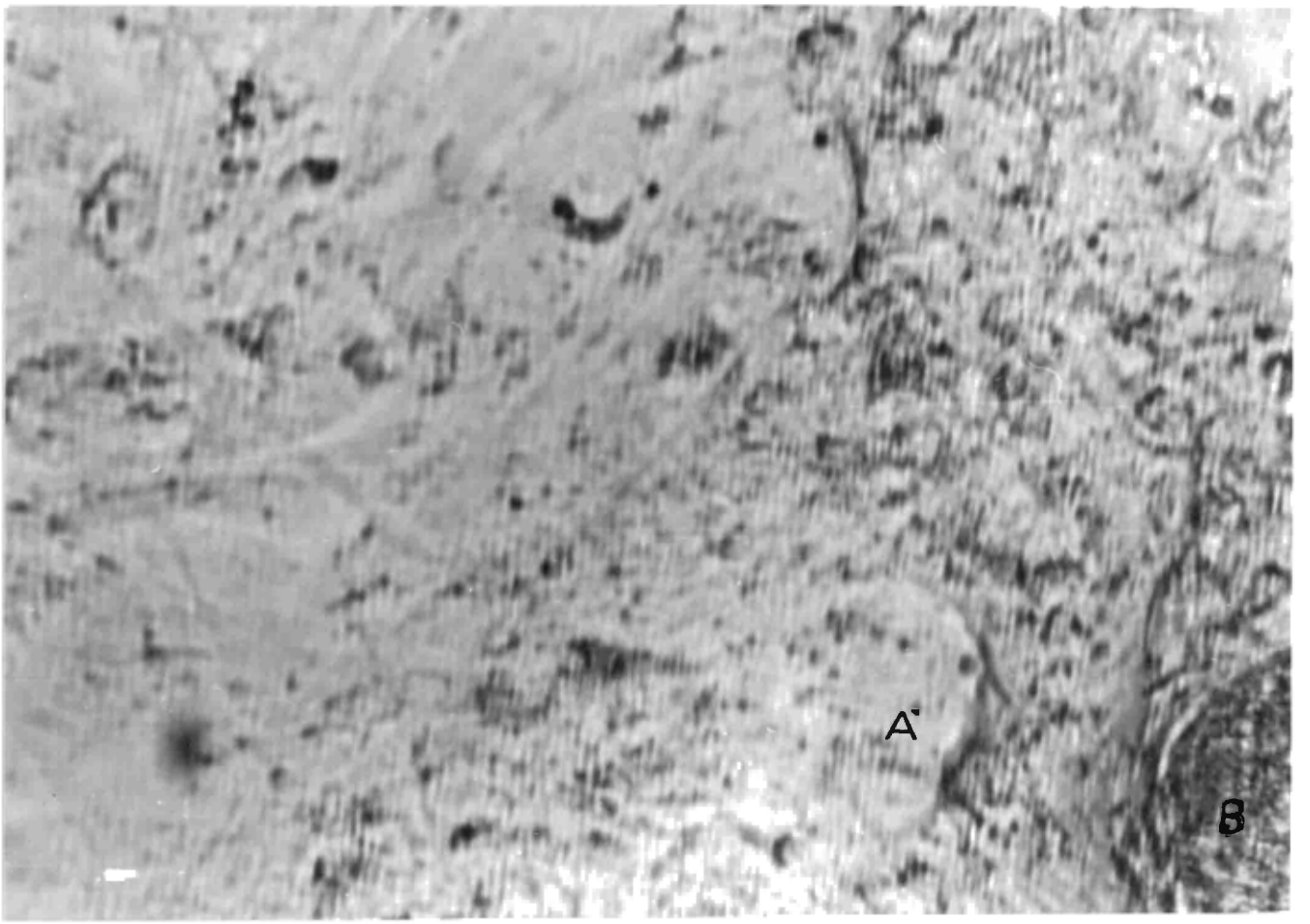

A
B
C


A

E. Giant cell (A.) contained many nuclei (B.) in monkey kidney cell tissue culture inoculated with Parainfluenza 3 virus

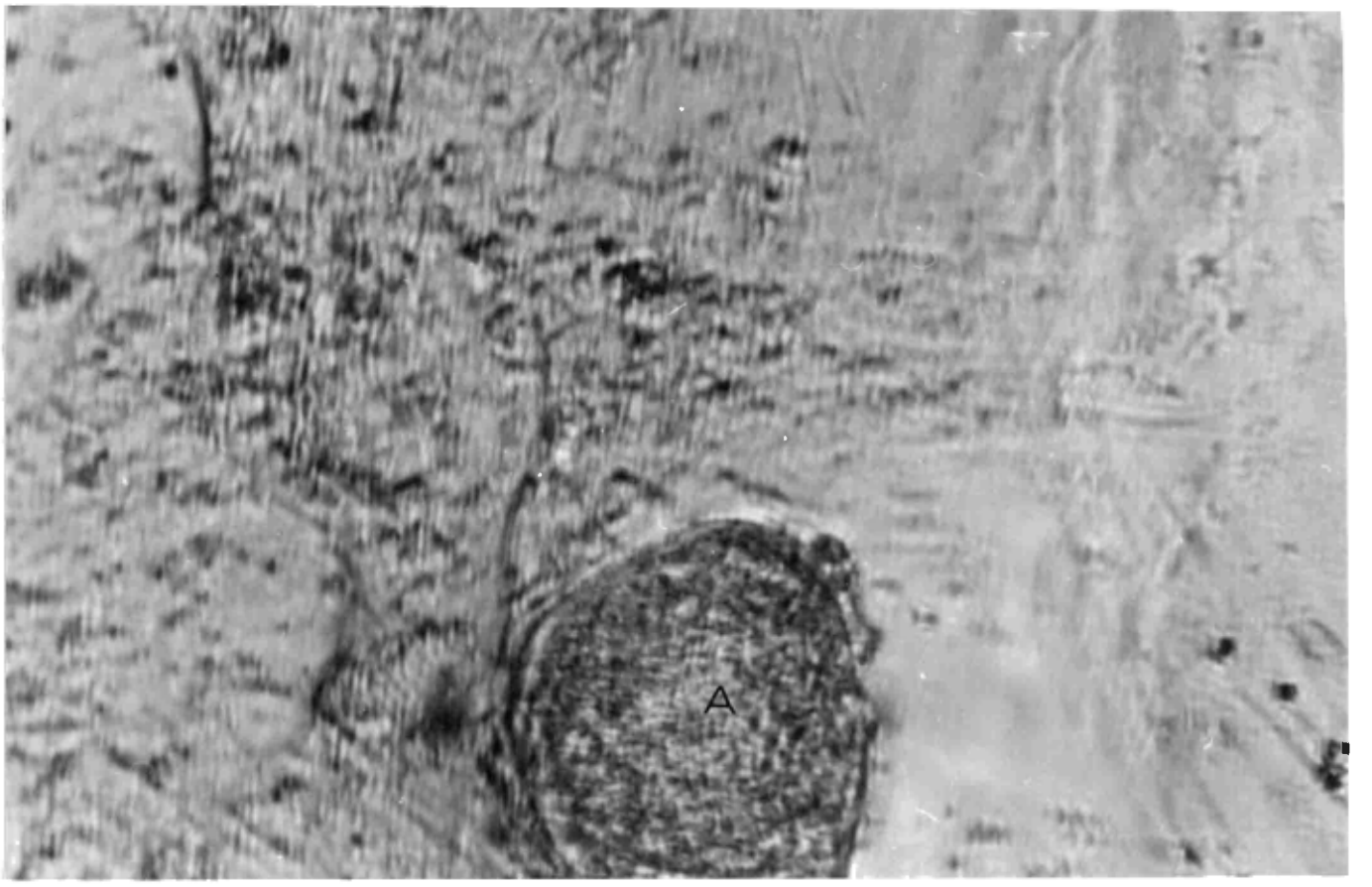
F. The area (A.) adjacent to the giant cell (B.) showed no readily visible cytopathic effect by Parainfluenza 3 virus

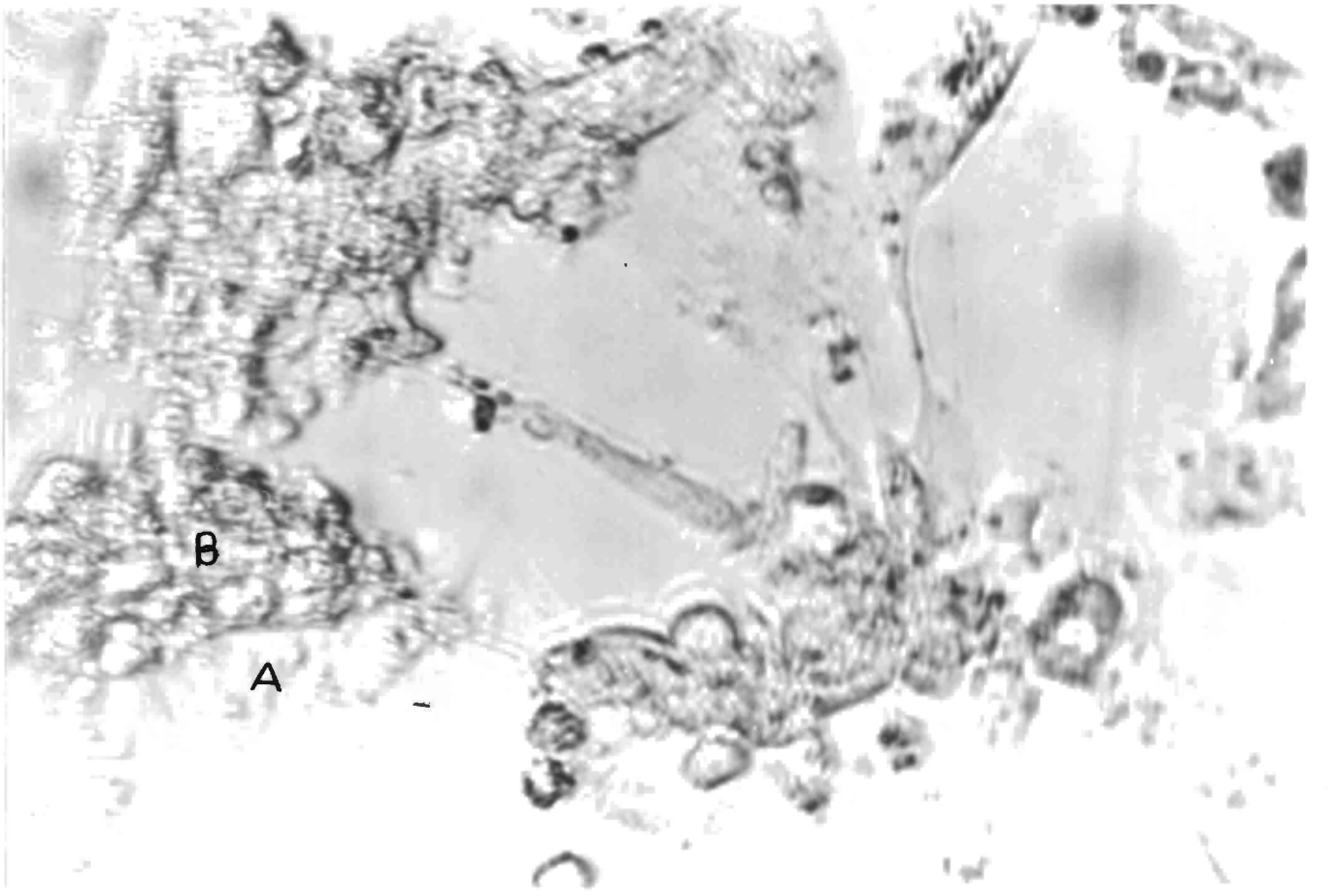

B
A


A'
B

G. A syncytium (A.) from a single giant cell, characteristic of the growth of Parainfluenza 3 virus in monkey kidney cell tissue culture

H. Third stage (3+ CPE) in which the monolayer was 75% destroyed, syncytia separated from the test tube wall, disintegrated into smaller entities (B.), and freed virus from host cells


A


B
A

I. Fourth stage (4+ CPE) in which the monkey kidney cell monolayer had neared total destruction

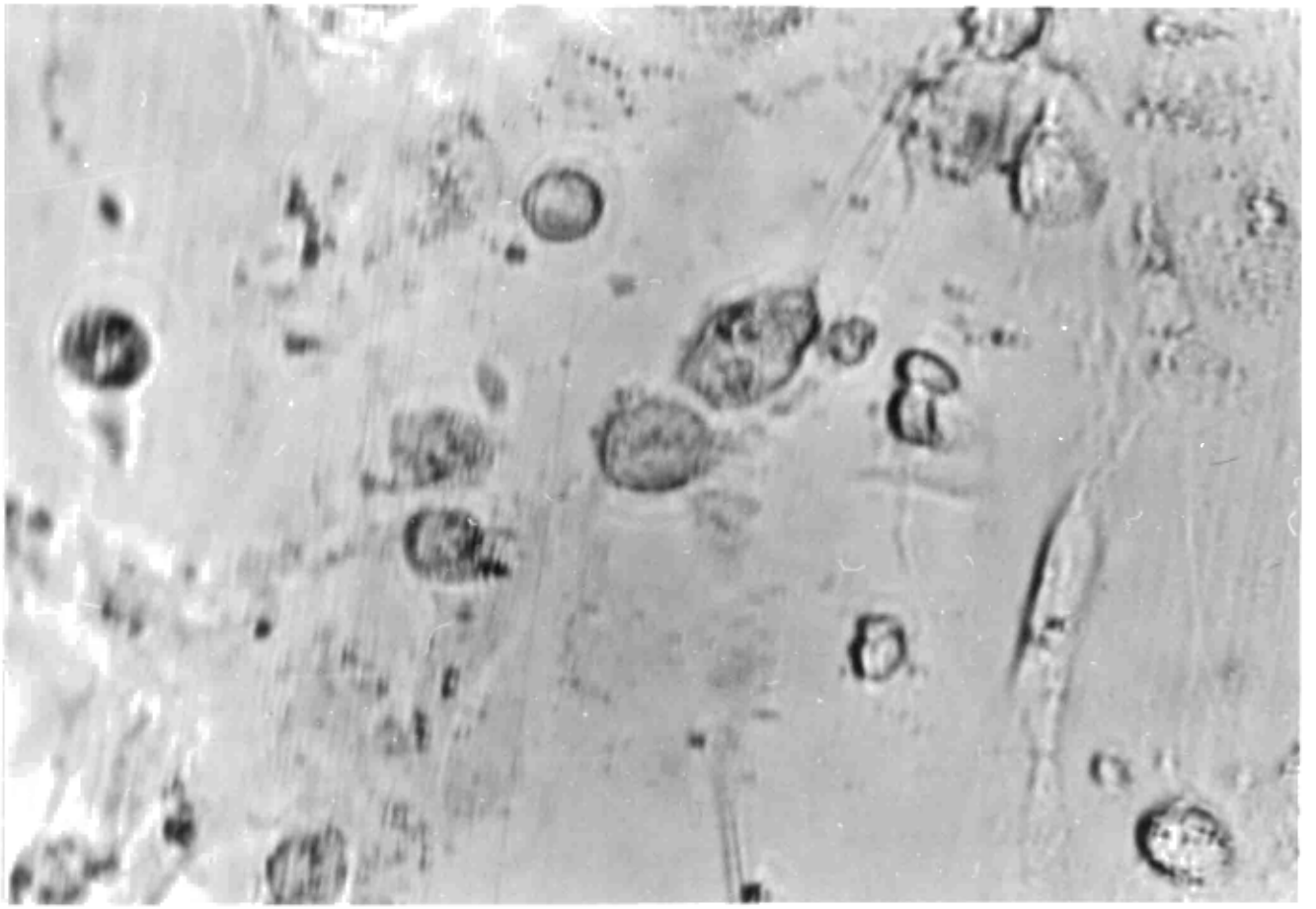
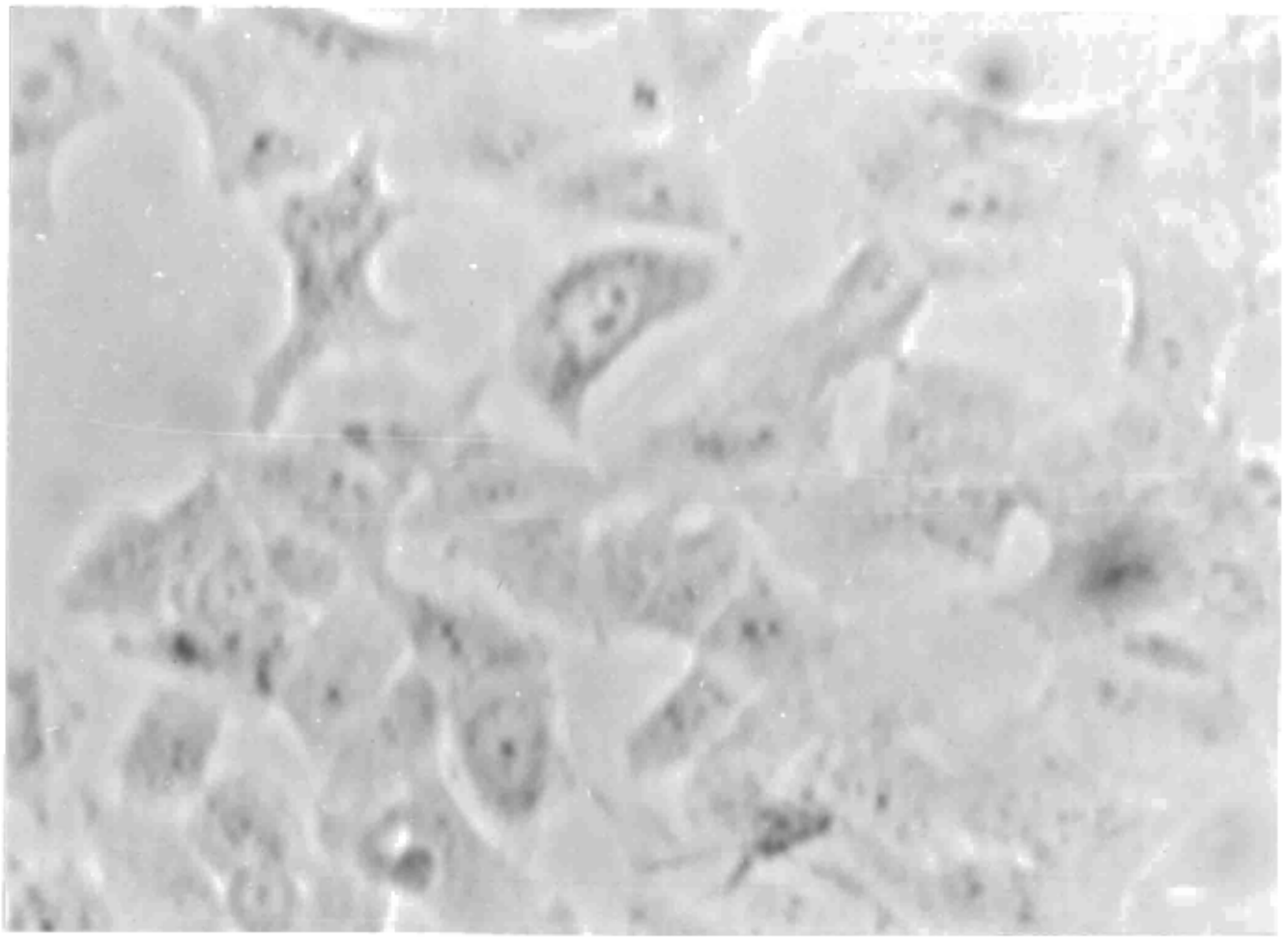


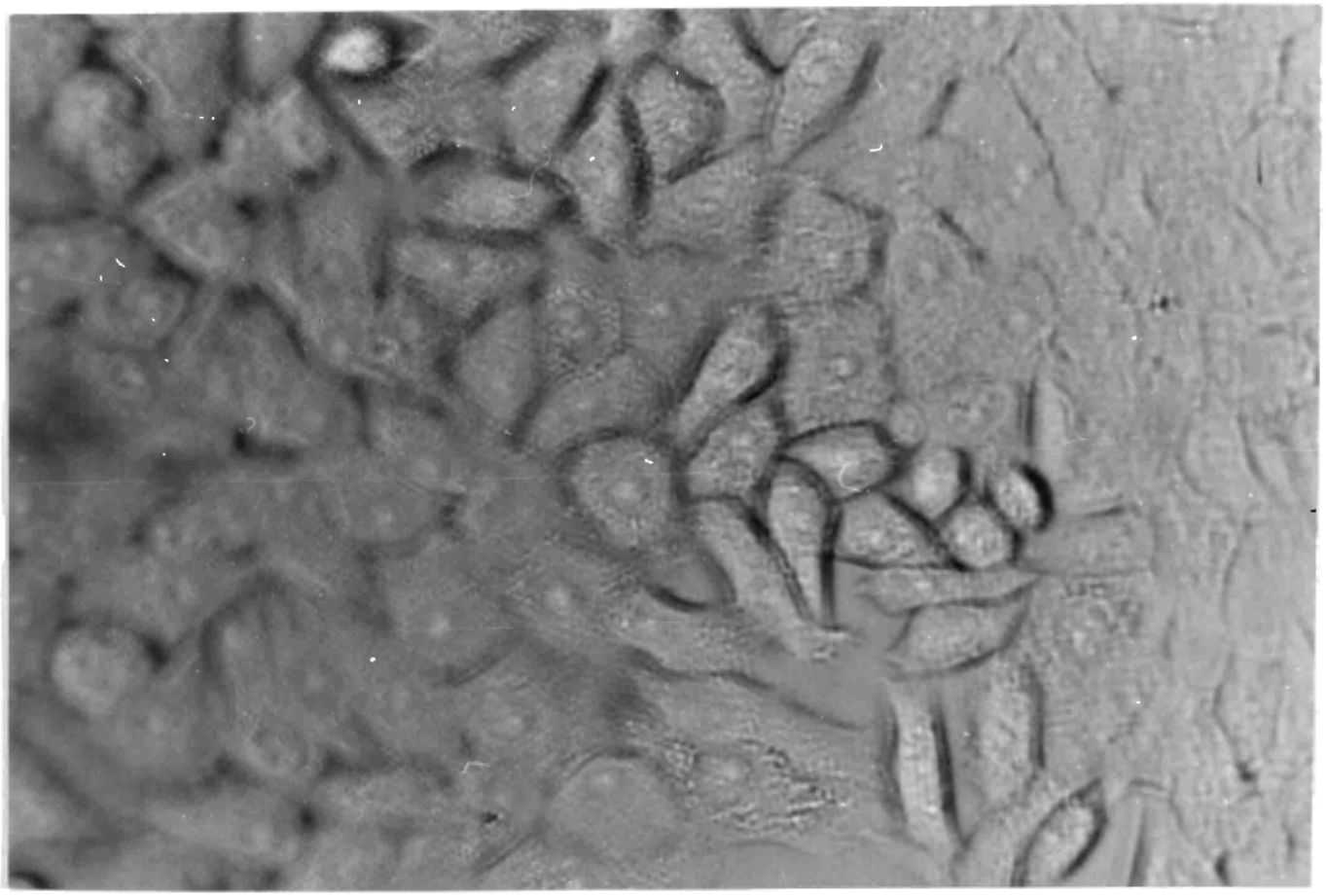
FIGURE 2

Stages in the Production of Cytopathic Effect by Parainfluenza 3 Virus in Human Epithelium (HEp-2) Tissue Culture

A. Virus-free human epithelium (HEp-2) cells in an early stage of monolayer formation on the test tube wall

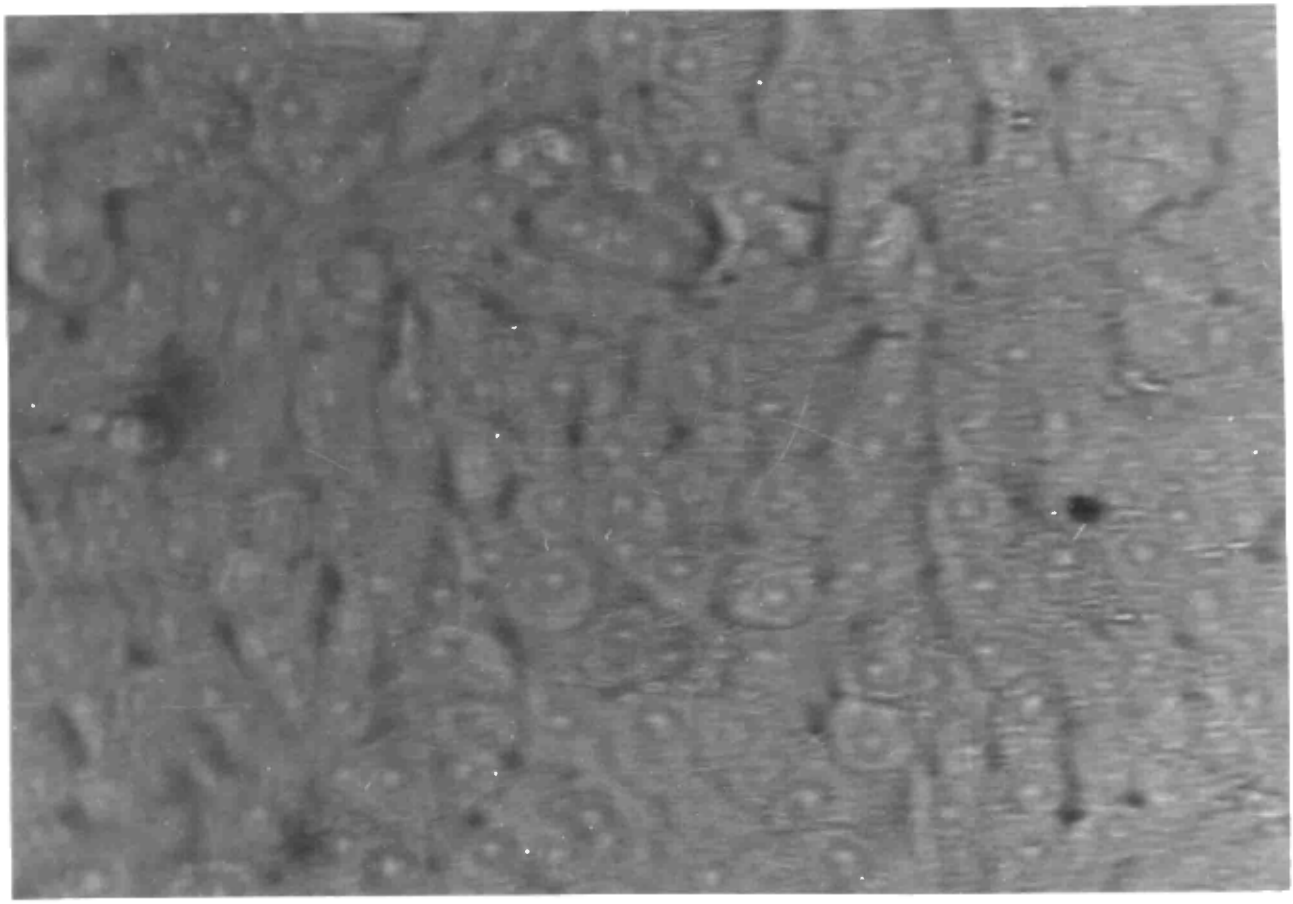
B. Virus-free monolayer of characteristically cuboidal cells near completion

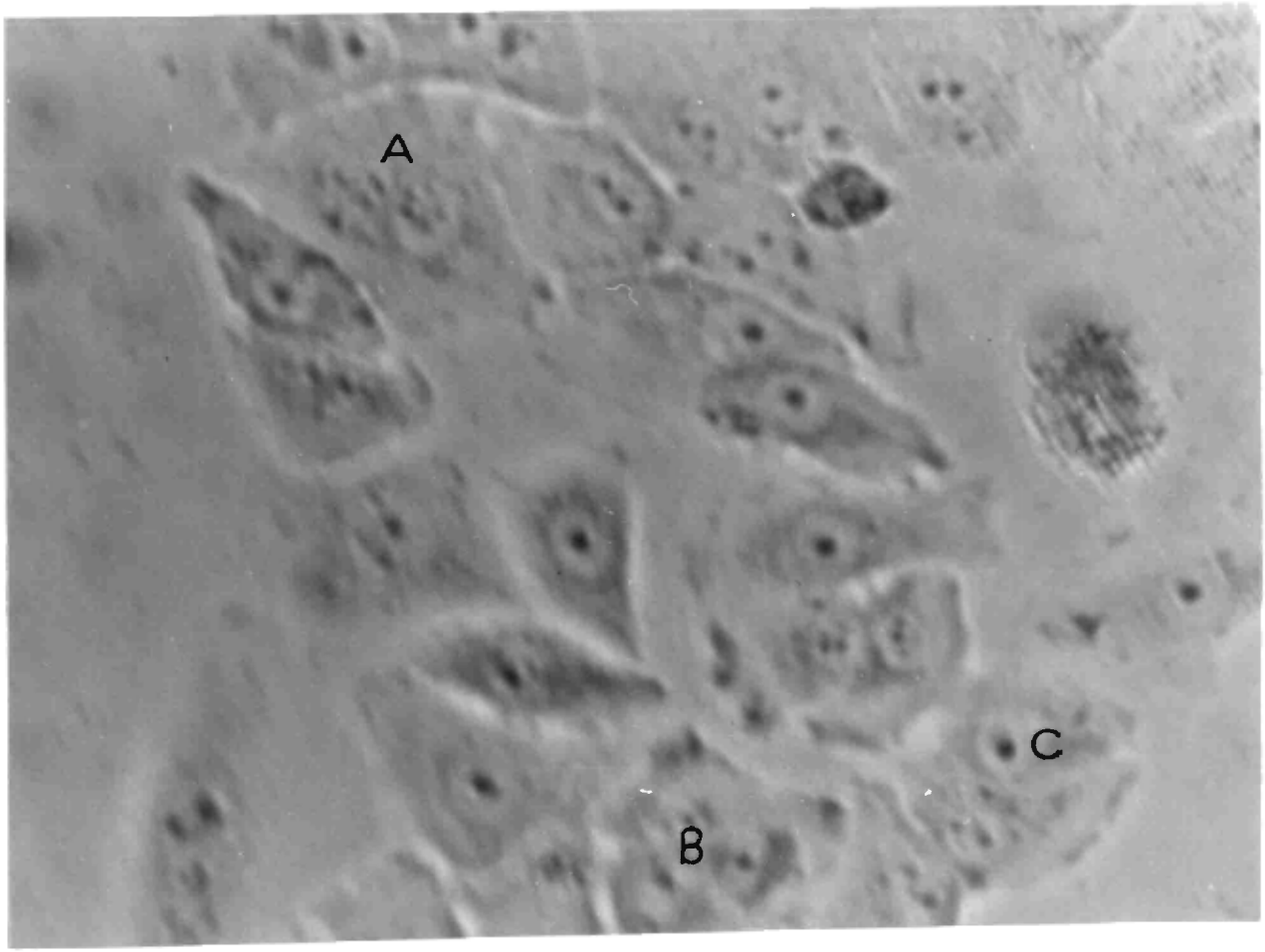




C. Human epithelium monolayer at the proper stage for virus inoculation

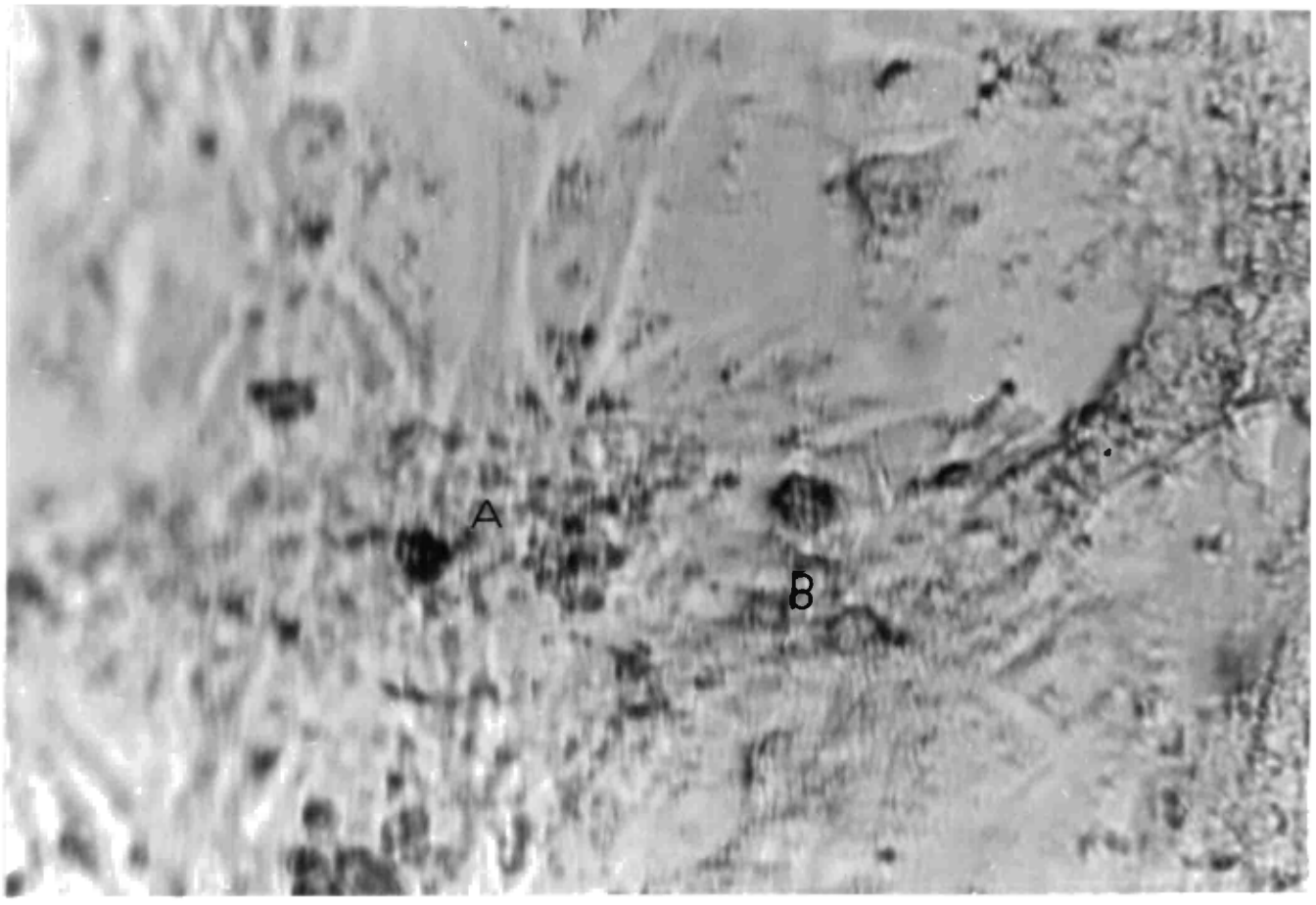
D. First stage (1+ CPE) in which multinucleate giant cells (A., B., and C.) were visible in the HEp-2 tissue culture

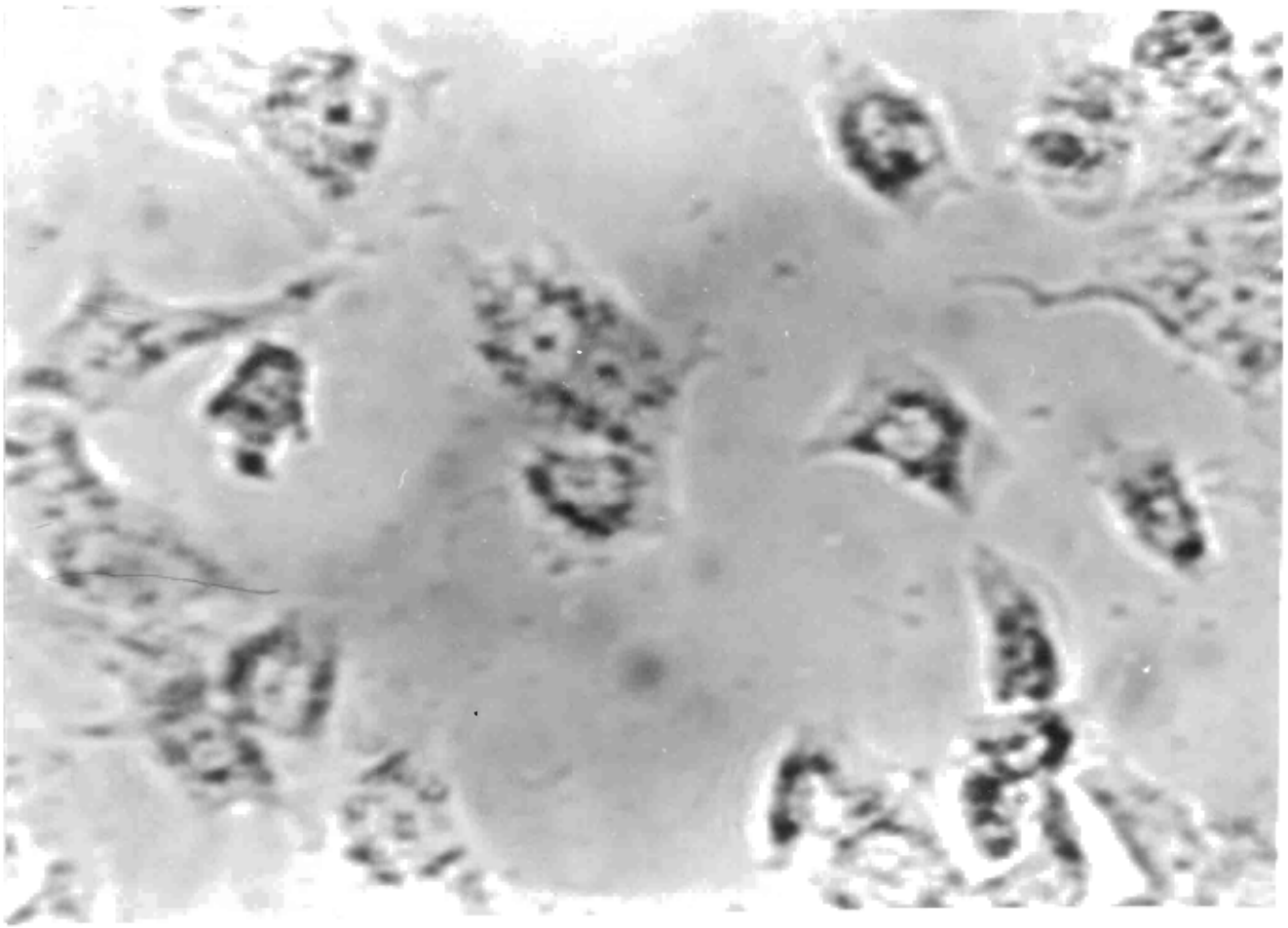



A
B
C

E. Areas of cellular destruction (A. and B.) by Parainfluenza 3 virus appeared in the HEp-2 tissue culture

F. The HEp-2 cells disintegrated and separated from the glass (3+ CPE)


A
B



G. A few necrotic, spindle-shaped HEp-2 cells remained attached to the wall of the test tube (4+ CPE)

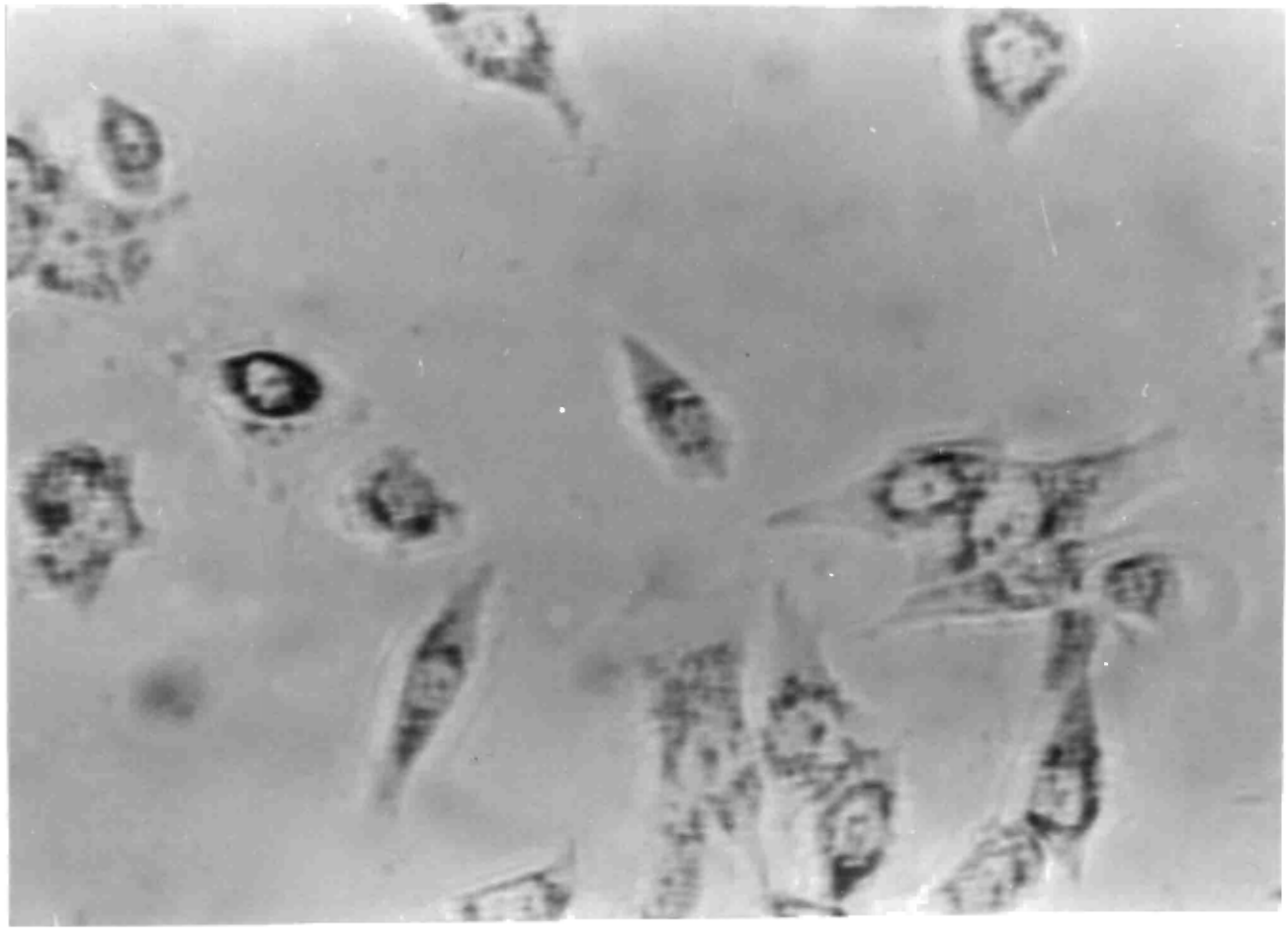
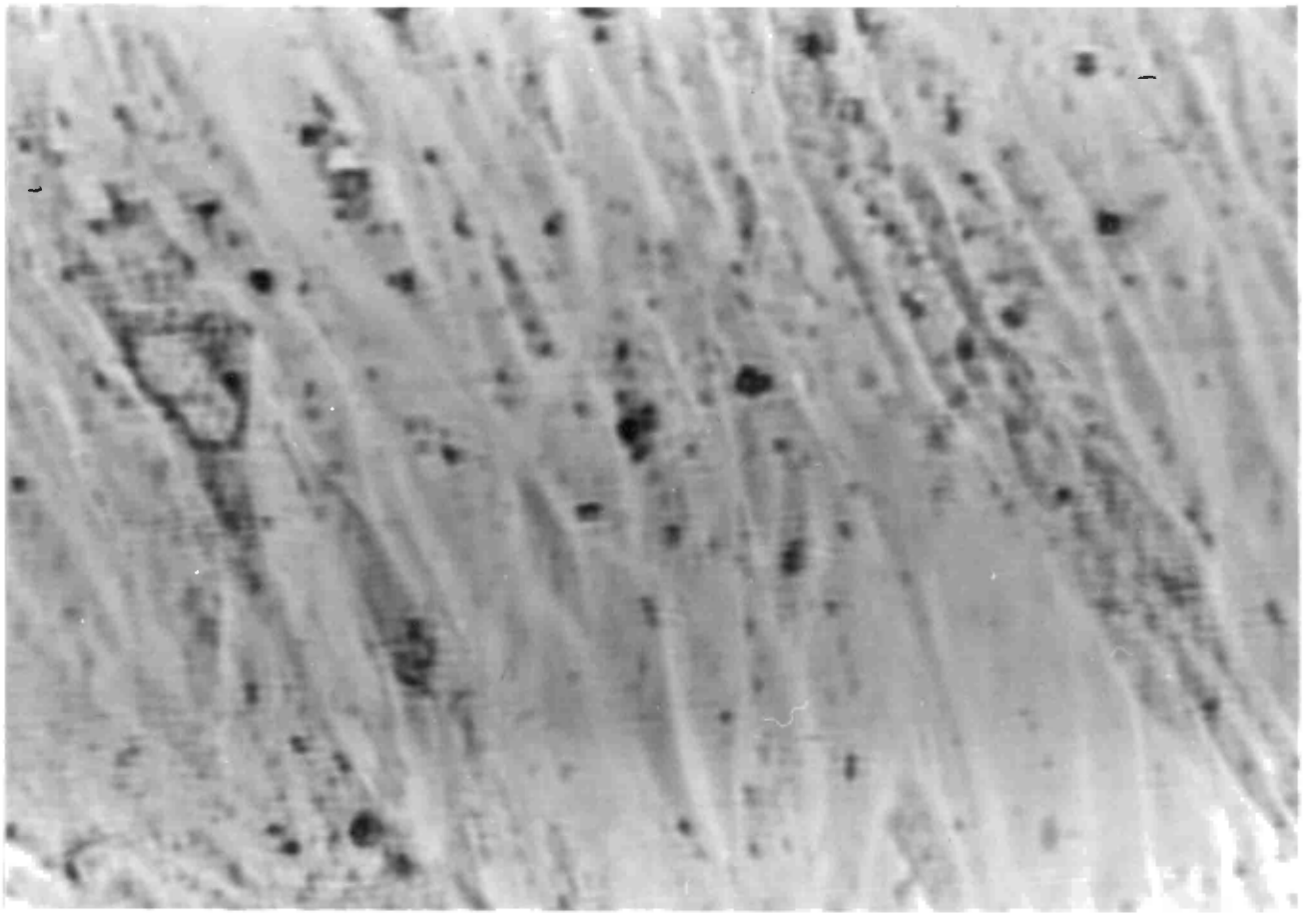


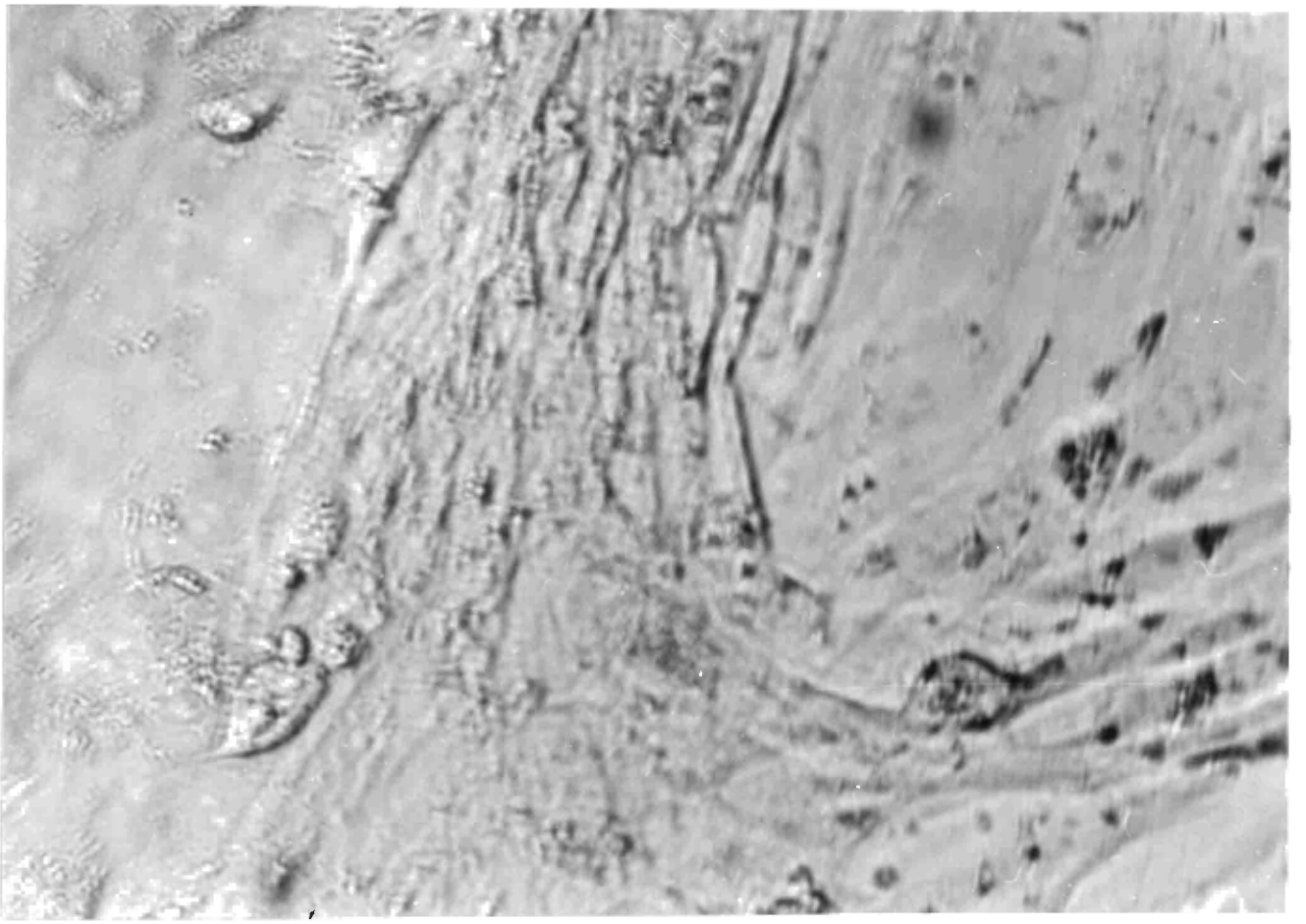
FIGURE 3

Stages in the Production of Cytopathic Effect by Echo 21 Virus in Rhesus Monkey Kidney Cell Tissue Culture

A. A rhesus monkey kidney cell monolayer which was at the proper stage for virus inoculation

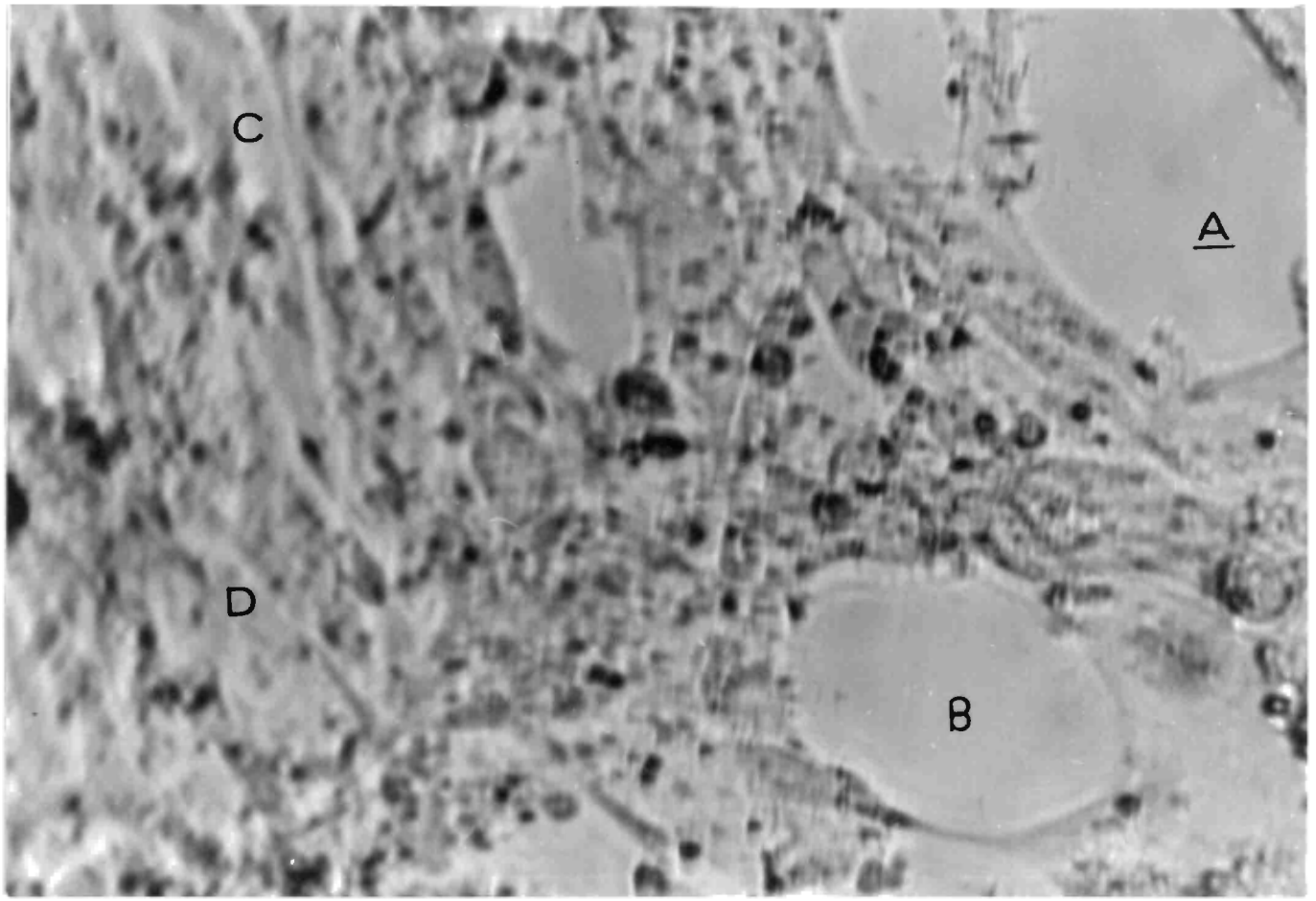
B. The first stage of cytopathic effect (1+ CPE) in which 25% of the monolayer was destroyed

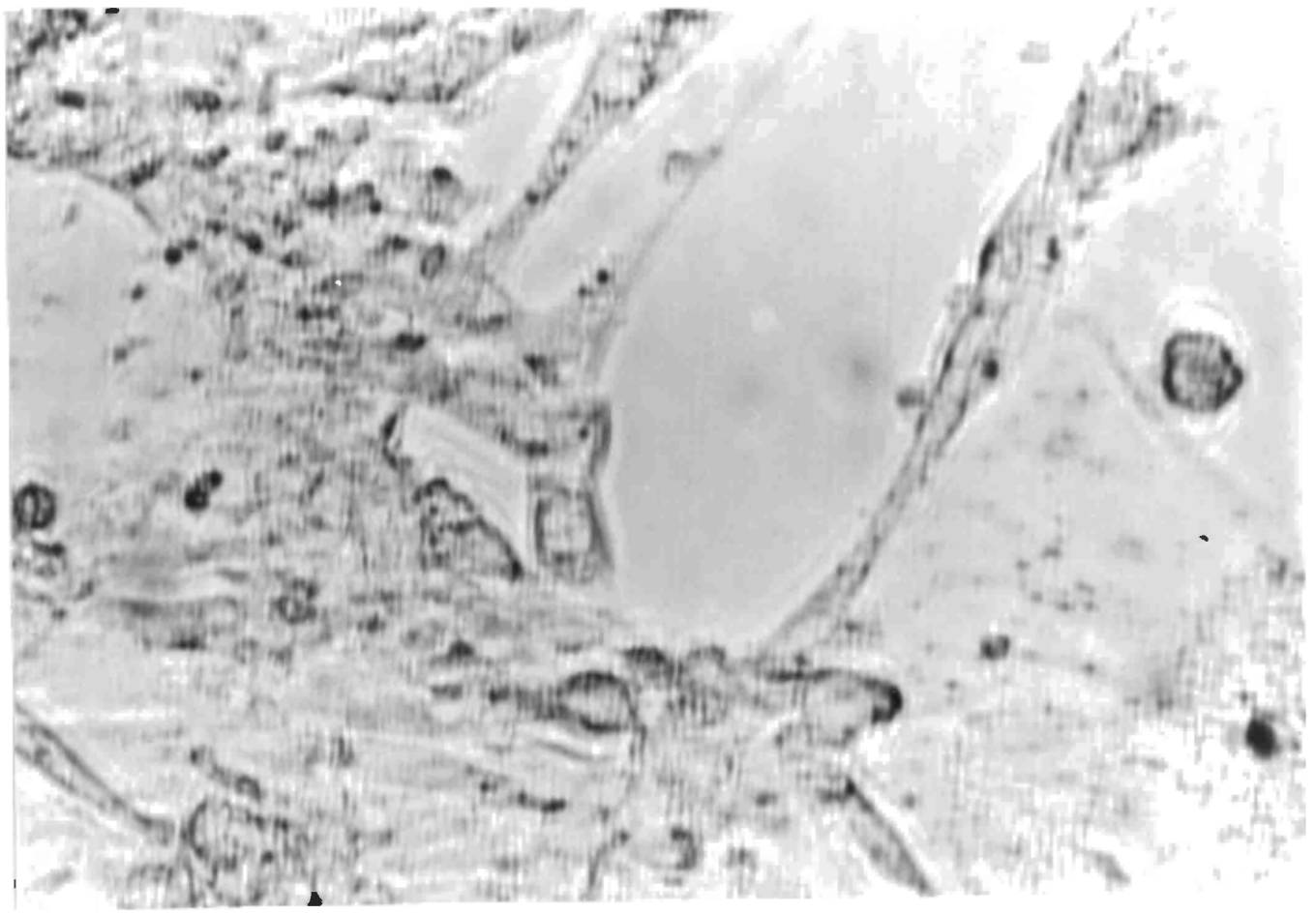




C. Areas void of cells (A. and B.) appeared in the monolayer where cells detached from the glass (2+ CPE) and areas of no apparent cytopathic effect (C. and D.) were present

D. Third stage cytopathic effect in which 75% of the monolayer had separated from the glass


C
A
D
B



VITA

William Ernest Steinmetz was born in Boca Raton, Florida. He attended elementary school, Chandler Junior High School, and John Marshall High School in Richmond, Virginia. In high school he was awarded the James Harwood scholarship.

Prior to his graduation from John Marshall he began a premedical college program at the University of Richmond where he received the Bachelor of Science degree with a major in biology and a minor in chemistry. He became president of the Canterbury Club, was a member of Circle K (Kiwanis) Club, and became a member of Beta Beta Beta National Honorary Biological Society.

He attended the University of Richmond Graduate School and is a candidate for the Master of Science degree in biology. As a graduate student he was the recipient of an A. D. Williams Fellowship, and was a laboratory assistant in bacteriology, mycology, and genetics. He has taken classroom and on-the-job training at the City of Richmond Public Health Laboratories. He has worked as a microbiologist at the Pathology Laboratory of Richmond Memorial Hospital.

APPENDIX

1. Ca^{++}- and Mg^{++}-Free Phosphate Buffered Saline (CMF-PBS, 10X) (Merchant, Kahn, and Murphy, 1964)

Sodium Chloride	80 g
Potassium Chloride	3.0 g
Glucose	20 g
Potassium Biphosphate	0.20 g
Disodium Phosphate·$2H_2O$	0.73 g
Glass Distilled Water	1000 ml

2. Trypsin (0.25%, 1X) (Merchant, Kahn, and Murphy, 1964)

Difco 1:250 Trypsin	0.25 gm
CMF-PBS (1X)	100 ml

3. Hanks Balanced Salt Solution (BSS, 10X) without sodium bicarbonate (Hanks, 1949). Source: Microbiological Associates

Sodium Chloride	80.0 g
Potassium Chloride	4.0 g
Calcium Chloride	1.4 g
Magnesium Sulfate·$7H_2O$	1.0 g
Magnesium Chloride·$6H_2O$	1.0 g
Desodium Phosphate·$2H_2O$	0.6 g
Monopotassium Phosphate	0.6 g
Dextrose	10.0 g
Phenol Red	0.2 g
Triple Distilled Water	1000 ml

Note: 2 g Magnesium Sulfate·$7H_2O$ may be substitued for

1 g Magnesium Sulfate·$7H_2O$ and 1 g Magnesium Chloride·$6H_2O$.

4. Minimal Essential Medium Eagle (MEM) Hanks Base (Eagle, 1959). Source: Microbiological Associates

l-Arginine	105 mg
l-Cystine	24 mg
l-Histidine	31 mg
l-Leucine	52 mg
l-Isoleucine	52 mg
l-Lysine	58 mg
l-Methionine	15 mg
l-Phenylalanine	32 mg
l-Threonine	48 mg
l-Tryptophan	10 mg
l-Tyrosine	36 mg
l-Valine	46 mg
Choline Chloride	1.0 mg
Biotin	1.0 mg
Folic Acid	1.0 mg
Inositol	2.0 mg
Pantothenic Acid	1.0 mg
Pyridoxal	1.0 mg
Thiamine	1.0 mg
Nicotinamide	1.0 mg

Riboflavin	0.1 mg
Glutamine	292 mg
Sodium Chloride	8.0 g
Potassium Chloride	0.4 g
Calcium Chloride	0.14 g
Magnesium Sulfate·$7H_2O$	0.1 g
Magnesium Chloride·$6H_2O$	0.1 g
Disodium Phosphate·$2H_2O$	0.06 g
Monopotassium Phosphate	0.06 g
Dextrose	1.0 g
Phenol Red	0.02 g
Sodium Bicarbonate	0.35 g
Triple Distilled Water	1000 ml

5. Fetal Calf Serum, (Normal, Sterile)
   Lot No. 11-496
   Date: 12 November 1963
   Control No.
   Source: Microbiological Associates

6. Melnick's Medium (Melnick, 1955). Source: Microbiological Associates
   a. Melnick's Medium, Hanks Base

Sodium Chloride	8.0 g
Potassium Chloride	0.4 g

Calcium Chloride	0.14 g
Magnesium Sulfate·$7H_2O$	0.1 g
Magnesium Chloride·$6H_2O$	0.1 g
Disodium Phosphate·$2H_2O$	0.06 g
Monopotassium Phosphate	0.06 g
Dextrose	1.0 g
Phenol Red	0.02 g
Sodium Bicarbonate	0.35 g
Lactalbumin Hydrolysate	5.0 g
Calf Serum	20.0 g
Triple Distilled Water	1000 ml

b. Melnick's Medium, Earle Base

Sodium Chloride	6.8 g
Potassium Chloride	0.4 g
Calcium Chloride	0.2 g
Magnesium Sulfate·$7H_2O$	0.2 g
Monosodium Phosphate	0.125 g
Dextrose	1.0 g
Sodium Bicarbonate	2.2 g
Phenol Red	0.02 g
Lactalbumin Hydrolysate	5.0 g
Calf Serum	20.0 ml
Triple Distilled Water	1000 ml

7. Earle's Balanced Salt Solution (1X) Earle, 1943)

Source: Microbiological Associates

Sodium Chloride	6.8 g
Potassium Chloride	0.40 g
Magnesium Sulfate.$7H_2O$	0.2 g
Monosodium Phosphate	0.125 g
Sodium Bicarbonate	2.20 g
Glucose	1.0 g
Calcium Chloride	0.2 g
Triple Distilled Water	1000 ml

8. Scherer's Medium (Maintenance) (Scherer, 1951)

Source: Microbiological Associates

Bacto-Casamino Acids	45 mg
DL-Tryptophane	20 mg
Glycine	20 mg
DL-Histidine	20 mg
L-Cystine	15 mg
Succinic Acid	10 mg
L-Malic Acid	5.0 mg
Riboflavin	0.5 mg
Calcium Pantothenate	0.5 mg
Choline	1.5 mg
Biotin	.01 mg
Inositol	1.5 mg

Folic Acid	.01 mg
D-Ribose	0.5 mg
Xanthine	1.0 mg
Guanine	1.0 mg
Uracil	1.0 mg
Adenine	2.5 mg
Glycerol	500 mg
p-Aminobenzoic Acid	0.1 mg
Thiamine	1.0 mg
Nicotinamide	0.5 mg
Pyridoxal	0.5 mg
Thymine	0.5 mg
Cytosine	0.5 mg
Sodium Acetate	500 mg
Sodium Pyruvate	500 mg
Bacto-Dextrose	2.0 g
Potassium Chloride	400 mg
Calcium Chloride	140 mg
Magnesium Sulfate$\cdot 7H_2O$	200 mg
Disodium Phosphate$\cdot 2H_2O$	60 mg
Monopotassium Phosphate	60 mg
Sodium Chloride	8.0 g
Sodium Bicarbonate	1.0 g
Phenol Red	20 mg
Triple Distilled Water	1000 ml

9. Cleaning Solution

Potassium Dichromate	63 g
Sulfuric Acid	902 ml
Distilled Water	25 ml

10. Echo 21 Antiserum

Lot No.	23285
Titer:	1:400

Source: Microbiological Associates

11. Parainfluenza 3 Antigen

Lot No.	3-1431
Titer:	1:400

Source: Microbiological Associates

12. Dulbecco Phosphate-Buffered Saline (PBS, 1X)
(Dulbecco and Vogt, 1954)

Sodium Chloride	8.0 g
Potassium Chloride	0.2 g
Disodium Phosphate	1.15 g
Potassium Phosphate	0.2 g
Magnesium Chloride $6H_2O$	0.1 g
Triple Distilled Water	1000 ml