CYTOPATHOLOGY OF CULTURED CELLS INFECTED WITH HERPES SIMPLEX VIRUS

by

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ABSTRACT

The cytopathology of herpes simplex virus (HSV) in H.Ep.2 and BHK-21 cells was studied using the techniques of light microscopy, immunofluorescence, electron microscopy, autoradiography and cytogenetics. Both cell types supported rapid growth cycles of HSV resulting in the production of maximum titres after 22 - 24 hours of infection. Cultures treated with 10 µg/ml ara-C or 100 µg/ml IDU at the time of infection showed a 99% decrease in infectious virus production.

HSV-infected H.Ep.2 and BHK-21 cells revealed typical virus-induced inclusion bodies and a generalized disorganization of the nucleus and cytoplasm. Syncytia formation was not observed but after 24 hours of infection, nearly 100% of the cells were rounded and often detached from the glass surface. Addition of 10 µg/ml ara-C or 100 µg/ml IDU failed to prevent virus cytopathology but did cause a characteristic cytoplasmic disruption and rounding of uninfected cells.

Virus-infected cells also revealed at least four separate immunofluorescent elements after exposure to hyperimmune serum prepared in guinea pigs. These elements included small nuclear granules, amorphous nuclear masses, diffuse cytoplasmic antigens, and intense surface fluorescence. The nuclear antigens and cytoplasmic fluorescence appeared after treatment with ara-C or IDU but the surface fluorescence was not

produced in the presence of the anti-viral agents.

Herpes simplex virus developed in the nucleus of infected H.Ep.2 and BHK-21 cells. The virions were enveloped at the inner lamella of the nuclear membrane and after passing into the cytoplasm, were released from the cells by a process of reverse phagocytosis. Ara-C and IDU allowed the synthesis of certain viral antigens and the development of nuclear cytopathology but completely prevented the formation of infectious HSV particles. Both drugs caused a marked distortion of the mitochondria and endoplasmic reticulum in uninfected cells.

DNA synthesis in HSV-infected cells, as measured by ^{3}H-thymidine incorporation, was almost completely inhibited by 4 hours of infection. This early inhibition of cellular DNA synthesis was followed by an immediate increase in ^{3}H-thymidine uptake corresponding to the synthesis of viral DNA. Both cell types showed a brief stimulation of mitosis prior to the complete inhibition observed after 20 hours of infection. Cellular and viral DNA synthesis and mitosis appeared to be inhibited in virus-infected and uninfected cells treated with ara-C or IDU.

Infection with HSV resulted in severe chromosomal damage to H.Ep.2 and BHK-21 cells. Chromosomal abnormalities included chromatid gaps and breaks, enhanced secondary constrictions, fragmentation, erosion, and endoreduplication, and were dependent on virus dose and time of infection. The capacity of the virus to induce chromosomal aberrations in cultured cells was UV-inactivated approximately five times less

rapidly than the infectious property. Ara-C acted synergistically with the virus to produce a large number of cells with multiple chromosome breaks and also caused a significant number of abnormalities in uninfected cells. In contrast, IDU treatment resulted in few aberrations over and above those produced by HSV and little damage in uninfected cells. It was concluded that HSV was capable of producing severe morphological and genetic alterations in cultured human and hamster cells. The antiviral agents ara-C and IDU were able to completely inhibit virus multiplication but were unable to prevent any of the virus-induced cytopathic effects *in vitro*.

ABBREVIATIONS

ara-C	Cytosine arabinoside (1-β-D arabinofuranosyl cytosine)
BHK-21	Baby hamster kidney line 21 (clone 13). Derived from a primary Syrian hamster kidney culture.
DNA	Deoxyribonucleic acid
HBSS	Hanks' balanced salt solution
H.Ep.2	Human epidermoid line #2. Derived from a carcinoma of the larynx.
IDU	5-iodo-2'-deoxyuridine
I.U.	international unit
MEM	Minimal essential medium (Eagle)
mg	milligram
nm	nanometer
pfu	plaque forming unit
pH	logarithm of the reciprocal of the hydrogen ion concentration
rpm	revolutions per minute
$TCID_{50}$	Infectious dose destroying 50% of the tissue cultures tested
µg	microgram

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INTRODUCTION

In the past, herpes simplex virus has mainly been of interest as a common disease agent of man, capable of producing infections of varying severity and persistence. However, present studies appear to be directed toward biochemically and cytologically defining the virus-host relationship in terms of potential mutagenesis and oncogenesis. This concern arose after a number of morphologically similar herpes-type viruses were implicated in cell transformation and tumor production in different animals. Shortly afterward, herpes simplex virus type 2 was epidemiologically associated with the occurrence of human cervical carcinoma and further implicated in mammalian cell transformation in vitro. As a result of these intriguing reports, present research has focused on virus-cell interactions at all levels of infection and at the same time is concerned with the development and examination of effective anti-viral agents. For these reasons, the purposes of this study are threefold:

1. To investigate the cytopathology of herpes simplex virus type 1 in cultured human and hamster cells with the aid of light and electron microscopy, immunofluorescence, autoradiography, and cytogenetics.
2. To examine the effects of two chemotherapeutic agents, IDU and ara-C, on herpes simplex virus replication and cyto-

pathology in the same human and hamster cell lines.

3. To observe and measure the biochemical and cytological effects of IDU and ara-C on normal, uninfected human and hamster cells in culture.

LITERATURE REVIEW

Herpes Simplex Virus

1. History

Over two thousand years ago, Hippocrates used the word "herpes" to describe a host of different skin diseases including eczema, skin cancer, erysipelas and herpes zoster (shingles). As late as the nineteenth century, medical texts still referred to many spreading, ulcerative lesions as herpes, but the term soon became restricted to certain vesicular eruptions of the skin and mucous membranes. By 1900, facial herpes, labial herpes, ocular herpes and genital herpes were generally recognized as clinical manifestations of the same human disease, herpes simplex (6).

In 1912, Grüter successfully transmitted herpetic keratitis to the cornea of a healthy rabbit and later reversed his procedure by transmitting experimentally induced ocular herpes to the cornea of a blind man. Following Grüter's initial work, Löwenstein reported that herpetic lesions of the eye, skin and mucous membranes yielded an infectious agent capable of producing a characteristic keratitis in rabbits. In 1921, examination of herpes-infected corneal cells led to the discovery of typical large intranuclear inclusion bodies. Later experiments confirmed that a single washed inclusion could successfully initiate a herpes infection. Interest in the herpes

simplex virus was subsequently spurred by the report of herpetic encephalitis in rabbits and man, and by Burnet and Williams' description of viral persistence in humans in 1939 (reviewed in 31).

Early characterization of the virus showed it to be 100-150 nm in diameter as estimated by membrane filtration (16). Infectivity was lost upon exposure to various detergents and electron microscopy in 1954 established the virus as a large enveloped particle with a dense inner nucleoid (52).

At present, herpes simplex is still of medical interest as a major cause of blindness in man but is perhaps of even more import as a disease model of latency and persistence. Recently, the genital strains of herpes simplex virus have also been epidemiologically associated with human cervical carcinoma and the virus is now the object of intensive scientific investigation (56,57,76).

2. Classification

Herpesviruses are formally defined as large enveloped virions with an icosahedral capsid of 162 capsomeres arranged around a DNA core. Included in this major group are herpes simplex virus, pseudorabies virus, varicella-zoster virus, the cytomegaloviruses, B virus, marmoset virus and various equine, bovine, canine, avian and feline herpesviruses (36,49). Future members may include the Epstein-

Barr virus found in Burkitt's lymphoma cell cultures, the Marek's disease agent of fowl, and the leopard frog virus isolated from Lucke's adenocarcinoma (24,59,85). At present, herpesviruses are either designated by their original descriptive names (i.e. herpes simplex virus, pseudorabies virus) or are classified according to Andrew's binomial system (i.e. Herpesvirus hominis, Herpesvirus suis).

The herpes simplex viruses have also been regrouped into two types or subtypes on the basis of immunological and biochemical differences recently detected in oral and genital strains. Type 1 isolates are generally from the mouth, eye and brain while the type 2 strains are derived from the perineum and genitalia. Apart from antigenic differences, type 1 viruses are less sensitive to heat, produce higher titres in rabbit kidney cells, and possess a lower particle: plaque forming unit ratio than type 2 viruses. On the other hand, type 2 strains are capable of producing plaques on chick embryo cells, have a higher buoyant density and guanine plus cytosine content, and are more neurotropic in mice (20,23,41,72).

3. Structure, Composition and Physical Properties.

The mature herpes simplex virion is a large enveloped particle 150 - 170 nm in diameter. It appears to consist of a dense core,

three concentric shells or capsids, and two outer envelopes (88). These three main architectural units have been concurrently studied with the electron microscope and the ultracentrifuge. The core is revealed as a 25 nm particle containing DNA; it is thought to be closely associated with the electron opaque inner capsid (92). The middle and outer capsids appear as electron translucent and electron dense shells in thin sections of infected cells; the outer capsid consists of 162 hollow protein capsomeres arranged in an icosahedral shape possessing 5:3:2 axial symmetry. The outer envelopes are composed of various lipids and glycoproteins, certain of which are necessary for viral infectivity (84).

Herpes simplex virus contains double-stranded DNA (4,95) of molecular weight 99 $\pm$ 5 x 10^6 daltons (2,41). Native DNA is reported to contain single-strand breaks and is linear and not cross-linked (41). No methylated or unusual bases are known to be present. The guanine plus cytosine content is 68% for type 1 and 70% for type 2 viruses (23). After acrylamide gel electrophoresis, partially purified virions yield up to 12 separate protein bands, at least 6 of which are glycosylated and associated with the viral envelope (106).

Infectious herpes simplex virions are relatively sensitive to heat, radiation, and lipid solvents (36). They are rapidly inactivated at 37°C in a first order reaction and are best stored in distilled

water at -70°C. Similarly, photosensitization, X-ray, and UV irradiation all destroy viral activity in an exponential manner. The virus is also sensitive to most lipid solvents such as sodium deoxycholate, chloroform and ethyl ether as well as enzymes such as trypsin and phospholipase C. Moreover, infectivity and structural integrity are rapidly lost after prolonged centrifugation in CsCl, where the virus generally bands at a density of 1.255 to 1.280 gm/cc (107).

4. Growth in Tissue Culture

(a) Host Range

Herpes simplex virus multiplies in a wide variety of primary and continuous cells cultivated *in vitro*. The virus replicates in most mammalian cell lines, chick embryo fibroblasts and tortoise kidney cells (36). Rabbit kidney cells are perhaps the most sensitive of the cell types found to support herpes simplex virus multiplication. It has been reported that the virus requires the amino acid arginine to complete assembly and maturation *in vitro* (3) and is best produced at temperatures ranging from 35°C to 37°C.

(b) Adsorption, Penetration and Uncoating

The rate of virus adsorption is unaffected by DNA and protein inhibitors and by changes in temperature, suggesting that the process does not require energy or active cell metabolism. Heparin

(30) and parathyroid hormone (80) inhibits virus adsorption while thyroid hormone (81) stimulates the process.

Using purely physical techniques, Huang and Wagner revealed that penetration of herpes simplex virus was 90% complete within 10 minutes of adsorption. They found the process to be temperature dependent and energy requiring (33). Penetration has also been studied by electron microscopy, although results have been confusing at best. For example, while one major report concluded that penetration was effected by pinocytosis of viral particles (32), another equally extensive study found that virus fusion with the cell membrane provided the only means of entry (50). Such conflicting data probably arise from the use of extremely high multiplicities of infection (i.e. 1000 particles per cell) with particle: plaque forming unit ratios of 10 or greater. As a result, the possibility exists that over 90% of the viruses observed in thin sections may not be going through an infectious process during entry and uncoating. It is known, however, that uncoated DNA appears in the nucleus within 30 minutes of virus penetration and that existing cell enzymes apparently uncoat the virus in the cytoplasm (33).

(c) Viral Synthesis

(i) DNA, RNA and Protein Synthesis

Virus-specific DNA is detected as early as 3 hours post infection in the nucleus of infected H.Ep.2 cells. DNA synthesis

reaches a maximum at about 7 hours and thereafter either declines or remains constant throughout the replication cycle (85, 89). Experiments with puromycin have revealed that protein synthesis is a definite requirement for initiation of viral DNA synthesis (86).

Virus-specific RNA is also detectable at 3-4 hours and reaches a maximum at 8 hours, paralleling the course of DNA synthesis (21). The nuclear RNA is heterogenous in size and contains at least one species with a sedimentation coefficient greater than 80s that does not appear in the cytoplasmic RNA profile. This and other evidence suggests that nuclear RNA is cleaved into pieces of lower molecular weight within 10-15 minutes of its synthesis and transported into the cytoplasm of the infected cell (121). Moreover, soon after infection, the cytoplasmic polyribosomes rapidly disaggregate and virus-specific polysomes containing RNA originally synthesized in the nucleus reform in their place (113). In a recent paper on herpes simplex virus RNA synthesis, Wagner also reported sequence differences in "early" and "late" RNAs from both the nucleus and cytoplasm, thus providing the first evidence of transcriptional control of the viral genome (120).

The bulk of virus-specific protein synthesis occurs within 4-6 hours post infection in the cytoplasm of infected cells (90). At least 9-11 different polypeptides are synthesized and incorporated

into the virions, although it is not yet known whether all of these are coded for by the virus genome (66). Synthesis of these structural proteins is asynchronous and their transport into the nucleus slow and relatively selective (67).

After infection of H.Ep.2 cells, Spear and Roizman found 25 virus-specific proteins ranging in molecular weight from 25,000 to 275,000. Of the proteins incorporated into the virions, two contained lipid and at least six were glycosylated and associated with the viral envelopes and cell membranes. These six glycoproteins varied in size and structure with the particular virus strain used, suggesting their synthesis was in part virus-directed (106).

(ii) Enzyme Production

Herpes simplex virus causes a marked increase in the activities of several cell enzymes with concomitant changes in their immunological and biochemical properties. Evidence supports the contention that virus-specific thymidine kinase (44), DNA polymerase (42) and alkaline DNA exonuclease (54) are all produced in infected cells, although conclusive proof awaits more rigorous examination of the virus-host system.

(iii) Virus-Specific Antigens

Immunofluorescent studies have revealed the presence of five separate antigenic elements in herpes simplex virus infected cells (22,45,91,94,124). Using hyperimmune serum prepared in rabbits

from unheated infected cell debris, Roizman et al. detected the presence of small and large nuclear granules, amorphous nuclear patches, cytoplasmic granules and diffuse cytoplasmic fluorescence in H.Ep.2 cells infected with the virus. They concluded that the diffuse staining represented early development of nucleocapsids and virions while the aggregates or granules found late in infection corresponded to large virus "factories" or crystal formations (91).

Roane and Roizman also discovered that the type of antiserum determines the number and kind of virus-specific antigenic elements found in infected cells. Thus, human convalescent serum produces only cytoplasmic and perinuclear staining, and hyperimmune serum prepared in laboratory animals from boiled infected cell debris reveals only specific nuclear fluorescence (78). In all cases, however, antigen production can be detected as early as 3 hours after infection and involves nearly the entire cell population by 24 hours.

(d) Virus Assembly and Release

The site of herpes simplex virus assembly is the nucleus of the infected cell. CsCl centrifugation studies and electron microscopy have suggested that a small core particle containing DNA and protein acts as the viral precursor (88). In the nucleus, the capsid subunits and inner envelope are assembled about the dense cores in a highly asynchronous manner, producing particles in various

stages of assembly throughout the replication cycle. Maturation is considered complete when the virus acquires its outer envelope by budding through the inner lamella of the nuclear membrane (13, 53). However, some particles may reach the cytoplasm before this final step, in which case envelopment usually occurs in the cytoplasmic membrane system. Once in the cell cytoplasm, singly and doubly enveloped virions, which are probably both infective (107), tend to aggregate near and around vacuoles and tubules (13,98).

For many years, it was thought that mature herpes simplex virus was released by budding through the cell membrane in a continuous process that did not lyse the infected cell (19). However, more recent studies have indicated that a network of cytoplasmic tubules transport the virions out of the cell (98). Controversy over the method of egress is still very apparent and will probably remain so until the inherent difficulties in thin section electron microscopy are circumvented.

5. Host Cell Response

(a) Macromolecular Synthesis and Mitosis

Upon infection with herpes simplex virus, the initial rates of cell DNA, RNA and protein synthesis decline. Cell DNA synthesis is rapidly inhibited by 2-3 hours of infection while RNA and protein

synthesis enjoy a gradual decline over the same period. Total macromolecular synthesis increases briefly for the peak 4-5 hours of viral synthesis and then decreases irreversibly until cell death (25).

In addition, the virus causes a rapid inhibition of the mitotic process and cell growth (112). This early event corresponds in time to the inhibition of cell DNA synthesis and is probably mediated by a structural component of the virion or a virus product made soon after infection.

(b) Cytopathology

Cells productively infected with herpes simplex virus display severe alterations in their morphology and social behavior. The first evidence of these alterations is usually seen in the infected cell nucleus (55,62). As early as 3-4 hours after infection, the network of normal cell chromatin becomes highly condensed and displaced to the periphery of the nucleus. Nucleoli also condense and gradually disintegrate and shortly afterwards, a large Feulgen-positive viral inclusion body forms (36). With the development of the inclusion, the cell nucleus becomes grossly enlarged and distorted while the cytoplasm vacuolates and shrinks in volume. Infected cells eventually round up completely and detach from the container surface.

Apart from such morphological changes, the virus also causes alterations in the social behaviour of cells (84). Infection with different strains may result in (i) the formation of polykaryocytes,

(ii) the production of clumped, rounded cells, or (iii) the formation of round "balloon" cells that do not aggregate.

Polykaryocytes result from the fusion of neighbouring cells to form syncytia containing up to 20-30 nuclei. Studies on their formation reveal that the fusion process can only take place after the initiation of viral DNA synthesis in infected cells (79).

In a series of papers dealing with the social behaviour of herpes-infected cells, Roizman concluded that fusion and clumping were the direct result of virus-induced cell membrane alterations. He observed that cells acquire new surface antigens and that cell membrane composition is changed after infection with herpes simplex virus (84,87,93). Presumably, these alterations which lead to the formation of aggregates and polykaryocytes aid in the cell-to-cell spread of the virus.

The type and extent of all alterations in cell morphology and behaviour are dependent on the genetic constitution of the virus strain. Variants that arise frequently after prolonged passage in vitro have been reported to differ markedly from the parent strain in the type of cytopathic effects produced in the same host culture (15). Moreover, the species of host cell and the age and character of the monolayer exert a profound effect on virus cytopathology. Thus, cell responses are influenced not only by the genotype but also by the phenotypic expression of the virus (118).

(c) Chromosome Alterations

Herpes simplex virus is one of a large number of mammalian viruses that induce chromosomal and mitotic irregularities in cultured cells (60,109). The first evidence of its mutagenic potential came in 1961 when Hampar and Ellison discovered that infection of the MCH Chinese hamster cell line with herpes simplex virus resulted in an increased incidence of chromatid breaks, accented secondary constrictions, and translocations. Most of the abnormalities appeared after the first cell division and were primarily located on chromosomes 1 and 2 of the aneuploid complement (27,28).

Since this initial work, many investigators have reported similar effects of virus infection in a variety of mammalian cells including diploid Chinese hamster cells (110), human embryonic lung cells (68,70), human peripheral leukocytes (68), rabbit kidney cells (97), BHK-21 cells (126), and monkey kidney cells (7). In all cases, save for the Chinese hamster cells, the chromosome aberrations appear to be random and non-specific. Virus effects depend on the host cell species and include chromatid and chromosome breaks and gaps, severe fragmentation, accented secondary constrictions, and C-mitosis. The lesions are without exception prevented by prolonged ultraviolet irradiation of the virus or neutralization with herpes simplex virus antibody. In addition, damage is detectable as early as 3 hours after

infection and is found to increase with time and initial viral dose (60, 109).

Early studies suggested that viral replication was necessary for the induction of chromosome abnormalities. However, zur Hausen found no evidence of viral DNA synthesis in BHK-21 cells with severely damaged metaphase plates and thus concluded that either a virion component or an early enzyme was responsible for the damage (126). At present, the specific cause of all virus-induced chromosome aberrations is still unknown.

(d) Cell Transformation and Oncogenesis

Transformation at the cellular level is generally defined as a stable, inheritable genetic alteration in a cell, usually resulting in a changed morphology and growth pattern. Cells transformed by viruses are very often oncogenic and are therefore studied in an effort to define the causes and conditions of carcinogenesis.

To date, there are at least four possible herpes-type viruses implicated in the oncogenic process - the human Epstein-Barr virus found in Burkitt's lymphoma cell cultures, the Marek's disease agent of chickens, the leopard frog adenocarcinoma virus, and Herpesvirus saimiri which induces lymphomas in marmoset monkeys (48,59,116). With the discovery of these viruses and the recent epidemiological evidence suggesting a relationship between the occurrence of herpes

simplex type 2 infections and human cervical carcinoma (56,57,76), the herpes simplex viruses have been re-examined for possible oncogenic and transforming capacities. So far, the virus has only been implicated as a co-carcinogen in live animal studies (114). However, early in 1971, Duff and Rapp reported the production of a hamster embryo fibroblast line transformed after exposure to ultraviolet-irradiated herpes simplex type 2. Thirty days after virus infection, the cells developed an altered morphology and unrestricted growth pattern. The resulting cell line produced herpes simplex virus antigens and was highly oncogenic in newborn and weaning hamsters (14). If this preliminary report is extended and verified by others, herpes simplex virus may join the ranks of DNA tumor viruses capable of interacting with their hosts to produce cell transformation as well as cytocidal and abortive infections.

6. Pathogenesis

(a) Animals

The natural host for herpes simplex virus is man. However, the virus can also be experimentally transmitted to rabbits, guinea pigs, mice, rats, chick embryos, geese and hedgehogs (36). Perhaps the most common experimental animals are the adult rabbit and newborn mouse.

Corneal inoculation of herpes simplex virus in rabbits has been

found to produce a typical keratitis beginning with the appearance of pinpoint dendritic lesions within 24 hours of infection. The lesions rapidly enlarge and invade the corneal stroma, producing a purulent exudate and ultimately, blindness. Viremias can be detected after 24 hours and virus can be successfully isolated from brain, spleen, kidney and lung homogenates. If the keratitis is left untreated, the disease often extends to the brain and causes a fatal encephalitis (40).

Using 5-day old mice, Wildy found that footpad inoculation resulted in the rapid development of posterior paralysis without evidence of viremia. He assumed that the normal route of virus invasion was exclusively via the peripheral nerves to the central nervous system (127). However, in 1964, extensive immunofluorescent studies of murine encephalitis provided evidence of both a neural and hematogenous spread. Johnson found that herpes simplex virus either produced a viremia that allowed infection of small cerebral vessels serving the central nervous system, or resulted in a centripetal infection of endoneural cells (not axons) leading to the vital cranial nerves. Intracerebral inoculation of the virus produced an exclusive direct neural spread while intranasal and intraperitoneal inoculation resulted in multiple pathways of hematogenous and neural invasion (35).

(b) Man

In man, primary herpes simplex virus infections usually occur between the ages of 6 months and 5 years. It is estimated that less than 10% of the infected individuals ever demonstrate any overt disease although the virus infection can occasionally be severe and even fatal (10). Primary infections can affect up to 60 - 100% of the population, producing a variety of vesicular lesions. The clinical expressions depend to a large degree on the portal of entry and include gingivostomatitis, keratitis, vulvovaginitis, eczema herpeticum and rarely, meningo-encephalitis. In most cases, the illnesses are relatively mild and self-limiting but in newborn and premature infants, generalized herpes simplex can be rapidly fatal (36).

Once the initial virus infection subsides, however, the majority of people suffer from a recurrent form of herpes simplex that invariably occurs in the presence of circulating antibody (10,40,83). Skin lesions tend to recur at or near the same site and appear following specific physical or emotional stimuli that include exposure to sunlight, illness, fever, menstruation, hormone treatment, administration of foreign proteins, specific types of neurosurgery and emotional stress. It is now generally accepted that the herpes simplex virus is harbored in a latent or inapparent form at some particular site in the body. Certain host factors reactivate the infection at various

times and cause the virus to manifest itself in typical herpetic lesions (36).

At present, there are two theories to explain the persistence of herpes simplex in man. The first proposes that virus multiplication occurs at a slow but constant rate at the site of the recurrent lesion. According to this dynamic state hypothesis, various stimuli provide a temporary permissive state in uninfected cells and thus allow brief recurrences of overt disease (83). The second theory envisions the existence in unknown cells of a noninfectious form of the virus. In this hypothesis, virus multiplication is reversibly interrupted at some stage and can be reactivated by certain agents and conditions of the host (83).

In an effort to distinguish between these two theories, many attempts have been made to isolate infectious virus from the healed site of lesions in the interim between recurrences. To date, no virus or infected cells have been recovered in this manner. However, a number of investigators have reported the isolation of infectious virus from the tears and saliva of apparently normal individuals as well as from the secretory glands and tears of rabbits with recurrent keratitis (10). Thus, the persistence of the herpes simplex virus may indeed be due to a low-grade, chronic infection of the host as envisaged by the dynamic state hypothesis.

On the other hand, an inapparent form of the virus has also been encountered in rabbits with recurrent ocular herpes. Examination of various neurons including the trigeminal ganglion revealed no apparent virus infection between recurrences. However, one to two weeks after organ cultures had been established, the trigeminal ganglion cells produced fully infectious herpes simplex virus (108). In man, surgery of the same ganglion frequently results in postoperative herpetic lesions (17). Thus, the experimental and clinical evidence appears to suggest that the virus exists in a latent, noninfectious form in specific neural ganglia of the host.

Persistent herpes simplex virus infections are rather unusual in that circulating antibody is normally present during the course of the recurrences. Moreover, there appears to be no change in the serum levels of this antibody either before or after overt infection (26). This apparent contradiction is probably a result of the method of virus spread. Herpes simplex virus can spread by direct cell-to-cell contact and thus remain unaffected by circulating antibody (84). However, the self-limiting nature of the lesions still requires satisfactory explanation from an immunological point of view.

A recent analysis of immunoglobulins in patients with persistent infections shows a significantly greater amount of IgA in the blood during a latent period than during an active recurrence (26).

Further studies showed that patients with persistent herpes also have impaired macrophage inhibition and lowered lymphocyte toxicity (128).

In summary, recurrent herpes simplex virus infections appear to be established and maintained by a complex interaction of a variety of host factors including IgA serum levels, cell-mediated immunity, temperature, interferon and body hormones (83).

(c) Chemotherapy

To date, IDU and ara-C have provided the best clinical results in treatment of herpes simplex virus infections (74). In clinical trials, Kaufman found that topical applications of IDU resulted in complete eradication of herpetic keratitis in man and animals (39). Subsequent double-blind studies confirmed that IDU had a significant effect on superficial lesions but failed to reduce the recurrence rate or prevent the development of deep stromal lesions. In most cases, the chemical had no obvious toxic effects, although at least one report of corneal "speckling" is recorded (36). However, in the intact animal, IDU is highly toxic and is used only in very severe diseases. Patients with acute necrotizing encephalitis and generalized herpes simplex are occasionally treated with massive doses of IDU. Most medical communications report an increased chance of recovery with the drug but little if any decrease in subsequent neurological damage (47). Moreover, toxic side-effects such as leukopenia and thrombocytopenia

are likely to occur after prolonged treatment (74).

In vitro, IDU is a potent inhibitor of herpes simplex virus. At least 99% inhibition of infectious virus production is obtained if 50 µg/ml IDU is added to an infected culture as late as 4 hours after infection (9,82,104,105). The halogenated pyrimidine appears to be incorporated into the newly synthesized viral DNA in place of thymidine, thus causing defective assembly of the virions (37).

Although repeated exposure to IDU results in the development of drug-resistant mutants, these viruses usually remain sensitive to the action of ara-C. Ara-C is equally effective against herpetic keratitis (117) and herpes simplex virus replication in vitro (9,46), but is highly toxic to all mammalian cells. Both IDU and ara-C prevent mitosis in culture and inhibit DNA synthesis (11,38,43,73,101) while causing concomitant chromosome abnormalities (8,61,69). As a result of the adverse effects of these drugs, chemotherapy of herpes simplex virus infections is generally restricted to localized lesions or severe cases of systemic disease.

MATERIALS AND METHODS

Cells and Medium

The H.Ep.2 continuous line of human epidermoid cells (51) and the BHK-21 (clone 13) line of Syrian hamster kidney cells (111) were obtained from Microbiological Associates, Bethesda, Md. The cells were maintained as monolayer cultures in milk dilution bottles or Leighton tubes on Eagles' minimal essential medium (MEM), AutoPow (Flow Labs., Rockville, Md.). The medium was routinely supplemented with 10% fetal calf serum, penicillin G (100 I.U./ml), streptomycin (100 µg/ml), and 0.025% glutamine. Cell cultures were divided regularly every three days by the use of 0.25% trypsin in Hank's balanced salt solution (HBSS).

Suspensions of approximately 10^6 cells in medium with 20% fetal calf serum and 10% dimethylsulfoxide were frozen and stored at -70°C to serve as stock cultures. Frozen cells were regularly revived every three months to ensure genetic and morphologic stability of the lines. Electron microscopy and tritiated thymidine autoradiography of normal H.Ep.2 and BHK-21 cells failed to reveal the particles and abnormal cytoplasmic labeling characteristic of mycoplasma infection (29,58).

Virus

1. Origin

The H4253 herpes simplex virus (HSV) strain was isolated by Dr. D.M. McLean from the throat of a patient with multiple lip lesions. Serological identification was made on the basis of specific neutralization tests in mice. Subsequent to its isolation, the virus was passaged five times in mice and a total of twelve times in H.Ep.2 monolayers. During this time, the virus was also plaque-purified three times by T. Mosmann. Electron microscopy revealed particle morphology typical of the herpes group of viruses.

2. Viral Preparation and Purification

Stock virus was prepared from 24 hour H.Ep.2 monolayers grown in dilution bottles and inoculated with 1.0×10^8 pfu HSV. The cultures were incubated at 37°C for 20 hours in MEM containing 2% fetal calf serum. At the end of this time, the medium was replaced with an equal volume of water, the cells scraped off the glass bottles, and the resulting suspensions frozen and thawed three times. The crude lysates were then centrifuged at 3500 rpm for 10 minutes to remove large cell debris and the supernatants recentrifuged at 4°C in a Beckman Model L2-65B Ultracentrifuge for one hour at 15,000 rpm. Pellets containing the virus were resuspended in MEM with 2% fetal

calf serum and were stored in serum bottles at -70°C. Stock preparations gave titres of 7.2×10^7 pfu/ml or 5.0×10^5 $TCID_{50}$/ml.

Virus Assays

1. End-point Dilution Technique

For rough assays of virus preparations, the end-point dilution method was used to calculate virus titres. The virus samples to be tested were serially diluted tenfold in MEM plus 2% fetal calf serum. Twenty-four hour H.Ep.2 monolayers grown in Leighton tubes were inoculated in duplicate with 0.1 ml of each dilution and incubated at 37°C for 48 hours. The monolayers were then examined microscopically for cytopathic effects and graded on a scale of +1 to +4. Virus titres were calculated by the Reed and Meunch method (77) and expressed as $TCID_{50}$ units.

2. Plaque Assay

Quantitative plaque assays were also performed using confluent H.Ep.2 monolayers grown in 35 x 10 mm Falcon plastic petri dishes. The cells were maintained under a 5% CO_2 atmosphere in Dulbecco's medium. Washed H.Ep.2 monolayers were infected in duplicate with 0.1 ml of each dilution and rotated periodically over 30 minutes

to ensure even inoculation. An agarose overlay was prepared by melting and mixing in equal parts 1% agarose in distilled water and serumless double-strength Dulbecco's medium. Each monolayer received 2.0 ml of the overlay before incubation at 37°C in a 5% CO_2 atmosphere for 3-4 days. The resulting plaques were stained for 3 hours with a 0.1% neutral red solution and observed under indirect light. The plaques appeared as small heavily stained spots surrounded by clear halos against a lightly stained background.

Chemicals and Radioisotopes

1. L-Arginine Hydrochloride was obtained from Sigma Chemicals, St. Louis, Missouri. A stock 5% solution in distilled water was stored at 4°C.

2. Colcemid was donated by the Ciba Co. Ltd., Dorval, Quebec. Stock 10 µg/ml solutions in HBSS were stored at 4°C in a light-tight container and were renewed every three weeks. A fresh 1.0 µg/ml preparation was made daily from the stock.

3. Cytosine Arabinoside Hydrochloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Stock solutions of 1000 µg/ml in HBSS were stored at 4°C and were renewed every three weeks. A fresh 10 µg/ml solution was prepared for each experiment.

4. 5-Iododeoxyuridine, purchased from Calbiochem, Los Angeles, California, was kindly supplied by Dr. D.M. McLean. Stock preparations in HBSS were stored at 4°C and were renewed every three weeks. A fresh 500 µg/ml solution was made daily from the stock.

5. Tritiated thymidine (^{3}H-thymidine), specific activity 49.2 Ci/mM, was obtained from New England Nuclear Corporation, Boston, Mass., and generously supplied by Dr. J.B. Hudson. Fresh preparations of the radioisotope were made in HBSS and held at 4°C until use.

UV Inactivation of Virus

The herpes simplex virus was irradiated in 60 mm Falcon plastic petri dishes (2.0 ml/dish) at a distance of 20 cm from a Sylvania G15T8 UV bulb. Irradiation was carried out at room temperature with constant agitation for periods of time ranging from 10 seconds to 240 seconds.

In Vitro Infection Procedure

Day-old cell cultures were washed once with HBSS and inoculated with HSV at an input multiplicity of about 1 pfu per cell. The input multiplicity was determined by calculating the average number of cells per culture in a Levy counting chamber (Max Levy, Philadelphia, Penn.). After 30 minutes adsorption at room temperature, the monolayers were rinsed with HBSS and incubated at 37°C in MEM with 10%

fetal calf serum.

Light Microscopy

Cell monolayers grown on Leighton tube coverslips were infected with 7.2×10^6 pfu HSV, treated with 10 µg/ml ara-C or 100 µg/ml IDU, or left to serve as controls. After 5, 12, 24 and 72 hours, the coverslips were removed and rinsed briefly in physiological saline. Fixation in absolute methanol for 3 minutes preceded staining in a Giemsa solution consisting of 3.0 ml stock Giemsa in 50 ml phosphate buffer pH 7.2 for 30 minutes. The stained coverslips were given a final rinse in saline, air-dried, and mounted on clean glass slides with Apochromount mounting fluid (Aloe Scientific, St. Louis, Missouri). The cells were examined and photographed using a Zeiss semi-automatic photomicroscope.

Indirect Fluorescent Antibody Technique

Monolayer cultures grown on Leighton tube coverslips were infected with 7.2×10^6 pfu HSV per tube (MOI = 1). In some cases, 10 µg/ml ara-C or 100 µg/ml IDU were added to both infected and non-infected cells. At 4, 7, 12 and 24 hours post infection, the coverslips were removed, rinsed in physiological saline, air-dried, and fixed in acetone for 10 minutes. Each coverslip was exposed to

0.1 ml of 1:2 dilution of guinea pig anti-HSV gamma globulin (Titre 1:32 Microbiological Associates, Bethesda, Md.) for 30 minutes in a moist chamber at 37°C. The coverslips were then rinsed in saline and treated with 0.1 ml fluorescein-labeled goat anti-guinea pig gamma globulin (Microbiological Associates, Bethesda, Md.) for 30 minutes in a moist chamber at 37°C. The cells were given a final rinse in saline and mounted on clean glass slides using phosphate-buffered glycerol pH 7.2 (Baltimore Biological Labs., Baltimore, Md.). Observation and photography was accomplished using a Reichert microscope equipped with an Osram HBO 200 high pressure mercury vapor lamp as a UV source.

Electron Microscopy

1. Negative Staining

A frozen stock HSV preparation containing 7.2×10^7 pfu/ml was rapidly thawed in a room temperature water bath and a drop of the virus suspension placed on a wax staining tray. A clean carbon and formvar coated copper grid was lowered onto the drop and allowed to remain in contact with it for one minute. The grid was then removed to a drop of 2% phosphotungstic acid pH 6.5 and stained for one minute before examination in a Phillips EM 300 electron

microscope. Grids were observed within 30 minutes of initial preparation in order to preserve virus integrity which was rapidly lost at room temperature.

2. Thin-Sectioning

Confluent monolayers of H.Ep.2 and BHK-21 cells grown in milk dilution bottles were used in all electron microscopy studies. Normal, infected and chemically treated cells were scraped from the glass with a rubber policeman, pelleted by low-speed centrifugation (1000 rpm for 5 minutes in an International Centrifuge Model CS), and immediately placed into 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 30 minutes at 4°C. The specimens were washed four times in 0.2 M phosphate buffered sucrose and post-fixed in 1% osmium tetroxide pH 7.2 for 30 minutes. Dehydration was accomplished by passing the samples through a graded series of ethanol concentrations followed by a step-wise transfer into propylene oxide. Specimens were embedded by placing the cells in increasing concentrations of Epon 812 resin dissolved in propylene oxide. After overnight incubation in plastic at room temperature, the embedded samples were placed in gelatin capsules and cured at 60°C for 24 hours.

Sections of all specimens embedded in Epon 812 were cut on a LKB III microtome and collected on carbon and formvar coated 150

mesh copper grids. They were stained with a saturated alcoholic solution of uranyl acetate for one minute and post-stained with Reynold's lead citrate for 30 seconds. The grids were examined on a Phillips EM 300 electron microscope at 60 kv.

Autoradiography

Twenty-four hour monolayer cultures grown on Leighton tube coverslips were infected with 7.2×10^6 pfu HSV (MOI = 1) and/or treated with either 10 µg/ml ara-C or 100 µg/ml IDU. At various times afterward, the cells were exposed to 2.0 µCi/ml ^{3}H-thymidine for 30 minutes at 37°C. Immediately following exposure to the radioisotope, the coverslips were washed with HBSS and fixed in a 3:1 mixture of methanol and acetic acid for 15 minutes. Residual fixative was removed by repeated washings in distilled water over a period for 20 minutes. The fixed cells were then air-dried and mounted on clean glass slides with Apochromount mounting fluid.

The slides were dip-coated with Ilford L-4 Nuclear Emulsion, drained, air-dried and stored for 7 days at 4°C in a light-tight box containing a small amount of Drierite.

The dipped slides were developed according to the following schedule: 12 minutes in Microdol-X developer (2 parts water and 1 part developer); 5 minutes in tap water; 5 minutes in fixer; 4 - 2 minute

washes in tap water. The developed slides were air-dried and examined with an Olympus light microscope for the presence of tritium label. To facilitate observation of the grains, coverslips were stained briefly in 1% eosin.

Metaphase Preparations

Cells growing logarithmically (i.e. 16 - 18 hour cultures) in Leighton tubes were used in all chromosome studies. Infected cells generally received 7.2×10^6 pfu HSV per tube and chemically treated cells either 10 µg/ml ara-C or 100 µg/ml IDU. Two hours prior to harvesting, infected, treated and control cultures also received 0.1 µg/ml colcemid. Cells were trysinized off the tubes with 0.25% trypsin in HBSS and centrifuged in an International Centrifuge Model CS for 5 minutes. The supernatant was decanted and the cells gently resuspended in 5.0 ml of 0.5% KCl solution for 15 minutes at room temperature. This suspension was recentrifuged and 4.0 ml of a 3:1 methanol-glacial acetic acid mixture added drop by drop over a period of 20 minutes. After two further changes of fixative, the cells were finally suspended in about 1.0 ml of the solution. Four or five drops from a Pasteur pipette were dropped on a precleaned glass slide from a height of 4 - 5 inches. After air-drying, the slides were stained in a Giemsa solution for 5 minutes. The stained slides were

then briefly rinsed in tap water, air-dried and examined preliminarily with an Olympus light microscope. Metaphase plates were scored for chromosomal aberrations under an oil immersion lens and photographed on Kodak Plus X panchromatic film on a Zeiss semi-automatic photomicroscope.

RESULTS

Growth Studies

Figure 1 shows the growth of HSV in H.Ep.2 and BHK-21 monolayers at 37°C. Both cell types supported viral multiplication but H.Ep.2 cells were clearly more susceptible to HSV infection. The first detectable increase in virus production occurred 8-12 hours after infection and maximum yields were obtained after 22-24 hours. Virus titres at 22 hours were $10^{5.0}$ $TCID_{50}$/ml in H.Ep.2 cells and $10^{3.8}$ $TCID_{50}$/ml in BHK-21 cells. Addition of 10 µg/ml ara-C or 100 µg/ml IDU at the time of infection reduced virus titres by 99.9% in both cell types. Further effects of the anti-viral drugs are documented in the various sections on light and electron microscopy, autoradiography and cytogenetics.

Light Microscopy

1. Cytopathology of HSV

Leighton tube cultures of H.Ep.2 cells were infected with an input multiplicity of 1 pfu HSV per cell and examined at 5, 12, 24 and 72 hours of infection.

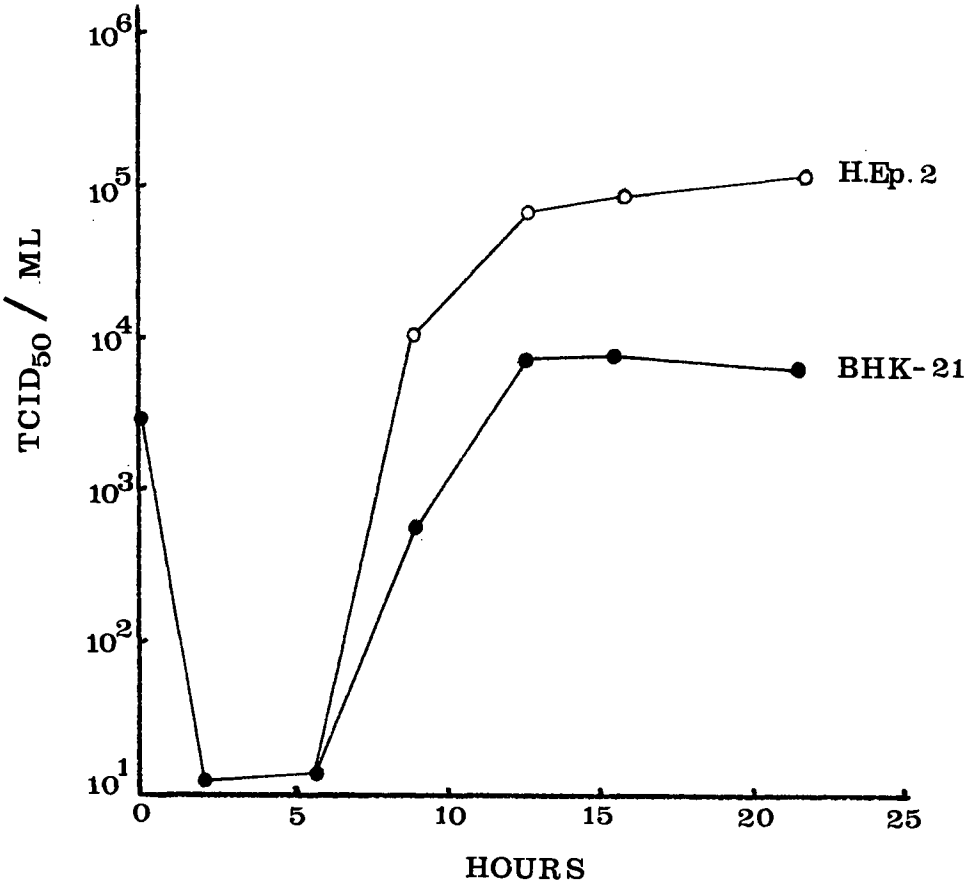

Figure 1. Representative growth curves of HSV in H.Ep.2 and BHK-21 cells. Total virus (extracellular and intracellular) was measured by the standard end-point dilution technique after initial infection with an input multiplicity of 1 pfu per cell.

Normal, uninfected H.Ep.2 monolayers were composed of closely packed epitheloid cells with large ovoid nuclei containing 2-4 well-defined nucleoli (Figs. 2,4). The first evidence of virus-induced cytopathology was observed in the nuclei of infected cells. Five hours after infection, many cells showed evidence of early nucleolar disintegration and peripheral chromatin displacement. At 12 hours, over one-half of the cells also demonstrated some degree of rounding and cytoplasmic vacuolization (Fig. 3). The empty-appearing nuclei of these cells were invariably swollen and usually contained dense viral inclusion bodies surrounded by clear halos (Fig. 3). After 24 hours of infection, large numbers of cells had detached from the glass surface and those remaining showed severe nuclear damage coupled with extensive cytoplasmic shrinking and rounding. The damaged cells had a slight tendency to aggregate in loose clumps but did not at any time form syncytia (Fig. 5). Monolayers were completely destroyed at 72 hours. A similar virus cytopathology was observed in BHK-21 cells infected with HSV.

2. Effect of Ara-C and IDU ON HSV Cytopathology

Cultures inoculated with HSV received either 10 µg/ml ara-C or 100 µg/ml IDU at the time of virus infection. Observations made at 5, 12, and 24 hours revealed no decrease in the severity or time of appearance of the virus-induced cytopathic effect in H.Ep.2 and BHK-21 cells.

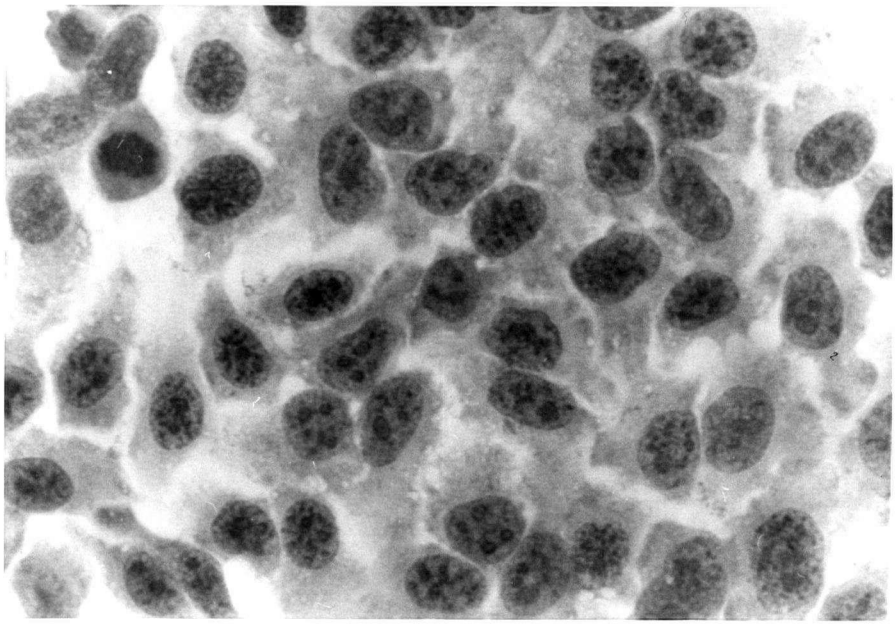

Figure 2. Uninfected culture of H.Ep.2 cells. Giemsa stain. X1750.

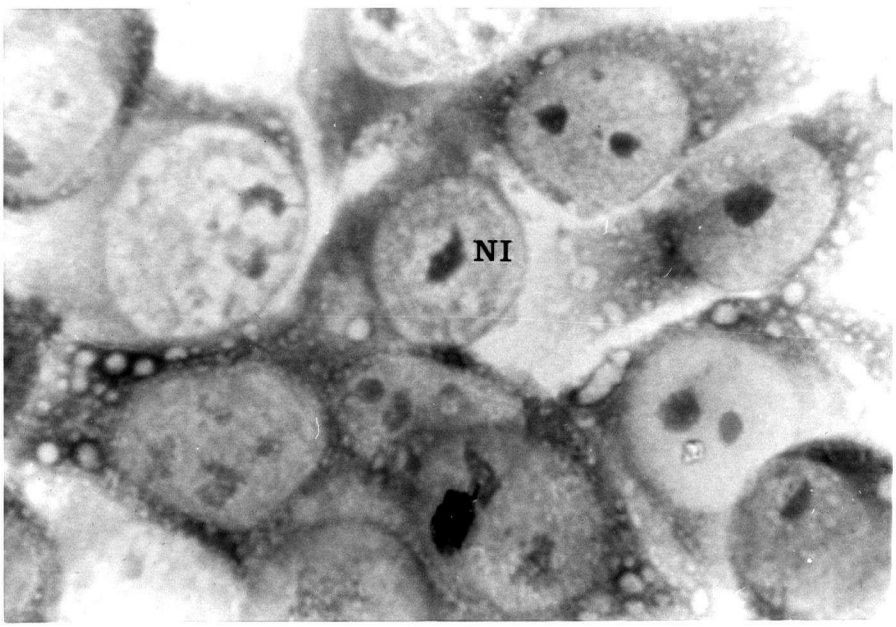

Figure 3. H.Ep.2 culture 12 hours after HSV infection. Giemsa stain. Note intranuclear inclusion (NI) and swollen degenerating nuclei of infected cells. X4400.

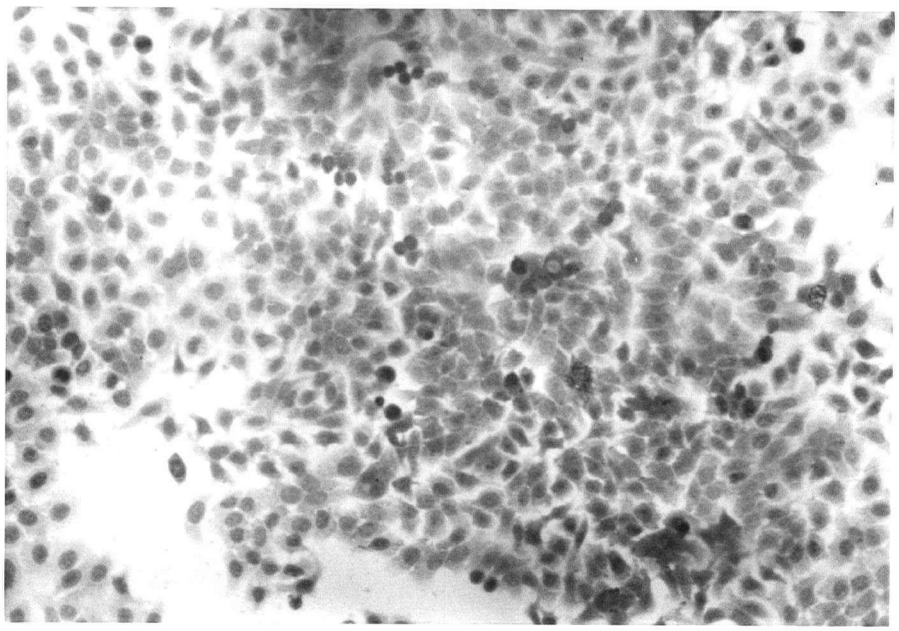

Figure 4. Uninfected culture of H.Ep.2 cells. X440.

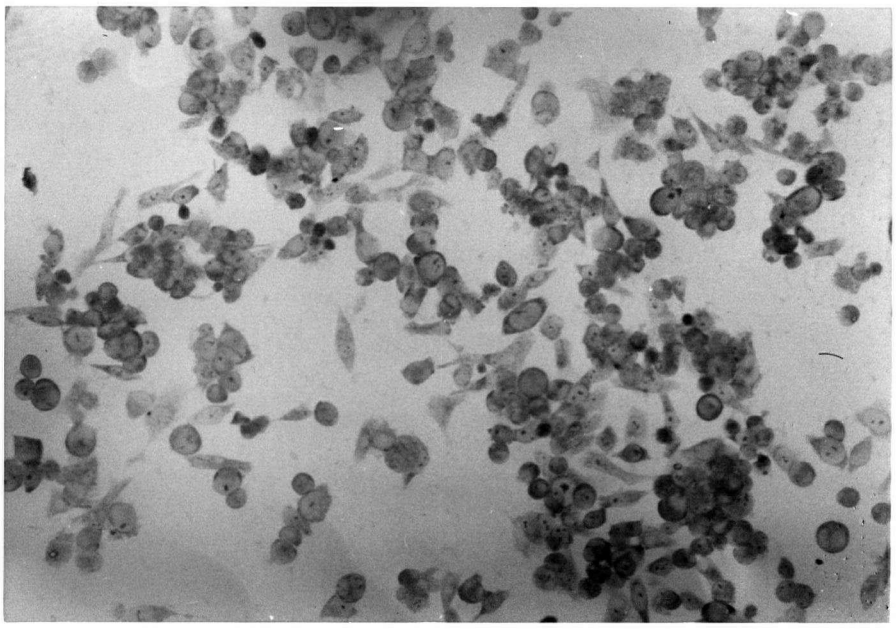

Figure 5. H.Ep.2 culture 24 hours after HSV infection. Note loose aggregates of rounded, shrunken cells. X440.

3. Effect of Ara-C and IDU on Uninfected Cells

Healthy H.Ep.2 and BHK-21 monlayers were treated with 10 µg/ml ara-C or 100 µg/ml IDU for 24 and 72 hours. Exposure to ara-C resulted in moderately severe damage to cultures after 72 hours. The monolayers were partially destroyed (Fig. 6) and individual cells highly vacuolized and often rounded (Fig. 7). IDU appeared to cause similar but less pronounced cellular alterations after 3 days of treatment.

Fluorescent Antibody Studies

1. HSV Antigen Production

Leighton tube cultures of H.Ep.2 and BHK-21 cells were infected with an input multiplicity of 1 pfu HSV per cell and observed for production of virus-specific antigens at 4, 7, 12 and 24 hours by the indirect fluorescent antibody method.

The first signs of viral infection appeared after 4 hours in the form of weak cytoplasmic and nuclear fluorescence (Fig. 8). The diffuse cytoplasmic fluorescence reached a maximum intensity at 7 hours and was particularly evident in the perinuclear regions of infected cells. Early nuclear antigens were characterized by small, irregularly-shaped granules (Fig. 8) that were later obscured

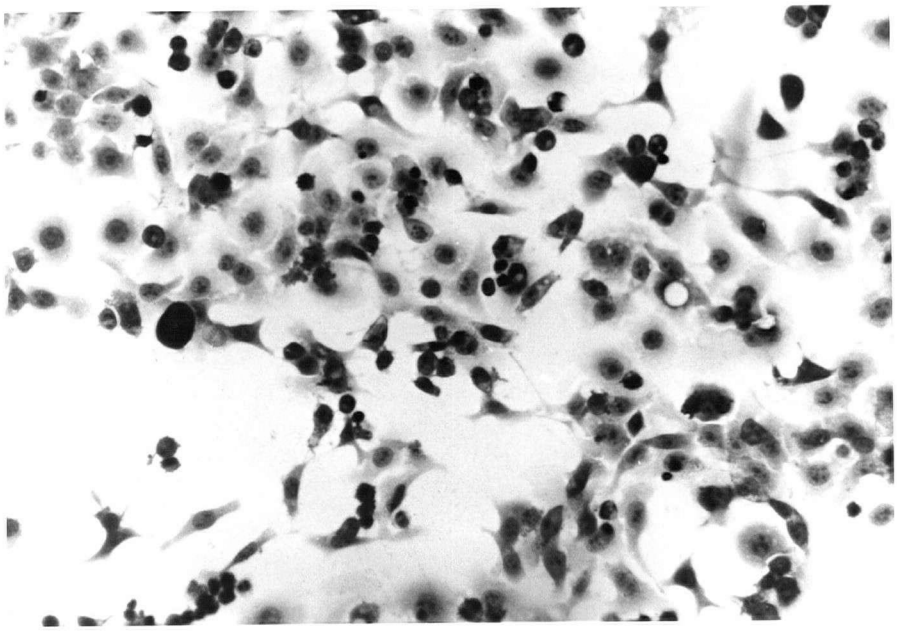

Figure 6. H.Ep.2 culture after 72 hours of ara-C treatment. X440.

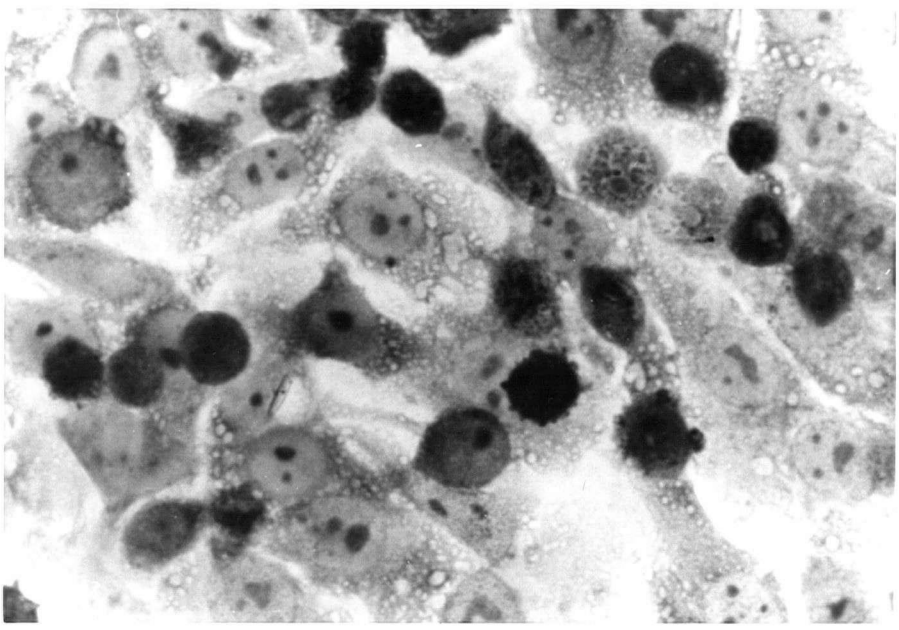

Figure 7. H.Ep.2 culture after 72 hours of ara-C treatment. Note cytoplasmic vacuolization and prevalence of rounded, shrunken cells. X1750.

by the development of large amorphous masses. Between 7 and 12 hours, the number of antigen-producing cells rose to over 70% of the population. Cells at this time commonly exhibited intense cytoplasmic staining together with patchy nuclear fluorescence (Fig. 9). By 24 hours, virus-specific fluorescence was observed in over 80-90% of the infected cell culture. Most of these cells fluoresced strongly in the cytoplasm and at the cell surface (Fig. 10).

2. The Effect of Ara-C and IDU ON HSV Antigen Production

The addition of 10 µg/ml ara-C or 100 µg/ml IDU to infected cultures failed to delay the appearance of HSV antigens or decrease the total number of fluorescing cells. Strong nuclear and perinuclear staining was evident at 4, 7 and 12 hours of infection but surface fluorescence was generally lacking in the chemically treated cells.

Electron Microscopy

1. Negative Staining

HSV morphology was studied by the negative stain method. Typical virus particles of mean diameter 170 nm were frequently observed in addition to the cell debris normally found in semi-purified virus preparations. The virions consisted of large envelopes enclosing

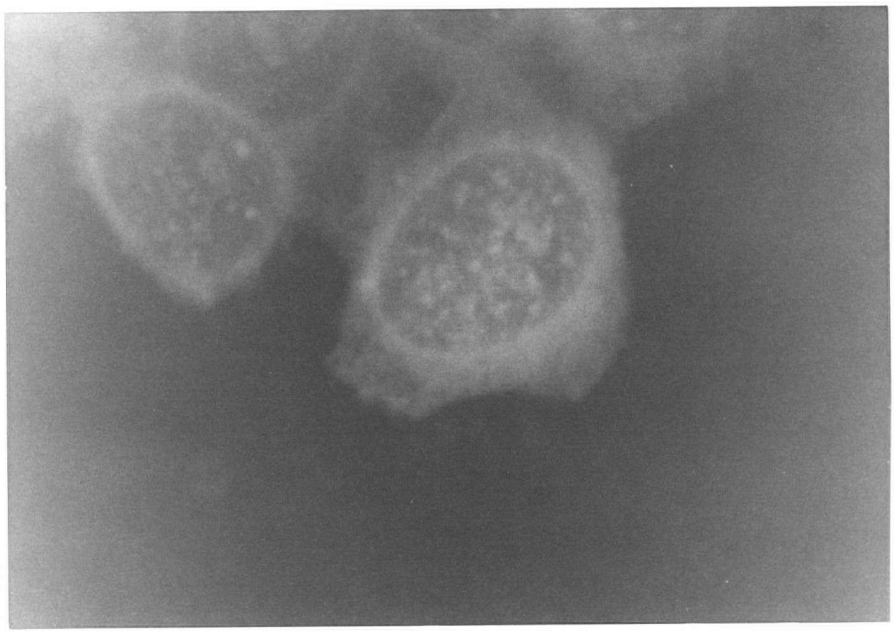

Figure 8. Fluorescent antibody study of H.Ep.2 cells 4 hours after HSV infection. Note weak perinuclear staining and fluorescent nuclear granules. X4400.

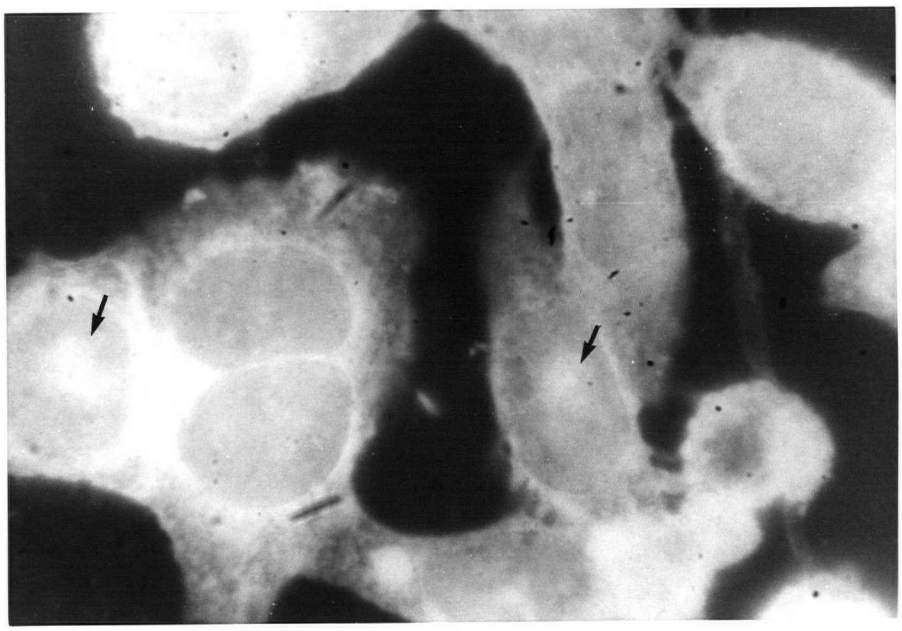

Figure 9. Fluorescent antibody study of H.Ep.2 cells 7 hours after HSV infection. Note amorphous nuclear masses and diffuse cytoplasmic fluorescence. X4400.

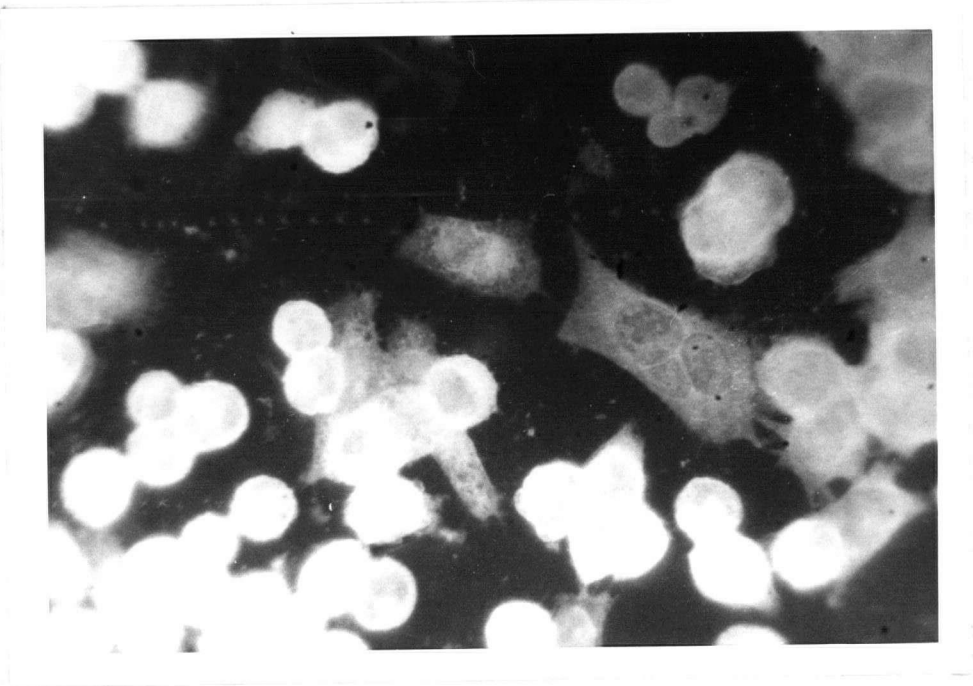

Figure 10. Fluorescent antibody study of H.Ep.2 cells 24 hours after HSV infection. X1750.

68-70 nm nucleocapsids. The capsids were clearly composed of hollow, polygonal capsomeres arranged in a regular icosahedral symmetry (Fig. 11). In cases where the phosphotungstic acid penetrated the external capsid, a dense inner "core" was sometimes observed. The core, which measured 30 nm in diameter, was encased in an outer shell composed of hollow subunits similar to those seen on the external capsid. Other particles appeared to contain no inner structure (Fig. 12).

2. Thin Sectioning

(a) Uninfected H.Ep.2 and BHK-21 Cells.

Uninfected H.Ep.2 and BHK-21 cells maintained in MEM plus 10% calf serum revealed well preserved microanatomy with intact membranes, undistorted mitochondria, and normal chromatin distribution (Fig. 13,14). Apart from an increased incidence of lipid granules in BHK-21 cells, the human and hamster lines appeared to possess very similar ultrastructure.

(b) Uninfected BHK-21 Cells: Abnormal Particle Formation

BHK-21 cells maintained in serumless MEM repeatedly gave rise to abnormal particles in the cytoplasm of affected cells. The 90 nm particles consisted of an electron dense core and a less-dense membrane-bound outer region (Fig. 15). Close examination revealed

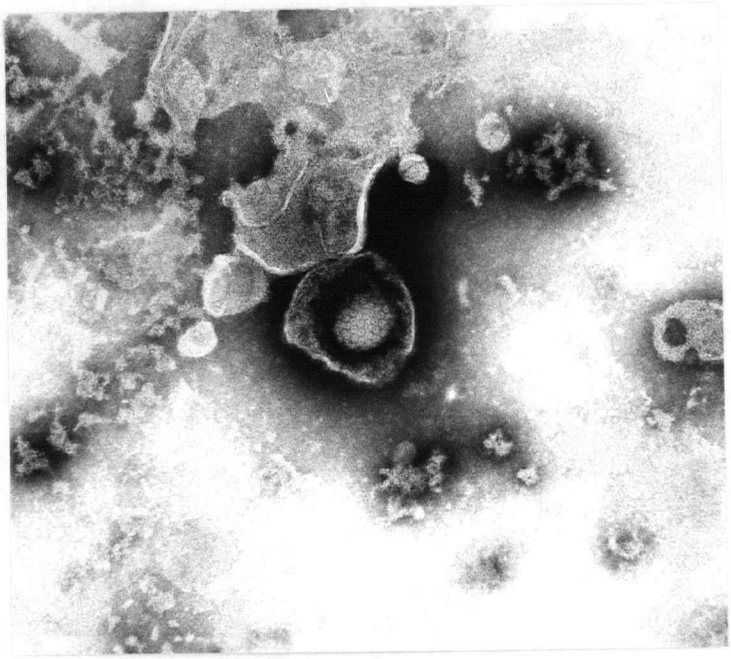

Figure 11. Electron micrograph of a negative stain preparation of HSV.
X90,700.

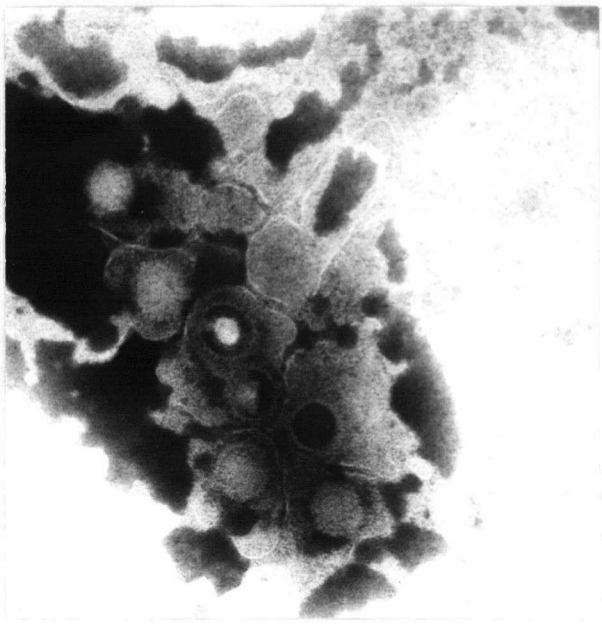

Figure 12. Electron micrograph of a negative stain preparation of HSV. The inner structure of two particles can be observed where the stain has penetrated the outer capsid.
X90,700.

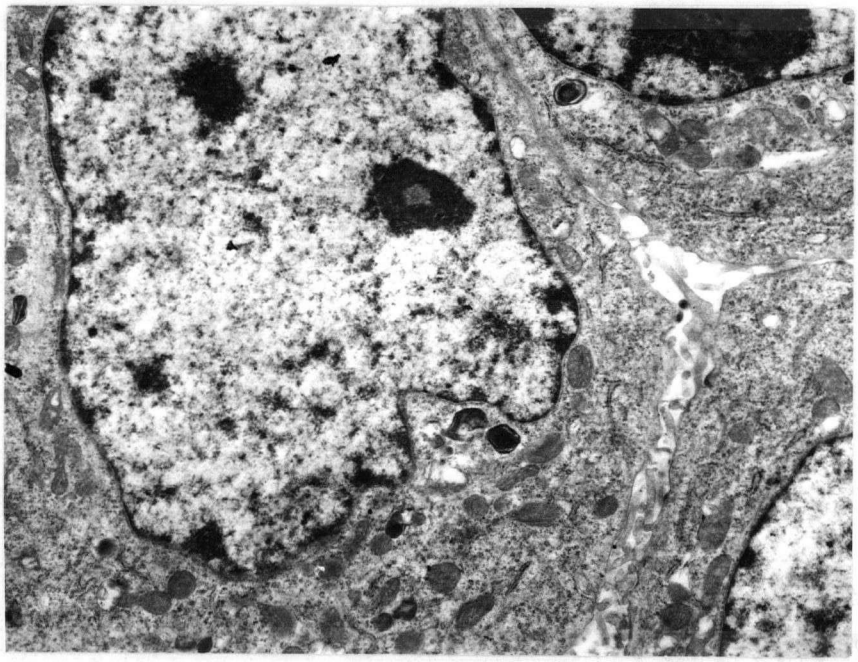

Figure 13. Electron micrograph of normal H.Ep.2 cells showing intact nuclear and cytoplasmic structure. X10,000.

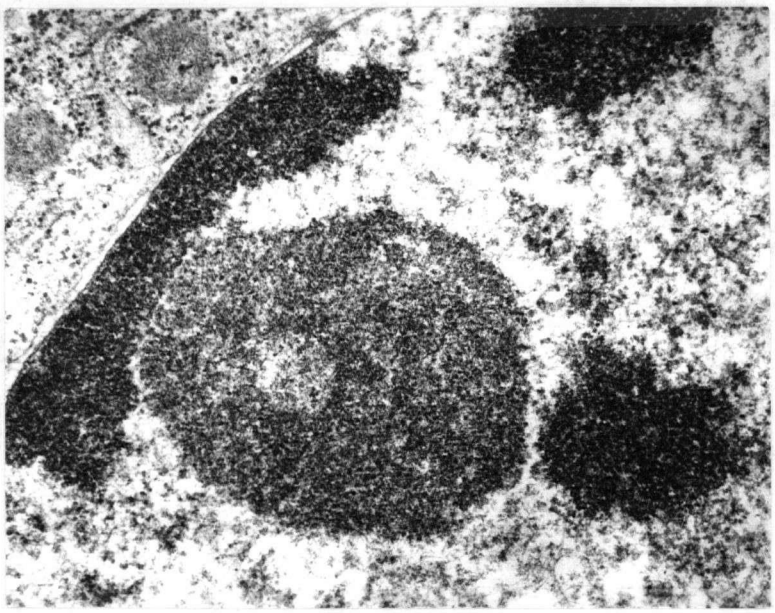

Figure 14. Electron micrograph of a normal BHK-21 cell showing intact nuclear and cytoplasmic structure. X42,600.

fine radial structures extending from the core to the external membrane (Fig. 16). The particles were normally observed in cytoplasmic spaces or between the swollen membranes of the endoplasmic reticulum. Numerous distorted mitochondria, ragged vacuoles, and myelin figures were also found in affected cells. Since serum starvation appeared to be the sole cause of these structural abnormalities, all electron microscope studies of BHK-21 cells were carried out on cultures provided with normal growth medium. No particles were observed in uninfected cells grown in the presence of 10% fetal calf serum.

(c) HSV Development in H.Ep.2 and BHK-21 Cells

H.Ep.2 and BHK-21 monolayers were inoculated with an input multiplicity of 20 pfu HSV per cell and examined at 4, 7, 12 and 20 hours after infection.

Evidence of viral replication was first detected as early as 4 hours after infection. At this time, infected cells developed irregularly condensed and marginated nuclear chromatin. In addition, dense granular aggregates were often observed scattered throughout the nuclei, although viral particles were not yet evident (Fig. 17).

By 7 hours, the first particles could be seen adjacent to reduplicated nuclear membranes in both H.Ep.2 and BHK-21 cells (Fig. 18). Membrane proliferation was usually extensive and fusion often resulted

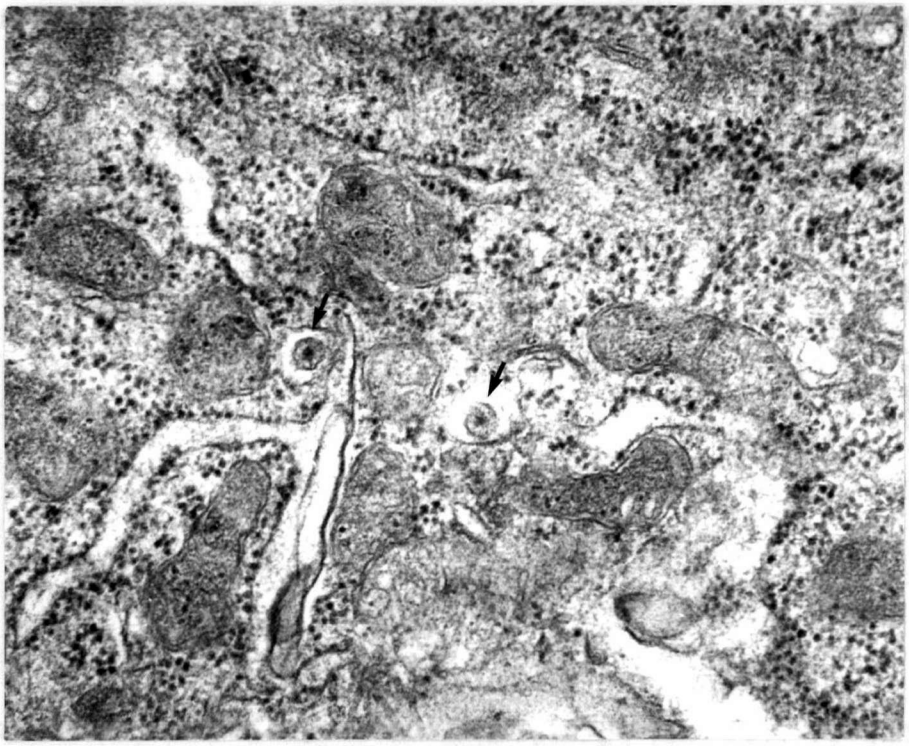

Figure 15. Electron micrograph of an uninfected BHK-21 cell maintained in serumless medium. Abnormal particles are present in the cytoplasm of the cell. X55,800

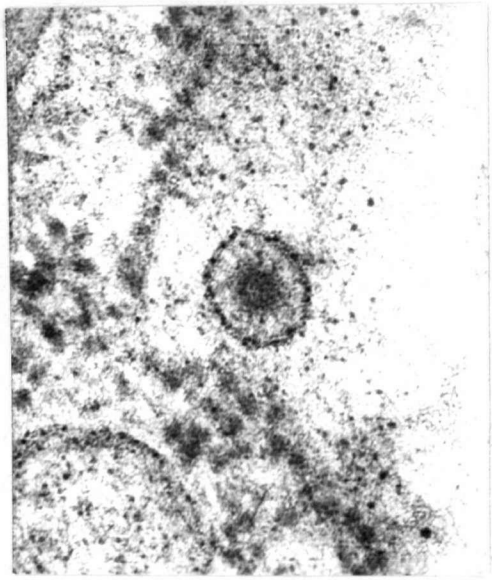

Figure 16. An abnormal particle in the cytoplasm of an uninfected BHK-21 cell maintained in serumless medium. X160,000

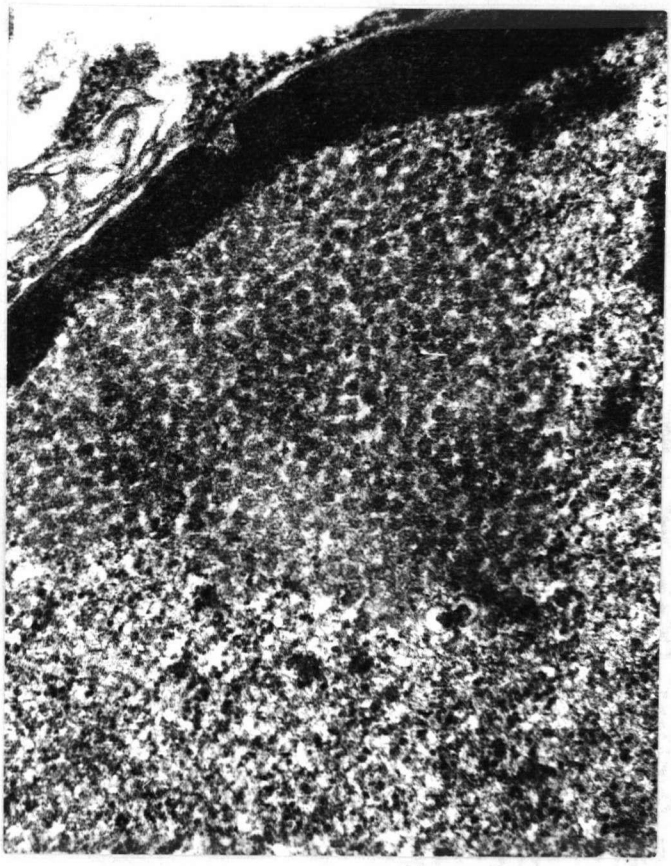

Figure 17. Electron micrograph of a BHK-21 cell 4 hours after HSV infection. Note the marginated chromatin and granular aggregate in the nucleus.
X50,000

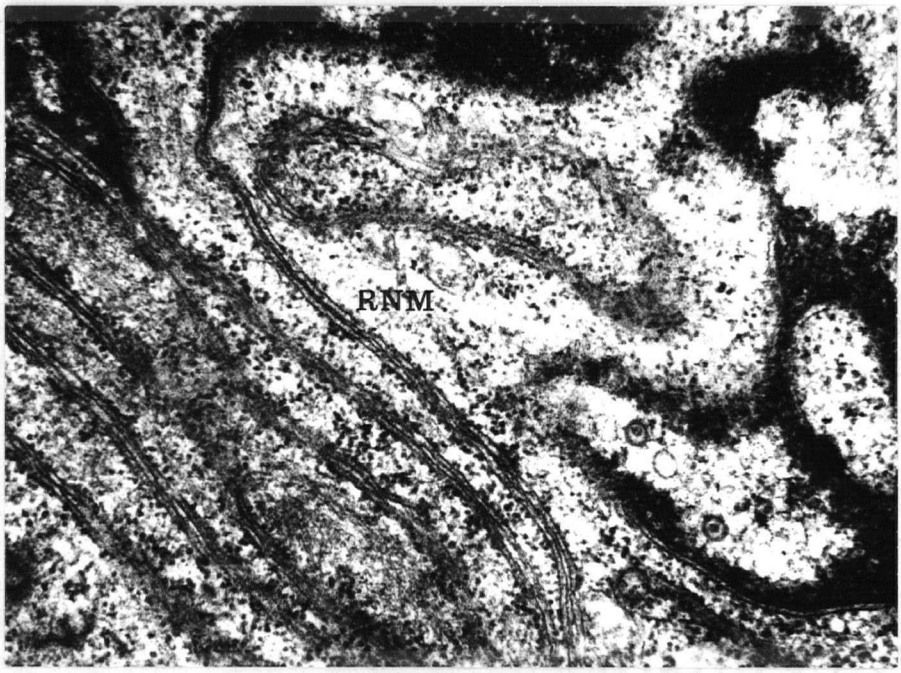

Figure 18. Reduplicated nuclear membranes (RNM) and immature virus particles in a BHK-21 cell 7 hours after HSV infection. X39,500.

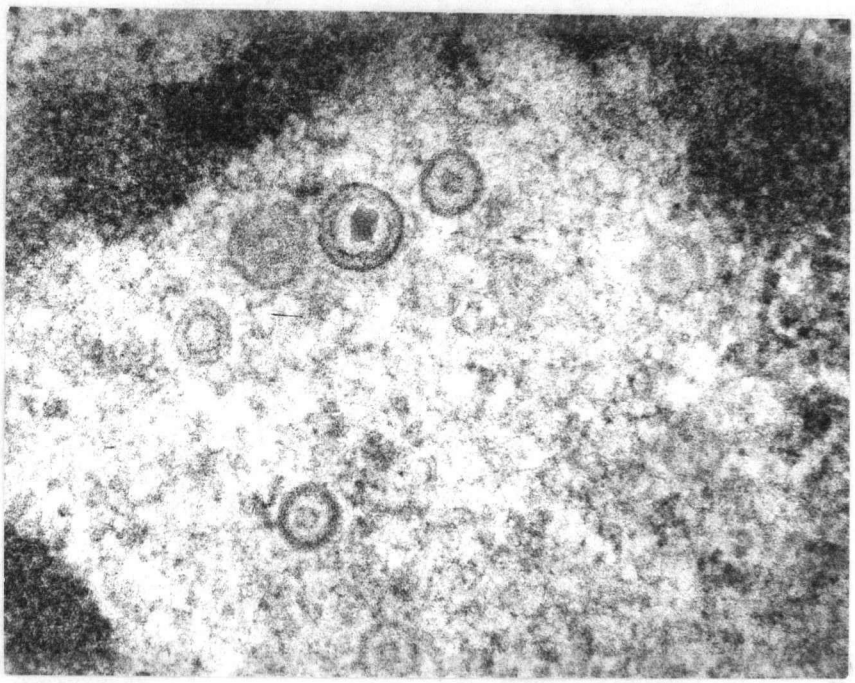

Figure 19. Immature virus particles in the nucleus of a BHK-21 cell 7 hours after infection. X112,000.

in the formation of bizarre concentric lamallae. Viral particles found in the nucleus were generally 90 nm in diameter and consisted of naked capsids enclosing electron dense or electron lucent cores. In addition, infected nuclei also contained a small number of enveloped particles 130 nm in diameter (Fig. 19). Very occasionally these immature virions could be seen acquiring a second envelope by budding through the inner lamella of the nuclear membrane (Fig. 20). A few mature particles with double membranes and a number of naked and immature virions were found in the cytoplasm at 7 hours of infection but no surface virus was detected at this time.

Extensive viral replication was evident at 12 hours after infection. Nuclear particles in various stages of assembly were observed in great numbers and cytoplasmic virus was frequently found associated with smooth membranous structures. The mature and immature viruses appeared to preferentially aggregate in cell vacuoles and within fine, branching tubules in the cytoplasm (Fig. 21). Mature particles measuring 170-175 nm were released by a process resembling reverse phagocytosis (Fig. 22). No evidence of budding virus was encountered in either cell type.

By 20 hours, widespread cell degeneration was evident with corresponding high levels of extracellular virus (Fig. 23). Enveloped

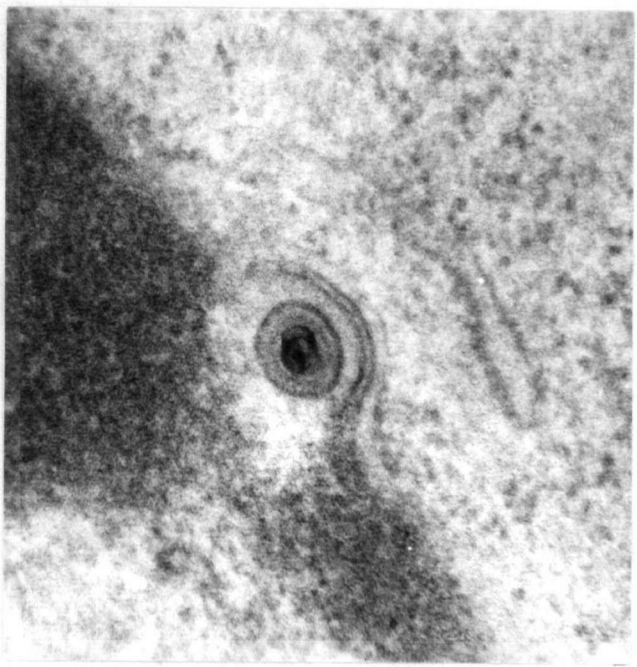

Figure 20. Immature virus particle budding through the nuclear membrane of a BHK-21 cell 7 hours after HSV infection. X91,000.

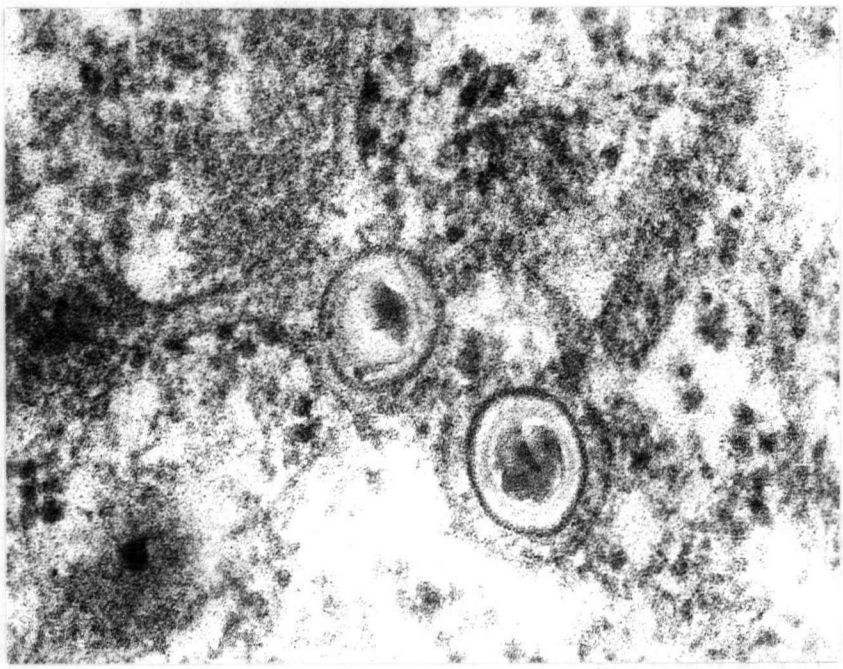

Figure 21. Mature virus particles near a branching tubule in the cytoplasm of a H.Ep.2 cell 12 hours after HSV infection. X123,000.

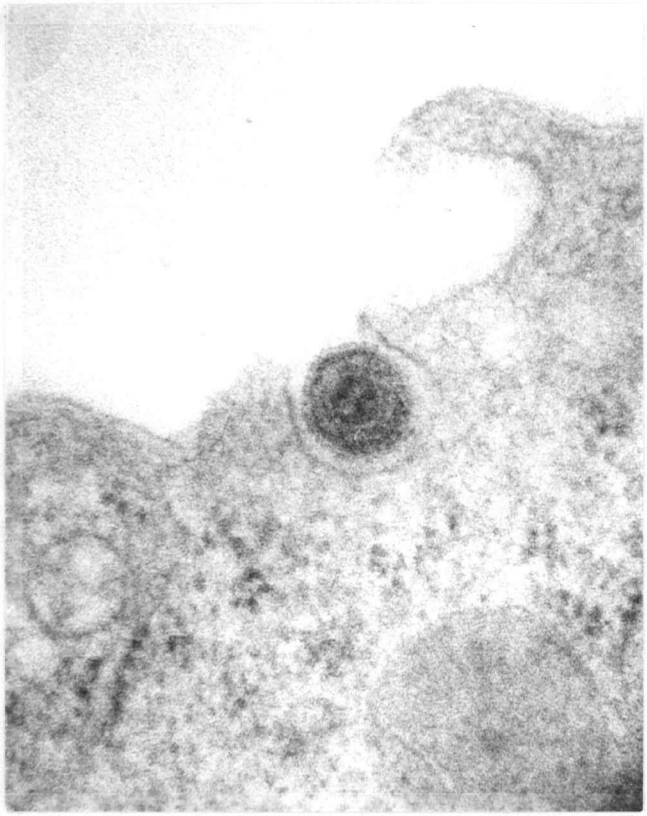

Figure 22. Release of a mature HSV particle from a H.Ep.2 cell 12 hours after infection. X112,000.

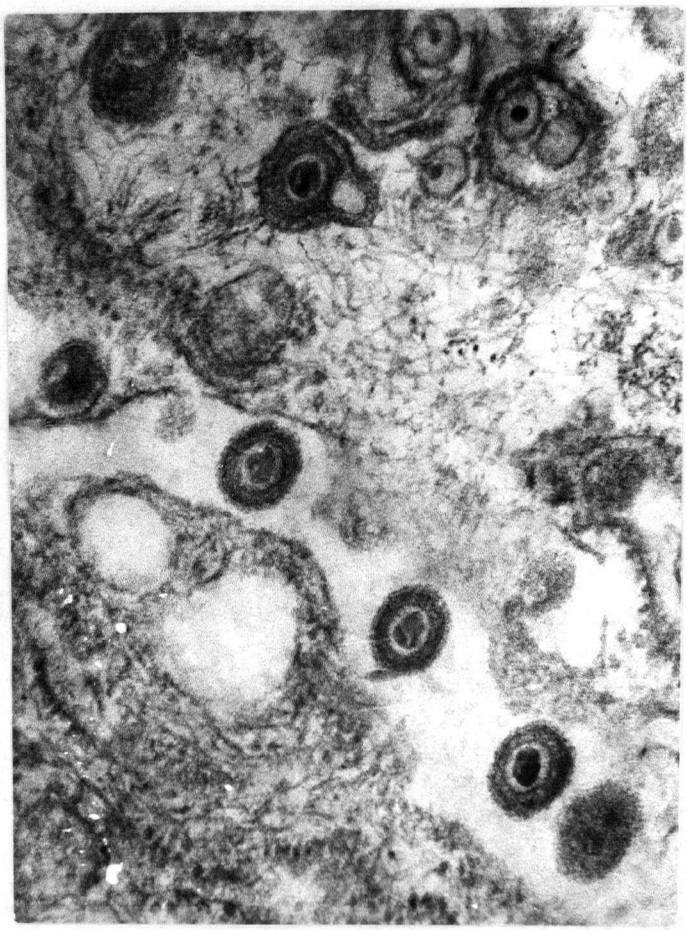

Figure 23. Intracellular and extracellular virus in a H.Ep.2 cell 20 hours after HSV infection. X62,500.

particles measuring 170 nm were often found at the cell surface lodged between prominent cytoplasmic processes. The surface itself was jagged and discontinuous and a great deal of cellular debris was found free in the medium. In addition, nuclear membranes were often broken and the nucleoplasm appeared granular and structureless. At this time, bizarre viral forms were also found in many infected cells. Large crystals composed of viral capsids enclosing a variety of electron dense components were frequently observed in the nuclei of BHK-21 cells (Fig. 24). Groups of membranous particles and mature virions were also found in the cytoplasm of degenerating cells. Cytoplasmic aggregates were enclosed in irregularly shaped vacuoles and existed adjacent to naked HSV particles and mature virus (Fig. 25).

In general, the development of HSV was very similar in both H.Ep.2 and BHK-21 cells. However, the hamster cultures appeared to give rise to a higher percentage of defective and unenveloped virus particles in the nucleus and cytoplasm of infected cells. Moreover, although bizarre viral aggregates were very common in BHK-21 cells, no crystal formations were ever observed in infected H.Ep.2 cells. Beyond these differences, virus replication appeared much the same in terms of assembly, envelopment and release.

(d) Effect of Ara-C and IDU on HSV Development

H.Ep.2 and BHK-21 monolayers were infected with 20 pfu HSV per cell. After receiving 10 µg/ml ara-C or 100 µg/ml IDU, the cells

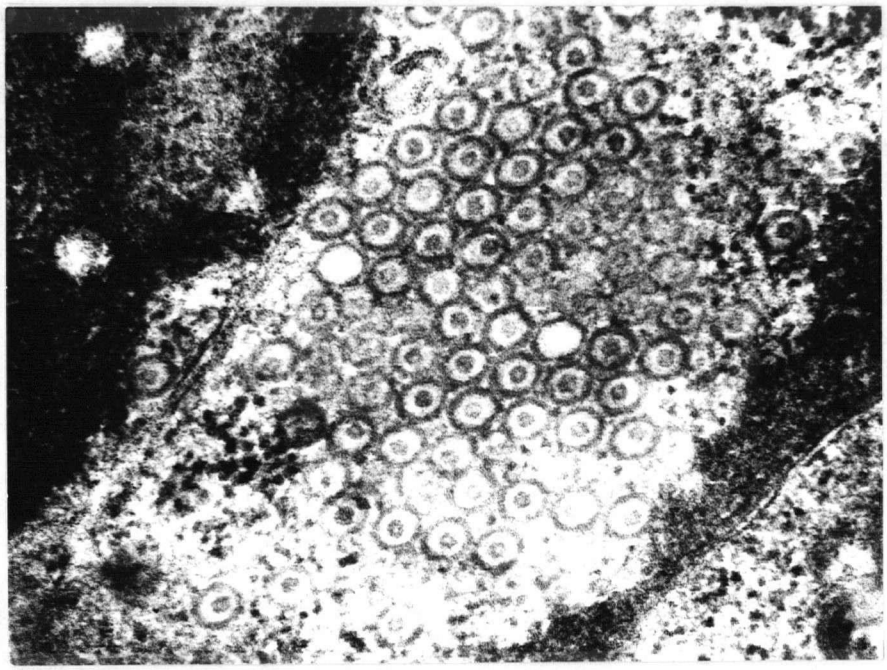

Figure 24. Intranuclear viral crystal in a BHK-21 cell 20 hours after HSV infection. X82,200.

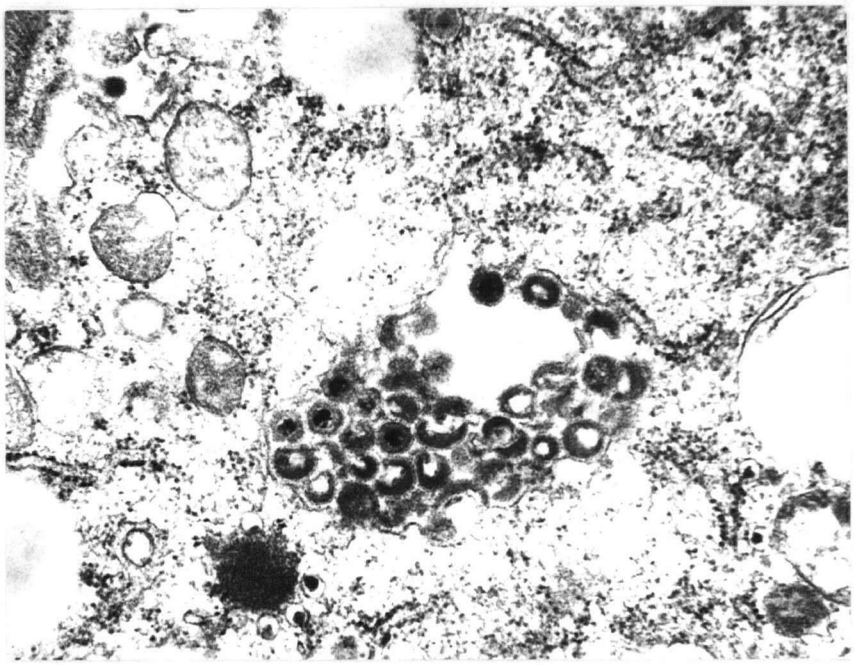

Figure 25. Cytoplasmic aggregate in a BHK-21 cell 20 hours after HSV infection. X39,500.

were incubated for 20 hours and examined under the electron microscope.

Ara-C prevented the formation of complete virus particles, but did not prevent the early nuclear alterations characteristic of HSV infection. Although membrane reduplication was not evident, condensed and marginated chromatin was readily observed in the nuclei of many infected cells. In addition, both cell types revealed a large number of dense intranuclear aggregates. The nuclear granules measured 30 nm in diameter and resembled viral cores in form and density (Fig. 26). No other virus-like particles were found in the nucleus or cytoplasm of cells treated with ara-C.

IDU had much the same inhibitory effect on HSV replication in cultured cells. In most cases, marginated chromatin and dense nuclear granules were observed in the chemically treated and infected cells. However, in addition to such particulate structures, a number of enveloped viruses measuring 130-135 nm in diameter were also found free in the cytoplasm. The visually defective particles were ragged in appearance and their cores were often of low density (Fig. 27). They appeared to possess only one envelope and were never seen at the surface of the infected cells.

(e) Effect of Ara-C and IDU on Uninfected Cells

Cells treated with 10 µg/ml ara-C were morphologically altered

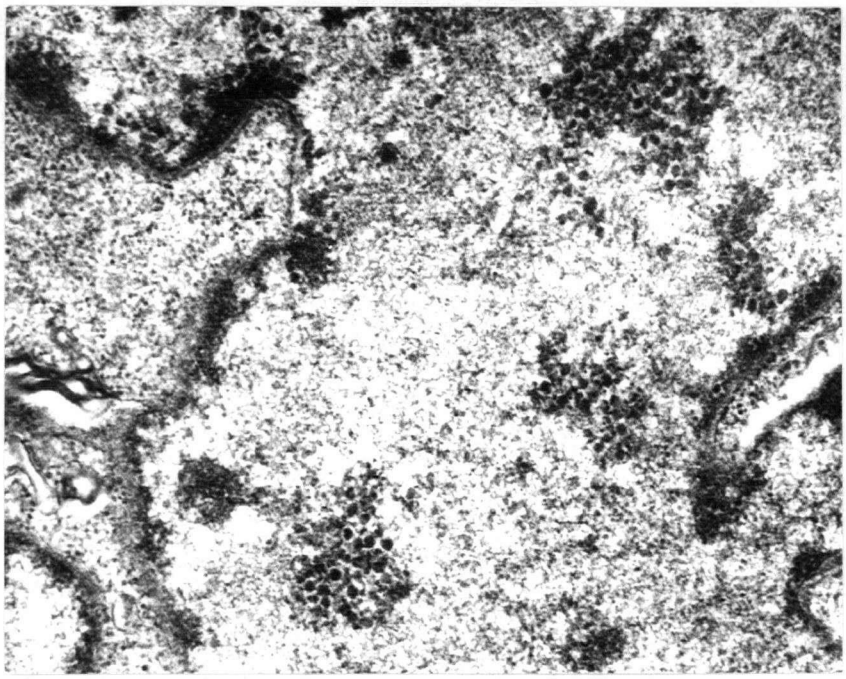

Figure 26. Intranuclear granules in a BHK-21 cell 20 hours after HSV infection and ara-C treatment. X35,300.

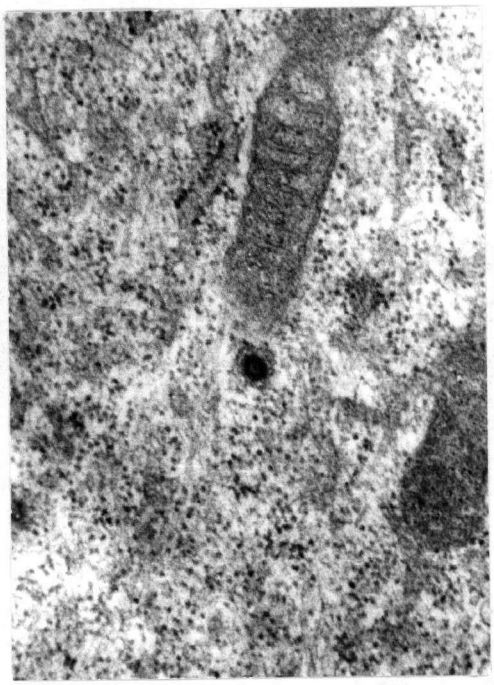

Figure 27. Cytoplasmic particle in a BHK-21 cell 20 hours after HSV infection and IDU treatment. X28,000.

by 24 hours. Early changes included marked distention of mitochondria (Fig. 28) and endoplasmic reticulum. Further treatment with ara-C resulted in conspicuous cytoplasmic vacuolization with loss of mitochondrial structure and distortion of the internal membrane systems (Fig. 29).

Exposure to 100 µg/ml IDU for 24 and 48 hours produced the same type of structural alterations in uninfected H.Ep.2 and BHK-21 cells. However, cytopathic effects were evident only after 48 hours and were restricted to a minimal distortion of cell organelles and vacuolization.

Autoradiographic Studies

1. DNA Synthesis in HSV Infected Cells

DNA synthesis in HSV infected cells was studied by pulse labeling the cells at various times after infection with 2.0 µCi/ml ^{3}H-thymidine for 30 minutes. HSV infection of H.Ep.2 and BHK-21 cells appeared to cause an immediate and almost complete inhibition of DNA synthesis. After 4 hours, however, the number of cells synthesizing DNA rose rapidly and reached a maximum of 28.2% after 8 hours of infection. Following this peak of synthetic activity, total DNA synthesis gradually declined to approximately one-sixth of the 0 time figure (Fig. 30).

The early decrease found in infected cells probably represented a virus-induced inhibition of cell DNA synthesis while the secondary

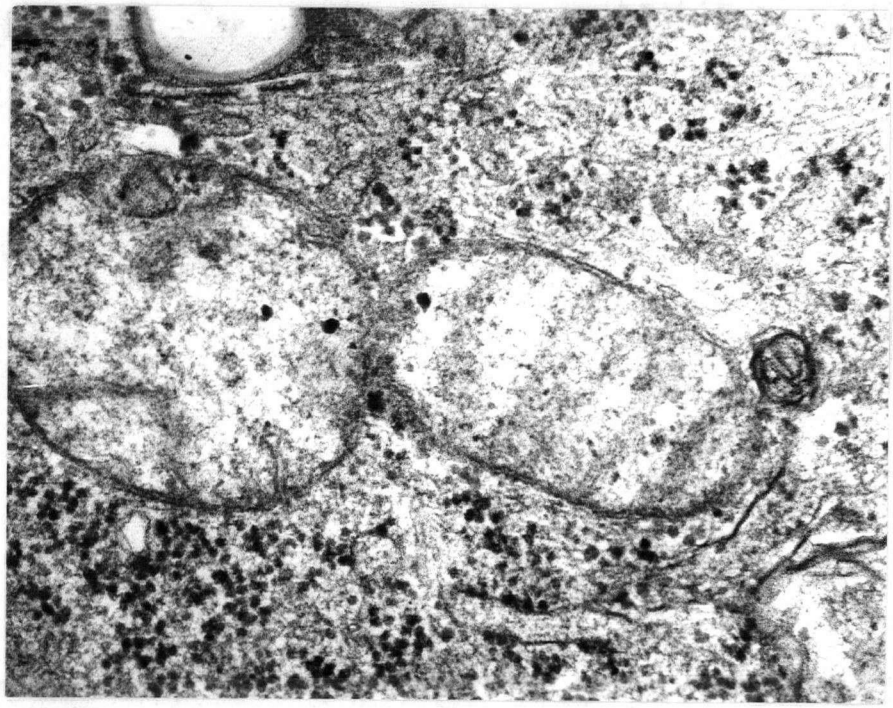

Figure 28. Mitochondria of a BHK-21 cell after 24 hours of ara-C treatment. X69,200.

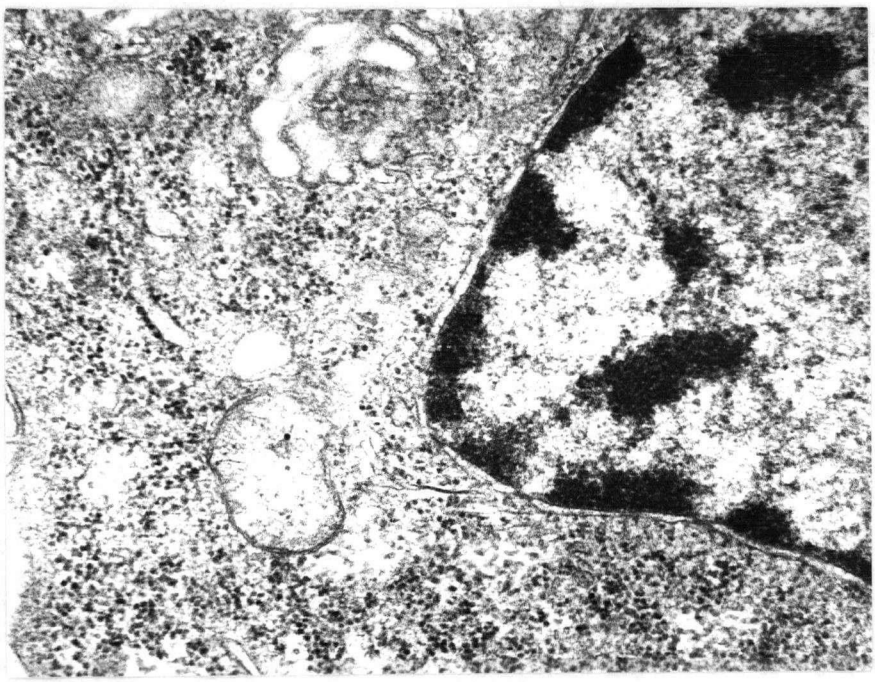

Figure 29. A BHK-21 cell after 48 hours of ara-C treatment. X44,500.

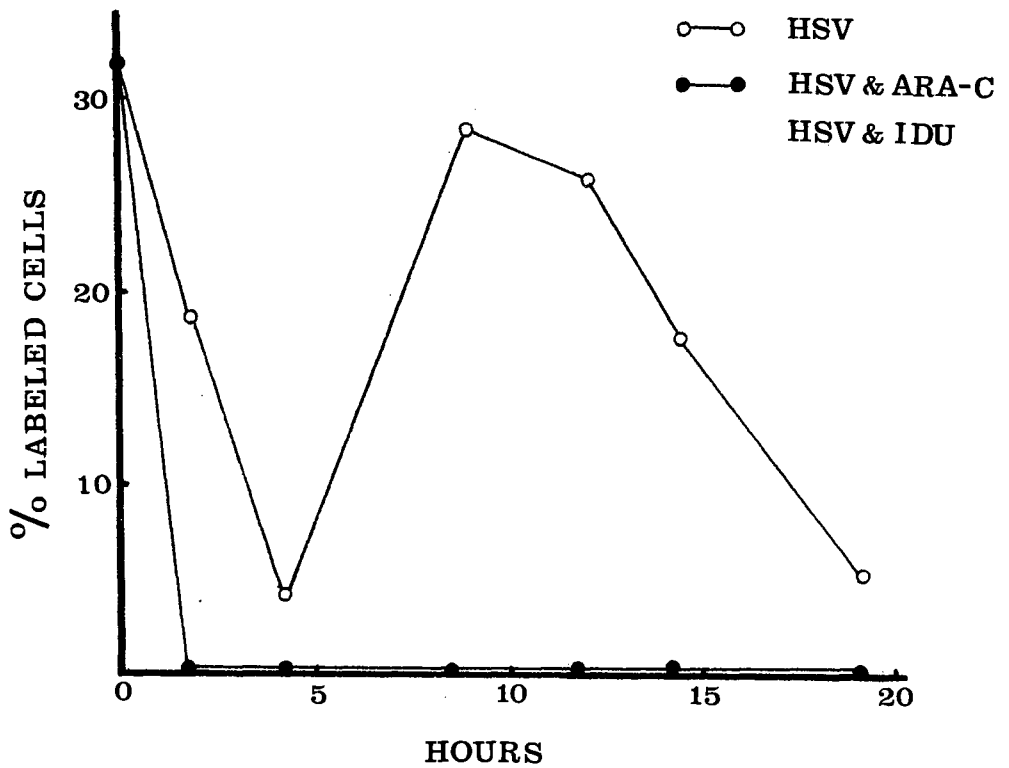

Figure 30. DNA synthesis in virus-infected and chemically treated BHK-21 cells. Cells were infected with an input multiplicity of 1 pfu/cell and treated with 10 µg/ml ara-C and 100 µg/ml IDU.

increase reflected the bulk of viral DNA synthesis and possibly repair of damaged host cell DNA (126).

2. DNA Synthesis in Cells Treated with Ara-C and IDU

Ara-C and IDU produced an immediate and complete inhibition of cell and viral DNA synthesis in HSV infected cells (Fig. 30). The anti-viral drugs also completely inhibited cellular DNA synthesis in uninfected H.Ep-2 and BHK-21 cells within 2 hours of their addition.

Cytogenetic Studies

1. Mitotic Rates

Mitotic rates were studied in HSV-infected and chemically treated H.Ep.2 and BHK-21 cells. HSV infection produced a rapid increase in the mitotic index of both cell types. In one experiment, the number of BHK-21 cells observed in metaphase tripled in the first six hours of infection and thereafter declined until the study was terminated. By 24 hours, mitosis was completely inhibited in all HSV-infected cells (Fig. 31).

The mitotic rate of cells exposed to ara-C or IDU at the time of HSV infection was similar to that of untreated virus infected cells.

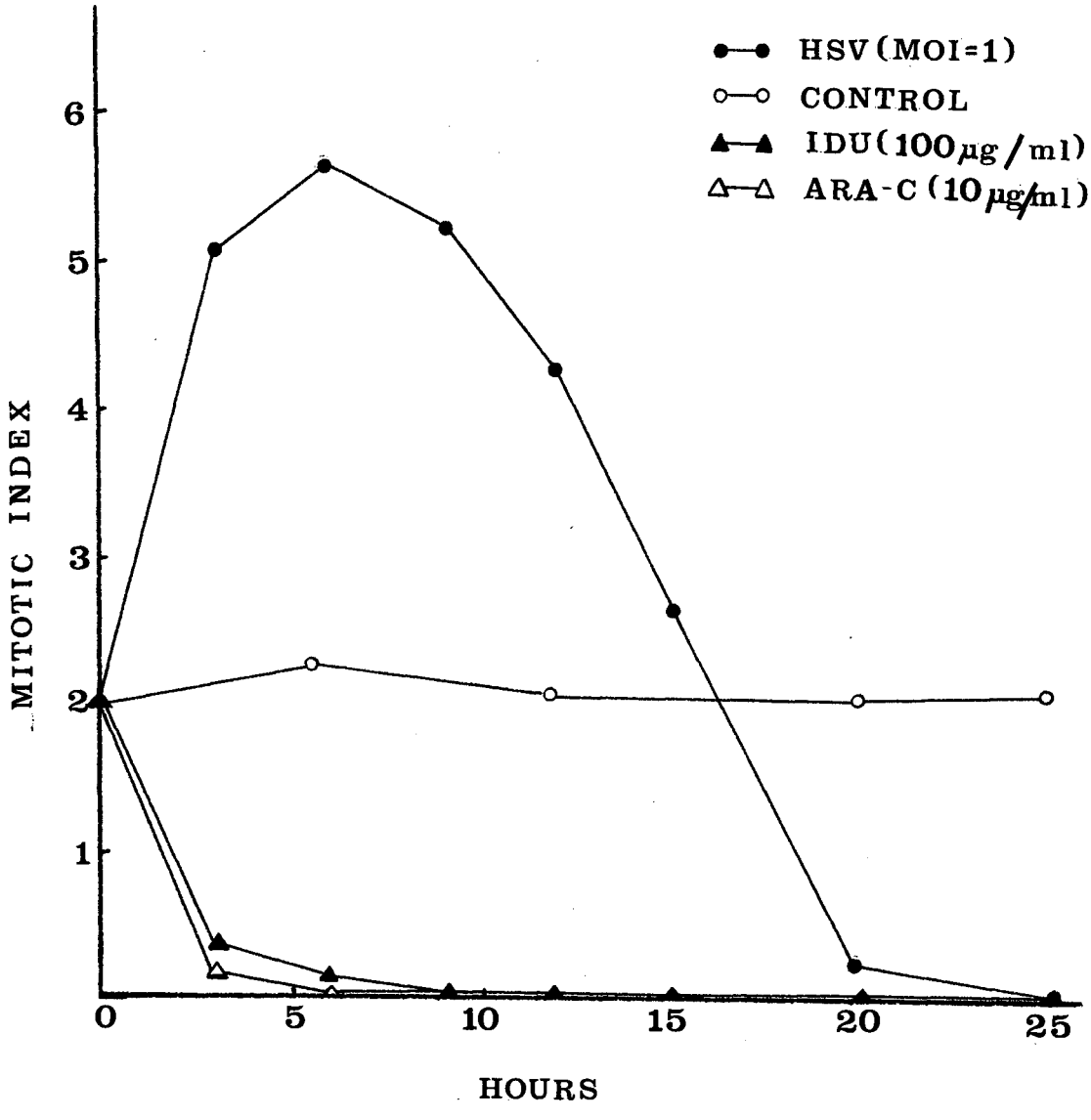

Figure 31. Mitotic rates of BHK-21 cells following virus infection and chemical treatment. Colcemid was omitted from the standard metaphase preparation technique.

However, chemically treated uninfected cells underwent a rapid and complete inhibition of mitosis within 6-8 hours of exposure to the drugs (Fig. 31).

2. Normal Cell Karyotypes

H.Ep.2 cells possessed a diploid male karyotype of 74 chromosomes (Fig. 32). The human cancer line had a relatively high percentage of aneuploidy in the diploid range and a low percentage of tetraploidy (Table I). BHK-21 cells displayed a normal male karyotype of 44 chromosomes with one metacentric marker chromosome (Fig. 33). A similar high percentage of aneuploidy and low percentage of triploidy and tetraploidy was observed in the hamster line (Table I).

3. HSV-Induced Chromosome Abnormalities

A comparative study was made concerning the effect of HSV on the chromosomes of H.Ep.2 and BHK-21 cells. Virus treated cultures showed a significant increase in the number of cells with chromosome aberrations when compared to control cultures (Fig. 34). The percentage of abnormal metaphases began to rise within 2 hours of infection and reached a maximum of 100% at 14-20 hours. Generally, there were fewer abnormalities observed in BHK-21 cells during the first 8 hours of infection. For example, after 4 hours, almost 60% of the H.Ep.2 metaphases were abnormal, while only 40% of the BHK metaphases revealed

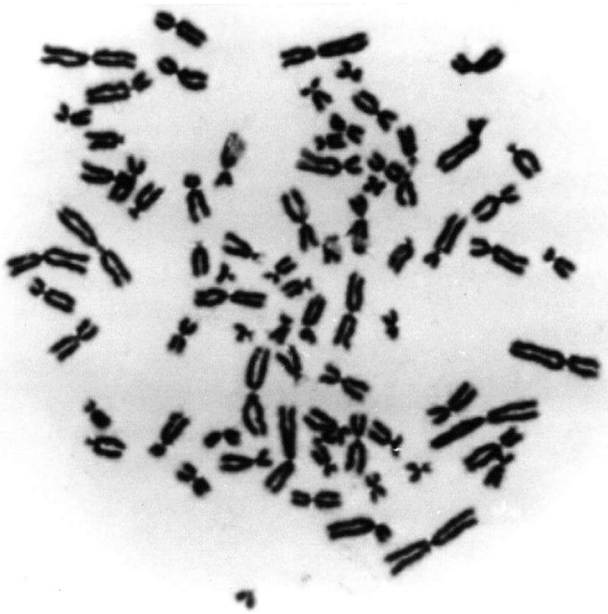

Figure 32. Karyotype of a normal H.Ep.2 cell. X4400.

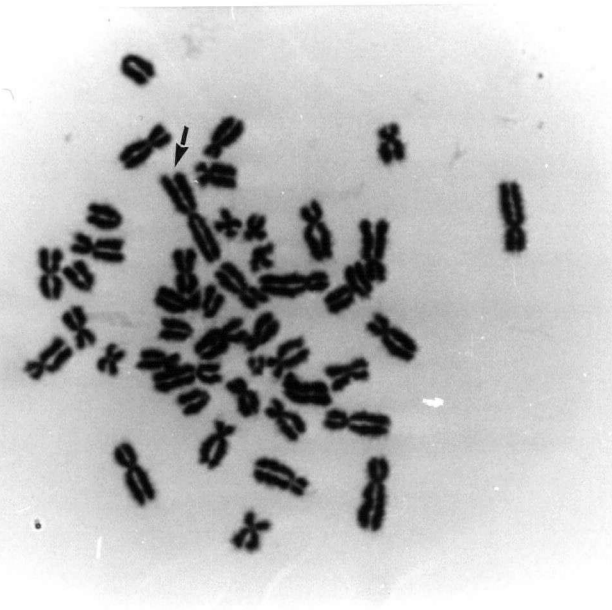

Figure 33. Karyotype of a normal BHK-21 cell. Note metacentric marker chromosome. X4400.

Table I. Frequency Distribution of Various Levels of Ploidy in H.Ep.2 and BHK-21 Cells.

	Approximate Chromosome Number						Total
*	Haploid n	Aneuploid <2n	Diploid 2n	Aneuploid >2n	Triploid 3n	Tetraploid 4n	Cells
H.Ep.2	8	28	137	55	-	42	250
BHK-21	5	34	145	48	15	3	250

* H.Ep.2 n = 37

BHK-21 n = 22

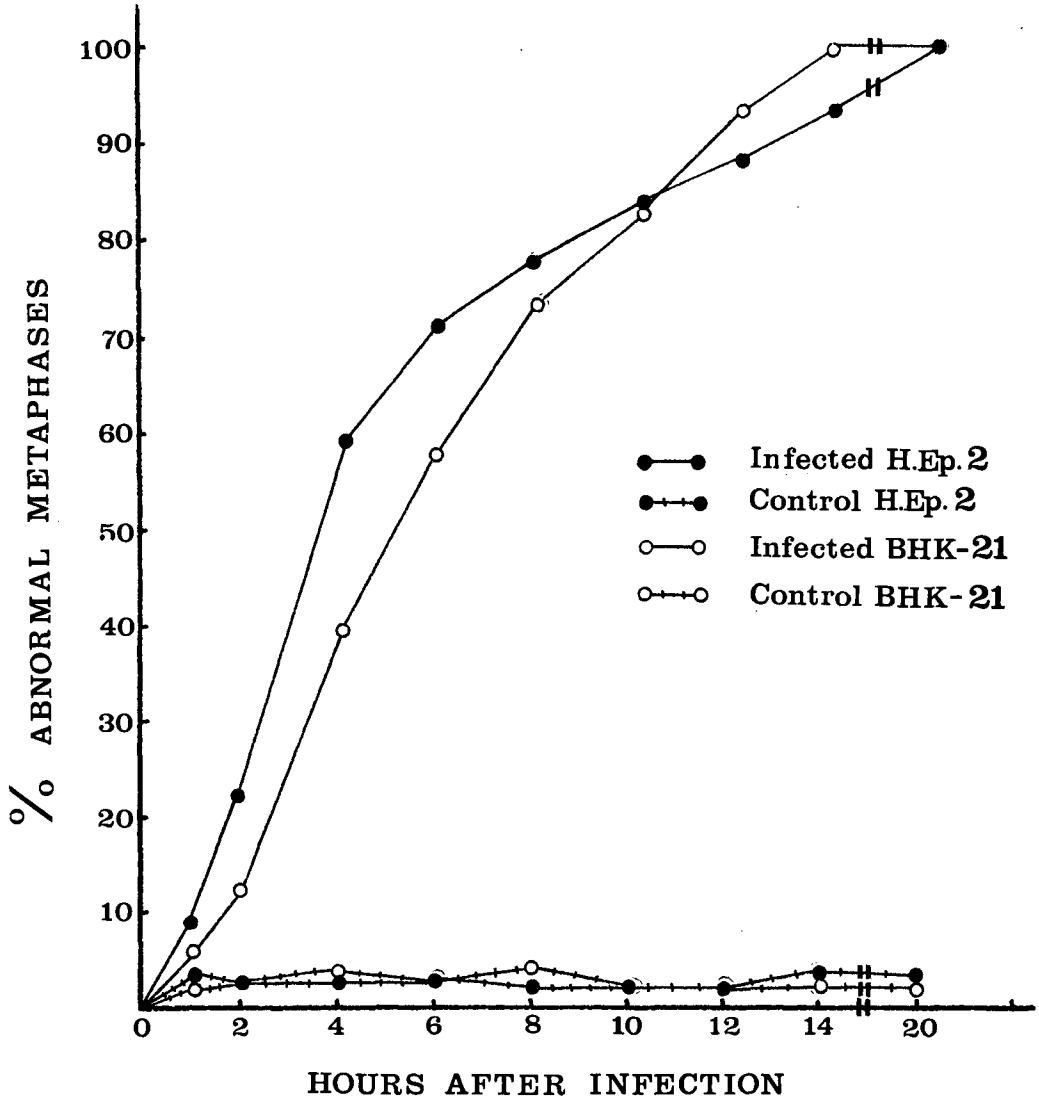

Figure 34. The effect of HSV infection on the chromosomes of H.Ep.2 and BHK-21 cells.

chromosome lesions. However, the total damage to the cell chromosomes after 20 hours was similar in both lines.

Quantitative analysis of the damage also showed that the same type of lesions were produced in the human and hamster cells. The lesions consisted of chromatid gaps or achromatic regions (Fig. 35), chromatid breaks (Fig. 36), secondary constrictions (Fig. 37), fragmentation (Fig. 38), erosion (Fig. 39), and endoreduplication (Fig. 40). Chromatid gaps, breaks and secondary constrictions were more frequent in the first 4 hours of infection while severe fragmentation and erosion appeared more often later in infection. In addition, H.Ep.2 cells revealed more extensive damage at an earlier time than BHK-21 cells (Table II, III). Chromosome abnormalities in both cell types seemed to be random and nonspecific, although extensive karyotyping was not performed in all cases. Moreover, all chromosome aberrations were completely prevented by the neutralization of the virus inoculum with specific HSV antiserum.

4. Effect of Multiplicity of Infection on HSV-Induced Chromosome Abnormalities.

Figure 41 demonstrates the relationship between initial multiplicity of infection and amount of chromosome damage found in BHK-21 cells. The percentage of abnormal metaphases increased in proportion to the virus inoculum. At an input multiplicity of 10 pfu per cell,

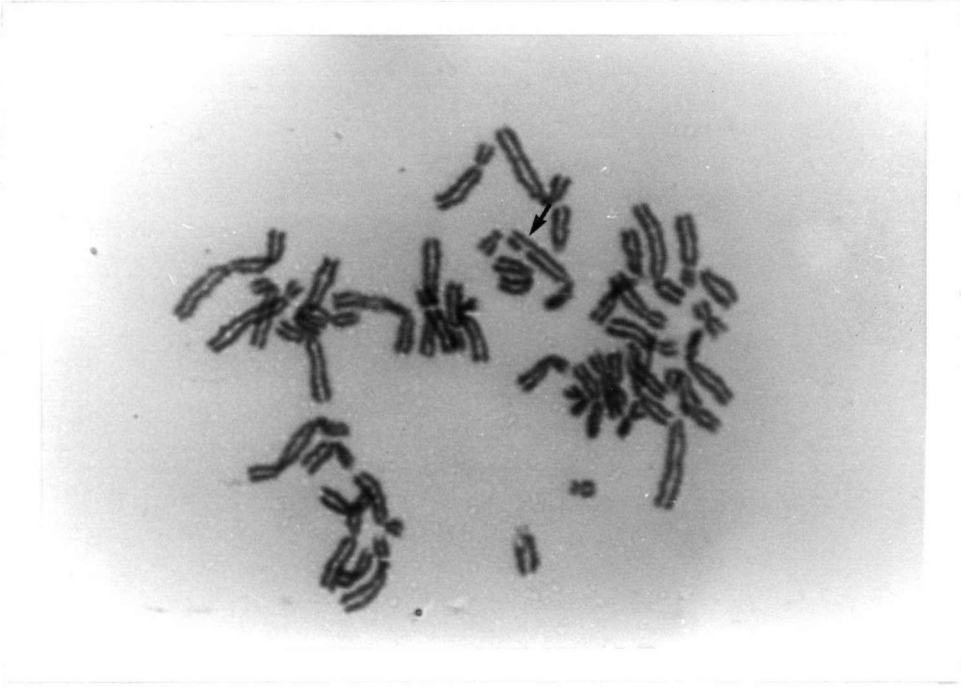

Figure 35. Chromosome complement of a BHK-21 cell 4 hours after HSV infection. Note prominent chromatid gap. X4400.

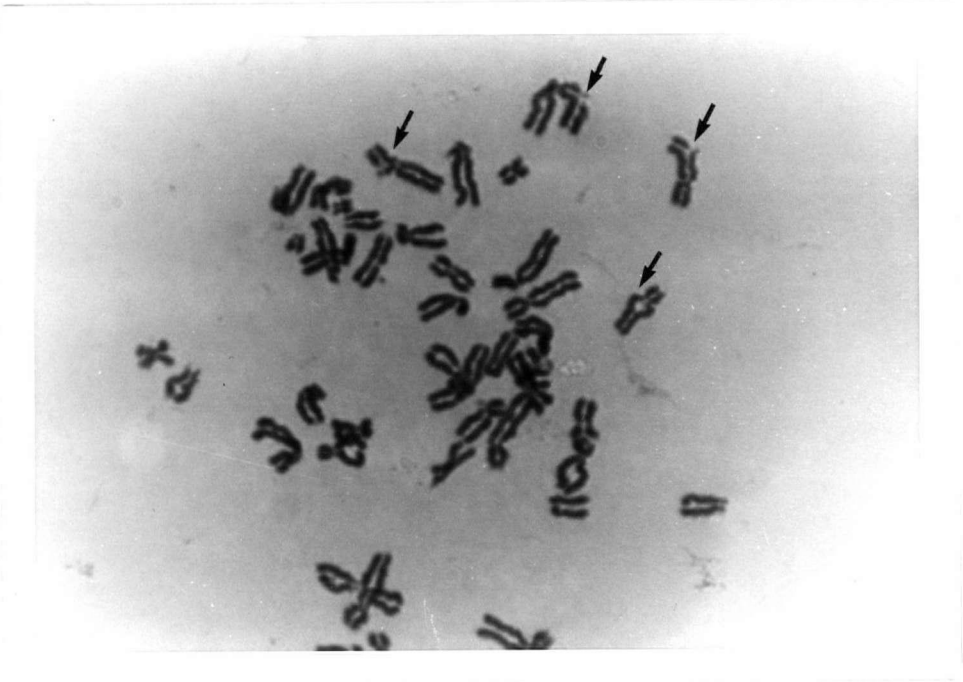

Figure 36. Chromosome complement of a BHK-21 cell 8 hours after HSV infection. Note multiple chromatid breaks and chromosome distortion. X4400.

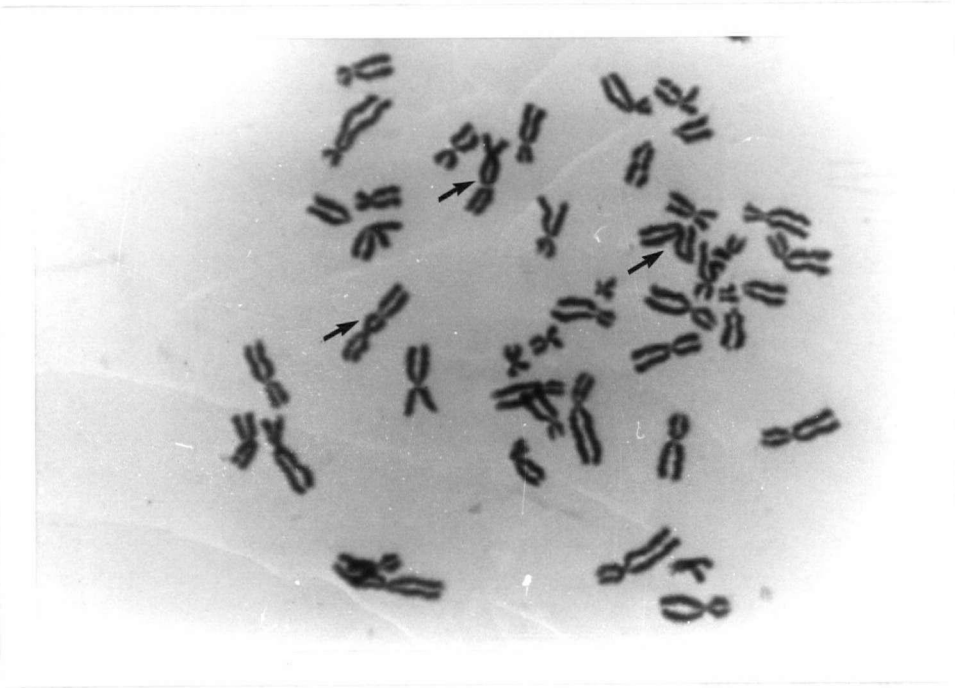

Figure 37. Chromosome complement of a BHK-21 cell 4 hours after HSV infection. Note chromatid break and prominent secondary constrictions. X4400.

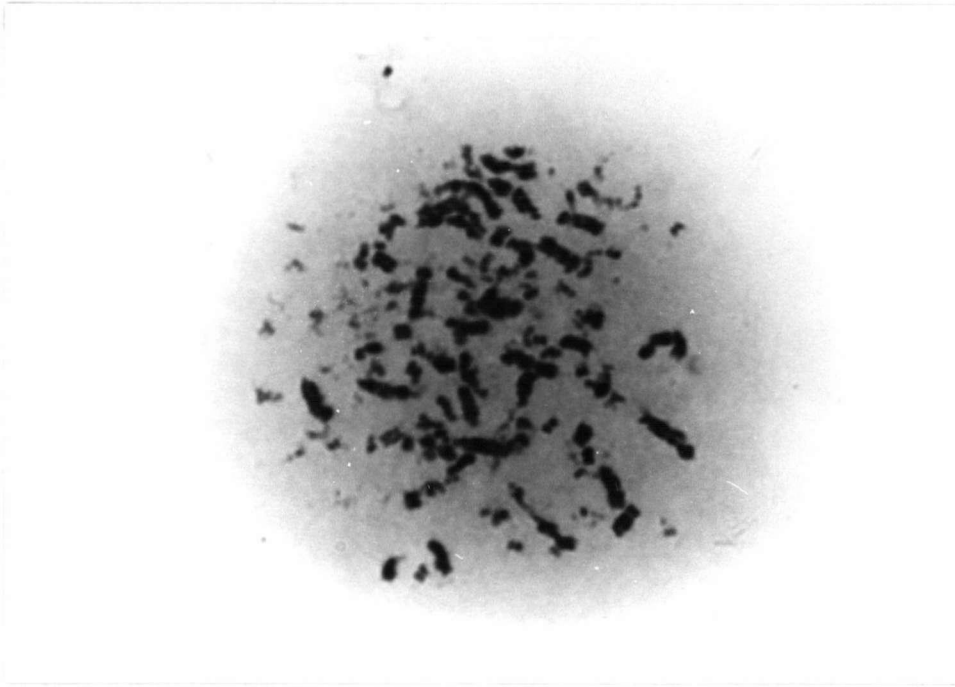

Figure 38. Chromosome complement of a BHK-21 cell showing complete fragmentation after 10 hours of HSV infection. X4400.

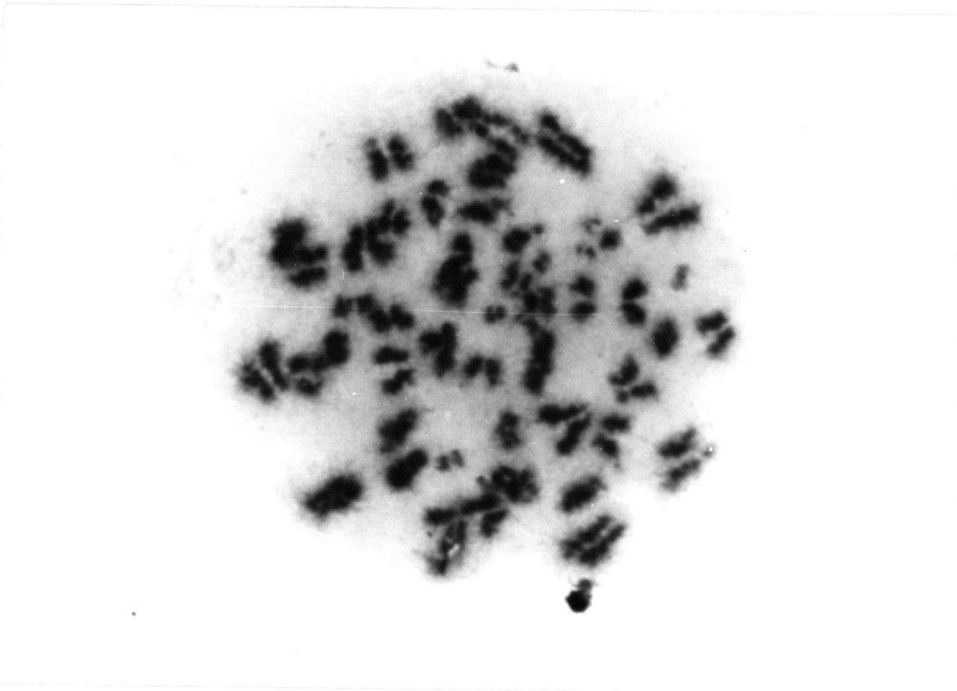

Figure 39. Erosion of BHK-21 complement after 10 hours of HSV infection. X4400.

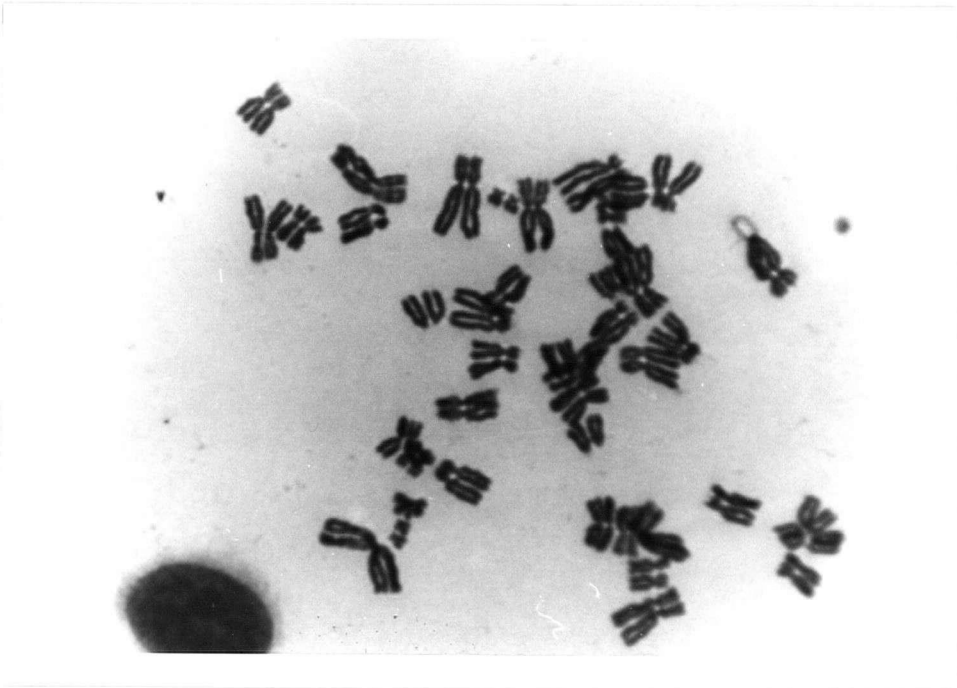

Figure 40. Endoreduplication of BHK-21 chromosomes 8 hours after HSV infection. X4400.

Table II. An Analysis of HSV-Induced[a] Chromosome Abnormalities in H.Ep.2 Cells

Hours after infection	No. cells scored	Single gaps and breaks	Multiple gaps	Secondary constrictions	Fragmentation	Erosion	Endoreduplication	Condensation
1	250	12	-	4	-	2	1	1
2	250	31	5	7	4	4	-	2
4	250	37	37	13	56	2	-	-
6	250	13	49	8	97	7	1	-
8	250	10	55	9	110	8	-	-
10	250	7	51	5	128	14	-	-
12	250	8	29	2	164	10	1	2
14	200	4	14	-	169		-	1
20	100	-	4	-	94	2	-	-

[a] Multiplicity of infection = 1 pfu per cell

Table III. An Analysis of HSV-Induced[a] Chromosome Abnormalities in BHK-21 Cells

Hours after infection	No. cells scored	Single gaps and breaks	Multiple gaps	Secondary Constrictions	Fragmentation	Erosion	Endoreduplication	Condensation
1	250	8	-	2	-	2	1	-
2	250	14	-	10	-	3	2	1
4	250	30	18	27	18	4	1	-
6	250	30	41	12	51	5	2	2
8	250	16	58	4	94	7	1	-
10	250	7	50	1	137	10	-	-
12	250	-	41	-	180	8	-	1
14	200	-	17	-	179	4	-	-
20	100	-	1	-	99	-	-	-

[a] Multiplicity of infection = 1 pfu per cell.

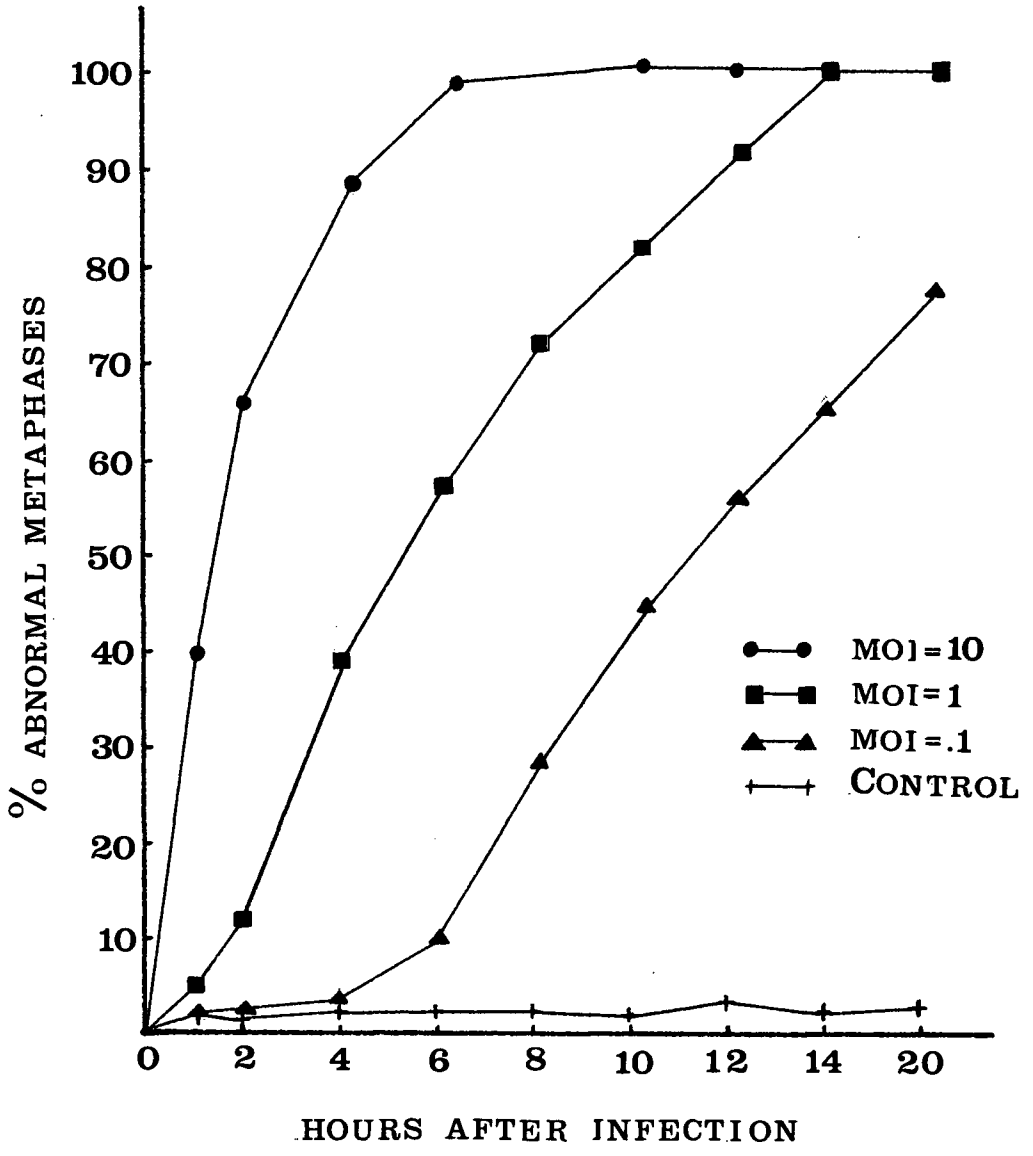

Figure 41. The relationship between multiplicity of infection and HSV-induced chromosome abnormalities in BHK-21 cells.

100% of the metaphases observed at 8 hours were abnormal, whereas for 10- and 100-fold lower doses of virus, 82% and 44% of the metaphases revealed chromosome lesions. The number of altered chromosomes per metaphase also increased in relation to the dose of virus and the time of incubation in both H.Ep.2 and BHK-21 cells.

5. Effect of UV Irradiation of HSV on Virus-Induced Chromosome Abnormalities.

The number of virus-induced chromosome abnormalities in H.Ep.2 and BHK-21 cells decreased logarithmically after UV irradiation of HSV. In one experiment using BHK-21 cells, the capacity of the virus to induce metaphase alterations was inactivated approximately five times more slowly than the infectivity (Fig. 42).

6. Effect of Excess Arginine on HSV-Induced Chromosome Abnormalities

Previous studies have established that a minimal concentration of arginine is necessary for HSV development in cultured cells (3). It is also known that mycoplasma infection generally results in arginine depletion and that such starvation can cause chromosome abnormalities in mammalian cells (60). Since mycoplasma infection has been ruled out in the H.Ep and BHK-21 lines, it was therefore of interest to determine if the abnormalities produced by HSV infection were in fact, due to virus-induced arginine starvation of the cells. This

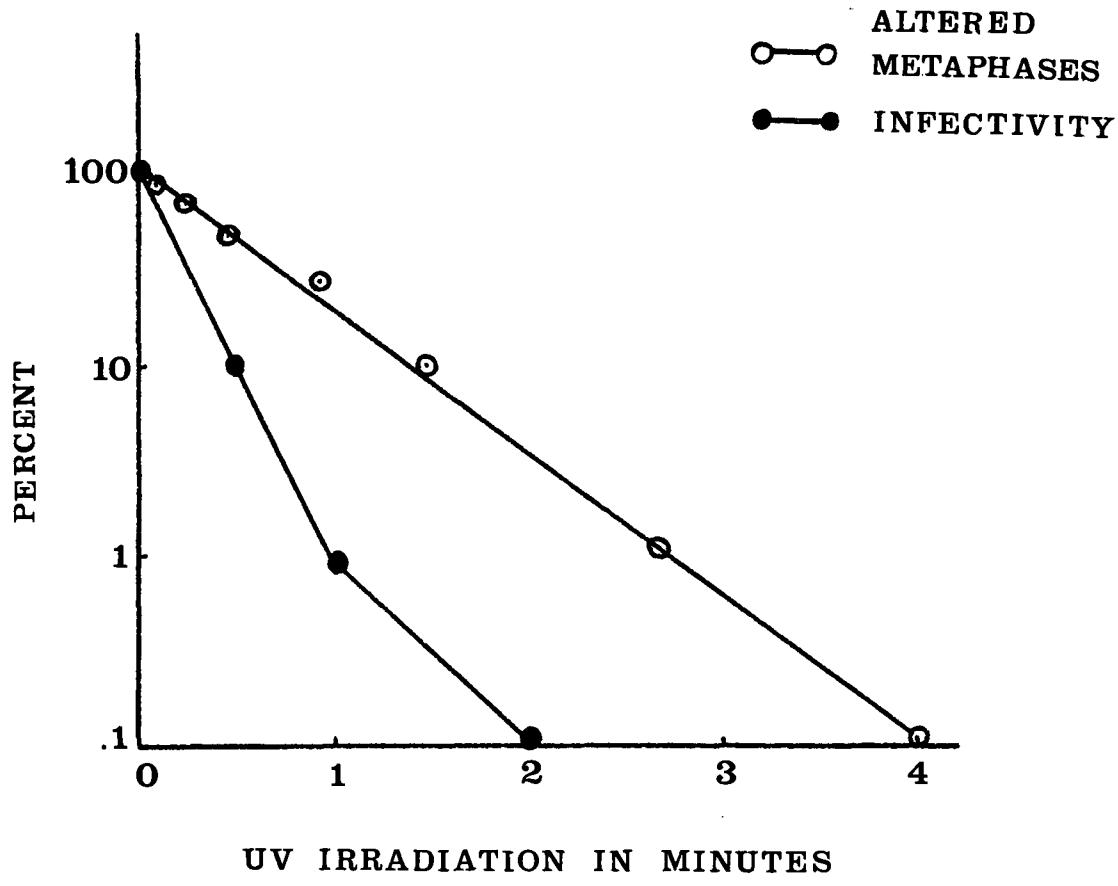

Figure 42. Effect of UV irradiation of HSV on viral infectivity and capacity to induce chromosome abnormalities in BHK-21 cells. The nonirradiated virus (1 pfu/cell) induced chromosomal damage at 12 hours in 92% of the observed metaphases, which was taken as 100%. Infectivity was measured by the end-point dilution technique.

was done by increasing the concentration of arginine in the growth medium and observing the effect on HSV-induced chromosome aberrations. Table IV shows the results of such an experiment carried out in BHK-21 cells. Increases in arginine varying from 5- to 15-fold did not significantly alter the number or type of aberrations produced in the cell chromosomes after HSV infection. Similar results were obtained in HSV-infected H.Ep.2 cells.

7. Effect of Ara-C on the Chromosomes of Uninfected and HSV-Infected Cells.

Rapidly dividing BHK-21 cells were exposed to various concentrations of ara-C for 4 hours. Chromosome analysis at the end of this time showed that ara-C caused a significant increase in the number of single chromatid gaps and breaks (Table V). The drug-induced damage was proportional to the concentration of ara-C used in the experiment (ie. 10 µg/ml ara-C induced abnormalities in 10% of the BHK-21 metaphases and 20 µg/ml ara-C induced lesions in 20% of the metaphases).

HSV-infected BHK-21 cells were also exposed to ara-C for 4 hours. Ara-C did not inhibit any of the HSV-induced chromosome abnormalities (Table V). Moreover, the drug appeared to act synergistically with the virus to produce an overly large number of cells with single and multiple gaps and breaks (Fig. 43). It was estimated that the number of affected

Table IV. The Effect of Excess Arginine on HSV-Induced Chromosome Abnormalities in BHK-21 Cells

Treatment	% Abnormal Metaphases[c]	Single gaps and breaks	Multiple gaps	Secondary Constrictions	Fragmentation	Erosion	Endoreduplication	Condensation
5 x Arg[a]	4	6	-	-	-	2	-	-
10 x Arg	6	7	-	-	-	3	1	1
15 x Arg	6	8	-	-	-	3	1	-
5 x Arg + HSV[b]	46	31	14	30	12	4	-	2
10 x Arg + HSV	44	31	15	26	11	3	-	1
15 x Arg + HSV	40	27	14	24	10	5	1	1
HSV	46	30	15	29	13	1	-	-
Control	4	7	-	-	-	-	-	-

[a] Arginine increases were based on normal medium content of 6.0×10^{-4} M

[b] Multiplicity of infections = 1 pfu per cell

[c] 200 cells were scored 4 hours after HSV infection and/or arginine treatment.

Table V. Chromosome Abnormalities in HSV-Infected[a] and Non-Infected BHK-21 Cells Treated with ara-C

Treatment	% Abnormal Metaphases[b]	Single Gaps and Breaks	Multiple Gaps	Secondary Constrictions	Fragmentation	Erosion	Endoreduplication
ara-C,5 µg/ml	2	4	-	-	-	-	-
ara-C,10 µg/ml	10	14	2	-	-	-	4
ara-C,20 µg/ml	20	28	4	-	-	4	4
ara-C, 5 µg/ml + HSV	45	8	30	4	44	4	-
ara-C,10 µg/ml + HSV	64	23	56	7	42	-	-
ara-C,20 µg/ml + HSV	76	13	52	7	70	5	5
HSV	40	7	20	5	36	12	-
Control	4	3	-	-	-	3	2

[a] Multiplicity of infection = 1 pfu per cell

[b] 200 cells were scored 4 hours after HSV infection and/or ara-C treatment.

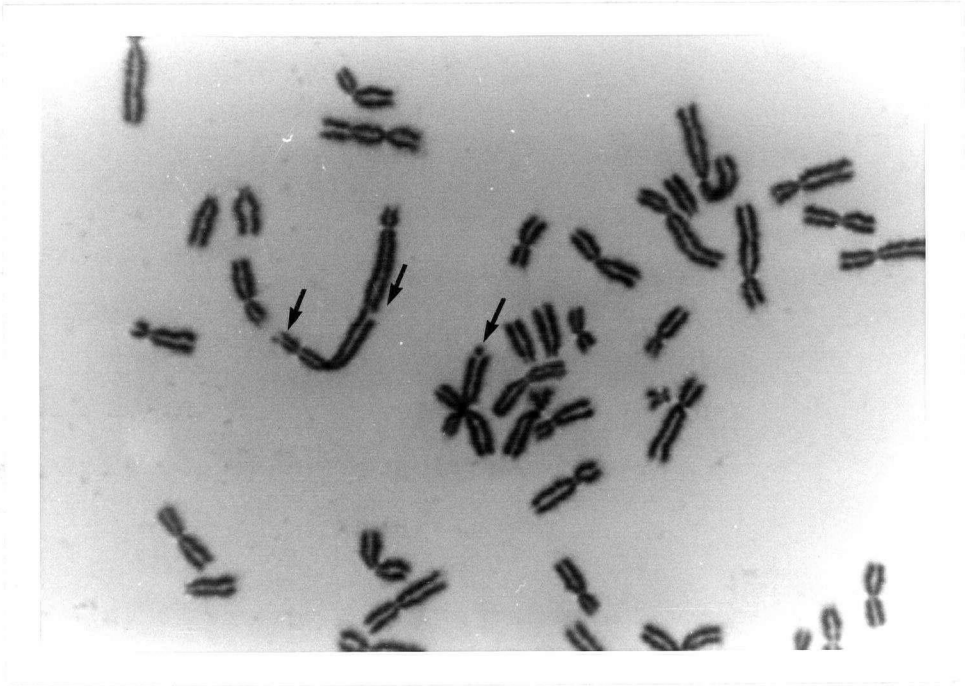

Figure 43. Chromatid gaps found in a H.Ep.2 cell 4 hours after addition of ara-C. X4400.

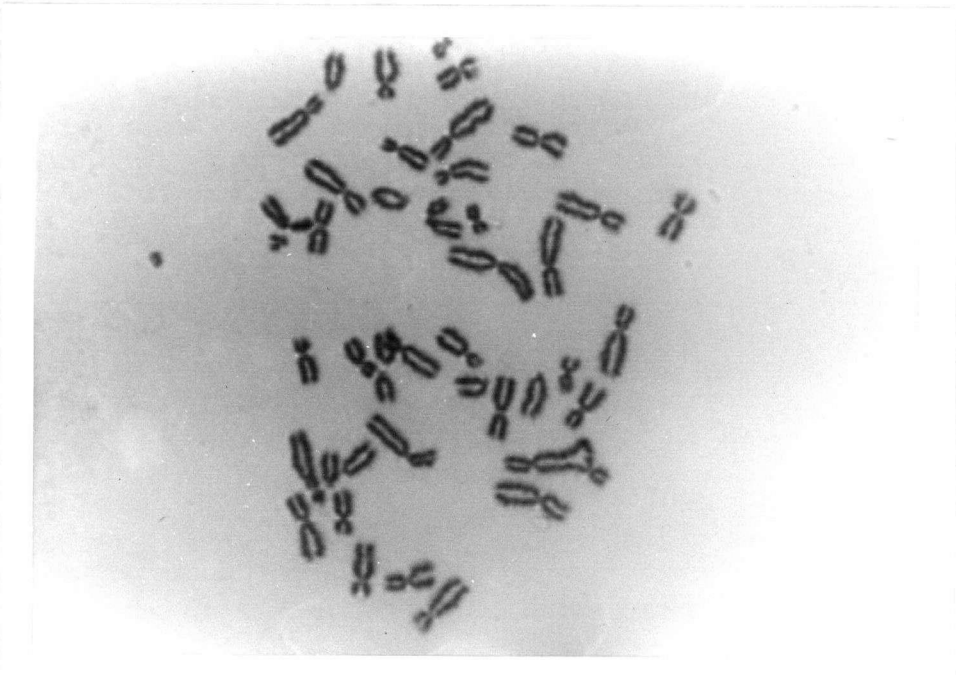

Figure 44. Translocation found in a BHK-21 cell 4 hours after addition of IDU. X4400.

cells in the HSV and ara-C treated cultures was approximately 1.3 times greater than the additive effects induced separately by the virus and the antimetabolite. The synergistic effect on chromosome damage could be observed if ara-C was added as late as 2 hours after virus adsorption. Exposure to the drug in the last 2 hours on infection, however, produced no increase in HSV-induced abnormalities. Similar results were obtained in H.Ep.2 cells.

8. Effect of IDU on the Chromosomes of Uninfected and HSV-Infected Cells.

Uninfected BHK-21 cells were exposed to concentrations of IDU ranging from 25 to 200 µg/ml. Low doses of IDU had little, if any, effect on the number of chromosome aberrations found in the cells after 4 hours of treatment. However, higher concentrations of the drug induced damage in 7-10% of the metaphases as compared to 3% in the control culture. IDU-induced abnormalities included single chromatid gaps, breaks, and translocations (Fig. 44).

HSV-infected BHK-21 cells were also exposed to IDU for 4 hours. IDU did not inhibit any of the virus-induced chromosome abnormalities nor did it act synergistically with the virus (Table VI). Infected cells treated with the lower concentrations (25 and 50 µg/ml) of the drug had the same number of aberrations as untreated infected cells. Cells receiving higher concentrations had damage that was equal to the

Table VI. Chromosome Abnormalities in HSV-Infected[a] and Non-Infected BHK-21 Cells Treated With IDU.

Treatment	% Abnormal Metaphases[b]	Single Gaps and Breaks	Multiple Gaps	Secondary Constrictions	Fragmentation	Erosion	Endoreduplication	Translocations
IDU, 25 µg/ml	2	1	-	-	-	-	-	-
IDU, 50 µg/ml	4	5	-	-	-	2	-	1
IDU, 100 µg/ml	7	9	-	-	-	2	2	1
IDU, 200 µg/ml	10	15	-	-	-	1	2	2
IDU, 25 µg/ml + HSV	40	14	16	8	35	5	2	-
IDU, 50 µg/ml + HSV	44	18	17	9	39	3	2	-
IDU, 100 µg/ml + HSV	47	21	19	8	41	3	1	1
IDU, 200 µg/ml + HSV	53	17	28	6	53	2	-	-
HSV	42	11	18	8	41	6	-	-
Control	3	3	-	-	-	2	1	-

[a] Multiplicity of infection = 1 pfu per cell

[b] 200 cells were scored 4 hours after HSV infection and/or IDU treatment

additive effects induced separately by IDU and HSV. Similar results were obtained in H.Ep.2 cells.

9. Effect of Ara-C and IDU on the Chromosomes on Uninfected and HSV-Infected Cells.

Infected and uninfected BHK-21 and H.Ep.2 cells were exposed to a combination of 10 µg/ml ara-C and 100 µg/ml IDU for 4 hours. The amount of chromosome damage incurred in uninfected cells was equal to the additive effects of ara-C and IDU. Damage in HSV-infected cells equalled the sum of the abnormalities induced by IDU alone and those induced by the synergistic action of ara-C and HSV. Aberrations were generally restricted to single and multiple gaps and fragmentation (Table VII).

Table VII. Chromosome Abnormalities in HSV-Infected and Non-Infected HBK-21 Cells Treated With ara-C and IDU

Treatment	% Abnormal Metaphases[d]	Single Gaps and Breaks	Multiple Gaps	Secondary Constrictions	Fragmentation	Erosion	Endoreduplication
ara-C[a]	11	5	-	-	-	4	2
IDU[b]	8	4	-	-	-	4	-
ara-C + IDU	20	15	1	-	-	4	-
HSV[c] + ara-C	62	17	6	8	29	2	-
HSV + IDU	49	11	4	7	25	1	1
HSV + ara-C + IDU	72	21	7	11	32	1	-
HSV	38	13	8	7	10	-	-
Control	3	2	-	-	-	1	-

[a] ara-C was used at a concentration of 10 µg/ml

[b] IDU was used at a concentration of 100 µg/ml

[c] Multiplicity of infection = 1 pfu per cell

[d] 100 cells were scored 4 hours after HSV infection and/or chemical treatment

DISCUSSION

These results show that HSV is capable of inducing severe morphological and biochemical alterations in infected human and hamster tissue culture cells. The anti-viral agents ara-C and IDU completely inhibit HSV replication in the same cells but are unable to prevent any of the cytopathic effects of the virus.

The rapid growth cycles of HSV in H.Ep.2 and BHK-21 cells have been documented in previous studies by Roizman et al. (89) and Russell et al. (96). Although both cell types supported a remarkably similar course of viral replication, the BHK-21 line was clearly less suitable for producing high titres of HSV.

The inhibition of HSV replication following addition of 10 µg/ml ara-C or 100 µg/ml IDU at the time of infection confirms earlier reports on the anti-viral nature of the drugs. Buthala (9) was the first to show that similar concentrations of ara-C and IDU were active in vitro against a select group of DNA viruses including HSV, pseudo-rabies, B-virus, swine pox, fowl pox and vaccinia virus. Prior to this work, Kaufman had reported that IDU was clinically effective against herpetic keratitis in rabbits (39) and subsequent studies showed that ara-C was equally active in curing ocular herpes in man and animals (36).

More recently, efforts have been focused on determining the mechanism of action of the two anti-viral agents. Levitt and Becker (46) found that ara-C appeared to prevent HSV replication *in vitro* by blocking the *de novo* synthesis of deoxycytidylate. Thus, infected cells treated with the drug showed no evidence of viral DNA synthesis or virion assembly. Similarly, Roizman *et al.* (89) reported that IDU completely inhibited HSV replication in human cells at a concentration of 5 µg/ml and appeared to prevent viral DNA synthesis by blocking the utilization of thymidine. On the other hand, later investigators found evidence of IDU substitution in viral DNA, suggesting that the drug affected production of defective late proteins involved in virus assembly rather than DNA synthesis itself (87,73).

Cytological examination of HSV-infected cells revealed the large inclusion bodies and nuclear disorganization typical of HSV infection (62). The HSV strain used in the present study produced marked rounding of both cell types with little aggregation of infected cells and no syncytia formation. A similar effect on the social behaviour of cells was described by Roizman (84) with the VR-3 strain of HSV.

Ara-C and IDU did not appear to reduce HSV cytopathology in either cell line. Previous reports have also described persistence of virus cytopathology in treated cells (9,43), indicating that the host damage is an early virus function independent of viral DNA synthesis or assembly of infectious particles.

Drug-induced cytotoxicity was observed in both H.Ep.2 and BHK-21 cells. The generalized degeneration of cells appeared to occur with increasing time of treatment and was more obvious in ara-C treated cultures. These results confirm Buthala's earlier work (9) on *in vitro* toxicity of ara-C and IDU. He observed marked vacuolization and mitochondrial disintegration in cells exposed to either drug for a prolonged period of time. In addition, ara-C appeared to be the more toxic of the two anti-viral agents although both were more active in rapidly growing cells than in stationary cultures. The latter observation is probably related to the fact that ara-C and IDU are known to inhibit DNA synthesis and mitosis in mammalian cells (43,73). Clinically, the two drugs are also capable of causing a marked disruption of the hematopoietic system of man (74). Thus, the present cytological and clinical evidence strongly suggests that ara-C and IDU act not as specific anti-viral agents but rather as mammalian antimetabolites. As a result, their usefulness as therapeutic agents in human disease is severely limited.

There are many reports in the literature concerning the large number of antigens formed in tissue culture cells infected with HSV. Using the agar gel precipitation test, Watson *et al.* (124) have detected 12 different precipitation bands with immune antiserum prepared against HSV-infected cell extracts. Several other investigators have

demonstrated at least five separate immunofluorescent elements in HSV-infected cells (45,78,91,94). In the present study, four different fluorescent antigens were detected in virus-infected H.Ep.2 and BHK-21 cells with a commercial HSV antiserum prepared in guinea pigs. Within 4 hours of infection, fluorescence was observed in the nucleus, perinuclear region, and cytoplasm of both cell types. The early nuclear antigens appeared as small, irregularly shaped granules and the cytoplasmic antigens as a diffuse fluorescence. Geder and Vaczi (22) described similar immunofluorescent elements in HSV-infected BSC-1 cells with antiserum prepared against virus-infected tissue culture extracts. After 4 hours, large fluorescent masses appeared in the nucleus of infected cells and late in infection, intense surface fluorescence was observed in over 90% of the cell population. These later antigens were previously described by Ross et al. (94) and Roane and Roizman (78), who also detected a second granular antigen in the cytoplasm after 5-6 hours of infection. The absence of cytoplasmic granules in the present study may be related to the use of different viral strains or different HSV antiserums.

Ara-C and IDU failed to prevent the formation of the nuclear or perinuclear antigens in infected cells but did prevent the appearance of surface fluorescence. Geder and Vaczi (22) reported similar results in BSC-1 cells after treatment with 10 µg/ml ara-C. This evidence

indicates that the nuclear granules and perinuclear elements are due to early antigenic components or products of HSV infection while the surface fluorescence is dependent on viral DNA synthesis and assembly.

It is apparent from this and other studies that the number and type of virus-specific antigens found in HSV-infected cells is dependent on the immunofluorescent technique, the virus strain, and the preparation of the antiserum. Moreover, most of the virus-induced antigenic components are not synthesized after in vitro exposure of the cells to the antiviral agents ara-C and IDU.

In the past, electron microscopy has provided much useful information concerning the morphology and development of HSV in cultured cells. However, various technical problems have also imposed certain restrictions on the technique. For example, the use of high titre samples containing large numbers of defective viruses does not permit the microscopic differentiation of infectious and noninfectious particles. Moreover, asynchrony of virus development makes it difficult to deduce the kinetic sequence of events from a series of static micrographs. As a result of these disadvantages, any evaluation of electron microscope studies must be tempered with the knowledge that the results constitute a biased view of virus structure and replication.

The HSV particles observed in this study after negative stain preparation were similar in size and morphology to the virions described by Watson et al. (125), Spring et al. (107) and Darlington and Moss (13). Present electron microscopy revealed four structural elements of the virus - the outer envelope, the outer capsid, the inner capsid and the dense core. Other studies have also described a second inner envelope and a middle capsid (85). The majority of the HSV particles appeared to be fully enveloped, indicating that the preparation used in this research contained a large number of morphologically mature virions. In addition, the rapid disintegration of particle structure observed at room temperature confirmed earlier reports of HSV thermosensitivity (122).

Under normal growth conditions, thin-sectioned H.Ep.2 and BHK-21 cells appeared quite healthy and intact. At the same time, severe morphological changes were commonly observed in BHK-21 cells maintained in serumless growth medium. These changes included the development of abnormal particles and marked cytoplasmic disruption in affected cells. Such alterations have been previously described in many continuous and oncogenic cell cultures. Bernhard and Tournier (5) were the first to detect virus-like particles in an apparently normal BHK-21 (clone 13) line. The spherical 85 nm structures were observed singly or in groups within cytoplasmic vacuoles or the swollen cisternae of the endoplasmic reticulum. The particles were further

distinguished by the presence of characteristic electron dense radial structures that appeared to emanate from the nucleoid. Subsequent studies have since confirmed the presence of similar "R" (radial) particles in BHK-21/13s, BHK-21/4, BHK-21/13/TC6/A, and BHK-21/F cells (71). Particles have also been found in BHK-21 cells transformed by SV_{40} and polyoma viruses, in hamster tumors induced by polyoma-transformed BHK-21 cells, and in a continuous calf kidney cell line (6). Thus, abnormal particle development appears to be a widespread phenomenon. Previously, however, the "R" particles were normally detected in actively growing cells while in the present study, they were observed only after serum starvation. Thus, it can be concluded that the conditions of serum deficiency induced or at least stimulated the formation of the abnormal virus-like structures in the BHK-21 system. A similar stimulatory effect has been described in BHK-21 cells infected with rubella virus (71).

Current theories tend to regard the "R" particles and other abnormal structures as manifestations of latent virus infections. However, to date, there is little evidence to support this hypothesis other than the obvious morphological similarity of the particles to known viruses. The structures may equally well arise in response to some medium deficiency or may represent another unknown infectious agent.

Thin-section electron microscopy of productively infected H.Ep.2 and BHK-21 cells revealed that HSV was assembled in the nucleus of infected cells and enveloped at the nuclear membrane. Previous investigations have demonstrated a similar course of development in a number of mammalian cell lines (13,53,63,99,102, 125). Present observations showed that actual virus assembly was preceded by alterations in the cell chromatin and nucleoli that were probably related to the early virus-induced inhibition of host DNA and RNA synthesis (99). The altered nuclei also exhibited a number of dense, granular aggregates peculiar to HSV-infected cells. Similar aggregates in KB cells tagged specifically with ferritin-conjugated HSV antibodies and were thought to be some form of early viral antigen or precursor particle (64).

In this study, naked viral particles were first observed in the nucleus of infected cells after 7 hours of infection. These scattered particles were soon supplanted by virions with a single envelope and large aggregations of viral capsids. In general, the viral assembly process appeared to be highly asynchronous and inefficient, especially during the later stages of infection.

The site of HSV envelopment has been previously reported to be the nuclear membrane of infected cells (13,53). In the present study, virus particles were occasionally seen budding through the inner lamella of the nuclear membrane of infected H.Ep.2 and BHK-21 cells.

However, this event was relatively rare. More common was the finding of extensively proliferated membranes extending into the cytoplasm or back into the nuclear matrix. Whether this reduplication was necessary for HSV envelopment or was merely the product of virus-induced alterations in cell membranes is not known. Furthermore, the possibility of other sites of virus envelopment in the cytoplasm could not be entirely ruled out. Epstein (19) demonstrated HSV budding into cytoplasmic vacuoles in infected HeLa cells while Siminoff and Menefee (102) observed HSV envelopment in the vicinity of the Golgi apparatus. Virus envelopment may thus occur randomly at a number of different cell membranes, including those of the smooth endoplasmic reticulum and the Golgi system, although recent evidence strongly implicates the nuclear membrane as the primary site.

Mature virus particles 170 nm in diameter were commonly observed in membrane-bound vacuoles and tubules in the cytoplasm of infected cells. These tubules resembled structures described by Schwartz and Roizman (98) in HSV-infected H.Ep.2 cells. In the previous study, mature HSV particles appeared to move out of the cell via a network of fine, branching tubules extending from the nuclear membrane to the cell surface. However, no evidence of a transport system could be seen in the present study. Virions were released from the cells by a form of reverse phagocytosis similar to the method of egress described by Darlington and Moss (13). Budding virus was not observed at the cell

surface despite repeated attempts to confirm Epstein's early report of HSV mode of release from HeLa cells (19).

The vast arrays of crystals and membranous aggregates found in BHK-21 cells after 20 hours of infection were very similar to the structures observed by Nii et al. (63) in infected FL cells. In both cases, bizarre viral forms were restricted to cells demonstrating extensive viral replication and degeneration. Moreover, they appeared to be composed to numerous viral components not incorporated into mature particles.

BHK-21 cells consistently gave rise to a larger number of morphologically defective viruses and aberrant viral forms than did H.Ep.2 cells. Previous growth studies also showed that the hamster cells produced lower titres of infectious virus. Thus, it would appear that inefficiencies in assembly and production of viral components give rise to the reduced yields of HSV found in BHK-21 cells.

The present study also revealed that the addition of 10 µg/ml ara-C at the time of infection prevented the formation of infectious particles in H.Ep.2 and BHK-21 cells. However, the drug did not prevent the early chromatin displacement or the production of granular aggregates in the nucleus of cells infected with HSV. These results confirm previous biochemical studies concerning the effect of ara-C on HSV replication (9,46). In virus-infected cells, ara-C appears to

permit formation of HSV structural units and antigens but completely inhibits particle assembly in vitro.

IDU similarly failed to prevent nuclear disorganization and precursor synthesis in HSV-infected H.Ep.2 and BHK-21 cells. However, ragged, morphologically abnormal particles were occasionally detected in the cytoplasm of treated cells. Smith (104) reported the presence of similar defective particles in HSV-infected cells treated with IDU. Biochemical studies have also shown that IDU is directly incorporated into HSV DNA and that defective late proteins are subsequently produced (37). As a result, virus assembly is severely impaired, and although a few abnormal particles are produced, no infectious virus can be detected. In contrast, HSV-infected cells treated with another halogenated pyrimidine, BudR, produced virus-specific antigens but no particles of any description (102).

The cytotoxicity of ara-C and IDU observed previously in this study under the light microscope was closely paralleled in the thin sections of H.Ep.2 and BHK-21 cells. After 48 hours of drug exposure, cells exhibited marked alterations including distortion of the mitochondria, swelling of the endoplasmic reticulum and vacuolization of the cytoplasm. Thus, the present electron microscope work confirmed and extended previous observations of ara-C and IDU-induced cytopathology.

HSV infection resulted in an immediate reduction of thymidine

uptake into the DNA of H.Ep.2 and BHK-21 cells. This early inhibition was followed by a sharp increase in DNA synthesis at 5 hours of infection. Similar results have been obtained by Roizman et al. (86,89) in H.Ep.2 cells and Russell et al. (96) in BHK-21 cells. Previous work has also shown that HSV inhibits host cell DNA synthesis within 2-3 hours and that viral DNA is first detected after 4-5 hours of infection. Viral DNA synthesis is virtually completed by 14 hours in most cell systems.

In the present study, the early inhibition of host DNA appeared chronologically related to the nuclear disruption and chromatin displacement observed in infected cells with the light and electron microscope. Furthermore, the onset of viral DNA synthesis closely preceded the appearance of immunofluorescent antigens and naked viral capsids in the nucleus. Maximum cytopathic effects were usually seen after the bulk of viral DNA had been synthesized in H.Ep.2 and BHK-21 cells.

Ara-C and IDU completely inhibited DNA synthesis as measured by ^{3}H-thymidine uptake in uninfected and HSV-infected cells (Fig. 30). These results correspond with earlier in vitro studies by Smith (104), Smith and Dukes (105), Levitt and Becker (46), and Cohen (11).

The mitotic index of H.Ep.2 and BHK-21 cells increased sharply after infection with HSV, reaching its peak at 6 hours and then declining to a level of complete inhibition at 24 hours of infection.

In the first hours of infection following inhibition of host DNA synthesis, cells already in mitosis appeared to be prevented from completing the division cycle and were thus detected in increasing numbers at metaphase. However, after initiation of virus replication and development of cytopathic effects, the number of cells in mitosis decreased until cell division ceased altogether in infected cultures. In contrast, Stoker and Newton (112) reported that mitosis was rapidly inhibited in parasynchronous HeLa cultures infected with HSV 2 hours prior to the calculated time of cell division.

Ara-C and IDU did not appear to alter the number of HSV-infected cells in mitosis. Mitotic effects were therefore an early viral function independent of ensuing viral DNA synthesis. On the other hand, ara-C and IDU completely inhibited mitosis in uninfected H.Ep.2 and BHK-21 cells within 6-8 hours (Fig. 31). Similar drug-induced effects have been observed in rabbit kidney (9) and HeLa cells (43).

A number of mammalian viruses are capable of inducing chromosome irregularities in cultured cells. The biological significance of these changes is not yet clear but is of potential importance in cell death, carcinogenesis, aging, teratogenesis, and somatic and germ cell mutation (60). HSV has been previously reported to cause chromosome abnormalities in a variety of mammalian cells (7,27,28,34,68, 97,110). In the present study, HSV infection of H.Ep.2 and BHK-21 cells resulted in visible damage to cell chromosomes. The virus-

induced aberrations included chromatid gaps, breaks, secondary constrictions, fragmentation, erosion and endoreduplication. These cytogenetic terms commonly refer to various lesions observed in stained metaphase preparations of cell chromosomes. Thus, chromatid gaps appear as unstained, loosely coiled chromosome segments while breaks constitute actual interruptions of the chromosome that lead to dislocated acentric fragments. Secondary constrictions represent partial chromosome lesions and fragmentation, a series of multiple breakages. The blurred outlines of eroded chromosomes are apparently caused by transient coiling anomalies and endoreduplication by chromosome replication in the absence of a spindle. The production of these virus-induced lesions has been variously attributed to inhibition of cell DNA synthesis, interference with cell protein synthesis, enzyme effects mediated by disrupted lysozomes, and a direct combination of viral and cellular nucleic acid. As yet, however, no one mechanism has been established on the single cause of chromosome abnormalities.

Any or all of the aforementioned lesions may occur in one or both chromatids of a given cell chromosome. Chromosomes behave as single structures in the G1 phase of the cell cycle before DNA synthesis has taken place. Therefore, if a defect is produced at this time, the lesion is replicated along with the second chromatid during the S or DNA synthesis period and the result is a full chromosome abnormality. However, if the injury occurs after the chromosome has

synthesized its DNA in the late S or G2 period when the chromosome is already a dual structure, a single chromatid lesion is the usual result. Most of the aberrations observed to date in virus-infected cells are of the chromatid variety, indicating that the chromosomes are affected in the late S or G2 phase of the cell cycle (60). In addition, virus-infected cultures rarely give rise to large numbers of exchanges or translocations. If Taylor (115) is correct in assuming that host DNA synthesis is necessary to a reunion of broken chromosomes, many of the viruses prevent exchanges by inhibiting cell DNA synthesis involved in repair.

In the present study, previous observations concerning the effect of virus dose, type of host cell and time of infection on the amount of HSV-induced damage in infected cells were essentially confirmed. The number of chromosome aberrations in infected H.Ep.2 and BHK-21 cells increased with time and virus multiplicity of infection. Defects were detected as early as 2 hours after infection and by 20 hours, 100% of the cells observed at metaphase exhibited some type of chromosome abnormality. Similar damage was observed in human and hamster cultures although the virus apparently produced more aberrations at an earlier time in H.Ep.2 cells. No evidence of specific chromosome alterations was seen in either cell line despite the fact that a number of investigators have reported non-random lesions in HSV-infected Chinese hamster cells (110) and mastomys (34) cultures.

Prolonged UV irradiation of HSV prevented the induction of all aberrations in infected cells. Moreover, the capacity of the virus to damage host chromosomes was significantly more resistant to UV inactivation than was the infectious property. Waubke et al. (126) reported similar results after UV irradiation of labeled HSV. Upon further investigation, they found evidence of impaired virus adsorption after irradiation although they were not able to conclude that the absence of chromosome damage was entirely due to this effect. Waubke's group also showed that the induction of chromosome lesions preceded and was thus independent of viral DNA replication.

Evidence for the interference of normal protein synthesis as a mechanism for induction of chromosome breaks has been obtained from various systems employing deficient media and mycoplasma (60). Mycoplasma-induced breaks in cultured cells were effectively prevented by the addition of excess arginine which is known to be depleted from infected cell media. Moreover, arginine-deficient media itself caused chromosome damage in a number of actively growing cells. Thus, although no evidence of mycoplasma infection was found in the H.Ep.2 or BHK-21 cells used in this research, it was of interest to determine if the HSV-induced chromosome abnormalities resulted from arginine depletion, since it is known that the amino acid is required for in vitro virus maturation (3). However, in the present study, large increases in arginine failed to prevent aberrations in HSV-infected

cells. Thus, it must be concluded that the virus does not injure cell chromosomes by interfering with the metabolism of arginine and the protein synthesis necessary for repair.

Ara-C has been previously reported to induce chromosome breakage in human leukocytes (8) and WI-38 cells (61) as early as 1-3 hours after inoculation. The present study confirms the production of chromatid breaks and secondary constrictions after exposure of H.Ep.2 and BHK-21 cells to various concentrations of ara-C. The amount of chromosome damage was significantly greater than the controls and appeared to have developed in the late S or G2 phase of the cell cycle when the chromosomes behave as dual structures. Since ara-C also acts as an inhibitor of DNA synthesis, the aberrations may have been related to the drug-induced inhibition of repair DNA synthesis in mammalian cells (60).

In HSV-infected cells treated with ara-C, the number of chromosome abnormalities was far greater than the combined effects of the two potential mutagens. O'Neill and Rapp (69) reported a similar synergism in HSV-infected human embryonic lung cultures. The drug and virus apparently act together to produce cells containing many multiple breaks. Simultaneous autoradiography performed in this study on uninfected and HSV-infected cells showed that ara-C completely blocked DNA synthesis. Therefore, since ara-C did not prevent the chromosome abnormalities induced by HSV, it can be concluded that

the lesions occurred in the absence of viral DNA synthesis. Previous studies have suggested that the antimetabolite and virus act to prevent the repair of cell DNA and thus induce visible chromosome damage.

In contrast to ara-C treated cultures, cells exposed to various concentrations of IDU did not generally exhibit a significant increase in the number of chromosome aberrations except at very high drug levels. Thus, despite its capacity to inhibit cell DNA synthesis as measured by ^{3}H-thymidine uptake, the anti-viral drug is not an active agent of chromosome damage.

Similarly, in the present study, HSV-infected cells exposed to IDU showed no evidence of synergism. The number of chromosome abnormalities in such cells equalled the sum of the lesions produced by HSV and IDU alone. As with ara-C, the virus-induced defects occurred in the absence of cell and virus DNA synthesis. Similar results were obtained by O'Neill and Rapp (70) in human embryonic lung cells.

The present work has revealed that uninfected and HSV-infected cells treated with a combination of ara-C and IDU showed a simple additive type of chromosome damage. It thus appeared that the molecular interactions responsible for the synergism of ara-C and HSV were not present in the ara-C/IDU system.

Although the significance of aberrations induced by viruses and chemicals is not known with certainty, there are at least four areas of actual or potential importance in mammalian cells. These are cell death, genetic damage that prevents further cell division, changes in chromosome number, and somatic and germline mutations. Virus-induced cell death and mitotic inhibition may be of greatest significance in the field of teratogenesis where it could lead to fetal abnormalities and spontaneous abortion. Most of the cells infected with HSV are normally expected to cease dividing and die. These include the small percentage of cells that exhibit visible chromosome defects at a metaphase and the large majority of cells that do not undergo mitosis. Thus, in light of the capacity of the virus to cause human genital infections (57), the mutagenic action of HSV could conceivably be involved in cell damage during embryogenesis.

Virus-induced aberrations theoretically could also result in changes in chromosome number or somatic mutations that subsequently might lead to malignancy. HSV has already been implicated in cell transformation (14) and possibly in the production of human cervical carcinoma (56,76). Thus, the visible and subvisible metaphase abnormalities induced by the virus may indeed be related to the process of oncogenesis and possibly integration and latency. Of even more interest at present, is the secondary finding that the chemotherapeutic

agents used to combat HSV infection induce similar chromosome damage and thus also exhibit a definite potential for disruption of normal cell growth and division.

In summary, HSV was found to cause profound metabolic and morphological alterations in infected H.Ep.2 and BHK-21 cells. These alterations included an inhibition of host DNA synthesis and mitosis followed by characteristic nuclear disruption, formation of intranuclear inclusion bodies, and production of various antigens and chromosome abnormalities. Ara-C and IDU failed to prevent the virus-induced cytopathic effects in vitro but did inhibit viral DNA synthesis and assembly of infectious particles. Moreover, exposure of the cells to the anti-viral agents themselves resulted in severe cytopathic alterations involving cytoplasmic disorganization, inhibition of DNA synthesis and mitosis, and induction of various levels of chromosome damage.

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