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Oncogenic Transformation by Herpes Simplex Virus

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ONCOGENIC TRANSFORMATION BY
HERPES SIMPLEX VIRUS

by

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Thesis

submitted in partial fulfillment of the requirements for the

Degree of Doctor of Philosophy in the Department of
..... Microbiology..... at the Medical College of Virginia

Virginia Commonwealth University

Richmond, Virginia

December, 1979

This thesis by James Christian Burns is accepted in its present form as satisfying the thesis requirement for the degree of Doctor of Philosophy

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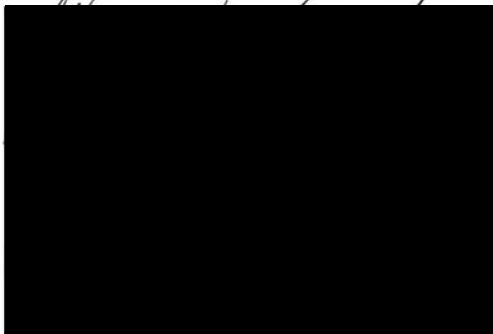
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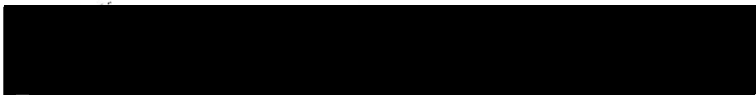


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APPROVED:

Chairman, MCV Graduate Council, Dean, School of Basic Sciences

DEDICATION

I would like to dedicate this dissertation to my son and his mother. Christian has been a continual source of happiness and a constant reminder of my personal priorities. Karen has provided understanding and encouragement in a patient atmosphere. Together they have given me the strength to complete this work.

ACKNOWLEDGMENT

The author wishes to thank Dr. Richard P. Elzay for supplying friendly advice, support, and, most of all, the freedom to learn and grow. His example is one that I will eternally strive to emulate.

The author would also like to thank the chairman, Dr. B. K. Murray, and other members of the graduate committee, Dr. P. Coleman, Dr. J. Tew, and Dr. G. Van Tuyle for suggestions, guidance, and technical assistance.

CURRICULUM VITAE

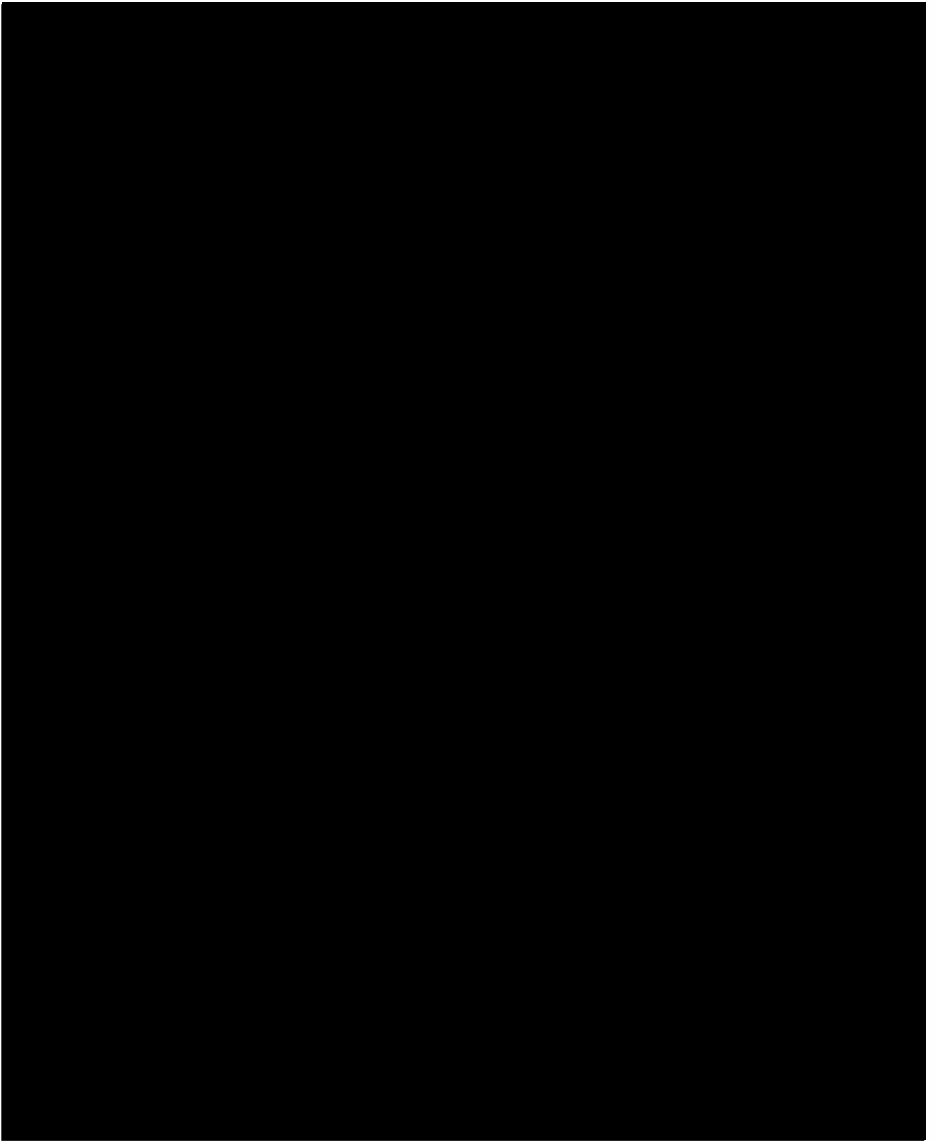


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INTRODUCTION

HISTORY

Since the earliest recorded description of herpetic lesions by the Roman physician Herodotus,¹ the herpes family of viruses has been associated with a myriad of clinical manifestations in man. By the end of the 1800's, Astruc had described the genital lesions of herpesvirus etiology² and Vidal had shown the virus to be infectious and communicable.³ Much of our knowledge of the pathogenesis of herpesviruses began with Gruter, who, in 1912, transmitted the disease to rabbits using vesicular fluid from a human lesion.⁴ Goodpasture, in the 1920's, demonstrated that herpes simplex virus (HSV) was capable of inducing a primary infection in rabbits and hypothesized sequential neural transport and establishment of a latent state within the nerve cells which could then be reactivated later to produce recurrent lesions.⁵ By 1971, Goodpasture's theories were demonstrated to be correct by the recovery of infectious HSV from sensory ganglia of latently infected animals and from humans with a history of recurrent herpetic lesions.⁶ The herpesviruses have subsequently been shown to be ubiquitous in nature and capable of infecting tissues derived from primitive ectoderm: skin, mucous membrane, eyes and nervous tissue. In humans, HSV, also called Herpes hominis, has been associated with gingivostomatitis, recurrent herpes labialis, herpes progenitalis, ocular keratoconjunctivitis, encephalitis, and most recently, a possible role in carcinoma.

CLASSIFICATION

The Herpetoviridae are a large family of double stranded deoxyribonucleic acid (DNA) viruses that have an icosahedral capsid composed of

162 capsomeres surrounded by a lipid soluble envelope. The virions contain a linear genome of approximately 100 million daltons molecular weight. Their ubiquity in nature is demonstrated by the fact that herpesviruses can infect over 30 different vertebrate species and some invertebrate species.⁷ In man, Epstein Barr virus, Cytomegalovirus, Varicella-Zoster virus, Herpes virus B, as well as HSV, can produce disease.

On the basis of antigenic, molecular and pathologic properties, HSV can be subdivided into two distinct types: type 1 (HSV-1), commonly associated with oral and facial lesions, and type 2 (HSV-2), associated with genital infections. Man is the only known host and exclusive reservoir for HSV-1 and HSV-2. Although less than 1% of the population has a history of clinically evident HSV-1 primary gingivostomatitis, 40 to 90% show serologic evidence of previous exposure.⁸ The genital lesions of HSV-2 etiology rank second to gonorrhea in prevalence as a venereal disease.⁹ These two virus types have different biological and biochemical properties. HSV-2 produces a larger pock on chick allantoic membranes than HSV-1. The buoyant densities of the viral DNAs in cesium chloride are different: 1.726 gm/cm^3 and 1.728 gm/cm^3 for HSV-1 DNA and HSV-2 DNA, respectively.^{10, 11} These respective densities correspond to a guanine and cytosine content of 67% for HSV-1 and 69% for HSV-2. There is more than a 50% genome homology between HSV-1 and HSV-2 as determined by DNA-DNA hybridization.¹² This degree of homology is reflected by antigens that produce cross-reacting as well as type-specific antibodies in man.¹³ Other differences between HSV-1 and HSV-2 include: host range susceptibility, variation in plaque size, and yield of infectious virions after exposure to elevated temperature, ultraviolet (UV) light or acid pH.^{14, 15, 16}

PHYSICAL AND CHEMICAL PROPERTIES

Neither of the two subtypes of HSV are particularly stable under various environmental conditions and thus their infectious properties can be inactivated. HSV is thermolabile and storage of the virus for long periods of time is difficult. Short-term storage necessitates a temperature of -70°C ; at warmer temperatures, infectivity is quickly lost. The half-life of viral infectivity at 37°C is approximately three hours.¹⁷ Maintenance of pH is very important in achieving optimal infectivity; for example, a pH of 7.0 - 7.6 results in maximal virus replication.¹⁸ The virus can also be inactivated by exposure to x-radiation or UV light. Infectivity is halved following 7 seconds of UV exposure at $42 \text{ ergs/cm}^2/\text{s}$.¹⁷ The viral envelope, which is obtained by budding from the nuclear membrane of the host cell, is easily disrupted following exposure to urea, sodium dodecyl sulfate, and lipid solvents such as ethyl ether. Since this envelope is necessary for adsorption to the host cell, exposure to these agents can greatly decrease infectivity. For example, a 30 minute exposure to ethyl ether completely eliminates the infectious capacity of HSV.⁷

HERPES SIMPLEX VIRUS - HOST CELL INTERACTIONS

REPLICATION OF HSV IN VITRO

The replicative cycle of HSV has been well documented. Initially, the virions attach to the host cell by an electrostatic interaction between the viral envelope and specific receptors on the cellular membrane. Following attachment, the virion is internalized into the cell by fusion or by phagocytosis. Uncoating of the virion is followed by migration of the viral DNA into the nucleus. There, transcription of

the viral DNA into viral RNA takes place via a host DNA dependent RNA polymerase. The viral transcript is then processed by capping, cleavage and adenylation. After leaving the nucleus, the mRNA is translated into proteins on polyribosomes. The viral proteins can be subcategorized into various groupings: (i) viral structural or non-structural proteins; (ii) early or late proteins (synthesis occurring before or after viral DNA synthesis)¹⁹; and (iii) the coordinately controlled alpha, beta, gamma protein groups²⁰ (alpha proteins induce the synthesis of beta proteins, beta proteins induce the synthesis of gamma proteins and repress the synthesis of alpha proteins, etc.). Thus, within a few hours postinfection, host cell protein synthesis is shut down in favor of viral mRNA translation. The early viral proteins are required in order to initiate synthesis of viral DNA which commonly occurs around six hours postinfection. The majority of HSV structural proteins are synthesized as early proteins. The remaining late proteins are then synthesized bringing the total of viral induced proteins to more than 50, with molecular weights ranging from 15,000 to 280,000 daltons.²⁰ Proteins are cleaved, phosphorylated and associated with cell lipids. Structural proteins are transported into the nucleus where assembly takes place. Accumulations of viral products within the nucleus are called Cowdry type A inclusion bodies.²¹ Other proteins are glycosylated and inserted into the nuclear and cytoplasmic membranes. Within these areas, the assembled nucleocapsid exits the nucleus and acquires its envelope. Newly assembled virions are seen within the cell between 7 and 12 hours postinfection, and are released from the host cell about 8 hours later.^{17, 22, 23} The virions leave the cell by lysis of the host cell, by transport through the endoplasmic reticulum system, or by spreading directly to an adjacent cell.^{13, 24}

Although the yield of infectious virus depends upon the cell type and growth conditions, HSV replication is generally quite inefficient. Less than 20% of the viral DNA is encapsulated and excess viral proteins are produced.²⁵ Many errors in assembly occur which result in unenveloped nucleocapsids and hollow envelopes. Some authors state that only between one and five percent of the total virus particles are infectious.^{22, 25}

Cytologically, HSV infected cells in culture first demonstrate a rounding up phenomenon associated with an increase in refractility. Within the nucleus, peripheral clumping of chromatin and Cowdry type A inclusion bodies can be seen. Some strains of HSV cause fusion of cell membranes between adjacent cells forming a syncytium. Ultimately, however, the host cells undergo lysis and cell death.

HSV INFECTIONS IN VIVO

In man, HSV-1 and HSV-2 induce a spectrum of diseases ranging from gingivostomatitis, recurrent herpes labialis or genital herpetic lesions, to fatal herpetic encephalitis. Biopsies of patients with herpetic infections show characteristic microscopic lesions. These include ballooning degeneration of the nuclei, Cowdry type A intranuclear inclusion bodies and formation of multinucleated giant cells. The primary site of HSV infection is stratified squamous epithelium. Within this epithelium, acantholysis of the spinous cell layer results in suprabasilar vesicles. Tzanck cells within the vesicles demonstrate cytologic abnormalities. Inflammatory cells infiltrate the underlying fibrous connective tissue. HSV can disseminate by viremia or along neurogenic pathways. Internal organs as well as the central nervous system can become infected. Histologic findings at these distal sites include coagulation necrosis and peri-

vascular cuffing by lymphocytes and plasma cells. With encephalitis, inclusion bodies are seen in neurons and oligodendrocytes.²⁶ Patients can succumb to disseminated herpes or encephalitis.

Numerous animal models have been developed to better elucidate the pathogenesis of HSV. Corneal infection of rabbits and foot pad inoculations of guinea pigs were utilized by early investigators to study neural transmission. More recently, mice have been infected by different routes to study pathogenesis: intracranial, intradermal, intraperitoneal or intravaginal. A murine lip model has been described previously and was utilized throughout the research described in this dissertation.²⁷ The histopathologic and virus isolation data from this model has documented the neurogenic spread of HSV from the lip into the central nervous system as follows: lip, day 1; ipsilateral trigeminal ganglion, day 2; contralateral trigeminal ganglion, day 4; and cerebrum and cerebellum, day 5.²⁷ Fatal encephalitis occurs within 8-12 days after infection and is dose-, as well as, age-dependent. Younger mice and larger doses of HSV yield greater percentages of fatal encephalitis. Mice surviving HSV infection develop HSV specific neutralizing antibodies and their regional ganglia characteristically demonstrate latent infection.

LATENT HSV INFECTION

One of the most intriguing aspects of HSV infection is the phenomenon termed latency. Latency involves the potential for recurrent active lesions caused by endogenous HSV. After the primary infection in a host that is seronegative, the virus becomes sequestered and when conditions permit, the virus reappears to cause a recurrent lesion. Goodpasture theorized that the trigeminal ganglia serve as reservoirs from which HSV

is reactivated to cause oral and facial lesions.⁵ Charlton later reported that patients suffered recurrent oral herpetic infections following surgical manipulation of the trigeminal ganglion.²⁸ Human, as well as latently infected animal ganglia are reactivated and shed infectious virus when explanted onto indicator cells.^{6, 29} However, no reliable in vivo model of reactivation that parallels the latent human condition exists. Nesburn used surgical stimulation of latently infected rabbit trigeminal ganglia to demonstrate peripheral release of infectious HSV from tear drops.³⁰ Two other accounts document HSV reactivation in mice using large quantities of cyclophosphamides and corticosteroids.^{31, 32} The mechanism of latency at the molecular level is unknown. However, two major theories are offered, the dynamic theory and the static state theory.

The dynamic theory of latency states that the infected tissues (ganglia, skin, or salivary glands) continually produce low levels of infectious virus. Recrudescence of clinical lesions is allowed only when specific systemic or local conditions exist. Proponents have documented that patients, who are clinically free of lesions, shed infectious viral particles in body secretions.³³ About 0.5% of the general population excrete HSV in genital secretions, whereas 5% shed HSV from oral secretions.³⁴ Also, Galloway, using in situ hybridization, has recently detected HSV mRNA in latently infected ganglion cells.³⁵ These results imply that some latent HSV DNA is transcribed. Finally, evidence from in vitro studies has shown that prostaglandins enhance cell to cell spread of HSV.³⁶ This implies that local immune and inflammatory prerequisites allow infectious virus to replicate and cause clinical disease.

Alternatively, the static state theory proposes that virus persists in the ganglia in a non-replicating state. This theory explains why it

has been so difficult to isolate infectious virus from latently infected ganglia and skin. Several lines of evidence support the static state theory of latency. First, viral DNA has been detected within neurons by in situ hybridization; however, extensive searches for viral antigens or virus particles utilizing immunofluorescence and electron microscopy have been inconclusive.³⁷ Secondly, assays utilizing renaturation kinetics have determined that only 0.11 ± 0.03 genome equivalents of viral DNA are present per latently infected ganglia cell.³⁸ Lastly, temperature-sensitive mutants of HSV have been shown capable of establishing latency, suggesting that viral DNA replication is not necessary for the establishment of latency.³⁹

In the final analysis, it seems that latent HSV could harbor itself within the ganglia in the form of free nucleic acid or nucleic acid integrated into the host's DNA. The virus could then reinitiate clinical disease according to either the dynamic theory, the static theory, or a combination. It seems that today's technology lacks extreme sensitivity and is incapable of delineating which form of latent virus or theory of activation is most correct.

HERPESVIRUSES AND ONCOGENICITY

If known malignancies in non-human species existed which were analogous to human oral or genital squamous cell carcinomas, and these were found to be virally induced, then the arguments for a role of HSV in oncogenesis would be strengthened. However, no analogous tumors are known to exist. There is ample evidence that members of Herpetoviridae other than HSV can and do induce malignancies. A neurolymphomatosis in chickens, known as Marek's disease, is caused by a herpesvirus. Significantly,

a vaccine has been developed against the disease; the first vaccine capable of controlling a neoplastic process.⁴⁰ Evidence exists that herpes induced lymphomas occur in monkeys, rabbits and guinea pigs.⁴¹ Lucke's tumor, an adenocarcinoma of frogs, is caused by a herpesvirus.⁴² In man, definite association exists between Epstein-Barr virus and three human diseases: infectious mononucleosis, nasopharyngeal carcinoma, and African Burkitt's lymphoma.^{43, 44} The latter two diseases are malignancies capable of killing their human hosts. In summary, guilt by association is frequently given as one piece of evidence linking HSV to the etiology of human squamous cell carcinomas.

HSV AND HUMAN CANCER

Attempts to implicate HSV as an oncogenic agent began soon after epidemiologic studies indicated a correlation between previous HSV-2 genital infection in women and the development of uterine cervical carcinoma.⁴⁵ About the same time, a British physician reported six cases of squamous cell carcinoma of the lip in locations that had suffered from recurrent herpes labialis.⁴⁶ Since these beginnings, many avenues of research have been followed investigating the oncogenic potential of HSV-1 and HSV-2.

Early epidemiologic evidence showed an increased risk of developing cervical carcinoma in females with a history of coitus at an early age and multiple sex partners. These data led epidemiologists to theorize that venereally transmitted factors were involved in the development of cervical carcinoma.⁴¹ Because herpes genitalis is a venereally transmitted disease and shows increased incidence in patients with histories of sexual promiscuity, HSV-2 was soon implicated as an etiologic agent. Neutralizing antibodies to HSV-2, assayed by either the kinetics of neutralization or

by neutralization titers, are significantly higher in women with cervical cancer as opposed to an age matched control group.⁴⁷ More impressive differences exist for the occurrence of antibodies to viral induced non-structural antigens. Three examples include Aurelian's AG-4 antigen, Hollinshead's HSV-TAA, and Anzai's VP134.⁴¹ Similar seroepidemiologic evidence exists linking HSV-1 to the etiology of squamous cell carcinomas of the head and neck. Measurements of antibody levels to HSV non-virion antigens have been made in cancer as well as non-cancer patients.^{41, 48, 49, 50}

Antibodies were observed in 90% of the laryngeal cancer cases, 91% of the head and neck squamous cell cancer cases, 11% of the non-squamous cell cancer cases (melanoma, breast, salivary gland, etc.), and 4% of normal subjects.^{48, 51} With both HSV-1 and HSV-2, antibodies to type specific antigens could simply denote repeated exposure of persons to viral antigens through recurrence and/or reinfection; however, the majority of researchers feel that the observed distribution of the antibodies in experimental cases as opposed to controls heavily favors an etiologic role for HSV in the development of genital and oral squamous cell carcinomas. To the contrary, it must be noted that not all patients with herpes infections develop cancer and that cancer can occur in patients with no serologic evidence of HSV infection.³⁴

Numerous studies have attempted to detect the presence of HSV-1 and/or HSV-2 specific markers (antigens, RNA, DNA, or virions) in human tumors. The unequivocal existence of viral antigens in cervical cancer cells has not yet been firmly established. Although Aurelian reported success utilizing immunofluorescence and specific antisera directed against HSV-2 antigens in staining neoplastic cells exfoliated from a cervical lesion, numerous other researchers have been unable to find specific herpes

antigens in their biopsies.⁴¹ Attempts are now in progress within our department to detect HSV-2 antigens in cervical carcinoma biopsies utilizing the immunoperoxidase stain (Dr. G. Cabral, personal communication). Hybridization techniques have been utilized to detect viral nucleic acids in human tumors. Frenkel was the first to report the detection of HSV-2 specific DNA fragments and RNA transcripts in human cervical carcinoma tissue.⁵² She concluded that each tumor cell contained 3.5 copies of a segment of DNA representing 39% of the HSV-2 genome. Recently, Jones reported detection of HSV-2 mRNA in 5 of 8 human cervical biopsies by in situ hybridization.⁵³ Unfortunately, studies performed by other workers have been unsuccessful in detecting HSV DNA in their biopsy material from cervical carcinomas.⁴¹ Besides HSV antigens and nucleic acids, entire virions have been found in tumors. A case from the Italian literature involved a carcinoma which arose in a location on the lip which had suffered recurrent HSV lesions. Virus was isolated from this tumor and characterized as HSV-1.⁵⁴ Finally, one report exists in which herpesvirus were observed within a squamous cell carcinoma of the urinary bladder by electron microscopy.⁵⁵ Thus, many anecdotal accounts document the presence of herpetic antigens, nucleic acids, or infectious virions within squamous cell carcinomas from the oral cavity, the uterine cervix and the bladder; however, the bulk of the molecular evidence has failed to provide unequivocal evidence for herpetic sequences within carcinomas.

In man, carcinoma has developed at the site of recurrent herpes labialis in a number of cases. Kvasnicka reported on a etiologic study of lower lip carcinoma in which 20% of the patients said that their carcinoma was a direct continuation of recurrent herpes labialis.⁵⁶ Later, he reported on five cases of carcinoma (four on the lips) that arose at the

site of recurrent HSV infection.⁵⁷ A localization site analysis of 283 lip carcinomas and 320 herpetic lesions by the same author revealed: "in the perioral region, both in men and women, cancer is frequent where the occurrence of herpes simplex is frequent and rarer at sites where the occurrence of herpes simplex is rarer."⁵⁸ An important observation in these cases is that the location of herpes labialis and lip carcinoma is frequently that area which receives most of the solar radiation, i.e. the skin surface of the upper lip and the vermillion portion of the lower lip. Marshall, studying a group of long-term immunosuppressed patients, noted an increased incidence of HSV infection and squamous cell carcinoma.⁵⁹ One case, a 14 year old boy with renal transplants, involved a lip carcinoma arising in the site of persistent herpes labialis. Lip carcinoma in a patient of this age is rare.

ONCOGENIC TRANSFORMATION BY HSV

Attempts to induce tumors in animals utilizing HSV-1 or HSV-2 have been unsuccessful. Nahmias inoculated newborn hamsters with infectious HSV-2 and with UV-irradiated HSV-2. Of the animals receiving infectious HSV-2, only 2-3% developed sarcomas while 1% of the control animals developed malignancies.⁴¹ Most researchers feel that the lytic nature of HSV must be in some way inactivated before the virus can become oncogenic. Alternatively, recurrent exposure of the host cell to HSV, instead of a single application, may be necessary to initiate the neoplastic process.

In studying the initial stages of tumor induction by viruses, many researchers utilize the technique of in vitro viral transformation of cultured cells. In order to distinguish virally transformed cells from spontaneously arising variants, it is necessary to show the presence of

specific viral markers as well as to demonstrate in vivo oncogenicity. One problem with studying transformation by HSV is that there are few cell lines which are non-permissive for HSV. Consequently, the lytic capability of HSV must be destroyed to prevent death of the host cell. The virus must be treated in a manner which eliminates viral infectivity without affecting its transforming capability. Originally, Duff and Rapp utilized UV-irradiation to inactivate the lytic functions of HSV-2 and induce the transformation of hamster embryo fibroblasts (HEF).⁶⁰ Since their description in 1971, numerous accounts documenting oncogenic, as well as biochemical transformation of human, rat, hamster and mouse cells, have appeared for both HSV1 and HSV-2.^{61, 62}

When UV-irradiated HSV was used to transform HEF cells, 7 of 15 HSV-2 isolates were described by Rapp and Duff as having transforming potential, as well as 2 of 12 HSV-1 strains.⁴¹ However, in most instances, the efficiency of transformation is low and not more than one or two transformed foci appear for every 5×10^6 cells infected at a multiplicity of infection (MOI) of 1.⁴¹ Two types of foci are noted: fibroblastic and epithelioid. When tested for oncogenicity in newborn hamsters, the fibroblastic type foci induce fibrosarcomas; whereas, the epithelioid foci produce adenocarcinomas. Infectious virus could not be isolated from either the transformed cells or the induced tumors. Significantly, the sera from the tumor bearing animals demonstrate neutralizing antibodies against HSV. Furthermore, immunofluorescence techniques using antisera against HSV show the presence of viral antigens in transformed cells (333-8-9 line).^{42, 63, 64, 65} Another method of destroying HSV lytic functions involves inactivation by photodynamic exposure of neutral red treated HSV infected cells. Photodynamic inactivation results in trans-

formed cell lines that are oncogenic when tested in newborn hamsters.⁴¹
Other techniques to inhibit the lytic gene functions of HSV and encourage transformation include: incubation of infected cells at suboptimal (20°C) temperatures,⁶⁶ incubation at supraoptimal temperatures,⁶⁷ and infection with temperature sensitive mutants.^{62, 68}

Nucleic acid hybridization has been used to detect the presence of HSV mRNA and DNA in transformed cell lines. Transformed cell lines contain about 10-13% of the mRNA present in lytically infected cells and 8-21% of the complete viral genome.^{41, 69} Thus, only a small portion of the complete viral genome appears to be present and actively transcribed in transformed cells. A recent report has documented that biochemical transformation of mouse cells is possible using restriction endonuclease fragments of HSV DNA.⁷⁰

Duff and Rapp also observed that endogenous type C RNA tumor virus was released after several passages from 3T3 cells transformed with UV-irradiated HSV.⁷¹ The possibility thus exists that oncogenic transformation by HSV involves the activation of an RNA tumor virus.

BIOCHEMICAL TRANSFORMATION BY HSV

Since the proof of oncogenic transformation involves steps that include the subcloning of foci, colony formation in soft agar, and tumor formation in susceptible animals, many researchers have attempted to devise a convenient biochemical assay to detect HSV transformation. To date, two assays have been reported. The most widely reported assay involves the conversion of thymidine kinase negative (TK⁻) mouse or human cells to the thymidine kinase positive (TK⁺) phenotype. The HSV-1 and HSV-2 thymidine kinases can be distinguished from one another and from

the thymidine kinase found in eukaryotic cytoplasm and mitochondria. This assay measures the transfer of HSV thymidine kinase to the mouse or human TK⁺ cells and their selection by growth in HAT (Hypoxanthine, Aminopterin, Thymidine) media. Biochemical transformation as measured by conversion to TK⁺ phenotype has been reported with UV inactivated HSV-1, UV inactivated HSV-2, temperature sensitive mutants of HSV and fragments of HSV DNA.⁴¹ Recently, another biochemical transformation assay has been reported which involves the detection of plasminogen activator utilizing a fibrin overlay method.⁷² Since many transformed malignant cells have been shown to produce the enzyme plasminogen activator, this report suggests that its presence provides a rapid detection of HSV specific cell transformation.

Other biochemical evidence exists which also argues in favor of a role for HSV in transformation. Deoxyribonucleases induced by HSV have both exonuclease and endonuclease activity.⁷³ These enzymes allow HSV to induce chromatid breaks and disrupt host DNA.⁷⁴ Repair of DNA breaks and increased DNA synthesis have been reported in HSV infected cells.⁷⁵ Host DNA breakage and repair could explain viral insertion mechanisms and mutational events, either alone or as a cofactor with known carcinogens.

HSV AND COCARCINOGENESIS

Joint action of HSV with known carcinogens has been reported in vivo and in vitro. Tanaka reported a greater incidence of skin papillomas induced by 3-methylcholanthrene (3-MC) application when HSV was injected intradermally in the same area.⁷⁶ Later he reported that the intraperitoneal injection of HSV on the third day after 3-MC application to the skin more than doubled the incidence of skin papillomas.⁷⁷ Also,

the rate of malignant conversion from papillomas to carcinomas was more frequent among mice given HSV. Howett reported enhanced biochemical transformation of TK⁻ mouse cells by UV-irradiated HSV following nitrosomethylurea treatment as compared to that seen with virus or chemical alone.⁷⁸ Recently, photodye therapy (light exposure following neutral red application) of genital herpetic lesions was held responsible for the development of three cases of intraepidermal carcinomas of the penis.⁷⁹

In summary, many lines of evidence exist associating HSV with carcinoma development. Many of these lines are well proven, but a greater number are incomplete in their documentation. Experimental studies include: in vitro transformation of human and non-human cells, induction of cervical carcinoma in mice and non-human primates, and virus stimulation of host DNA alterations and synthesis.⁴³ The data emanating from studies with humans include: seroepidemiology, retrospective studies of venereal viral diseases, and virus markers (proteins, RNA, or DNA) in human squamous cell carcinomas.⁸⁰ Important theoretical differences exist between association, correlation and etiology. No evidence implying association or correlation of HSV with squamous cell carcinoma development can be interpreted as definite evidence of a cause and effect relationship. A purist could argue that the available information to date reflects merely as association of HSV with neoplasia due to the preferential growth of the virus in tumor cells. Although the specific role of HSV in the induction of tumors in man has not been established with any degree of certainty, the many findings that HSV infections contribute in a positive way to the carcinogenic processes in several experimental systems indicates HSV's importance to the final outcome.

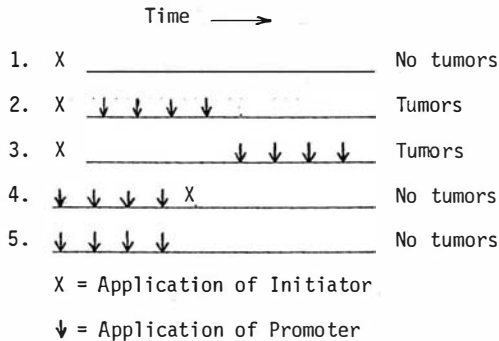
TWO-STAGE CARCINOGENESIS

INTRODUCTION

The biologic processes leading to malignant neoplasms in general are multifactorial in terms of etiology and multisteped in terms of evolution, rather than simple "one-hit" mutagenesis. Furthermore, the sequential nature in the induction of neoplasia can be critically modified by exogenous as well as endogenous factors. The interaction of multiple environmental factors may increase the incidence of cancer more than the exposure to a single carcinogen and gains tremendous importance from a public health standpoint. In the broadest sense, then, the development of tumors can be thought of as a series of cocarcinogenic events.

Many of the current concepts concerning the natural history of the neoplastic process in vivo stem from the pioneering studies of Berenblum in 1941.⁸¹ He successfully demonstrated what has now become a classical mouse skin tumorigenesis model. The development of neoplasia in this model is divided into two stages. The first stage, termed initiation, follows the application of a single subthreshold dose of a chemical carcinogen. Then compounds, termed promoters, are administered repeatedly to the same site. Most of this early work employed polycyclic aromatic hydrocarbons as the initiating agents and Croton oil as the promoting agent. Tumorigenesis is uniformly induced by repeated application of the promoter to mouse skin treated previously with a single application of carcinogen. The first tumors arose 30-70 days after the beginning of promoter treatments.⁸² Significantly, if the order of the treatments is reversed, no tumors are induced. Also, if the promoter is given by itself repeatedly or if the carcinogen is given alone, no tumors emerge.

The process of initiation is irreversible; by beginning promoter exposure as long as one year after the application of the initiator, tumorigenesis is still possible. Thus, a type of permanent but unexpressed damage is done during the process of initiation. This damage requires promotion in order to express itself as a clinical neoplasm. The following figure demonstrates the scheme for two-stage epidermal carcinogenesis in mice and underscores the importance of the sequence of treatments.⁸³



Considerable interest and importance is now attached to the two-stage carcinogenesis model. Recent demonstrations exist of similar sequences in carcinogenesis of tissues other than skin. In every instance, the carcinogen is delivered in minute amounts and the promoter, lacking its own carcinogenicity, is subsequently and repeatedly administered. Representative systems of initiation-promotion are listed in the following table:

<u>Tissue</u>	<u>Initiator</u>	<u>Promoter</u>
Mouse epidermis	3 Methylcholanthrene Benzopyrene Nitrosoguanidine UV-Irradiation	Tetradecanoyl-Phorbol-Acetate (TPA) TPA Croton oil Croton oil
Rat epidermis	Beta radiation	Cigarette smoke condensate
Rat bladder	Methylnitrosourea	Saccharin
Rat liver	Dimethylnitrosamine	Phenobarbital
Rat mammary gland	Dimethylbenzanthracene	Phorbol
Mouse liver	Dimethylnitrosamine	Phorbol
Mouse lung	Dimethylnitrosamine Urethan	Phorbol Hydroxytoluene
Mouse embryo fibroblasts in culture	3 Methylcholanthrene UV-Irradiation	TPA TPA
Mouse stomach	Dimethylbenzanthracene	TPA

Thus, initiation-promotion sequencing and tumorigenesis can be demonstrated in vivo in various animal systems as well as in vitro.^{84, 85}

TUMOR PROMOTING AGENTS AND TETRADECANOYL-PHORBOL-ACETATE

Agents capable of promotion in two-stage carcinogenesis, comprise a wide variety of chemical agents. Phorbol esters, detergents, iodoacetic acid, phenobarbital, artificial sweeteners, and numerous other chemicals are implicated as exogenous promoting agents. Endogenously, hormones may act as promoters. Much of the research is centered around the phorbol esters, derivatives of Croton oil. The seed oil of Croton tiglium, a leafy shrub of the family Euphorbiaceae, is the source of the phorbol esters.⁸⁶ These macrocyclic diterpene esters were isolated and characterized by Hecker.⁸⁷ Tetradecanoyl-phorbol-acetate (TPA) is the component

of Croton oil active as a promoter.

TPA, sometimes also called phorbol-myristate-acetate (PMA), is the 12, 13 diester of phorbol with tetradecanoic and acetic acids as side groups. The agent has a melting point of 72°C and is usually obtained in crystalline form. TPA is soluble in organic solvents including acetone and dimethylsulfoxide (DMSO). TPA is highly irritating and is the strongest tumor promoter known on mouse skin.⁸⁸ After initiation with dimethylbenzathracene (DMBA), repeated application (twice weekly, 0.02 umole TPA/application) onto mouse skin produces a large number of papillomas and squamous cell carcinomas.⁸⁸ The mechanism of action of TPA is not known, but extensive molecular research is ongoing. In the past, the preponderance of experimental effort investigating the mode of action has been performed on mouse skin. However, in that system it is difficult to identify, from an array of biochemical alterations, that one which is linked with promotion. Many of these in vivo observations may be pleiotropic responses to cell division. These in vivo alterations will be briefly discussed before the more recent in vitro investigations are examined.

TPA application causes certain histologic effects on mouse skin. These include edema, erythema, leukocyte infiltration and, eventually, hyperplasia. Raick demonstrated that following a single exposure to TPA, mouse epidermis shifts to an active growth state. The mitotic index increases as well as the rate of precursor incorporation into protein, RNA and DNA.⁸⁹ Maximum synthesis of RNA occurs at 6 hours, protein at 12 hours and DNA at 18 hours. There is an enlargement of nucleoli, as well as an increased number of polysomes. There is altered morphology of the spinous cells taking on the appearance of basal cells. Rapid stimulation of phospholipid synthesis is noted. In addition, alteration in

membrane permeability and structure, reduction in epidermal histidase activity, as well as an increase in ornithine decarboxylase (ODC) activity have been reported. Significantly, Balmain has documented rapid histone phosphorylation and suggested that this might be responsible for the increased synthesis of DNA and RNA.⁹⁰ Troll has reported detecting a trypsin-like protease as early as 30 minutes after TPA application onto mouse skin. This protease may modify or remove histones thereby activating a specific region of the genome.⁹¹ Alternatively, this protease may activate DNA in a manner similar to that observed in sex hormone activation; Slaga has reported that TPA binds to a cytosol receptor protein.⁹² Other interesting in vivo observations include those of VanDuuren, who reported that minor changes in the molecular structure of TPA result in diminished, or complete loss of, tumor promoting activity on mouse skin.⁸² Tumor promoting activity is also inhibited by prior application of exogenous agents, including fluocinolone acetonide (a steroidal anti-inflammatory agent) or retinoids (Vitamin A derivatives).^{93, 94} Many of the aspects of the biologic nature of tumor promotion by TPA are characterized; however, the molecular events that take place during promotion remain unclear. Attention to this problem has now turned to in vitro evaluation of promotion by TPA.

Many in vitro investigations have focused on the stage of promotion in the development of the neoplastic state. An initial observation showed TPA to be effective in enhancing the outgrowth of Swiss 3T3 cells transformed by SV40 virus. Good correlations exist between various promoting agents in that system and the classical in vivo mouse skin assays.⁹⁵ Other cell culture systems, utilizing various initiators and promoters, are also similar to in vivo two-state carcinogenesis; cells exposed to

subtransforming doses of carcinogens and phorbol esters produce transformed foci. Transformed foci are not produced by treatment with carcinogen or phorbol esters alone. Mondal reported transformation of a mouse fibroblast cell line using phorbol esters as promoters and aromatic hydrocarbons or UV light as initiators.⁹⁶ TPA causes a two to three fold enhancement in transformation of rat embryo cells infected with a temperature sensitive mutant of adenovirus type 5. In the presence of TPA, these transformed foci appeared earlier and were significantly larger in diameter.⁹⁷ Thus, a two-stage protocol utilizing polycyclic hydrocarbons, UV light, or virus as initiators and phorbol esters as promoters can result in increased efficiency of transformation of cells in vitro and an enhanced ability of these cells to grow in soft agar. The effect of phorbol esters alone has been investigated within these in vitro systems and three general areas of activity will be discussed: the effect on cell differentiation, cell membrane alteration, and the effect on cellular enzymology.

Many effects by TPA upon cellular differentiation have recently been reported. Most of the evidence documents a dramatic inhibitory effect. Under the influence of TPA, chicken myoblasts do not enter G₀ phase at the same frequency as normal cells, do not synthesize normal myosin chains, and do not exhibit normal fusion of myotubules. Besides its mitogenic effect on chicken chondroblasts, TPA transforms these cells from sessile polygonal cells into mobile fibroblastic cells that form multilayers. These chondroblasts cease to synthesize two macromolecules needed in mature cartilage.⁹⁸ TPA also decreases collagen synthesis in chicken fibroblasts. This effect is reversible and is not the result of permanent transformation nor the selection of a population subset.⁹⁹ Explanted rat tracheal epithelium shows an increased rate of

cellular division and capacity to grow out in primary cultures in the presence of TPA. Significantly, these cells are nontumorigenic when inoculated into immunosuppressed hosts.¹⁰⁰ Other examples of the inhibitory effect on cellular differentiation by TPA include erythroid conversion of Friend erythroleukemic cells, adipose conversion of lipogenic mouse cells and neurite conversion of mouse neuroblastoma cells.^{101, 102} Two generalities that have been noted are: (i) positive correlation exists between tumor promoting activity in vivo on mouse skin and a promoter's ability to inhibit differentiation of cells in vitro; (ii) upon removal of the promoter the in vitro effects of the phorbol esters are reversible.⁹⁵ Rovera reported that TPA has more than an inhibitory effect on cellular differentiation. TPA can induce a differentiation of HL-6 human promyelocytic leukemic cells to become adherent to plastic and to undergo a morphologic change into a monocytic cell. He concluded that TPA can have at least three different effects upon cellular differentiation: (i) stimulation of the normal pathway; (ii) inhibition of normal differentiation; and (iii) induction of an alternate pathway of differentiation. Furthermore, he believed the effect of tumor promoting agents may depend upon the target cell (unipotent vs. multipotent).¹⁰³

A critical target for tumor promotion by the phorbol esters may be the cell membrane. TPA can bind to membranes due to its lipophilic-hydrophilic nature.¹⁰⁴ Examples of TPA's effect upon the cytoplasmic membrane include: (i) reduction of electrophoretic mobility; (ii) blockage of stimulation by isoproterenol of epidermal cAMP; (iii) increased agglutinability of 3T3 cells by concanavalin A; (iv) release of 3T3 cells from contact inhibition; (v) synergism with phytohemagglutinin or concanavalin A in stimulating lymphocyte DNA synthesis; and (vi) various effects on

lysosome disruption, microtubule assembly and cytoplasmic membrane permeability.^{84, 95} Chicken embryo fibroblasts in the presence of TPA demonstrate a decrease in mean cellular volume, a change in cellular morphology, and the loss of a large external transformation sensitive glycoprotein (LETSP).¹⁰⁵ TPA also inhibits epidermal growth factor (EGF) from binding to cell membrane receptors on HeLa cells.¹⁰⁶ Although TPA and EGF have dissimilar chemical structures, both can induce similar phenomena in HeLa cells, namely: (i) increase the synthesis of plasminogen activator; (ii) stimulate growth; (iii) increase sugar transport; (iv) increase the synthesis of ODC and prostaglandins; and (v) act as a promoter in mouse skin carcinogenesis.¹⁰⁷ Thus, TPA may mimic the natural effect of EGF. Similarly, TPA may act like a chalone.⁸⁴

Lists of enzymatic alterations that are secondary to in vitro exposure of cells to TPA are found in recent reviews.^{84, 95} Some include: (i) stimulation of enzymes for histone phosphorylation; (ii) stimulation of epidermal cAMP, cGMP, and ATPase; and (iii) induction of ODC and plasminogen activator. Plasminogen activator is a serine protease that cleaves plasminogen to plasmin, but its significance to promotion remains unclear. ODC induction has been noted in mouse skin in vivo, and in mouse epidermal cells and hamster embryo cells in vitro.^{86, 95, 108, 109, 110} The decarboxylation of ornithine by ODC to form putrescine is the first and rate-limiting step in polyamine biosynthesis. Elevated putrescine and spermidine concentrations are present in transformed chick embryo cells, mouse leukemic cells and many neoplastic tissues. A positive correlation exists between a promoter's ability to induce ODC in vitro and its ability to promote tumorigenesis in vivo.¹¹¹ Since metabolism of TPA does not occur within cells, alterations observed cannot be due to degradation of TPA.¹¹²

Numerous theories for the action of promoting agents have been offered. Boutwell proposed that TPA acts as a gene activator and derepresses segments of the genome which otherwise would remain dormant.⁸³ Another theory suggests that TPA interferes with DNA repair thereby increasing the possibility of error. Suppression by TPA of host immune responsiveness and surveillance is another theory. Finally, phorbol esters may interact with specific receptors on the cell surface and, analogous to hormonal-receptor interactions, influence the metabolism of the affected cell.⁸³

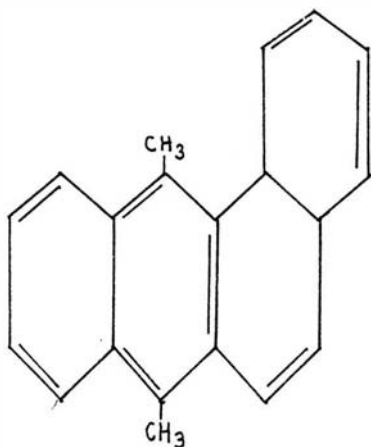
INITIATING AGENTS AND DIMETHYLBENZANTHRAcene

In 1775, Pott theorized that chimney soot is a cause of skin cancer in man.¹¹³ By 1922, Passey demonstrated the experimental production of skin cancer in laboratory animals by the application of extracts of soot.¹¹⁴ Finally, Kennaway isolated and characterized polycyclic hydrocarbons as the factors involved in carcinogenesis.¹¹⁵ The recent conclusion of epidemiologists that 60-90% of human cancers are environmental in etiology underscores the importance of chemical carcinogens, in general, and polycyclic hydrocarbons, specifically.¹¹⁶

Polycyclic hydrocarbons are relatively stable compounds which are easily formed by combustion of organic matter. Consequently, they are found in air, soil and water as products of smoke and combustion. Examples include cigarette smoke, automobile exhaust and barbequed food products. Various polycyclic hydrocarbons are carcinogenic in models utilizing mice, rats and hamsters. This thesis will limit its attention to one of the polycyclic hydrocarbons, namely DMBA.

DMBA has a colorless, crystalline structure which is soluble in organic solvents. DMBA has a molecular weight of 258, a melting point of

160°C, a boiling point of 400°C and the following chemical structure:¹¹⁷



DMBA has been studied extensively as a carcinogen. The location and route of application are important in determining the histologic type of malignancy that arises. Intravenous administration into rats can yield tumors of the pilosebaceous unit and in females can produce mammary carcinomas.^{118, 119} Subcutaneous injection yields fibrosarcomas.¹¹⁹ Intragastric deposition of DMBA followed by the repeated administration of TPA produces carcinomas of the forestomach.⁸⁵ DMBA can produce squamous cell carcinomas when applied to the skin or mucous membranes of laboratory animals.^{120, 121} Salley showed that DMBA was capable of inducing oral squamous cell carcinomas of the cheek pouch of Syrian hamsters.^{122, 123, 124} Wallenius produced palatal and buccal mucosal squamous carcinomas by oral gavage with DMBA.¹²⁵ Recently, squamous cell carcinomas of the tongue have been produced by the repeated application of DMBA.¹²⁶ Only one attempt at inducing carcinomas of the lip is in the literature and in this instance, a single painting of DMBA produced no tumors.¹²⁷

Investigations of initiating agents revealed that their chemical natures vary and bare no relationship to one another. In 1950, Boyland suggested that polycyclic hydrocarbons underwent biotransformation in tissues to become active carcinogens.¹²⁸ Gelboyn showed that the cellular microsomal fraction is the site of biotransformation into hydroxylated derivatives.¹²⁹ The microsomal mixed function oxidase systems are located on the endoplasmic reticulum.⁸³ These enzyme systems take inactive polycyclic hydrocarbons through an epoxide intermediate step to a dihydrodiol.¹³⁰ Many chemical carcinogens are active only after this conversion.

A key mixed function oxidase is aryl hydrocarbon hydroxylase (AHH). This enzyme is present in human lung, human lymphocytes, and skin from mice, rats and humans.^{131, 132} AHH may determine susceptibility to polycyclic hydrocarbon carcinogenesis. This enzyme's degree of inducibility in man and mouse is genetically determined. The higher the degree of inducibility, the greater the susceptibility to polycyclic hydrocarbon carcinogenesis.¹³³

Chemical carcinogens have strong electrophilic regions.¹¹⁶ These regions can react with nucleophilic areas, relatively abundant in DNA, RNA and proteins. Although the nature by which chemical carcinogens initiate transformation is unknown, it may be that binding to DNA leads to their intercalation into the helical structure. This insertion, if maintained, can produce "frame shift" mutations. Chemical carcinogens can bind to nucleic acids in cells in vitro causing transformation. These transformed cells produce carcinomas when injected into laboratory animals.¹³⁴ A positive correlation exists between the DNA binding capability of various polycyclic hydrocarbons and their oncogenicity in vivo.¹³⁵ Two observations support a genetic mechanism of action: (i) initiation in vivo is

irreversible and permanent; and (ii) initiation in vitro requires at least one round of cell division after the carcinogen treatment.⁸⁴ However, an epigenetic mechanism of action proposes that the active carcinogen binds with RNA or proteins to control their expression.¹¹⁶

ULTRAVIOLET LIGHT AND CARCINOGENESIS

The electromagnetic spectrum extends from cosmic rays to radio waves. Near the center of the spectrum lies the visible field of light. Within the visible spectrum, the shortest wave lengths perceivable are violet and the longest are red. The neighboring invisible regions are the UV and infrared regions. The UV spectrum consists of shorter wave lengths and higher energy photons than violet light. The UV spectrum has 200 to 400 nm wave lengths and is subdivided into three portions (UV-A, UV-B, and UV-C in order of decreasing wave lengths.)¹³⁶

Both procaryotic and eucaryotic cells show molecular damage following UV exposure. Cellular damage may be oxygen dependent or oxygen independent. Oxygen dependent damage is mediated by free-radicals and includes single strand DNA breaks or DNA-protein cross-links. Oxygen independent damage leads to the formation of pyrimidine dimers and thymine glycols. Dimers can be formed between thymine-thymine, cytosine-cytosine, or cytosine-thymine. All types of dimers have been isolated from in vivo UV-irradiated guinea pig and mouse skin.¹³⁷ Evidence exists that these dimers can give rise to tumors.¹³⁸

Following UV induced damage to the cell, particularly the DNA, repair must be completed before normal cellular replication and functions can resume. A number of different DNA repair mechanisms are known, namely: photoreactivation, excision repair and postreplication repair.¹³⁶ In the

absence of normal repair, cellular alterations and possibly death will follow. The human disease, xeroderma pigmentosum is an example. This disease is genetically transmitted. It shows an increased incidence in skin cancers following UV damage due to defective DNA repair mechanisms.¹³⁹

Evidence exists that implicates UV-irradiation as a cause in the development of some human cancers, particularly squamous cell carcinoma, basal cell carcinoma and melanoma. This evidence can be summarized as follows: (i) superficial skin cancers occur most frequently on surfaces chronically exposed to sunlight; (ii) protection from UV damage exists among dark pigmented races; (iii) persons in occupations, such as fishing and farming, with increased sun exposure time have a greater incidence of skin cancer; (iv) near the equator where sun exposure is greater, an increased prevalence of skin cancer exists; (v) genetic diseases (albinism, xeroderma pigmentosum) that show photosensitivity are associated with increased skin cancer development; and (vi) skin cancer can be produced in animals by repeated exposures of UV radiation.^{136, 140} Skin tumors have been induced in Swiss, Hairless, C3Hf, and T-cell deficient Nude mice.^{141, 142, 143, 144, 145}

The major conclusions of these in vivo studies can be summarized as follows: (i) wave lengths between 280 and 320 nm are the most effective in producing skin cancer; (ii) skin pigmentation and hair protect against UV induced tumorigenesis; (iii) UV induction is a cumulative process; (iv) the latent period is inversely related to the dose of UV radiation; and (v) the induced carcinomas are of the squamous cell type.¹⁴⁶

Several chemicals can potentiate UV induced transformation in vitro and in vivo. Bingham demonstrated a cocarcinogenic effect using n-paraffins in combination with UV light.¹⁴⁷ Epstein reported tumor formation on mouse

skin subjected to UV light and bi-weekly applications of Croton oil. UV exposure or Croton oil application alone produced no tumors after 18 months of observation.¹⁴⁸ Croton oil is the promoter while UV light acts as an initiator. Mondal demonstrated the in vitro transformation of mouse embryo fibroblasts using UV-irradiation while a phorbol ester was present in the media. UV-irradiation alone causes no transformed foci; however, transformation frequencies of $1.3 - 37.0 \times 10^{-3}$ result when TPA is added to the media 48 to 96 hours postirradiation.⁹⁶ Thus, TPA potentiates the effect of subthreshold doses of UV light in vitro.

Although sun exposure is recognized as a common reason given by patients for recurring herpes labialis, few studies combining UV light and HSV are documented. Most of these studies are involved with the UV treatment of HSV prior to in vitro transformation assays (discussed under Oncogenic Transformation by HSV). Harbour showed that UV exposure two days before HSV infection of mouse skin, has no effect on the viral titer in the tissue three days postinfection. However, UV exposure, two days after HSV infection, enhances the viral titer in the tissues.¹⁴⁹ Coppey noted that human skin cultures exposed to UV light before infection with HSV-1 show impaired viral production.¹⁵⁰ Finally, Hamper demonstrated that infection of nontransformed mouse cells with UV inactivated HSV results in release of type C virus.¹⁵¹

MATERIALS AND METHODS*

EXPERIMENTAL ANIMALS

Five to nine week old male BALB/c or Swiss ICR mice were obtained from Laboratory Supply Company (Indianapolis, Indiana), Simonson Laboratories (San Diego, California) or Flow Laboratories (Dublin, Virginia). Mice were housed at a maximum of 10/cage and supplied tap water and Purina Lab Rodent Chow ad libitum. Animals were acclimated for five to seven days prior to experimental manipulations. HSV infected and uninfected mice were housed in separate biohazard facilities; carcinogen treated animals were housed in a third biohazard location.

The LSH strain of golden Syrian hamsters (Charles River Laboratories) was used for hamster embryo fibroblast production, antisera production, and oncogenic determination of cells transformed in vitro. Hamsters were housed in biohazard facilities. New Zealand white rabbits were used for antisera production and were housed in the central animal facilities.

VIRUS

Two strains of HSV were used. The Patton strain of HSV-1 and the 333 strain of HSV-2 (333) originally isolated from human lesions, were obtained from Dr. B. K. Murray, Medical College of Virginia. Virus stocks were prepared by infecting monolayers of HEp-2 cells (MOI = 0.1) and harvesting when 80% of the cells showed HSV induced cytopathic effects. The cell associated virus was obtained by lysing the infected cells in distilled water (2 ml/T-75 flask for 30 minutes) and subsequent sonication for 30 seconds. Supernatant fluids were clarified by low speed centrifugation. Aliquots of virus pools were stored at -70°C. Virus infectivity

*Refer to Appendix I for the supplier of each reagent or chemical.

was assayed on green monkey kidney cells and expressed as plaque forming units per ml (PFU/ml).

CELLS AND MEDIA

Several cell lines were used, namely: Vero cells (African green monkey kidney), HEp-2 cells (Human Epidermoid), HEF cells (primary Hamster Embryo Fibroblast), and transformed hamster embryo cells (333-8-9, 12F#1, or 12F#2). Vero cells were used for PFU assays of virus pools. HEp-2 cells were used to grow virus pools and as controls for immunoperoxidase stains and in situ hybridizations. HEF cells were used for UV inactivated HSV transformation experiments and as controls for immunoperoxidase stains and cell sorter analysis. 333-8-9 cells were obtained from Baylor College of Medicine. They were initially transformed by UV inactivated 333 in Dr. F. Rapp's laboratory. These cells were used as controls in immunoperoxidase stains and in situ hybridizations. 333-8-9 cells were also grown under various concentrations of TPA for analysis by cell sorting techniques and two-dimensional gel electrophoresis. 12F#1 and 12F#2 cells were obtained from Dr. B. K. Murray. They were initially transformed by UV inactivated 333 in his laboratory. These cells were evaluated by immunoperoxidase stains.

Cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, gentamycin (50 ug/ml) and 0.15% NaHCO_3 (for cells in stoppered vessels) or 0.22% NaHCO_3 (for cells in a CO_2 atmosphere). Cells were maintained at 37°C and passed every third day. At the time of passage, cell monolayers received a 5 minute rinse with 0.02% ethylenediaminetetraacetic acid (EDTA) followed by a 2 minute treatment with 0.25% trypsin in EDTA. Cells were dispersed by pipetting and

seeded into two culture flasks.

Primary explants were obtained from lip tumors, including DMBA induced, HSV + TPA induced, UV + TPA induced and HSV + UV + TPA induced tumors. Tumor explants (1 mm³) were individually layered between one drop of chicken embryo extract and one drop of chicken plasma. During the out-growth phase, explants were incubated in Waymouth's medium containing 10% fetal bovine serum, gentamycin, penicillin and NaHCO₃. The cellular out-growths were evaluated by immunoperoxidase stains.

ULTRAVIOLET IRRADIATION

UV exposure was delivered to the ventral surface of mice and to inactivate virus pools. Two UV bulbs (General Electric G15T8, 15 watt) delivered 42 ergs/mm²/s of exposure when held 21 cm above the surface to be irradiated.

CHEMICALS AND SOLUTIONS

Scopolamine was used to inhibit salivary secretions when chemical carcinogens were applied to the lips. It was diluted with sterile saline to a concentration of 0.02 mg/0.1 ml. Mice were injected intraperitoneally with 0.1 ml (approximately 1 mg/kg of body weight). This produced xerostomia within 15 minutes. Water bottles were removed from the cages for the four hours following injection so that the mouth remained dry.

3-methylcholanthrene (3-MC) and dimethylbenzathracene (DMBA) were dissolved in dimethylsulfoxide (DMSO) and used to induce tumors on mouse lips. Concentrations of 3-MC used were 5 ng/application and 15 ng/application. Concentrations of DMBA used were 15 ng/application and 25 ng/application.

The tumor promoting agent, 12-O-Tetradecanoyl-Phorbol-13-Acetate (TPA)

was used in both in vivo and in vitro studies. In the in vivo studies, 10 mg of TPA was dissolved in 7 ml of DMSO. Ten ul applications (0.02 umole) were delivered to the right maxillary lip three times a week (Mon., Wed., Fri.). During in vitro experiments, TPA was used at concentrations of 1 ug/ml, 0.1 ug/ml, 0.01 ug/ml and 0.001 ug/ml of media.

Nembutal and Metophane were used as anesthetic agents. Nembutal, a short acting barbiturate, was diluted 1:10 in sterile saline and 0.1 ml injected intraperitoneally. Eight to ten minutes of anesthesia resulted. Metophane, an inhalation agent, was delivered to animals in a closed chamber and provided 3-5 minutes of anesthesia.

LABIAL INOCULATION OF MICE

Mice were anesthetized in a closed chamber in which the atmosphere was saturated with Metophane fumes. Anesthesia was obtained after 5 minutes in this environment. The right maxillary lip of the anesthetized mouse was lightly abraded by a slow speed dental drill with a steel brush attachment. Light pressure on the mucosa with the dental brush produced erythema, but active bleeding was not produced. The abraded lip was painted with (i) a cotton tipped stick moistened in a virus solution (approximately 100 ul), (ii) 10 ul of chemical carcinogen solution (3-MC or DMBA), or (iii) 10 ul of DMSO control solution according to the individual experimental protocol. Lips were examined weekly under 2X magnification for herpetic and/or neoplastic lesions.

TISSUE REMOVAL AND EVALUATION

At the termination of the in vivo experiments, mice were sacrificed by chloroform inhalation and cardiac exsanguination. Sera were saved for neutralizing antibody assays. External macroscopic evaluation was care-

fully performed to document the presence of lip or skin tumors. Autopsy was performed whenever lymphadenopathy or organomegaly were present and sporadically on normal appearing mice. Photographs of tumors at 1:1 magnification were obtained. Sterile forceps and scissors were used to remove lip tumors. Large lip tumors were trisected and portions were treated as follows:

- A. Placed in a tissue embedding bag, identified by mouse number, fixed in 10% neutral buffered formalin, and submitted for histologic evaluation with hematoxylin and eosin (H&E) staining (see Appendix II for details);
- B. Identified by mouse number, embedded in frozen section embedding compound (OTC), and evaluated by immunoperoxidase staining;
- C. Explanted onto a coverslip in a 60 mm petri dish, embedded in a plasma clot, and cultured under Waymouth's media.

LIGHT MICROSCOPIC EVALUATION

Specimens were obtained from biopsied lip tumors at the time the mice were sacrificed. The tissue was fixed in 10% neutral buffered formalin and blocked in paraffin for sectioning at 6 micron intervals. Sections were H&E stained and observed for the following:

- A. Evidence of viral infection (ballooning degeneration, multinucleated giant cells, eosinophilic nuclear inclusions);
- B. Evidence of anaplasia (hyperchromaticity, nuclear or cellular pleomorphism, inverse nuclear-cytoplasmic

ratio, abnormal mitotic figures, loss of polarity or anisocytosis);

- C. Tumor type (papilloma, epithelial dysplasia, squamous cell carcinoma).

Other specimens for immunoperoxidase staining were also obtained from biopsied lip tumors. The tissue was embedded in OTC and sectioned on a cryostat at 6 micron intervals. Sections were fixed in acetone for 10 minutes at room temperature and air dried. Sections were immunoperoxidase stained and evaluated for the presence of HSV antigens.

Light microscopy was performed on a Nikon microscope. Photomicrographs were taken with a 35 mm camera utilizing Ektachrome film (ASA 64).

PREPARATION OF HYPERIMMUNE ANTISERA

Antisera for the immunoperoxidase stain were produced in mice, hamsters and rabbits. For hamster α -333 antisera, each of five adult hamsters received 1 ml of an inoculum containing 0.5 ml of complete Freund's adjuvant and 0.5 ml of UV inactivated 333. Fourteen and 28 days later, the hamsters received 1 ml injections of infectious 333. All injections were bilateral and given subcutaneously near the popliteal lymph nodes. Hamster α -tumor antisera were obtained from those hamsters who developed tumors following injection of the transformed hamster cell line 12F#2. Antisera raised in rabbits included α -333, α -HSV-2 strain MS, α -VP 143 of 333, and α -4 hour antigen of 333. Rabbit α -333 antisera were prepared in a manner similar to that used for the hamster α -333. The 333 early four hour antigen was prepared by infecting HEp-2 cells with 333 and harvesting the soluble protein four hours postinoculation. Two ml of this preparation was injected into each of two rabbits on days

0, 14, 28 and 42. The injections were given subcutaneously near the popliteal, axillary and submandibular lymph nodes. Rabbit α -HSV-2 strain MS antiserum was obtained from the Center for Disease Control. Rabbit α -VP 143 antiserum was obtained from Dr. Guy Cabral. Mouse α -tumor antisera were obtained from those mice which developed tumors following HSV + UV + TPA treatment, and whose neutralizing antibody titer was equal to or greater than 1:40.

Blood was drawn from these animals by cardiac puncture 14 days after the last immunization. Serum was separated by centrifugation and stored at -20°C . Optimal dilutions of antisera (1:2 to 1:10) were determined by microcytology double immunodiffusion. Antisera were absorbed by incubation with 10^8 HEp-2 cells/ml of antiserum and/or 10^3 HEF cells/ml for one hour at 37°C . Following overnight storage in the refrigerator, the absorption was repeated. The supernatant fluid collected after centrifugation was passed through a 0.45 millipore filter. This was the primary antiserum for the immunoperoxidase stain.

IMMUNOPEROXIDASE STAINING PROCEDURE

Demonstration of viral antigens in cultured cells or clinical specimens was accomplished using the immunoperoxidase stain as described by DeLellis.¹⁵² Cover slips, upon which cells were grown, were fixed by: (i) three saline rinses of 5 min. each, (ii) air drying for 30 min., and (iii) fixation in acetone for 5 min. at room temperature. Biopsied tissue specimens were either embedded in OTC (for later sectioning and acetone fixation) or explanted for cellular outgrowth in vitro on glass coverslips (for later acetone fixation).

The staining procedure began with a rehydration of the fixed speci-

men for 10 min. in 0.1 M PBS, pH 7.6. Primary antiserum from hamsters, rabbits or mice was flooded onto the tissue and incubated for 45 min. at 37°C in a humidified chamber. Endogenous peroxidase was blocked by a 30 min. rinse in methanol, a 5 min. rinse in 3% H₂O₂, and a 30 min. rinse in a 10% egg albumin. All blocking procedures were performed at room temperature. The specimens were washed in PBS for 10 min. A second layer of antiserum, consisting of a different species' antiserum against the primary antibody, (e.g. goat α -rabbit antibody) was diluted 1:4 in PBS and flooded onto the glass slide or cover slip. A 30 min. period at 37°C in a humidified environment followed. If the technique used was two layered, the antibody for the second layer was purchased conjugated to peroxidase (Cappel Incorporated); if the three layer technique was used, a third antiserum was added after the second incubation. This third layer (same species as the primary) consisted of anti-horseradish peroxidase serum bound to its antigen (Cappel Incorporated). This antiserum was diluted 1:100 in PBS and applied for a 30 min. incubation period at 37°C. The specimen was washed in PBS for 30 sec. Following the peroxidase containing layer, the material was incubated in the dark for 10 min. in Tris buffered saline (pH 7.6) containing 0.05% diaminobenzidine and 0.01% hydrogen peroxide. The tissues were rinsed in distilled water, dehydrated through a series of ethanols and xylene, and mounted in Permount for observation with light microscopy.

Necessary controls that were run in parallel included known-positive HSV infected HEp-2, HEF and Vero cells as well as uninfected HEp-2 and HEF cells. Additional controls consisted of replacing individual antiserum layers with PBS, using non-HSV-specific serum as the primary antiserum, and absorbing the primary antiserum with high titer HSV.

MICRO-TITER ASSAY OF SERUM NEUTRALIZING ANTIBODY

Sera were obtained by cardiac puncture or retroorbital plexus bleeding. Following retroorbital bleeding, 100 μ l of blood was added to 0.45 ml of GLB (a 1:10 dilution of serum assuming a hematocrit of 50%). Following clot formation, the sera were separated by centrifugation at 1000 x G for 10 min. Sera were drawn off and stored at -30°C . Sera were heat inactivated at 56°C for 30 min. and two-fold serial dilutions (1:10 to 1:320) were made in a 96 well plate (Costar, Cambridge, Mass.). A suspension of 333 containing 100 TCID₅₀ was added to each well and incubated at 36°C for one hour. Then, 4.0×10^5 Vero cells were added to each well and incubated for 2-3 days. Necessary controls included a high titer rabbit α -333 anti-serum, a normal mouse serum and cells without serum. When 50% of the normal mouse serum control wells developed cytopathic effects (CPE), the remaining wells were graded for the presence or absence of CPE. The neutralizing antibody titer of the experimental serum was expressed as the reciprocal of the highest dilution of serum which prevented viral CPE.

IN VITRO TRANSFORMATION BY UV INACTIVATED HSV

A 333 virus inoculum was exposed to $42 \text{ ergs/mm}^2/\text{s}$ of UV light for various periods of time, ranging from 0 to 10 min. Aliquoted HEF cell pellets were infected with the UV inactivated 333 at an MOI of 3 and incubated at 37°C on a rotary shaker for 1 hour. Control cells were mixed with GLB in place of virus. The cells were then suspended in EMEM and seeded 1.1×10^6 cells/T-75 flask. The media were changed weekly for two weeks. On day 21 postinoculation, the experiment was terminated by (i) picking selected foci for subculturing, (ii) fixation in acetone and analysis by immunoperoxidase staining, or (iii) fixation in 10% formalin, stain-

ing with 0.2% Toluidine blue and counting the number of transformed foci per flask. Cells from picked foci were analyzed by immunoperoxidase staining, cell sorting techniques and inoculation into newborn hamsters. In later experiments, TPA (0.1 ug/ml) was added to the media at various times, ranging from 0 to 96 hours postinoculation.

ONCOGENICITY OF TRANSFORMED HEF

For oncogenicity testing, 5×10^5 cells suspended in 50 μ l of HBSS were injected subcutaneously in the interscapular area of newborn hamsters. These animals were examined weekly for tumor growths. The tumors that developed were surgically removed and portions explanted to cell culture or fixed in 10% formalin. The fixed tissue was examined by light microscopy utilizing H&E staining. The cell line grown from explants was analyzed by immunoperoxidase staining.

PURIFICATION AND RADIOLABELING OF CELL AND HSV DNA

In preliminary studies, radiolabeled HEp-2 DNA was prepared by in vitro labeling of the cells with ^3H -Thymidine (10 $\mu\text{C}/\text{ml}$) for 24 hours. The labeled HEp-2 cells were washed, trypsinized, collected in medium, and pelleted by centrifugation (1500 x G for 10 min.). The cells were lysed with 1% SDS and treated with Proteinase K (1 mg/ml). The nucleic acids were extracted three times with an equal volume of chloroform:isoamylalcohol (24:1) and precipitated in 90% ethanol. Following treatment with RNAase, the DNA was extracted again with chloroform:isoamylalcohol, precipitated in ice cold 90% ethanol and resuspended in 0.5 ml 1X SSC. This HEp-2 DNA had an activity of 7.8×10^5 cpm/ml. In later studies, radiolabeled HSV DNA was prepared by nick translation of purified 333 DNA. The 333 DNA was obtained by the "Hirt" extraction method described by

Rapp.¹⁵³ The supernate fraction was banded twice in an ethidium bromide-cesium chloride gradient (density = 1.566 g/cm³). The viral DNA band was collected and exhaustively dialysed against TNE buffer for 48 hours. DNA was precipitated in 2 volumes of 90% ethanol and resuspended in 1X SSC. For nick translation, 100 ul of 333 DNA was mixed with 25 uc of each of the four H³-dNTP in 100 ul of 50 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 10 mM 2-mercaptoethanol (ME), 50 ug/ml of bovine serum albumin. The reaction, as described by Maniatis,¹⁵⁴ was started by the addition of 10⁻⁸ ug of DNAase I and 12 units of DNA polymerase 1, was incubated at 15⁰C for 1 hour, and was terminated by chloroform:isoamylalcohol extraction. The radio-labeled 333 DNA was precipitated with ice cold ethanol and resuspended in 0.5 ml SSC. Its activity was 5.8 x 10⁶ cpm/ml.

IN SITU HYBRIDIZATION

The methods used to localize viral transcripts by in situ hybridization were similar to those described by Brahic.¹⁵⁵ Uninfected HEp-2, 333 infected HEp-2, and 333-8-9 cells were grown on acid cleaned microscope slides and prepared for hybridization as controls. The slides were rehydrated in PBS and fixed for 20 min. in ethanol/acetic acid (3:1). They were treated with 0.2 M HCl for 20 min., 300 mM NaCl/30 mM Na-citrate for 30 min. at 70⁰ C and 10 mM Tris HCl/2 mM CaCl₂/1 ug Proteinase K/ml for 15 min. at 37⁰C. These treatments were interspersed with distilled water rinses and terminated by dehydration in a series of 70% and 95% ethanol rinses.

The hybridization medium contained the radiolabeled DNA in a solution of 40% formamide, 10 mM Tris HCl, 1 mM EDTA, 600 mM NaCl, 0.02% (wt/vol) Ficoll, 0.02% polyvinylpyrrolidone and 1 mg of bovine serum

albumin/ml. This hybridization mixture was heated to 100°C for 20 sec. and immediately cooled to 0°C. Fifty ul were used to cover each cytological preparation which was then covered with a glass cover slip. The hybridization chamber consisted of a 150 mm petri dish whose bottom was covered with 2X SSC. The hybridizing slides were on a stage above this solution and maintained at 28°C for 60 hours. Following hybridization, the slides were washed twice in chloroform and twice in 50% formamide/10 mM Tris HCl/1 mM EDTA/ and 600 mM NaCl for 5 min. each. Extensive washing for the next 24 hours utilizing the same buffer preceded two 5 min. rinses in 300 mM NaCl/30 mM Na-citrate. This was followed by dehydration in 70% and 90% ethanol. The slides were then dipped in a 45°C solution of 1 part Kodak NTB-3 nuclear track emulsion and 3 parts of 400 mM ammonium acetate. The slides were allowed to dry in the upright position and were transferred to a desiccator for storage in the dark at 4°C. After the appropriate exposure time (6-8 days), the slides were developed in Dektol developer for 2 min., rinsed in distilled water and fixed in 30% sodium thiosulfate, After washing in water, the slides were counterstained with Toluidine blue (0.2% in 60% ethanol) for 1 hour. The slides were dehydrated in 95% ethanol and a coverslip was applied with Permount. The slides were then examined and photographed using a Nikon light microscope.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

Radiolabeled soluble cell proteins were analyzed by two dimensional (2-D) gel electrophoresis. 333-8-9 cells under various concentrations of TPA (0.0 ug/ml, 1.0 ug/ml, 0.1 ug/ml, 0.01 ug/ml, 0.001 ug/ml) and HEF cells were cultured in 60 mm petri dishes with S³⁵ methionine (20 uc/ml).

Following incubation for 48 hours, the cells were rinsed with distilled water and treated with 100 μ l of lysis buffer (9.5 M urea, 5% ME, 2% NP-40, 2% ampholines pH 3-10). The protein concentration was approximately 100,000 cpm/ μ g of protein as determined by the recently described Bernlohr method.¹⁵⁶ The radiolabeled proteins in lysis buffer were collected and stored at -70°C .

The 2-D gel electrophoretic procedure used was similar to that described by O'Farrell.¹⁵⁷ For the first dimension, isoelectric focusing (IEF) gels were prepared in glass tubes with an overall length of 13 cm and an internal diameter of 2.5 mm. The gel mixture consisted of 5.5 gm urea, 1.33 ml of acrylamide stock (29.2% acrylamide, 0.8% bis in 100 ml of deionized water), 2.0 ml of NP-40, 1.95 ml of water and 0.5 ml of ampholines (0.4 ml of pH 5-7, 0.1 ml of pH 3-10 for equilibrium conditions) (0.5 ml of pH 3-10 for nonequilibrium conditions). The mixture was swirled in a 37° water bath and degased under negative pressure. TEMED and ammonium persulfate (5 μ l and 10 μ l, respectively) were added. The gel mixture was poured into each glass tube and polymerized for one hour. The glass tubes were then loaded into an electrophoretic chamber with the lower chamber (positive terminal) filled with 10 mM H_3PO_4 and the upper chamber (negative terminal) with 20 mM NaOH. The gels were prerun for 15 min. at 100 V, 30 min. at 300 V, and 30 min. at 400 V. For nonequilibrium conditions, the terminals and buffers were reversed and the gels were not prerun. Three sets of 2-D gels were performed on each protein sample, namely: (i) 25 μ l of sample per gel under equilibrium conditions, (ii) 145,960 cpm of sample per gel under equilibrium conditions, and (iii) 145,960 cpm of sample per gel under nonequilibrium conditions. IEF was performed for 18 hours at 300 V for equilibrium conditions and 3 hours at 400 V for nonequilibrium conditions. Following this, each glass tube

was removed and the gel extruded by air pressure. Each gel was placed in a 15 ml tube and covered with 5 ml of SDS sample buffer (2.3% SDS, 5% ME, 10% glycerol and 62.5 mM Tris-HCl, pH 6.8). IEF gels were stored at -70°C until the 2-D gels were run.

The 2-D protein separation was performed by electrophoresis in an exponential 7% to 20% SDS-polyacrylamide gradient (SDS-PAGE). The gradient was made with a gradient maker containing 5 ml of dense gel solution in the constant side and 16 ml of light gel solution in the inconstant side. The dense gel solution (20%) contained 2.0 ml lower gel buffer, 5.3 ml acrylamide stock, 0.7 ml 75% glycerol, 15 μl ammonium persulfate and 4 μl TEMED. The light gel solution (7%) contained 4.0 ml lower gel buffer, 3.73 ml acrylamide stock, 8.27 ml deionized water, 25 μl of ammonium persulfate and 8 μl TEMED. Fluids were pumped from the gradient maker by an automatic pumping device at a flow rate of 3 ml/min. into a slab gel apparatus (0.75 mm x 15 cm x 14 cm). After overnight polymerization, a stacking gel was layered on top. The stacking gel solution contained 1.25 ml stacking gel buffer (0.4% SDS, 0.5 M Tris-HCl, pH 6.8), 0.75 ml acrylamide stock, 3 ml water, 15 μl ammonium persulfate and 5 μl TEMED. The stacking gel polymerized for one hour. Meanwhile, the cylindrical IEF gel was equilibrated in fresh SDS sample buffer for one hour. Melted 1% agarose was added to the top of the stacking gel and the IEF gel placed horizontally in it. The apparatus was then connected to the power source. Each gel was run at 20 mA constant current for approximately $2\frac{1}{2}$ hours at 20°C until the tracking dye reached the bottom of the slab gel. The gels were separated from the apparatus, fixed, stained, dried and autoradiographed on Kodak XR-1 film. The autoradiographs from the individual samples were then compared with one another for quantitative and qualitative differences.

CELL SORTER ANALYSIS

For cell sorter analysis, 10^6 cells/sample were harvested and washed in Ca Mg free PBS. The cells were pelleted by centrifugation and resuspended in 3 ml of PBS. While vortexing, 7 ml of 100% ethanol was added. This 10 ml cell suspension was used for cell sorter analysis.

The analysis involved quantitative fluorescent staining of the cellular DNA and rapid analysis of the fluorescence emission signal obtained as each cell traversed a laser beam. This technique has been presented in detail by Crissman.¹⁵⁸ The cell suspension was centrifuged, washed in PBS and resuspended in 4.5 ml PBS containing 10 ug/ml of RNAase. Incubation at 37°C progressed for 30 min. The cells were then centrifuged, washed in 5 ml PBS and incubated for 30 min. in 5 ml of 6.9×10^{-5} M propidium iodide/ 3.8×10^{-2} M Na-citrate. The cells were then centrifuged, washed, resuspended in PBS, filtered through a 74 um filter and introduced into the cell sorter. The flow chamber allowed cells to proceed at a rate of 1000 cells/s past electrical and optical sensors that measured the cell volume and fluorescence. Passage through a 75 um diameter orifice produced an electric signal which was proportional to the cell volume. Passage through a laser beam excited the propidium iodide stained DNA and the emitted fluorescence was measured, amplified and displayed as histograms utilizing a multichannel pulse height analyzer. The histograms were bimodal: the peak on the left represented cells in G_1 phase, the peak with twice the DNA content of G_1 cells represented cells in $G_2 + M$ phase, and distributed between the two peaks were cells in S phase with varying degrees of DNA replication completed. The number of cells in $G_2 + M$ phase for each of the submitted samples were compared with one another for significant differences.

STATISTICAL EVALUATION OF DATA

Chi-square test with Yate's correction factor and Student's t test were employed where applicable to analyze the data. Serum neutralizing antibody titers from control, tumor bearing and non-tumor bearing mice were analyzed using the Student's t test.

RESULTS

INDUCTION OF LABIAL HERPETIC LESIONS

Since infectious HSV has been identified in some human tumors and indirect evidence has inferred an etiologic role for HSV in the development of squamous cell carcinomas, the initial phases of this project were designed to develop a suitable animal model for inducing herpetic lesions. The maxillary lip of a mouse was chosen as the site for infection due to its analogy to human herpes labialis, its access to topical application of UV light or chemicals, and its prior use by Kitces, et al., in this laboratory.²⁷ Abraded right maxillary lips of male BALB/c mice were infected with the 333 strain of HSV-2 (333). Vesicles developed at the inoculation site 2-3 days later. These vesicles soon ruptured and formed ulcers (Fig. 1a). By the tenth to sixteenth day, these lesions had healed and regained normal macroscopic and microscopic appearance.

Histopathologic features of herpetic lip lesions include fluid filled vesicles surrounded by multinucleated giant cells and an intense inflammatory infiltrate (Fig. 1b). Microscopic examination with higher magnification revealed ballooning degeneration within many of the Tzanck cells. The presence of HSV antigens within the herpetic lip lesions was shown by immunoperoxidase staining of cryostat sections using rabbit α -333 antiserum as the primary serum (Fig. 1c). The majority of the HSV antigens were located within the surface epithelial cells; however, positive staining was often evident along the hair shaft epithelium and within the sebaceous glands. Also, when various groups of mice between 5 and 8 wks of age (16 to 100 mice/group) were inoculated with HSV, the incidence of herpetic lesions varied with the titer of the inoculum, ranging from 60% to 100% as titers increased from 1×10^6 PFU/ml to 1.3×10^7 PFU/ml (Table 1). This

titer-related finding is not due to an age related factor since it has previously been shown in this laboratory that 5 wk. old mice develop a higher incidence of lesions than 8 wk. old mice when inoculated with identical virus preparations.

FIGURE 1

Macroscopic and microscopic pathology of HSV-2 induced lip lesions. The right maxillary lip of a 5 week old male BALB/c mouse was inoculated with HSV-2 as described in Table 1.

- (a) Within 5 days, a vesicular ulcerative lesion appeared on the lip.
- (b) Microscopic examination of hematoxylin and eosin stained paraffin embedded sections revealed an intraepithelial vesicle surrounded by inflammatory cells (40X magnification).
- (c) Microscopic examination of immunoperoxidase stained cryostat sections showed the presence of HSV antigens within the epithelium and along the hair shafts (40X magnification).

FIGURE 1

a.



b.

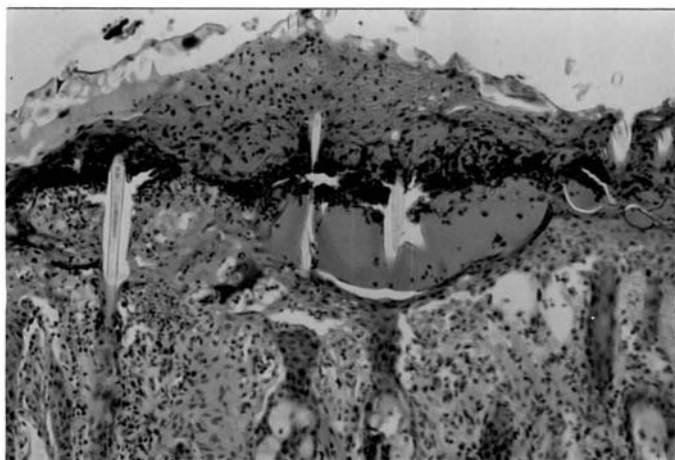


FIGURE 1 (continued)

c.

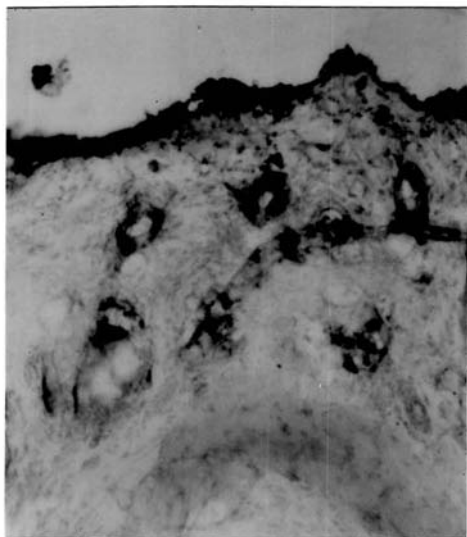


TABLE 1 EFFECT OF HSV TITER ON THE INCIDENCE OF LABIAL HERPETIC LESIONS^a

Mouse Age ^b	HSV Titer (PFU/ml) ^c	Mice with Herpetic Lip Lesions/ Total Mice ^d
5 wk	1.0×10^6	12/20 (60)
8 wk	3.9×10^6	99/100 (99)
7 wk	5.0×10^6	16/16 (100)
7 wk	5.0×10^6	74/75 (99)
7 wk	1.3×10^7	36/37 (97)

- a. Male BALB/c mice were infected on the abraded right maxillary lip with approximately 100 ul of a HSV-2 strain 333 solution.
- b. Age of the mice when infected with HSV.
- c. Titer of virus pool used to inoculate the mouse.
- d. Each animal was evaluated 5 days postinoculation with a 2x scope for the presence of herpetic lip lesions. The number in parentheses represents the percentage of mice with herpetic lip lesions.

Since recurrent herpes labialis has been associated with the onset of squamous cell carcinoma of the lip in humans, attempts were made to reinfect mouse lips with HSV. Repeated reinfection was designed to mimic recurrent HSV infection in humans since no recurrent HSV model in mice exists. Repeated applications of infectious virus to the same site on the mouse's lip failed to induce tumors (Table 2). In fact, when mice were reinfected with HSV-1 or HSV-2, the incidence of herpetic lip lesions decreased with subsequent inoculations. Also, the severity of lesions and the duration before healing decreased. The apparent immunologic protection following primary infection was further shown by the failure to develop herpetic lip lesions on the left maxillary lip after three infections on the right maxillary lip (Table 2).

CHEMICAL CARCINOGENS AND ULTRAVIOLET IRRADIATION AS TUMOR INDUCERS

Single or multiple labial infections of mice with HSV failed to induce lip tumors in our model. However, the possibility exists that HSV may act as a cocarcinogen in concert with known chemical or environmental agents. In the next series of experiments, models for the induction of labial tumors by chemical carcinogens or ultraviolet irradiation were investigated. These models were designed for eventual use with concomitant HSV infection in order to determine if HSV plus carcinogen could (i) act synergistically to increase tumor yield, or (ii) effect the latent period of tumor emergence. Thus, dosages and exposure protocols for carcinogen application were varied in order to determine which protocol would allow HSV superimposed infection to express its effect.

In one series of experiments, 3-Methylcholanthrene (3-MC), a polycyclic hydrocarbon, was applied to abraded mouse lips in two different

TABLE 2 EFFECT OF REPEATED INOCULATIONS WITH HSV ON THE INCIDENCE OF LABIAL HERPETIC LESIONS

Virus Inoculum	(PFU/ml)	% Mice With Herpetic Lip Lesions ^a				% Mice With Lip Tumors ^e
		1st Inoculation	2nd Inoculation	3rd Inoculation	4th Inoculation	
HSV-1 ^b	3 x 10 ⁷	100	0	20	0	0
HSV-1 ^c	3 x 10 ⁷	100	10	10	0	0
HSV-2 ^d	1 x 10 ⁶	60	47	--	-	0

- a. Each animal was evaluated 5 days postinoculation with a 2x scope for the presence of herpetic lip lesions on the most recent inoculation site.
- b. Ten male BALB/c mice (6 wks. of age) were infected on the abraded right maxillary lip with approximately 100 ul of a virus solution on each of the first three occasions. The fourth inoculation was delivered to the abraded left maxillary lip. The consecutive treatments were 4, 6 and 4 wks. apart respectively.
- c. Same protocol as "b" above. The fourth inoculation was delivered to the abraded right maxillary lip.
- d. Twenty male BALB/c mice (5 wks. of age) were infected on the abraded right maxillary lip with approximately 100 ul of a virus solution on both occasions. The second inoculation followed the first by 23 wks.
- e. Each animal was evaluated with a 2x scope for the presence of tumors 34-40 wks. after the first inoculation.

concentrations for one to six applications (Table 3). Twenty-five weeks after 3-MC application, tumors had failed to appear. Apparently, these concentrations and/or application frequencies were not enough to act as initiators and promoters. Consequently, a more potent carcinogen, dimethylbenzanthracene (DMBA), was used in the next series of experiments. DMBA, also a polycyclic hydrocarbon, was applied to abraded mouse lips in two different concentrations for one to thirty applications (Table 4). The lower concentration of DMBA (15 ng/application) failed to induce tumors when applied from 1 to 10 times. However, at a higher concentration and with more frequent applications, tumors began to emerge 5 weeks after cessation of DMBA application. Subsequently, these tumors grew to grotesque sizes (Fig. 2). The incidence of tumors increased as the number of applications increased. From these data, it was determined that DMBA application for 3, 4 or 5 weeks would result in a tumor yield (11.75, 18.8, 40.0% respectively) that would permit the effect of concomitant HSV infection to be effectively measured. Thus, these regimens were used in later studies combining HSV infection prior to and during DMBA application.

Ultraviolet (UV) irradiation, often associated with clinical reactivation of herpes labialis and the development of lip cancer in humans, was investigated in another series of experiments. A group of 10 BALB/c male mice (6 wks. of age) were anesthetized, abraded on the right maxillary lip, and exposed on their ventral surface to six minutes of UV-irradiation at $42 \text{ ergs/mm}^2/\text{s}$. Six weeks later the procedure was once again repeated. Another group of 19 BALB/c male mice (5 wks. of age) were then exposed to the UV-irradiation daily for 7 days. Daily exposure (6 minutes at $42 \text{ ergs/mm}^2/\text{s}$) for 7 days was once again repeated 23 weeks later. In both experiments, tumors failed to develop and the lips retained their normal appearance.

TABLE 3 RESPONSE OF MICE TO SUBCARCINOGENIC DOSAGES OF 3-METHYLCHOLANTHRENE^a

CONCENTRATION OF 3-MC/ APPLICATION	ANIMALS IN GROUP	NUMBER OF APPLICATIONS ^b	LATENT PERIOD ^c	MICE WITH TUMORS/ SURVIVING MICE	% MICE WITH TUMORS ^d
5 ng	7	1	-	0/7	0
15 ng	10	1	-	0/10	0
15 ng	10	2	-	0/9	0
15 ng	10	3	-	0/10	0
15 ng	10	6	-	0/8	0
DMSO sham	10	6	-	0/9	0

- a. Five wk. old male ICR mice were used in all groups except the first (5 ng), where BALB/c mice were used. Each anesthetized mouse received 10 ul of 3-Methylcholanthrene (3-MC) in DMSO on the lightly abraded right maxillary lip.
- b. Frequency of application as follows: 2 (days 0, 12); 3 (days 0, 7, 14); 6 (days 0, 2, 4, 7, 9, 11).
- c. Average time span until emergence of clinically evident tumors.
- d. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors 25 wks. after 3-MC application.

TABLE 4 INDUCTION OF LIP TUMORS IN MICE FOLLOWING DIMETHYLBENZANTHRACENE APPLICATION^a

CONCENTRATION OF DMBA/ APPLICATION	ANIMALS IN GROUP	NUMBER OF APPLICATIONS ^b	LATENT PERIOD ^c	MICE WITH TUMORS SURVIVING MICE ^d	% MICE WITH TUMORS ^e
15 ng	10	1	-	0/10	0
15 ng	10	2	-	0/10	0
15 ng	10	3	-	0/10	0
15 ng	10	6	-	0/10	0
15 ng	10	10	-	0/8	0
25 ng	18	15	7 wks	2/16 (1)(1)	12.5
25 ng	24	15	6 wks	2/18 (0)(2)	11.0
25 ng	18	20	6 wks	3/16 (1)(2)	18.8
25 ng	18	25	5 wks	4/10 (1)(3)	40.0
25 ng	100	25	6 wks	32/80 (10)(22)	40.0
25 ng	18	30	7 wks	7/14 (2)(5)	50.0
DMSO SHAM	18	30	-	0/16	0
SALINE CONTROL	10	12	-	0/10	0

- a. Five wk. old BALB/c mice were used in all groups except the 15 ng/dose studies where ICR mice were used. Each anesthetized animal received 10 ul of dimethylbenzanthracene (DMBA) in DMSO on the lightly abraded right maxillary lip.
- b. Frequency of application as follows: 2 (days 0, 12); 3 (days 0, 7, 14); 6 (days 0, 2, 4, 7, 9, 11); 10 (Mon.-Fri. for 2 wks.); 12 (Mon., Wed., Fri. for 4 wks.); 15 (Mon.-Fri. for 3 wks.); 20 (Mon.-Fri. for 4 wks.); 25 (Mon.-Fri.. for 5 wks.); 30 (Mon.-Fri. for 6 wks.).
- c. Average time span until emergence of clinically evident tumors.
- d. Histologic determination of tumor type: number within first parentheses represents # of papillomas; number within second parentheses represents # of squamous cell carcinomas.
- e. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors 20 wks. after DMBA application.

FIGURE 2

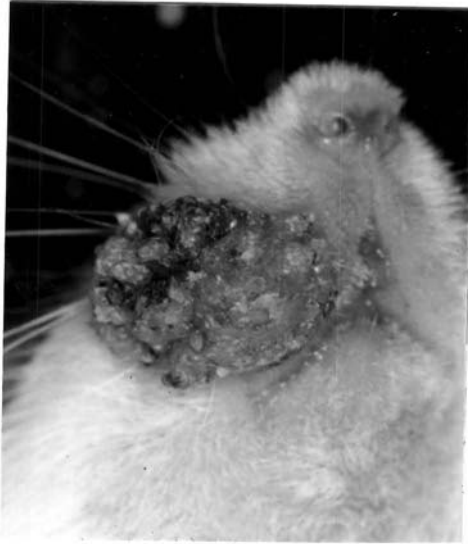
Macroscopic and microscopic pathology of DMBA induced lip lesions. The right maxillary lips of 5 week old male BALB/c mice were repeatedly inoculated (25 times) with DMBA as described in Table 4.

- (a) Within 7 weeks an exophytic tumor appeared on the lip.
- (b) By the termination of the experiment, 20 weeks after DMBA application, many of the tumors had grown to appreciable size.
- (c) Microscopic examination of hematoxylin and eosin stained paraffin embedded sections revealed malignant invasive squamous epithelial cells (20X magnification).

Diagnosis: Squamous Cell Carcinoma

FIGURE 2

a.

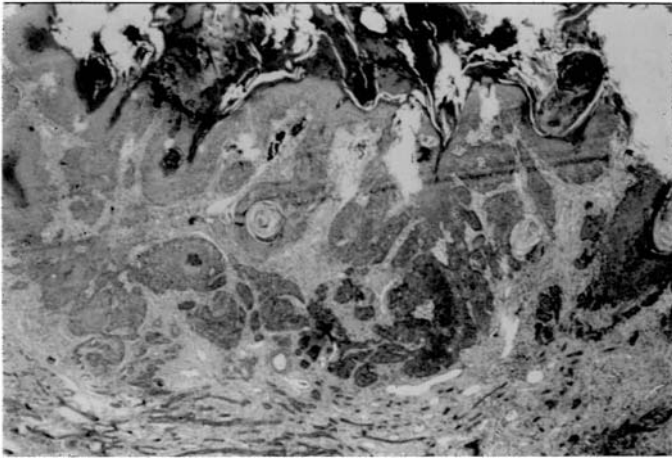


b.



FIGURE 2 (continued)

c.



CONCOMITANT HSV INFECTION AND DMBA APPLICATION

Due to the lack of tumor emergence when HSV was used alone and in order to investigate possible synergism, the effect of HSV infection accompanying DMBA application was investigated in several groups of mice. In two different experiments, HSV was inoculated prior to DMBA application. When HSV infection was initiated two days before the application of a 2.5% DMBA solution five days a week for four weeks, 6% of the animals developed tumors by the termination of the experiment at 22 weeks (Table 5). Although from different studies, a comparison of these data to that of a four week application of DMBA alone (Table 4) (6% and 18.8%, respectively) shows that HSV infection did not cause an increase in tumor incidence. In another experiment, when a 2.5% DMBA solution was applied to the lips of mice daily for a period of five weeks, the incidence of tumors at 20 weeks was no different ($p = .06$) between that group which was infected with HSV on the lip two days prior to the initiation of DMBA applications and the other group that received no virus inoculation (Table 6). The percentages of animals with papillomas as compared to those with squamous cell carcinomas varied a little but not significantly ($p = .3$).

Since HSV infection prior to DMBA application failed to increase the tumor yield, HSV infection during the actual DMBA application period was investigated. A decrease in tumor incidence was seen when HSV infection was superimposed on day 5, 12, and 19 after the initiation of the 21 day DMBA application period (Table 7), however it was not statistically significant ($p = .14$). Eleven percent of the mice receiving DMBA developed squamous cell carcinomas; whereas, those mice with superimposed HSV infections failed to show tumors. It was impossible to evaluate for the presence typical vesicular-ulcerative herpetic lesions after inoculation

TABLE 5 INDUCTION OF LIP TUMORS IN MICE FOLLOWING HSV INFECTION PRIOR TO THE APPLICATION OF DMBA FOR 4 WEEKS^a

GROUP	% MICE WITH HERPETIC LESIONS ^b	MICE WITH TUMORS/ MICE SURVIVING ^c	% MICE WITH TUMORS ^d
DMSO	100%	0/16 (0)(0)	0%
DMBA	99%	4/65 (1)(3)	6%

- a. Both groups received 100 ul of a 5×10^6 PFU/ml 333 solution to the right maxillary lip. Two days later, 10 ul of 2.5% DMBA or DMSO was applied to the right maxillary lip of 7 wk. old BALB/c male mice (16 in control group; 75 in experimental group) 5 days wk. for 4 wks.
- b. Each animal was evaluated 5 days postinoculation with a 2X scope.
- c. Histologic determination of tumor type: number within first parentheses represents # of papillomas; number within second parentheses represents # of squamous cell carcinomas.
- d. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors 22 wks. after DMBA application.

TABLE 6 INDUCTION OF LIP TUMORS IN MICE FOLLOWING HSV INFECTION PRIOR TO THE APPLICATION OF DMBA FOR 5 WEEKS^a

HSV INOCULATION GROUP	% MICE WITH HERPETIC LIP LESIONS ^b	MICE WITH TUMORS/ MICE SURVIVING ^c	% MICE WITH TUMORS ^d
Uninfected	0%	32/80 (10)(22)	40%
Infected	99%	29/71 (8)(21)	41%

- a. The infected group received 100 μ l of 3.9×10^6 PFU/ml 333 solution to the right maxillary lip. Two days later, both groups received 10 μ l of 2.5% DMBA application to the right maxillary lip of 8 wk. old BALB/c male mice (100 mice per group).
- b. Each animal was evaluated 5 days postinoculation with a 2X scope.
- c. Histologic determination of tumor type: number within first parentheses represents # of papillomas; number within the second parentheses represents # of squamous cell carcinomas.
- d. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors 15 wks. after DMBA application.

TABLE 7 INDUCTION OF TUMORS IN MICE FOLLOWING APPLICATION OF DMBA AND SUPERIMPOSED HSV INFECTION^a

HSV INOCULATION GROUP	MICE WITH TUMORS/ SURVIVING MICE ^b	% MICE WITH TUMORS ^c
No Inoculation	2/18 (2)	11%
Day 5 Inoculation	0/19	0%
Day 12 Inoculation	0/16	0%
Day 19 Inoculation	0/15	0%

- a. All groups received 10 ul of 2.5 % DMBA, 5 days/wk. for 3 wks. to the abraded right maxillary lip of 6 wk. old male BALB/c mice (24 mice per group). In addition, 3 groups received 100 ul of a 1×10^6 PFU/ml 333 solution to the same site on the indicated day after carcinogen application initiation.
- b. Histologic determination of tumor type: number within parenthesis represents # of squamous cell carcinomas.
- c. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors 20 wks. after DMBA application.

because the lips had already been made erythematous, hairless, swollen and ulcerated by repeated DMBA applications. However, at the termination of this study, serum was obtained from HSV infected animals and shown to contain neutralizing antibodies directed against HSV. Thus, HSV infection initiated prior to or during the DMBA application period did not increase the tumor yield, shorten the latent period, or change the papilloma to squamous cell carcinoma ratio.

CONCOMITANT HSV INFECTION AND PHYSICAL IRRITATION

Physical irritation is often listed as a contributing factor to the initiation of the neoplastic process. In addition, trauma often precedes the lesions of herpes labialis in humans. Thus, abrasion was investigated in conjunction with HSV infection.

Four weeks after HSV infection, the right maxillary lips of mice were lightly abraded by a slow speed dental handpiece with a steel brush attachment. This abrasion was once again repeated six weeks later. When the experiment was terminated 34 weeks postinfection, none of the animals had developed lip tumors (Table 8, Groups 3 & 4). In a parallel experiment, an equal number of animals received the same treatment except for the addition of six minutes of UV-irradiation at $42 \text{ ergs/mm}^2/\text{s}$ immediately following the light abrasion. The results, however, were similar in that none of the mice developed tumors and the lips retained normal appearance (Table 8, Groups 5 & 6).

CONCOMITANT HSV INFECTION AND UV-IRRADIATION

As previously pointed out, the delivery of UV exposure alone or the inoculation of HSV by itself did not induce lip tumors. Therefore, a series of experiments were designed to investigate the effect of UV-irra-

TABLE 8 RESPONSE OF BALB/c MICE TO REPEATED UV, HSV, OR ABRASION TREATMENTS

GROUP	FIRST TREATMENT ^a	% MICE WITH HERPETIC LIP LESIONS ^b	SECOND TREATMENT ^c	% MICE WITH HERPETIC LIP LESIONS ^b	THIRD TREATMENT ^d	% MICE WITH HERPETIC LIP LESIONS ^b	FOURTH TREATMENT ^e	% MICE WITH HERPETIC LIP LESIONS ^b	% MICE WITH LIP TUMORS ^f
1	HSV right	100%	HSV right	0%	HSV right	20%	HSV left	0%	0%
2	HSV right	100%	HSV right	10%	HSV right	10%	HSV right	0%	0%
3	HSV right	100%	Abrasion	0%	Abrasion	0%	HSV left	25%	0%
4	HSV right	100%	Abrasion	0%	Abrasion	0%	HSV right	22%	0%
5	HSV right	100%	Abrasion & UV	0%	Abrasion & UV	0%	HSV left & UV	78%	0%
6	HSV right	100%	Abrasion & UV	0%	Abrasion & UV	0%	HSV right & UV	80%	0%
7	Media right	0%	Abrasion & UV	0%	Abrasion & UV	0%	HSV right & UV	100%	0%
8	Abrasion	0%	Abrasion	0%	Abrasion	0%	Abrasion	0%	0%

- a. Each group of 10 males (6 wks. of age) were anesthetized, abraded on the maxillary right lip and the first six groups received 100 ul of HSV-1 inoculation (3×10^7 PFU/ml). The seventh group received a "media only" inoculation, whereas the eighth group received only abrasion.
- b. Each animal was evaluated 5 days postinoculation with a 2X scope for lesions on the most recent inoculation site.
- c. Each animal was anesthetized, abraded on the maxillary right lip and the first two groups received 100 ul of HSV inoculation (3×10^7 PFU/ml). The fifth, sixth and seventh groups received six minutes of UV irradiation at 42 ergs/mm²/s. The second treatment followed the first by four weeks.
- d. Same protocol as "c" above. The third treatment followed the second by six wks.
- e. Each animal was anesthetized, abraded on the maxillary right or left lip (as indicated) and the first seven groups received 100 ul of HSV-1 inoculation (3×10^7 PFU/ml). In addition, groups five, six and seven received six minutes of UV-irradiation at 42 ergs/mm²/s. The fourth treatment followed the third by four weeks.
- f. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors 20 wks. after the fourth treatment. In addition, histopathologic evaluation of representative lips from each group revealed only a mild chronic inflammatory cell infiltrate underlying a normal-appearing epithelium.

diation in conjunction with HSV inoculation in our mouse model.

Since Rapp has reported in vitro transformation of hamster cells by infection with HSV-2 irradiated for six minutes at $42 \text{ ergs/mm}^2/\text{s}$, an analogous protocol was attempted in vivo. In one experiment, a virus inoculum was irradiated prior to its application onto abraded lips of mice. Fifteen male BALB/c mice (5 wks. of age) were anesthetized, lightly abraded on the right maxillary lip, and swabbed with approximately 100 μl of a 333 solution (1×10^6 PFU/ml) that had just been UV-irradiated for 6 minutes at $42 \text{ ergs/mm}^2/\text{s}$. Five days later, when HSV induced lip lesions would normally be present, no lesions were seen. Daily evaluation of the lips continued to show the lack of herpetic lesions; whereas in a group of mice inoculated with the same virus pool, but not irradiated prior to application, 60% of the animals developed herpetic lesions by the fifth day (Table 9) ($p = .02$). Apparently, the UV exposure had destroyed the infectious properties of the HSV-2 inoculum. Twenty-three weeks later, the lips appeared normal and no tumors were evident. The procedure of inoculation with a UV-irradiated virus pool was repeated. Once again, herpetic lesions failed to develop by the fifth day and tumors were not evident seventeen weeks later. Microscopic examination of these lips at that time revealed normal stratified squamous epithelium and fibrous connective tissue. Consequently, another series of experiments were designed to investigate the effect of UV exposure delivered to mouse lips infected with normal unirradiated HSV-2. Three different combinations of HSV inoculation on day 0 followed by UV-irradiation (6 minutes daily at $42 \text{ ergs/mm}^2/\text{s}$) on days 0 through 6, 2 through 6 or 4 through 6 in three groups of 15 BALB/c mice each were investigated. The incidences of herpetic lip lesions for the three groups were 64%, 86% and 87% respectively and are depicted in

TABLE 9 INDUCTION OF DYSPLASIA IN MICE BY UV EXPOSURE TO HSV INOCULATED LIPS

HSV INOCULATION ON DAY 0 ^a	UV EXPOSURE ON DAY			% MICE WITH HERPETIC LIP LESIONS ^c	HSV INOCULATION ON DAY 162 ^d	UV EXPOSURE ON DAY			% MICE WITH HERPETIC LIP LESIONS ^c	MICE WITH TUMORS/MICE SURVIVING	% MICE WITH LIP TUMORS ^e
	0-6	2-6	4-6			162-168	164-168	166-168			
	Yes			0%		Yes			0%	0/15	0%
Yes				60%	Yes				47%	0/16	0%
Yes			Yes	87%	Yes			Yes	38%	0/12	0%*
Yes		Yes		86%	Yes		Yes		50%	0/11	0%*
Yes	Yes			64%	Yes	Yes			20%	0/10	0%
Yes ^f				0%	Yes ^f				0%	0/15	0%
					Yes			Yes	80%	0/20	0%
					Yes		Yes		74%	0/20	0%
					Yes	Yes			59%	0/25	0%

*Histopathologic examination of biopsied lips revealed hyperkeratosis, acanthosis and epithelial dysplasia in 4 of the 23 animals (p < 0.01 when compared to remaining 75 UV + HSV treated animals).

- a. BALB/c male mice, approximately 5 wks. of age, were anesthetized, abraded on the right maxillary lip, and swabbed with 100 ul of a 1×10^6 PFU/ml solution of 333. At the beginning of the experiment, the number of animals/group, reading from the top to the bottom, were 19, 20, 15, 15, 15, 15, 30, 30 and 30 respectively.
- b. Animals were anesthetized and their lips exposed to 6 minutes of UV-irradiation at $42 \text{ ergs/mm}^2/\text{s}$ on each day.
- c. Each animal was evaluated 5 days postinoculation with a 2X scope. Weekly evaluations of healing lesions are shown in Fig. 3.
- d. Same as "a" except 333 inoculum had a titer of 6.1×10^6 PFU/ml.
- e. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors on day 283.
- f. This virus inoculum was irradiated with 6 minutes UV light at $42 \text{ ergs/mm}^2/\text{s}$ prior to skin application.

Table 9. Apparently, since the UV delivery on days 0 and 162 was given within minutes after the HSV inoculum was applied, much of the infectious behavior of the virus was inhibited. Weekly examination of the healing lips demonstrated an increase in herpetic lesion incidence and severity with prolonged healing periods (Fig. 3a). When the different subgroups of UV administration were examined separately, delivery on days 4 through 6 and 2 through 6 increased the incidence and severity of the herpetic lesions to a greater extent than delivery on days 0 through 6 (Fig. 3b). After 23 weeks, delayed healing was well documented, but tumors had failed to emerge. Consequently, the procedures were repeated. In addition, three other groups of mice were subjected to similar viral infections and UV exposures (Table 9). Once again, that group of animals receiving UV exposure on days 0 through 6 had less severe and a smaller incidence of herpetic lesions as compared to those receiving UV exposure on days 2 through 6 or 4 through 6 postinfection (20%, 50%, 38%, respectively). At the time of sacrifice on day 283, macroscopic evidence of tumors was lacking. However, several animals of the group that received HSV infection and UV exposure on days 2 through 6 or 4 through 6 postinfection demonstrated histologic evidence of hyperkeratosis, acanthosis, and epithelial dysplasia (Table 9, Fig. 4a & b). Although many of the experimental animals died secondary to anesthesia during HSV inoculation and UV exposure, the histologic evidence was encouraging and led to an expansion of the experiments investigating concomitant UV exposure and HSV infection.

CONCOMITANT HSV INFECTION AND UV IRRADIATION WITH PROMOTION BY TPA

Because epithelial dysplasias developed following UV-irradiation of herpetic lip lesions, it was felt that the addition of a tumor promoting

FIGURE 3

Delayed healing of HSV-2 induced lip lesions following UV irradiation. The right maxillary lips of 5 week old male BALB/c mice were (i) inoculated with HSV-2 (HSV only), (ii) UV-irradiated on days 0-6 (UV only), or (iii) inoculated with HSV-2 and UV-irradiated on days 0-6, 2-6, or 4-6 postinoculation (HSV + UV) as described in Table 9. Figure 3a represents the presence of lip lesions over the healing period. In addition, the HSV + UV group was divided into its three subgroups (UV-irradiation delivered on days 0-6, 2-6, or 4-6 postinoculation) and the presence of lip lesions during the healing period is presented in Figure 3b.

FIGURE 3a

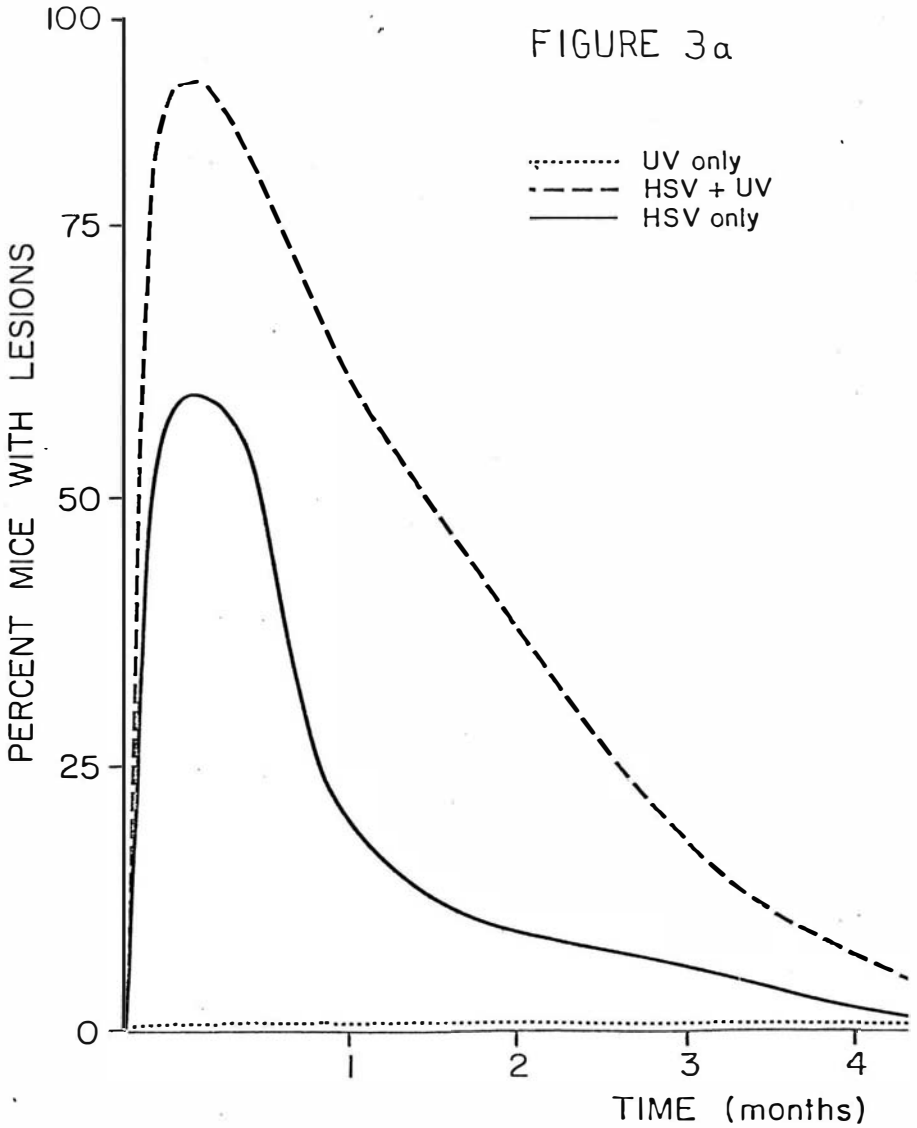


FIGURE 3b

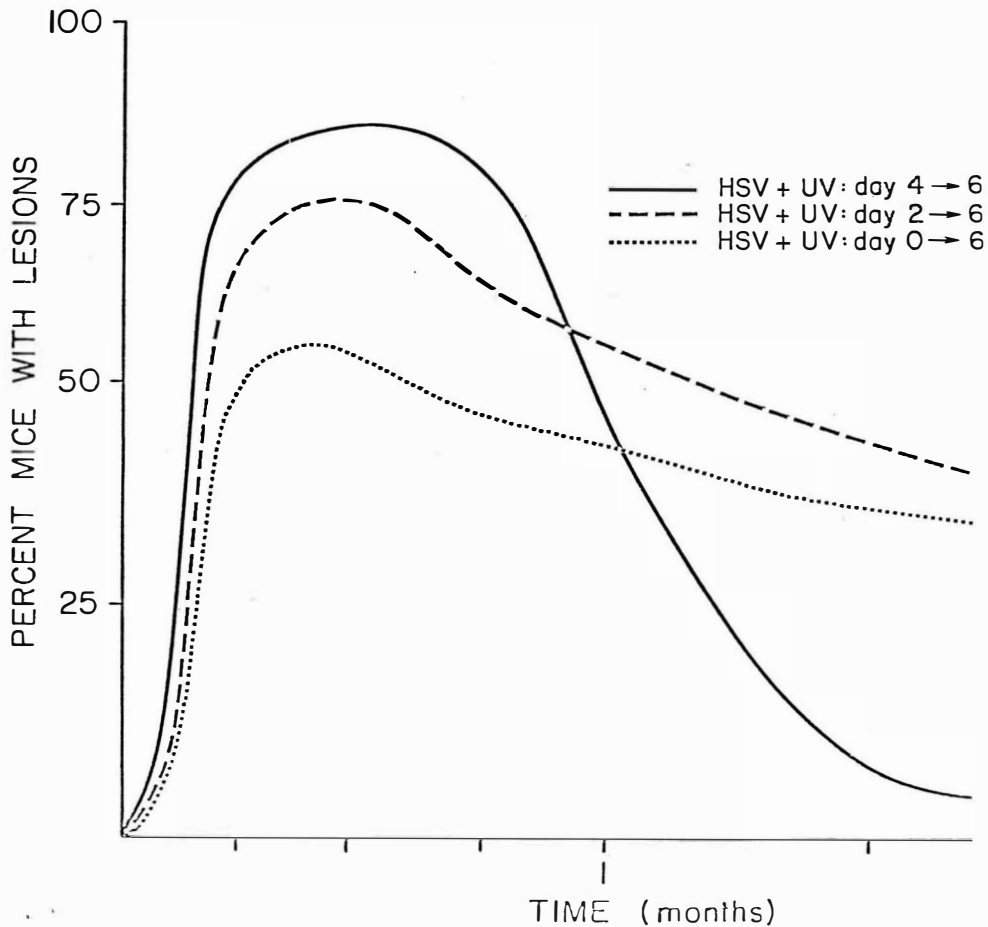


FIGURE 4

Macroscopic and microscopic pathology of HSV-2 infected, UV-irradiated lip lesions. The right maxillary lip of a 5 week old male BALB/c mouse was inoculated with HSV-2 and UV-irradiated on days 2-6 and 164-168 postinoculation as described in Table 9.

- (a) At autopsy on day 283 postinoculation, the lip was erythematous but lacked macroscopic tumors.
- (b) However, microscopic examination of hematoxylin and eosin stained paraffin embedded sections revealed hyperkeratosis, acanthosis and epithelial dysplasia (40X magnification).

FIGURE 4

a.



b.



agent to the regimen might increase cellular replication rates and promote tumor formation. Tetradecanoyl-phorbol-acetate (TPA) has been widely utilized as a promoter of the neoplastic process while investigating possible carcinogenic agents and consequently was chosen for use in this study. One hundred eighty-two mice were subjected to HSV lip inoculation, UV exposures on days 3, 4, 5, 6, and TPA applications three times a week beginning on day 9 for the duration of the experiment (Table 10). Ninety-six percent of these animals developed typical herpetic lip lesions by five days postinoculation. Within this group, 32 mice developed lip tumors at the site of HSV infection. The average period of time for these tumors to emerge was 10.8 weeks. At the termination of the experiment 193 days after HSV inoculation, histologic examination of these tumors revealed 24 benign papillomas, 2 epithelial dysplasias, and 6 squamous cell carcinomas (Fig. 5 & 6). The various control groups of animals are listed in Table 10. Of statistical significance, the application of TPA alone three times a week produced no tumors as compared to the group receiving HSV + UV + TPA ($p = .013$). However, HSV inoculation plus the application of TPA three times a week produced two papillomas, whereas UV exposure plus TPA three times a week yielded three papillomas (Fig. 7). Since the TPA was dissolved in DMSO, one control group received HSV inoculation, UV exposure and DMSO applied three times a week. This group developed one papilloma. A comparison of the experimental group with the remaining 152 control animals revealed that squamous cell carcinomas developed only in the group receiving HSV, UV, and TPA (Table 10) ($p = .04$). Also, papillomas developed more frequently in the experimental group ($p = .001$). In addition, tumors arose earlier in this group (10.8 wks.) as compared to the control groups (13-24 wks.)

TABLE 10 INDUCTION OF LIP TUMORS IN MICE FOLLOWING HSV INFECTION,
UV EXPOSURE, AND TPA REPEATED APPLICATION

ANIMALS IN GROUP ^a	TREATMENT	% MICE WITH HERPETIC LIP LESIONS ^h	LATENT; PERIOD ⁱ	MICE WITH TUMORS/ SURVIVING MICE ^j	% MICE WITH TUMORS ^k
30	TPA only ^b	0%	-	0/30	0%
37	HSV & TPA ^c	97%	13 wks.	2/33 (2)	6%
40	UV & TPA ^d	0%	13 wks.	3/34 (3)	9%
40	UV & TPA ^e & Abrasion	0%	-	0/36	0%
20	HSV & UV & DMSO ^f	100%	24 wks.	1/19 (1)	5%
182	HSV & UV & TPA ^g	96%	10.8 wks.	32/156 (24)(2)(6)	21%

- a. BALB/c male mice were approximately 7 wks. of age when first treated.
- b. Each animal was anesthetized and abraded on the maxillary right lip on day 0 and then received 0.02 μ mole TPA/10 μ l of DMSO on the site three times every wk.
- c. Each animal was anesthetized, abraded on the maxillary right lip and swabbed with 100 μ l of a 1.3×10^7 PFU/ml solution of 333 on day 0 and then received TPA as in "b" above.
- d. Each animal was anesthetized and abraded on the maxillary right lip on day 0, exposed to 6 min. of UV-irradiation at 42 $\text{ergs/mm}^2/\text{s}$ daily on days 3, 4, 5 & 6, and then received TPA as in "b" above.
- e. Each animal was treated as in "d" above and then lightly abraded on the maxillary right lip on day 25.
- f. Each animal was anesthetized, abraded on the maxillary right lip and swabbed with 100 μ l of 1.3×10^7 PFU/ml solution of 333 on day 0, exposed to 6 min. of UV-irradiation at 42 $\text{ergs/mm}^2/\text{s}$ daily on days 3, 4, 5 & 6, and then received 10 μ l of DMSO on the site three times every wk.
- g. Same as "f" above except that each 10 μ l of DMSO contained 0.02 μ mole TPA.
- h. Each animal was evaluated 5 days postinoculation with a 2X scope.
- i. Average time span until emergence of clinically evident tumors.
- j. Histologic determination of tumor type: number within first parenthesis represents # of papillomas; number within second parenthesis represents # of epithelial dyaplasias; number within third parenthesis represents # of squamous cell carcinomas.
- k. Each animal was evaluated weekly with a 2X scope and then graded for the presence of tumors on day 193.

FIGURE 5

Papilloma induction by HSV infection, UV-irradiation and repeated TPA application. The right maxillary lips of 7 week old male BALB/c mice were inoculated with HSV-2, UV-irradiated on days 3-6 postinoculation and exposed to repeated TPA applications as described in Table 10 (HSV & UV & TPA).

- (a) At autopsy, 28 weeks postinfection, 24 mice had developed papillomas at the original herpetic lesion site.
- (b) Microscopic examination of hematoxylin and eosin stained paraffin embedded sections revealed benign stratified squamous epithelium arranged in finger-like projections over a fibrous connective tissue core (20X magnification).

Diagnosis: papilloma.

FIGURE 5

a.



b.

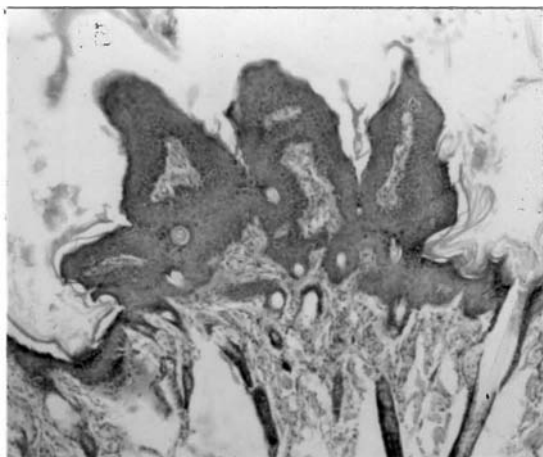


FIGURE 6

Squamous cell carcinoma induction by HSV infection, UV-irradiation and repeated TPA application. Refer to the legend of Figure 5 for details.

- (a) At autopsy, 28 weeks postinfection, 6 mice had developed squamous cell carcinomas at the original herpetic lesion site.
- (b) Microscopic examination as described for Figure 5 revealed invasive islands of malignant squamous epithelium. Pleomorphism, inverse nuclear:cytoplasmic ratios and keratin pearl formation are evident (40X magnification). Diagnosis: squamous cell carcinoma.

FIGURE 6

a.



b.

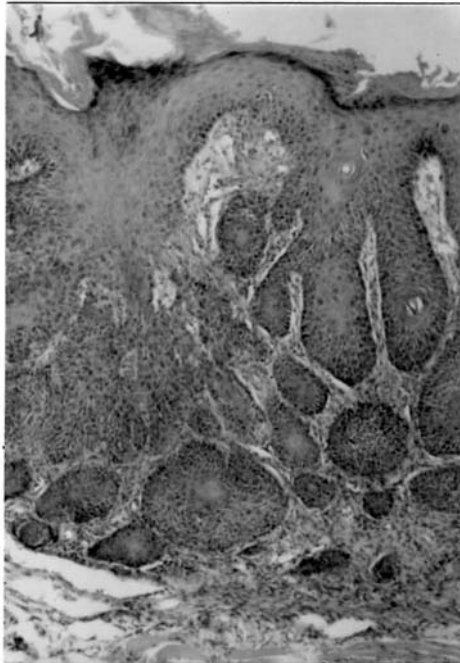
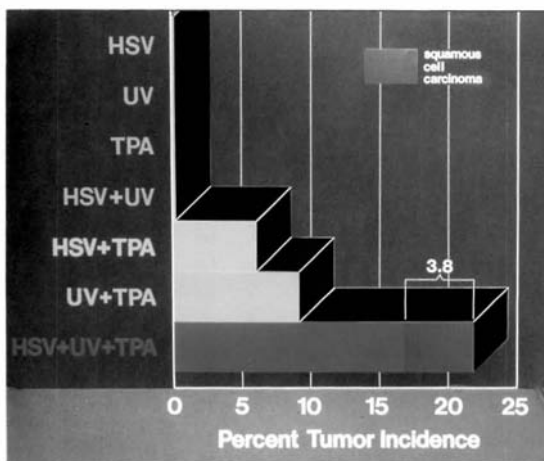


FIGURE 7

Tumor induction with various combinations of HSV infection, UV-irradiation, and TPA application. The right maxillary lips of 7 week old male BALB/c mice were either inoculated with HSV-2, UV irradiated on days 3-6 postinoculation, exposed to repeated TPA applications, or treated with combinations of these agents as described in Table 10. The homogram depicts the tumor incidence following each of these treatments.

FIGURE 7



At the termination of this experiment, a total of 38 lip tumors that had developed in the experimental as well as control groups were surgically removed. Many of the tumors were not large enough to trisect for: (i) formalin fixation and paraffin embedding, (ii) cryostat sectioning, and (iii) explantation to tissue culture. Therefore, priorities for evaluations were set. The first priority was to obtain a histologic diagnosis utilizing H&E stained paraffin embedded tissues. The diagnoses are depicted in Table 10. The next priority was to examine the tumor cells for the presence of HSV antigens utilizing the immunoperoxidase stain. Preliminary studies, staining HSV infected mouse lips, indicated that formalin fixation and paraffin embedding often yielded false negative results. Best results were obtained in these preliminary studies utilizing cryostat sections of frozen specimens. Consequently, the search for HSV antigens in experimental tissue was performed upon cryostat sections or explanted tissue in culture. Twenty-two of the 38 lip tumors were large enough to hemisect and freeze one portion for cryostat sectioning. Eleven of the tumors were even larger and were trisected; one portion explanted to tissue culture in vitro. Five of these attempted explants successfully grew out onto cover slips. In both instances, cells were eventually fixed and stained utilizing the immunoperoxidase stain. Results are listed in Table 11. When the primary antiserum of the immunoperoxidase stain was rabbit α -HSV-2 strain MS, four tissue specimens stained for HSV antigens. These specimens were from three animals of the HSV + UV + TPA group. When hamster α -333 antiserum was used as the primary antiserum, six specimens stained positive (Fig. 8). Once again, these specimens came from animals of the HSV + UV + TPA group. In addition, these six specimens stained positive when mouse α -333 or mouse α -HSV + UV + TPA tumor

TABLE 11 PRESENCE OF HSV ANTIGENS IN MOUSE LIP TUMORS AS DETERMINED BY IMMUNOPEROXIDASE STAINING UTILIZING VARIOUS ANTISERAS

ANIMAL NUMBER	TREATMENT GROUP ^a	HISTOPATHOLOGIC DIAGNOSIS ^b	TISSUE TYPE ^c	RABBIT α -HSV-2-MS ^d	HAMSTER α -HSV-2-333 ^d
82	UV + TPA	Papilloma	C	-	-
91	"	"	C	-	-
91	"	"	E	-	-
132	HSV + TPA	"	C	-	-
155	HSV + UV + TPA	"	C	+	+
155	"	"	E	+	+
168	"	"	C	-	-
169	"	"	C	-	-
177	"	"	C	-	-
217	"	Squamous Cell Ca.	C	-	-
225	"	Papilloma	C	-	-
235	"	Squamous Cell Ca.	C	-	-
236	"	Epithelial Dysplasia	C	-	-
250	"	Papilloma	C	-	-
251	"	Squamous Cell Ca.	C	-	-
255	"	Papilloma	C	-	-
256	"	"	C	+	+
256	"	"	E	-	+
257	"	Squamous Cell Ca.	C	-	-
265	"	"	E	+	+
272	"	"	C	-	-
272	"	"	E	-	-
273	"	Papilloma	C	-	-
275	"	"	C	-	+
278	"	"	C	-	-
285	"	"	C	-	-
297	HSV + UV + DMSO	"	C	-	-

- a. Details of the treatment are found in Table 10.
- b. Light microscopic examination of H&E stained 6 micron sections of the formalin-fixed paraffin embedded portions of biopsied tumor.
- c. "C" represents 6 micron cryostat sections of portions of biopsied tumor. "E" represents outgrowths from explanted tumor tissue cultured in vitro on cover slips.
- d. This antiserum was used as the primary serum in the immunoperoxidase staining.

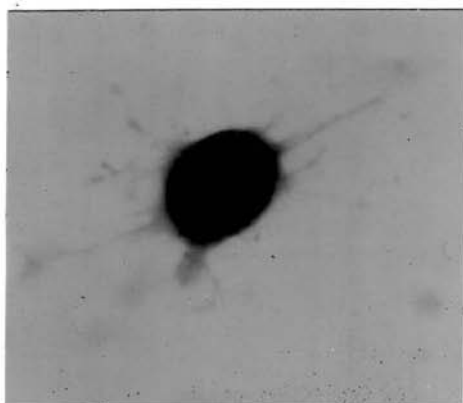
FIGURE 8

Presence of HSV antigens in HSV + UV + TPA induced tumors as determined by immunoperoxidase staining. Details of the tumor induction procedure are described in Tables 10 and 11.

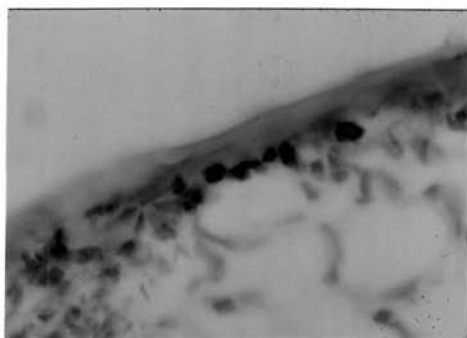
- (a) Positive immunoperoxidase staining for HSV antigens in a cell grown out from the explanted papilloma removed from animal #155 (400X magnification).
- (b) Positive immunoperoxidase staining for HSV antigens within five or six basal epithelial cells from the papilloma removed from animal #256 (100X magnification).
- (c) Negative immunoperoxidase staining for HSV antigens within normal uninfected mouse lip epithelium (100X magnification). Refer to Figure 1c for an example of positive staining within HSV-2 strain 333 infected mouse lip epithelium.

FIGURE 8

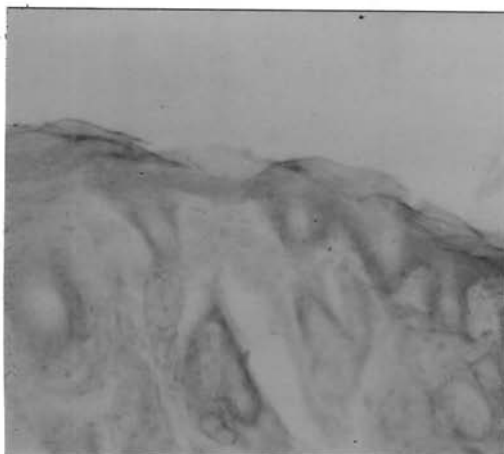
a.



b.



c.



antiserum was used as the primary antiserum. Since only tumors from the HSV + UV + TPA group showed the presence of HSV antigens as determined by immunoperoxidase staining with these four antisera, these results suggest that HSV may play a role in the development of these tumors.

COMPARISON OF HSV-ANTIBODY TITERS

At the termination of each of the aforementioned in vivo carcinogenesis experiments, the animals were sacrificed by cardiac exsanguination. The serum was analyzed by micro-titer assay for neutralizing antibodies. The mean serum HSV neutralizing antibody titer for each of the groups is listed in Table 12. Antibodies to HSV were not found in mice which had not been infected with HSV. Of those groups that had been inoculated on the lip with HSV, the mean antibody titer ranged from 12 to 18.4. Statistical analysis of these titers utilizing Student's t Test revealed no significant differences between the groups. However, within one group (HSV + UV + TPA), when the titers from the tumor bearing animals were compared to those of non-tumor bearing animals, a highly significant difference emerged ($p < 0.001$). Those animals which developed papillomas and carcinomas had an increased antibody titer against HSV (28.6) as compared to those animals without tumors (16.3). Further subdivision and comparison of titers from papilloma bearing (27.6) versus carcinoma bearing (30.0) animals failed to yield significant differences, although the carcinoma group had slightly greater titers. Although there was a significant difference between tumor bearing and non-tumor bearing in the HSV + UV + TPA group, the same was not true for the tumor bearing (17.2) and non-tumor bearing (14.0) subdivisions of the HSV + DMBA group. Thus, the tumor bearing characteristic alone cannot be held responsible for the

significant increase in HSV antibody response seen in the HSV + UV + TPA tumor bearing animals.

TRANSFORMATION IN VITRO BY UV-INACTIVATED HSV

The induction of malignant tumors in BALB/c mice following infection with HSV-2, exposure to UV-irradiation and topical application of TPA reinforced the concept that HSV may act as a cocarcinogen. However, the molecular mechanisms of these variables (HSV, UV and TPA) and their functions in the transforming events were impossible to deduce from the in vivo data. Consequently, a series of in vitro experiments were designed to investigate some of the molecular events and interactions of HSV, UV and TPA during cell transformation.

The effect of infection with HSV on cellular metabolism and macromolecular biosynthesis has been well documented. Within hours after HSV infection the cell's macromolecular biosynthetic machinery is shut down in favor of viral transcription, translation and virus assembly. Viral replication generally leads to cell death by cytolysis. For example, one ml of the HSV-2 pools used in these studies can cause as many as 10 million plaques (areas of cell destruction) to form in a monolayer of Vero cells within 48 hours after infection. It has been clearly demonstrated, however, that viral infectivity is optimal at specific pHs and temperatures and can be inhibited by various agents, including UV-irradiation.

In the first series of experiments, the effect of UV-irradiation on HSV-2 infectivity was investigated. Petri plates containing HSV-2 were subjected to various exposure times of UV-irradiation at $42 \text{ ergs/mm}^2/\text{s}$. At intermittent periods, ranging from 0 minutes to 10 minutes, samples

TABLE 12 COMPARISON OF HSV-ANTIBODY TITERS

TREATMENT GROUP ^a	MEAN SERUM NEUTRALIZING ANTIBODY TITER \pm S.D. ^b
3-MC (3)	0.0
DMBA (4)	0.0
DMBA + HSV (7)	12.0 \pm 12
HSV + DMBA (5)	15.0 \pm 10
HSV + DMBA (6)	15.6 \pm 9
(i) Tumor bearing	17.2 \pm 9
(ii) Non-Tumor bearing	14.0 \pm 9
TPA only (10)	0.0
HSV + TPA (10)	16.7 \pm 13
UV + TPA (10)	0.0
UV + TPA + Abrasion (10)	0.0
HSV + UV + DMSC (10)	14.7 \pm 5
HSV + UV + TPA (10)	18.4 \pm 13
(i) Non-Tumor bearing	16.3 \pm 10*
(ii) Tumor bearing	28.6 \pm 18*
a. Papilloma	27.6 \pm 20
b. Squamous Cell Carcinoma	30.0 \pm 11

*Analysis by Student's t Test reveals $p < 0.001$. All other combinations of comparisons were not significant ($p > .22$).

- a. The number in parentheses represents the table number where details of treatment are found.
- b. Serum neutralizing antibody titers for each mouse were determined by microtiter analysis as described in Materials and Methods.

were removed and assayed for infectivity (PFU/ml) on Vero cells. The effect of UV-irradiation is depicted in Figure 9. Generally for each minute of UV exposure, the viral titer decreased one log. After six minutes of UV exposure, little or no infectious virus remained. Rapp had previously suggested that the viral infectivity of HSV needed to be inhibited before the oncogenic potential could be expressed in transformation experiments and he used 6 and 8 minutes of UV exposure in his studies.⁷¹ In cognizance of Rapp's data and the data presented in Figure 9, an endeavor was made to transform HEF cells in vitro using HSV-2 irradiated for 6, 7 and 8 minutes.

In numerous preliminary transforming experiments, the critical nature of the pH of the medium during the virus adsorption phase and during attachment of the infected HEF cells was evident. Extreme acidic or basic conditions during virus adsorption or during cell attachment severely diminished the number of cells that grew after being seeded in T-75 flasks. If a subthreshold number of cells were seeded (e.g. 5.5×10^5 cells/T-75 flask) few, if any, cells survived and they failed to divide. Thus, the number of cells seeded per flask proved to be crucial and 1.1×10^6 HEF/flask appeared to be optimal. In addition, constant surveillance was necessary in order to maintain a pH of 7.0-7.2 throughout the duration of the experiments.

The amount of UV-irradiation received by the virus was very important in determining the efficiency of transformation (Table 13). Six minutes and eight minutes of UV exposure yielded an average of 0.95 and 1.30 foci per T-75 flask, respectively. Seven minutes of UV exposure generated an average of 3.27 foci per flask (Fig. 10). This represents 9.9×10^{-7} focus forming units (FFU) per PFU or, conversely, 1×10^6 PFU/FFU.

FIGURE 9

Effect of UV-irradiation on HSV infectivity. Two ml volumes of HSV-2 strain 333 (1.0×10^7 PFU/ml) in 35 mm Petri dishes were exposed to $42 \text{ ergs/mm}^2/\text{s}$ of UV-irradiation. At various times during UV exposure, samples were removed and assayed for infectivity (PFU/ml) on Vero cells.

FIGURE 9

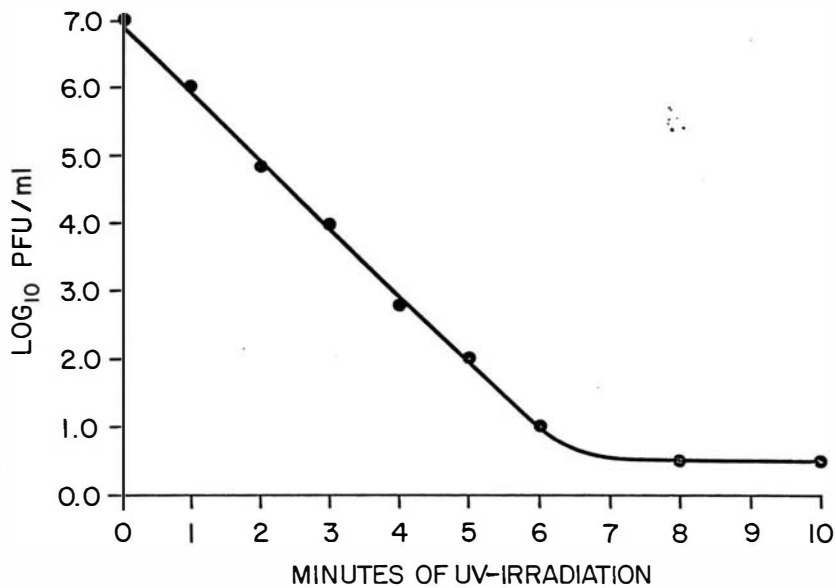


TABLE 13 TRANSFORMATION OF HAMSTER EMBRYO FIBROBLASTS IN VITRO BY UV INACTIVATED HSV^a

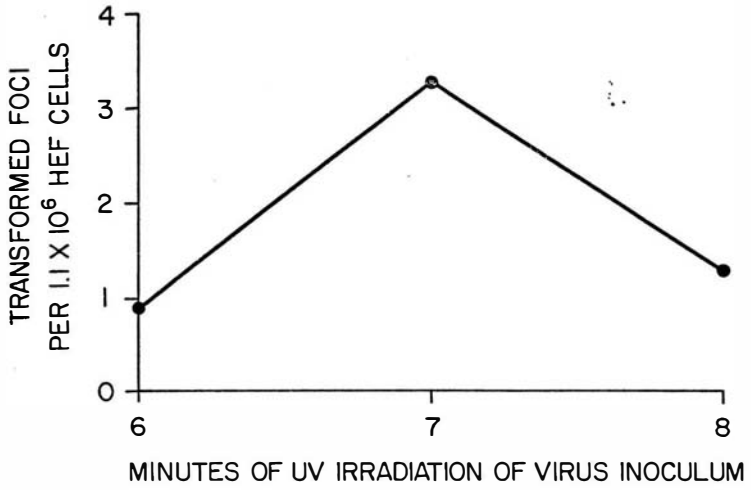
NUMBER OF T-75 FLASKS	333 INOCULUM PFU/ml ^b	MINUTES OF UV IRRADIATION OF 333 INOCULUM	AVERAGE # OF TRANSFORMED FOCI/1.1 x 10 ⁶ CELLS ^c	TRANSFORMATION EFFICIENCY FFU/PFU ^d
35	-	-	0.09	
15 ^e	-	-	0.03	
20	5.2 x 10 ⁷	6	0.95	2.9 x 10 ⁻⁷
65	1.7 x 10 ⁷	7	3.27	9.9 x 10 ⁻⁷
40	5.2 x 10 ⁷	8	1.30	3.9 x 10 ⁻⁷

- a. Each flask was seeded with 1.1 x 10⁶ hamster embryo fibroblasts (HEF) after a one hour incubation at 37°C with either GLB or UV-irradiated 333 (MOI=3).
- b. Assayed on Vero cells prior to UV irradiation for the indicated number of minutes at 42 ergs/mm²/s.
- c. Flasks were fixed in 10% formalin and stained with 0.2% Toluidine blue on day 21 postinoculation. Utilizing a 2X scope, foci were counted based upon size, morphology and staining characteristics.
- d. Focus Forming Units (FFU) per Plaque Forming Unit (PFU) of viral inoculum (eg. 3.27 foci/3.3 x 10⁶ PFU = 9.9 x 10⁻⁷ FFU/PFU).
- e. Media contained DMSO (0.1 ml/ml).

FIGURE 10

Efficiency of transforming HEF cells by infection with HSV-2 strain 333 UV-irradiated for various periods of time. A virus inoculum was UV-irradiated for 6, 7 or 8 minutes prior to infecting HEF as described in Table 13. The efficiency of transformation, in terms of average number of transformed foci per 1.1×10^6 cells, was maximal at 7 minutes of UV exposure.

FIGURE 10



In addition, this represents 3.0 FFU per 10^6 cells. Transformation efficiencies of this magnitude have not previously been reported; generally, the transformation efficiencies reported for HSV are 1.5-25.0 times less efficient in terms of foci per cell. However, only 6 and 8 minute UV exposure times have been reported and, therefore, the 7 minute UV exposure is one explanation for the high efficiencies noted in these experiments. Slight variation in virus preparations is another possible explanation.

PROPERTIES OF UV-INACTIVATED HSV TRANSFORMED HAMSTER EMBRYO CELLS

Following the demonstration that UV-inactivated HSV was capable of transforming HEF cells into foci that had an increased rate of cell division and had the ability to proliferate without the addition of new growth media every 2-3 days, it was necessary to further characterize these foci. Foci generally could be visualized 10-14 days postinfection as small areas of increased cell density per unit area surrounded by a monolayer of degenerating HEF cells. However, not all foci appeared morphologically identical by 21 days postinfection, when the experiment was terminated by either fixation in 10% formalin and staining with Toluidine blue or by picking and subculturing individual foci. Many of the foci appeared fibroblastic with long slender cytoplasmic processes that intertwined, overlapped and piled up in a haphazard fashion (Fig. 11a). Other foci appeared epithelioid with pleomorphism and angular cytoplasmic boundaries (Fig. 11b). Rapp has previously reported that two major morphological variants of foci exist, namely: fibroblastic and epithelioid.⁶⁰

In an effort to characterize transformed cells in more detail, a focus picked from a flask and passaged to establish a stable cell line

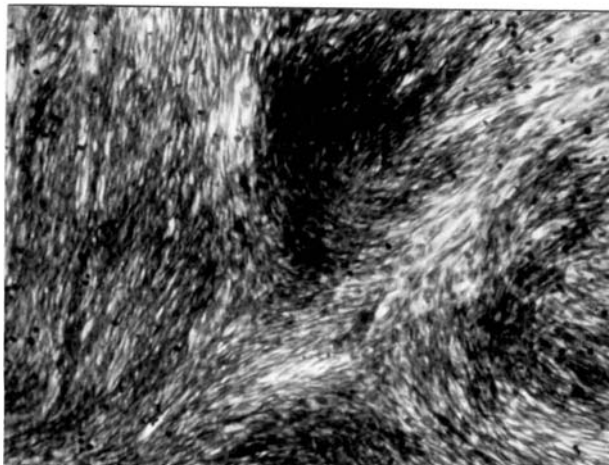
FIGURE 11

Morphological variants of HEF cells transformed by UV-inactivated HSV-2. HEF cells were infected with HSV-irradiated for 7 minutes as described in Table 13.

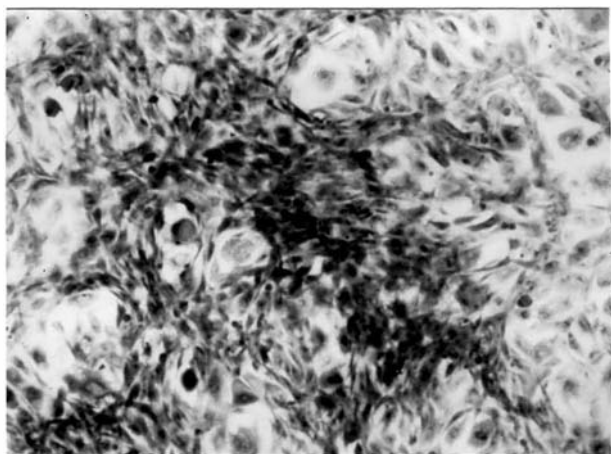
- (a) A fibroblastic type foci demonstrating intertwined fusiform cells with a tendency to overlap and pile up on one another (20X magnification).
- (b) An epithelioid type foci demonstrating pleomorphic cells with angular cytoplasmic boundaries (20X magnification).

FIGURE 11

a.



b.



was studied. The fibroblastic cell line that resulted was designated 3-26-7#1 representing the foci from flask #1 of the transformation experiment performed on 3-26-79 utilizing 7 minutes of UV exposure to inactivate the HSV inoculum. The cell line was evaluated (i) for oncogenicity, (ii) by immunoperoxidase staining and (iii) by cell sorter analysis.

In order to examine the oncogenic nature of 3-26-7 #1 cells, 5×10^5 cells in 50 ul of HBSS were injected subcutaneously in the interscapular area into each of 16 newborn (5-14 days old) hamsters. Six weeks later, 4 of 16 hamsters had developed large tumors (2 cm x 1 cm x 1 cm) at the site of injection (Fig. 12a). Although the skin was freely moveable over the tumors, surgical exploration showed them to be fixed to the underlying muscle layers. Histologic examination of H&E stained tissue sections revealed that all the tumors were fibrosarcomas (Fig. 12b). At autopsy, no evidence of metastasis was found in tumor bearing animals and the remaining 12 hamsters demonstrated no evidence of tumors. Sera were obtained from the tumor bearing animals. By microtiter techniques, the pooled sera were found to have a HSV neutralizing antibody titer of 10 which indicated that the hamsters had developed antibodies capable of recognizing HSV virion envelope antigens. In addition, these sera (termed hamster α -tumor) was capable of detecting HSV specific antigens in (i) cells productively infected with HSV-2 (HEF, HEp-2 or Vero cells), (ii) parental transformed (3-26-7#1) cells, (iii) other transformed HEF cells (12F#1 & 12F#2) and (iv) cells derived from outgrowths of hamster tumor tissue explanted in vitro onto glass cover slips (Table 14). Apparently, the 3-26-7#1 cells express HSV glycoprotein antigens in the cytoplasmic membrane that are antigenically related to the HSV envelope glycoproteins. It has previously been reported that HSV transformed cells express several HSV virus

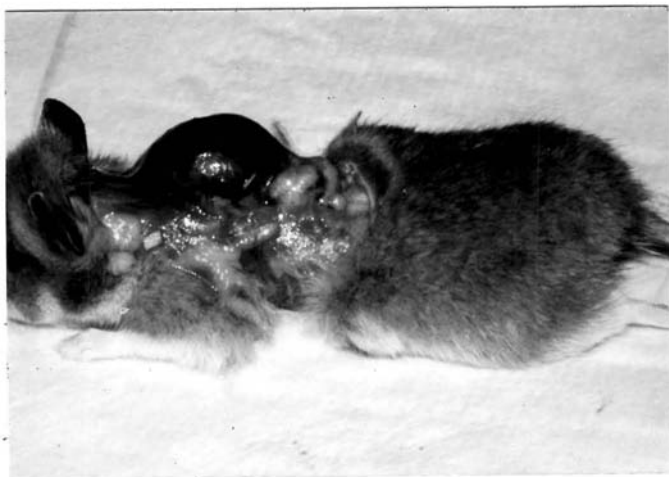
FIGURE 12

Oncogenicity of 3-26-7#1 cells. The UV-inactivated HSV transformed cell line 3-26-7#1 was tested for oncogenicity by injecting 5×10^5 cells in 50 μ l of HBSS into the interscapular area of newborn hamsters (5-14 days old).

- (a) Six weeks later, 4 of 16 hamsters developed tumors at the site of injection. These tumors were fixed to the underlying muscle layers.
- (b) Histopathologic examination of H&E stained paraffin embedded sections revealed malignant spindle cells demonstrating pleomorphism and anisocytosis (100X magnification). Diagnosis: fibrosarcoma.

FIGURE 12

a.



b.

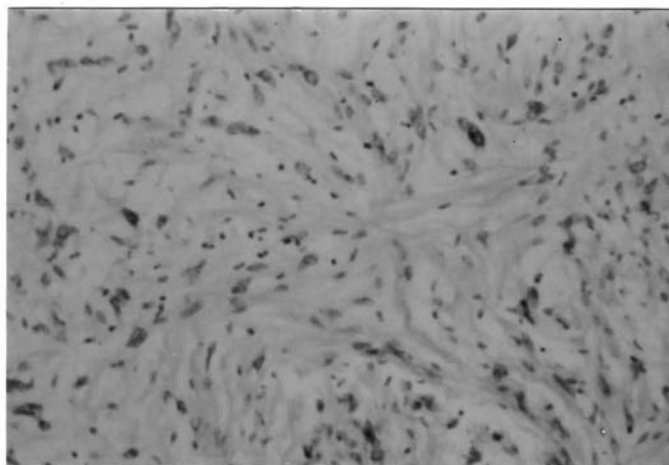


TABLE 14 PRESENCE OF HSV ANTIGENS IN CULTURED CELLS AS DETERMINED
BY IMMUNOPEROXIDASE STAINING UTILIZING VARIOUS ANTISERAS^a

CELLS ^b	RABBIT α - HSV-2/MS	RABBIT α -333	RABBIT α -333 EARLY 4 HR. ANTIGEN	RABBIT α - VP 143 (333)	HAMSTER α -333	HAMSTER α -TUMOR	MOUSE α - HSV+UV+TPA TUMOR
HEF Uninfected	-	-	-	-	-	-	-
HEF Infected with 333	+(35)	+(30)	+(30)	+(25)	+(40)	+(3)	+(15)
HEp-2 Uninfected	-	-	-	-	-	-	-
HEp-2 Infected with 333	+(70)	+(60)	+(50)(C) ^c	+(50)(C) ^c	+(70)	+(2)	+(30)
Vero Infected with 333	+(50)			+(30)	+(50)	+(2)	
3-26-7#1	+(5)	+(1)	+(1)	+(.5)	+(.5)	+(.5)	-
12F#1		+(.5)	+(1)	+(2)	+(2)	+(3)	-
12F#2		+(2)	+(1)	+(1)	+(2)	+(2)	-
Hamster Tumor Explant			+(1)	+(2)	+(2)	+(10)	-
Mouse DMBA Tumor Explant	-	-	-	-	-	-	-

- a. Rabbit, hamster and mouse sera were used as the primary antisera in the immunoperoxidase stain. The staining technique and development of antisera are described in Materials and Methods. The number in parentheses after the "+" represents the percentage of cells showing presence of positive staining.
- b. HEF, HEp-2 and Vero cells were infected with 333(MOI=1) and incubated for 15 hrs. at 37°C. UV-inactivated HSV transformed cell lines were 3-26-7#1, 12F#1 and 12F#2. A hamster tumor, created by the subcutaneous injection of 12F#2 cells was explanted and the in vitro outgrowths analyzed. A mouse squamous cell carcinoma, induced by DMBA, was explanted and the in vitro outgrowths analyzed.
- c. Generally, the location of positive staining was nuclear or perinuclear; however "(C)" represents prominent cytoplasmic staining.

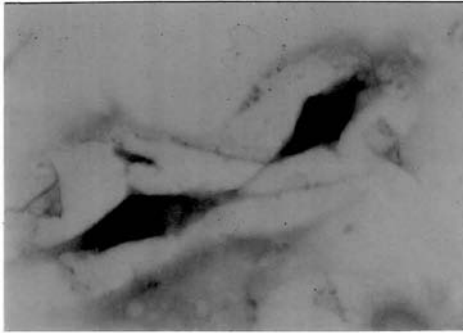
polypeptides and glycoproteins. In order to test this hypothesis, 3-26-7#1 cells were stained by the immunoperoxidase method utilizing various primary α -HSV sera (Table 14).

HSV antigens present in 3-26-7#1 cells were determined with antisera specific for different HSV proteins. The presence of HSV-2 strain 333 (333) viral protein VP 143 (an early nonstructural protein with a molecular weight of 143,000) was demonstrated in 0.5% of the 3-26-7#1 cells by staining with primary rabbit α -VP 143 antiserum. The presence of one or more of the early 4 hour proteins of 333 was demonstrated in 1% of the 3-26-7#1 cells by staining with rabbit α -early 4 hour antigen of 333 antiserum (Fig. 13). The presence of some of the proteins of the 333 virion was demonstrated in 1% and 5% of the 3-26-7#1 cells by staining with rabbit α -333 and hamster α -333 antiserum, respectively. As previously mentioned, 3-26-7#1 cells were capable of inducing fibrosarcomas when injected into hamsters and the sera from these animals (hamster α -tumor) proved capable of staining 3-26-7#1 cells. Thus, these 3-26-7#1 cells expressed tumor specific antigens; some of which could be 333 VP 143, early 4 hour proteins of 333 or 333 envelope proteins. Before leaving this topic, mention must be made of the immunoperoxidase staining of the control and other HSV transformed cells (Table 14). Two other UV-inactivated HSV transformed cell lines, 12F#1 and 12F#2, were evaluated. Generally, the staining of these cells was similar to 3-26-7#1 cells. The cell lines, 12F#1 and 12F#2 had previously been shown by another researcher in our laboratory to possess HSV antigens as measured by immunofluorescence staining. However, since those results were not quantitatively evaluated, the putative greater sensitivity of immunoperoxidase staining as compared to immunofluorescence staining could not be tested. The negative control cells, uninfected HEP-2

FIGURE 13

Presence of early 4 hour antigens of HSV-2 strain 333 in 3-26-7#1 cells. The UV-inactivated HSV transformed cell line 3-26-7#1 was stained with the immunoperoxidase method utilizing rabbit antiserum to the early 4 hour antigens of 333 as described in Table 14. Cells demonstrating positive staining are shown (400X magnification.).

FIGURE 13



and HEF cells consistently stained negative for HSV antigens irrespective of the primary antisera utilized (Fig. 14a). This indicated the absence of HSV antigens as well as adequate absorption of the antisera. 333 infected HEp-2, HEF and Vero cells consistently stained positive for HSV antigens (Fig. 14b). DMBA induced mouse lip tumors explanted to cell culture failed to show the presence of HSV antigens. Outgrowths from explanted hamster tumors, created by subcutaneous injection of UV-inactivated HSV transformed cells into hamsters, stained positive for HSV antigens with four different primary antisera. Finally, the antiserum from mice bearing HSV + UV + TPA induced tumors stained only 333 infected HEF and HEp-2 cells, indicating that if tumor specific antigens exist on the surface of HSV + UV + TPA induced mouse tumors they are different from (i) HSV antigens on the surface of UV-inactivated HSV transformed cells or their in vivo induced hamster tumors or (ii) tumor specific antigens on the surface of DMBA induced mouse squamous cell carcinomas.

After demonstrating that the cell line, 3-26-7#1, was oncogenic and expressed HSV specific antigens, attention was turned to investigating the cell DNA content by cell sorting analysis and comparing it to the DNA content of normal hamster embryo cells. The computer generated histograms plotting DNA content per cell vs. number of cells are presented in Figures 15 and 16. Surprisingly, considering the fact that the 3-26-7#1 cells were oncogenic in hamsters, the histogram analysis of 3-26-7#1 cells was nearly identical to that of normal hamster cells. Most of both cell types were in $G_0 - G_1$ phase at channel number 40 and few cells were in S, G_2 or M phase. These data and the fact that 12 of the 16 newborn hamsters injected with 3-26-7#1 cells failed to develop fibrosarcomas suggested that the cell line was a mixture of transformed and nontransformed cells and

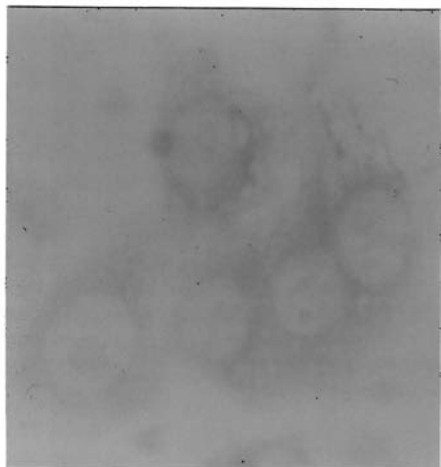
FIGURE 14

Immunoperoxidase stained control cells. HSV positive and negative control cells were stained with the immunoperoxidase method as described in Table 14.

- (a) Uninfected HEp-2 cells stained with rabbit α -333 as the primary antiserum demonstrate the absence of HSV antigens (400X magnification).
- (b) HSV-2 strain 333 infected Vero cells stained with rabbit α -VP 143 as the primary antiserum demonstrate the presence of HSV antigens (400X magnification).

FIGURE 14

a.



b.

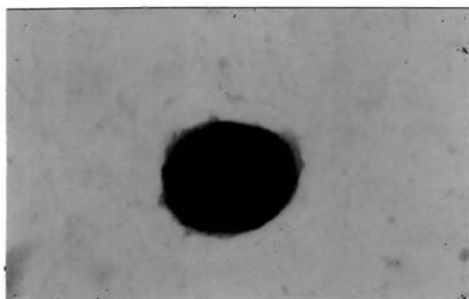


FIGURE 15

Cell sorter analysis of hamster embryo cells. The computer generated histogram, plotting DNA content per cell vs. number of cells, reveals that the majority of cells are diploid and in $G_0 - G_1$ phase.

HAMSTER EMBRYO FIBROBLAST CONTROL, 654/20, PIC 9/20/79 NUM = 35415
TP: COLLINS 11 FILE 12

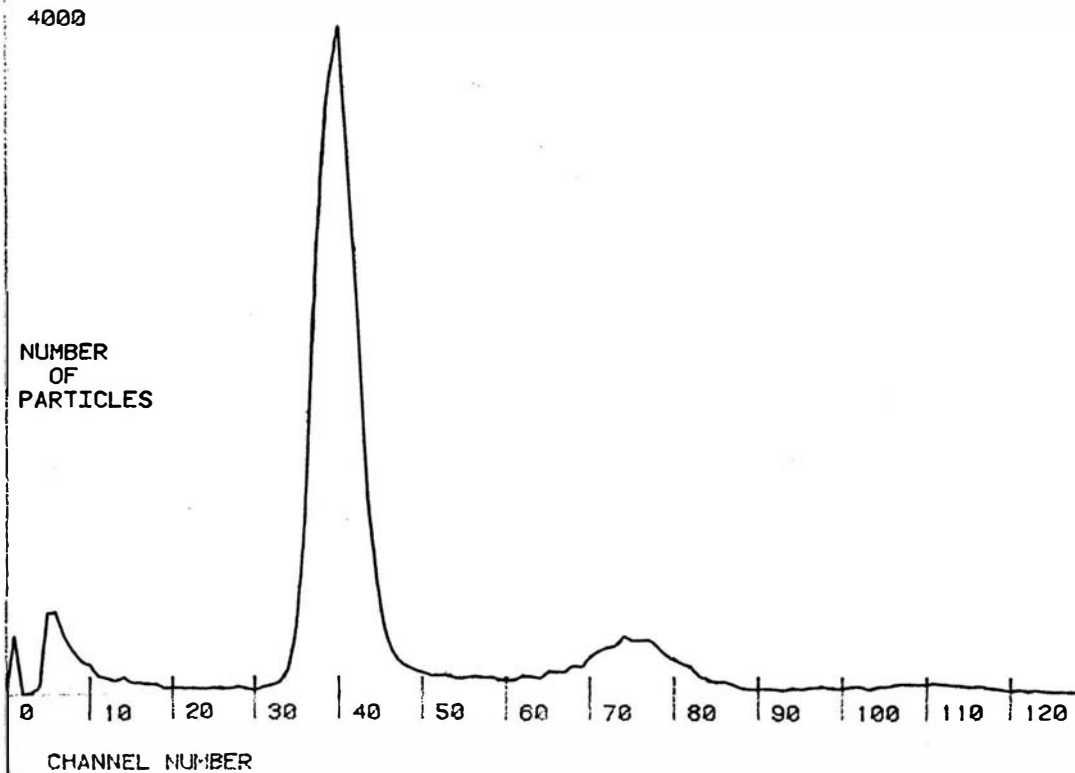
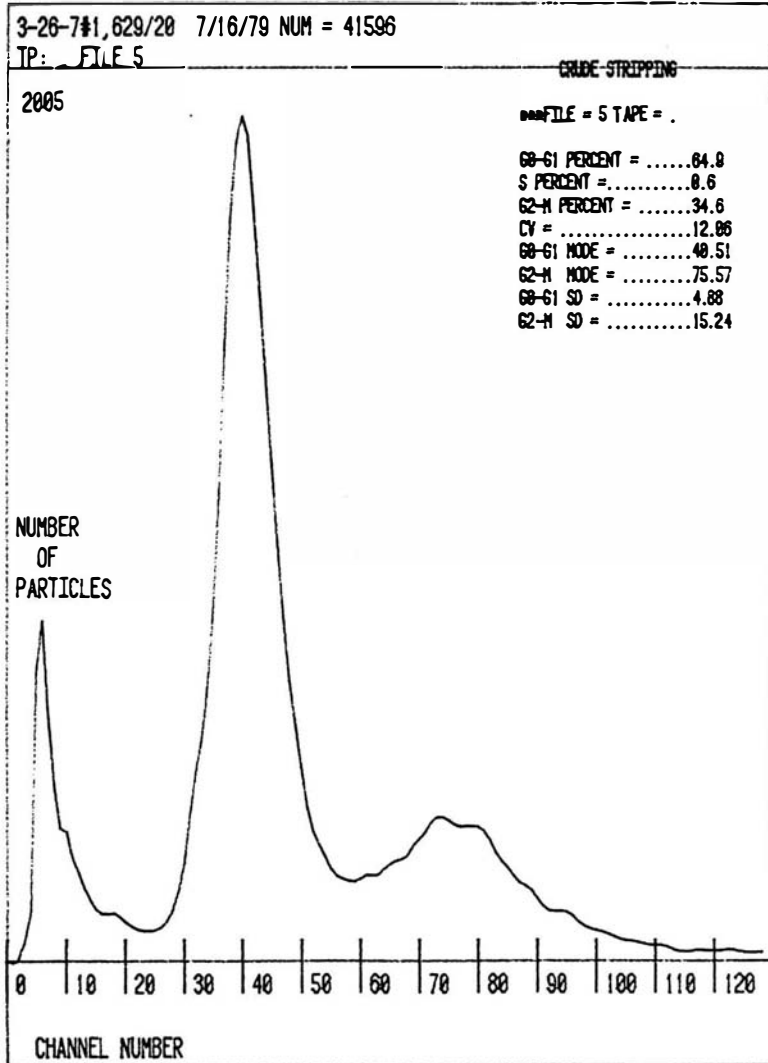


FIGURE 15

FIGURE 16

Cell sorter analysis of 3-26-7#1 cells. The computer generated histogram, plotting DNA content per cell vs. number of cells, reveals that the majority of cells are diploid and in $G_0 - G_1$ phase.

FIGURE 16



not homogenous. Consequently, 3-26-7#1 cells were serially diluted and samples seeded onto a 96 well plate for the purpose of subcloning the cell line. Three different subcloned cell lines originating from single cells of the parental 3-26-7#1 were successfully initiated: 3-26-7#1a, 3-26-7#1b, 3-26-7#1c. These three cell lines were then evaluated by cell sorting analysis. The histograms for these three subcloned cell lines are presented in Figures 17, 18 and 19, respectively. These analyses showed that the parental cell line was, indeed, heterogenous in cell type. Although 3-26-7#1a and 3-26-7#1b cells were similar to the parental 3-26-7#1 cells with the majority of the cells in $G_0 - G_1$ phase, many of the 3-26-7#1c cells had twice as much DNA per cell. Two possible explanations existed: (i) rapid cellular replication placed these cells in the tetraploid G_2-M phase, or (ii) these G_0 aneuploid cells had approximately twice the chromosome number as the other cell lines. However, the point was made that this transformed focus was not a homogenous cell population. Unfortunately, these cell lines were lost to further study due to bacterial contamination.

EFFECT OF TPA ON TRANSFORMATION IN VITRO BY UV-INACTIVATED HSV

Since the addition of TPA application to the HSV + UV in vivo regimen was so successful in promoting the emergence of tumors on mouse lips, a series of experiments was designed to investigate the activity of TPA in vitro. Particular attention was paid to the effect that TPA might have on UV-inactivated HSV transformation of hamster cells. However, prior to investigating that, preliminary experiments were necessary to evaluate the effect of TPA itself upon cells and to arrive at proper dosages for incorporation into growth media.

The first series of experiments were designed to investigate the long-

FIGURE 17

Cell sorter analysis of 3-26-7#1a. The computer generated histogram, plotting DNA content per cell vs. number of cells, reveals that the majority of cells are diploid and in $G_0 - G_1$ phase.

3-26-7 #1A, 054/20, PIC 9/20/79 NUM = 47156
TP: COLLINS 11 FILE 13

4000

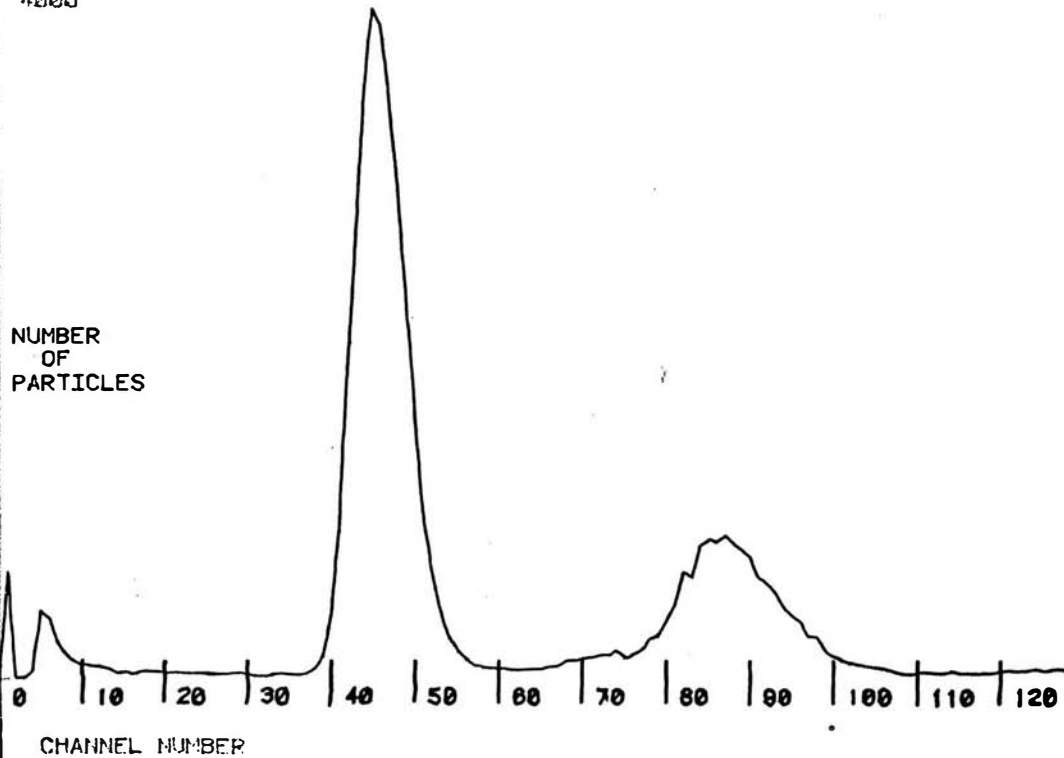


FIGURE 17

FIGURE 18

Cell sorter analysis of 3-26-7#1b cells. The computer generated histogram, plotting DNA content per cell vs. number of cells, reveals that the majority of cells are diploid and in $G_0 - G_1$ phase.

3-26-7 #1B, 654/20, PIC 9/20/79 NUM = 41062
TP: COLLINS 11 FILE 14

4000

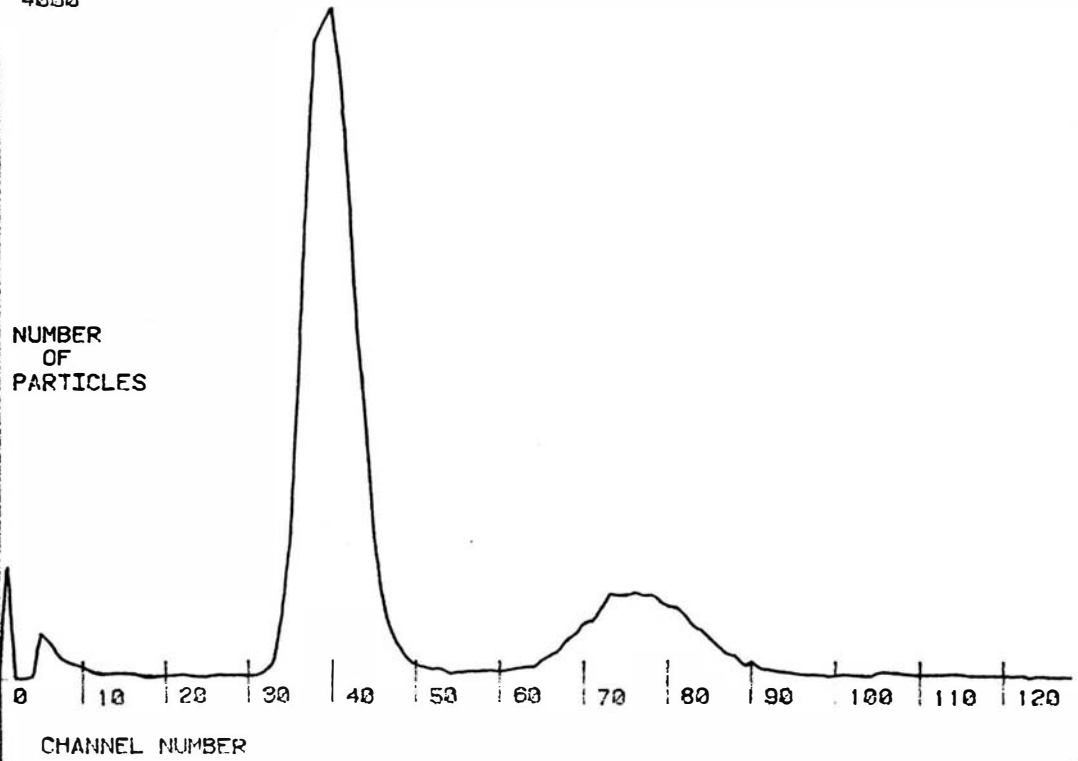


FIGURE 18

FIGURE 19

Cell sorter analysis of 3-26-7#1c cells. The computer generated histogram, plotting DNA content per cell vs. number of cells, reveals that the majority of cells have twice the DNA content per cell as $G_0 - G_1$ cells.

3-26-7 #1C, 654/20, PIC 9/20/79 NUM = 7726
TP: COLLINS 11 FILE 15

4000

NUMBER
OF
PARTICLES

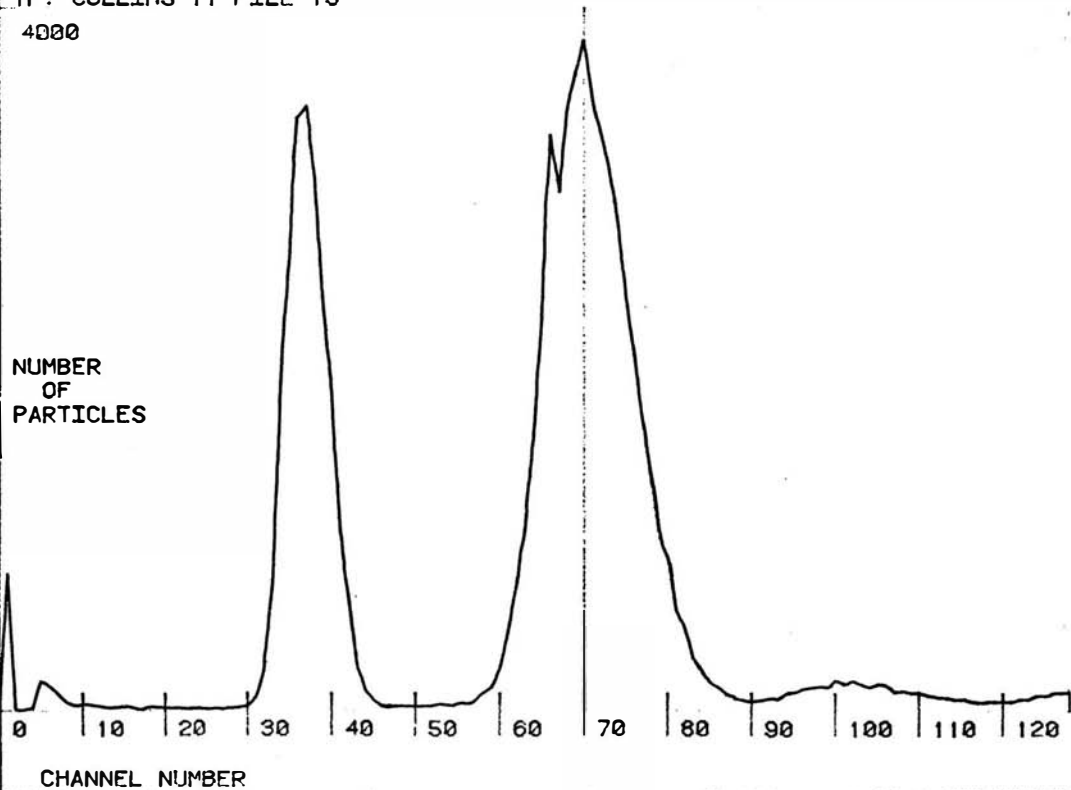


FIGURE 19

term effect (one month or more) of various concentrations of TPA in the growth media on the expression of HSV genome and on protein synthesis in the transformed cells 333-8-9. This knowledge was essential for its use in later 21 day transformation experiments. The concentrations of TPA tested were 0.0 ug/ml, 1.0 ug/ml, 0.1 ug/ml, 0.01 ug/ml and 0.001 ug/ml of media. Three separate parameters in 333-8-9 cells were investigated, namely: (i) quantitative or qualitative variations in soluble protein production as measured by two-dimensional gel electrophoresis, (ii) changes in DNA content per cell as analyzed by the cell sorter and (iii) variations in HSV antigen expression as determined by immunoperoxidase staining.

For two-dimensional (2-D) gel electrophoresis, samples of HEF cells and the five samples of 333-8-9 cells grown under various concentrations of TPA were seeded in equal numbers (1×10^4 cells) onto separate Petri dishes. The cells were incubated at 37°C in the presence of 20 μ c of S^{35} methionine/ml of media. After 48 hours, the cells were harvested in lysis buffer and 5 μ l of this sample was TCA precipitated and counts per minute (cpm) determined in a scintillation counter. An equal number of cpm was not obtained for the different samples (Table 15). Apparently, maximal protein synthesis occurred when TPA was present at a concentration of 0.1 ug/ml of media. Since this increase could be induction of new proteins or simply an increase in normal proteins, other aliquots of the same six samples were subjected to 2-D gel electrophoresis. Autoradiographs from equilibrium (25 μ l/sample and 145,960 cpm/sample) as well as non-equilibrium (145,960 cpm/sample) 2-D gels were scrutinized for qualitative or quantitative changes in radiolabeled protein content. Comparisons of the autoradiographs from the 2-D gels performed on HEF and 333-8-9 cells revealed most of the labeled proteins to be similar in their pI points and

TABLE 15 EFFECT OF TPA IN THE MEDIA OF CULTURED 333-8-9 CELLS

<u>SAMPLE^a</u>	<u>T.C.A. PRECIPITABLE cpm/25 u1^b</u>
HEF	218,260
333-8-9	279,640
333-8-9 with TPA 1/ug/ml	274,145
333-8-9 with TPA 0.1 ug/ml	736,670
333-8-9 with TPA 0.01 ug/ml	435,200
333-8-9 with TPA 0.001 ug/ml	145,960

- a. Each cell sample (1×10^4 cells/60 mm Petri dish) was incubated for 48 hours at 37°C in the presence of 20 μ c of S^{35} methionine per ml of media.
- b. The cells were harvested in 100 μ l of lysis buffer and 5 μ l of this sample was evaluated for cpm following TCA precipitation.

molecular weights. However, consistently there appeared two proteins in the HEF cells that were not present in the 333-8-9 cells (Fig. 20a, b, c, d). These proteins had molecular weights of 68,000 and 43,000. In contrast, comparisons of the autoradiographs of the gels performed on 333-8-9 cells maintained under various concentrations of TPA in the media revealed no qualitative differences. However, there appeared to be an increase in the amount of one protein in the samples grown under TPA concentrations of 0.1 ug/ml and 0.01 ug/ml (Fig. 20b, c, d). This protein had a molecular weight of 50,000. A similar quantitative increase was not seen in those samples grown under TPA concentrations of 1.0 ug/ml or 0.001 ug/ml. Thus, the increase in TCA precipitable counts among 333-8-9 cells grown under TPA at concentrations of 0.1 ug/ml or 0.01 ug/ml was not a qualitative induction of new protein but rather a quantitative increase of at least one major protein.

Interest in TPA at a concentration of 0.1 ug/ml of media for future in vitro transformation studies began during the 2-D gel experiments. That interest was increased by the cell sorter analysis of 333-8-9 cells cultured under the same five concentrations of TPA as used in the previous experiment. The DNA histograms are presented in Figures 21a-e representing the five increasing concentrations of TPA, respectively. By summing the percentages of cells in S, G₂ and M phases, it is possible to get an indication of the replicative activity of each of the five cell samples. In order of increasing concentrations of TPA from 0.0 ug/ml to 1.0 ug/ml, these replicative indexes are 54.8, 54.7, 59.6, 62.2 and 53.9, respectively. The replicative activity of 333-8-9 cells was, therefore, maximal at a TPA concentration of 0.1 ug/ml of media.

FIGURE 20

Autoradiographs from the 2-D gels of HEF and 333-8-9 cells. HEF cells and 333-8-9 cells were radiolabeled in culture and subjected to 2-D gel electrophoresis as described in Table 15 and in its section of Materials and Methods.

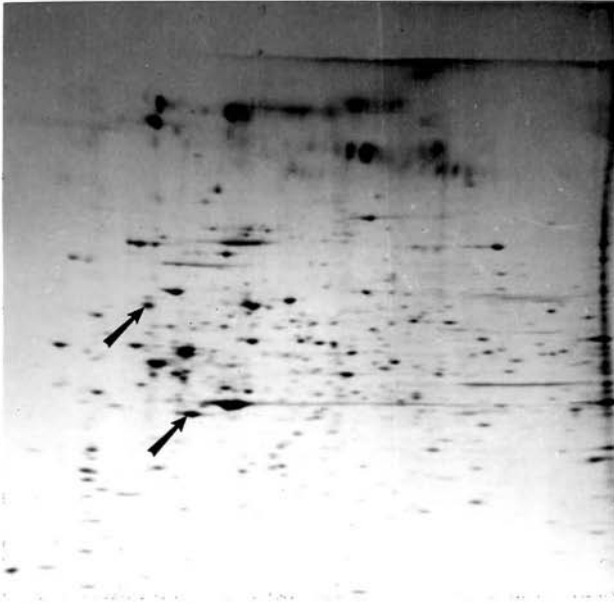
- (a) The autoradiographs of normal HEF cells demonstrate the presence of two proteins not seen in any of the 333-8-9 protein gels (arrows).
- (b,c,d.) The autoradiographs of 333-8-9 cells grown under TPA at concentrations of 0.0 ug/ml, 0.1 ug/ml and 0.01 ug/ml, respectively. The qualitative increase in one particular protein is demonstrated in the later two gels (arrows).

Each IEF gel was loaded with 145,960 cpm/ sample and run under equilibrium conditions (approximately 100,000 cpm/ug of protein).

The upper right corner of each figure represents the top (basic end, pH7) of the 1st dimension gel and the upper left corner was the bottom (acidic end, PH5). The isoelectric focusing was from right to left. The SDS-PAGE separation was from top to bottom through an exponential gradient of 7% to 20% acrylamide.

FIGURE 20

a.



b.

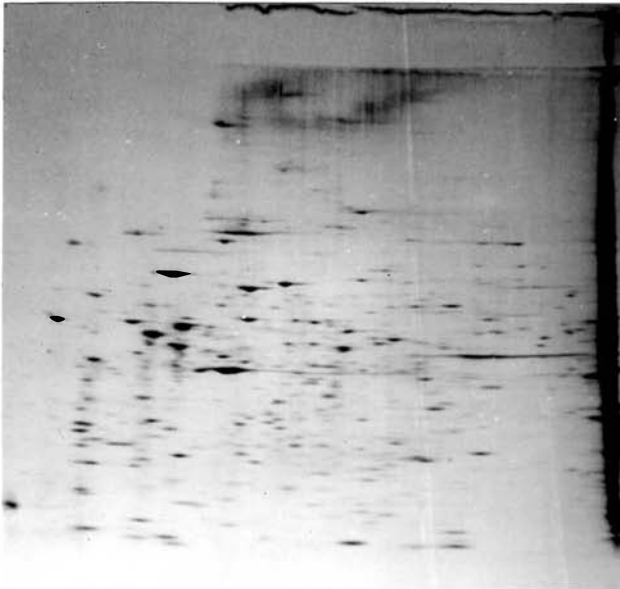
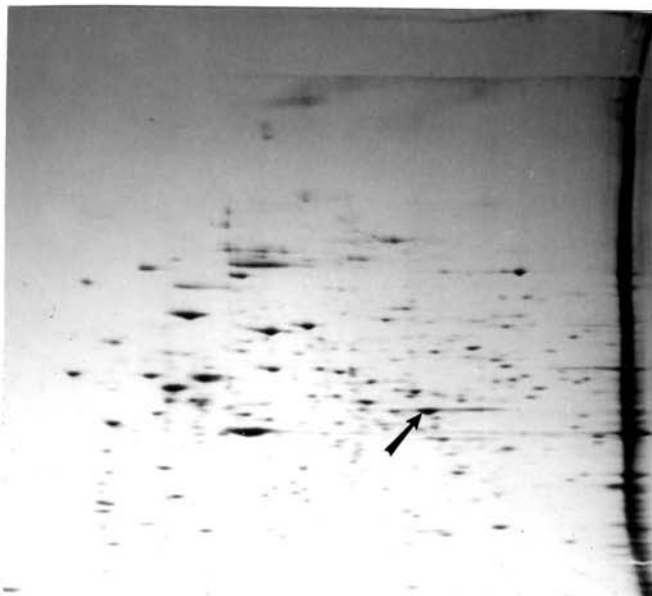


FIGURE 20 (continued)

c.



d.



FIGURE 21

Cell sorter analysis of 333-8-9 cells cultured with various concentrations of TPA. The computer generated histograms, a-e, represent 333-8-9 cells cultured with TPA in the media at concentrations of 0.0 ug/ml, 0.001 ug/ml, 0.01 ug/ml, 0.1 ug/ml or 1.0 ug/ml, respectively. The replicative activities (S percent + G_2M percent) are 54.8, 54.7, 59.6, 62.2 and 53.9, respectively.

FIGURE 21a

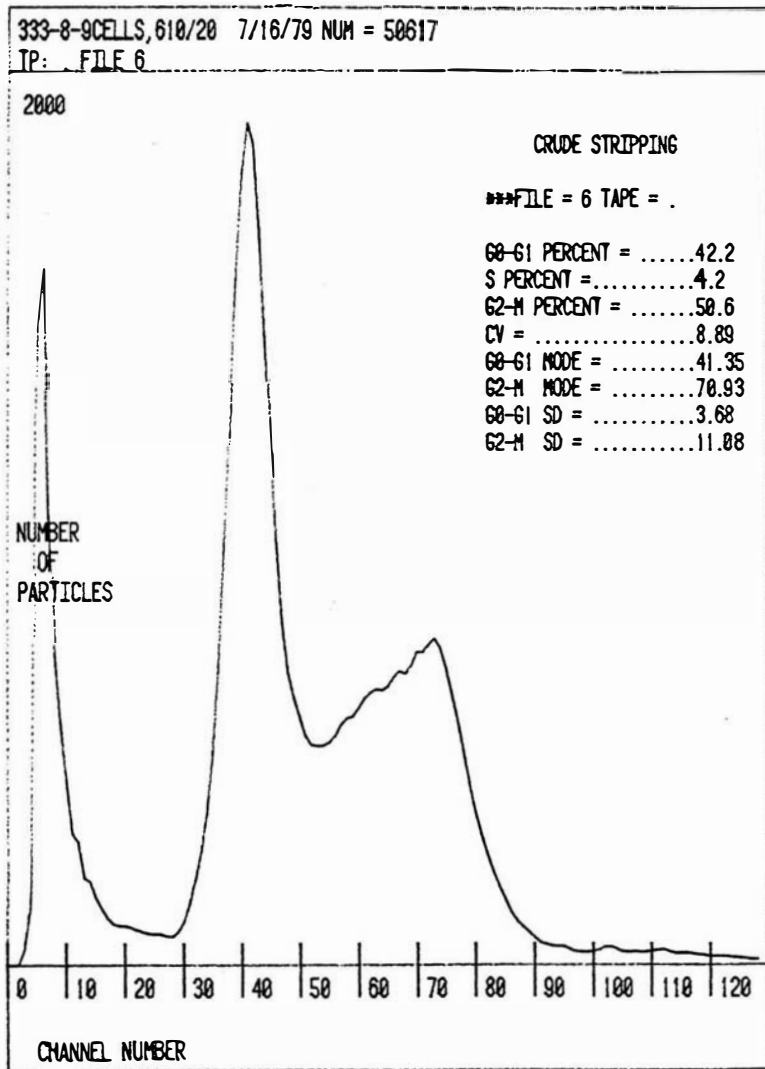


FIGURE 21b

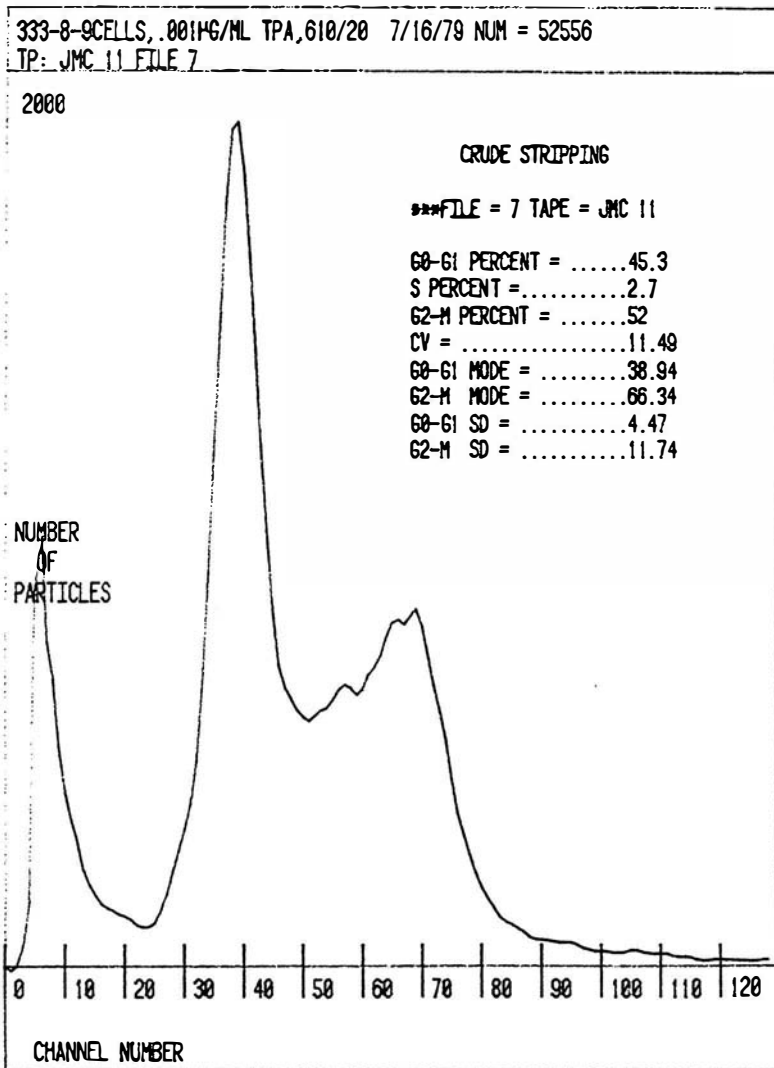


FIGURE 21c

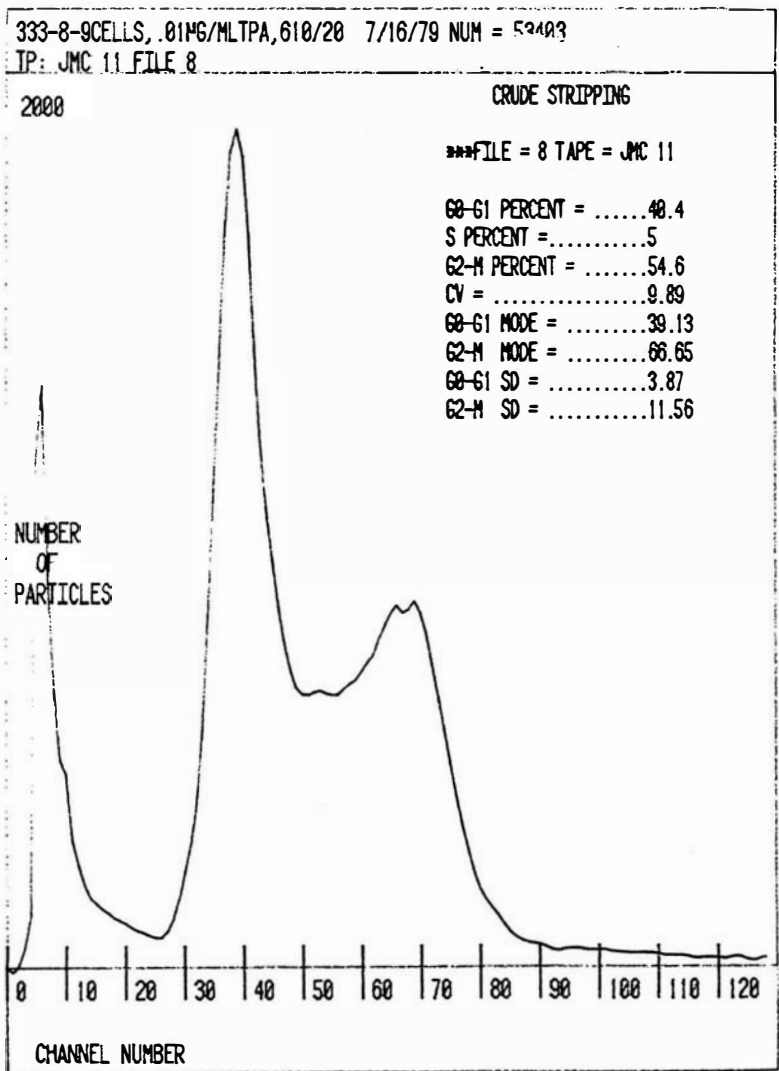


FIGURE 21d

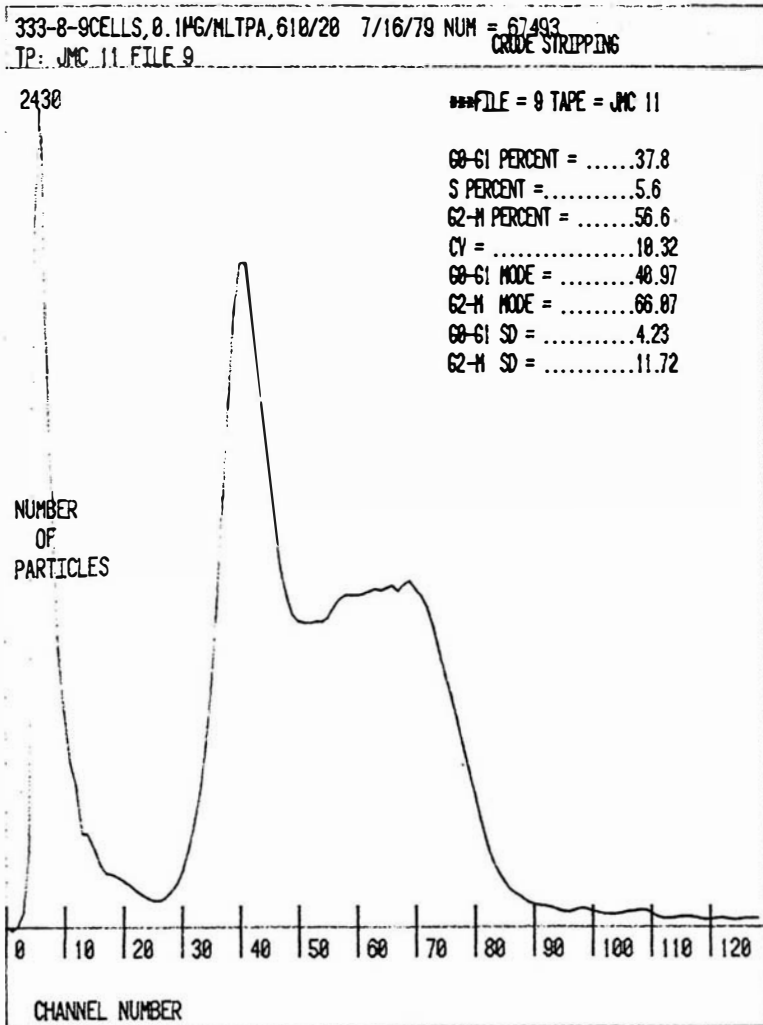
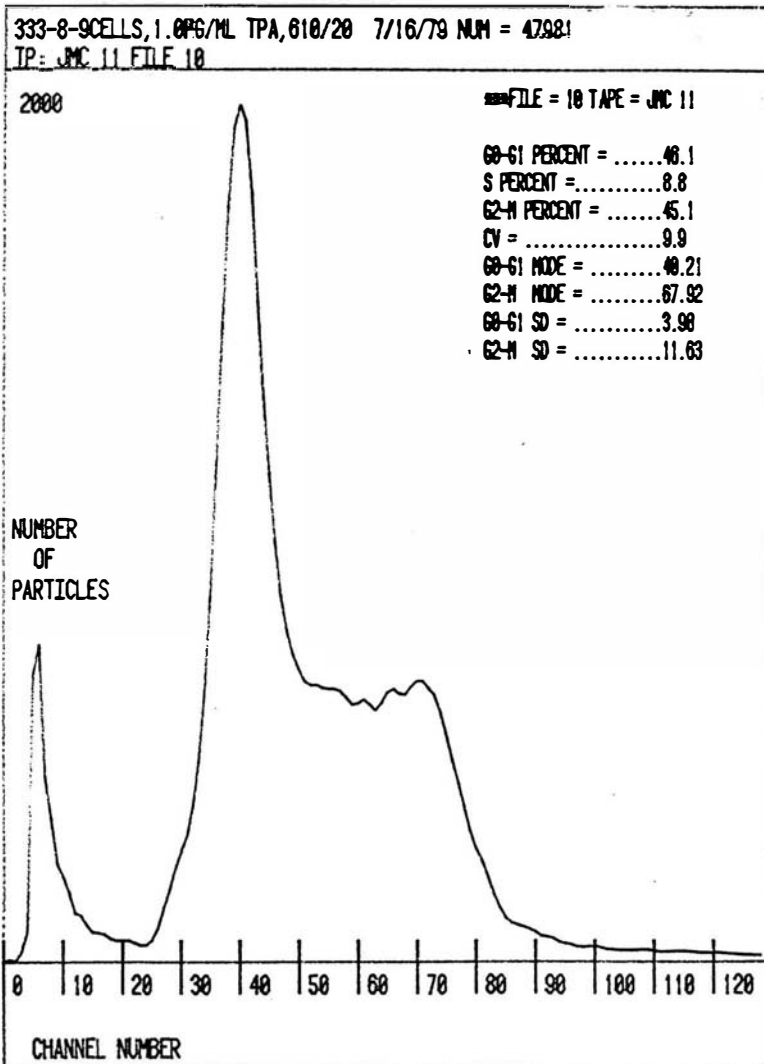


FIGURE 21e



Following investigations of the effect of TPA upon cultured 333-8-9 cells by 2-D gel electrophoresis and cell sorter analysis, the third and final sets of experiments were performed. These were designed to evaluate any variations in HSV expression in 333-8-9 cells cultured with TPA at the same five concentrations as in the previous two studies. The presence of HSV antigens was determined by immunoperoxidase staining using seven different antisera (Table 16). All but one of the antisera, stained every sample (Fig. 22); however, trends were not obvious. Apparently, any quantitative or qualitative differences in HSV antigen expression among these cells were not detectable by immunoperoxidase staining or, conversely, the presence or absence of TPA in the growth media had little effect on HSV antigen expression by 333-8-9 cells. Since HSV antigens were present in 333-8-9 cells, it was also possible to detect HSV mRNA within these cells by in situ hybridization (Fig. 23).

At the termination of these three series of experiments investigating the effect of TPA by itself on transformed cells, it was clear that the agent caused increased protein synthesis and cellular replication. This effect was maximal at TPA concentration of 0.1 ug/ml of media. This concentration of TPA had been used in previous in vitro studies for unexplained reasons and was chosen for use in UV-inactivated HSV transformation experiments within this dissertation because of data just presented.¹⁶⁰

In those experiments designed to measure the effect of TPA on UV-inactivated HSV transformation of hamster cells, a protocol was followed exactly as described earlier for transformation experiments in vitro except for the addition of TPA at a concentration of 0.1 ug/ml to the media. The variable then became the lapsed time postinfection that the TPA was added and this varied from 1 hour to 96 hours. Results are presented in Table 17.

TABLE 16 PRESENCE OF HSV ANTIGENS IN CELLS CULTURED WITH TPA AS DETERMINED
BY IMMUNOPEROXIDASE STAINING UTILIZING VARIOUS ANTISERA^a

CELLS ^b	RABBIT α - HSV-2/MS	RABBIT α -333	RABBIT α -333 EARLY 4 HR. ANTIGEN	RABBIT α - VP 143 (333)	HAMSTER α -333	HAMSTER α -TUMOR	MOUSE α - HSV+UV+TPA TUMOR
333-8-9	+(1)	+(4)	+(3)	+(6)	+(4)	+(2)	-
333-8-9 with TPA 1 ug/ml	+(2)	+(3)	+(2)	(1)	+(6)	+(2)	-
333-8-9 with TPA 0.1 ug/ml	+(2)	+(3)	+(2)	+(4)	+(6)	+(4)	-
333-8-9 with TPA 0.01 ug/ml	+(3)	+(5)	+(1)	+(7)	+(5)	+(2)	-
222-8-9 with TPA 0.001 ug/ml	+(2)	+(3)	+(3)	+(3)	+(4)	+(1)	-

a. Refer to Table 14 for details.

b. The UV-inactivated HSV transformed cell line, 333-8-9, was cultured under various concentrations of TPA prior to fixation in acetone.

FIGURE 22

Presence of HSV antigens in 333-8-9 cells. The transformed 333-8-9 cells were stained with the immunoperoxidase method utilizing hamster α -tumor antiserum as described in Table 16. One cell demonstrating positive staining adjacent to two negatively stained cells are shown (400X magnification).

FIGURE 22

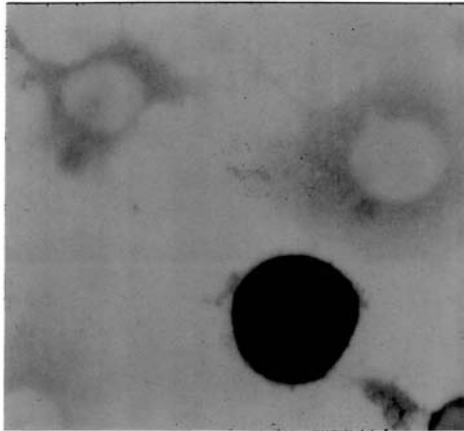


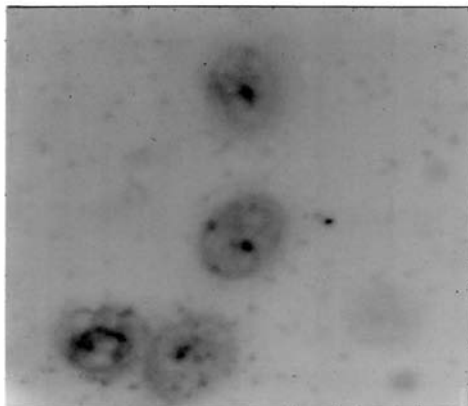
FIGURE 23

Presence of HSV specific mRNA in 333-8-9 cells. The transformed 333-8-9 cells, along with HSV positive and negative controls, were subjected to in situ hybridization with radiolabeled HSV DNA as described in its section of Materials and Methods.

- (a) Uninfected HEp-2 cells were used as negative control cells and lacked HSV mRNA in the cytoplasm.
- (b) HSV-2 strain 333 infected HEp-2 cells were used as positive control cells and showed the presence of mRNA in the cytoplasm.
- (c) Transformed 333-8-9 cells also showed the presence of mRNA in the cytoplasm.

FIGURE 23

a.



b.

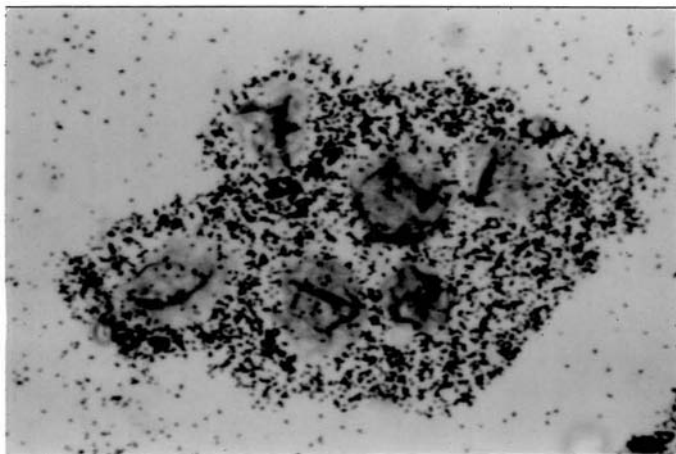


FIGURE 23 (continued)

c.

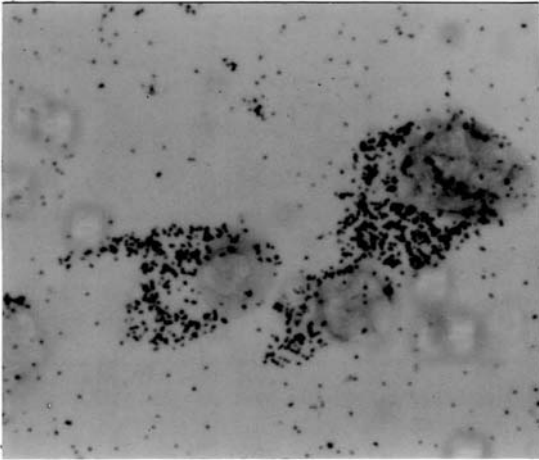


TABLE 17 EFFECT OF TPA UPON TRANSFORMATION OF HAMSTER EMBRYO FIBROBLASTS IN VITRO BY UV INACTIVATED HSV^a

NUMBER OF T-75 FLASKS	333 INOCULUM PFU/ml	MINUTES OF UV-IRRADIATION OF 333 INOCULUM	HOURS POSTINOCULATION TPA ADDED TO MEDIA	# OF TRANSFORMED FOCI/ 1.1 x 10 ⁶ CELLS	TRANSFORMATION EFFICIENCY FFU/PFU
15	-	-	1	0.00	
15	-	-	24	0.07	
20	1.7 x 10 ⁷	-	1	0.60	1.8 x 10 ⁻⁷
45	1.7 x 10 ⁷	7	24	2.10	6.4 x 10 ⁻⁷
10	1.7 x 10 ⁷	7	48	5.62	1.7 x 10 ⁻⁶
10	1.7 x 10 ⁷	7	72	3.80	1.2 x 10 ⁻⁶
10	1.7 x 10 ⁷	7	96	2.91	8.8 x 10 ⁻⁷
20	5.2 x 10 ⁷	8	1	0.30	9.0 x 10 ⁻⁸

a. See footnotes of Table 13 for details. Media contained TPA (0.1 ug/ml) dissolved in DMSO (1 mg/ml).

The effect of TPA's addition to the media was dependent upon the time of its administration (Fig. 24). When the TPA was added immediately after the 1 hour viral attachment period as the cells were seeded onto the T-75 flasks, the number of foci that emerged was smaller than the number derived from non-TPA containing experiments (Table 13). However, as the addition of the TPA to the media was delayed, increasingly greater numbers of foci per flask emerged. The effect peaked 48 hours postinoculation and then began to decrease. The maximum average number of foci per flask in these experiments utilizing TPA in the media was 5.62 (Fig. 25). This represented almost a two-fold increase as compared to non-TPA containing flasks (Table 13). Thus, TPA not only promoted tumor induction in vivo with concomitant HSV infection and UV exposure, but also increased transformation efficiency in vitro with UV-inactivated HSV.

FIGURE 24

Efficiency of transforming HEF cells with the presence of TPA in the media. HSV-2 strain 333 was UV-irradiated for 7 minutes and used to infect HEF cells as described in Table 17. The efficiency of transformation, in terms of average number of transformed foci per 1.1×10^6 cells, was maximal when the TPA (0.1 ug/ml) was added 48 hours postinoculation.

FIGURE 24

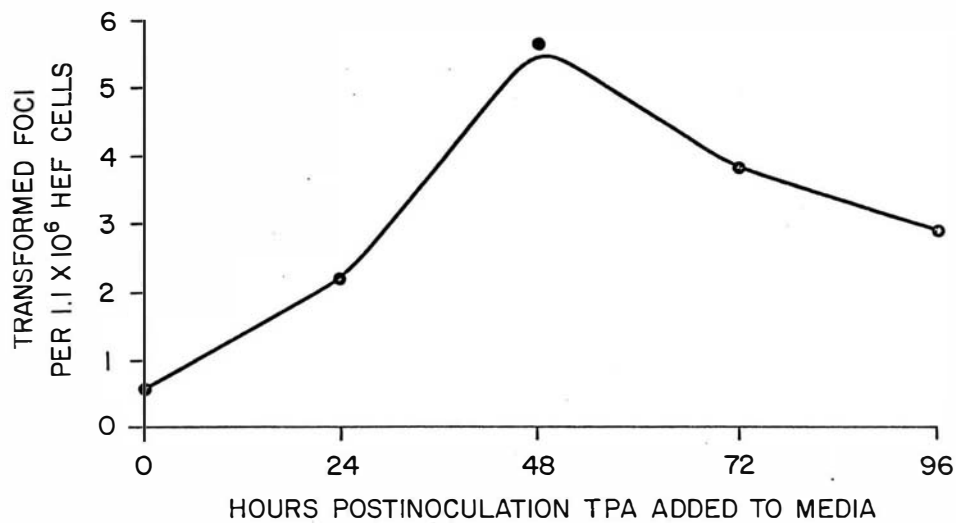


FIGURE 25

Comparison of flasks from UV-inactivated HSV transformation experiments. On the left is a flask that was seeded with uninfected HEF cells and maintained in culture for 21 days. No foci were evident and microscopic examination revealed only degenerated cellular debris. The middle flask was seeded with HEF cells infected with UV-inactivated (7 min.) HSV. Foci were evident that microscopically appeared fibroblastic or epithelioid. On the right is a flask that was seeded with HEF cells infected with UV-inactivated (7 min.) HSV and maintained in media containing TPA (0.1 ug/ml). An increased number of foci were evident.

FIGURE 25



DISCUSSION

IN VIVO TRANSFORMATION

Certain Herpesviruses have long been associated with several human neoplasias (uterine cervical carcinoma, Burkitt's lymphoma, and nasopharyngeal carcinoma)⁴³, but the role of HSV in the development of human cancer has been very difficult to demonstrate. The neoplastic transforming process is a very complex series of events and HSV may act in concert with other factors to induce cancer. Therefore, an in vivo model was designed combining HSV infection with exposure to certain environmental cofactors (a chemical carcinogen or UV-irradiation with or without a tumor promoting agent). Prior to this model, the only in vivo evidence for HSV's direct role in oncogenesis was the subcutaneous injection of cells transformed in vitro. Thus, an appropriate model was designed and, in addition, many interesting observations relating to peripheral topics were made during the process. For example, Table 1 implies that the incidence of macroscopically observable herpetic lip lesions in mice was related to the titer of the viral inoculum as long as one accepts the fact that younger mice generally develop a higher incidence of lesions than older mice when inoculated with identical virus preparations.²⁷ This titer-dependent response might explain how 40-90% of the human population have serologic evidence of prior HSV-1 infection in the apparent absence of a primary HSV-1 induced gingivostomatitis by theorizing that the titer of the virus during primary exposure was low and the subsequent infection was subclinical.⁸ Another example of an interesting observation relates to the fact that physical trauma and/or UV exposure are common causes given by patients for the recrudescence of herpes labialis. However, in our mouse model

repeated abrasion and UV exposure following a herpetic lip infection failed to activate recurrence of clinically evident lesions (Table 8). Thus, a difference may exist between HSV latency in the mouse as compared to latency in humans and is in agreement with other reports on HSV latency in that a good animal model that mimics human latency is not available. The remaining observations relate more directly to the field of interest investigated in this dissertation.

Oncogenic transformation of a host cell requires the survival and uncontrolled replication of that cell. Therefore, an alteration in the normal cytolytic course of HSV infection must occur in order for the host cell to survive. When an unaltered HSV infection was initiated prior to or during the application of DMBA, a decrease in tumor yield resulted as compared to uninfected DMBA treated animals (Table 5, 6, 7). One possible explanation is that the lytic infection by HSV destroyed the cells committed by DMBA to oncogenic transformation. In a similar vein, repeated HSV infections failed to induce tumor development on mouse lips (Table 2), even though repeated exposure or persistent infections by HSV have been hypothesized as a possible mechanism for oncogenic transformation. The hypotheses state that HSV latency, repeated reactivation or defective viral production might produce alteration of the viral genome, thus inhibiting the lytic functions of HSV and allowing expression of the oncogenic capability. In the studies reported in this thesis, it was necessary to inhibit the lytic functions of HSV by UV-irradiation. The data presented in Figure 9 demonstrate that an optimal exposure time to UV radiation exists in order to inactivate HSV infectivity. Rapp has previously presented similar data and shown that 6 to 8 minutes of UV exposure results in maximal transformation in vitro.⁷¹ When HSV was irradiated for 6 minutes prior to inoculation

on the lips of mice, no herpetic lesions or tumors arose (Table 9). Thus, a gene function necessary for HSV infectivity was inactivated by exposure to UV radiation. In addition, repeated HSV infections with superimposed UV exposure on days 3, 4, 5 and 6 when viral titers were maximal in the tissue failed to induce tumor development (Table 9). These data, however, do not rule out the possibility that HSV may be oncogenic for at least two reasons: (i) the duration of observation may have been too short and (ii) known carcinogens applied to abraded mouse lips also failed to induce tumors under certain circumstances. Mice that were subjected to (i) UV-irradiation daily for 7 days on two separate occasions (Table 9), (ii) various concentrations of 3-MC for as many as six applications (Table 3) and (iii) 15 ng of DMBA per application for as many as 10 applications (Table 4) did not develop tumors. With DMBA as the carcinogen, tumors emerged only when higher concentrations and more frequent applications were used (Table 4). This dose dependent response is analogous to human data with cigarette smoking which shows an increased incidence of lung cancer as the consumption of tobacco increases. Since DMBA and cigarette smoke have been shown capable of initiation and promotion, it is reasonable that lower dosages and less frequent exposures of DMBA are subcarcinogenic. A similar situation may exist in the studies of HSV + UV induced neoplasia in the mouse model. At lower frequencies of exposure and without adequate promotion, HSV + UV treatments in vivo were subcarcinogenic (Table 9). Although the combination of HSV + UV resulted in microscopic dysplasia, malignant tumors developed only when the repeated application of TPA was added to the HSV + UV protocol. This emphasizes the possible multisteped and multifactorial nature of carcinogenesis, as well, as the common requirement for initiation and promotion delivered in a particular sequence.

The results obtained in these studies show that squamous cell carcinomas do develop after HSV + UV + TPA exposure (Table 10). This represents the first data implicating HSV as a cocarcinogen in an animal model. Analogous to the classical two stage chemical carcinogenesis model, the combination of HSV infection and UV-irradiation on the days of maximum tissue titer was considered the initiator and TPA the promoter. However, different latent periods before tumor emergence existed in the two models: 35-49 days for DMBA (Table 4) and 76 days for HSV + UV + TPA (Table 10). In addition, the ratio of papilloma development to squamous cell carcinoma development varied between the two models: 15 to 35 (70% cancers) for DMBA (Table 4) and 24 to 6 (20% cancers) for HSV + UV + TPA (Table 10). Thus, comparison of the two models indicates that, under the conditions of this study, DMBA was capable of inducing a higher incidence of squamous cell carcinomas with a shorter latent period than HSV + UV + TPA treatments ($p < .001$). In agreement with previous studies,¹¹⁹ these studies showed that DMBA represents a potent carcinogen.

Important theoretical explanations emerge when HSV + UV is considered the initiating agent since most studies currently view alterations of cellular DNA as the most attractive mechanism for the initiation of the carcinogenic process. One explanation would be that UV-irradiation acts as a mutagen inducing DNA alterations as described by Parrish¹³⁶ and that HSV acts as an inhibitor of DNA repair by disruption of host DNA as described by Aurelian.⁷⁴ However, another explanation could be that UV-irradiation simply destroys the cytolytic capabilities of HSV (Fig. 9) and allows the virus to express its inherent oncogenic capability. The author prefers the later explanation for several reasons. In these studies, UV exposure on those days immediately following HSV infection

increased the severity and delayed the healing of lip lesions (Fig. 3). Furthermore, analysis of the serum neutralizing antibody titers from the mice in the HSV + UV + TPA group showed a significant increase among those animals bearing tumors (Table 12). In addition, four of these tumors (3 papillomas and 1 squamous cell carcinoma) stained positive for the presence of HSV antigens by immunoperoxidase staining using hamster α -333, mouse α -333 and mouse α -HSV + UV + TPA tumor antisera. The increased antibody titers in tumor bearing mice and the presence of HSV antigens in these tumors implied a relationship between HSV and tumor development. However, HSV + TPA and UV + TPA treatments resulted in development of a few papillomas but no squamous cell carcinomas (Table 10). In summary, these data suggest that HSV contributed in a positive way to the development of squamous cell carcinomas following HSV + UV + TPA treatment and, in that regard, may be considered a cocarcinogen.

One final hypothesis of the mode of action of HSV as a cocarcinogen is that HSV infection activates endogenous type C RNA tumor viruses. Proving or disproving this hypothesis was peripheral to the studies documented within this thesis. Type C RNA tumor viruses are released from mouse and hamster cells after infection with UV-inactivated HSV.¹⁵¹ Proof that HSV infection or HSV transformation activates RNA tumor viruses is difficult to demonstrate since expression of the type C tumor viruses is not required for transformation, and activation of the virus is not necessary for the clinical induction of leukemia. Research to date has always linked these viruses to the induction of leukemias and lymphomas and never to the development of squamous cell carcinomas or sarcomas. In addition, Hampar has suggested that the release of type C RNA virus may be the result of transformation and not the cause.¹⁵⁹

IN VITRO TRANSFORMATION

Previous studies have shown that hamster, mouse and human cells are capable of undergoing biochemical as well as oncogenic transformation after infection with UV-inactivated HSV. These studies have also elucidated that the optimal time for UV-inactivation of HSV is six or eight minutes at $42 \text{ ergs/mm}^2/\text{s}$. The efficiency of transformation following these exposures yielded a maximum of 4×10^{-7} FFU/PFU.⁴¹ In comparison, our studies have shown the following efficiencies of transformation (FFU/PFU): 2.9×10^{-7} , 9.9×10^{-7} and 3.9×10^{-7} for 6, 7 and 8 minutes of UV exposure at $42 \text{ ergs/mm}^2/\text{s}$ of the HSV inoculum, respectively (Table 13). This represents 0.9, 3.0, and 1.3 FFU/ 10^6 cells, respectively. Furthermore, if TPA (0.1 ug/ml) was added to the growth medium 48 hours postinoculation, a transformation efficiency (FFU/PFU) of 1.7×10^{-6} was obtained (Table 17). This represents 5.1 FFU/ 10^6 cells. Thus, with 7 minutes of UV exposure which has not previously been reported, the transformation efficiency was improved two and one-half times that reported previously as the maximum in terms of FFU/PFU. If TPA was added 48 hours postinoculation to this 7 minutes regimen, the transformation efficiency was nearly doubled again, reaching five times that previously reported. Since other studies have used MOIs of 1-5 and Rapp has reported 1 FFU/ 5×10^6 cells, these transformation efficiencies represent a 1.5-25 fold increase in terms of FFU/cell. Although the reasons for this increased efficiency was most probably the critical nature of the 7 minute UV exposure time and the addition of TPA to the medium, the importance of the strict maintenance of neutral pH and the number of cells seeded per T-75 flask cannot be understated. Without the knowledge of the minute experimental protocols followed by others, comparison of the many variables is impossible.

Foci derived from the UV-inactivated HSV transformation experiments were subcultured and shown to possess properties similar to other transformed cell lines. Rapp has previously reported that fibroblastic foci and epithelial foci produce fibrosarcomas and adenocarcinomas, respectively, when tested for oncogenicity in newborn hamsters.⁶⁰ The fibroblastic transformed cell line, 3-26-7#1, was shown in these studies to produce fibrosarcomas when injected into hamsters, thus agreeing with Rapp's findings. The pooled serum from these animals contained antibodies directed against HSV and was shown to be capable of staining HSV infected HEF, HEP-2 and Vero cells when used as the primary antiserum of the immunoperoxidase stain (Table 14). Furthermore, outgrowths from explanted hamster tumors as well as the 3-26-7#1 cells demonstrated the presence of HSV antigens when stained with a variety of antisera against 333 or its early proteins (Table 14). These results, agreeing with previous studies,⁴³ demonstrate that UV-inactivated HSV transformed cells carry on their surface HSV antigens that are numerous enough to be immunogenic. However, as can be seen in Table 14, not all of the cells at any given time expressed HSV antigens. This may be explained by the hypothesis that antigen expression is cell cycle dependent. Although this hypothesis was not directly tested in these studies, it was observed that most of the cells expressing HSV antigens were rounded up as if they were entering G₂-M phase. This observation combined with cell sorter data showing less than 34.6% of the cells in G₂-M phase at a given time (Fig. 16) supports this hypothesis. In addition, later studies (Figs. 17, 18, 19) showed that the 3-26-7#1 cell line was heterogenous in cell type and may explain, further, why so few of the cells expressed HSV antigen.

Another UV-inactivated HSV transformed cell line, 333-8-9, had many properties similar to 3-26-7#1 cells. It had previously been shown to be oncogenic in hamsters and to express HSV antigens on its surface. In our studies, HSV antigen expression by 333-8-9 cells was reiterated (Table 16, Fig. 22) and the cells were shown to possess HSV specific mRNA in their cytoplasm by in situ hybridization (Fig. 23). The presence of HSV specific protein and mRNA implies that at least a fragment of HSV DNA is present, transcribed and translated within these cells. Furthermore, the 333-8-9 cells were shown by 2-D gel electrophoresis to lack two major proteins that are possessed by normal hamster cells. However, identification and characterization of these proteins is necessary before their importance can be determined.

Two final topics deserve mention. These are the molecular actions of TPA and the phenomenon of "spontaneous" foci arising in vitro. It was clearly shown in these studies that TPA was capable of inducing a qualitative increase in protein synthesis (Table 15, Fig. 20) and facilitating cellular replication (Fig. 21) when used at a certain concentration in vitro (0.1 ug/ml). However, at higher concentrations (1.0 ug/ml), these effects were not observed. TPA, noted for its irritating effect on skin, may have toxic activities at higher concentrations. The enhancement by TPA at optimal concentrations of UV-inactivated HSV transformation of hamster cells has already been discussed (Table 17). In this action, TPA may assist in the incorporation of HSV genome into the host cell or may depress a persisting HSV genome as previously suggested by Zur Hausen;¹⁶⁰ but certainly TPA does not simply increase cellular replication in vitro. For example, "spontaneous" foci, possibly representing some form of smooth surface tumorigenesis,¹⁶¹ arose in uninfected flasks but at the very low

incidence of 0.09 foci per 1.1×10^6 cells (Table 13); this incidence decreased to 0 and 0.07 foci when TPA (0.1 ug/ml) was added to the media 1 hour and 24 hours postinoculation, respectively (Table 17). In summary, a 36 fold increase in the "spontaneous" rate of foci development was seen when the hamster cells were infected with UV-irradiated HSV-2 (7 min) and a 62 fold increase emerged when TPA was added to the growth medium at 48 hours postinoculation (Tables 13 & 17).

CONCLUSIONS

The significance of these results rests with their ability to be applied to the human situation. Humans suffer from recurrent herpes labialis and can release infectious HSV in the absence of clinical lesions. We are exposed to UV-irradiation in the forms of sunlight and artificial illumination. We are in daily contact with environmental chemicals that can act as initiators as well as promoters. Cigarette smoke and certain dietary substances, both capable of initiation and promotion, come in contact with our lips repeatedly. Thus, HSV, UV and promoter exposure to humans is not rare; to the contrary, the incidence of lip cancer relative to these exposures is. However, it is well recognized that after initiation with a small dose of carcinogen, if no promoter is applied, no tumors develop. Thus, combinations of many factors influence the likelihood of neoplastic development and actual tumorigenesis is a rare phenomenon. However rare, this research has brought us one step closer to showing an etiological role for HSV in the development of squamous cell carcinomas in man; but important theoretical differences between association and etiology exists and deserve mention. Association or correlation of HSV with carcinoma development cannot be interpreted as cause and effect. Although these results showed HSV + UV + TPA treatments capable of causing squamous cell

carcinomas in mouse lips, the possibility exists that the UV-irradiation was the inducer and not the UV-inactivated HSV. Although the tumor bearing mice had increased HSV antibody titers as compared to non-tumor bearing mice, treated in a similar fashion, the possibility exists that this may be activation of a latent HSV infection by an immunosuppressive disease (squamous cell carcinoma). Although HSV antigens were shown to be present in outgrowths from explanted tumors as well as tumor biopsies, this may represent a passenger role for HSV or preferential growth of HSV in tumor cells. HSV antigens were also present in UV-inactivated HSV transformed cells in vitro and the same possibility exists. The formation of these transformed foci, although 36 to 62 times more numerous with UV-inactivated HSV infected cells as compared to uninfected control cells, may represent enhanced "spontaneous" in vitro transformation. Localization of HSV DNA within cancer cells is difficult and may still not be ultimate proof of the etiological role for HSV in the development of squamous cell carcinoma. For example, "hit and run" transformation is possible where the viral DNA sequences responsible for the malignant conversion are lost after initiation. Furthermore, if the quantity of HSV DNA necessary for transformation is similar to that required by SV 40 virus (1-2%), detection of this DNA in a sea of host DNA will be difficult. Problems exist with biopsied tumor tissue since variation exists between sections and nontumorous tissue (necrotic and/or normal) often pollutes the biopsy's value. Outgrowths from tumor biopsies may not truly represent the tumor since some cellular subsets might show preferential growth in vitro. In the future, the approach of using those radiolabeled fragments of the HSV genome specific for transformation, in conjunction with in situ hybridization, may hold the greatest promise of detecting HSV nucleic acids in

malignant cells.

Ironically, the ultimate proof for the etiological link between HSV and carcinogenesis may come in the next 30 years from human prospective studies. With the rapidly expanding epidemic of genital herpetic infections that is presently ongoing in the world, a parallel rise in the incidence of cervical carcinoma should occur. Conversely, numerous laboratories have reported the development of a vaccine to prevent HSV infections and this prevention should be followed by a decrease in the incidence of carcinoma if a true link exists between HSV and carcinogenesis.

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APPENDIX I

Each chemical or reagent used in this study is listed below its respective supplier.

Amersham Radiochemical Center, England

S^{35} Methionine, 4.56 μ Ci/ml
 H^3 dGTP, 6.8 Ci/m mol
 H^3 dTTP, 48.0 Ci/m mol
 H^3 dATP, 16.6 Ci/m mol
 H^3 dCTP, 19.0 Ci/m mol

Baker, J. T., Co., Phillipsburg, NJ

acetone
ammonium acetate
DMSO
 H_2O_2
isoamylalcohol
sodium bicarbonate
sodium citrate
sodium hydroxide

Beckman Instruments, Fullerton, CA

Proteinase K
Scintillation toluene

Bethesda Research Lab., Rockville, MD

agarose
DNA polymerase I

Bio-Rad Lab., Richmond, CA

Acrylamide
Ampholines
Bis
Glycine
NP-40
SDS
TEMED
Urea

Calbiochem, San Diego, CA

2-ME
ethidium bromide
Ficoll
polyvinylpyrrolidone

Consolidated Midland Corp., Brewster, NY

TPA

Difco, Detroit, MI

gelatin
lactalbumin hydrolysate
Freud's complete adjuvant

Eastman Kodak Co., Rochester, NY

Dektol
DMBA
3-MC
sodium thiosulfate
formamide

Fisher Scientific Co., Fair Lawn, NJ

acetic acid
ammonium persulfate
calcium chloride
egg albumin
eosin
hematoxylin
methylcellulose
Permout
Toluidine blue

Grand Island Biological Co., Grand Island, NY

chick embryo extract
chicken plasma heparinized
Eagles minimal essential medium
Waymouth's medium

Kawecki Berylco Industries, Inc., Cleveland, OH

cesium chloride

Lab-Tek Products, Naperville, IL

frozen tissue embedding compound (OTC)

Mallinckrodt Chemicals, McGraw Park, IL

chloroform
glycerol
phosphoric acid

Medical College of Virginia, Richmond, VA.

ethanol
methanol
neutral buffered formalin
nembutal
scopolamine

Miles Lab., Kankakee, IL

bovine serum albumin, fraction V powder

North American Biological Inc., Miami, FL

fetal bovine serum
glutamine
goat serum
HBSS
trypsin

Pitman-Moore Inc., Washington Crossing, NJ

methoxyfluorane (Metophane)

Polyscience, Bay Shore, NY

DAB

Sigma Chemical Co., St. Louis, MO

EDTA
gentamycin sulfate
sodium chloride
Tris base

Worthington Chemicals, Freehold, NJ

DNAase
RNAase

APPENDIX II

HEMATOXYLIN AND EOSIN STAIN
(HARRIS HEMATOXYLIN)

1. Xylene	2 minutes
2. Xylene	2 minutes
3. Abs. Alcohol	2 minutes
4. Abs. Alcohol	2 minutes
5. 95% Alcohol	2 minutes
6. 95% Alcohol	2 minutes
7. Dist. H ₂ O	Dip
8. Hematoxylin	7 minutes
9. Tap H ₂ O	Dip
10. Tap H ₂ O	Dips
11. Acid Alcohol	1 or 2 dips
12. Tap H ₂ O	Dip
13. Ammonia H ₂ O	1 or 2 dips
14. Wash under tap	10 minutes
15. Eosin	3 minutes
16. 95% Alcohol	2 to 3 dips
17. 95% Alcohol	2 to 3 dips
18. Abs. Alcohol	5 minutes
19. Abs. Alcohol	5 minutes
20. Xylene	Clear - 5 minutes
21. Xylene	Clear

ABBREVIATIONS LIST

<u>ABBREVIATION</u>	<u>MEANING</u>
AHH	Aryl Hydrocarbon Hydroxylase
C	Degrees Centigrade
cAMP	Cyclic Adenosine 5'. - Monophosphate
cGMP	Cyclic Guanosine 5'. Monophosphate
DMBA	Dimethylbenzanthracene
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal Growth Factor
EMEM	Eagles' Minimum Essential Media
GLB	Gelatin Lactalbumin Hydrolysate buffer
HAT	Hypoxanthine, Aminopterin, Thymidine medium
HBSS	Hank's Balanced Salt Solution
H&E	Hematoxylin and Eosin
HEF	Hamster Embryo Fibroblast (primary)
HeLa	Cervical Carcinoma Cell Line
HEp-2	Human Epidermoid Cell Line
hr	Hour
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus Type 1
HSV-2	Herpes Simplex Virus Type 2
LETSP	Large External Transformation Sensitive Protein
2-ME	2-Mercaptoethanol

<u>ABBREVIATION</u>	<u>MEANING</u>
Min	Minutes
MOI	Multiplicity of Infection
MOL	Mole
mRNA	Messenger Ribonucleic Acid
ODC	Ornithine Decarboxylase
OTC	Frozen Section Embedding Compound
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
p. i.	Postinfection
PFU	Plaque-Forming Units
PMA	Phorbol Myristate Acetate
RNA	Ribonucleic Acid
s	seconds
s. c.	Subcutaneous
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SLS	Sodium Lauryl Sulfate
3-MC	Three-Methylcholanthrene
333	HSV-2 Strain 333
333-8-9	Dr. Rapp's UV inactivated HSV-2 Strain 333 Transformed Cell Line
3-26-7#1	Dr. Burns' UV inactivated HSV-2 Strain 333 Transformed Cell Line
TK ⁻	Thymidine Kinase Negative Cells
TK ⁺	Thymidine Kinase Positive Cells
TPA	Tetradecanoyl Phorbol Acetate

ABBREVIATIONS

MEANING

12F#1	Dr. Murray's UV Inactivated HSV-2 Strain 333 Transformed Cell Line
12F#2	Dr. Murray's UV Inactivated HSV-2 Strain 333 Transformed Cell Line
UV	Ultraviolet Light
Vero	African Green Monkey Kidney Cell Line
V	Volt
wk	week

ABSTRACT

ONCOGENIC TRANSFORMATION BY HERPES SIMPLEX VIRUS

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Many lines of evidence exist associating herpes simplex virus (HSV) with the development of carcinoma. Data from human studies includes seroepidemiologic studies of carcinoma patients and the localization of viral markers in human cancers. Experimental studies include in vitro transformation of cultured cells and viral induced alterations of host DNA. Much of this evidence is anecdotal or associative in nature and does not prove a cause and effect. The purpose of this research was to investigate the oncogenic potential of HSV type 2 (HSV-2) in vivo and in vitro.

An in vivo mouse model for lip carcinogenesis was designed to combine HSV-2 infection, ultraviolet (UV) exposure and tetradecanyl-phorbol-acetate (TPA) application. Preliminary studies showed that HSV-2 inoculation onto abraded mouse lips was capable of causing vesicular ulcerative lesions. These lesions healed completely after 10-14 days. Repeated herpetic lip infections failed to induce tumors. UV-irradiation delivered to the lesion site daily for 6 minutes at $42 \text{ ergs/mm}^2/\text{s}$ on days 3 through 6 postinfection caused hyperkeratosis, acanthosis and dysplasia to develop in several lips; while the same UV exposure delivered by itself failed to alter the histologic appearance. The addition of repeated TPA application to the

HSV + UV regimen promoted tumor emergence. Thirty-two of 156 BALB/c mice developed tumors. Although the majority were papillomas, six were squamous cell carcinomas. These tumor bearing mice had increased HSV specific antibody titers. HSV antigens were shown to be present in outgrowths from explanted tumors as well as in tumor biopsies by immunoperoxidase staining with HSV specific antisera.

Another series of in vivo studies showed that HSV lip infection initiated prior to or during repeated chemical carcinogen (dimethylbenzanthracene/DMBA) application was capable of reducing the incidence of tumors as compared to DMBA application without superimposed HSV infection. Comparison of results from this system and those from the HSV + UV + TPA system emphasized the fact that the lytic capability of HSV must be inhibited for the virus to express its oncogenic potential. It was hypothesized that the in vivo UV-irradiated HSV acted as the inducer and TPA as the promoter, analogous to the classical two-state carcinogenesis model. Since neither the HSV infection by itself, the UV exposure by itself, the TPA application by itself nor any combination of two induced the development of squamous cell carcinomas, HSV was considered a cocarcinogen with UV-radiation.

An analogous tripartite system was devised in vitro. Hamster embryo cells were infected with HSV-2 irradiated for 6, 7 or 8 minutes at 42 ergs/mm²/s. Twenty-one days postinoculation transformed foci had developed with frequencies (FFU/PFU) of 2.9×10^{-7} , 9.9×10^{-7} , and 3.9×10^{-7} , respectively. This represents 0.9, 3.0 and 1.3 FFU/10⁶ cells. The 7 minute transformation frequencies were further increased to 1.7×10^{-6} by the addition of TPA to the growth media (0.1 ug/ml) 48 hours postinoculation. This represents 5.1 FFU/10⁶ cells. Thus, TPA was capable of tumor promotion in

vivo and increasing transformation efficiency in vitro. Furthermore, TPA was shown by two dimensional gel electrophoresis to increase protein synthesis in transformed cells and by cell sorter analysis to increase cellular replicative activity.

A transformed cell line, 3-26-7#1, derived from a UV-inactivated HSV transformation experiment, was shown to possess HSV antigens by immunoperoxidase staining with specific antisera and to be oncogenic when injected into newborn hamsters. Sera from the tumor bearing animals had HSV neutralizing antibodies and when used as the primary antisera in the immunoperoxidase stain could detect antigens in HSV infected cells and outgrowths from explanted hamster tumors. Another transformed cell line, 333-8-9, was shown by in situ hybridization to have HSV specific mRNA in its cytoplasm.

These data reveal UV-irradiated HSV capable of tumor induction in vivo and of transforming hamster cell in vitro. In these systems, HSV can be considered a cocarcinogen.