velopment (i) binds but does not initiate regression, (ii) binds but the interaction initiates a totally different cellular response, or (iii) does not bind at all. It could be that the receptor is exclusively fetal, but can be reactivated in a tumor originating from cells that had constitutive receptors to MIS during fetal life. Fetal enzymes, isoenzymes, antigens, proteins, and hormones, such as alpha fetoprotein (*16*), carcinoembryonic antigen (*17*), and galactosyltransferase (*18*), are known to occur spontaneously in adult tumors. Müllerian duct tumors, occurring in the cervix, endometrium, fallopian tube, and vagina, as well as tumors of Müllerian duct nature (in the ovary) may have receptors for MIS, and the receptor-ligand complex may initiate cellular regression.

The epithelial morphology of earlier Müllerian duct structures predominates in ovarian tumors (*3*). For example, in the well-differentiated types, serous tumors are characterized by papillary ciliated cells of fallopian tube character; mucinous tumors have epithelium that resembles the endocervix, and endometrioid carcinomas have epithelium resembling the lining of endometrial glands. Clear cell tumors of the ovary (*19*), like those of the vagina (*20*), have been shown to be related to an endometrial type of epithelium. Most of the more poorly differentiated adenocarcinomas and undifferentiated carcinomas are thought to arise from the same coelomic surface epithelium.

The cytotoxic effect was not observed unless synchrony of the cell cycle and the density of cell seeding were carefully controlled. Noncoercive partial synchrony of the cell cycle was obtained by subculture with a 1:2 division 3 days prior to and again at the time of harvesting and seeding the microtest plates, in each case just before confluency was reached. It has been shown (*21*) that DNA synthesis (S phase), as measured by thymidine uptake, is maximal just before confluency. Test fractions were added 20 hours later, coincident again with the S phase of the cell cycle, and allowed to incubate for a subsequent 24 hours. Preliminary experiments demonstrated that cells subcultured more than 5 days before seeding, when thymidine uptake falls to negligible levels (*21*), failed to respond to biologically active MIS fractions. Presumably, cells in the S phase are more responsive to MIS. This finding correlates with previous observations with the electron microscope of active, euchromatic nuclei occurring in all Müllerian duct epithelial cells during the initial phases of regression (*13*).

Cells seeded at densities of greater than 100 cells per well failed to respond to MIS. The effect of cell density on MIS responsiveness may be related (i) to diminished surface receptors for the cytotoxic material, since a dense population might cover receptor sites that would become more exposed if the cells were allowed to spread more fully on a monolayer, or (ii) to the uncoupling of DNA synthesis usually seen with cell adherence and spreading, as observed by Folkman and Moscona (*22*).

The finding that a human ovarian cancer (HOC-21) can respond to fractions that cause Müllerian duct regression in the embryo implies the presence of MIS receptors on the tumor and provides an opportunity for small quantities of MIS to be detected.

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2 March 1979; revised 12 April 1979

Trigeminal Ganglion Infection by Thymidine Kinase–Negative Mutants of Herpes Simplex Virus

Abstract. *The incidence of trigeminal ganglion infection after corneal inoculation of guinea pigs with thymidine kinase-negative mutants of herpes simplex virus was markedly reduced compared to infection after inoculation of thymidine kinase-positive virus. Thymidine kinase-negative herpes simplex virus replicated well in ocular tissues in which dividing or potentially dividing cells were present, but not in trigeminal ganglion infection of nondividing neurons. Thymidine kinase-positive virus, however, replicated well in ocular tissues as well as in trigeminal ganglion. These results suggest that thymidine kinase expression of herpes simplex virus may be important in infections of sensory ganglia.*

Infection of the trigeminal ganglion of guinea pigs with thymidine kinase-negative (TK^-) mutants of herpes simplex virus (HSV) was studied to further examine the mechanisms of latent HSV infection of sensory ganglia. Latent infection of sensory ganglia appears to be an infection of neurons (*1*, *2*), that is, cells that do not replicate beyond the neonatal period. HSV codes for a virus-specific TK (deoxypyrimidine kinase) (*3*), which is expressed in infected cell cultures (*4*), in latent in vivo infection (*5*), and in HSV-transformed cells (*6*). Since viral TK expression is important for virus replication in nondividing cells (*3*), it seemed that HSV TK might be important in the pathogenesis of in vivo infections in nonreplicating sensory ganglion neurons.

To compare TK^- and TK^+ HSV infections, we performed experiments using standard TK^+ HSV type 1 (HSV-1) strain KOS, and a TK^- mutant derived from the TK^+ parental stock. Arabinofuranosylthymine (ara-T) was used to select for the TK^- phenotype since the

0036-8075/79/0831-0915$00.50/0

drug is phosphorylated by HSV TK but not by cellular TK and inhibits replication of TK^+ but not TK^- HSV (*7*). Standard TK^+ HSV (KOS) and TK^- mutant stocks were grown in primary rabbit kidney (RK) cells; titers were 2×10^7 to 4×10^7 plaque-forming units (PFU)/ml and 3×10^7 to 5×10^7 PFU/ml, respectively. Results obtained with TK^- (KOS) virus were subsequently confirmed with the use of an HSV-1 TK^- mutant derived from HSV-1 strain Glasgow 17 (obtained from Dr. N. Wilkie). The titer of this stock TK^- virus grown in human fibroblastoid cells (Flow 5000 cells, Flow Laboratories, Rockville, Maryland) was 8×10^7 PFU/ml.

The TK phenotypes of the parental TK^+ (KOS), the TK^- (KOS) mutant, and TK^- (Glasgow 17) HSV's were determined by ara-T testing and also by direct enzyme analysis of homogenates of lytically infected cells according to modifications of the methods of Munyon (*6*) and Kit (*4*). With ara-T testing, titers of TK^+ HSV were reduced by 3 to 4 log units, whereas TK^- HSV was inhibited by less than 1 log unit (*8*). In biochemical assays of TK^- HSV, phosphorylation of thymidine was 2 to 5 pmole per milligram of protein, whereas for TK^+ HSV, it was 910 to 1065 pmole/mg (*9*).

Acute and latent HSV infection of the trigeminal ganglion was studied in random-bred Hartley guinea pigs. Animals were anesthetized, and bilateral corneal inoculation was performed (*10*). Guinea pigs were killed after 3 days (during the period of acute infection) or 24 to 40 days (during the period of latent infection). Trigeminal ganglia were removed from each animal and tested. In animals killed during the period of latent infection, ganglia were minced and cocultivated with RK cells. Virus isolation from ganglia was demonstrated by the development of typical cytopathic effect (CPE) in the RK cells, and that the virus was HSV was confirmed by neutralization with specific antiserum. Isolates from latently infected animals were characterized for TK phenotype with ara-T.

Death from HSV encephalitis was reduced in TK^--inoculated compared to TK^+-inoculated guinea pigs. In animals corneally inoculated with TK^- (KOS) virus, there were no mortalities and in those inoculated with TK^- (Glasgow 17) virus the mortality rate was only 8 percent (Table 1). In guinea pigs inoculated with the parental TK^+ HSV (KOS), however, the mortality rate was 35 percent. A decrease in acute pathogenicity of TK^- HSV has been reported (*11*). The mortality rate for TK^+ HSV (KOS) was similar to that noted after corneal inoculation of guinea pigs with HSV-1 strain McIntyre (*10*).

In animals inoculated with TK^+ (KOS) virus, latent ganglionic infection was detected in 9 of 13 (69 percent) animals surviving to the period of latency. After corneal inoculation of TK^- HSV (KOS), latent infection was found in only 4 of 15 (27 percent) (Table 1). Latent infection was detected in 3 of 12 (25 percent) guinea pigs inoculated with the HSV (Glasgow 17) TK^-. Phenotypes of the latent period isolates were determined with the ara-T assay. From the seven latently infected TK^--inoculated guinea pigs nine of ten isolates were TK^-. One isolate was intermediate, as determined by multiple ara-T and enzyme assays. This isolate was obtained from an animal that yielded two TK^- isolates. Isolates from five TK^+-inoculated latently infected animals were TK^+ (nine of nine separate isolates).

We then investigated TK^- HSV during the acute period of ganglionic infection. These animals, inoculated corneally, were killed 3 days after inoculation, and the amount of HSV present in ganglionic tissue was quantified. Each ganglion was homogenized; after centrifugation, the cell-free supernatants were titrated in plaque assays. An excess of 100-fold or more virus was present in ganglia of TK^+-inoculated animals than in animals inoculated with TK^- HSV (Table 2, right); TK^- HSV was not isolated from homogenate sediment, thus eliminating the possibility that highly cell-associated TK^- virus was present. This titration experiment indicated that in the acute stage of disease (3 days after corneal inoculation), TK^- HSV was present in lesser amounts than TK^+ HSV in trigeminal ganglion tissue. Virus was not isolated from ganglion tissue in several animals killed 5 days after corneal inoculation with TK^- HSV (data not shown).

Decreased amounts of TK^- HSV in trigeminal ganglia after corneal inoculation may be due to (i) impaired survival or replication of virus in ocular tissues, (ii) decreased axonal transport of TK^- HSV, or (iii) impaired survival of virus in ganglion neurons. To evaluate the amount of TK^+ and TK^- HSV present in ocular tissue 3 days after corneal inoculation, we studied HSV taken from eye swabs. Eye swabs taken 3 days after corneal inoculation permitted isolation of HSV from all TK^--inoculated guinea pigs (15 of 15) and from all TK^+-inoculated guinea pigs (11 of 11) tested. Nearly equivalent amounts of TK^+ and TK^- HSV were isolated from bilateral eye swabs of five TK^+-inoculated ani-

Table 1. Latent trigeminal ganglion infection in newborn guinea pigs after corneal inoculation of TK^+ and TK^- HSV.

Virus inoculated (latent infection)	Inoculated animals (No.)	Survival to period of latency		Positive at time of killing		Mean days ± S.D. after cocultivation to CPE
		No.	Percent	No.	Percent	
TK^+ (KOS)	20	13	65	9	69	16.0 ± 3.3
TK^- (KOS)	15	15	100	4	27	17.2 ± 2.6
TK^- (Glasgow 17)	13	12	92	3	25	13.7 ± 3.6

Table 2. Isolation of TK^+ and TK^- HSV from eye swabs and from trigeminal ganglia after corneal inoculation of guinea pigs. Results are means and ranges are given in parentheses.

Virus inoculated	Samples (No.)	HSV isolated from eye swabs (PFU/0.6 ml)*	HSV isolated from ganglion homogenates (PFU)†	
			Per ganglion	Per milligram of ganglion tissue
TK^+ (KOS) (2×10^6 PFU)	10	1.6×10^4 (5.1×10^1 to 5.1×10^4)	3.3×10^2 (1.2×10^1 to 1.7×10^3)	5.5×10^0 (2×10^{-1} to 3×10^1)
TK^- (KOS) (2×10^6 PFU)	10	1.8×10^4 (2.1×10^2 to 5.2×10^4)	$< 3 \times 10$	$< 5 \times 10^{-2}$

*Three days after corneal inoculation of TK^+ or TK^- HSV, guinea pigs were anesthetized with pentobarbital. Bilateral eye swabs were made with sterile cotton moistened with tissue culture medium containing 10 percent calf serum. Swabs were transported in a total volume of 0.6 ml of medium, and this fluid was titrated by standard procedures. From the titration, the total number of HSV PFU's in each 0.6-ml volume was calculated. †Both trigeminal ganglia from each animal were removed. Each was washed in Hanks balanced salt solution and homogenized in sterile Ten Broeck homogenizers in 0.6 ml of tissue culture medium containing 10 percent calf serum (heat-inactivated). Homogenates were centrifuged at 2000 rev/min for 10 minutes at 4°C, and the supernatants were titrated. The sensitivity of the assay was 3 PFU per ganglion and 5×10^{-2} PFU per milligram of ganglion tissue. Each ganglion weighed approximately 60 mg.

mals (ten eye swabs) and five TK$^-$-inoculated animals (ten eye swabs) (Table 2, left).

The isolation of similar amounts of TK$^+$ and TK$^-$ HSV from eye swabs but decreased amounts of TK$^-$ HSV in ganglion homogenates suggests that TK$^-$ virus may not be transmitted via the trigeminal nerve to the ganglion as efficiently as TK$^+$ virus or, more likely, that TK$^-$ virus may not survive or replicate as well as TK$^+$ virus in ganglionic neurons. These results indicate that HSV-induced TK may play a role in viral pathogenesis in neural but probably not in nonneural tissues and that the presence of the TK gene in HSV may be important in the pathogenesis of natural HSV infection of ganglion neurons.

The TK$^+$ HSV phenotype was found to be important in in vitro infection of nondividing cells (*3*), and may be analogous to infection in vivo in nondividing cells, that is, neurons. While TK$^-$ HSV was easily isolated from ocular tissues where many dividing or potentially dividing cells are present, TK$^-$ HSV was not isolated from ganglion tissue. Nonneuronal supporting and sheathing cells are capable of division, but for virus to reach these cells, it probably must first pass through ganglionic neurons. While the role of other possible defects in the TK$^-$ viruses cannot be excluded, our results indicate the probable importance of the HSV TK$^+$ phenotype in infection of the trigeminal ganglion.

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9. Homogenates of uninfected TK$^+$ and TK$^-$ mouse fibroblastic cells (N clA and N clA cl10, respectively, provided by R. Goldberg of NIH) and of TK$^-$ cells lytically infected with TK$^+$ and TK$^-$ HSV were tested. Monolayer cells in 60-mm dishes were infected at a multiplicity of 1 to 2. After being allowed to absorb for 40 minutes at 37°C, virus was removed and 5 ml of Dulbecco's medium supplemented with antibiotics and 5 percent fetal calf serum was added. After the cells were incubated at 37°C for 16 hours, medium was decanted and homogenates of infected cells as well as those of uninfected TK$^+$ and TK$^-$ cells were prepared. To each dish, 0.5 ml of homogenization buffer (tris, 0.01*M*, pH 7.9; 2-mercaptoethanol, 0.0014*M*; KCl, 0.025*M*; $MgCl_2$, 0.02*M*) was added. Cells were harvested with a rubber policeman, put into centrifuge tubes, and frozen at −70°C. After thawing, the homogenized cells were centrifuged at 16,000 rev/min (31,000*g*) for 1 hour at 4°C. Supernatants were tested for TK activity. Supernatant (20 μl) and reaction buffer (20 μl) (homogenization buffer plus 0.1*M* adenosine triphosphate, 0.05 m*M* thymidine, and ^{3}H at 2 μCi/ml) were inoculated on 1.25-cm squares of DEAE-81 filter paper and incubated for 20 minutes at 37°C in a humidified incubator. Filter squares were washed three times with 2-ml portions of 0.03*M* ammonium formate, three times with distilled water, and once with absolute ethanol. Filters were then dried, and residual ^{3}H activity was determined after addition of 5 ml of toluene-based scintillation fluid and counting in a liquid scintillation counter (Beckman LS-250). The amount of thymidine phosphorylated was determined after subtracting background activity.
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22 February 1979; revised 25 May 1979

Digitalis Genin Activity: Side-Group Carbonyl Oxygen Position Is a Major Determinant

Abstract. *The Na$^+$,K$^+$-adenosine triphosphatase-inhibiting activity of digitalis genins and their analogs is a function of side-group carbonyl (C=O) oxygen position. For each 2.2 angstroms that this oxygen is displaced from its position in digitoxigenin, activity drops by one order of magnitude. This quantitative relation resolves previously proposed models which have attempted to describe the molecular basis of genin activity. A multidisciplinary (crystallographic, conformational energy, synthetic, biological) approach to structure-activity relations is described.*

Digitalis preparations remain a major therapeutic modality in the management of cardiovascular disease. The pharmacological effects of the digitalis glycosides [digoxin (**1**) and digitoxin (**2**)] and their genins [digitoxigenin (**3**) and digoxigenin (**4**)] appear to be the result of inhibition of membrane-bound Na$^+$, K$^+$-adenosine triphosphatase (Na$^+$, K$^+$-ATPase) (*1, 2*).

An array of often conflicting models has been proposed to describe the chemical and geometric characteristics that determine the capacity of a given genin to inhibit the Na$^+$,K$^+$-ATPase (*3*). However, the activity of a number of modified genins has been unexplained by, or is inconsistent with, important portions of these models. For example, aldehyde **5** was predicted to be up to 123 times more active than digitoxigenin, on the basis of proposed models, but it was found to be slightly less active (*4*). The C20 stereoisomers **8** and **9** were unexpectedly found to have greatly different activities (*5, 6*). The glucose glycoside actodigin (AY-22,241) with an unusual genin lactone linkage (**7**) has been of interest because of its unpredicted rapid reversibility of Na$^+$,K$^+$-ATPase inhibition and toxicity (*7*). Finally, although it has been presumed that the 14β-hydroxyl group enhances inhibition of Na$^+$, K$^+$-ATPase, the 14-anhydro analog **8** was found to be more active than the 14β-hydroxyl analog **6** and its C20 stereoisomer (*8, 9*).

A multidisciplinary approach, including x-ray crystallography, conformational energy calculations, organic synthesis and Na$^+$,K$^+$-ATPase inhibition studies, has been used to find the straightforward relation between genin structure and activity which we report. Nine genins (**3** to **11**) (Fig. 1) differing widely in construction, stereochemistry, and geometry were selected for study (*10*). In compounds **3** to **7** and **11**, C14 is tetrahedral and (except for **6**) C20 is planar. The reverse is true for **8** and **9**. In **10**, C14 and C20 are both planar. Aldehyde **5** is illustrative of the group of active genins that do not have a lactone ring for a side group. Six have the presumably important 14β-hydroxyl, and three do not. The genin portion (**7**) of AY-22,241 was included in our study, as was strophanthidin (**11**), with its additional oxygen substituents on C5 and C19.

Although the steroid backbone of these molecules is quite rigid, the 17β side group can rotate about the C17–C20 bond. In order to explore this conformational freedom, relative conformational

0036-8075/79/0831-0917$00.50/0