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Growth characteristics of Herpesvirus hominis type 2 in Vero, HeLa, and MA111 stable cell lines

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GROWTH CHARACTERISTICS OF HERPESVIRUS HOMINIS TYPE 2 IN VERO, HeLa, AND MA111 STABLE CELL LINES

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CHAPTER I

INTRODUCTION

SOME GENERAL AND BIOLOGICAL AND PHYSICAL CHARACTERISTICS OF HERPESVIRUSES

Every species of animal is suspected of harboring at least one herpesvirus. However, since herpesviruses are often difficult to culture, only about twenty viruses have been reported to be in the herpesvirus group, which includes animal and human viruses (16, 25, 34). Melnick, et al. (1964) subdivided the herpesviruses into subgroups A and B. Group A viruses are released readily from infected cells, whereas Group B viruses tend to remain cell-associated (17, 34).

Group A viruses include Herpesvirus hominis (formerly herpes simplex virus) and several similar animal viruses. Group B viruses include the varicella-zoster virus (chicken pox in children; shingles in adults), cytomegalovirus, which causes a fatal pneumonia of infants, and several other human and animal viruses (34).

Herpesviruses are relatively large viruses with an overall size estimated between 140 and 250 nm (6, 8, 34), with an average size of 180 to 200 nm (8). The virus is enclosed by a lipid-rich membrane or envelope which is apparently of host cell origin (6, 8) and which accounts for the overall particle size. Because of essential lipids in the envelope, herpesviruses are inactivated by ether or chloroform (8, 12, 34). Group A viruses can obtain their envelopes at the nuclear membrane but may add additional membranes at the cell surface (6). Serological studies show that anti-host cell antibodies (e.g., rabbit anti-HeLa) will agglutinate only enveloped viruses grown in that particular cell line (HeLa). Anti-herpesvirus antibodies agglutinate mostly unenveloped viruses (8, 33). Therefore, the envelope may serve to

mask the antigenic determinants. Both enveloped and non-enveloped viruses are released during infection, but enveloped particles attach to host cells more readily and, once attached, have a much higher viability or infectivity than do non-enveloped viruses. It is possible that non-enveloped viruses are sensitive to enzymes which may enter through holes that appear to be in the capsomeres (structural subunits) (7).

Herpesvirus deoxyribonucleic acid (DNA) is double stranded with a molecular weight ranging from 54 to 92 x 10^6 daltons (6). According to current quantitative methods, herpesvirus DNA is estimated to contain enough information to specify the sequence of up to 140,000 amino acids. Assuming that genes are not duplicated, only 10 to 20% of the total genetic information is expressed in tissue culture. Compared to other viruses, herpesviruses contain large amounts of genetic information for viruses of their size and complexity. Since herpesviruses tend to establish lifelong infections in their natural hosts, it is thought that the extra genetic information contributes to the total infective process in the natural host (16).

SOME CLINICAL ASPECTS OF GROUP A HERPESVIRUSES

Clinically, among Group A herpesviruses, it is important to note that there is a marked change in virulence when a virus is introduced into an immature or unnatural host. Cytomegalovirus generally causes a subclinical infection in adults, but it is generally fatal for infants. Herpesvirus hominis (HVH) usually causes a mild infection in adult humans but causes severe central nervous system damage in rabbits and mice (8).

In man, HVH infections may take several forms and are often subclinical. The clinical response is usually a mild vesicular eruption of the skin or mucous membranes. The virus may also cause severe illnesses such as stomatitis, Kopsis' varicelliform eruption, meningoencephalitis, and a generalized, usually fatal, neonatal disease (12).

Herpesvirus hominis has been categorized into two subtypes: 1 and 2. The type 1 virus strain causes localized recurrent lesions

about the lips and mouth. It can also be involved over wider areas of skin and cause meningoencephalitis or keratoconjunctivitis (12). The type 2 virus strain causes recurrent genital lesions in both men and women (21), and frequently causes a systemic infection of infants exposed congenitally (transplacentally) or neonatally via herpetic lesions in the birth canal. It can also be involved in keratoconjunctivitis or meningoencephalitis (13).

IN VITRO STUDIES OF HERPETIC INFECTIONS

Biological Aspects of In Vitro Herpetic Infections

In vitro studies (i.e., in tissue culture) have yielded much information about the infectious process of herpesviruses. In tissue culture, HVH has a wide host range, but not all cell lines are equally susceptible. Human, simian, rabbit, hamster, mouse, chicken embryo, and other cell lines are infected with varying virulence (34). Exposure to HVH induces an abortive infection in dog kidney cells and inhibits the infective process of pseudorabies virus (PRV) in dog kidney cells (2).

The reproductive cycle can be best described in terms of factors affecting four aspects of the replicative cycle. (i) the duration of the eclipse or latent period, (ii) the duration of the replicative cycle, (iii) the yield of virus per cell, (iv) the release of virus from the infected cells.

(i) The duration of the latent period varies from 3 to 8 hours for most herpesviruses. It is affected by the temperature of incubation, by the multiplicity of infection, and by prior infection of the cells by another mutant strain of virus. Each cell has a minimum latent period when infected with a given virus (e.g., 3 hours for PRV in rabbit kidney cells, or 5 hours for HVH in HEp-2 cells). The period cannot be shortened by increasing the multiplicity of infection.

(ii) The duration of the replicative period of herpesviruses varies with the virus and the infected cell. It is also affected by the multiplicity of infection and the temperature of incubation. The

reproductive cycle of HVH in HEp-2 cells varies from 13 to 19 hours; at 37°C with an infective dose of 50 plaque-forming units per cell, the cycle lasts 17 hours (28). In HeLa at 37°C with an infective dose of 5 plaque-forming units per cell, the replicative cycle of HVH lasts 12 hours (30).

(iii) The virus yield from infected cells increases exponentially from the end of the latent period until almost the end of the replicative cycle. Under optimal conditions the yield of HVH from HEp-2 is 10,000 to 100,000 virons per cell, but only about 0.1 to 10% of these virons are viable as indicated by their plaque-forming ability (28). In HeLa about 40,000 virons per cell are produced, of which 0.025% are infective as indicated by their plaque-forming ability (31). Cells in monolayer usually yield more virus than cells in suspension. High virus titers are more readily obtained in rapidly growing cells maintained after infection in enriched medium at pH and temperature optimal for the particular strain of virus.

(iv) After infectious herpesvirons appear inside infected cells, release is generally slow and temperature-dependent. Herpesvirus hominis is released more readily from suspended cells at 37°C than from cells adhering to glass surfaces or cells incubated at 34°C. Freezing and thawing or sonication release aggregates of virus from infected cells (28).

Cytopathic effect (CPE), as observed in the light microscope, for HVH type 2 (strain 196) is generally "ballooning" and detachment of infected cells, with occasional formation of small syncitia (giant cells formed by the fusion of several cells). Certain strains of PRV normally cause extensive syncitia in infected monolayers of cells (25). Many authors have generalized the PRV infective pattern to apply to all herpesviruses. Some strains of HVH in the presence of specific antibody do form extensive syncitia (11, 25), but strain 196 (used in this study) forms syncitia only occasionally.

Observations made with the electron microscope have shown that after attachment the viruses are phagocytized into vaculoles in the peripheral cytoplasm. The envelope is removed and the naked capsid passes into the cytoplasm (6). It is likely that two particle types

are necessary to initiate infection. Miyamoto and Morgan (1971) report two types of capsids, designated light and dense, based on electron density. Light capsids constitute 53%, and dense capsids constitute 47% of the total number of capsids observed. Shortly after penetration, dense capsids were observed to be in various stages of disruption; light capsids remained stable for at least one hour after infection. However, after 20 to 30 minutes most of the light capsids were devoid of cores.

It was suggested that the DNA released from the dense capsids near the cell surface transcribes a virus-specific messenger ribonucleic acid (mRNA) which codes for an enzyme which renders light capsids permeable to their core contents as they approach the nuclear membrane (20). Parental capsids have not been observed in the nucleus. Naked capsids appear in the nucleus within 5 to 6 hours after infection (18, 23), and enveloped virons appear in the cytoplasm about an hour later. Envelopment of the capsid occurs as the virus buds through the nuclear membrane or into cytoplasmic vacuoles (6, 23). Late in the infection, crystal arrays of capsids appear in the nucleus just prior to cell death (6).

Molecular Aspects of In Vitro Herpetic Infection

During the course of infection, viral mRNA, which is more stable than cellular mRNA, is synthesized; this new mRNA is complementary to viral DNA (7). The activity of DNA metabolism within the cell is increased. During the period 2 to 8 hours after infection the activity of thymine kinase, thymidylate kinase, DNA polymerase, and DNAase increase; afterward their activities decrease (28). The increased activity is due in part to the stabilization of host cell enzymes. The HVH-coded enzymes differ from host cell enzymes and poxvirus-coded enzymes in heat stability, response to excess substrate, response to allosteric reactants, and in Michaelis constant (8, 14). Viral protein synthesis increases during the period 3 to 8 hours after infection, after which it declines (28). Host cell protein synthesis decreases by one-half within 4 hours. The enzymes which

appear early in the infection decrease in activity as the rate of assembly of virus particles from accumulated subunits increases. Assembly and active release continue until the cell dies, usually within 24 hours (8).

GROWTH CURVE STUDIES WITH HERPESVIRUS HOMINIS

Purpose

It was noted by Dr. R. N. Ushijima that when stock pools of HVH were prepared in different cell lines there were differences in the rates of appearance of CPE and in the peak yields of virus. Primary rabbit kidney cells showed rapid CPE but yielded low titers of virus. HeLa showed delayed CPE but produced higher titers of virus. Vero showed rapids CPE and produced the highest virus yield. Since these cell lines are used routinely in this laboratory, it was decided that growth curve information on HVH in these cell lines would be valuable.

Statement of the Problem

Experiments were designed to determine the time and rate of synthesis of HVH type 2 (strain 196) in the stable cell lines, Vero, HeLa, and MA111 (a stable rabbit kidney line) and to determine the correlation, if any, between the appearance of CPE and virus production by the respective cell lines.

Experiments were designed to determine the stability of HVH type 2 (strain 196) at 37°C and to repeated freeze-thaw cycles, since the virus was exposed to both temperature and freeze-thaw degradation during preparation.

Early trials showed that the titer of the released virus remained constant for up to 24 hours after the cells detached from the glass culture surface. An experiment was designed to determine whether this release of virus by detached cells was active or passive, i.e., whether the detached cells were alive or dead.

Literature Review

There appear to be no previous studies reported in the literature using either Vero or MA111 with HVH. Both cell lines have been used extensively to study RNA viruses (26, 27) and, to some extent, SV-40 and adenoviruses (29). HeLa cells have been used extensively in studies of HVH. Most studies were either electron microscope examinations of the infectious process or biochemical studies of infected cells, but some growth curve information in HeLa cells has been reported. Waterson (1968), summarizing Newton and Stoker (1958) and Siminoff (1964), reports yields ranging from 10 to 20 plaque-forming units per cell--the *in vitro* plaque method of virus assay in tissue culture is analogous to the plaquing of bacteriophage on susceptible bacteria (4, 5). Peak yields were obtained 16 hours later using an inoculum of 5 plaque-forming units per cell and incubating at 37°C.

The above studies were of interest but were not directly applicable to this study. Three different strains of HVH were used in the above studies, none of which were strain 196. The plaque assay method was not used in this study and lower multiplicity inocula were used. However, similar medium, incubation temperatures, infecting methods, and sampling methods were employed.

Kaplan (1957) conducted stability tests similar to the ones done in this study. He conducted both temperature (37°C) and freeze-thaw stability tests. Although he used the plaque assay method, his results were reported in survival percentages and could, therefore, be directly compared to the results in this study. Again, there were virus strain differences to be considered. From Kaplan's results, up to 99% loss of virus was expected after exposure to 37°C for 24 hours; little loss of virus was expected after up to five freeze-thaw cycles.

CHAPTER II

MATERIALS AND METHODS

ROUTINE MATERIALS AND METHODS

General Comments

Work with tissue culture of animal cells requires more rigorously sterile conditions and quantitatively cleaner glassware than does routine bacteriological work. Tissue culture medium is an ideal medium capable of supporting the growth of many contaminant organisms: bacteria, fungi, and mycoplasma. Antibiotics can control contaminating organisms of they are few in number and sensitive to the antibiotics used. In nearly all laboratories there is some degree of airborne microbial contamination, but if certain precautions are taken, airborne contamination can be minimized and controlled by the penicillin and streptomycin routinely present in tissue culture medium.

An isolation or "sterile" room is of prime importance. The isolation room should have minimal in-and-out traffic and should be equipped with high intensity ultraviolet (U.V.) tubes which are activated when the room is not in use. A hood with its own U.V. source should be in the isolation room to provide additional protection to the main work area. Minimal in-and-out traffic allows dust to settle in the room and minimizes entry of outside contamination. Therefore, all glassware and equipment routinely used for handling cells should be stored in the isolation room. The U.V. serves to inactivate both contaminant airborne microbes and any virus that invariably escapes when infected culture containers are opened.

Sterilization and Decontamination Methods

In addition to maintaining a semi-sterile environment, any

equipment which comes into contact with the cells or cell medium must also be sterile. Heat is the most common means of sterilization. The hot air oven, employing a temperature of 180°C, is useful for certain glassware and other materials which can tolerate the heat. Since dry heat depends upon desiccation to kill microbes, the time needed for sterilization depends upon the object to be sterilizied. The autoclave, employing pressurized steam at 121°C, is the choice for materials which will tolerate that temperature. A serious disadvantage of an autoclave operated by centrally supplied stram (the type at the University of Montana) is that the additives in the steam to prevent rust and mineral deposits in the steam lines are toxic to mammalian cells. However, microbial contamination is generally more of a problem than toxicity, and methods to minimize autoclave toxicity will be discussed as they apply. Filtration is used to sterilize liquids which will not tolerate autoclave temperatures. Treatment of filters will be discussed below. Ethylene oxide gas is used to sterilize plastic objects which cannot be autoclaved. Ultraviolet irradiation is used to sterilize certain plastic objects and powdered insoluble antibiotics.

Two filtering systems were available: the Seitz and the Millipore. Both employ air pressure or vacuum to force filtrate through the filter pad. The major advantage of the Seitz system is its relatively low cost. The Seitz system employs asbestos pads of low porosity, resulting in low flow rates of filtrate. The Seitz filter is cleaned after use and rinsed well in distilled water. It is sterilized in the hot air oven for 40 minutes (any longer chars the pad) to avoid autoclave toxicity.

The Millipore system, which is more expensive, employs synthetic pads of high porosity and more uniform size, resulting in high flow rate of filtrate at low pressure. Millipore pads cannot tolerate dry heat and must be autoclaved.

Both filter systems must be pre-treated with glass-distilled water prior to use. The pre-treatment in the Seitz filter helps to seal the pad and flushes away any debris from the pad. The Millipore pads have a wetting agent which is toxic and must be removed. To

remove wetting agent from a 293 mm pad, pass four liters of hot glass-distilled water (95 to 100°C) through the pad followed by two liters of cold (25°C) water. This treatment also seems to remove any autoclave toxicity factors. Specific uses of filters will be discussed as they apply.

In addition to routine sterilization of equipment, there is the problem of disposal of contaminated (intentional and unintentional) materials. Disposable plastic materials can be merely autoclaved. Metal containers should be available for storing contaminated materials prior to autoclaving. Glassware can be soaked in a dilute bleach (Chlorox or Purex) solution for about 10 minutes. In culture bottles, this treatment also detaches any cells from the glass, making cleaning easier. The pipette disposal jars should also contain a dilute bleach solution. Metal caps, plastic caps, and rubber stoppers, which will not tolerate bleach or which tend to absorb bleach, can be soaked in 70% ethanol.

Cleaning of Glassware

Glassware must be quantitatively clean to support the life of animal cells. Animal cells will not tolerate even the small amounts of metal ions normally found in distilled water. Glass-distilled water must be used in the final rinse of any glassware used to grow cells. A satisfactory supply of glass-distilled water is prepared by redistilling tap-distilled water in a Bellco single stage distilling unit with a long column.

The general procedure for washing glassware is as follows:

1. Rinse in tap water (if it has been soaked in bleach.
2. Wash well in tap water and Detergex brand powdered cleanser (available from Pharmaseal Laboratories, Glendale, California).
3. Rinse well in tap water.
4. Soak for about twenty minutes in dilute hydrochloric acid in distilled water to remove any residual detergent.
5. Rinse well in distilled water.

6. Rinse in glass-distilled water if container will be used to grow cells or to store medium.

The general procedure is modified somewhat to fit the glassware being cleaned, but the basic steps are the same.

Pipettes are drained, the cotton plugs removed, the pipettes placed in a Detergex solution and autoclaved for 10 minutes. The pipettes are rinsed in a commercial rinser with tap water for 30 minutes, placed in an acid rinse for 5 minutes and finally rinsed three times in tap-distilled water. After drying, the pipettes are plugged with cotton, placed in metal cans and sterilized in the hot air oven for 60 minutes.

Prescription and media bottles are washed according to the general procedure and given a final rinse in glass-distilled water and dried. To minimize autoclave toxicity, a square of Reynolan plastic, cut from Brown-in-Bag cooking wrap, is used as a gasket under the screw cap to minimize the amount of steam which enters the bottle. They are autoclaved 10 minutes and put on the drying cycle.

The Cornwall automatic pipette--an adjustable syringe with a one-way siphon--is disassembled and cleaned. The entire components receive a final rinse in glass-distilled water, then are wrapped in foil and autoclaved 10 minutes on fast exhaust to protect the surgical tubing siphon.

Caps and rubber stoppers are boiled in distilled water. Caps are sterilized with the bottle or tube. Rubber stoppers are wrapped in foil "candlesticks" and autoclaved 10 minutes on fast exhaust.

Culture Media and Solutions

This section describes and discusses media, buffers, and other solutions needed to handle cells in tissue culture. To prepare versene-trypsin (Table 1), mix part A for 30 to 45 minutes on a magnetic stirrer with about half the $NaHCO_3$ and most of the water; add B, then mix for 10 to 15 minutes. The solution is ready to filter when clear. Filter through a pad with 0.22 or 0.25 um pores. Dispense in 8-ml amounts in screw-capped tubes and store at $-70^{\circ}C$. Versene-trypsin is used to remove monolayered cells from the glass or plastic culture surface.

Table 1. Chemical Composition of Versene-Trypsin Solution

Solution	Material	gm/1. glass-distilled H_2O
A.	NaCl	8.0
	KCl	0.4
	Glucose	1.0
	Trypsin	1.5
B.	$NaHCO_3$	0.58
	Versene	0.2

Phosphate buffered saline (PBS) (Table 2) without Mg^{++} and Ca^{++} ions is designated Pd. Since these cations inhibit the action of trypsin, Pd is used instead of PBS as a wash for monolayered cells prior to trypsinization for further passage. The cation-deficient buffer is prepared as a 10x stock which is diluted prior to use. The resultant isotonic solution is decanted into 100 ml milk dilution bottles and autoclaved 15 minutes with the caps snug to minimize mixing with steam. The autoclave is set on slow exhaust cycle.

Table 2. Chemical Composition of Phosphate Buffered Saline (Dulbecco and Vogt, 1954, a & b)

Solution	Material	gm/1. glass-distilled H_2O
A.	NaCl	8.0
	KCl	0.2
	$Na_2HPO_4 \cdot 7H_2O$	2.15
	KH_2PO_4	0.2
B.	$MgCl_2 \cdot 6H_2O$	0.1
	$CaCl_2$	0.1

Minimal Essential Medium (MEM) is prepared from the materials listed in Table 3, then filtered through a 0.22 or 0.25 um filter pad. Powdered MEM is available through Grand Island Biological Company. Potassium penicillin C and streptomycin sulfate are available through

Calbiochem. Other antibiotics used were Chlorotetracycline, Kanamycin, and Neomycin (anti-mycoplasma agent) and Mycostatin or Nystatin (anti-fungal agent). These are available from Grand Island Biological Company, DIFCO, and others. Product information is provided by the suppliers.

Calf serum can be collected at a local slaughter house. Fetal calf blood is preferred, but blood from animals under 8 months of age is acceptable. These animals have minimal amounts of gammaglobulins in their serum.

Glass containers are most appropriate for collecting blood since they minimize hemolysis. One-gallon mayonnaise jars work well. The blood is allowed to clot for several hours, then the clot is cut in quarters and allowed to stand overnight in the cold. The serum decanted from the jars is put in 250 ml centrifuge tubes, spun at 2,000 rpm for 15 minutes in an International H9070 size 2 centrifuge with a swinging bucket head, and allowed to free wheel to a stop. The serum is carefully pipetted from the tubes by suction apparatus. It is then cleared by filtration through a filter pad with 4 um pores. If filter-sterile serum is desired, it should be refiltered through a 0.22 or 0.25 um pad. The complement in all sera, sterile or non-sterile, should be inactivated at 56°C for 30 minutes prior to use.

In this study, MEM with 3 and 5% calf serum was used. Thirty milliliters of 50 ml of serum were added to one liter and designated 3 and 5%, respectively.

Serum was stored in 100 and 250 ml amounts in whirl bags if non-sterile, and in 500 ml amounts in bottles if sterile. The media were prepared in large volumes both for this project and other projects in the laboratory. Stocks of media containing 5 or 10% serum or no serum were prepared monthly and stored at 4°C.

Table 3. Composition of the Medium Prepared with Eagle's Minimal Essential Medium

Material	Amount of Material Used
Glass-distilled H_2O	1.0 liter
Commercial powder	9.9 gm
$NaHCO_3$	1.5 gm (varies)
Penicillin	100 units/ml
Streptomycin	100 ug/ml
Serum	20 to 200 ml*

*Serum may be omitted and sterile serum added as needed.

The bottles used to store the medium or serum were either 500 ml bottles which originally contained commercial fetal calf serum or 500, 1000, or 1500 ml bottles which originally contained isotonic saline and were obtained from a local hospital.

For general laboratory equipment, John Paul's Cell and Tissue Culture (1959) contains basic lists of laboratory apparatus and materials as well as a partial list of companies which supply research equipment.

EXPERIMENTAL MATERIALS AND METHODS

The Virus

Herpesvirus hominis type 2 (strain 196) was obtained from Dr. W. E. Rawls of Baylor University College of Medicine. Stock pools of virus were prepared in Vero (stable Green monkey kidney) cells. After the culture medium was removed from monolayers of Vero cells in 8 oz. prescription bottles containing about 1×10^7 cells, 1.6 to 4.0×10^6 TCID units of virus in 1 ml culture fluid were allowed to adsorb to the cells for one hour at 37°C. At the end of one hour, 15 to 20 ml of MEM with 3% calf serum were added to the bottle. After 30 to 36 hours, or when most of the cells had detached from the glass, the cultures were frozen and thawed twice to disrupt

the cells and to release cell-associated virus. The cellular debris was pelletted in an International H9070 size 2 centrifuge at 1,000 rpm for 10 minutes. The supernatant culture fluid was collected and stored in flame-sealed glass ampules in 1-ml amounts at -70°C until use. The stock pool was titered by pooling two ampules of virus and titration by the 50% Tissue Culture Infective Dose ($TCID_{50}$) method.

The $TCID_{50}$ by the Microtiter Method[1]

Special materials used for this method were Falcon 3040 Microtest II tissue culture plates obtained from Falcon Plastics, Oxnard, California; Microdiluters (0.05 ml volume), available from Cooke Engineering Company; and Micropipettes (0.05 ml volume), obtained from Microbiological Associates, Bethesda, Maryland.

The Falcon Microtest II tissue culture plate has 96 wells in an 8 by 12 arrangement. Using a 0.05 ml micropipette, in which each drop is calibrated at 0.05 ml, one drop of MEM with 3% calf serum was added to each well. Appropriate serial 10-fold dilutions of virus in quadruplicate were diluted with a 0.05 ml microdiluter in three step 2-fold series. Three dilutions of each sample were plated.

The microdiluter was dipped in 95% ethanol and rotated between the fingertips. After flaming, and starting with the 10^{-3} dilution, an entire sample was plated before sterilizing the microdiluter again. A diagrammatic representation of the tissue culture plate is shown in Figure 1.

The microdiluter was allowed to cool for about 15 seconds, then dipped into the 10^{3} dilution. The microdiluter was rotated for 10 cycles back and forth with the fingertips, then placed into the 2^{-1} well in the extreme right of the 10^{-3} series and rotated for 10 cycles. The microdiluter was removed from the 2^{-1} well, placed into the 2^{-2} well, and rotated for 10 cycles. The microdiluter was removed from the 2^{-2} well, placed into the 2^{-3} well, and rotated for 10 cycles. Finally, the microdiluter was removed from the last well and reloaded in the 10^{-3} dilution. The above was repeated until the

[1]Microtiter is a trademark of Cooke Engineering Company of Alexandria, Virginia.

three dilutions were plated, the microdiluter was sterilized in alcohol and flamed, and the next sample was started. The process was made faster when two or more microdiluters were used simultaneously. With dilutions in test tubes, two microdiluters was the practical limit. When all samples were plated, 0.05 ml of a suspension of 2×10^5 Vero cells per ml of MEM plus 3% calf serum was added to each well, or 1×10^3 cells per well. The plates were read at 48 hours by noting the presence of absence of CPE in the monolayers in the well.

	10^{-1}				10^{-2}				10^{-3}				
Sample #1	⊖	⊖	⊖	⊖	⊖	⊖	⊖	⊖	0	0	0	0	2^{-1}
	⊖	⊖	⊖	⊖	⊖	⊖	⊖	0	0	0	0	0	2^{-2}
	⊖	⊖	⊖	⊖	0	0	0	0	0	0	0	0	2^{-4}
Controls	0	0	0	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	0	0	0	0	0	0	
Sample #2	⊖	⊖	⊖	⊖	⊖	0	0	0	0	0	0	0	2^{-1}
	⊖	⊖	⊖	⊖	0	0	0	0	0	0	0	0	2^{-2}
	⊖	⊖	⊖	0	0	0	0	0	0	0	0	0	2^{-4}

Figure 1. Diagrammatic Representation of a Microtest II Plate

To calculate the $TCID_{50}$, the Reed-Muench formula was modified from the original described by Lennette and Schmidt (15). Data was arranged according to Table 4 (e.g., Sample 1, from Figure 1).

The accumulated values for the number of wells showing CPE (column e) or not showing CPE (column f) are obtained by adding columns c and d in the direction indicated by the arrows. The accumulated mortality ratio (column g) represents the accumulated number of wells showing CPE (column e) over the total number of wells accumulated (column e + column f). For example, in the 4×10^2 reciprocal of the virus (RV) dilution, there were three wells showing CPE in a total of four wells.

In the example, the percentage CPE in the 4×10^2 RV dilution is higher than 50%; the percent CPE in the next RV dilution, 8×10^2, is lower than 50%. The 50% endpoint lies between these two RV dilutions.

The proportionate distance of the 50% endpoint from the dilution next above 50% is obtained as follows:

$$\text{Proportionate Distance} = \frac{(\text{Percent CPE at RV dil. next above 50\%}) - (50\%)}{(\text{Percent CPE at RV dil. next above 50\%}) - (\text{Percent CPE at RV dil. next below 50\%})}$$

$$= \frac{75 - 50}{75 - 0}$$

$$= \frac{25}{75}$$

$$= 0.33$$

Since logarithmically the distance between any two dilutions is a function of the incremental steps used in preparing the series, it is necessary to correct (i.e., multiply) the proportionate distance by the logarithm of the dilution factor. In a 2-fold dilution series, the $\log_{10}(2) = .301$ is used as the correction factor. Therefore:

$$\text{The logarithm of the } TCID_{50} \text{ endpoint titer} = \begin{array}{c}(\text{The logarithm of the RV dilution next above 50\% CPE}) \\ + \\ (\text{The proportionate distance multiplied by the logarithm of the dilution series used})\end{array}$$

or

$$[\log_{10}(4 \times 10^2)] + [(0.33)(\log_{10}(2))]$$

$$[(0.33)(.301)]$$

$$[2.602] + [0.100] = 2.702$$

Since the volume of the culture fluid sample is 0.05 ml, the

$$[\log_{10}(TCID_{50})] = [2.702/0.05 \text{ ml}]$$

$$[TCID_{50}] = [5.4 \times 10^2 \text{ units}/0.05 \text{ ml}]$$

$$= [1.08 \times 10^3 \text{ units/ml}]$$

$$= [1.1 \times 10^3 \text{ units/ml}]$$

In cases such as Sample #2 in Figure 1, where the RV dilution next above 50% is 8×10^1, and the next below 50% is 2×10^2, which is

not a 2-fold increment, the average of 8×10^1 and 1×10^2, or 9×10^1, was used to minimize error.

Table 4. Data Arrangement for Reed-Muench Formula

Reciprocal* of Virus Dilution	CPE Ratio	CPE	No CPE	Accumulated Values			
				CPE	No CPE	Mortality	
						Ratio	Percent
a	b	c	d	e	f	g	h
2×10^1	4/4	4 ↓	0	19	0	19/19	100
4×10^1	4/4	4 ↓	0	15	0	15/15	100
8×10^1	4/4	4 ↓	0	11	0	11/11	100
2×10^2	4/4	4 ↓	0	7	0	7/7	100
4×10^2	3/4	3	1 ↑	3	1	3/4	75
8×10^2	0/4	0	4 ↑	0	5	0/5	0
2×10^3	0/4	0	4 ↑	0	9	0/9	0
4×10^3	0/4	0	4 ↑	0	13	0/13	0

*The reciprocal of the virus dilution is abbreviated as the RV dilution in this procedure.

Cells

The cells used for this experimental series were Vero, HeLa, and MA111. The few laboratory rabbits available for this study carried a vacuolating virus which appeared about 72 hours or more after their kidney cells were cultured. Because of this and other difficulties, primary rabbit kidney cultures were not included in the study.

The Vero cell line was derived from a culture from an African Green monkey kidney line by Yasumura and Kawakita (1). The HeLa line originated from a human carcinoma of the cervix and was established by Gey, et al. (9). The MA111 line is a stable rabbit kidney line stabilized after numerous passages by Vincent (29).

The MA111 line was received from Microbiological Associates in Medium 199 and 10% fetal calf serum. It was transferred to MEM with 10% calf serum and adapted to MEM with 5% calf serum which was the standard medium used to grow stock cultures of stable cell lines.

On initial passages, MA111 was split 1:2 as recommended by Microbiological Associates. It was found that cells could be split 1:5 on clean glass and 1:10 on "used" glass, as was standard procedure for Vero and HeLa cells. Used glass is glassware that was used for growing cells and which has not been reprocessed. Glassware was used until the cells began sticking to the glass during passage.

The cells lines, which were grown in 8 oz. prescription bottles, were passed by trypsinization of monolayers and reseeding part of the cells. Prior to the addition of 1 ml of versene-trypsin solution to each bottle, the monolayer was washed twice with about 5 ml of Pd, incubated in 10 to 15 ml of Pd for 5 minutes, and washed once more with 5 ml Pd. The Pd wash served to remove Mg^{++} and Ca^{++} ions and any other serum factors which inactivate trypsin. When the cells detached from the glass (5 to 10 minutes), the suspension was mixed with 10 ml of growth medium. For maintaining stock cultures, 1 to 2 ml of this cell suspension was left in each bottle and 20 ml of fresh MEM with 5% calf serum was added to each bottle.

Cells seeded on clean glass were gassed with air containing 5% CO_2 to complete the Earle's HCO_3^-/CO_2 buffer used in MEM. Cells seeded on used glassware generate sufficient CO_2 to complete this buffer. Microtest plates were placed in a gas-tight box which was gassed with air with 5% CO_2. The temperature of incubation for growing cells and for conducting the growth curve studies was 37°C.

When known numbers of cells were desired, the cells were counted by using a hemocytometer. A hemocytometer has a grid of 16 large squares measuring 1 mm^2. A glass coverslip restricts the chamber depth to 0.1 mm. Therefore, each square represents 0.1 mm^3 of cell suspension. The cells are counted in the 4 corner squares which are in turn subdivided into 16 smaller squares for ease of counting. The total number of cells in the 4 sqaures is divided by 4 to get the average cell count per 0.1 mm^3. There are 1000 mm^3 per 1.0 cm^3, and 1.0 cm^3 is approximately 1.0 ml. Therefore, the average cell count per 0.1 mm^3 multiplied by 10^4 is a good estimate of the number of cells per ml. Needless to say, if any other dilutions are made before the cell count is taken, they must be considered in the calculations.

CHAPTER III

RESULTS

For the growth curve studies, 3 oz. prescription bottles were seeded with 5×10^5 cells on 10 to 12 ml of MEM with 5% calf serum. The cells were allowed to grow monolayers of 4×10^6 cells as determined by trypsinizing random cultures and counting the cells in a hemocytometer. The monolayers were infected with 1.6 to 2.0×10^6 $TCID_{50}$ units of HVH type 2 (strain 196) in 1.0 ml medium. The virus was adsorbed for 60 minutes at 37°C. After the adsorption period the cultures were washed three times with 10 ml Pd to remove unadsorbed virus. Finally, 8 ml of MEM with 3% calf serum was added to the cultures. The samples taken at this time were plotted at Time = 0.

Released virus was sampled by adding 0.5 ml of MEM with 3% calf serum to the culture, mixing, then removing 0.5 ml of culture fluid. Total virus was sampled by freezing and thawing the culture three times to disrupt the cells and to release cell-associated virus. If released and total virus were sampled simultaneously from the same culture, the released virus sample was taken as above prior to freezing the culture. All samples were assayed for virus by the $TCID_{50}$ method described in the Materials and Methods section.

The growth curves of HVH type 2 in all three cell lines were superficially similar to each other although different in detail. All showed latent periods where the residual virus titer fell. After sufficient time to initiate assembly of virus, the titers rose, passing quickly into log growth phase. Foci of CPE appeared toward the end of the latent period. Finally, there were decelerated growth and terminal growth phases. All cells had detached from the glass surface by the end of the decelerated growth phase. During the terminal phase the virus was not being replicated, but cell-associated virus

was released slowly as the cells broke up due to autolysis.

For Vero cells (Table 5, Figure 2), the latent period for total virus was about three hours. After four hours the titer had risen and replication was in log phase. Between twelve and fourteen hours replication had slowed, and by sixteen hours terminal phase was reached. The latent period for virus release was about four hours. Release reached log phase by six hours, slowed by twenty hours and was terminal by twenty-four hours. The peak total virus titer was about 1.6×10^6 $TCID_{50}$ units per ml of culture fluid. The peak titer of virus released was about 5.2×10^5 $TCID_{50}$ units per ml of culture fluid. Peak titer was determined by taking the mean of the titer of all samples taken after terminal phase was reached.

For HeLa cells (Table 6, Figure 3), the latent period to total virus replication was about four hours. After six hours the titer had risen and replication was in log phase. By twenty hours replication had slowed, and by twenty-four hours replication was in terminal phase. The latent period for virus release was about four hours. After six hours the titer had risen and release was in log phase. The release slowed between eighteen and twenty hours and was terminal by twenty-four hours. Peak total virus titer was about 7×10^5 $TCID_{50}$ units per ml of culture fluid. Peak released virus titer was about 3.0×10^5 $TCID_{50}$ units per ml of culture fluid.

For MA111 cells (Table 7, Figure 4), the latent period for total virus replication was about six hours. Log phase was reached shortly after eight hours. Replication slowed by eighteen hours and was terminal by twenty hours. The latent period for virus release was about nine hours. Log phase was reached by twelve hours. Release slowed by eighteen hours and was terminal by twenty hours. Peak total virus titer was about 8.1×10^5 $TCID_{50}$ units per ml of culture fluid. Peak released virus titer was about 1.1×10^5 $TCID_{50}$ units per cell.

Herpesvirus hominis type 2 (strain 196) tended to remain cell-associated in all three cell lines, as indicated by the differences between total and released virus titers. In Vero cells 67.5% of the virus remained cell-associated; in MA111 cells 87.3% of the virus remained cell-associated. In HeLa cells 57% of the virus remained cell-associated.

A thermal stability test at 37°C was conducted by suspending an ampule of stock virus in a total of 10 ml of MEM with 3% calf serum in a screw capped test tube. At desired times, 0.5 ml of virus suspension was removed and assayed by the $TCID_{50}$ method. The tube was gassed with air plus 5% CO_2 each time it was opened to maintain pH 7.

It was found (Figure 5) that after a relatively stable period of about five hours the virus became unstable. About 70% of the virus was lost in ten to twelve hours and about 95% of the virus was lost in twenty-four hours. By extrapolation, the half-life of HVH type 2 was about eight hours.

Freeze-thaw stability was determined by suspending stock virus in 10 ml of MEM with 3% calf serum. A dry ice-alcohol bath was used to freeze the sample, and warm water was used to thaw it. The screw capped test tube was gassed with air each time it was opened to flush the CO_2 which entered the tube from the dry ice bath. After each freeze-thaw cycle 0.5 ml of the virus suspension was removed and assayed by the $TCID_{50}$ method.

The freeze-thaw stability test (Figure 6) showed a slight rise in titer after the first cycle, then a stable period of two cycles, followed by loss of virus on subsequent cycles. Half the peak titer of virus was lost by the seventh cycle.

A trial was conducted to verify that release of virus by cells after they detach from the culture surface were passive (i.e., that the cells were dead and virus was released when the cells disrupted due to autolysis). A 3 oz. prescription bottle containing 4×10^6 cells was infected with 2×10^6 $TCID_{50}$ units of virus by the same methods used in the growth curve studies. After twenty-two hours the detached cells were pelletted at low speed (1,000 rpm in an International H9070 centrifuge). The cells were washed twice by resuspending them in 10 ml MEM with 3% calf serum and 1.0 ml samples were removed, after gently rocking the tube to resuspend the cells at two-hour intervals for six hours. Of the sample, 0.5 ml was used to assay for released virus by the $TCID_{50}$ method, and the balance was used to make cell counts in a hemocytometer.

The trial (Table 8) shows a slight increase in released virus

titer accompanied by a slight loss in cell count, suggesting that virus was released from the detached cells as the cells were disrupted by autolysis.

Table 9 shows the peak total and released virus titers expressed as yield per cell in the infected monolayer. This comparison makes it possible to relate this study with those in the literature. These data were calculated by taking the average yields per ml of culture fluid, and dividing by 4×10^6 cells. Vero cells released 1.1 $TCID_{50}$ units per cell and produced 3.2 $TCID_{50}$ units per cell. HeLa cells released 0.6 $TCID_{50}$ units per cell and produced 1.4 $TCID_{50}$ units per cell. MA111 cells released 0.22 $TCID_{50}$ units per cell and produced 1.6 $TCID_{50}$ units per cell.

Table 5. Herpesvirus hominis type 2 (strain 196) Replication in a Monolayer of 4 x 10^6 Vero Cells in 8 ml Minimal Essential Medium with 3% Calf Serum; Infective Dose 1.6 to 2.0 x 10^6 $TCID_{50}$ Units; Incubation Temperature 37°C.

Time*	Released		Total		
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 3
0	1.6 x 10^3	2.3 x 10^3	8.0 x 10^4	5.5 x 10^3	2.3 x 10^4
3	8.0 x 10^2	---	---	1.4 x 10^3	---
4	---	1.1 x 10^3	---	---	5.6 x 10^3
6	1.1 x 10^3	---	4.0 x 10^4	2.3 x 10^4	---
8	---	2.0 x 10^3	2.0 x 10^5	---	1.6 x 10^4
9	5.7 x 10^3	---	---	1.6 x 10^4	---
12	1.6 x 10^4	6.4 x 10^4	2.3 x 10^6	4.0 x 10^5	1.4 x 10^5
15	1.0 x 10^5	---	---	8.0 x 10^5	---
16	---	6.8 x 10^4	---	---	1.3 x 10^6
18	1.1 x 10^5	---	---	8.0 x 10^5	---
20	---	2.0 x 10^5	---	---	2.3 x 10^6
21	5.7 x 10^4	---	6.4 x 10^6	5.7 x 10^5	---
24	8.0 x 10^5	3.6 x 10^5	---	6.4 x 10^5	2.5 x 10^6
28	---	4.5 x 10^5	---	---	1.4 x 10^6

*Time expressed in hours.

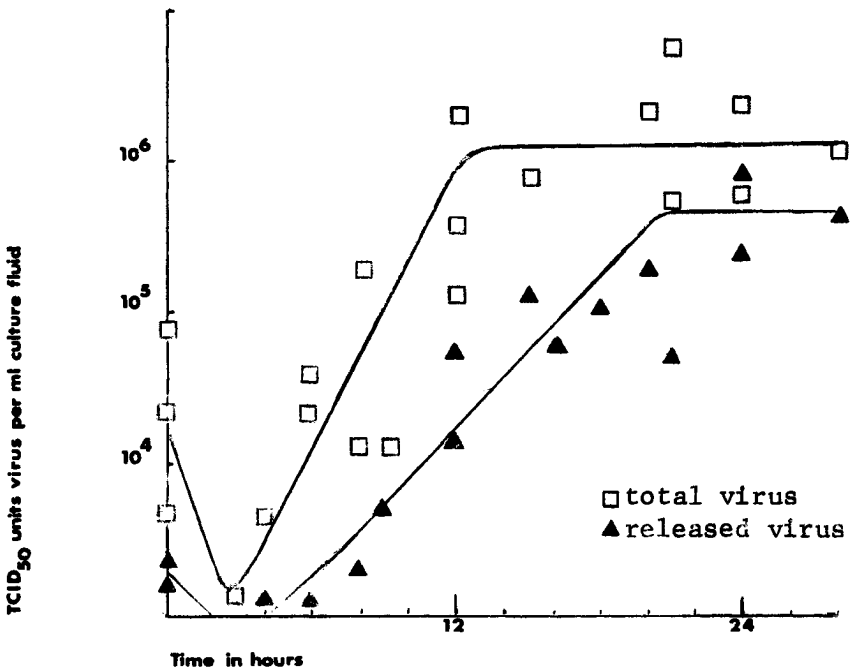

Figure 2. Herpesvirus hominis type 2 (strain 196) Replication in a Monolayer of 4 x 10^6 Vero Cells in 8 ml Minimal Essential Medium With 3% Calf Serum; Infective Dose 1.6 to 2.0 x 10^6 $TCID_{50}$ Units; Incubation Temperature 37°C.

Table 6. Herpesvirus hominis type 2 (strain 196) Replication in a Monolayer of 4 x 10^6 HeLa Cells in 8 ml Minimal Essential Medium With 3% Calf Serum; Infective Dose 1.6 to 2.0 x 10^6 $TCID_{50}$ Units; Incubation Temperature 37°C

Time*	Released		Total	
	Trial 1	Trial 2	Trial 1	Trial 2
0	6.0 x 10^3	3.6 x 10^3	4.7 x 10^3	1.6 x 10^4
3	2.0 x 10^3	---	---	---
4	---	2.0 x 10^3	---	5.7 x 10^3
6	3.0 x 10^3	---	8.0 x 10^3	---
8	---	5.0 x 10^3	---	2.5 x 10^4
9	1.4 x 10^4	---	---	---
12a**	3.6 x 10^4	1.1 x 10^4	1.6 x 10^4	5.7 x 10^4
12b**	2.0 x 10^4	---	9.3 x 10^4	---
15	2.5 x 10^4	---	---	---
16	---	1.0 x 10^5	---	2.9 x 10^5
18	5.6 x 10^4	---	2.6 x 10^5	---
20	---	5.7 x 10^4	---	2.2 x 10^5
21	1.0 x 10^5	---	---	---
24	2.1 x 10^5	4.4 x 10^5	2.0 x 10^5	1.1 x 10^6
28	---	2.5 x 10^5	---	8.0 x 10^5

*Time expressed in hours.

**The growth curve was split at 12 hours. T = 12a was the end of one half, and T = 12b was the start of the other half.

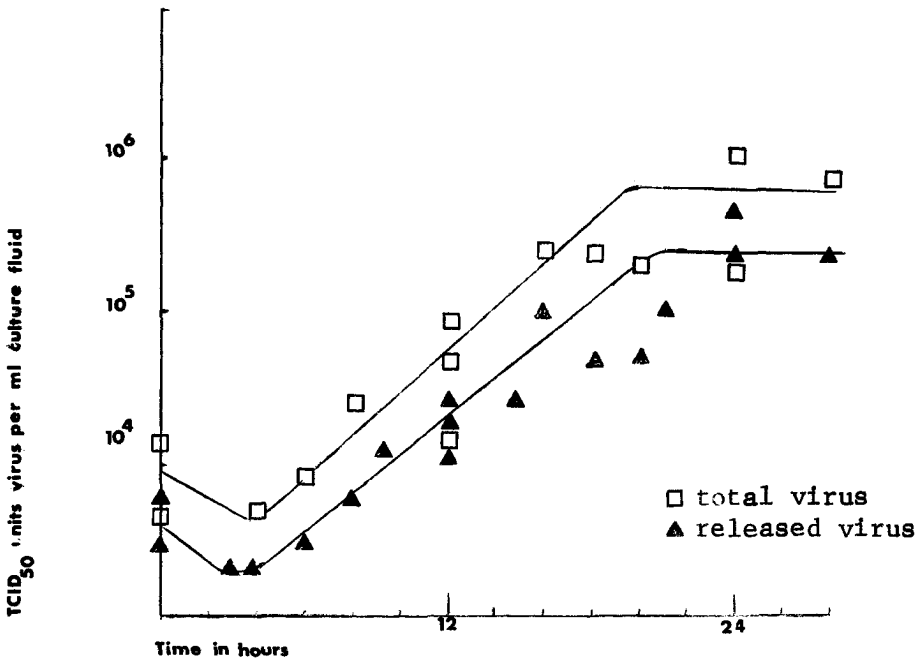

Figure 3. Herpesvirus hominis type 2 (strain 196) Replication in a Monolayer of 4 x 10^6 HeLa Cells in 8 ml Minimal Essential Medium With 3% Calf Serum; Infective Dose 1.6 to 2.0 x 10^6 $TCID_{50}$ Units; Incubation Temperature 37°C

Table 7. Herpesvirus hominis type 2 (strain 196) Replication in a Monolayer of 4 x 10^6 MA111 Cells in 8 ml Minimal Essential Medium With 3% Calf Serum; Infective Dose 1.6 to 2.0 x 10^6 $TCID_{50}$ Units; Incubation Temperature 37°C

Time*	Released		Total	
	Trial 1	Trial 2	Trial 1	Trial 2
0	1.1 x 10^4	6.4 x 10^3	7.7 x 10^3	2.0 x 10^4
3	4.0 x 10^3	---	---	---
4	---	3.0 x 10^3	---	6.8 x 10^3
6	3.0 x 10^3	---	2.0 x 10^3	---
8	---	2.3 x 10^3	---	4.5 x 10^3
9	2.5 x 10^3	---	---	---
12a**	8.0 x 10^3	5.0 x 10^3	6.4 x 10^4	3.0 x 10^4
12b**	4.5 x 10^3	---	2.2 x 10^4	---
15	1.6 x 10^4	---	---	---
16	---	1.6 x 10^4	---	2.0 x 10^5
18	8.0 x 10^4	---	1.3 x 10^6	---
20	---	9.3 x 10^4	---	4.7 x 10^5
21	8.0 x 10^4	---	---	---
24	1.3 x 10^5	1.6 x 10^5	1.6 x 10^6	5.7 x 10^5
28	---	1.8 x 10^5	---	6.4 x 10^5

*Time expressed in hours.

**The growth curve was split at 12 hours. T = 12a was the end of one half, and T = 12b was the start of the other half.

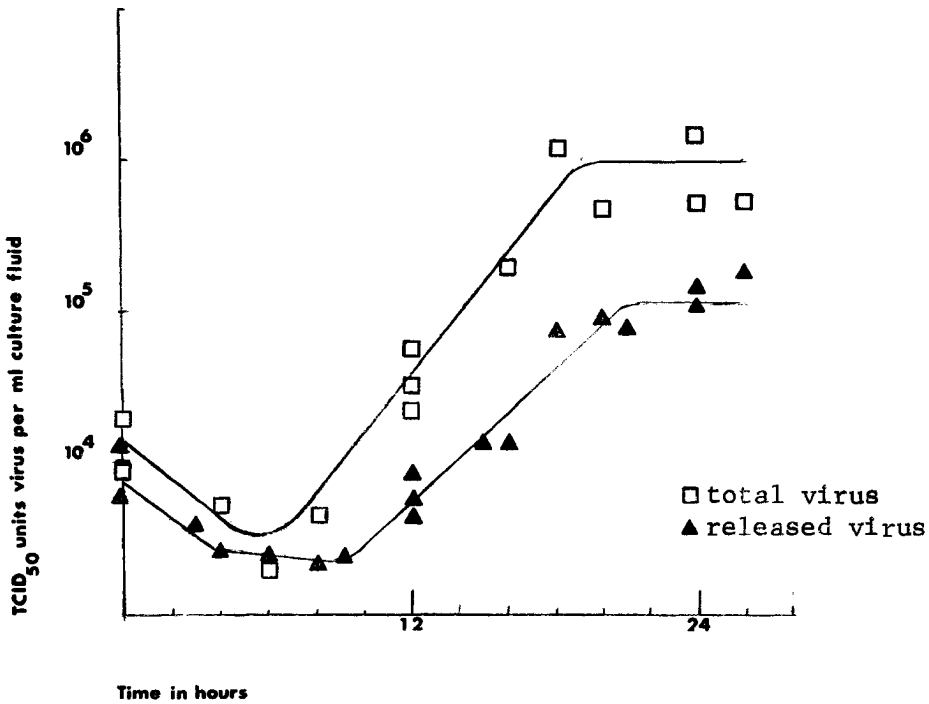

Figure 4. Herpesvirus hominis type 2 (strain 196) Replication in a Monolayer of 4 x 10^6 MAlll Cells in 8 ml Minimal Essential Medium With 3% Calf Serum; Infective Dose 1.6 to 2.0 x 10^6 $TCID_{50}$ Units; Incubation Temperature 37°C

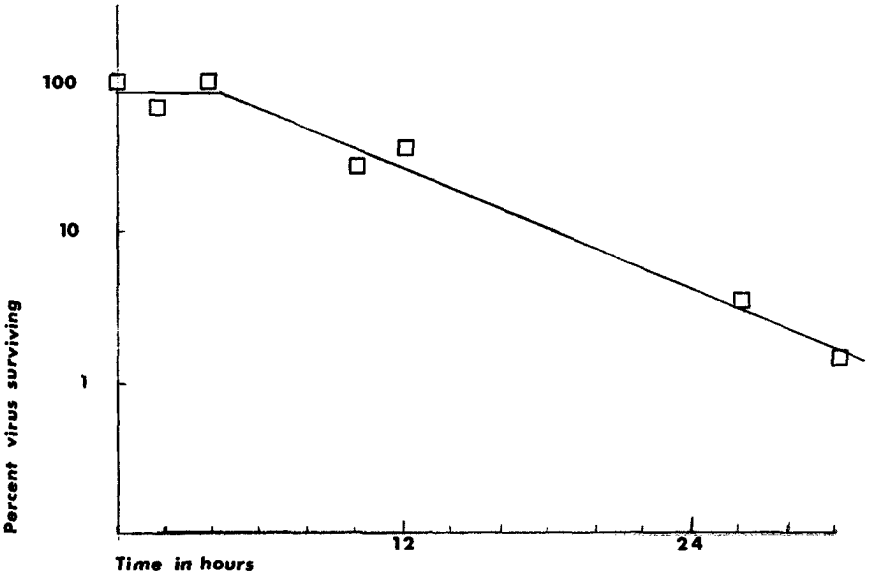

Figure 5. Thermal Stability of Herpesvirus hominis type 2 (strain 196) at 37°C

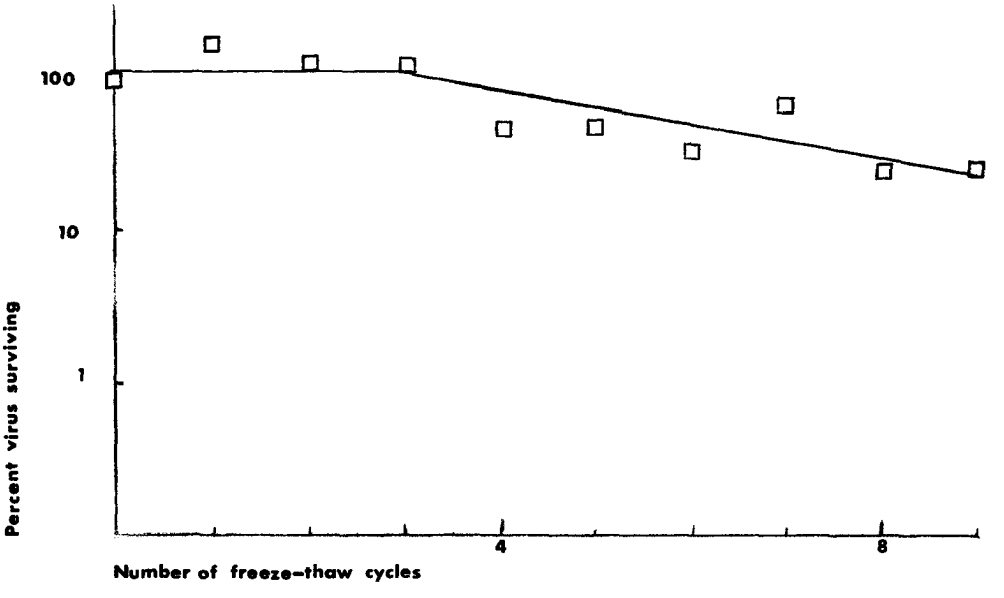

Figure 6. Freeze-Thaw Stability of Herpesvirus hominis type 2 (strain 196)

Table 8. Extended Release of Herpesvirus hominis type 2 (strain 196) by Detached Vero Cells in 10 ml Minimal Essential Medium With 3% Calf Serum; Incubation Temperature 37°C

Time in Hours	$TCID_{50}$ of Released Virus	Cell Count
0	6.36×10^5 units/ml	7×10^5/ml
2	6.36×10^5 units/ml	5×10^5/ml
4	6.00×10^5 units/ml	6×10^5/ml
6	8.00×10^5 units/ml	5×10^5/ml

Table 9. Peak Herpesvirus hominis type 2 (strain 196) Titers in Different Stable Cell Lines Expressed in Terms of $TCID_{50}$ Units per Cell in the Infected Monolayer; Incubation Temperature 37°C

Cell Line	Released Virus Titer	Total Virus Titer
Vero	1.10	3.2
HeLa	0.60	1.4
MA111	0.22	1.6

CHAPTER IV

DISCUSSION

There appears to be no precedent in the literature for discussing the Vero or MA111 culture and HVH systems. Both cell lines have been used extensively for the study of RNA viruses (26, 27) and to some extent with SV-40 and adenoviruses (29). This study indicated that Vero was superior to HeLa cells in its capacity to support the reproduction of HVH type 2. Vero was also found to be superior to HeLA as an indicator cell for the assay of HVH type 2 because it showed CPE sooner than HeLa, espeically under low multiplicity conditions. Furthermore, in pilot studies, it was found that Vero could be maintained in monolayer for longer periods of time than HeLa. The MA111 cell line was at least equal to HeLa in its capacity to support HVH type 2 reproduction, but it was not equivalent to primary rabbit kidney cells as it was hoped to be. The initial observations which prompted this study indicated that primary rabbit kidney did not support the growth of HVH type 2 as well as HeLa did.

There has been extensive work with the HeLa and HVH system, and some growth curve data has been published. Waterson (1968), summarizing a paper by Newton and Stoker (1958), reports that HeLa produced 10 plaque-forming units per cell. Siminoff (1964), working with two strains of HVH, reports yields of 16 and 50 plaque-forming units per cell. In both studies 5 plaque-forming units per cell were used as initial inocula. The differences in yield of this study compared to yields reported in the literature are probably due to a combination of initial infective dose, strain differences in the virus, and to the relative sensitivity of the assay methods.

The stability tests (Figures 5 and 6) compared closely with those done by Kaplan (1957). He reports less than 1% survival of his

H-4 strain of HVH after 24 hours at 37°C. In this study, HVH type 2 (strain 196) had a survival rate of less than 5% after 24 hours. Kaplan's freeze-thaw material shows a rise in titer that he attributes to disruption of virus aggregations, a stable period of two or three freeze-thaw cycles, and finally, loss of virus on subsequent cycles. The results of this study (Figure 6) show a similar pattern, with 50% loss by cycle 7 and 70% loss by cycle 9. After the first three cycles, roughly 10% loss of virus titer can be expected with each subsequent freeze-thaw cycle.

This study showed different growth patterns in the different cell lines. The log growth phase in both Vero and MAlll had a steeper slope than did the log phase of HeLa. As mentioned in the introduction, Group A viruses show changes in virulence when introduced into unnatural hosts. Herpesvirus hominis infection in man is usually mild, but in the absence of immune mechanisms the virus is able to infect all the cells in the culture. The steeper slopes of the log growth phases of Vero and MAlll and their subsequent earlier death may indicate part of the reason for the severity of HVH infection in monkeys and rabbits. The virus had a longer latent period in MAlll than in either Vero or HeLa. Since rabbits are not as phylogenetically close to humans as monkeys, HVH may not be able to use the enzyme systems present in rabbit cells as efficiently as it uses the enzyme systems in human or monkey cells. After the latent period, virus-coded enzymes play a more important role in the infection (8, 14). Once the virus-coded enzyme systems are established, there are no genetic factors, as found in human cells, to retard the infection in either Vero or MAlll.

Initial trials showed that the released virus titer remained stationary for about 48 hours before falling. It was presumed that cell-associated virus was being released from the detached cells at a rate to compensate for thermal lability. Detached Vero cells resuspended in fresh MEM plus 3% calf serum showed a slight rise in virus titer and a slight loss in cell count (Table 6). The growth curve studies showed the presence of a viable cell-associated virus which is released when the cells are disrupted. This test verified a slight

rise in virus titer with a slight loss in cell count. This study was conducted within the time limit of the stable period in the viral thermal lability curve, and the cells were put under more stress for this test than in the pilot growth curve studies when they were not pelletted and resuspended. All factors considered, it is feasible that enough virus was cell-associated and could be released by cell break-up due to autolysis to keep the released virus titer relatively stable. Pilot studies with Vero also showed that the total virus titer fell within 36 hours. From the above, it can be concluded that detached cells are dead and are no longer replicating virus.

In all three cell lines, ballooning and detaching of cells seemed to be directly related to virus production; as more cells ballooned, more virus was detected. All cells in all three cell lines detached within 18 to 24 hours, Vero being the first to detach completely and HeLa and MA111 detaching later.

In the preparation of stock pools, it was noted that most HeLa cells detach from the glass culture surface after 48 to 60 hours. Vero cells detach after 26 to 30 hours. Since virus production seems to be related to the appearance of CPE, the lower titers observed in HeLa are probably a result of the slower rate of virus replication in HeLa, the resultant lower peak titer, and to thermal inactivation of the virus at $37^{o}C$.

CHAPTER V

SUMMARY

It was noted by Dr. R. N. Ushijima that when Herpesvirus hominis was prepared in different cell lines, there were differences in peak titers of virus produced. It was decided to do growth curve studies to determine the rate and time of release of HVH type 2 (strain 196) from the cell lines, HeLa, Vero, and MA111, and to correlate, if possible, the appearance of CPE with the production of virus. Stability tests were done; a determination of the viability of detached cells was done.

Monolayers of 4×10^6 cells in 3 oz. prescription bottles were infected with 0.5 $TCID_{50}$ units of virus. The cultures were sampled for total and released virus. The virus was assayed by the Microtiter[1] method, and the 50% Tissue Culture Infective Dose ($TCID_{50}$) was calculated by a modified Reed-Muench method.

Cell-free virus was resuspended in MEM with 3% calf serum, and the virus was tested for stability at 37°C and for stability when exposed to repeated freeze-thaw cycles.

Detached infected Vero cells were pelletted and resuspended in fresh MEM. Virus assays and cell counts were done at two-hour intervals for six hours.

The growth pattern of HVH was different in each cell line. With respect to total virus, the latent period in Vero was about 3 hours, in HeLa about 4 hours, and in MA111 about 7 hours. Peak titers were produced in 14 hours by Vero, in 24 hours by HeLa, and in 16 hours by MA111. The virus tended to stay cell-associated in all cell lines. In Vero, 67.5% of the virus remained cell-associated, and in HeLa 57% of the virus remained cell-associated; 87.3% of MA111 virus

[1]Trademark of Cooke Engineering Co., Alexandria, Virginia.

remained cell-associated. The virus remained cell-associated for a relatively longer time in MA111 than in Vero or HeLa. Cytopathic effect appeared in foci at the end of the latent period, and all Cells had detached by the time peak titers were produced.

Peak total virus titers were 3.2 $TCID_{50}$ units per cell for Vero, 1.4 $TCID_{50}$ units per cell for HeLa, and 1.6 $TCID_{50}$ units per cell for MA111. Peak titers for released virus were 1.1 $TCID_{50}$ units per cell for Vero, 0.6 $TCID_{50}$ units per cell for HeLa, and 0.22 $TCID_{50}$ units per cell for MA111.

Both the thermal stability test and the freeze-thaw stability test showed brief stability followed by lability. The virus was stable for about five hours at 37°C and over three freeze-thaw cycles. The half-life of the virus at 37°C was about eight hours, and half the virus titer was lost after seven freeze-thaw cycles.

It was concluded that detached cells were dead and released cell-associated virus passively as they broke up due to autolysis.

It was also concluded that the lower peak titers produced in HeLa with respect to Vero when stock pools were prepared were a function of the slower replication rate in HeLa and the lower peak titer produced by HeLa reduced further by thermal inactivation of the virus.

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APPENDIX

PARTIAL LIST OF SUPPLY COMPANIES

Bellco Glass Company, Inc.
340 Edrudo Road
Vineland, New Jersey 08360

Bioquest
P.O. Box 243
Cockeysville, Maryland 21030

Calbiochem
P.O. Box 12087
San Diego, California 92112

Cole-Parmer Instrument Company
745 North Oak Park Avenue
Chicago, Illinois 60648

Cooke Engineering Company
Medical Research Division
735 N. St. Asaph Street
Alexandria, Virginia 22314

Corning Glass Works
Laboratory Products Division
Corning, New York 14380

Difco Laboratories
P.O. Box 5058A
Detroit, Michigan 48232

Flow Laboratories
1710 Chapman Avenue
Rockville, Maryland 20852

Grand Island Biological Company (GIBCO)
3175 Stanley Road
Grand Island, New York 14072

Kimble Products, Division of Owens-Illinois
Toledo, Ohio 43601

Miles Laboratories, Inc.
Research Division
P.O. Box 272
Kankakee, Illinois 60901

Microbiological Associates
4733 Bethesda Avenue
Bethesda, Maryland 20014

Sargent-Welch Scientific Company
7300 North Linder Avenue
Skokie, Illinois 60076

Van Waters and Rogers Scientific Company
P.O. Box 320
San Francisco, California 94119

or

P.O. Box 23
High Brdge Station
Bronx, New York 10452