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Encapsidation Defectiveness of Herpes Simplex Virus Type 2 During Replication at Acid pH Condition

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SUMMARY **The maximal yield of herpes simplex virus type 2 (HSV-2) grown at pH 6.5 decreased 10^2-10^3 fold compared to that recovered at pH 7.5. Electron microscopic observation of the infected cells maintained at these 2 pH conditions indicated that approximately equal amounts of immature virions were synthesized 6 hours after infection. However, at 18 hours post infection the majority of viruses present in the nucleus of infected cells maintained at pH 6.5 were empty or partially cored capsids with some particles enveloped and present in the cytoplasm, whereas at pH 7.5 mature virions already appeared at the cytoplasmic membrane. Analysis of viral polypeptides by radioimmunoprecipitation indicated that the synthesis of p40, a family of polypeptides closely involved in viral DNA encapsidation, was significantly impaired in infected cells maintained at pH 6.5.**

It is well recognized that physiological alterations brought about by fever, emotional change, menstruation, hormone or being under immunosuppressive therapy play role in the recurrence of herpes simplex virus (HSV) infection.[1] The influence of a variety of environmental condition, viz. pH, nutrient and temperature on the productive type of HSV infection *in vitro* has also been demonstrated.[2-4] Since the pH of vaginal discharge in adult female genitalia is also changed during menstrual cycle,[5] the *in vitro* model might be very useful for study to reveal whether such change influenced the expression of HSV. A few studies have indicated an inhibition of plaque formation by HSV during growth at acid pH environment. This has been suggested to be due to an inhibition of the spread of virus from cell to cell[6] or that the expansion of infectious foci was interrupted.[7] However, this phenomenon has not been thoroughly investigated. It is possible that a number of steps in the replication cycle of the virus may be affected, including an impairment in macromolecular synthesis. The present report describes studies *in vitro* to elucidate such a possibility. Results of an electron microscopic (EM) observation of infected cells and immunoprecipitation analysis using monoclonal antibodies revealed that at least the synthesis of virus protein involved in encapsidation of HSV-2 was inhibited during growth at acid pH condition.

MATERIALS AND METHODS

Cell culture

Vero cells were grown in growth medium (GM) composed of M199 with Earle' salts supplemented with 10% heat-inactivated fetal calf serum (both from Gibco, Grand Island, NY, USA), 100 units/ml penicillin G and 100 μg/ml streptomycin at 37°C in 5% CO_2 environment. Cells were maintained in maintenance medium (MM) in which the amount of serum was reduced to 2% and 10 mM N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid (HEPES) was added. MM was adjusted to pH 7.5, unless indicated otherwise.

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Virus

Stocks of HSV-2 (Baylor 186 strain) were prepared from Vero cells infected with the virus at an approximate multiplicity of infection (MOI) of 0.5 PFU/cell. The virus in MM, pH 7.5, was allowed to adsorb for 1 hour (adsorption condition used throughout this study). Infected cell cultures which exhibited >75% cytopathic effects (CPE) were frozen and thawed twice; after eliminating cell debris by centrifugation at 1,100 × g, the infected cell lysate containing virus was stored at -70°C.

Plaque assay was used for determination of the titre of HSV-2. In brief, 0.5 ml of Vero cell suspension, containing 1.5-2.0 × 10^5 cells was plated in each well of a 24-well tissue culture plate (Falcon, Oxnard, California, USA). Confluent cell monolayers were infected in quadruplicate with 0.1 ml of appropriate dilutions of virus. After adsorption, the cells were washed with phosphate buffered saline (PBS), pH 7.5, and overlaid with 0.5 ml of GM containing 0.8% gum tragacanth. The medium was discarded 4-5 days later; the infected cultures were stained with 1% crystal violet in 10% formalin for 30 minutes and the number of plaques was counted.

Electron microscopy

Cell monolayers were washed with fresh MM at 37°C for 10 minutes and then with PBS. They were fixed in 2.5% glutaraldehyde in PBS at room temperature for an hour and washed 3 times with PBS. The pH of MM, PBS and fixative used was the same as that for virus cultivation. The cells were scraped, pelleted, postfixed in 1% osmium tetroxide in PBS, dehydrated and embedded in EM bed-812 (Electron Microscopy Sciences, Washington, PA, USA). Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a HU-12A Hitachi electron microscope at 75 KV.

Antiviral antibodies

The immunoglobulin fraction of rabbit antibodies to HSV-2 MS strain (rabbit anti-HSV-2) was from Dakopatts, Copenhagen, Denmark. The preparation and characterization of monoclonal antibody (MAb), $6E_8.F_{11}$, directed to HSV glycoprotein D (gD) have been described elsewhere.[8] Another MAb, $6C_5E_{10}$, reactive to p40, a major component involved in packaging of viral DNA[9] was similarly prepared. These 2 MAbs, in mouse ascites form, were combined before use.

Radioimmunoprecipitation assay for viral proteins

A subconfluent Vero cell monolayer was infected with HSV-2 strain 186 in MM at 10 PFU/cell. After 1 hour adsorption period, MM (either at pH 6.5 or 7.5) was added. Four hours after infection, the medium was replaced with one containing 50 μCi/ml of ^{35}S-methionine (>800 Ci/mM, Amersham International, Buckinghamshire, England) and the cells were cultured for 18 hours at 37°C. The cells were then harvested, washed, solubilized and processed further for

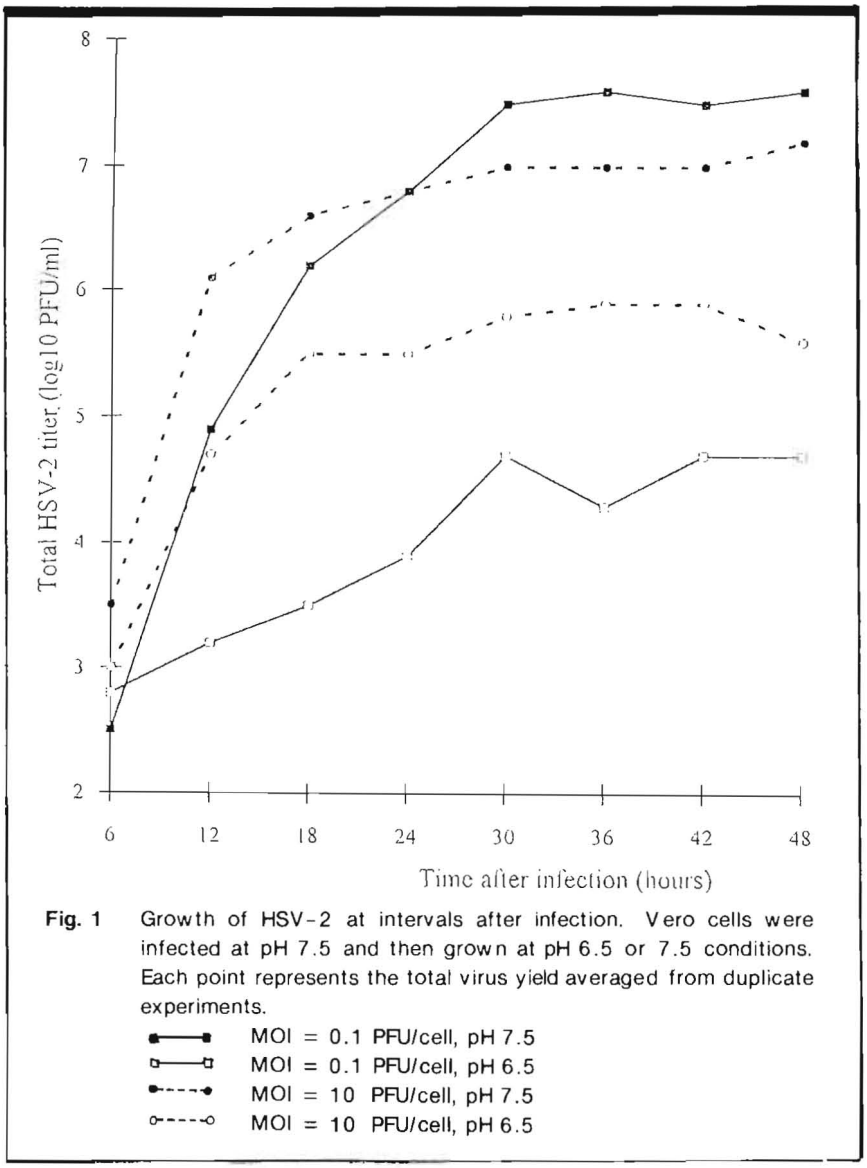

Fig. 1 Growth of HSV-2 at intervals after infection. Vero cells were infected at pH 7.5 and then grown at pH 6.5 or 7.5 conditions. Each point represents the total virus yield averaged from duplicate experiments.

- MOI = 0.1 PFU/cell, pH 7.5
- MOI = 0.1 PFU/cell, pH 6.5
- MOI = 10 PFU/cell, pH 7.5
- MOI = 10 PFU/cell, pH 6.5

immunoprecipitation using the method described previously.[8,10] The solubilized immune complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 9.5% acrylamide gel cross-linked with N,N′ diallyltartardiamide. Equal amounts of the samples were electrophoresed, unless indicated otherwise. The gels were treated for fluorography,[11] dried and exposed to Kodak X-OMAT AR film at -70°C.

RESULTS

Yield of HSV-2 under different pH conditions of growth

The effect of pH on the replication of HSV-2 was investigated in Vero cells monolayer grown in 24-well tissue culture plates. Cells were infected with the virus at an MOI of 0.1 PFU/cell. After 1 hour of adsorption, the inoculum was replaced with 1 ml of MM adjusted to various pH values ranging from 6.0 to 8.5, with an incremental pH difference of 0.5 unit. Infected cells were observed and the total (ie intra- and extracellular) virus yields were assayed 24 hours after infection. Cell fusion or polykaryon formation was a characteristic of HSV-2 strain 186-infected cells grown at pH $\geq$ 7.5 but was rarely or could not be seen at pH $<$ 7.5. Maximal virus yields were observed at pH 7.5 to 8.0 but started to decline at pH $\leq$ 7.0 (data not shown). At pH 6.5, the yield of HSV-2 was lower by approximately 3 log orders compared to those grown at the optimal pH condition; no alteration of the infected cells was seen under light microscopic examination. Fig. 1 illustrates growth kinetics of HSV-2 in cells infected with an MOI of 0.1 PFU/cell, at pH 6.5 and 7.5, and also in cells infected at an MOI of 10 PFU/cell, ie increasing the amount of inoculum 100 times. The difference in virus yield in cells infected with an MOI of 10 was apparent at 12 hours after infection when HSV-2 yields at pH 6.5 were approximately 1 to 1.5 log orders lower than those observed during growth at pH 7.5. Thus, the pH condition of growth affected the yield of HSV-2 in Vero cells infected with both low and high amounts of HSV-2. However, it should be noted that the ability of replication was resumed to normal whenever the environment was favorable. For instance, at 24 hours after infected, if the pH of the culture medium was shifted from 6.5 to 7.5, the yield of virus would significantly increase and reach the maximal titre within 24 hours (data not shown).

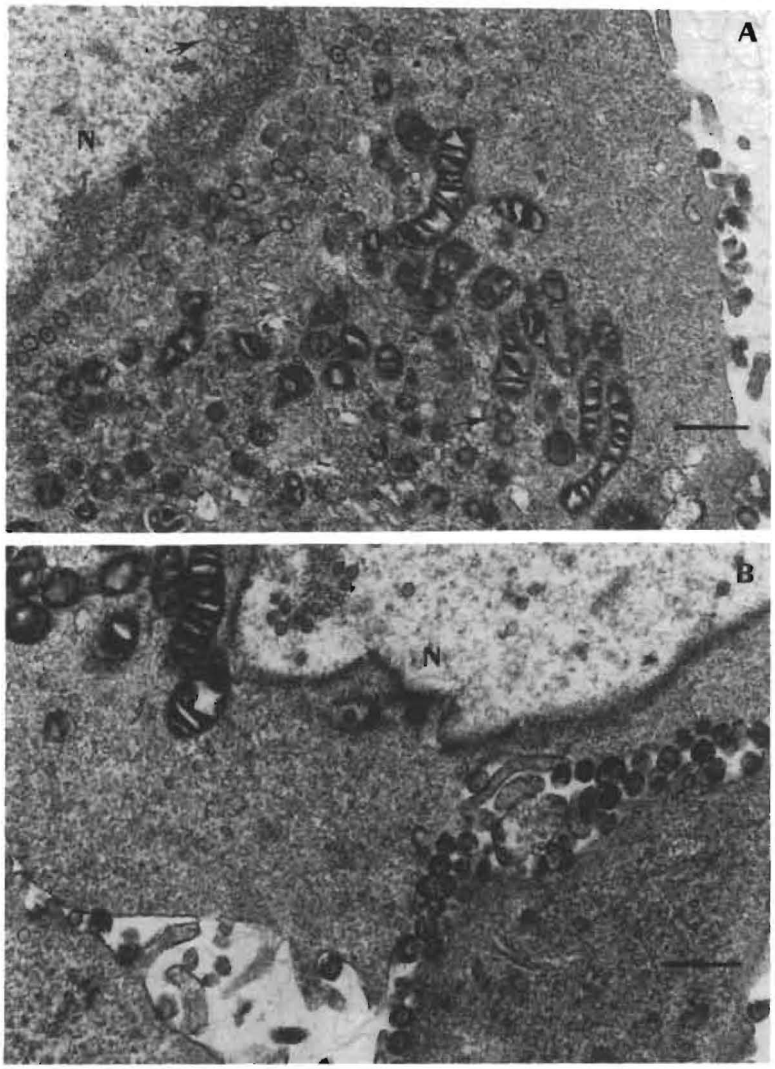

Fig. 2 Electron micrographs of Vero cells infected with HSV-2 and grown at either pH 6.5 (A) or pH 7.5 (B) for 18 hours. Cells were infected at MOI = 10 PFU/cell. Arrow indicates empty capsid or immature particle with empty capsid. N = nucleus, bar = 0.5 μm.

Electron microscopic observation of HSV-2 infected cells

A reduction in HSV-2 yield during growth at acid pH environment could be due to pH-mediated inhibition at any number of steps of the replication cycle. Thus, EM was applied to study the morpho-

genesis of HSV-2 at pH 6.5 and pH 7.5 conditions. Vero cells infected with the virus at an MOI of 10 PFU/cell were maintained either at pH 6.5 or 7.5, harvested at 6, 12 and 18 hours after infection and processed further for EM examination. At 6 hours after infection, the number of virus nucleocapsids observed in the nucleus seemed to be approximately the same at both pH 6.5 and 7.5. At 12 to 18 hours, virus particles were more prominent at pH 7.5 than at pH 6.5 condition of growth. Complete virions could be seen at the cytoplasmic membrane at pH 7.5 whereas these were rarely observed at pH 6.5. At 18 hours after infection at pH 6.5, a relatively high number of empty and partially cored capsids were seen in the nucleus; numerous virus particles in the cytoplasm were with empty and partially cored capsids (Fig. 2).

Virus-specific protein synthesis

Protein synthesis in infected cells maintained at pH 6.5 and 7.5 was determined by measuring ^{35}S-methionine incorporation. Although the amount of cellular uptake of ^{35}S-methionine at pH 6.5 was 30% compared to that at pH 7.5 (Table 1), the percentage of radioactivity incorporated into proteins was the same (47%), indicating that pH condition had no marked effect on overall protein synthesis.

Immunoprecipitation of virus-specific polypeptides was performed using rabbit anti-HSV-2 antibodies. To correct for differences in the specific activity of radiolabelled proteins obtained under pH 6.5 and 7.5 conditions, equal radioactive amounts of protein were employed for immunoprecipitation. Table 1 shows that there were no apparent differences in the quantity of virus-specific proteins synthesized. However, when the proteins were analyzed by SDS-PAGE (Fig. 3), a number of protein bands were reduced in amount or undetectable in the virus-

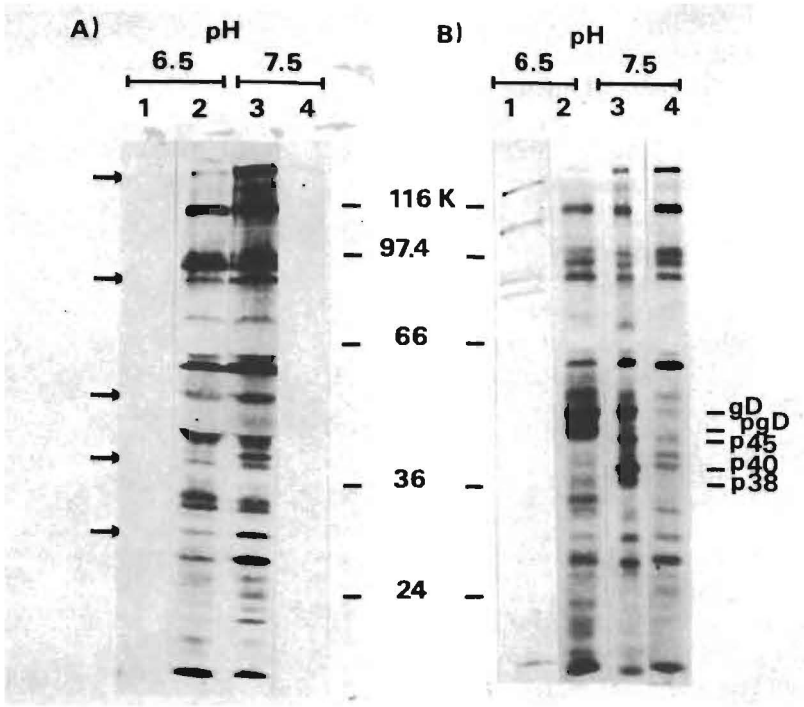

Fig 3 Fluorograms of SDS-PAGE of ^{35}S-methionine labelled antigens extracted from Vero cells infected with HSV-2 for 18 hours. Adsorption of virus was carried out at pH7.5, thereafter, cells were grown at either pH 6.5 or 7.5. (A) Equivalent amounts of antigens were immunoprecipitated using rabbit anti-HSV-2 antibodies (lanes 2 and 3) or with non-immune rabbit serum (lanes 1 and 4). Virus antigens present in reduced amount or absent in infected cells grown at pH 6.5 are indicated by arrows. (B) Equivalent amounts of antigens were immunoprecipitated using a mixture of MAbs ($6E_8.F_{11}$ and $6C_5.E_{10}$) (lanes 2 and 3 or with mouse ascites fluid (lanes 1 and 4). Numbers refer to MW of protein markers. See text for identity of the antigens.

Table 1. Incorporation of ^{35}S-methionine into cellular and HSV-2 specific polypeptides, 18 hours after infection, at different pH conditions of growth.

	Infected cells (cpm/10^6 cells)		
pH	Cellular extract	TCA precipitated polypeptides	Virus-specific polypeptides* (cpm)
6.5	4,038,968	1,938,604	359,900
7.5	13,621,860	6,253,000	349,850

* Precipitated by rabbit anti-HSV-2 antibodies from equal cpm (3,126,500) of total protein.

infected cells maintained at pH 6.5. The identity of some of these viral components were determined using a mixture of 2 MAbs recognizing viral gD and p40 antigens; gD but not p40 could be detected in HSV-2 antigens extracted from infected cells cultured at pH 6.5 whereas both gD and p40 were present at pH 7.5 (Fig. 3, lanes 2 and 3). Anti-gD MAb precipitated glycosylated gD molecule and its precursor (pgD) while anti-p40 MAb additionally precipitated proteins having molecular weights (MW) of 45 and 38 kDa similar to that observed previously by other investigators.[12,13]

DISCUSSION

The duration of successive steps in the replication cycle of HSV depends upon various factors.[14] In our studies, when the growth of HSV-2 strain 186 in Vero cells at different pH conditions was compared it was found that pH $<$ 7.5 interfered with the replication of this virus. The yield of virus was at its lowest when infected cells were grown at pH 6.5 and cell fusion was also inhibited. However, the effect could be reversed when the pH was returned to 7.5. The results were similar to those previously shown in HSV-1 infected Vero cells.[15] The effect could be observed both at the MOI of 0.1 PFU/cell and 10 PFU/cell but it was more prominent when the cells were infected with the lower inoculum size (Fig. 1). Related studies in our laboratory indicated that, at pH 6.5, there was a decrease in the number of cells synthesizing specific viral antigens; moreover, cell surface antigen expression attributed to polykaryon formation was impaired (unpublished observation). It is not known whether polykaryon formation which was rarely observed in cells infected at low MOI under acid pH was also due to a drop in intracellular pH, thereby requiring an increased expenditure of ATP; since fusion is energy dependent[16] the process may be blocked due to a depletion of ATP.[17] However, an increase in polykaryon formation seen in cells infected at high MOI might be related to the higher level of virus proteins which promote cell fusion. Acidic pH condition has also been previously shown to exert an adverse effect on the replication of other viruses, viz. vesicular stomatitis virus,[18] infectious avian bronchitis virus,[19] Semliki forest virus[20] and poliovirus.[21]

The inhibition of HSV-2 production at pH 6.5 did not appear to be due simply to an extended replication cycle since the kinetics of virus growth at this pH indicated that the virus titres reached plateau level at approximately 24 to 30 hours after infection, similarly to that at pH 7.5 (Fig. 1). At 6 hours after infection, the replication of HSV-2 in infected cells at pH 6.5 as observed under EM was not apparently affected, suggesting that early steps of replication cycle, ie virus entry and translocation of viral DNA to the nucleus, were not affected by pH. Nevertheless, an argument may be made about the instability of the secreted virions bathed in environmental acid pH. Low pH has been shown to deform the envelope of influenza virus, however, this could be reversed if the pH was returned to neutral.[22] Our study on inactivation kinetics of HSV-2 strain 186 at 37°C, for up to 4 hours, indicated that the fractional survival numbers (ie virus titres at time t/virus titres at time zero) were not different between the virions prepared by diluting in MM pH 6.5 or pH 7.5 (data not shown).

Although the overall viral protein synthesis, as measured by immunoprecipitation, was not affected, however, metabolic labelling of polypeptides from HSV-2 infected cells revealed that there was a defect in the synthesis of a number of virus-specific proteins in cells maintained at pH 6.5. Immunoprecipitation using MAbs indicated that the virus protein, p40,[23] which is associated with packaging of viral DNA into preformed capsids was absent or synthesized in very low amount in virus-infected cells grown at pH 6.5 (Fig. 3). This result was consistent with EM observation of empty and partially cored capsids at 18 hours after infection (Fig. 2). Therefore, one reason for the reduction in the total number of infectious and/or mature virions observed at pH 6.5 may be due to the impairment of virus DNA packaging. In our study, a moderate number of empty capsids were seen to be partially enveloped; other investigators have demonstrated that capsids lacking virus DNA are rarely enveloped.[12] However, the complete absence of p40 at pH 6.5 could not be excluded since a few infectious virions were also detected. Virus proteins associated with HSV-DNA packaging, similar to p40, have been demonstrated by others.[9,24] In HSV-1 infected cells grown at pH 6.7, the rate of synthesis and cell-surface expression of many virus-specified glycoproteins were reduced but could be accumulated later.[15] Other investigators have shown that HSV-encoded ribonucleotide reductase exerted its optimum activity at an alkaline pH range.[25] The nature of other viral proteins which might also be suppressed could not be revealed in the present study since a battery of specific MAbs was not available. Analysis of specific polypeptides in cells infected with this virus at pH 6.5 compared to pH 7.5, using polyclonal antibodies and extracts from an equal number of infected cells, revealed a lack or quantitative difference in the level of synthesis and expression of various proteins including those responsible for cell fusion (unpublished observation). Proteins of MW higher than 116 kDa which were depleted (Fig. 3) might be major DNA-binding proteins and structural or viral capsid components involved in the formation of complete virions.

Inhibition of HSV-2 replication observed in the present study may also be mediated by mechanisms other than that indicated above. Moreover, differences may exist between *in vitro* culture studies and growth of virus *in vivo*. Of relevance in the context of the present observations is that the extent of HSV infection may depend upon varying physiological status of the host. It should be mentioned that the pH value of the vaginal discharge is between 4.0-5.0[5] and the pH of the vaginal wall is in a similar acid range.[26] During menstruation there is a rise in the vaginal pH, approaching that of blood which is approximately 7.4, the extent of such rise being correlated with greater menstrual flow. Although periodic recurrence of HSV infection in adult women is known to be linked to the menstrual cycle[27] the extent to which physiological pH *in situ* may be involved in the occurrence of herpetic lesions in female genitalia remains to be demonstrated.

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