THE INTERACTION OF HERPES SIMPLEX VIRUS WITH MURINE LYMPHOCYTES

I. Mitogenic Properties of Herpes Simplex Virus*

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Many acute viral diseases can be contained by vaccination, however, there exist a number of slow, latent, and chronic viral diseases for which such control is not possible. Such viruses exhibit a number of relationships with the host cell. They may replicate so slowly they are undetectable in the host cell, or they may be replicated with the host genome. The mechanisms by which the expression of the host cell genome and the viral genome influence each other are unknown and they form a unique area of eukaryotic gene regulation.

Herpes simplex viruses (HSV)[1] cause many common diseases in humans. There are two major serotypes of HSV, designated type 1 and type 2 (1, 2). HSV-1 is associated with recurrent fever blisters, while HSV-2 is closely associated with venereal diseases. The two serotypes show minor differences in several biological, physical, and immunological properties, however, only 20–30% of their DNA sequences are held in common (3). Interest in the oncogenic potential of HSV originates primarily from the suspected role of another Herpes group virus in several human malignancies, especially Burkitt's lymphoma and nasopharyngeal carcinomas (for review see 4, 5). The oncogenic potential of HSV has been demonstrated by morphological transformation of cells in human, hamster, and mouse fibroblast cultures and by tumor formation in animals (6–10). Target cell transformation is observed only after the inactivation of infectious HSV by ultraviolet irradiation (6–10) which reduces the lytic activity of HSV, and the HSV-transformed cells appear to propagate only fragments of the original HSV genome (11, 12).

Infection of both humans and mice with HSV is followed by the disappearance of virus, and sustained latent viral infections are later manifest by the reappearance of infectious virus (13). A major problem in studying the expres-

[1] *Abbreviations used in this paper:* LPS, lipopolysaccharide; PFC, plaque-forming cell; PFU, plaque-forming unit; SRBC, sheep erythrocytes; TNP, trinitrophenyl; UV, ultraviolet.

sion of the oncogenic potential or latency of HSV is to find cells that can be infected with virus in culture and then undergo cell transformation without being destroyed by virus replication. In other words, host cells that are nonpermissive for virus replication, but permissive for the expression of transformation genes, are not available. In this report we describe the effects of infectious HSV in mouse spleen cultures.

Materials and Methods

Mice.   C57BL/6J and C3H/HeJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c mice were obtained from Strong Laboratories, Del Mar, Calif. Congenitally athymic (nu/nu) and heterozygous (nu/+) mice were from our breeding colony (14).

Spleen Cultures.   Mouse spleen cells were prepared as described elsewhere (15). Cells were resuspended at a density of 5×10^6 cells/ml in Eagles medium (Gibco F-14) supplemented with 5% fetal calf serum (Irvine Scientific, Irvine, Calif.), 10^{-5} M 2-mercaptoethanol, 1 mM glutamine, and antibiotics (15). Aliquots of 0.2 ml (10^6 cells) were plated into each well of a microtitre plate (Microtest II, Falcon Plastics, Division of BioQuest, Oxnard, Calif.) and incubated at 37°C in a gas mixture of 7% oxygen, 10% carbon dioxide, and 83% nitrogen. Control cultures were treated with *Escherichia coli* K235 LPS (generously provided by Abbott Laboratories, N. Chicago, Ill.). After 24, 48, 72, or 96 h, cultures were radioactively-labeled for 6 h with 0.5 μCi [^{3}H]thymidine (12 c/mmol, New England Nuclear, Boston, Mass.) and harvested with the Mash II (Microbiological Associates, Bethesda, Md.) harvester.

Spleen cultures were depleted of macrophages by using Sephadex G-10 (16). Polyclonal antibody responses were determined with a microscope slide assay (17), in which sheep erythrocytes (SRBC) (Colorado Serum Co., Denver, Colo.), or TNP-SRBC were used as indicator cells (18). The difference in the number of PFC determined with SRBC and TNP-SRBC as indicator cells yields the number of TNP-specific PFC present.

Cell and Viruses.   The HSV-1 strains used in this study were either primary isolates made in this laboratory (Muckbeck, JH) or were obtained from Dr. Berge Hampar (Nii, Savage). All virus stocks were prepared on diploid human foreskin fibroblasts originating in this laboratory. The cells were propagated in complete MEM containing 5% fetal calf serum and gentamycin. The cells were mycoplasma free and used a passage level of less than 10 from the primary culture. Virus stocks were prepared by infection at a multiplicity of 0.1 plaque-forming unit (PFU)/cell followed by incubation at 37°C for 48–60 h. After this incubation, the cell debris were removed from the culture supernate by centrifugation and the resulting fluid used without further treatment. Only extracellular virus was used in these studies.

Results

Mitogenic Responses to HSV.   The data presented in Fig. 1 show the effect of different concentrations of infectious HSV-1 (Muckbeck) on DNA synthesis in BALB/c spleen cultures. Three points should be emphasized. First, HSV-1 stimulates DNA synthesis, optimal responses being observed with 5×10^6 to 10^6 PFU/culture (Fig. 1). Second, when cultures are assayed for polyclonal antibody responses to TNP determinants, an increase in PFC specific for TNP is observed (Fig. 1). Third, with an optimum concentration of HSV-1, a kinetic analysis of the DNA synthetic responses show a peak is reached after 48 h, and thereafter decreases (Fig. 2). Similar results have been obtained by using four other isolates of HSV-1, JH, Nii, and Savage, (data not presented). Under optimal conditions, the stimulation index in both the DNA and polyclonal responses, ranges in our experiments from 10- to 50-fold.

The data presented in Fig. 3 show the effect of HSV-1 (JH) on DNA synthesis in normal, T-cell-depleted, and macrophage-depleted spleen cultures, and in
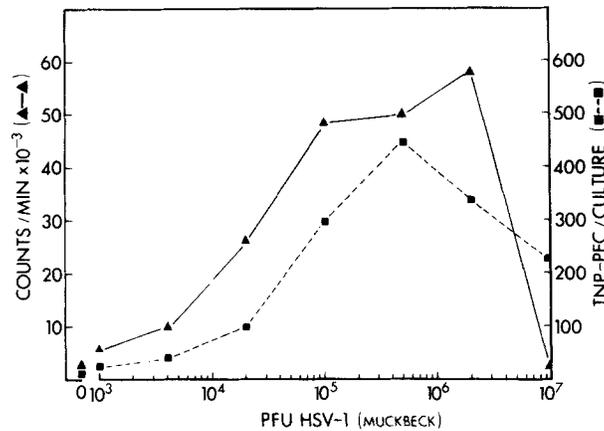
FIG. 1. The effect of HSV-1 (Muckbeck) on DNA synthesis in BALB/c spleen cultures. Each culture contained 10^6 spleen cells in 0.2 ml medium and the titer of virus indicated. After 48 h, cultures were labeled with 0.5 μCi [^{3}H]thymidine for 6 h, and then harvested. Each point represents the mean of quadruplicate cultures. The polyclonal responses were assayed after 72 h in culture. Quadruplicate cultures were harvested and assayed for PFC directed against SRBC or SRBC conjugated with TNP, by using a modified Jerne assay (17). The difference between SRBC-PFC and TNP-SRBC-specific PFC gives the number of TNP-PFC, which serves as the indicator of a polyclonal antibody response. Each figure represents the mean of quadruplicate microcultures.

thymocyte cultures. The stimulation of DNA synthesis was similar in both normal and T-cell-depleted (nu/nu) spleen cultures (Fig. 3), however, HSV-1 does not stimulate DNA synthesis in thymocyte cultures. Removal of macrophages by passaging through Sephadex G-10 columns (16), had little effect on the DNA synthesis response to HSV-1 (Fig. 3). As a control, we also describe the response of these various cultures to 0.5 μg $E.$ $coli$ K235 LPS (Fig. 3). Since HSV-1 and LPS stimulated DNA synthesis in normal, T-cell-depleted and macrophage-depleted cultures, but not in thymocyte cultures, the cells responding to HSV-1 appear to be the B lymphocytes. Since polyclonal responses are also observed in these cultures (Fig. 1), we consider the target cell the B lymphocyte.

Nature of DNA Synthesis. The addition of infectious HSV to spleen cultures could result in either viral DNA replication, or host cell DNA synthesis. Since HSV DNA and mouse DNA have different densities, they can be easily separated by equilibrium sedimentation (2). Experiments were performed to determine whether the DNA synthesized in spleen cultures treated with HSV was viral or cellular in origin. Spleen cultures were prepared from BALB/c mice and incubated with 5 μg/ml K235 LPS as a control, or 5 × 10^6 PFU/ml HSV-1 (Nii). After 48 h, 1.25 μCi [^{3}H]thymidine was added to cultures for 6 h, then DNA was separately prepared from each culture. The DNA was examined by cesium chloride density sedimentation. As a marker for HSV DNA, a sample of ^{32}P-labeled *Pseudomonas aeruginosa* DNA, which is identical in density to HSV, was added to each density gradient. The results of these experiments are shown in Fig. 4. The ^{3}H-labeled DNA prepared from both the lipopolysaccharide (LPS)-and HSV-stimulated cultures appear identical in
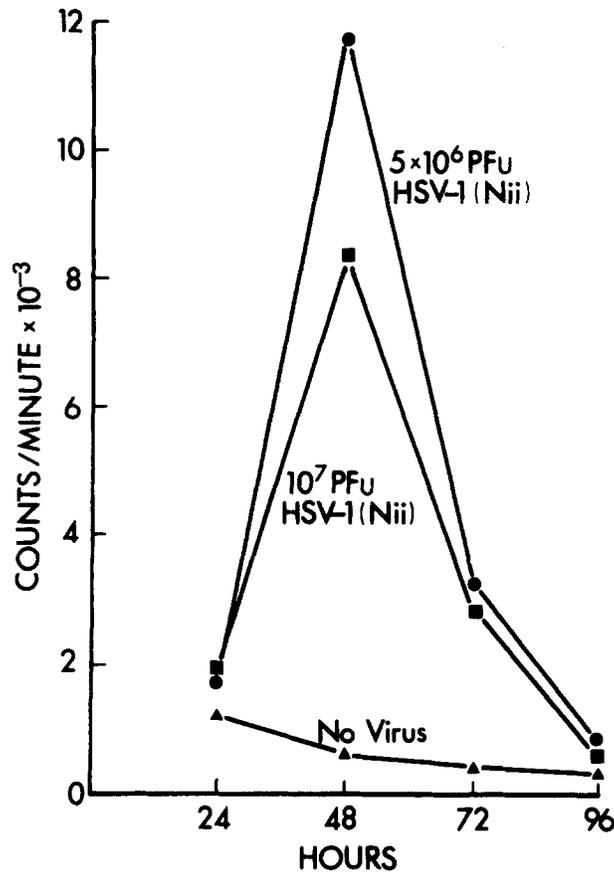
Fig. 2. The kinetics of DNA synthesis after the addition of HSV-1 (Nii) to spleen cultures. Spleen cultures were prepared from BALB/c mice and plated in microwells (10^6 cells in 0.2 ml medium). Either 10^7 PFU or 5×10^6 PFU HSV-1 (Nii) was added to cultures. After 24, 48, or 72 h, triplicate cultures were assayed for the incorporation of [^{3}H]thymidine as described in the legend of Fig. 1.

density (ρ = 1.700 g/cm^3) to mouse DNA. No newly synthesized DNA is observed co-sedimenting with the ^{32}P-labeled P. $aeruginosa$ DNA (ρ = 1.728 g/ cm^3). These data imply that most of the DNA synthesized after the addition of HSV to mouse spleen cultures is host cell and not viral in origin (Fig. 4).

The Mitogenic Response Requires Infectious Virus. A number of agents have been found to be specific murine B lymphocyte mitogens (15, 18). Therefore, it is necessary to show that the mitogenic properties of the HSV-1 preparations are due to the virus, rather than a contaminant. Such a contaminant could be LPS, or something produced by the human diploid fibroblasts during the preparation of the HSV-1 stocks. Since the DNA synthesized in spleen cultures after addition of HSV is host rather than viral in origin, the mitogenic activity may be due to a viral protein, rather than the expression of the viral genome.

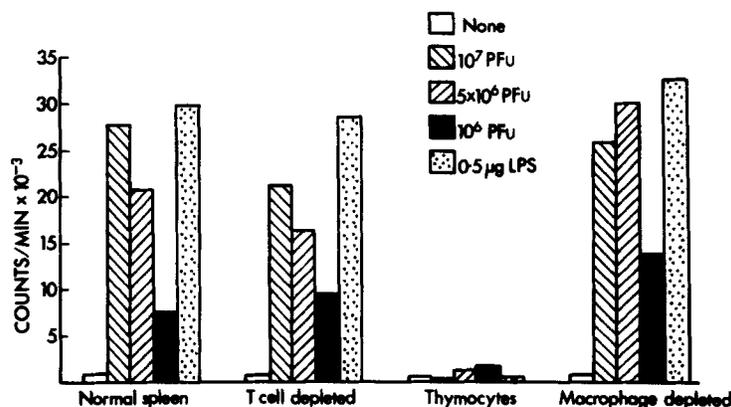B lymphocytes from C3H/HeJ mice are refractory to the mitogenic effects of

FIG. 3. Effect of HSV-1 on DNA synthesis in normal, T-cell-depleted, macrophage-depleted, and thymocyte cultures. The normal spleen cultures and thymocyte cultures were prepared from BALB/c mice. The T cell-depleted cultures were prepared by using spleen cells from congenitally athymic (nude) mice, in which the genes responsible for the lack of thymus cells had been bred in a BALB/c background (14). Triplicate cultures contained 10^6 cells and the titers of HSV-1 (JH) indicated. After 48 h the uptake of [^{3}H]thymidine into DNA was assayed as described in the legend of Fig. 1.
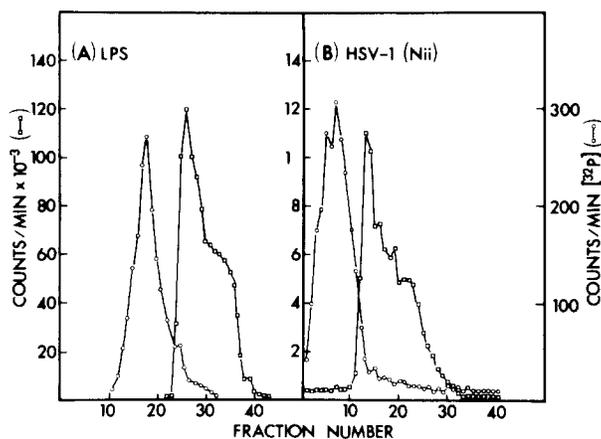

FIG. 4. Cesium chloride density equilibrium gradients of DNA extracted from LPS and HSV-1 (Nii) stimulated BALB/c spleen cultures. Cells were labeled with [^{3}H]thymidine as detailed in the text. As a density marker, ^{32}P-labeled P. aeruginosa DNA (ρ = 1.728 g/cm^3) was added to each gradient before centrifugation.

LPS (15, 18). The data presented in Fig. 5 show that two stocks of HSV-1 (Muckbeck and Nii) both stimulate DNA synthesis in C3H/HeJ spleen cultures. Thus, it is unlikely that LPS is responsible for the mitogenic activity of HSV.

To distinguish between the requirement for infectious versus noninfectious virus for induction of DNA synthesis in B lymphocytes, we have made use of findings that heat and ultraviolet (UV) treatments have been shown to have different effects on viral expression in other systems. Heat treatment can be used to inactivate HSV by preventing adsorption of HSV to the cell. UV treatment leads to an inhibition of HSV replication in human fibroblast
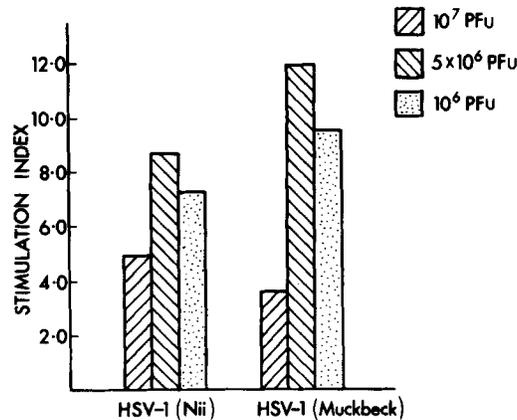
FIG. 5.   The stimulation of DNA synthesis by HSV-1 (Nii and Muckbeck) in C3H/HeJ spleen cultures. Cells were prepared and assayed as described in the legend to Fig. 1. C3H/HeJ spleen cells do not support a mitogenic response to LPS (15, 18).
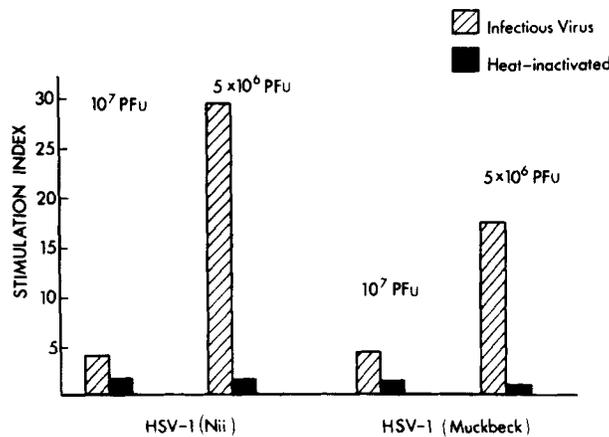

FIG. 6.   The effect of infectious HSV and heat-inactivated HSV on DNA synthesis in BALB/c spleen cultures. Cells were cultured as described in the legend for Fig. 1. HSV-1 (Nii and Muckbeck) were heat inactivated as detailed in Materials and Methods.

cultures, but does not prevent the oncogenic potential of HSV as shown by morphological transformation of cells in culture, or by tumor formation in animals (6-10). Heat treatment (56°C for 1 h) destroys the mitogenic properties of HSV-1. The data for two virus stocks (Nii and Muckbeck) are described in Fig. 6. The effect of UV treatment on the infectious activity of HSV-1 (Muckbeck) is described in the experiment of Fig. 7. A stock of virus was exposed to UV irradiation and at various periods, aliquots of a constant volume were assayed for PFU in human diploid fibroblasts. A decrease in virus titer is observed with increasing length of UV treatment (Fig. 7). The mitogenic activity of UV-inactivated HSV-1 (Muckbeck) was then compared to that of nontreated infectious HSV-1 in mouse spleen cultures. The data are shown in Fig. 8. The solid line represents the mitogenic response to the titered, UV-
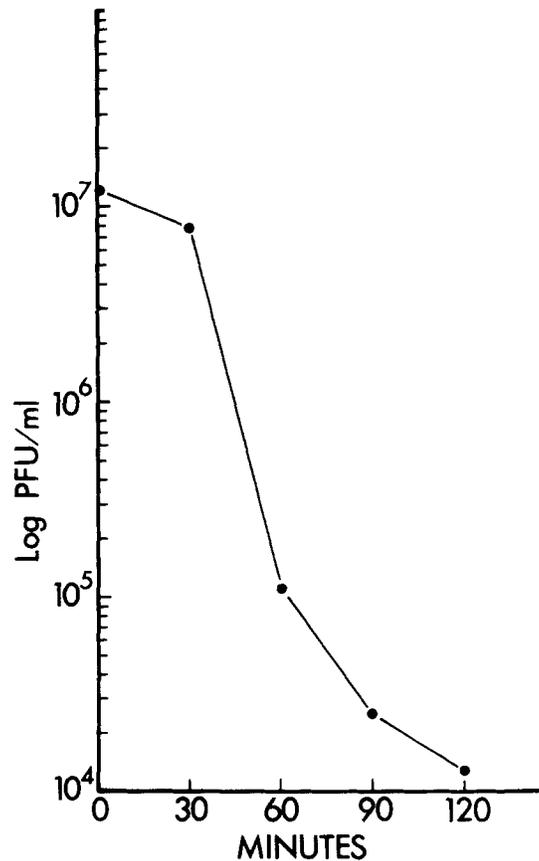
Fig. 7. Kinetics of inactivation of HSV-1 by UV irradiation. A virus stock of HSV-1 (Muckbeck) suspended in complete MEM plus 5% FCS was exposed to UV light from an MR-4 lamp (George Gates and Co., Long Island, N.Y.). The virus suspension (2 ml) was placed in a 60-mm Petri dish and was slowly rotated throughout the irradiation period. At various intervals small samples were removed and the infectious titer determined by a standard plaque assay on diploid human fibroblasts.

treated HSV-1. The mitogenic response is plotted as a function of the virus titer determined in the previous experiment (Fig. 7). The broken line represents the control, which is the mitogenic response of spleen cultures to infectious HSV-1 (Muckbeck). This control virus stock was not UV treated, but added to cultures at corresponding PFU titers (Fig. 8). The mitogenic responses elicited by these two virus stocks are virtually identical when related to their infectious virus content. The mitogenic response is also proportional to the amount of infectious virus present, decreasing as the amount of infectious virus decreases (Fig. 8). These experiments show that the mitogenic response requires the presence of infectious HSV-1.

We have also prepared a stock of HSV-1 which contains predominantly defective virus particles. This is done by repeated high multiplicity infections of human diploid fibroblasts. This virus stock was not mitogenic in mouse spleen cultures (data not shown). These experiments show that it is unlikely a
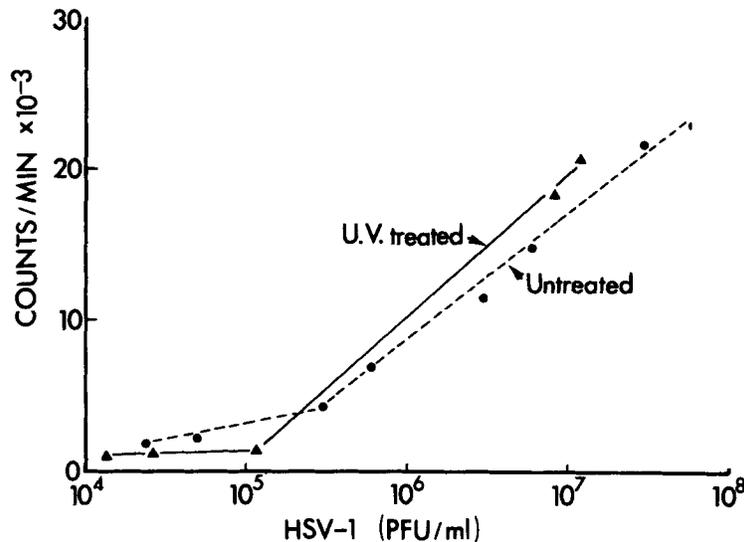
FIG. 8. Dependence of mitogenic activity on the infectious titer of HSV. Both UV irradiated and nonirradiated HSV-1 (Muckbeck) were compared for their mitogenic activity as a function of the infectious virus titer. In the case of the irradiated virus, the titer remaining in the undiluted suspension after irradiation was used while, in the case of the control virus, the stocks were diluted to obtain the necessary reduction in infectious titer. The cells were cultured as described in the legend for Fig. 1. The labeling for all the cultures was done after 48 h of incubation after infection. The infectious viral titer was determined by plaque assays on low passage diploid human fibroblasts.

viral protein is the mitogenic agent, and confirm our previous findings that infectious HSV-1 is required for the mitogenic response.

Discussion

The addition of HSV-1 to mouse spleen cultures results in the induction of DNA synthesis. The rate of radioactive thymidine incorporation reaches a maximum 48 h after addition of virus to cultures, and then decreases. The stimulation observed is generally 10- to 50-fold above the background response, and is comparable in magnitude to the responses elicited by LPS in spleen cultures (Fig. 1). The DNA that is synthesized bands at a density in cesium chloride which is characteristic of mouse cellular DNA (ρ = 1.700 g/cm^3), and not viral DNA (ρ = 1.728 g/cm^3) (Fig. 4). The stimulation of DNA synthesis by HSV-1 occurs in spleen cell cultures from congenitally athymic mice and in cultures which have been depleted of adherent cells by passage over Sephadex G-10, but not in thymocyte cultures (Fig. 3). In addition to the stimulation of radioactive thymidine incorporation which is the measure of cells synthesizing DNA, HSV-1 stimulates a polyclonal antibody response (Fig. 1). The magnitude of mitogenic and polyclonal responses induced by different concentrations of HSV-1 in culture are similar. These observations indicate that the B lymphocytes in mouse spleen cultures are induced by HSV-1 to undergo DNA synthesis and differentiation to antibody-forming cells.

Infectious HSV-1 appears to be the mitogenic agent. Heat treatment, which

prevents viral adsorption to the cell, inactivates the mitogenic properties of HSV-1. There was a direct correlation in the loss of activity of HSV-1 when assayed for PFU in human diploid fibroblasts and for mitogenicity with murine lymphocytes, after UV irradiation (Figs. 7 and 8). These experiments directly implicate the expression of the HSV-1 genome in the initiation of the mitogenic process. Other possibilities as the causitive agents of mitogenesis, such as LPS, viral protein, or some nonspecific product of the human diploid fibroblasts used to prepare virus stocks, appear to be ruled out (Fig. 5).

In view of the known oncogenic properties of HSV-1 (6–10, 19), it is of interest to question whether the viral DNA that induces the morphological transformation of hamster and mouse fibroblasts in culture is responsible for the stimulation of DNA synthesis in B lymphocytes. Unless HSV-1 is UV-inactivated, it replicates in fibroblasts and leads to cell destruction (6-10). Since infectious HSV-1 is required for the stimulation of B lymphocytes, it appears the response of fibroblasts and lymphocytes to HSV-1 differs.

There have been two reports of successful in vitro transformation of tissue culture cells by infectious HSV. The first report (20) demonstrated the morphological transformation of human embryonic lung cells. The second report showed that the XC cell line (21), a rat fibroblast line carrying a Rous sarcoma virus genome, was biochemically transformed by HSV in the absence of viral inactivation (21). Unfortunately, neither of these reports have been confirmed. If the transforming region of the HSV-1 genome is responsible for the observed mitogenicity in murine B lymphocytes, HSV-1 may be capable of transforming B lymphocytes into malignant cells. However, proof that transformation has occurred requires either establishment of continuous cell lines or, growth as a malignant tumor in syngeneic recipients. If malignancy is demonstrated, then the possibility exists that the B-cell tumors produced would be found to be plasmacytomas.

Spleen cell cultures infected with HSV-1 do not appear to synthesize viral DNA (Fig. 4). This means that if viral replication is required for mitogenesis, it must be very low. In a later report, we will show that infectious HSV-1 is not produced in lymphocyte cultures after the mitogenic response induced by HSV-1.[2] These observations are in agreement with those of Kirchner et al. (22, 23), who have shown that HSV is mitogenic in mouse spleen cultures, and that HSV replication can only be demonstrated in mouse spleen cultures which have been prestimulated with LPS or Poly I:C for 48 h before infection. Every strain tested appeared to be permissive for viral replication except C57BL/6 mice. This observation awaits confirmation, however, it presents an interesting way of studying the host control of HSV infection. By using genetic analyses of a permissive and nonpermissive host, our studies may provide a basis for probing the complex regulation of the expression of the HSV genome in eukaryotic cells.

Summary

Herpes simplex virus (HSV) stimulates DNA synthesis in mouse spleen

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[2] S. Hedrick, D. T. Kingsbury, and J. Watson. The Interaction of herpes simplex virus with murine lymphocytes. II. Kinetics of the host cell response, and virus replication. Manuscript in preparation.

cultures prepared from normal, macrophage-depleted, and T-cell-depleted spleen cells, but not from thymocytes. In addition, a polyclonal antibody response is observed in HSV-infected spleen cultures. These findings indicate that the cells stimulated to undergo DNA synthesis after HSV infection appear to be the bone marrow-derived lymphocytes. The newly synthesized DNA is host cell and not of viral origin. Heat treatment and ultraviolet irradiation of HSV before addition to spleen cultures prevents the induction of DNA synthesis. We consider the use of this system as assay for the study of cell transformation by HSV and also for the study of host cell control of the expression of the viral genome.

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References

1. Dowdle, W. R., A. J. Nahmias, R. W. Harwell, and F. P. Pauls. 1967. Association of antigenic type of *Herpesvirus hominis* with site of viral recovery. *J. Immunol.* **99:**974.
2. Plummer, G., C. R. Goodheart, M. Miyagi, G. R. B. Skinner, M. E. Thouless, and P. Wildy. 1974. Herpes simplex viruses: discrimination of types and correlation between different characteristics. *Virology.* **60:**206.
3. Sugino, W. M., and D. T. Kingsbury. 1976. DNA homologies between strains of herpes simplex virus. *Virology.* **71:**605.
4. Zur Hausen, H. 1975. Oncogenic herpes viruses. *Biochim. Biophys. Acta.* **417:**25.
5. Epstein, M. A. 1970. Aspects of the EB virus. *Adv. Cancer Res.* **13:**383.
6. Duff, R., and R. Rapp. 1971. Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. *Nat. New Biol.* **233:**48.
7. Duff, R., and F. Rapp. 1973. Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type 1. *J. Virol.* **12:**209.
8. Duff, R., and F. Rapp. 1975. Quantitative assay for transformation of 3T3 cells by herpes simplex virus type 2. *J. Virol.* **15:**490.
9. Kimura, S., V. L. Flannery, B. Levy, and P. A. Schaffer. 1975. Oncogenic transformation of primary hamster cells by herpes simplex virus type 2 (HSV-2) and an HSV-2 temperature-sensitive mutant. *Int. J. Cancer.* **15:**786.
10. Boyd, A. L., and T. W. Orme. 1975. Transformation of mouse cells after infection with ultraviolet irradiation-inactivated herpes simplex virus type 2. *Int. J. Cancer.* **16:**526.
11. Davis, D. B., and D. T. Kingsbury. 1976. Quantitation of the viral DNA present in cells transformed by ultraviolet irradiated herpes simplex virus. *J. Virol.* **17:**788.
12. Kraiselburd, E., L. P. Gage, and A. Weissbach. 1975. Presence of a Herpes simplex virus DNA fragment in an L cell clone obtained after infection with irradiated herpes simplex virus type 1. *J. Mol. Biol.* **97:**533.
13. Oldstone, M. B. A., M. V. Haspel, M. A. Pellegrino, D. T. Kingsbury, and L. Olding. 1976. Histocompatibility complex and virus infection, latency and activation. *Transplant. Rev.* **31:**225.
14. Watson, J., R. Epstein, I. Nakoinz, and P. Ralph. 1973. The role of humoral factors in the initiation of *in vitro* primary immune responses II effect of lymphocyte mitogens. *J. Immunol.* **110:**43.
15. Watson, J., and R. Riblet. 1974. Genetic control of responses to bacterial lipopolysaccharides in mice I. Evidence for a single gene that influences mitogenic and immunogenic responses to lipopolysaccharides. *J. Exp. Med.* **140:**1147.
16. Ly, I., and R. I. Mishell. 1974. Removal of adherent cells by Sephadex G-10 chromatography. *J. Immunol. Methods.* **5:**239.

17. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* 126:423.

18. Watson, J., and R. Riblet. 1975. Genetic control of responses to bacterial lipopolysaccharides in mice. II. A gene that influences the activity of a membrane receptor for lipopolysaccharides. *J. Immunol.* 114:1462.

19. Nahmias, A. J., Z. M. Naib, W. E. Josey, F. A. Murphy, and C. F. Luce. 1970. Sarcomas after inoculation of newborn hamsters with Herpes virus hominis type 2 strains. *Proc. Soc. Exp. Biol. Med.* 134:1065.

20. Darai, G., and K. Munk. 1973. Human embryonic lung cells abortively infected with herpes simplex virus hominis type 2 show some properties of cell transformation. *Nat. New Biol.* 241:268.

21. Svoboda, J. 1963. Presence of chicken tumor virus in the sarcoma of the adult rat inoculated after birth with Rous sarcoma tissue. *Nature (Lond.).* 186:980.

22. Kirchner, H., M. Schwenteck, H. Northoff, and H. Ruhl. 1977. Effects of herpes simplex virus (HSV) on human and murine lymphocyte cultures. *Fed. Proc.* 36:1228.

23. Kirchner, H., H. M. Hirt, C. Kleinicke, and K. Munk. 1976. Replication of herpes simplex virus in mouse spleen cell cultures stimulated by lipopolysaccharide. *J. Immunol.* 117:1753.