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# In vitro stimulation of human umbilical cord blood lymphocytes by ultraviolet-irradiated herpes simplex virus

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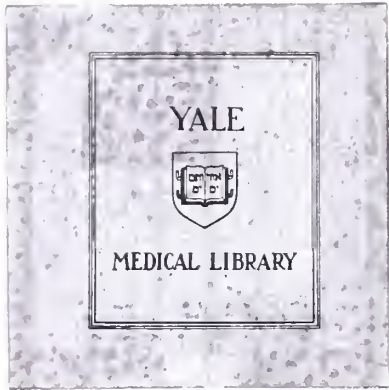
IN VITRO STIMULATION OF HUMAN UMBILICAL CORD BLOOD  
LYMPHOCYTES BY ULTRAVIOLET-IRRADIATED HERPES SIMPLEX VIRUS

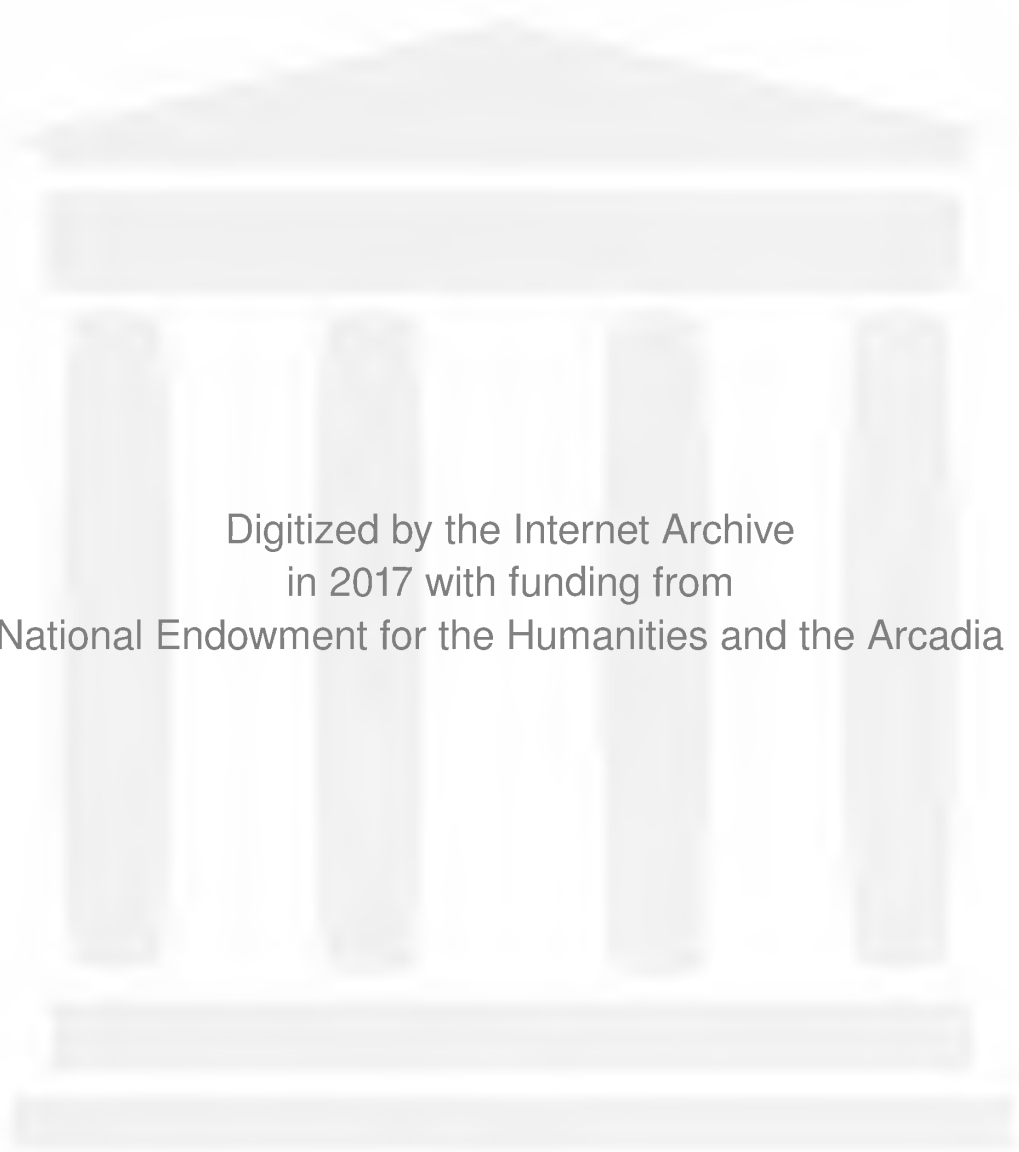


THOMAS R. MOORE

1979







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IN VITRO STIMULATION OF HUMAN  
UMBILICAL CORD BLOOD LYMPHOCYTES  
BY ULTRAVIOLET-IRRADIATED HERPES SIMPLEX VIRUS

THOMAS R. MOORE '79

A Thesis Submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the  
Requirement for the Degree of Doctor of Medicine

February 1979



## DEDICATION

To Dr. George Miller MD whose interest, enthusiasm and dedication to the things of science and the life of the soul have made this project and my association with him satisfying, enjoyable and even inspiring.





## HERPESVIRUSES AND ONCOGENESIS

I. Epidemiological and In Vivo Evidence.

The concept that herpesviruses are intimately associated with, if not indeed etiologic agents of, some forms of human cancer is scarcely two decades old. The demonstration in 1964 by Epstein et al.<sup>1</sup> that a herpesvirus could be found in cultured lymphoblasts of African Burkitt lymphoma was highly suggestive.

Work with the Lucke carcinoma of the frog earlier established the presence of herpesvirus particles in the tumor cells<sup>2</sup>, but it was not until 1969 that Mizell et al. were able to induce tumors in vitro in frog tissue using the Lucke virus.<sup>3</sup> This work was later confirmed in vivo by Naegle et al.<sup>4</sup>

Similarly, Marek's tumor of chickens, long known to be a contagious lymphoma, was found to harbor a herpesvirus in 1967.<sup>5</sup> The discovery that immunizing chickens with a related herpesvirus of turkeys (1970) could prevent tumor formation<sup>6</sup> added compelling evidence that herpesviruses may be oncogenic in animals. Efforts to develop more direct associations for herpesviruses and malignancies in mammals ensued.

In primates, studies established that Herpes saimiri, a non-pathogenic herpesvirus in its natural squirrel monkey



host, induced lymphomas in owl monkeys and cottontop marmosets<sup>7</sup> and fatal leukemias in other sub-human primates.<sup>8</sup> In 1972, another simian herpesvirus, Herpes ateles, was shown to be oncogenic in South American monkeys.<sup>7,9</sup> These investigations have not only provided considerable evidence of herpesvirus oncogenicity in primates but also supported a concept which evolved from the Lucke virus studies:<sup>4</sup> that a latent, benign viral infection in one setting might result in malignant tumor formation in another.

Efforts to demonstrate oncogenesis by human herpesviruses have been nearly as fruitful. Epidemiological association of Epstein-Barr virus (EBV) with Burkitt's lymphoma was provided by Henle's report of the correlation of high EBV antibody titers in lymphoma patients; geometric mean titer in the tumor patients was 275 while control patients' titers averaged 37.<sup>10</sup> Subsequently, EB virus was cultured from many lymphoblastoid cell lines derived from Burkitt lymphoma tissue;<sup>11,12</sup> EBV antigens<sup>13</sup> and EBV specific DNA sequences were identified<sup>14-17</sup> in the nuclei of Burkitt lymphoma cells. Similar antigenic associations<sup>18,19</sup> and nucleic acid homologies<sup>19,20</sup> have been described in the tissues of nasopharyngeal carcinoma.

More direct evidence of oncogenicity was obtained by inoculation of EBV into sub-human primates. Malignant lymphomas produced by EBV in cottontop marmosets<sup>21</sup> and owl monkeys<sup>22,23</sup> yielded EBV-producing cell lines in vitro;





and EB nuclear antigen<sup>24</sup> and EBV genomes<sup>25</sup> were detected in the primary tumor cells as well.

Implicating other herpesviruses in human malignancy has been less rewarding. To date, there is no in vivo evidence linking cytomegalovirus (CMV) or varicella zoster virus (VZV) with oncogenesis<sup>26</sup> although Albrecht et al.<sup>27</sup> induced in vitro malignant transformation of hamster cells with ultraviolet-irradiated CMV. Studies with Herpes simplex virus (HSV) have been more suggestive, and are recently well reviewed.<sup>26,28-30</sup>

Naib<sup>31</sup> first suggested the association of HSV type 2 with cancer of the uterine cervix. Nahmias et al. found that the incidence of cervical dysplasia and in situ carcinoma was increased 2 to 8-fold among HSV type 2 seropositive individuals compared to seronegative controls,<sup>32</sup> strengthening Gagnon's observation some fifteen years earlier that not a single case of cancer of the cervix was found among 13,000 Catholic nuns over a 20 year survey period.<sup>33</sup>

Attempts to identify HSV-2 specific antigens in cervical tumor tissue have been generally unsuccessful.<sup>34</sup> Aurelian was able to detect immunity to an HSV-2-induced tumor-specific antigen in cervical cancer patients while no such antibody was found among controls.<sup>35</sup> Several investigators reported finding HSV antigens in 2-20% of exfoliated cells in 60% of Papanicolau smears of patients with invasive cancer, versus 0-6% in control smears.<sup>34,36</sup>



Evidence documenting HSV DNA in tumor cells has been contradictory,<sup>37,38</sup> although Frenkel et al. detected a fragment of Herpes simplex type 2 DNA in the cells of a cervical carcinoma;<sup>39</sup> Aurelian et al. (1971) reported recovery of HSV-2 from cervical tumor cells in tissue culture<sup>40</sup> and complete virus particles were observed in cells of 2 of 14 cervical tumors by Herrera et al.<sup>41</sup>

Compared to the weight of data supporting the role of EBV in oncogenesis, the evidence relating HSV to human cervical cancer is highly suggestive, but by no means conclusive.<sup>27,30,42</sup>

## II. Herpesvirus Infection: Lytic and Latent Mechanisms.

Interpretation of the epidemiological and biological associations of herpesviruses and malignancy depends on understanding the dynamics of cell-virus interaction during infection. For example, a high proportion of individuals with cervical cancer have serological and histologic history of HSV-2 infection, yet it is clear that oncogenic transformation of infected cells never occurs in the majority of affected persons.<sup>43</sup> Similarly, Epstein-Barr virus infection is extremely prevalent world-wide<sup>44</sup> but only a tiny fraction can be associated with Burkitt's lymphoma or nasopharyngeal carcinoma.<sup>45</sup> Is there, then, more than one way in which the infecting virus influences the cell?





It is likely that all herpesviruses are capable of establishing both lytic<sup>46</sup> and latent<sup>47</sup> infections and that the specific mode of infection depends to a considerable degree on the native characteristics of the host cell. In the case of Epstein-Barr virus, human umbilical cord lymphocytes transformed by EBV into cell lines with the ability to grow continuously in culture produce no detectable virus despite the presence of EB nuclear antigen (EBNA) and EBV DNA in the transformants.<sup>48</sup> A non-productive lymphoblastoid line (Raji) with similar characteristics has been isolated from Burkitt lymphoma.<sup>49</sup> Infection of marmoset lymphocytes with the same strain of EBV results in transformation and considerable virus production.<sup>48</sup>

Epithelial cells are characteristically permissive for the replication of Herpes simplex virus: the outcome is cell lysis and death.<sup>38,46</sup> Other cells, e.g. those of neural origin, appear to be non-permissive for replication and the usual outcome of HSV infection is latency.<sup>49</sup>

Stevens has summarized the data on this point.<sup>47,50</sup> Intact virus and hybridizable DNA sequences of latent HSV have been demonstrated in trigeminal ganglion neurons after experimental chronic herpetic eye infection in rabbits<sup>51</sup> and mice;<sup>52</sup> reactivation of dormant infection has been achieved by neurectomy<sup>53</sup> or imposition of septic shock.<sup>50</sup> Baringer<sup>54</sup> documented recovery of infectious HSV-1 from 31 of 71 human cadaver trigeminal ganglia examined, and HSV-2



from 5 of 46 sacral ganglia, suggesting that 50% of the human population may harbor latent HSV in their sensory ganglia.<sup>55,56</sup>

The ability of HSV to establish latent infection in human cells is now well-documented but it is less certain whether HSV may be capable of oncogenic transformation in human disease. The demonstration of HSV DNA and HSV antigens in cervical carcinoma tissue has bolstered speculation that HSV may also be a "transforming virus" in the production of human cervical cancer.<sup>57</sup> Attempts to support this thesis with in vitro techniques are discussed below.

### III. Oncogenicity and Herpesviruses: In Vitro Evidence.

Insights from in vivo and epidemiological studies led to experiments combining cells and herpesviruses in vitro with hopes of inducing oncogenic transformation under controlled circumstances.

#### A. Transformation by Epstein-Barr Virus.

The first report by Henle et al.<sup>11</sup> in 1967 of transformation by EBV of human leukocytes co-cultivated with X-irradiated cells from a Burkitt's lymphoma tumor was followed shortly by Pope et al. (1968) who transformed





fetal leukocytes using the filtered extracts of an EBV-producing leukemia cell line.<sup>12</sup> This work was confirmed by Miller et al. in 1969.<sup>58</sup>

In these and the numerous subsequent reports, the in vitro transformation of leukocytes by EBV exhibits the following characteristics:

1) EBV from several sources produces transformation, including Burkitt cells,<sup>11</sup> leukemia,<sup>12</sup> infectious mononucleosis<sup>59</sup> and previously transformed monkey leukocytes.<sup>48</sup>

2) a single target cell--probably a "B" lymphocyte<sup>60</sup>--can be derived from several sources, including fetal bone marrow<sup>61</sup>, umbilical cord blood<sup>52</sup> and peripheral adult blood.<sup>58</sup>

3) morphologically and biologically, the transformed cells are lymphoblasts<sup>63</sup> which are capable of surviving and dividing continuously in culture.

4) the EBV DNA is associated with the host cell's genome and can be detected by the presence of viral antigens (EBNA)<sup>13</sup> and hybridizable DNA sequences.<sup>14</sup>

Certain aspects of the in vitro EBV transformed cell are strikingly similar to those of the Burkitt lymphoma cell, i.e. the presence of EBV DNA and antigens in lymphoid cells with altered growth characteristics. On the strength of these similarities, the EBV model of oncogenesis is a convenient standard by which other "transformation" systems can be measured.



## B. Transformation by Herpes Simplex Virus.

Efforts to induce in vitro transformation by other human herpesviruses are in general frustrated by the tendency of the virus to undergo lytic infection in the host, resulting in cell death before transformation.<sup>64</sup> A number of experimental techniques have been brought to bear on this problem:

1) development of temperature-sensitive mutant viruses (Ts) which are infectious at physiological (permissive) temperatures but able to infect cells without lytic reproduction at elevated (non-permissive) temperatures.<sup>65-69</sup>

2) partial inactivation of virus by pre-treatment with sub-lethal doses of ultraviolet (UV) light,<sup>70-72</sup> chemicals<sup>73-76</sup> or light-activated chemicals.<sup>72,77</sup>

3) suppression of viral reproduction after infection by maintaining infected cell cultures at elevated temperatures.<sup>78</sup>

4) use of specific viral DNA fragments defective for lytic functions to "transfect" cells directly.<sup>79,80</sup>

The thesis behind these maneuvers is that if a portion of the herpesvirus genome which directs viral reproduction and cell lysis can be suppressed, deleted or bypassed, oncogenic functions of the virus may be unmasked.<sup>81</sup>

Owing to the lytic behavior of HSV in epithelial tissues, transformation attempts were begun with partially-



inactivated virus, using a variety of non-lymphoid cell types as targets. These studies are summarized in Table I.

Using ultraviolet-irradiated HSV, in which infectivity had been reduced by  $10^{-4}$ , Duff and Rapp<sup>38,82,83</sup> were able to transform hamster embryo cells at a frequency of  $10^{-5}$  transformants per normal cell. The transformants had the following properties:

- 1) transformation was observed with a minority of virus strains tested (2 of 12);<sup>35</sup>
- 2) transformants grew out into stable tissue culture cell lines;<sup>38,68,72,84,85</sup>
- 3) transformed lines expressed HSV surface antigens;<sup>38,86</sup>
- 4) transformed cells induced tumors in Syrian hamsters on inoculation;<sup>38,68,82</sup>
- 5) transformants were morphologically poorly differentiated.<sup>80,82</sup>

It should be noted that the transformed lines yielded no infectious virus or cytopathic effect in culture with susceptible cells.

HSV DNA fragments in these transformants have been reported to represent 10%<sup>87</sup> and 8-32%<sup>88</sup> of the genome, although Summers<sup>37</sup> could find no sequences in seven strains of HSV-transformed cell lines. Morphological and tumorigenic transformation of rodent cells was also achieved by Duff, Rapp et al. using photodynamic inactivation of





TABLE I

TRANSFORMATION OF NON-LYMPHOID CELLS BY HSV

<u>Cell Type</u>	<u>Virus Inactivator</u>	<u>Cell Lines</u>	<u>Outcome: Tumors</u>	<u>TK<sup>+</sup></u>	<u>Reference</u>
Hamster Embryo Fibroblast	UV (5'-12' exposure)	+	+		38,67,82 83,102
	Temperature-Sensitive Mutant (Ts)	+	+		67
	Chemical	+			74
	Photodynamic	+	+		77
Rat Embryo Fibroblast	Ts	+	+		68
Mouse Fibroblast	UV (10'-12' exposure)	+	+	+	85,103
	DNA Fragments			+	79
Human Embryo Lung	Cell Temperature = 42°C	+			78



HSV. 73,78,89

Biochemical transformation, in which the transformants are morphologically unchanged but exhibit altered enzymatic characteristics, has been accomplished with HSV in mouse "L" cells. Culturing infected cells in medium lethal for the absence of thymidine kinase activity<sup>85</sup> selects transformants possessing this enzyme (TK<sup>+</sup>) while uninfected or untransformed cells lacking the enzyme (TK<sup>-</sup>) do not survive. Biochemical transformation of rodent cells lacking thymidine kinase was induced with ultraviolet-irradiated HSV (UV-HSV),<sup>85</sup> with live HSV in non-permissive Rous-sarcoma transformed rat cells<sup>90</sup> and more recently with specific HSV DNA fragments alone.<sup>80</sup> In subsequent work, hybridization techniques have detected 6 copies of HSV DNA fragments representing 15% of the viral genome in the TK<sup>+</sup> transformed cells, with none reported in the TK<sup>-</sup> cells.<sup>91</sup> The newly induced enzyme is considered to be of viral origin, in that the transformed cell enzyme's substrate specificity and electrophoretic mobility are identical to the native HSV enzyme.

#### IV. Interaction of Lymphoid Cells and Herpes Simplex Virus.

Interest in transforming lymphoid cells with HSV grew out of the experience with EBV, although in vivo evidence that HSV infects lymphocytes latently or lytically is lacking.



## A. Replication.

Experiments demonstrating the extent to which HSV can infect and replicate in lymphoid cells are summarized in Table II. It will be readily recognized that in contrast to epithelial tissues, primary unstimulated lymphocytes support HSV replication very poorly.<sup>89,95-97</sup> However, lymphocytes stimulated with mitogens (phytohemagglutinin (PHA), concanavalin A (Con A), antilymphocyte globulin) support HSV replication over a short (2 to 5 day) period before the lytic effects of the virus presumably kill the culture.<sup>95,96-99</sup> The virus reportedly grows well in both T and B lymphocyte cell lines<sup>97,99,100</sup> and the initial belief that only T cells replicate virus<sup>101</sup> appears now to be unfounded.<sup>97,99</sup>

Why lymphocytes are especially resistant to productive infection by HSV is not clear. The fact that cells actively synthesizing DNA (mitogen stimulated or cell lines) support replication much more readily suggests that the state of cellular metabolism may play an important part.<sup>96</sup> Of interest is the report by Daniels et al.<sup>95</sup> of HSV replication in human macrophages in vitro. Attempts to infect freshly isolated macrophages resulted only in "abortive infection" in which no infectious virus was produced but empty viral capsids and HSV antigens were detected in the cells. In fresh cultures only  $1/10^5$  cells was productively infected,



TABLE II

REPLICATION OF HERPES SIMPLEX VIRUS IN HUMAN LYMPHOCYTES

<u>Cell Type</u>	<u>Replication</u>		<u>Peak Titer/Culture Interval</u>	<u>Reference</u>
	<u>Mitogen (+)</u>	<u>Mitogen (-)</u>		
Adult Peripheral Lymphocytes:				
Mixed	+	-	2-3 days/ 10 days	96-99,104
B-Enriched	+	+ (3/5 Donors)	3 days/ 7 days	95,97
T-Enriched	+	- (5/5 Donors)	" "	95,97
Umbilical Cord Lymphocytes, Mixed				
		+ (4/8 Donors)	5 days/ 5 days	105
Cell Lines:				
T-Cell		+	3 days/ increasing	97,99,100
B-Cell		+	" "	97,99,100





but in cultures "aged" in vitro for 3 days, susceptible cells increased to 1% and rising viral titer was noted over the next four days. Confirmation of this work or elucidation of the fate of the abortively infected cells has not been reported.

#### B. Antigenic Stimulation.

Efforts to induce transformed lymphocyte cell lines with HSV have been unsuccessful to date, but several investigators have succeeded in transient stimulation of lymphocytes pre-immunized to HSV in vivo. These experiments are summarized in Table III.

Rosenberg's work<sup>106-108</sup> with lymphocytes of HSV-immune rabbits documented that significant stimulation (evidenced by increased uptake of tritiated thymidine (<sup>3</sup>HTdr)), occurred 3 days after infection with HSV, an effect which was increased by UV treatment of the virus. The stimulation was transitory (3 to 5 days) and was associated with increased synthesis of cellular, not viral DNA.<sup>107</sup> No stimulation occurred in cells from antibody negative animals.

Similar results were achieved with both live and inactivated HSV in lymphocytes of immune human donors.<sup>75,76, 109-112</sup> Corey et al.,<sup>110</sup> and Lopez et al.<sup>111</sup> have studied the stimulation of lymphocytes from persons with active or recurrent herpes labialis infection by UV irradiated HSV in



some detail. They found stimulation indices ( = ratio of  $^3\text{HTdr}$  uptake by HSV-infected lymphocytes compared with the uptake by uninfected controls) as high as 69 in patients with symptomatic herpes infection. The magnitude of lymphocyte stimulation correlated with the stage of virus infection: the peak of lymphocyte stimulation index was observed to coincide with the maximal rate of virus shedding.<sup>110</sup> Stimulation decreased to lower levels in convalescent, but antibody positive individuals.<sup>110-111</sup>

Corey observed that cells from neutralizing-antibody negative donors produced stimulation indices of  $0.8 \pm 0.2$ . This finding was confirmed by Starr<sup>109</sup> and Rasmussen<sup>113</sup> and by Shillitoe et al.<sup>75,76</sup> who also noted that two seronegative control patients whose stimulation index was unexpectedly greater than 2.0, developed active vesicular disease and seroconversion within two weeks.

The latter observation may help explain the divergent finding by Lopez et al.<sup>111</sup> of an average stimulation index of 4.5 in 11 asymptomatic, seronegative controls but nevertheless, this result is clearly at odds with those of the other studies cited.

The bulk of the data suggest, then, that UV-irradiated HSV is antigenic to lymphocytes in immune humans and rabbits but the resulting stimulation is transitory and probably linked to a cellular immune response to antigens on the virion rather than mediated by the viral genome. The in-



TABLE III

## ANTIGENIC STIMULATION OF LYMPHOCYTES BY HERPES SIMPLEX VIRUS IN VITRO.

Cell Type	Antigen	Stimulation Index*		Reference
		Donor Antibody (+)	Antibody (-)	
Rabbit Spleen	HSV + UV	9.4	0.45	106-108
	Live HSV	4.3	0.03	
Human Peripheral:				
Mixed	HSV + UV	69	4.5	111
		17	0.8	116
	HSV + Heat	1.4-1.8	0.5-1.0	109,113
	HSV "Antigens"	6-12	2-5	75,76,112
B-Enriched	Live HSV	2.0	1.0	75,76
T-Enriched	Live HSV	7.5	1.0	75,76

\*Stimulation Index =  $\frac{^3\text{HTdr uptake by infected cells}}{^3\text{HTdr uptake by uninfected cells}}$





creased <sup>3</sup>HTdr uptake is due to increased cellular, not viral DNA synthesis, as evidenced by cesium chloride gradient DNA analysis<sup>107</sup> and the finding that live HSV infected cultures do not exhibit increased thymidine uptake despite ongoing viral synthesis.<sup>97</sup>

### C. Mitogenic Stimulation.

Mitogenic stimulation is distinguished from antigenic stimulation by the ability of the mitogen to cause stimulation of both immune and non-immune cells, whereas antigens stimulate only pre-immunized cells.<sup>114</sup> Demonstration of stimulation of non-immune lymphocytes by HSV would be a first step toward the goal of oncogenic transformation.

Studies of non-immune lymphocytes and HSV have been few. Review of the experiments cited in Table III shows that stimulation of lymphocytes from HSV-antibody negative humans is generally unsuccessful. In the cases where stimulation was reported,<sup>111,112</sup> interpretation is confounded by the possibility of cross-reaction of lymphocytes immunized to related antigens.

Experiments with lymphocytes derived from the spleens of nude mice conducted in separate laboratories<sup>115,116</sup> have documented that live HSV is strongly mitogenic in mice (Table IV). Moreover, the stimulation is maximized in "B" enriched cultures and abolished by heat, UV or antiserum



MITOGENIC STIMULATION OF LYMPHOCYTES BY HERPES SIMPLEX VIRUS IN VITRO.

<u>Cell Type</u>	<u>Mitogen</u>	<u>Stimulation Index*</u>	<u>Remark</u>	<u>Reference</u>
Mouse (Nu-Nu) Spleen:				
Mixed	Live HSV	10-50	1) SI 1.0 for HSV + UV, heat, antiserum	115,116
B-Enriched	Live HSV	20-50	2) No infectious virus production.	
T-Enriched	Live HSV	1.0	3) SI Maximum @ 2-3 days	
			4) 6/13 strains of HSV were mitogenic	
			5) Cellular DNA stimulated, not viral.	
			6) Macrophage depletion required for stimulation	
Human Umbilical Cord:				
Mixed	HSV + Heat	1.0		109

\*Stimulation Index (SI) =  $\frac{{}^3\text{HTdr uptake by infected cells}}{{}^3\text{HTdr uptake by uninfected cells}}$



inactivation of the virus. Only a minority of the virus strains tested<sup>116</sup> possessed stimulatory activity, however.

These results are in contrast to the "antigen" stimulation studies cited in Table III: the effect requires live virus, and maximal stimulation occurs at 3 days versus 7 days in the antigen studies.<sup>110,111</sup>

Investigation of lymphocyte response to HSV in immune-deficient/compromised individuals has not been reported but one experiment involving heat-inactivated HSV and human umbilical cord lymphocytes found no stimulation.<sup>109</sup>

#### V. Modes of Latent Infection and Transformation.

Transformation of cells by both Epstein-Barr virus and Herpes simplex virus is well-documented, although the inability to recover infectious virus from any HSV transformants<sup>82</sup> and to demonstrate viral genome sequences in all HSV-induced cell lines<sup>38</sup> attests to clear differences between HSV-induced and EBV-induced transformation. When detectable, HSV DNA in transformed cell lines is only a small fragment, in contrast to the multiple copies of complete genomes found in EBV lymphoblastoid lines. Intact EBV transforms cells but HSV must be partially inactivated to perform this function. The striking dissimilarity in the UV doses used to achieve maximum transforming efficiency



(10-15,000 ergs/mm<sup>2</sup>-sec) for both tumorigenic and biochemical transformation may be indicative of the target sizes of the "lytic" and "transforming" parts of the HSV genome, but no such differentiation of functions has been clearly demonstrated in EBV.

Regarding the setting in which latent herpesvirus infection may occur, four prominent possibilities have been proposed:

1) viral genome is present in cells but unexpressed; it is capable of reactivation into a lytic, productive cycle,<sup>47,53</sup> e.g. recurrent herpes labialis, h. pro genitalis, h. zoster.

2) viral genome, or part of it, is present and expressed as oncogenic transformation of the host cell, with malignancy as the outcome; lytic functions of the genome are either missing or suppressed<sup>16,17</sup> e.g. EBV in Burkitt lymphoma, Marek's virus and chicken lymphoma.

3) viral genome is present during the initial transformation of the cell, but continued presence of the genome is not required for ongoing reproduction of the transformant progeny e.g. the so-called "hit and run" model of carcinogenesis.<sup>92</sup>

4) viral genome or fragment is present in the infected cell as the inducer/promotor of a latent C-type RNA virus which in turn transforms the cell.<sup>93,94</sup> The studies cited in Table I provide support for each of the first three





proposals although the evidence is inconclusive for all. The last possibility, that of oncornavirus activation, was suggested by the finding of Hampar et al.<sup>94</sup> that infection of rodent cells with UV-HSV resulted in the induction of a xenotropic C-type virus. Kraiselburd et al.<sup>93</sup> extended these observations by noting that the optimum UV dose for xenotropic virus induction closely paralleled that for maximum thymidine kinase transformation and proposing that the genes of HSV causing this effect represent a small fraction of the genome. HSV DNA synthesis was not required for activation of the C-type virus.<sup>93</sup>

Although it may be helpful to conceptualize transformation by herpesviruses occurring in latent infection as occurring by one or more of the mechanisms cited above, it is not at all clear which, if any, of these is pertinent to transformation by Herpes simplex virus. Further studies are required to elucidate the details of HSV transformation.

## VI. Rationale for Experiments with Herpes Simplex Virus and Non-Immune Lymphocytes.

The purpose of the experiments presented in this paper was to investigate the possibility that primary lymphocytes could be stimulated or transformed by UV-irradiated HSV.

Choice of the lymphocyte was based on several consid-



erations. First, lymphocytes had been shown to be relatively non-permissive for HSV replication.<sup>96-99,109</sup> Successful stimulation and transformation experiments in the past have been conducted in non-permissive cells (cf. umbilical cord lymphocyte with EBV<sup>62</sup> and the mouse spleen lymphocyte and HSV<sup>115,116</sup>). Further, the existence of lymphoblastoid cell lines derived from cancer patients in which no virus can be identified suggested that perhaps HSV could be a "transforming virus" for lymphocytes. Use of non-immune, washed lymphocytes collected from umbilical cord blood at the time of fetal delivery would minimize confusion about lymphocyte-antigen cross reaction or mitogenesis associated with antigen-antibody complexes.<sup>114</sup>

Similarly, partial inactivation of the virus was considered likely to maximize stimulatory properties of the virus since lymphocytes had been shown to support limited replication of HSV over the time period in which stimulation was expected to be evident (3 to 7 days).<sup>109</sup> Choice of ultraviolet irradiation was based on the documented ability of UV-irradiated HSV to transform rodent cells,<sup>38,40</sup> and in accordance with the hypothesis that the lytic functions of the HSV genome have a larger UV target size than the transforming functions.<sup>70,82</sup>

Assays for stimulation of DNA synthesis, manifested by the level of thymidine uptake, determinations of the



infectivity of live and inactivated virus, and attempts to induce transformation of cells in different conditions of tissue culture were conducted.



## MATERIALS AND METHODS

Virus. HSV type 1, strain No. 15077, was derived from a brain specimen obtained from a patient with encephalitis. It was isolated in the Vero line of African Green Monkey cells and subsequently passaged three times in the AH-1 Green Monkey Kidney (GMK) cell line. The virus stock titer was  $5 \times 10^5$  plaque-forming units (pfu)/0.1 ml in Vero cells.

Prior to use the virus was passaged once more in GMK AH-1 cells at a multiplicity of infection of 1 pfu/cell. After three or four days, the supernatant fluid was collected and the cellular debris removed by low-speed centrifugation. The titer of this stock, designated "unpurified virus" was  $10^6$  pfu/0.1 ml.

"Purified virus" was subjected to the following further processing: the supernatant was centrifuged in a Beckman Type 19 rotor for 3 hours at  $5^{\circ}\text{C}$ , 20,000 rpm; the pellet was resuspended in 1 ml of Tris .05M and NaCl .15M buffer, layered on a 20-60% continuous sucrose gradient and centrifuged in an SW 27 rotor at 23,000 rpm for 90 minutes. A discrete, light-scattering band was collected and dialyzed against RPMI 1640 medium for 48 hours at  $5^{\circ}\text{C}$ . The virus was divided into 1.0 ml aliquots and stored at  $-70^{\circ}\text{C}$  until use. "Purified virus" stock titer was  $10^9$  pfu per 0.1 ml. By electron microscopy<sup>48</sup> this stock consisted





of 82% enveloped virus particles, 18% capsids, and contained no cellular debris. All virus stocks were shown to be free of mycoplasma by culturing on mycoplasma agar.

UV Irradiation of Virus. Virus suspensions in growth medium (2 ml) were irradiated in 6 cm plastic culture dishes with a 25 watt GE Germicidal Lamp at a distance of 12 cm. Irradiation at this distance produced an ultra-violet light intensity of  $25 \text{ ergs/mm}^2\text{-sec}$  as determined by a dosimeter constructed and calibrated by R. Latarjet,<sup>117</sup> and kindly loaned by D. Rupp. During irradiation the dishes were kept on ice and rotated 100 revolutions per minute on a rotary platform shaker, while exposed to atmospheric oxygen.

Plaque Titration of Virus. Stock and UV-treated virus (0.1 ml), at various dilutions in growth medium, were inoculated onto confluent monolayers of GMK AH-1 cells in 6 cm plastic culture dishes. After one hour, 3 ml of 1.5% methylcellulose in growth medium was added and the dishes were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in air. After 3 days, the dishes were drained, fixed in absolute methanol for 5 minutes and stained by the method of Giemsa. Characteristic plaques were counted and titer was expressed as plaque-forming units per 0.1 ml.



Cell Isolation. Human umbilical cord leukocytes (HUCL) derived by the Ficoll-Hypaque method<sup>118</sup> were used.

To obtain cell populations depleted of T lymphocytes, mononuclear cells ( $5-10 \times 10^6$  leukocytes) were mixed with a 1% suspension of sheep erythrocytes pre-treated with 25 units/ml of neuraminidase (*Vibrio cholerae*, Calbiochem), centrifuged for 10 minutes at 200 g and held at room temperature for one hour.<sup>119</sup> The resulting mixture of rosetting cells (E rosettes) and nonrosetting cells was then layered over a second Ficoll-Hypaque gradient and centrifuged for 30 minutes at 400 g. The population at the interface then contained less than 5% of cells which formed E rosettes. This population, designated "B" enriched cells, was concentrated to  $4 \times 10^6$  cells / ml for thymidine assays.

To obtain cell populations enriched for macrophages, freshly isolated human umbilical cord lymphocytes were suspended in growth medium and cultured flat in tissue culture flasks. Non-adherent cells were washed off three times during 7 days. The cells were harvested by washing in calcium-free medium several times<sup>122</sup>, assayed for purity by demonstration of phagocytosis of EAC-IgG reagent-treated erythrocytes<sup>123</sup> and infected as described above. These cells consisted of greater than 98% phagocytosing cells.

Cell Culture. Before exposure to virus, some leukocyte populations were maintained for 12-24 hours at  $4 \times 10^6$  cells



per ml in tissue culture flasks. This had the effect of removing most of the mononuclear phagocytic cells (population designated "macrophage depleted."<sup>120</sup>) Medium, RPMI 1640, was supplemented with penicillin (50 U/ml), streptomycin (50 ug/ml), amphotericin B (1 ug/ml) and various concentrations of heat-inactivated (56<sup>0</sup>C, 30 minutes) fetal calf serum. 0.1 ml aliquots of cells were placed in wells of microtiter plates (Costar) at  $4 \times 10^6$  cells/ml. 0.1 ml virus or medium was added as inoculum. All cells were cultured at 37<sup>0</sup>C in an atmosphere of 5% CO<sub>2</sub> and air.

Cell Counts. At the end of the culture interval, 2-4 wells of cells from each infected and sham-infected group were pooled and assayed for viability (Trypan Blue exclusion) and cell number (Coulter Counter).

Infectious Centers Assay. To determine the fraction of leukocytes in which viral replication occurred, varying numbers of infected cells were plated onto a monolayer of HSV-1 susceptible cells (GMK AH-1): leukocytes in growth medium were inoculated with HSV at a multiplicity of 10:1 pfu per cell; after one hour an equal volume of rabbit antiserum to HSV (titer 1:40, E. Grogan) was added; after an additional hour the cells were washed twice in Hank's Balanced Salt Solution (HBSS) and plated onto GMK cell monolayers. The plates were cultured for 3 days at 37<sup>0</sup>C



under a layer of 1.5% methylcellulose, following which they were drained, fixed in absolute methanol and stained with Giemsa. Plaques were counted and the results expressed as infected cells/total cells plated  $\times 10^{-3}$ .

Lymphocyte Stimulation Assay. Incorporation of tritiated thymidine ( $^3\text{HTdr}$ ) into acid-insoluble material was measured in infected and sham-infected cultures during the last four hours of incubation by the addition of 0.1 ml of  $^3\text{HTdr}$  (specific activity 6.7 Ci/mM) to each well. The final concentration was 5 uCi/ml. The reaction was stopped by placing the microtiter plate on ice for 20 minutes.

Acid insoluble material was collected by pipetting the contents of each well onto a 2.5 cm membrane filter (Millipore Corp., type HA, 0.45  $\mu\text{m}$ ) and washing each well with a small volume of HBSS to which 1.0 ml of trichloroacetic acid (w/vol) was added. The filters were then rinsed twice in 5 ml of water, dried and placed in vials with scintillation fluid (Econofluor, New England Nuclear). Radioactivity was determined in a liquid scintillation counter.<sup>121</sup> Separate experiments demonstrated that  $^3\text{HTdr}$  added to cells and collected on filters without incubation resulted in counts per minute less than 10% of the values from incubated cultures.

Assays were done with 6 to 8 wells per group; average activity was expressed as the mean of counts per minute





per well of each group and the standard error of the mean of these groups was consistently less than 10% of the mean. Lymphocyte stimulation was expressed as "stimulation index", i.e., the mean counts/minute of infected cultures divided by mean counts/minute of sham/infected cultures. Statistical significance of differences was determined by the Student 't' test.



## RESULTS

Infected Centers Assay. Previous studies established that HSV replication in lymphocytes is a limited, transient phenomenon,<sup>96-99,109</sup> but the fraction of cells productively infected in culture has not been determined. This can be approximated by calculation of the fraction of lymphocytes exposed to HSV which will induce a characteristic plaque after incubation on a monolayer of permissive cells such as GMK.

Table V shows that fewer than 1/1000 freshly isolated umbilical cord lymphocytes will support lytic viral replication. However, if these cells are maintained in culture for more than 5 days, the number of susceptible cells increases almost ten-fold.

To determine if one type of lymphocyte was more susceptible, populations enriched for "B" cells and macrophages were prepared and assayed for infectious centers. As shown in Table V, the number of infectious centers formed by B-enriched cells and macrophages were not significantly different from the mixed cultures. Tritiated thymidine incorporation in parallel cultures of mixed umbilical cord lymphocytes increased almost two-fold after 7 days of in vitro culture without virus.<sup>121</sup>

UV Inactivation of HSV-1. Results of titration of virus



subjected to UV irradiation are shown in Figure 1. It will be noted that infectivity is rapidly inactivated during the first minute of exposure but a relatively resistant region is encountered from 1 to 6 minutes; essentially complete inactivation is achieved by 20 minutes of exposure. These results are nearly identical to those obtained by other investigators.<sup>82,124</sup>

Stimulation of Umbilical Cord Lymphocytes by HSV. Preliminary thymidine uptake assays revealed a small but significant stimulation of lymphocytes infected by HSV irradiated for 6' to 10' (stimulation index = 1.15). This effect was apparent only on the third and fourth days of incubation. Increasing the multiplicity of infection from 1:1 to 100:1 pfu/cell, the use of purified virus and enriching the cells for B lymphocytes did not improve the stimulation achieved, although the effect was consistently observed in several experiments.

A number of attempts by other investigators to demonstrate induction of cellular DNA synthesis by virus have been successful only when the cells were "metabolically arrested." Yamanishi et al. demonstrated a five-fold stimulation of hamster cells exposed to a temperature-sensitive mutant HSV only when the serum concentration was less than 1%.<sup>66</sup> Human embryo fibroblasts exhibited a stimulation index of 9 with HSV-2 when maintained at 42°C



in 0.2% fetal calf serum.<sup>73</sup> St. Jeor et al. induced cellular DNA synthesis with live CMV (stimulation index = 9) when human embryonic lung cells were incubated in 0.2% fetal calf serum, but not in higher concentrations of serum.<sup>125</sup>

Umbilical cord lymphocytes maintained in culture medium supplemented with 20% fetal calf serum exhibit a marked spontaneous increase in <sup>3</sup>HTdr uptake during the first 7 days of culture.<sup>121</sup> This increase in background activity in uninfected cells may mask transient stimulatory phenomena which may be occurring simultaneously in the infected culture. Accordingly, stimulation assays were conducted in medium supplemented with 0.2% fetal calf serum; these are compared to parallel experiments in 20% fetal calf serum in Figure 3.

Significant stimulation ( $p < .001$ ) in the 0.2% serum cultures was observed on the fourth day of incubation for all UV-treated virus; the highest values occurred with 6' and 10' UV doses (stimulation index = 1.7). Stimulation was significant ( $p < .001$ ) in the 20% fetal calf serum cultures only for 10' UV-HSV infected cells, but the stimulation index was small (= 1.11). Thymidine uptake in cells exposed to unirradiated HSV was significantly depressed below control cultures in both serum concentrations tested.

Results similar to those of Figure 3 with mixed lymphocytes were repeated in five consecutive umbilical cord





blood specimens (mean stimulation index  $\pm$  SEM =  $1.58 \pm 0.12$ ).

Enrichment of lymphocytes for "B" cells significantly increased the stimulation index, as shown in Table VI. These cells had also been depleted of macrophages prior to inoculation. The values obtained for 0', 6' irradiated UV-HSV were unchanged, but 10' UV-HSV resulted in a stimulation index of 2.02, compared with 1.67 in mixed cells ( $p < .001$ ).

Stimulation assays done on the seventh day after infection in 0.2% fetal calf serum resulted in a stimulation index (SI) of 1.43 ( $p < .001$ ) for 10' UV-HSV and SI = 1.08 ( $p =$  non-significant) for 6' UV-HSV. Uptake assays for periods longer than 7 days have not been conducted in 0.2% fetal calf serum. Experiments with 20% serum at 14 days showed thymidine uptake less than 50% of controls for all virus preparations tested (0', 6', 10'). Continued stimulation of lymphocytes in reduced-serum medium at 7 days suggests that longer term activity of the UV-treated virus may be present.

To help exclude the possibility that the producer cells (GMK) elaborate a soluble mitogen which was responsible for the observed stimulation, both purified and unpurified virus suspensions were centrifuged for 1 hour at 23,000 rpm (SW 27 rotor), and the supernatant fluid was tested for stimulation. As shown in Table VI, centrifuged



supernatants of purified virus ( $5 \times 10^3$  pfu/0.1 ml) produced a stimulation index of 0.89 in B-enriched cells. Unpurified supernatant ( $2 \times 10^3$  pfu/0.1 ml) actually depressed thymidine uptake (stimulation index = 0.76) of B-enriched cells over control. Similar results were observed in mixed lymphocytes (Table VI). Stimulation assays in which lymphocytes were incubated with the supernatant culture fluid (SNF) removed from the producer cells (GMK) before inoculation with virus produced  $^3\text{HTdr}$  uptake essentially identical to cultures incubated in RPMI 1640 medium alone (Figure 3).

Attempts to Isolate Transformed Cell Lines. Limited attempts to develop transformed cell lines from umbilical cord lymphocytes infected with UV-HSV were conducted in 20% fetal calf serum only. Although several of these cultures survived longer than 60 days, no difference was noted in survival time or morphology between infected and uninfected cultures. No transformed cell lines were isolated.

Cell Counts of Cultured Lymphocytes Infected with UV-HSV.

It was important to determine if virus infection affected the survival of cells in culture over time, in order to interpret stimulation of lymphocytes after 4 days of incubation. Infection with HSV (multiplicity 100:1 pfu/cell) was conducted in fresh, mixed umbilical cord lymphocytes supplemented with 20% and 0.2% fetal calf serum. Cell



counts over days after infection, shown in Figure 3, were identical for all groups. Greater than 85% of the cells in each group were viable by Trypan Blue exclusion. Separate experiments with B-enriched populations indicate that the loss of 50-60% of the initial mixed inoculum of mixed cells in the first 1-2 days is due to the death of T lymphocytes, which do not survive well in the culturing methods used.



TABLE V.

Infected Centers in Lymphocyte Subpopulations  
Infected with HSV-1.

Infected Centers/ $10^3$  Cells

<u>Cell Population</u>	<u>Pre-Infection Culture Interval</u>	
	<u>0-5 Days</u>	<u>6+ Days</u>
Mixed Cells	0.6	4.8
B-Enriched Cells	---	8.0
Macrophages	---	6.0
Mean $^3\text{HTdr}$ uptake,		
Mixed Cells	1956	3541
(counts per minute).		





TABLE VI.

Comparison of Stimulation by UV-HSV:  
Mixed vs "B" Enriched Leukocytes.

Stimulation Index\*

<u>Cell Population</u>	<u>UV Time</u>		
	<u>6'</u>	<u>10'</u>	<u>0' + Centrifugation**</u>
Mixed Cells	1.60	1.67	1.03
"B" Enriched Cells	1.54	2.02	.89

$$* \text{ Stimulation Index} = \frac{{}^3\text{HTdr uptake, infected cells}}{{}^3\text{HTdr uptake, uninfected cells}}$$

Assay on fourth day after infection of freshly isolated lymphocytes

Serum: 0.2% fetal calf serum

Multiplicity of infection: 1:1 pfu/cell

\*\* UV-HSV centrifuged 20,000 (SW 27 rotor) for 1 hour.



FIGURE 1

INACTIVATION OF HSV-1 BY ULTRAVIOLET (UV) LIGHT

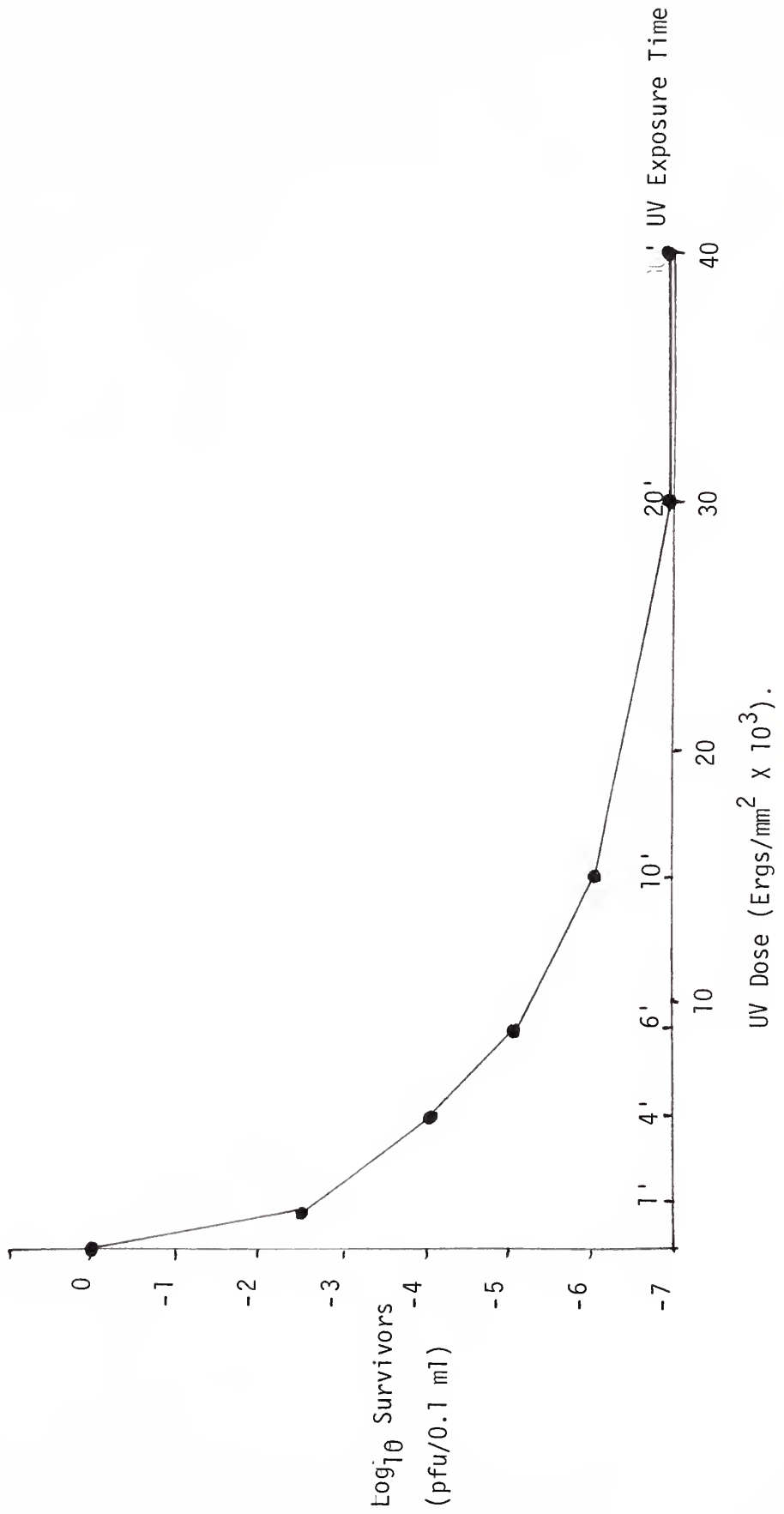




FIGURE 2

CELL COUNTS OF HSV-INFECTED LEUKOCYTES

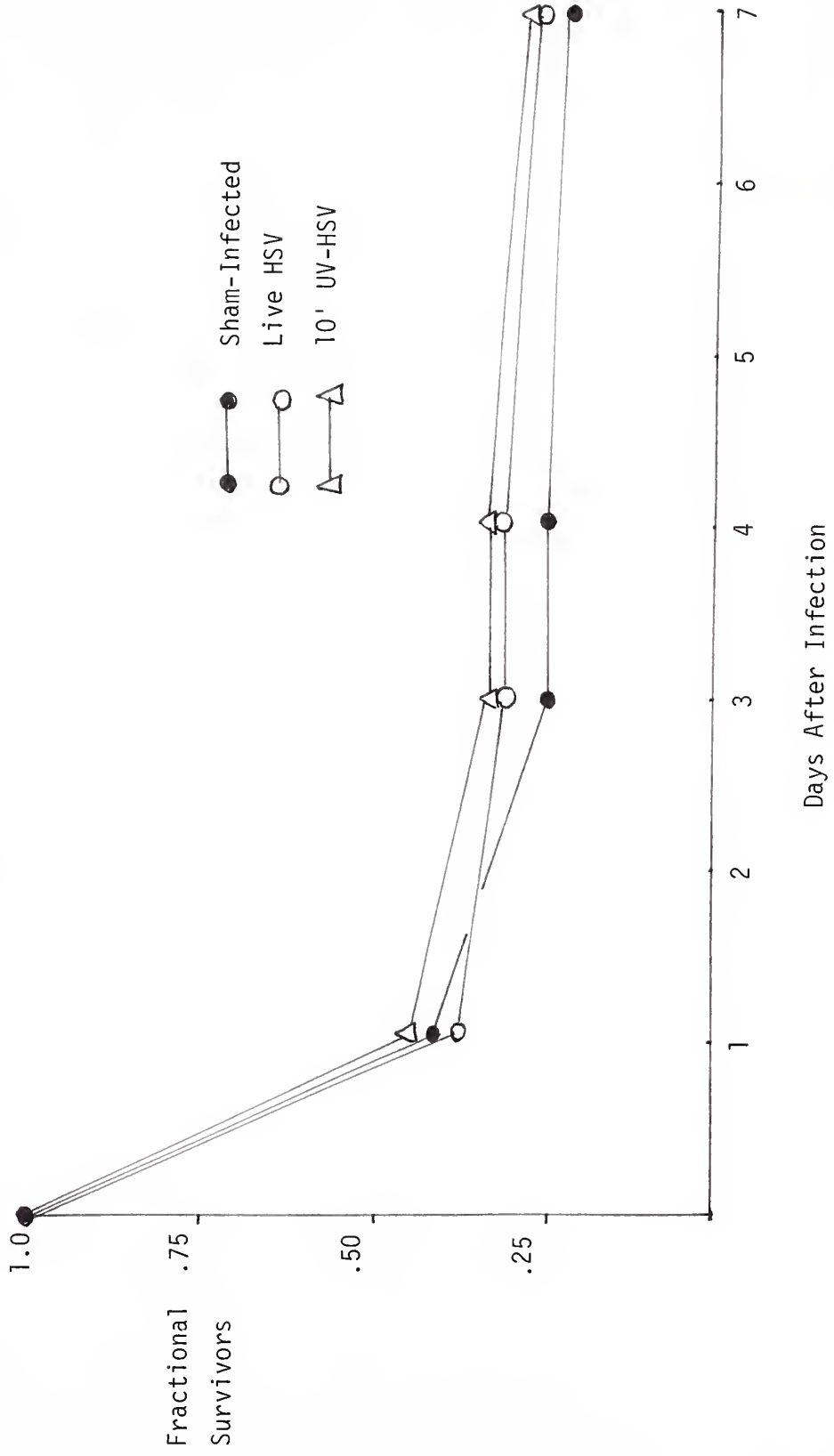
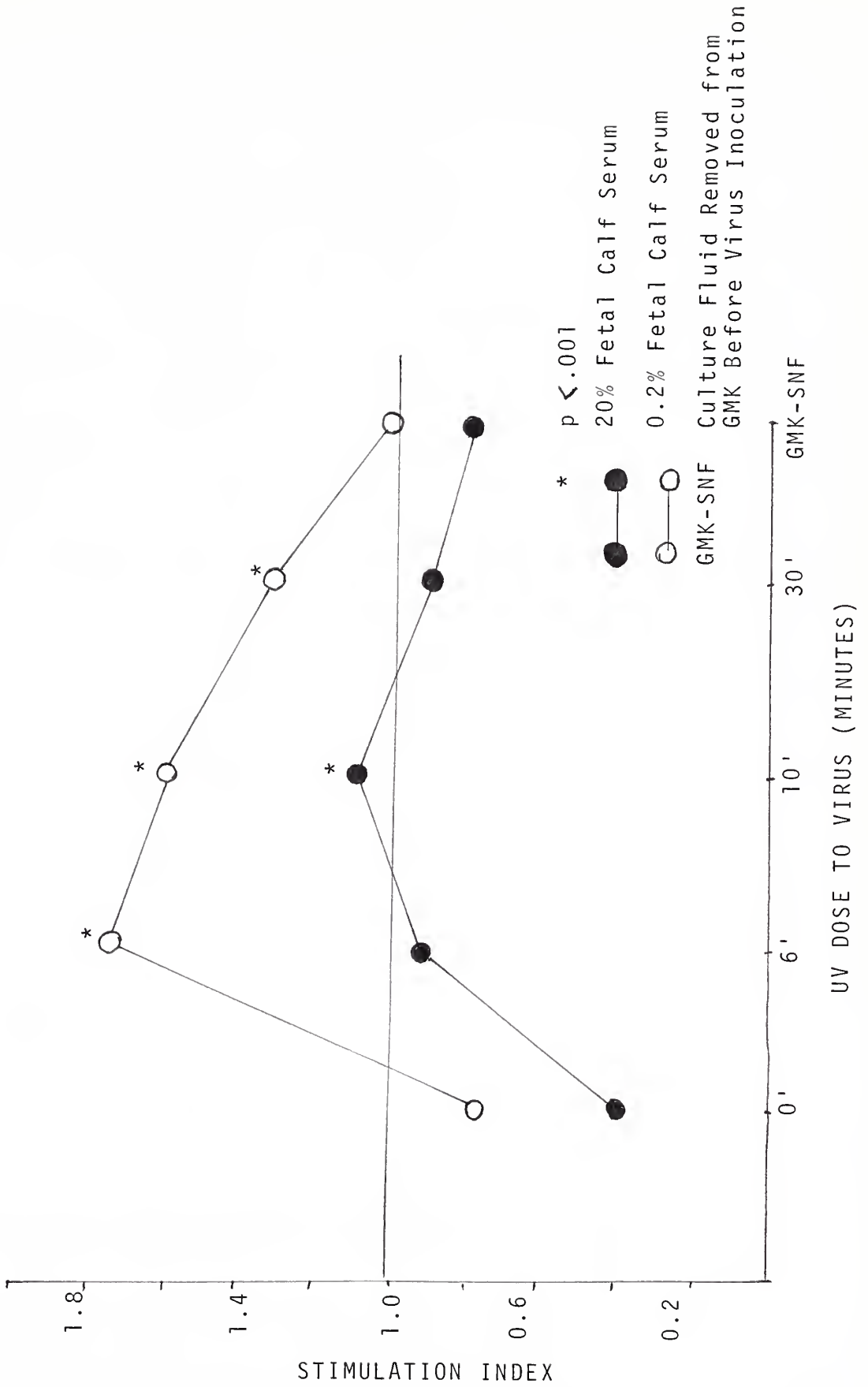




FIGURE 3

LYMPHOCYTE STIMULATION BY UV-HSV







## DISCUSSION

Summary. These experiments have shown that live HSV-1 productively infects only a small fraction (1-6/1000) of freshly-isolated human umbilical cord lymphocytes. Maintaining these cells in culture for 5 days or more is associated with a ten-fold increase in productively infected cells. Umbilical cord lymphocytes (HUCL) infected with UV-irradiated HSV exhibit significant increases in thymidine uptake compared with sham-infected controls, after four days in culture. This effect is intensified by the use of reduced serum concentration in the incubation medium and by the enrichment of the cell population for "B" cells. Sucrose gradient purified virus causes the stimulation while unpurified stocks do not; producer-cell (GMK) growth medium and virus suspensions depleted of virus by centrifugation do not cause stimulation; and the virus preparation itself was shown to be free of cellular debris or mycoplasma contamination. Maximal stimulation is caused by virus exposed to ultraviolet light for 6' to 10' and occurs 3 to 4 days after infection.

Association of Lymphocyte Infectivity and Aging in Culture.

The increase in cells susceptible to infection by HSV over



5 to 7 days of in vitro culture is similar to the result found in macrophages by Daniels et al.<sup>95</sup> The correlation with the increase in DNA synthesis in cultures over time may suggest that cell cycle and/or metabolism influence susceptibility to HSV infection.<sup>96,97,104</sup> It is also possible that in vitro culture selects susceptible cells, and loss of certain cells (e.g. "T" cells) in the first days after isolation inactivates the infection resistance exhibited by the culture when all cell populations are represented in in vivo proportions.

Stimulation of Lymphocytes by UV-HSV. A number of uncertainties about the meaning of the demonstrated increase in thymidine uptake persists.

1) Viral Antigen or Genome as Lymphocyte Stimulator?

Whether the stimulation is due to antigens on the virus surface or due to the viral genome itself has not been shown. However, the use of lymphocytes presumed to be non-immune and washed free of autologous humoral factors suggests that immune mechanisms do not play a part. Starr<sup>109</sup> produced a stimulation index of 25 in adult immune lymphocytes exposed to heat-inactivated HSV antigens but found no stimulation of umbilical cord lymphocytes. The fact that selective UV irradiation of virus, a technique known to function by damaging viral DNA,<sup>70</sup> was required for stimulation in



these experiments further supports the notion that the partially inactivated genome is responsible. If a surface antigen were the stimulatory principle, one might expect increasing stimulation with increasing doses of UV, rather than the convex curve observed (Figure 3).

The possibility that a small number of immunized maternal cells in the umbilical cord blood are responsible for the effect cannot be entirely excluded. Data concerning the extent of maternal cell contamination of human umbilical cord blood is scant. Sharpe<sup>126</sup> examined the metaphases of peripheral lymphocytes in four male neonates and found 0.6% of lymphocytes in one infant had a female karyotype; he concluded that transplacental lymphocyte migration is a rare event.

Fujikura<sup>127</sup> surveyed blood from placentas obtained after delivery for the presence of sickle cells in the offspring of hemoglobin (SS) mothers. He found 8.4% of erythrocytes in the umbilical cord vein were sickled, but Olding<sup>128</sup> found no maternal lymphocytes in fourteen separate samples of umbilical vein blood collected intrapartum. Grogan et al. reported a case of fatal graft-versus-host reaction to lymphocytes of maternal origin in a neonate with severe combined immune deficiency<sup>129</sup> which indicates that although rare, transplacental maternal-fetal lymphocyte transfusion does occur.



Lawler<sup>130</sup> has suggested that the immune-competant fetus may be able to suppress maternal-lymphocyte activation. He demonstrated in mixed maternal-HUCL culture (1:1) that the ratio of mitoses favored fetal cells 6-8:1. Even more convincing is the observation by Olding<sup>131</sup> that 98% of the mitoses in maternal/HUCL cultures stimulated with phytohemagglutinin were in fetal cells. This work was confirmed by Adinolfi.<sup>132</sup>

Thus the possibility that UV-HSV stimulation of umbilical cord lymphocytes is antigenically mediated is remote. The intensification of stimulation in B-enriched and macrophage-depleted cell populations also mitigates against an immune phenomenon: HSV is known to be a "T-dependent" antigen.<sup>114,133</sup> Oppenheim et al. observed that a variety of antigens activated pre-immunized lymphocytes but not umbilical cord lymphocytes; in non-immune cells, only mitogens (PHA, Con A, antilymphocyte globulin) were capable of causing activation.<sup>134</sup>

It is possible that UV-inactivated HSV possesses a mitogen on its surface. Future experiments to determine if stimulation is affected by maternal antibody status or the neutralization of HSV by heat or antiserum will help clarify this issue.

## 2) Induced Cellular or Viral DNA Synthesis? Uptake





of  $^3\text{HTdr}$  in UV-HSV exposed cells is considered to be due to increased incorporation of thymidine into DNA and, therefore, increased DNA synthetic activity. Bain reported that uptake of  $^3\text{HTdr}$  in mixed adult lymphocyte cultures was linear over periods of up to five hours, and that the incorporation was directly into DNA.<sup>135</sup> He documented the formation of numerous degradation products of  $^3\text{HTdr}$ , but these were not included into acid-insoluble material.

Whether thymidine incorporation is associated with viral or cellular DNA synthesis in these experiments has not been determined. Indirect evidence suggest, however, that UV-HSV probably stimulates cellular DNA synthesis: numerous reports have documented HSV replication in lymphocytes over 3-5 days after infection<sup>97,101,109</sup> but Rinaldo<sup>97</sup> found no increase in  $^3\text{HTdr}$  uptake in infected cultures versus controls; Yamanishi et al.<sup>66</sup> compared  $^3\text{HTdr}$  uptake by hamster cells exposed to live and temperature-mutant HSV and observed stimulation only in the mutant-infected cells.

Future experiments should determine, via cesium chloride density gradients, whether the  $^3\text{HTdr}$  is incorporated into viral or cellular DNA.

3)  $^3\text{HTdr}$  Uptake: DNA Repair or Semi-Conservative Replication?



Thymidine incorporation may be involved in the replication of DNA (mitogenesis) or in the repair of damaged DNA.

These experiments have not addressed this issue, but differentiation of these possibilities is important because the introduction of UV-damaged viral DNA into the cell may of itself stimulate endogenous repair mechanisms for correction of defects in the viral genome. Radman<sup>92</sup> has proposed that the introduction of damaged viral DNA may provoke an "error prone" cellular DNA repair system; during such repair, mutagenesis of the cell occurs through incorrectly repaired genome sequences. Future experiments to determine if stimulated cultures are undergoing semi-conservative DNA synthesis or repair should help resolve this important question.

4) <sup>3</sup>Htdr Uptake: Increased DNA Synthesis or Increased Thymidine Kinase Activity?

DNA replication in eukaryotic cells involves the incorporation of thymidine (as thymidine triphosphate (TTP)), mainly derived from the conversion of uridine monophosphate via the so-called endogenous pathway.<sup>136</sup> A very small fraction of the TTP is derived from pre-formed thymidine; pre-formed thymidine enters the DNA synthetic cycle via the "salvage" pathway and the enzyme thymidine kinase. Stimulation assays utilizing <sup>3</sup>HTdr depend on the salvage pathway to



measure thymidine integration into DNA and thus to infer relative rates of DNA synthesis.

Numerous studies cited above<sup>79,85,103</sup> documented the transfer of the thymidine kinase (TK) gene from the HSV genome to mouse cells lacking that enzyme. It is also possible that importation of the TK gene on HSV during infection of lymphocytes induces increased activity of this enzyme. Increased uptake of <sup>3</sup>HTdr could then occur without a net increase in DNA synthesis.

Arguing against this notion is the report by Starr et al. that no increased <sup>3</sup>HTdr uptake occurred in HUCL exposed to heat-inactivated HSV<sup>109</sup> although heat-inactivation of the TK gene in that experiment cannot be excluded. Yamanishi reported a net increase in cellular DNA synthesis in hamster cells infected with a temperature-sensitive mutant of HSV.<sup>66</sup> However it is important that future work be able to show that UV-HSV infected cultures exhibit thymidine kinase activity similar to controls and that net DNA synthesis is increased.

##### 5) Effect of Serum Starvation: Reduction of Background or Enhanced Susceptibility to HSV?

Reduction of the serum supplementation in other cell systems has had the effect of reducing the background DNA synthesis



and repair, and presumably unmasking low-level stimulatory effects,<sup>66,74,125,137</sup> but this technique has not been applied to human leukocytes. In the present studies, counts per minute (cpm) of medium-inoculated cultures after four days were 30% lower in 0.2% fetal calf serum (FCS) than in 20% FCS, although in one experiment no difference was noted. By 7 days, activity in the 0.2% FCS cultures was 50% less than that of the 20% FCS. It is likely that the lowered serum concentration "arrested" the cells. Johnson<sup>138</sup> has suggested, however, that fetal calf serum contains a mitogenic substance and Alford et al.<sup>139</sup> reported that lymphocyte transformation by mitogens was more efficient when autologous serum was added than with heterologous or calf serum.

The last two reports indicate that fetal calf serum in the medium may inhibit transformation or at least increase the baseline mitogenesis in the culture. It may be that the resistance of lymphocytes to infection and transformation by HSV is attributable to the metabolic state conferred by the fetal calf serum. Further investigation of the varying effects of fetal calf serum and autologous serum is warranted.

#### Lymphocytes and HSV: Prospects for the Future.

The basis for the striking resistance of the lymphocyte





to infection and transformation by HSV remains unknown. It is likely that this resistance transcends the immune functions of these cells, for even isolated primary "B" lymphocytes are refractory.

Some of the findings in the present experiments ring true to work done in other cell systems: the optimal stimulation dose of UV (6' to 10') is very similar to the dose used in transforming rodent cells (Table I); the use of serum starvation enhanced detectable stimulation although the cells remained viable.<sup>66,74,125,137</sup> These similarities suggest that certain intrinsic properties of the HSV genome are unmasked by limited UV damage to the DNA, and that damaged virus is capable of interacting with cells, probably all cells, in a characteristic fashion which may, in the right setting, involve oncogenic transformation.

Extension of the present findings will require careful exclusion of the possibilities of induced TK and DNA-repair activity and antigenic interaction with maternal HUCL contaminants, as discussed above. Several virus strains should be screened for stimulatory ability (cf. Rapp and Duff's finding of 2/12 transforming strains<sup>38</sup>). Assays conducted over longer term (21 days) may assist in determining if the stimulation in 0.2% fetal calf serum is transitory or the harbinger of transformation of a small clone of cells. Finally, if dedicated effort to induce



outgrowth of a transformed cell line is successful, these cells should be tested for the presence of HSV antibodies and/or HSV genomes.

Further elucidation of the effects documented in this paper may prove fruitful in demonstrating new aspects of the action of herpesviruses in oncogenesis, and at the very least may shed light on the peculiar ability of the lymphocyte to abort viral infection and replication.



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## REFERENCES

1. Epstein, MA et al., Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* i: 702-3 (1964)
2. Fawcett, DW, Electron microscope observations of intracellular virus-like particles associated with the cells of the Lucke renal adenocarcinoma. *J. Biophys. Biochem. Cytol.* 2: 725-42 (1956)
3. Mizell JS et al., Tumor induction in developing frog kidneys by a zonal centrifuge purified fraction of the frog herpes type virus. *Science* 165: 1134-7 (1969)
4. Naegle RF et al., The presence of the Lucke herpesvirus genome in induced tadpole tumors and its oncogenicity: Koch-Henle postulates fulfilled. *PNAS* 71: 830-4 (1974)
5. Churchill AE et al., Agent of Marek's Disease in tissue culture. *Nature* 215: 528-30 (1967)
6. Okazaki W et al., Protection against Marek's Disease by vaccination with a herpesvirus of turkeys. *Avian Dis.* 14:423-8 (1970)
7. Hunt RD et al., Clinical, epidemiological and pathological features of cytotoxic and oncogenic herpesviruses in South American monkeys. *J. Nat. Canc. Inst.* 49:261-71 (1972)
8. Melendez LV et al., Acute lymphocytic leukemia in owl monkeys inoculated with Herpesvirus saimiri. *Science* 171: 1161-71 (1971)
9. *Ibid.*, Herpesvirus ateles, a new lymphoma virus of monkeys. *Nature New Biol.* 235:182-84 (1972)





10. de Schryver A et al., Epstein-Barr virus associated serology in malignant disease: antibody levels to viral capsid antigens (VCA) membrane antigen (MA) and early antigens (EA) in patients with various neoplastic conditions. *Int. J. Cancer* 9:353-74 (1972)
11. Henle W et al., Herpes-like virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science* 157: 1064-74 (1967)
12. Pope JH et al., Identification of the filterable leukocyte transforming factor of QMIR-WIL cells as herpes-like virus. *Int. J. Cancer* 4: 225-60 (1969)
13. Lindahl T et al., Relationship between Epstein-Barr virus (EBV) DNA and the EBV-determined nuclear antigen (EBNA) in Burkitt lymphoma biopsy and other lympho-proliferative malignancy. *Int. J. Cancer* 13: 764-72 (1974)
14. Adams A et al., Linear association between cellular DNA and Epstein-Barr virus DNA in a human lymphoblastoid cell lines. *PNAS* 70: 2888-2893 (1973)
15. zur Hausen H et al., Presence of EB virus nucleic acid homology in a "virus free" line of Burkitt tumor cells. *Nature* 227:245 (1970)
16. Nonoyama M et al., Detection of Epstein-Barr viral genome in non-productive cells. *Nature* 233:103-5 (1971)
17. Nonoyama M et al., DNA of E.B. virus detected in tissue of Burkitt's lymphoma and nasopharyngeal carcinoma. *PNAS* 70: 3265 (1973)
18. Klein G et al., Direct evidence for the presence of Epstein-Barr viral DNA and nuclear antigen in malignant epithelial cells from



patients with anaplastic carcinoma of the nasopharynx.  
PNAS 71: 4737-41 (1974)

19. Ho HC, Current knowledge of the epidemiology of nasopharyngeal carcinoma: a review, in Oncogenesis and Herpesviruses, Lyon, IARC, de The ed., 1972, pp.357-66
20. Wolf H et al., Epstein-Barr viral genomes in epithelial nasopharyngeal carcinoma cells. *Nature New Biol.* 244: 245-7 (1973)
21. Shope T et al., Malignant lymphoma in cotton-top marmosets after inoculation with Epstein-Barr virus. *PNAS* 70: 2487-91 (1973)
22. Epstein MA et al., Pilot experiments with EB virus in owl monkeys (Aotus trivirgatus). I. Reticuloproliferative disease in an inoculated animal. *Int. J. Cancer* 12: 309-318 (1973a)
23. Ibid, Pilot experiments with EB virus in owl monkeys (Aotus trivirgatus). II. EB virus in a cell line from an animal with reticuloproliferative disease. *Int. J. Cancer* 12: 319-332 (1973b)
24. Miller G. et al., Epstein-Barr viral nuclear antigen (EBNA) in tumor cell imprints of experimental lymphoma of marmosets. *Trans. Assoc. Am. Phys.* 87: 205-218 (1974)
25. Wolf H et al., EBV DNA in non-lymphoid cells of nasopharyngeal carcinoma and in malignant lymphoma obtained after inoculation of EBV into cottontop marmosets. *Cold Spring Harbor Symp. Quant. Biol.* 39: 791-96 (1974)
26. Pope JH, Transformation in vitro by herpesviruses--A review. in Oncogenesis and Herpesviruses II, Lyon, IARC, de The G ed., 1975, p.368



27. Albrecht T et al., Malignant transformation of hamster embryo fibroblasts following exposure to UV-irradiated human cytomegalovirus. *Virology* 53: 53-61 (1973)
28. Nahmias AJ et al., Association of genital herpesviruses and cervical cancer. *Int. Virol.* 1: 187-88 (1969)
29. Mumford DM et al., Immunity, herpes simplex virus and cervical carcinoma. *Surg. Clin. North America* 58: 39-54 (1978)
30. Munoz N, Antibody to herpesviruses in patients with cervical cancer and controls, in Oncogenesis and Herpesviruses II, 1975 pp. 47-50
31. Naib ZM et al., Cytology and histopathology of cervical herpes simplex infection. *Cancer* 19: 1026-31 (1966)
32. Nahmias AJ et al., Antibodies to Herpesvirus hominis type 1 and 2. II. Women with cervical cancer. *Am. J. Epidem.* 89: 547-64 (1969)
33. Gagnon F, Contribution to the study of the etiology and prevention of cancer of the cervix of uterus. *Am. J. Byne.* 60: 516-22 (1950)
34. Aurelian L, Possible role of Herpesvirus hominis type 2 in human cervical cancer. *Fed. Proc.* 31: 1651-59 (1972)
35. Ibid., Herpesvirus type 2-induced tumor-specific antigen (AG-4) and specific antibody in patients with cervical cancer and controls, in Oncogenesis and Herpesviruses II, 1975, pp. 79-81
36. Nahmias AJ et al., Antigenic relationship between herpes simplex virus, human cervical cancer and HSV-associated hamster tumors. in Oncogenesis and Herpesviruses II, 1975, pp. 309-311



37. Summers WC et al., Use of restriction endonucleases to analyze the DNA of herpesviruses, in Oncogenesis and Herpesviruses II, 1975, pp. 143.
38. Rapp F and Duff R, Transformation of hamster embryo fibroblasts by herpes simplex virus type 1 and type 2. *Canc. Res.* 33: 1527-32 (1973)
39. Frenkel et al., DNA fragment of HSV 2 and its transcription in human cervical cancer tissue. *PNAS* 69:3784-89 (1972)
40. Aurelian L et al., Herpes simplex type 2 isolated from cervical tumor tissue cells grown in tissue culture. *Science* 174: 704-7 (1971)
41. Herrera et al., On finding virus-like structures in uterine cervical carcinoma. *Acta Cytology* 18: 45-50 (1974)
42. Rawls WE et al., The association of herpes simplex type 2 and cancer of the uterine cervix. *Am. J. Epidem.* 89: 547-54 (1969)
43. Naib ZM et al., Relation of cytohistopathology of genital herpesvirus infections to cervical anaplasia. *Cancer Research* 33: 1452-53 (1973)
44. Deinhardt F et al., Distribution of antibodies to EBV in various American population groups. *Bact. Proc.* 178-9 (1969)
45. de The, G et al., A prospective sero-epidemiological study to investigate the role of EBV in Burkitt lymphoma, in Unifying Concepts of Leukemia, Ducher RM ed., Basel, Karger, 1973, pp.448-50
46. Fridlender B et al., Interaction of ultraviolet-irradiated herpes simplex virus type 1 with BSC-1 cells. *J. Gen Virol.* 39:371-75 (1978)





47. Stevens JG, Herpes simplex viral latency--A review, in Oncogenesis and Herpesviruses II, 1975, pp. 67-72
48. Miller G et al., Comparison of yield of infectious virus from clones of human and simian lymphoblastoid lines transformed by Epstein-Barr virus. J. Exp. Med. 138: 1398-42 (1973b)
49. Epstein MA et al., Morphological and virological investigations on cultured Burkitt tumor lymphoblasts (strain Raji). J. Nat. Canc. Inst. 37: 547-9 (1966)
50. Stevens JG et al., Latent characteristics of selected herpesviruses. Adv. Canc. Res. 26: 227-56 (1978)
51. Ibid., Latent HSV from trigeminal ganglia of rabbits with recurrent eye infection. Nature 235: 216-7 (1972)
52. Cook ML, Evidence that neurons harbor latent Herpes simplex virus. Infect. Immunol. 9: 946-51 (1974)
53. Walz NH et al., Latent infection with Herpes simplex virus type 1 and type 2: viral reactivation in vivo after neurectomy. Science 184: 1185-7 (1974)
54. Baringer JR, Herpes simplex virus in human sensory ganglia, in Oncogenesis and Herpesviruses II, 1975, pp. 73-77
55. Bastian FO et al., Herpesvirus hominis: isolation from human trigeminal ganglia. Science 178: 306-7 (1972)
56. Baringer JH et al., Recovery of herpes simplex virus from human trigeminal ganglia. New Engl. J. Med. 288: 648-50 (1973)



57. Roizman B et al., The transcription and state of Herpes simplex virus DNA in productive infection and in human cervical cancer tissue. *Cancer Res.* 33: 1402-16 (1972)
58. Miller G et al., Establishment of lines from normal human blood leukocytes by co-cultivation with a leukocyte line derived from a leukemic child. *Proc. Soc. Exp. Biol.* 132: 247-62 (1969)
59. Chang RS, Leucocyte-transforming agent from mononucleosis is ether-stable. *Nature New Biol.* 237: 273-4 (1972)
60. Jondal M et al., Surface markers on human B and T lymphocytes II. Presence of Epstein-Barr virus receptors on B lymphocytes. *J. Exp. Med.* 138: 1365-78 (1973)
61. Pope JH et al., Transformation of foetal human leukocytes in vitro by filtrates of a human leukaemic cell line containing herpes-like virus. *Int. J. Cancer* 3; 857-66 (1968)
62. Chang RS, Umbilical cord leukocytes transformed by lymphoid cell filtrates from healthy people. *Nature New Biol.* 237: 273-4 (1973)
63. Nilsson K et al., The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV. *Int. J. Cancer* 8: 443-50 (1971)
64. Rapp F et al., Transformation of mammalian cells by DNA-containing viruses following photodynamic inactivation. *Virology* 33: 339-46 (1973)
65. Kimura et al., Enhanced replication of temperature-sensitive mutants of herpes simplex virus 2 at the non-permissive temperature in cells transformed by HSV-2. *Intervirology* 3: 162-9 (1974b)



66. Yamanishi K et al., Induction of cellular DNA synthesis by a temperature sensitive mutant of HSV type 2. *Virology* 67: 450-62 (1975)
67. Kimura et al., 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: 786-98 (1975)
68. Macnab, JCM Transformation of rat embryo cells by a temperature sensitive mutant of herpes simplex virus. *J. Gen. Virol.* 24: 143-53 (1974)
69. Shcaffer PA, Temperature-sensitive mutants of herpesviruses. *Current Topics Microb. Immunol.* 70: 51-100 (1975)
70. Benjamin T, Relative target sizes for the inactivation of the transforming and reproductive abilities of the polyoma virus. *PNAS* 54: 121-24 (1965)
71. Duff R et al., Induction of oncogenic potential by herpes simplex virus. *Prospect. Virol.* 8: 189-209 (1973)
72. Jarrat M, Photodynamic inactivation of herpes simplex virus. *Photochem. Photobiol.* 25: 339-40 (1977)
73. Kucera LS et al., Herpes simplex virus type 2 (HSV-2) functions expressed during stimulation of human cell DNA synthesis. *Abstracts of Herpesvirus Workshop, St. John's College, Cambridge, 1978.*
74. Docherty JJ et al., Differential susceptibility to herpes simplex viruses of hamster cell lines established after exposure to chemically inactivated herpesvirus. *J. Gen. Virol.* 13:337-84 (1971)



75. Shillito EJ et al., Sequential changes in T and B lymphocyte responses to herpes simplex virus in man. *Scand. J. Immunol.* 7: 357 (1978)
76. Ibid., Sequential changes in cell-mediated immune response to herpes simplex virus after recurrent herpetic infection in humans. *Inf. Immunol.* 18: 130-7 (1977)
77. Rapp F, Oncogenic potential of herpes simplex virus in mammalian cells after photodynamic inactivation. *Photochem. Photobiol.* 25: 335-7 (1977).
78. Darai G et al., Human embryonic lung cells abortively infected with herpes virus hominis type 2 show some properties of cell transformation. *Nature New Biol.* 241: 268-9 (1973)
79. Portugal FH, Cell transformation with herpes simplex virus. *Nature* 269: 196 (1977)
80. Camacho A, Transformation of hamster embryo fibroblasts by a specific fragment of the herpes simplex virus genome. Abstract of the Herpesvirus Workshop, St. John's College, 1978
81. Rapp F et al., Biochemical transformation of mouse cells by herpes simplex virus type 1 and 2: comparison of different methods of inactivation of viruses. *Arch. Virol.* 56: 77-87 (1978)
82. Duff R et al., Properties of hamster embryo fibroblasts transformed in vitro after exposure to ultraviolet irradiated herpes simplex virus type 2. *J. Virol.* 8: 469 (1971)
83. Duff R et al., Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type 1. *J. Virol.* 12: 209-217 (1973)





84. Kutimora L et al., Increased oncogenicity and synthesis of herpesvirus antigens in hamster cells exposed to herpes simplex type 2 virus. N. Nat. Cancer Inst. 50: 759-65 (1973)
85. Munyon W et al., Transfer of thymidine kinase to thymidine kinaseless L cells by infection with ultraviolet irradiated herpes simplex virus. J. Virol. 7 813-20 (1971)
86. Rapp F. et al., In vitro cell transformation by herpesviruses. Fed. Proc. 31: 1660-8 (1972)
87. Davis DB et al., Quantification of the viral DNA present in cells transformed by UV-irradiated herpes simplex virus. J. Virol. 17: 788-93 (1976)
88. Frenkel N et al., Herpes simplex DNA in transformed cells: sequence complexity in five hamster cell lines and one derived hamster tumor. J. Virol. 18: 885 (1976)
89. Melnick JL et al., Photodynamic inactivation of herpes simplex virus and its DNA. Photochem. Photobiol. 25: 341 (1977)
90. Garfinkle B et al., Transformation of cultured mammalian cells by viable herpes simplex virus subtypes I and II. PNAS 71: 220 (1974)
91. Kraiselburd E et al., Presence of a herpes simplex virus type 1 genome fragment in HSV-transformed cells, in Oncogenesis and Herpesviruses II, 1975, pp. 417-9
92. Radman M, On the mechanism and genetic control of mutagenesis by carcinogens and mutagens, in Origins of Human Cancer, Cold Spring Harbor Laboratory, 1977, pp. 903-21



93. Kraiselburd E et al., Xenotropic virus induction by ultraviolet irradiated herpes simplex virus. Abstracts of Herpesvirus Workshop, 1978.
94. Hampar B et al., Activation of an endogenous mouse type C virus by ultraviolet irradiated herpes simplex virus type 1 and 2. PNAS 73: 646 (1976)
95. Daniels MD et al., Abortive and productive infection of human mononuclear phagocytes by type 1 herpes simplex virus. Am. J. Path. 91: 119-29 (1978)
96. Nahmias AJ et al., Viral-leukocyte interrelationships. I. Multiplication of a DNA virus--herpes simplex virus in human leukocyte cultures. J. Immunol. 93: 69 (1964)
97. Rinaldo CR et al., Replication of herpes simplex virus and cytomegalovirus in human leukocytes. J. Immunol 120: 130 (1978)
98. Bouroncle BA et al., Replication of herpes simplex virus in cultures of phytohemagglutinin-stimulated human lymphocytes. J. Nat. Cancer Inst. 44: 1065 (1970)
99. Plaeger-Marshall S et al., Experimental infection of subpopulations of human peripheral blood leukocytes by Herpes simplex virus. Proc. Soc. Exp. Biol. Med. 158: 263-78 (1978)
100. Bedoya V, Growth in vitro of herpes simplex virus in human lymphoma cell lines. J. Nat. Cancer Inst. 41: 635 (1968)
101. Pelton BK et al., Susceptibility of human lymphocyte populations to infection by herpes simplex virus. Immunol. 32: 803 (1977)
102. Kutimora L et al., Increased oncogenicity and synthesis of herpesvirus antigens in hamster cells exposed to herpes simplex



type 2 virus. J. Nat. Cancer Inst. 50: 759-63 (1973)

103. Duff R. et al., Quantitative transformation of Swiss/3T3 cells by UV-irradiated herpes simplex virus type 2, in Oncogenesis and Herpesviruses II, 1975, p.421
104. Kleinman L et al., Herpes simplex virus replication in human lymphocyte cultures stimulated with phytohemagglutinin and anti-lymphocyte globulin. Proc. Soc. Exp. Biol. Med. 141: 1095 (1972)
105. Trofatter KF et al., Growth of herpes simplex virus (HSV) type 2 in human mononuclear leukocytes from umbilical cord blood. Fed. Proc. 36: 1076 (1977)
106. Rosenberg GL et al., In vitro stimulation of sensitized lymphocytes by herpes simplex virus and vaccinia virus. PNAS 69: 756-60 (1972)
107. Ibid., Induction of cellular immunity to herpes simplex virus: relationship to humor immune response. J. Immunol. 112: 1019 (1974)
108. Ibid., Differentiation of type 1 and type 2 herpes simplex virus by in vitro stimulation of immune lymphocytes. J. Immunol. 109: 413-4 (1972)
109. Starr SE et al., Stimulation of human lymphocytes by herpes simplex virus antigens. Inf. Immunol. 11: 109 (1975)
110. Corey L et al., Cellular immune responses in genital herpes simplex virus infection. New England J. Med. 299: 986-91 (1978)
111. Lopez C et al., Cell mediated immune response in recurrent herpesvirus infection. I. Lymphocyte proliferation assay. J. Immunol. 118: 895 (1977)



112. Wilton JMA et al., Cell mediated immunity in herpesvirus hominis infection. Br. Med. J. i: 723 (1972)
113. Rasmussen LE et al., Lymphocyte interferon production and transformation after herpes simplex infection in humans. J. Immunol. 112: 728-36 (1974)
114. Ling NR and Kay JE, Lymphocyte Stimulation, American Elsevier, New York, 1975, pp. 320-400
115. Mochizuki D et al., The interaction of herpes simplex virus with murine lymphocytes. I. Mitogenic properties of herpes simplex virus. J. Exp. Med. 146: 1500-10 (1977)
116. Kirchner H et al., In vitro mitogenic stimulation of murine spleen cells by herpes simplex virus. J. Immunol. 120: 641-45 (1978)
117. Latarjet RP et al., Un appareil simple pour les dosage des rayonnements U.V. eims par les lampes germientes. Ann. Inst. Pasteur 85: 174-84 (1953).
118. Boyum A, Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Invest. 21:(Suppl.97):77-89 (1968)
119. Weiner MS et al., Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. Blood 42: 939-46 (1973)
120. Henderson E et al., Efficiency of transformation of lymphocytes by Epstein-Barr virus. Virology. 76: 152-63 (1977)
121. Robinson J et al., Assay for Epstein-Barr virus based on stimulation of DNA synthesis in mixed leukocytes from human umbilical cord blood. J. Virology. 15: 1065-72 (1975)





122. Blaese et al., Lymphocyte-macrophage interaction in antigen-induced in vitro lymphocyte transformation in patients with Wiskott Aldrich syndrome and other diseases of anergy. *Cell Immunol* 4: 228 (1972)
123. Robinson J et al., Host determined differences in expression of surface markers on human and simian lymphoblastoid cell lines transformed by Epstein-Barr virus. *PNAS* 74: 749-53 (1977)
124. Ross L J N et al., Ultraviolet irradiation of herpes simplex virus: reaction processes and delay in viral multiplication. *J. Gen. Virol.* 16: 299 (1972)
125. St. Jeor SC et al., Stimulation of cellular DNA synthesis by human Cytomegalovirus. *J. Virol.* 13: 353-62 (1974)
126. Sharpe HBA, Human foetal-maternal barrier. *Nature* 226: 453 (1970)
127. Fujikura T et al., Transplacental passage of maternal erythrocytes and sickling. *J. Pediatr.* 7: 781 (1975)
128. Olding LB et al., Possibility of maternal-fetal transfusion of lymphocytes in man. *Acta Pediatr. Scand.* 61: 73-5 (1972)
129. Grogan T et al., Graft versus host reaction in a severe combined immune deficient patient. *Arch. Pathol.* 99: 330 (1975)
130. Lawler SD et al., Interaction of maternal and neonatal cells in mixed leukocyte culture. *Lancet* ii: 1185-6 (1975)
131. Olding LB et al., Lymphocytes from human newborns abrogate mitosis of mother's blood. *Nature* 249: 161 (1974)



132. Adinolfi M et al., Inhibition of mitosis of maternal lymphocytes by fetal cells, in 1st International Congress of Immunology-- in Obstetrics and Gynecology, Padua, 1973, Centaro A et al ed., Amsterdam, 1973, p. 177
133. Yoshinaga M et al., Regulation of lymphocyte responses in vitro. I. Regulating effect of macrophages and thymus-dependent (T) cells on the response of thymus-independent (B) lymphocytes to endotoxin. J. Exp. Med. 136: 956-67 (1972)
134. Oppenheim JJ et al., Signals regulating in vitro activation of lymphocytes. Prog. Allergy, 20: 65 (1976)
135. Bain B, Tritiated thymidine uptake in mixed leukocyte cultures: effect of specific activity and exposure time. Clin. Exp. Immunol. 6: 255 (1970)
136. Lehninger AL, Biochemistry, Worth, New York, 1970, p.576
137. Fried M et al., Replication of polyoma virus DNA. I. A resting cell system for biochemical studies on polyoma virus. Virol. 34: 761-770 (1968)
138. Johnson G et al., Reaction of human lymphocytes in culture to components of the medium. Nature 208: 343 (1965)
139. Alford RH, The effects of autologous plasma on lymphocyte transformation. Proc. Soc. Exp. Biol. Med. 133: 1443-6 (1970)













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