INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value; however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
HARRIS, Lester Floyd, 1939-  
SPECIFICITY OF IMMUNE LYMPHOCYTES FOR IN VITRO  
DETECTION OF VIRAL INDUCED TUMOR ASSOCIATED  
ANTIGEN(S).  
The Ohio State University, Ph.D., 1975  
Microbiology

Xerox University Microfilms, Ann Arbor, Michigan 48106
SPECIFICITY OF IMMUNE LYMPHOCYTES FOR IN VITRO DETECTION OF VIRAL INDUCED TUMOR ASSOCIATED ANTIGEN(S)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

by

Lester Floyd Harris, B.S., M.S.

* * * * * *

The Ohio State University

1975

Reading Committee:  
Henry G. Cramblett  
Vincent V. Hamparian  
Raymond W. Lang  
Donald C. Thomas

Approved by

Vincent V. Hamparian
Advisor
Department of Medical Microbiology
ACKNOWLEDGMENTS

I wish to express thanks to the faculty members of the Department of Medical Microbiology for providing a stimulating environment for graduate education.

Professor Vincent V. Hamparian, my advisor, was especially helpful in all stages of this research. I am sincerely appreciative of his personal encouragement, warmth, and understanding.

My committee members, Drs. D. C. Thomas, J. H. Hughes, R. W. Lang, and H. G. Cramblett, were helpful with provocative discussion and constructive criticism. I thank them for this.

Elizabeth A. Young provided expert technical assistance throughout the study, to her goes a special note of gratitude. I am also appreciative of the excellent technical assistance of Kristi L. Fowler during the preliminary stages of this work.

Phyllis LeMasters contributed professional secretarial assistance in typing this manuscript, to her I am grateful.

Finally, I am thankful for the understanding and encouragement of my wife, Julanne and my two sons, Andrew and Matthew.

This work was supported by The American Cancer Society Grants 3954-T1 and 3981-A1.
VITA

December 15, 1939 • • • • • • Born - New Boston, Ohio

1966 ........................................ B.S., Ohio University, Athens, Ohio

1966-1968 ................................ Teaching Assistant, Department of Zoology, Ohio University, Athens, Ohio

1968-1971 ................................ Chief Research Associate, Virology Laboratory, Children's Hospital, Columbus, Ohio

1969 ........................................ M.S., Ohio University, Athens, Ohio.

1971-1972 ................................ Microbiologist-Immunologist G.S. II, The Veterans Administration Hospital, Sepulveda, California

1972-1973 ................................ Chief Research Associate, Virology Laboratory, Children's Hospital, Columbus, Ohio

1973-1974 ................................ Research Associate, Department of Medical Microbiology, The Ohio State University, Columbus, Ohio
"An Investigation of Several Lots of Commercially Available Selective Media for Quantitative Recovery of Clostridium perfringens", L. F. Harris, Master's Thesis on file at the Ohio University Library, Ohio.


"Characterization of Hamster Lymphocyte Reactivity to Phytohemagglutinin in Microculture", L. F. Harris, V. V. Hamparian, V. V., E. A. Young, J. H. Hughes, and H. G. Cramblett. (Manuscript In Preparation).

"Detection of Cellular Immunity to Herpes Simplex Virus Type 2 Transformed Fibrosarcomas in Syngeneic LSH Hamsters", L. F. Harris, V. V. Hamparian, E. A. Young, J. H. Hughes, and H. G. Cramblett. (Manuscript in Preparation).
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Acknowledgements</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vita.</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables.</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
</tbody>
</table>

Introduction and Review of the Literature

- Expression of Neoantigens in DNA Virus Transformed Cells
- Other Surface Antigens on Transformed Cells (Embryonic Antigens)
- Biochemical Composition of TSTA
- State of the Viral Genome in Transformed Cells
- Lymphocyte Transformation Assay for Detecting TAA
- The Role of Herpesviruses in Human Malignancies

Materials and Methods

- Animals
- Tumor
- Tumor Transplantation
- Cell Cultures
- Antigens Used to Stimulate Lymphocytes
- Cell-Free Antigens
- Mitomycin-C Treated Cells
- Antigens From HSV-2 Infected Cells
- Phytohemagglutinins
- Antigens Used for Immunization of Hamsters
- Immunization of Animals
- Preparation of Hamster Lymphocytes
- Lymphocyte Transformation Assay
- Harvesting Procedure

Results

- Characterization of Hamster Lymphocyte Transformation With Phytohemagglutinin (PHA)
- Effects of Varying Cell Concentrations
- Effects of Varying PHA Concentrations
Comparative Mitogenic Effects of PHA on Hamster Blood and Splenic Lymphocytes ........................................... 25
Reactivity of Hamster Splenic Lymphocytes to PHA in Media Containing Either Fetal Bovine Serum (FBS) or Autologous Hamster Serum ........................................... 30
Effect of PHA on Splenic Lymphocytes from Hamsters Bearing Herpes Simplex Virus Type 2 Tumor ................. 31
Temporal Relationship of Tumor Cell Input and Tumor Development .......................................................... 35
Tumor Cell Neutralization by Immune Splenic Lymphocytes ................................................................. 35
Stimulation of Lymphocytes From HSV-2 Tumor Bearing Hamsters by Cell-Free Tumor Antigens .............. 42
Comparison of Immune Splenic and Peripheral Blood Lymphocytes from the Same Animals for Reactivity to HSV-2 Cell-Free Tumor Antigens ........................................... 42
Response of HSV-2 Tumor Immune Lymphocytes to Cell-Free HSV-2 Tumor Antigen According to Length of Incubation ................................................................. 47
Stimulation of HSV-2 Tumor Immune Splenic Lymphocytes With Cell-Free Antigen From HSV-2 Infected Human Embryonic Lung (WI-38) Cell Cultures ........................................... 47
Specific Transformation of Immune Splenic Lymphocytes With Cell-Free Tumor Antigens ................................. 47
Specific Transformation of Immune Splenic Lymphocytes From Hamsters Receiving Multiple Intradermal Injections .... 52
Effect of Mitomycin-C on Tritiated-Thymidine Uptake by Tumor Cells ...................................................... 54
Blastogenic Response by Immune Splenic-Lymphocytes in the Mixed Lymphocyte-Mitomycin-C Treated Tumor Cell Reaction MLTR ............................................................... 58
Stimulation With Mitomycin-C Treated HSV-2 Tumor Cells According to the Time of Incubation ................. 58
Comparison of Tumor Immune Splenic and Blood Lymphocytes for Reactivity to Mitomycin-C Treated HSV-2 Tumor Cells ................................................................. 67
In Vitro Sensitization of Normal Hamster Splenic Lymphocytes With Mitomycin-C Treated Syngeneic HSV-2 Tumor Cells ................................................................. 67
Specific Transformation of Immune Splenic Lymphocytes in the Mixed Lymphocytes Mitomycin-C Treated Tumor Cell Reaction ................................................................. 67

DISCUSSION ........................................................................................................................................ 74

SUMMARY ................................................................. 82

BIBLIOGRAPHY ....................................................... 84
<table>
<thead>
<tr>
<th>TABLE</th>
<th>DESCRIPTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Specific Transformation of Immune Splenic Lymphocytes With Cell-Free Antigens</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>Specific Transformation of Immune Splenic Lymphocytes From Hamsters Receiving Multiple Intradermal Injections</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>Specific Transformation of Immune Splenic Lymphocytes in the Mixed Lymphocyte-Mitomycin-C Treated Tumor Cell Reaction</td>
<td>73</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Title</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Characterization of Hamster Lymphocyte Transformation with PHA - Effect of Varying Cell Concentrations</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>Characterization of Hamster Lymphocyte Transformation with PHA - Effect of Varying PHA Concentrations</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>Characterization of Hamster Lymphocyte Transformation with PHA - Comparative Effect of Varying PHA Concentrations on Blood and Splenic Lymphocytes</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Reactivity of Hamster Splenic Lymphocytes to PHA-P in Medium with Either Fetal Bovine Serum (FBS) or Autologous Hamster Serum (HS)</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>Characterization of Hamster Lymphocyte Transformation with PHA - Effect on Splenic Lymphocytes from Hamsters Bearing Herpes Simplex Virus Type 2 (HSV-2) Tumors</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Cumulative Incidence of Tumor Development in LSH Hamsters Following Subcutaneous Injection of Varying Numbers of HSV-2 Tumor Cells</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>Suppression of Tumor Development in Hamsters Inoculated with a Mixture of Immune Hamster Lymphocytes and HSV-2 Tumor Cells</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>Stimulation of Immune Splenic Lymphocytes with Cell-Free HSV-2 Tumor Antigens</td>
<td>43</td>
</tr>
<tr>
<td>9</td>
<td>Comparison of Immune Splenic and Peripheral Blood Lymphocytes from the Same Animal for Reactivity to HSV-2 Cell-Free Tumor Antigen</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>Response of HSV-2 Tumor Immune Splenic Lymphocytes to Cell-Free HSV-2 Tumor Antigen According to Length of Incubation</td>
<td>48</td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION AND REVIEW OF THE LITERATURE

Expression of Neoantigens in DNA Virus Transformed Cells

It is well established that oncogenic deoxyribonucleic acid (DNA) viruses can transform cells in vivo and in vitro (74). It is also known that virus-specific neoantigens are present in cells transformed by these viruses. These neoantigens include the transplantation rejection or tumor specific transplantation antigens (TSTA) (29,30,43,49,50,113). Tumors induced by the inoculation of polyoma virus, simian virus-40 (SV-40) and adenoviruses express these antigens. With the exception of the cross reactivity found among certain adenovirus types, the antigens are virus-specific and are not a structural part of the virion (93). The TSTA is located on the membrane of transformed cells (74,128) and is similar to histocompatibility-type antigen(s) since a homograft-type injection response is evoked in the host to tumor cell transplantation (74). Animals immunized with either live virus or virus-transformed syngeneic, allogeneic, or xenogeneic cells develop specific resistance to transplantation of homologous virus-free syngeneic tumor cells (29,43,50,70,71).

Tumor specific transplantation antigens are known to be distinct from other virus-specific neoantigens found in DNA virus transformed cells including the tumor (T) antigen(s) located in the nuclei (12,93)
and cytoplasm (18) and the surface (S) antigen(s) of SV-40 transformed cells (127). It has been suggested that the S antigen is of host-cell origin (126).

In addition to transplantation rejection, a second in vivo test has been described for detection of tumor associated antigens (137). This test commonly known as the Winn test or tumor cell neutralization test is performed by mixing in vitro tumor cells with lymphoid cells from specifically sensitized donors. These mixtures are injected into appropriate syngeneic recipients and the animals are observed for tumor development. Inhibition or delay in tumor development is interpreted as an indication of specific cell-mediated immunity. Using the tumor cell neutralization test, Tevethia et al. (125) demonstrated that spleen cells from mice immune to SV-40 TSTA elicited specific tumor cell neutralization. Furthermore, immune splenic lymphocytes responsible for neutralization were shown to be thymus dependent (T) lymphocytes since the neutralizing activity of the lymphocytes was specifically abrogated with antibodies directed against membrane markers (theta antigens) present on (T) lymphocytes.

Other Surface Antigens on Transformed Cells (Embryonic Antigens)

Coggin et al. (23,24) reported that hamsters immunized with 10 day old hamster or mouse embryo cells developed cytostatic antibodies which temporarily stopped the replication of SV-40 transformed cells. In addition, immunization of male hamsters with irradiated embryo cells produced tumor immunity to challenge with either SV-40 or adenovirus 31 transformed cells. Ting et al. (129) studied SV-40 and polyoma induced tumors in mice and found two separate neoantigens at the cell surface of
each tumor. By differential absorption of antibody from syngeneic antisera to fetal or tumor cells, it was found that the TSTA and embryonic antigens were different. The origin of TSTA has not been completely resolved. However, neoplasia induced by DNA viruses and permissive cells infected with these viruses express TSTA which are specific for the transforming virus indicating an intimate causal relationship of virus infection and antigen expression.

Biochemical Composition of TSTA

Recently, Drapkin et al. (32) described the isolation of a papain soluble SV-40 TSTA in a syngeneic mouse system. Immunization with a crude membrane preparation, and a soluble, chromatographed fraction of this preparation elicited specific tumor immunity by the tumor rejection assay. The active soluble fraction of SV-40 TSTA had an elution profile with sephadex G-150 chromatography similar to that found with mouse H-2 alloantigens (120) and a soluble TSTA isolated from a RNA virus-transformed mouse line (75). The molecular weight for each antigen was estimated at 50,000 daltons.

Both RNA and DNA virus transformed cells have been shown to contain cell surface glycoproteins which differ from untransformed cells (134). Pronase digests of trypsinates or cell membrane preparations of \( ^3\)H-fucose labelled transformed cells and \( ^{14}\)C-fucose labelled control cells differ in elution profile on Sephadex G-50. Transformed cell membranes contained a larger, earlier eluting material than similarly prepared control cell material. Neuraminidase treatment of transformed cell pronase digests removes the early eluting material. The transformed cells were found to contain a higher level of sialyl transferase
than control cells and more sialic acid residues in their cell membrane polysaccharides. The sugar residues of the polysaccharides have not been determined.

Investigators have reported differences in membrane glycolipids between transformed and control cells (14); however, other investigators have not detected such differences (134). Although it is apparent that the TSTA of transformed cells is a surface membrane neoantigen, the specific biochemical makeup of the TSTA has not been determined.

State of the Viral Genome in Transformed Cells

Non-permissive cells transformed by DNA viruses do not produce infectious virus or structural proteins of the virion; however, the persistence of the viral genome in these cells is evident by the expression of virus-specific neoantigens. These antigens are also expressed early in permissive cells infected with either polyoma virus or SV-40 (44,66, 132). It has now been demonstrated, using hybridization techniques with SV-40 specific and polyoma specific RNA, that specific viral DNA sequences are associated with the chromosomal DNA of the transformed cells (136,138). In addition, infectious virus has been recovered from SV-40 transformed cells by co-cultivation with permissive cells or by fusion of transformed cells and permissive cells with an irradiated paramyxovirus.

Lymphocyte Transformation Assay for Detection of Tumor Associated Antigens

There is little question that cell-mediated immunity is of prime importance in tumor resistance and suppression of tumor growth in the host. Although the immune response to TSTA can be detected in vivo by
tumor rejection, the semiquantitative nature of the assay and its in-applicability to human studies are major limitations. Carefully devised \textit{in vitro} tests to detect cell-mediated immunity to TSTA are now available. Most of the techniques currently used were originally developed to study cell-mediated immunity (CMI) to histocompatibility antigens. The assays can be grouped in three general categories: (1) assays based on the ability of sensitized lymphocytes to lyse target cells (55,122, 130), (2) assays detecting soluble biological factors released by lymphocytes after contact with antigen (11,28), and (3) assays involving lymphocyte transformation by antigen (8). Using these \textit{in vitro} assays, specific tumor associated antigens (TAA) have been detected in animal tumors induced by chemicals and viruses (13,23,52,56,57,76,86,97) and also in human neoplasia (48,61,80,118,131).

The lymphocyte transformation assay offers some advantages over other \textit{in vitro} assays of CMI in that soluble or whole cell antigens can be used as stimulators of transformation and the reactants can be readily quantitated. However, there are many variables to consider in the lymphocyte transformation assay. These include purity of lymphocytes, variations in media, isotopic labeling, and lymphocyte-antigen ratios. Some of these variables have been studied and methods are available for their control. Recently Hartzman \textit{et al.} (53,54) described a multiple automated sample harvestor (MASH) for harvesting human mixed lymphocyte cultures from microculture plates. Several investigators using the MASH have reported that this method greatly reduces variability, while increasing productivity and reproducibility of the lymphocyte blastogenesis assay.
The uptake of \(^3\)H-thymidine by lymphocytes in response to antigen stimulus has been shown to be a reliable and quantitative means to assay lymphocyte transformation. Shons et al. (108) reported on variables encountered with \(^3\)H-thymidine labeling in a micromixed leukocyte culture technique. They found that \(^3\)H-thymidine was rapidly taken up in the first 8 hours, then leveled off between 8-24 hours. It was also shown that pulsing with \(^3\)H-thymidine of high specific activity (17.3 C/mM) for a period of 18-24 hours gave optimum quantitative and qualitative discrimination.

The most frequently used method to purify lymphocytes is the Ficoll-Hypaque gradient technique (53) reports have been favorable supporting purity obtained using this technique. Lymphocyte purity greater than 90% has been the usual finding (53,76).

Several different media have been used in lymphocyte transformation assays, but RPMI-1640 medium has consistently been shown to be satisfactory for lymphocyte culture. The variables of lymphocyte-antigen ratios, length of culture, and serum supplements differ depending on the species of lymphocytes and assay conditions. These variables should be considered and standardized for meaningful interpretation of any lymphocyte transformation assay.

The lymphocyte transformation assay has been applied to the study of human neoplasia as a reliable means for detecting TAA. Stjernsward et al. (118) reported on lymphocyte transformation response of patients with a variety of different active cancers. He applied techniques originally described by Bach and Voynow (8) in the one-way mixed leukocyte culture. DNA synthesis of neoplastic or non-neoplastic
cells was blocked with mitomycin-C treatment, and the cells were used as antigens in the lymphocyte transformation assay. Lymphocyte transformation was quantitated by uptake of $^3$H-thymidine among test and control cultures. Positive blastogenic responses were found in 33% of all patients with active cancer. The reactivity of lymphocytes to tumor cells was dose dependent and specific for malignant cells as opposed to control non-malignant cells from the same donor. Lymphocyte blastogenesis could be blocked by prior incubation of tumor cells with autochthonous serum. In some instances, tumor cells would stimulate if serum blocking factors were eluted at pH 3.1, thus supporting one proposed mechanism of in vivo tumor enhancement (57). Other investigators (80) using mitomycin-C treated tumor cells have confirmed the findings of Stjernsward. Tumor associated antigens on human tumors have also been detected using soluble membrane extracts. Guttermann et al. (48) demonstrated that human leukemic tumor antigens solubilized with three molar potassium chloride stimulated autologous lymphocytes to undergo a specific blastogenic response. Their data suggests that this in vitro responsiveness of lymphocytes represents specific tumor immunity.

Soluble tumor antigens have also been detected in non-lymphoid human tumors by the lymphocyte transformation assay (79).

In human studies, a question has been raised as to whether the lymphocyte response represents a primary reaction to tumor antigen or a secondary response of memory lymphocytes. In lower animals, studies in syngeneic tumor models have convincingly shown the reaction to be a secondary response of lymphocytes in recognition of homologous antigen in vitro.
Poon and Cauchi (97) studied in vitro spleen cell transformation in a syngeneic Ehrlich ascites carcinoma (EAC) model in BALB/c mice. Spleen cell transformation was assayed by the incorporation of $^3$H-thymidine into the cells. Normal and tumor immune spleen cells underwent blastogenesis in response to stimulation with mitomycin-C treated EAC cells. However, immune spleen cells responded earlier and to a greater degree than normal spleen cells. The response was dose-dependent; optimum stimulation occurred on days 3 to 4 with $2 \times 10^6$ spleen cells and $5 \times 10^4$ tumor cells for both normal and immune spleen cells. In addition, normal spleen cells could be sensitized in vitro to membrane extracts of EAC cells. This response was also dose-dependent and maximal stimulation occurred on days 3 and 4.

Recently, Colgrove and Shifrine (25) demonstrated antigenicity of a methylcholanthrene-induced mammary carcinoma in syngeneic BALB/c mice. Spleen cells from mice with no prior exposure to tumor cells were stimulated with syngeneic mitomycin-C treated tumor cells to incorporate increased amounts of $^{14}$C-thymidine. The response was variable; maximum incorporation of isotope occurred after 5-7 days incubation of spleen cells with tumor cells. Stimulation ratios varied from 2.35 to 3.10. The stimulation of unimmunized spleen cells with tumor cells in this syngeneic system was interpreted as detection of tumor associated antigens.

Littman et al. (76) studied cell-mediated immunity to tumor-specific antigens in syngeneic guinea pigs with transplantable ascites variants of diethylnitrosamine-induced hepatomas, designated line 1 and line 10. Immunity was measured by macrophage migration inhibition (MIF),
lymphocyte transformation, and delayed cutaneous hypersensitivity (DCH). Immunity was compared in tumor-bearing animals and animals immunized to the tumors by multiple intradermal injections. Cell-mediated immunity to soluble 3M KCL tumor cell extract was found for tumor-bearing and immune guinea pigs by the MIF assay. The response was greater with immune animals. Delayed cutaneous hypersensitivity was not detected in animals with tumors. However, immunized animals gave positive reactions. Soluble antigen and mitomycin-C treated tumor cells were compared for antigenicity in the lymphocyte transformation assay. Both antigens stimulated blastogenesis equally well. The reaction was dose-dependent and specific; line 10 tumor immune lymphocytes were not stimulated with line 1 tumor cells and vice versa. Maximal blastogenesis occurred after 3 days incubation of lymphocytes, after which the response decreased. Normal lymphocytes were not stimulated with antigen even after 5 days incubation. The lymphocyte transformation reactivity was similar with that found for MIF in that immune animals were more responsive. Although DCH could not be detected in tumor-bearing animals, lymphocytes from these animals were responsive to PHA stimulation indicating that the animals were not immunosuppressed.

Thus, studies in syngeneic animal models have clearly demonstrated the utility of the lymphocyte transformation assay for specific detection of TAA. This assay has potential for investigations of etiology in certain human neoplasia by comparative stimulation with antigens found in known virus induced tumor models and antigens found on human tumors.
The Role of Herpesviruses in Human Malignancies

Among the human DNA viruses, the herpesviruses are considered to be prime candidates in the etiology of certain human malignancies. Herpesviruses are a family of DNA containing viruses that have similar chemical, physical, and biological properties (68). Several herpesviruses of lower animals are known to be oncogenic. Herpesviruses have been shown to cause neoplasia in frogs (37,77,84,115), chickens (Marek's disease) (21,22), guinea pigs (65,91), rabbits (62), and subhuman primates (81,82,83,139). Research with Marek's disease of chickens has led to an attenuated virus vaccine which prevents the expression of cancer due to a herpesvirus found naturally in chickens (22).

For human herpesviruses, it appears likely that Epstein-Barr virus (EBV) is a tumor virus. The virus was first isolated from cultured cells of Burkitt's lymphoma (36), and it was subsequently demonstrated that cells of the tumor contained virus-specific DNA (45,92,140). However, the virus has not been recovered from the tumor itself. Furthermore, non-virus producing cell lines derived from Burkitt's lymphoma can be chemically induced to synthesize virus particles (41,51). Henle et al. (58) found higher antibody titers to EBV antigens in the sera of patients with Burkitt's lymphoma than in sera from matched controls. In addition, higher titers to EBV early antigen were found in Burkitt's patients (60). The EBV directed tumor membrane antigen is apparently also found in the viral envelope (95,110). Human lymphocytes can be transformed in vitro by the addition of EBV (42) and malignant lymphoma has been induced in marmosets following inoculation of EBV (31,109). These findings are consistent with the belief that EBV is indeed
a tumor virus. Further, it is a reasonable certainty that EBV is the etiologic agent of infectious mononucleosis (47,59,96). Viruses resembling EBV have been found in the upper respiratory tract of patients with acute infectious mononucleosis (47,96). Recently, a non-human primate model of Herpesvirus samiri (HVS) was described for studying as an analogy the role of EBV in human neoplasia (1). The characteristics of HVS induced neoplasia are very similar to lymphomas believed to be of EBV etiology in humans.

Until recently, herpes simplex virus was considered to be an agent causing acute, localized cytolytic infections in man. The primary infections are usually self limiting; however, herpes simplex virus is well known for establishing latent infections which can persist for the life of the host. Nahmias and Dowdle (88) were able to demonstrate by serological techniques that this virus could be divided into two groups, designated herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). It has been shown that these viruses differ in nucleotide sequences (16,69) and biological properties (88,68). Most strains of HSV-2 have been isolated from the genital tract, while strains of HSV-1 are usually isolated from other body sites. Herpes simplex virus type 2 is now considered to be venereally transmitted (20). Both HSV-1 and HSV-2 cause latent infections. Herpes simplex virus (HSV) has been recovered from spinal and trigeminal ganglia from mice and rabbits latently infected with HSV (116,117). Similar reports of human studies have shown that HSV-2 can be recovered from sacral ganglia (9) and HSV-1 from trigeminal ganglia (10). These studies suggest that neural tissue can harbor HSV latently in a non-productive state between clinical episodes. The
intimate association of HSV with host cells in itself has prompted many investigators to suspect these viruses as oncogenic agents. However, during the past 6 years considerable evidence favoring the association of HSV-2 in human cervical carcinoma has been found. Seroepidemiological studies have demonstrated a higher incidence of antibodies to HSV-2 in women with cervical carcinoma as compared to control groups (2,4,5, 90,103,114). Aurelian et al. (7) isolated a virus from cultured cells of cervical carcinoma which was antigenically and biologically identical to HSV-2. In addition, HSV-2 antigens were detected by immunofluorescence in exfoliated anaplastic cells from cervical carcinoma patients (107). Virus has not been found in uncultured cervical tumors; however, a fragment of HSV-2 DNA was recently detected in a human cervical carcinoma (39).

Tarro and Sabin (123) described a non-virion antigen of HSV-1 produced 3 hours after infection of guinea pig kidney cells, and 12-24 hours after infection of rabbit or Hep-2 cells. Antibodies to this non-virion antigen could be detected in animal sera after absorption with virion antigen. These investigators (124) subsequently reported that HSV-1 non-virion antigen prepared in guinea pig kidney cells was apparently distinct from HSV-2 non-virion antigen prepared in identical manner. Although HSV-1 virion immune guinea pig serum cross reacted with both HSV-1 and HSV-2 virion antigen, HSV-1 non-virion immune guinea pig serum was specific. However, HSV-2 non-virion immune serum cross reacted with HSV-1 non-virion antigen. This is reasonable since HSV-1 and HSV-2 have been reported to share 40% DNA base pairs by homology studies (69).
Hollingshead et al. (63) reported that hyper-immune guinea pig serum to HSV-2 virion antigen reacted with a soluble membrane antigen isolated from vaginal, vulvar, and cervical cancers of humans. In addition, they found that 95% of sera from cervical cancer patients reacted in complement-fixation (CF) tests with soluble membrane antigens from genital cancer cells. However, 24% of matched control sera also reacted. The antigens found in genital cancer cells may be analogous to the non-virion antigens described by Tarro and Sabin (124).

Recently, it was reported that herpes simplex virus types 1 and 2 produce early antigens in permissive cells that cross react serologically with antigens found in squamous cell cancers of humans. Hollingshead and Tarro (64) demonstrated with guinea pig hyper-immune serum that soluble "non-virion" antigens from HSV-1 or HSV-2 infected HE-p-2 cells cross reacted with soluble membrane antigen from human lip and cervical carcinomas, but not with soluble extracts of normal vaginal tissue or intestinal carcinoma.

Aurelian et al. (6) reported that patients with cervical carcinoma have complement fixing antibody to HSV-2 induced non-virion antigens prepared in HE-p-2 cells; they designated the antigen Ag-4. This CF antibody is apparently not neutralizing antibody directed against the virion. The antibody is tumor-specific, in that 90% of patients with invasive carcinoma had antibody which reacted with Ag-4 while only 10% of matched control were positive. Patients which were treated for cervical cancer and cured did not have antibody to Ag-4 but did have antibody to HSV-2 virion antigens. Recurrent neoplastic disease was associated with antibody to Ag-4. The Ag-4 antigen does
not appear to be a derepressed cellular antigen such as the carcino-
embryonic (CEA) antigen reported by Gold, et al. (46). Sera from patients
with adenocarcinoma of the stomach and pancreas did not react to Ag-4;
however, patients with adenocarcinoma are known to produce circulating
antibodies to CEA.

Although herpes simplex viruses have been associated with human
cancers by serological means, it has been difficult to directly demon-
strate their oncogenicity in experimental animals. However, recently
the transforming potential of these viruses has been demonstrated.
Munyon, et al. (85) showed that mouse L cells could be transformed with
UV-irradiated HSV-1. With similar methods, Duff and Rapp (33,35) were
able to transform hamster embryo fibroblasts first with HSV-2 and later
with HSV-1. Kutinova et al. (72) have also succeeded in similar trans-
formation attempts with UV-irradiated HSV-2. Darai and Munk (27) re-
cently reported success in transforming human embryonic lung cultures
with HSV-2, by maintaining the cultures at a non-permissive temperature,
42°C. Finally, Rapp et al. (101) reported photodynamically inactivated
HSV-1 and HSV-2 also would transform hamster embryo fibroblasts. It is
now apparent that herpes simplex viruses do have oncogenic potential.
The lytic phase can be uncoupled from the transforming potential by means
of physical treatment. Both intact and defective (UV-irradiated) herpes-
viruses have transformed cells. These findings have provided for the
first time a practical animal model for investigating these viruses as
etiologic agents of neoplasia.
Herpes simplex virus type 2 transformed LSH hamster embryo fibroblasts have been extensively studied by Duff and Rapp (33,34,98, 99,100). These transformed cells produced fibrosarcomas when injected into syngeneic LSH hamsters. By indirect immunofluorescence, the tumor cells were reported to contain HSV specific cytoplasmic and surface antigens. Incomplete HSV particles have been detected in a few cells by electron microscopy and herpes simplex virus specific neutralizing antibody has been found in sera from tumor-bearing hamsters. However, infectious HSV-2 has not been recovered from the transformed cells using either co-cultivation with permissive cells or fusion techniques. The transformed cells were shown to be free of oncornaviruses by a variety of established techniques for detection of such RNA tumor viruses. Recently, using hybridization techniques with RNA from HSV-2 transformed cells and DNA from the homologous virus, Collard et al. (26) demonstrated that approximately 10% of the HSV-2 genome was transcribed in HSV-2 transformed cells. Finally, attempts to detect tumor specific transplantation antigens on the HSV-2 tumor cells with established in vivo methods have not been successful. There is need for further immunological characterization of the HSV-2 tumor system particularly as a possible model for etiologic studies of certain human malignancies.

We have investigated cell-mediated immunity to HSV-2 tumor antigens in syngeneic LSH hamsters with an in vitro lymphocyte transformation assay and an in vivo tumor cell neutralization test. In order to determine the utility of the hamster system and for purposes of standardization, we first studied the response of splenic and blood lymphocytes to a non-specific stimulator of blastogenesis, phytohemagglutinin (PHA).
We applied the results obtained with PHA to investigate the response of splenic lymphocytes from hamsters immune to HSV-2 tumor; cell-free tumor antigens and mitomycin-C treated tumor cells were used as stimulants. The specificity of the response was investigated by differential stimulation of separate lymphocyte cultures with homologous HSV-2 tumor antigens, heterologous SV-40 tumor antigens and normal antigens from syngeneic tissue. The dose relationship and the temporal response to each antigen was determined. In addition, splenic lymphocytes from hamsters with no prior experience to tumor antigen were used to study in vitro sensitization with mitomycin-C treated HSV-2 tumor cells.
MATERIALS AND METHODS

Animals

Weanling inbred strain LSH male Syrian hamsters were obtained from the Lakeview Hamster Colony, Newfield, N.J. Animals for each experiment were matched according to age. Animals ranged in age from 6 weeks to 3 months.

Tumor

One LSH hamster carrying a tumor of herpes simplex virus type 2 (HSV-2) transformed LSH hamster embryo fibroblasts, 333-8-9 TI, was obtained from Drs. Duff and Rapp of the Milton S. Hershey Medical Center of The Pennsylvania State University. Biologic properties of the tumor have been described (33). Herpes simplex virus type 2 tumor cells were tested in the laboratories of Dr. Norman Somerson and found to be free of mycoplasma species contamination.

Tumor Transplantation

Prior to surgical procedures, animals were asphyxiated with carbon dioxide. Tumor tissue was surgically excised, debrided of fat and necrotic tissue, and washed two times with Earles Minimal Essential Medium (EMEM; Flow Laboratories, Rockville, Maryland). The tissue was finely minced and disaggregated in EMEM by repeated gentle flushing with a 5 ml pipette. The cells were filtered through sterile 2 x 2 gauze
sponges and washed two times. Viable cell counts were determined by trypan blue dye exclusion. Tumor cell transplantation was routinely accomplished by subcutaneous injection of $1 \times 10^6$ viable HSV-2 tumor cells contained in 0.1 ml of EMEM.

**Cell Cultures**

The following cells were used in this study. An established Simian virus-40 (SV-40) induced tumor cell line (BTH) was supplied by Dr. Anthony J. Girardi of the Wistar Institute. This cell line was developed from a tumor induced in newborn Syrian hamsters by the injection of SV-40. The cells are tumorigenic and express SV-40 directed Tumor Specific Transplantation Antigens (TSTA) (personal communication, Anthony J. Girardi). Also a SV-40 transformed mouse cell line (TCMK-1) obtained from the American Type Culture Collection was used. A human cell strain, WI-38, was supplied by Dr. Vincent V. Hamparian. These cells were originally obtained from Dr. Leonard Hayflick. In addition, primary cultures of HSV-2 tumor and hamster lung were initiated. Tissue was processed with methods described above with the exception that the primary tissue for culture was disaggregated by exposure to 0.25% trypsin for 1 hour at 37°C. The cells were filtered, washed, and counted as above. All cell cultures were grown and maintained in rubber stoppered prescription bottles in EMEM supplemented with 10% Fetal Bovine Serum (FBS, Flow Laboratories, Rockville, Md.), 12 ml of 7.5% sodium bicarbonate, 250 units of penicillin, 125 units of polymyxin, 200 μg of streptomycin sulfate, and 25 μg of Chlorotetracycline per ml. This medium was used to grow all cell cultures. Primary cultures were initiated in
3 ounce prescription bottles by seeding with approximately $5 \times 10^6$ cells in 20 ml of EMEM growth medium. For cell passages, the residual trypsin technique was used to remove the cells from the glass; approximately $5 \times 10^6$ cells were transferred in 100 ml of growth medium to 32 ounce prescription bottles. Cell cultures were passed on a weekly schedule.

**Antigens Used to Stimulate Lymphocytes**

**Cell-free antigens.** Cell-free antigens were prepared from either freshly excised tissue or cultured cells. Single cell suspensions were prepared from HSV-2 tumor and normal syngeneic hamster lung tissue without trypsinization as described above. Monolayers of BTH and TCMK-1 cultured cells were washed twice with EMEM without FBS and maintained for 24 hours in 50 ml of this medium prior to use. The medium was decanted and the cells were harvested by scraping into 10 ml of fresh medium, washed twice with EMEM and counted by trypan blue dye exclusion. Freshly excised cells and cultured cells were resuspended separately in 3 ml of RPMI-1640 medium without FBS (Flow Laboratories, Rockville, Md.). The final concentration of cells was approximately $10 \times 10^6$ per ml. The suspensions were frozen at $-80^\circ$C and quickly thawed in a 37°C waterbath; this process was repeated twice. The cell lysate was clarified by centrifugation at 175 g for 25 minutes at room temperature in an International Centrifuge Model K. The supernatant was removed and stored at $-80^\circ$C until used.

**Mitomycin-C treated cells.** Cells treated with mitomycin-C to prevent incorporation of $^3$H-thymidine were also used as antigens. The optimal concentration of mitomycin-C was determined essentially as described by Littman, et al. (76).
Antigens from HSV-2 infected cells. Herpes simplex virus type 2 strain 333 was obtained from Drs. Duff and Rapp (33). This virus was used to productively infect monolayer cultures of WI-38 cells for preparation of cell-free antigens. Thirty-two ounce prescription bottles were seeded with WI-38 cells in EMEM growth medium. After 3 days incubation at 37°C, when cultures were approximately 80% confluent, the medium was removed and each bottle received $1 \times 10^6$ TCID$_{50}$ of HSV-2 strain 333 contained in 1.0 ml of EMEM. After a 45 minute absorption period, 50 ml of EMEM without serum was added to each culture. The cultures were incubated for 24 hours, at which time approximately 25% of the monolayer showed cytopathology. The cultures were washed three times with 25 ml of EMEM and were harvested by scraping into 10 ml of EMEM. The cell suspension was washed by centrifugation at 175 g and the pellet was resuspended in 3 ml of RPMI-1640 diluent. The suspension was frozen at -80°C and quickly thawed in a 37°C waterbath. The freeze-thaw process was repeated twice, and the suspensions were clarified by centrifugation for 25 minutes at 175 g. The supernatant was removed and stored at -80°C until used.

Phytomitogens. Phytohemagglutinin (PHA) was obtained from three commercial sources: PHA-P (Difco Laboratories, Detroit, Michigan), PHA-M (General Biochemicals, Chagrin Falls, Ohio), PHA (Burroughs-Wellcome, Beckenham, England). PHA-P was weighed and distributed in 2000 µg aliquots in 1.0 ml of RPMI-1640 medium without FBS. PHA (Burroughs-Wellcome) was prepared similarly. PHA-M was prepared as recommended by the manufacturer; µl aliquots of this PHA were used. All PHA preparations were
stored at -80°C until used. These PHA preparations were used interchangeably.

**Antigens Used for Immunization of Hamsters**

Prior to use, cultured BTH and TCMK-1 cells were incubated for 24 hours in EMEM without FBS. The monolayers were harvested by scraping into 25 ml of EMEM medium and washed twice by centrifugation at 175 g. Single cell suspensions were prepared by pipette dispersion. The cells were counted for viability by trypan blue dye exclusion and suspended in EMEM without FBS at the appropriate cell concentration. Freshly excised HSV-2 tumor cells were processed as described above for tumor transplantation.

**Immunization of Animals**

Hamsters were immunized in two ways: separate groups of animals received a single subcutaneous injection of various numbers of viable TCMK-1, BTH, or HSV-2 tumor cells contained in 0.1 ml of EMEM without FBS. These animals were assayed for immunity at different time intervals post-inoculation. Other groups of hamsters were immunized to HSV-2 tumor, TCMK-1, or BTH cells by 3 weekly intradermal injections of $3 \times 10^6$ viable cells in 0.1 ml of EMEM without FBS. These animals were sacrificed 5 days after the last injection.

**Preparation of Hamster Lymphocytes**

Immune and normal LSH hamsters were sacrificed by carbon dioxide asphyxiation; spleens were removed aseptically and rinsed with RPMI-1640 medium without FBS. This medium was used for processing all lymphocyte preparations. Single cell suspensions were prepared by gently teasing
the spleens apart with forceps, and repeatedly pipetting the suspensions with a Pasteur pipette. The spleen cell suspensions were filtered through a 60 mesh wire screen to remove connective tissue and passed through a 23 gauge needle to break up any clumps. Blood was obtained by cardiac puncture and mixed with preservative free heparin at 100 units per ml. Both blood and spleen cell preparations were washed twice and suspended separately in 25 ml of medium prior to separation on a Ficoll Hypaque gradient.

The procedure for separating lymphocytes was essentially that described by Hartzman, et al. (54). Briefly, a Ficoll-Hypaque gradient consisting of one part of 34% Hypaque (Wintrop Laboratories, New York, N.Y.) and 2.4 parts of 9% Ficoll (pharmacia) was prepared and sterilized by filtration. The gradient was carefully layered under splenic or blood cells suspended in 25 ml of RPMI-1640 medium in 50 ml glass centrifuge tubes. The tubes were centrifuged at room temperature for 25 minutes at 400 x g. Lymphocytes banding at the top of the gradient were carefully removed with a Pasteur pipette along with some of the gradient and suspending medium. The lymphocytes were washed three times and resuspended. Viable counts were determined by exclusion of trypan blue dye; viability was always greater than 95%. Approximately 10 - 20 x 10^6 lymphocytes were obtained from each spleen, and 1 - 3 x 10^6 lymphocytes per ml of blood.

Lymphocyte Transformation Assay

Lymphocytes were suspended in RPMI-1640 medium containing either 10% FBS or 15% hamster serum, and 8 ml of 7.5% sodium bicarbonate/L, 2mM L-glutamine/ml, 250 units of penicillin/ml, 200 μg streptomycin sulfate/ml,
and 125 units of polymyxin/ml. Lymphocytes and antigen were diluted separately in medium so that the appropriate concentration for each was contained in 0.1 ml. These materials were dispensed separately into wells of microtest II plates (Falcon-Plastics, Oxnard, Calif., Plate 3040) giving a final volume of 0.2 ml/well of the mixtures. All reagents were dispensed with microdroppers (Cooke Engineering). The plates were sealed with adhesive backed film (Falcon Plastics) and incubated for the appropriate time at 37°C. Eighteen hours prior to harvesting, each culture was pulsed with 1 μCi of tritiated thymidine (specific activity 20 Ci/mMole, New England Nuclear) contained in 25 μl of RPMI-1640 medium.

**Harvesting Procedure**

The cultures were harvested onto glass fiber filters (Whatman GF/A) with a multiple automated sample harvester (MASH) as described by Hartzman, *et al.* (53). The filters were washed free of extraneous isotope with 12 saline rinses. The filters were dried in a hot air oven at 80°C for 30 minutes and placed in plastic scintillation vials along with 10 ml of a toluene base cocktail consisting of 4 grams of 2,5-bis-[2-(5-tert-Butylbenzoxazoly1)] - Thiophene (Scintillation Grade) (BBOT; Packard; Downers Grove, Illinois)/liter. The amount of radioactivity incorporated was determined by counting in a Packard Tri-Carb liquid scintillation counter. All samples were counted for sufficient time to allow for less than 2% counting error. Quenching was found to be uniform among the samples; counting efficiency was 42%.
Lymphocyte transformation was determined by the amount of tritiated thymidine incorporated. The blastogenic response was expressed in two ways, either as the mean counts per minute (CPM) of lymphocytes with antigen compared to the mean CPM of lymphocytes without antigen or as a stimulation index. The results were analyzed by Student's t test (135) with an Olivetti Underwood Programma 101.
RESULTS

Characterization of Hamster Lymphocyte Transformation With Phytohemagglutinin (PHA)

Preliminary studies to standardize optimum culture conditions for transformation of hamster lymphocytes were conducted with phytohemagglutinin (PHA), a non-specific mitogen of lymphocyte blastogenesis.

Effects of Varying Cell Concentrations

Splenic lymphocytes in varying numbers (2 x 10^6, 1 x 10^6, 5 x 10^5, and 2.5 x 10^5) were stimulated with 2 μg of PHA. The results presented in Figure 1 indicate that maximal incorporation of \(^{3}H\)-thymidine occurred with 1 x 10^6 splenic lymphocytes. Based on these findings, all subsequent experimentation with PHA was conducted with 1 x 10^6 lymphocytes.

Effect of Varying PHA Concentrations

The results in Figure 2 show the response of splenic lymphocytes to various concentrations of PHA. Maximal incorporation of \(^{3}H\)-thymidine occurred with 2 μl of PHA. Complete inhibition of incorporation of \(^{3}H\)-thymidine occurred at concentrations of 20 μl and above.

Comparative Mitogenic Effects of PHA on Hamster Blood and Splenic Lymphocytes

Peripheral blood and splenic lymphocytes from the same animals were reacted with varying concentrations of PHA. The stimulation curves
Splenic lymphocytes in varying numbers were stimulated with 2 μg of PHA. Cultures were pulsed with 1 μCi of tritiated thymidine for the final 18 hours prior to harvesting at 72 hours. Mean values plus or minus the standard error of the mean are based on quadruplicate determinations for each lymphocyte concentration.
FIGURE 1

<table>
<thead>
<tr>
<th>STATISTICAL COMPARISON</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Student's T Test</strong></td>
</tr>
<tr>
<td>1x10^6 vs 2.5x10^5</td>
</tr>
<tr>
<td>1x10^6 vs 5x10^5</td>
</tr>
<tr>
<td>1x10^6 vs 2x10^6</td>
</tr>
</tbody>
</table>

NORMAL SPLENIC LYMPHOCYTES x 10^5 / CULTURE

CPM x 10^-3
Varying concentrations of PHA were added to a constant number of splenic lymphocytes (1 x 10^6). Cultures were pulsed with 1 μCi of tritiated thymidine for the final 18 hours prior to harvesting at 72 hours. The results are based on quadruplicate determinations. Stimulation Index S.I. = (CPM) lymphocytes with PHA/(CPM) lymphocytes without PHA.
FIGURE 2

<table>
<thead>
<tr>
<th>PHA</th>
<th>P-Value</th>
<th>S.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>&lt; .05</td>
<td>.82</td>
</tr>
<tr>
<td>0.5</td>
<td>&gt; .05</td>
<td>1.22</td>
</tr>
<tr>
<td>1</td>
<td>&lt; .001</td>
<td>1.54</td>
</tr>
<tr>
<td>2</td>
<td>&lt; .001</td>
<td>1.84</td>
</tr>
<tr>
<td>4</td>
<td>&lt; .005</td>
<td>1.67</td>
</tr>
<tr>
<td>8</td>
<td>&lt; .001</td>
<td>1.32</td>
</tr>
<tr>
<td>20</td>
<td>&lt; .05</td>
<td>.82</td>
</tr>
<tr>
<td>40</td>
<td>&lt; .001</td>
<td>.39</td>
</tr>
<tr>
<td>80</td>
<td>&lt; .001</td>
<td>.21</td>
</tr>
</tbody>
</table>

CPM x 10^-2

PHA CONCENTRATION (µl/0.2 ml)
obtained are shown in Figure 3. The pattern of reactivity was similar. Maximal isotope incorporation for both occurred with 2 μl of PHA. The overall incorporation of 3H-thymidine was lower for the blood lymphocytes. However, the stimulation index (SI) was consistently greater for most concentrations of PHA.

Reactivity of Hamster Splenic Lymphocytes to PHA in Media Containing Either Fetal Bovine Serum (FBS) or Autologous Hamster Serum

Preliminary experiments with PHA-P (Difco) in RPMI-1640 medium supplemented with 10% FBS indicated that normal splenic lymphocytes did not respond in the usual manner to this lot of PHA. The stimulation observed for all concentrations of PHA-P, 0.5 μg - 8.0 μg, either did not differ significantly from control lymphocytes or showed significant inhibition. However, when normal splenic lymphocytes were incubated with PHA-P in medium supplemented with 15% autologous hamster serum, significant stimulation occurred, see Figure 4. The results illustrate that the response of normal hamster splenic lymphocytes to PHA-P was influenced by the serum. Lymphocytes in medium containing 15% hamster serum showed significant stimulation with 2 μg of PHA-P while lymphocytes in medium containing 10% FBS were inhibited.

Effect of PHA on Splenic Lymphocytes from Hamsters Bearing Herpes Simplex Virus Type 2 Tumor

This experiment was done to determine if lymphocytes from hamsters with large HSV-2 tumors could be stimulated by PHA. Lymphocytes were obtained from hamsters which had received a subcutaneous inoculation of
FIGURE 3

CHARACTERIZATION OF HAMSTER LYMPHOCYTE TRANSFORMATION WITH PHA - COMPARATIVE EFFECT OF VARYING PHA CONCENTRATIONS ON BLOOD AND SPLENIC LYMPHOCYTES

Lymphocytes at a constant concentration of $1 \times 10^6$ cells per culture were exposed to varying dilutions of PHA. The cultures were pulsed with 1 μCi of tritiated thymidine for the final 18 hours prior to harvesting at 72 hours. The results are based on quadruplicate determinations. S.I. = (CPM) lymphocytes with PHA/(CPM) lymphocytes without PHA; • = $1 \times 10^6$ splenic lymphocytes; • = $1 \times 10^6$ blood lymphocytes.
FIGURE 3

<table>
<thead>
<tr>
<th>PHA µl/0.2</th>
<th>LYMPHOCYTES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPLenic</td>
<td>BLOOD</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>S.I.</td>
</tr>
<tr>
<td>0.5</td>
<td>&gt;.05</td>
<td>1.22</td>
</tr>
<tr>
<td>1.0</td>
<td>&lt;.001</td>
<td>1.54</td>
</tr>
<tr>
<td>2.0</td>
<td>&lt;.001</td>
<td>1.84</td>
</tr>
<tr>
<td>4.0</td>
<td>&lt;.005</td>
<td>1.67</td>
</tr>
<tr>
<td>8.0</td>
<td>&lt;.001</td>
<td>1.32</td>
</tr>
<tr>
<td>20.0</td>
<td>&lt;.05</td>
<td>0.82</td>
</tr>
</tbody>
</table>

CPM x 10^{-3}

PHA CONCENTRATION
(µl/0.2 ml)
Splenic lymphocytes obtained from a pool of four hamster spleens were dispensed at $1 \times 10^6$ cells per culture. These lymphocytes were exposed to varying concentrations of PHA-P in medium supplemented with either 10% FBS or 15% HS. The cultures were pulsed with 1 μCi of tritiated thymidine for the final 18 hours prior to harvesting at 72 hours. All cultures were done in quadruplicate. $\bullet\bullet\bullet\bullet = \text{medium} + 10\% \text{ FBS}; \bullet\bullet\bullet\bullet = \text{medium} + 15\% \text{ HS}$. S.I. = (CPM) lymphocytes with PHA/(CPM) lymphocytes without PHA.
**FIGURE 4**

**PHA-P CONCENTRATION (µg/0.2 ml)**

<table>
<thead>
<tr>
<th>PHA-P</th>
<th>15% HS P-Value</th>
<th>S.I.</th>
<th>10% FBS P-Value</th>
<th>S.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>&lt;.005</td>
<td>3.15</td>
<td>&gt;.05</td>
<td>1.06</td>
</tr>
<tr>
<td>2.0</td>
<td>&lt;.005</td>
<td>4.07</td>
<td>&lt;.002</td>
<td>0.43</td>
</tr>
<tr>
<td>20.0</td>
<td>&gt;.05</td>
<td>1.56</td>
<td>&lt;.001</td>
<td>0.16</td>
</tr>
</tbody>
</table>
of $1 \times 10^6$ tumor cells 35 days earlier. Tumors were approximately $2 \times 2$ cm in diameter. The results in Figure 5 indicate that lymphocytes from tumor-bearing hamsters are reactive to PHA. However, in the unstimulated control, the incorporation of $^3$H-thymidine appeared to be greater (7400 CPM) than in normal lymphocytes (1100 CPM, Figure 2). Furthermore, the reactivity differed from results obtained with normal lymphocytes in that maximal stimulation occurred at 4 μl and appeared to plateau.

**Temporal Relationship of Tumor Cell Input and Tumor Development**

Hamsters in groups of five were inoculated subcutaneously with varying numbers of viable HSV-2 tumor cells suspended in 0.1 ml of medium without FBS. The animals were observed for 17 days. Tumors were palpable as early as day 10 in animals receiving tumor brei, and as late as day 17 in animals receiving $1 \times 10^4$ cells (Figure 6).

**Tumor Cell Neutralization by Immune Spenic Lymphocytes**

Splenic lymphocytes from hamsters inoculated subcutaneously 15 days earlier with HSV-2 tumor brei were tested for ability to prevent or delay tumor growth. Splenic lymphocytes ($5 \times 10^7$) from tumor bearing animals and $5 \times 10^5$ HSV-2 tumor cells were mixed in a total volume of 1 ml of EMEM and incubated for 45 minutes in a 37°C waterbath. The mixture was washed twice by low-speed configuration, 175 g, and resuspended in 0.5 ml of EMEM. Normal lymphocytes were incubated with tumor cells in an identical manner. Animals in groups of five were inoculated subcutaneously with 0.1 ml of the various mixtures or with $1 \times 10^5$ tumor cells alone. The results are shown in Figure 7. All animals receiving tumor cells only or tumor cells mixed with normal lymphocytes developed tumors
Splenic lymphocytes were obtained from tumor bearing hamsters 35 days post inoculation with $1 \times 10^6$ tumor cells. Lymphocytes at a concentration of $1 \times 10^6$ cells per culture were stimulated with varying concentrations of PHA. The cultures were incubated for 72 hours at $37^\circ$C. Eighteen hours prior to harvesting, the cultures were pulsed with 1 $\mu$Ci of tritiated thymidine. The results are based on quadruplicate determination for each PHA concentration. S.I. = (CPM) lymphocyte with PHA/ (CPM) lymphocytes without PHA.
FIGURE 5

<table>
<thead>
<tr>
<th>PHA μl</th>
<th>P-Value</th>
<th>S.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>&lt;.05</td>
<td>1.23</td>
</tr>
<tr>
<td>2.0</td>
<td>&lt;.001</td>
<td>1.66</td>
</tr>
<tr>
<td>4.0</td>
<td>&lt;.001</td>
<td>2.23</td>
</tr>
<tr>
<td>8.0</td>
<td>&lt;.001</td>
<td>2.11</td>
</tr>
</tbody>
</table>

CPM x 10^-3

PHA CONCENTRATION
(μl/0.2ml)
Hamsters in groups of five were injected subcutaneously with varying numbers of viable tumor cells suspended in 0.1 ml of EMEM without FBS. Concentration of tumor cells: ■—■ = tumor brei (40% cells by volume), ■—■ = 1 x 10^6; ▲—▲ = 2 x 10^5, □—□ = 2 x 10^4, ○—○ = 1 x 10^4.
FIGURE 6

Percent Hamsters with Tumors

Day of Tumor Appearance

0 6 8 10 12 14 16 18
FIGURE 7

SUPPRESSION OF TUMOR DEVELOPMENT IN HAMSTERS INOCULATED WITH A MIXTURE OF IMMUNE HAMSTER LYMPHOCYTES AND HSV-2 TUMOR CELLS

$5 \times 10^7$ splenic lymphocytes from either normal or tumor bearing hamsters were mixed with $5 \times 10^5$ HSV-2 tumor cells. The mixtures were incubated for 45 minutes at $37^\circ C$, washed by centrifugation at 175 g and resuspended in 0.5 ml of medium. Animals in groups of five were inoculated subcutaneously with 0.1 ml of these mixtures. Tumor cells alone, treated similarly, were inoculated into a separate group of animals. △—△ = $1 \times 10^5$ tumor cells alone. □—□ = $1 \times 10^5$ tumor cells and $1 \times 10^7$ normal lymphocytes, △—△ = $1 \times 10^5$ tumor cells and $1 \times 10^7$ immune lymphocytes.
FIGURE 7

PERCENT HAMSTERS WITH TUMORS

DAY OF TUMOR APPEARANCE
by 18 days. Hamsters inoculated with a mixture of immune lymphocytes and tumor cells showed a 2-week delay in tumor development. However, all of these animals developed tumors by day 32. The results indicate that immune lymphocytes inhibited HSV-2 tumor cells when inoculated together at a ratio of 100:1.

**Stimulation of Lymphocytes From HSV-2 Tumor Bearing Hamsters by Cell-Free Tumor Antigens**

To study the optimum response of tumor immune lymphocytes to HSV-2 tumor cell-free antigens, varying dilutions of antigen were incubated with $1 \times 10^6$ immune lymphocytes. Incorporation of $^3$H-thymidine was measured after 72 hours incubation. As seen in Figure 8, maximal stimulation was induced by the 1:100 dilution of antigen; there was no stimulation with undiluted antigen. A subsequent experiment using a 1:1000 dilution of antigen did not increase $^3$H-thymidine uptake. The results paralleled the findings with PHA.

**Comparison of Immune Splenic and Peripheral Blood Lymphocytes from the Same Animals for Reactivity to HSV-2 Cell-Free Tumor Antigens**

Suspensions of splenic and peripheral blood lymphocytes from immune hamsters were prepared, and $1 \times 10^6$ lymphocytes were incubated with a 1:100 dilution of HSV-2 tumor cell-free antigen. Tritiated thymidine incorporation was measured after 72 hours incubation. See Figure 9. Splenic lymphocytes were stimulated significantly, Student's t test $p < 0.005$, while peripheral blood lymphocytes were not, $p > 0.05$. 
Varying dilutions of HSV-2 tumor cell-free antigen were incubated with $1 \times 10^6$ lymphocytes obtained from hamsters inoculated 15 days earlier with $1 \times 10^6$ tumor cells. The cultures were incubated for 72 hours. Eighteen hours prior to harvesting, the cultures were pulsed with 1 μCi of tritiated thymidine. The results are based on quadruplicate cultures for each antigen dilution. Stimulation Index = (CPM) lymphocytes plus antigen/(CPM) lymphocyte alone.
FIGURE 9

COMPARISON OF IMMUNE SPLENIC AND PERIPHERAL BLOOD LYMPHOCYTES FROM THE SAME ANIMAL FOR REACTIVITY TO HSV-2 CELL-FREE TUMOR ANTIGEN

Splenic and peripheral blood lymphocytes were obtained from hamsters injected subcutaneously with $1 \times 10^6$ tumor cells 15 days earlier. These lymphocytes were incubated at $1 \times 10^6$ cells per culture with a 1:100 dilution of cell-free HSV-2 tumor antigen. The test was incubated for 72 hours at 37°C. Eighteen hours prior to harvesting, the cultures were pulsed with 1 µCi of tritiated thymidine. The results are based on quadruplicate cultures. Stimulation Index = (CPM) lymphocytes plus antigen/(CPM) lymphocytes alone.
HSV-2 TUMOR CELL-FREE ANTIGEN (1:100)

STIMULATION INDEX

- Splenic Lymphocytes
- Blood Lymphocytes

FIGURE 9
Response of HSV-2 Tumor Immune Lymphocytes to Cell-Free HSV-2 Tumor Antigen According to Length of Incubation

The optimal time for maximal stimulation of HSV-2 tumor immune lymphocytes was determined by incubating $5 \times 10^5$ immune lymphocytes with a 1:100 dilution of HSV-2 tumor cell-free antigen. Incorporation of $^{3}$H-thymidine was assayed from 72-144 hours. Figure 10 shows that significant stimulation, Student's t test $p<0.001$, occurred at 72 hours, and $p<0.05$ at 96 hours. Although not shown, subsequent experiments using $1 \times 10^6$ lymphocytes indicated that maximal stimulation occurred as late as 120 hours. However, significant stimulation always occurred as early as 72 hours.

Stimulation of HSV-2 Tumor Immune Splenic Lymphocytes With Cell-Free Antigen From HSV-2 Infected Human Embryonic Lung (WI-38) Cell Cultures

The following experiment was done to determine if immune lymphocytes could recognize HSV-2 virus induced antigens obtained from WI-38 infected cell cultures. The results of incubating $1 \times 10^6$ tumor immune lymphocytes with varying dilutions of HSV-2 WI-38 cell-free antigen are shown in Figure 11. Maximal incorporation of $^{3}$H-thymidine occurred after 72 hours incubation with a dilution of 1:100 of cell-free antigen. The results were similar to those obtained with tumor immune splenic lymphocytes and syngeneic HSV-2 tumor cell-free antigen.

Specific Transformation of Immune Splenic Lymphocytes With Cell-Free Tumor Antigens

The following experiment was done to determine if HSV-2 tumor immune lymphocytes were specific in antigen recognition. Separate groups of hamsters were immunized to HSV-2 tumor, mouse SV-40 (TCMK-1), and
FIGURE 10

RESPONSE OF HSV-2 TUMOR IMMUNE SPLENIC
LYMPHOCYTES TO CELL-FREE HSV-2
TUMOR ANTIGEN ACCORDING TO LENGTH OF INCUBATION

$5 \times 10^5$ splenic lymphocytes from hamsters injected 15 days earlier with
$1 \times 10^6$ HSV-2 tumor cells were incubated for varying time intervals with
a 1:100 dilution of cell free HSV-2 tumor antigen. For each time interval,
18 hours prior to harvesting, 1 $\mu$Ci of tritiated thymidine was added
to each culture. The results are based on quadruplicate determinations
for each time interval. Stimulation Index = (CPM) lymphocytes plus
antigen/(CPM) lymphocytes alone.
FIGURE 11

STIMULATION OF IMMUNE SPLENIC LYMPHOCYTES FROM
HSV-2 TUMOR BEARING ANIMALS BY CELL-FREE ANTIGEN
FROM HSV-2 INFECTED WI-38 CELL CULTURES

Splenic lymphocytes were obtained from hamsters injected subcutaneously
15 days earlier with $1 \times 10^6$ HSV-2 tumor cells in 0.1 ml of EMEM without
FBS. $1 \times 10^6$ lymphocytes were incubated for 72 hours with varying dilu­
tions of HSV-2 WI-38 cell-free antigen. Eighteen hours prior to har­
vesting, the cultures were pulsed with 1 $\mu$Ci of tritiated thymidine.
All cultures were done in quadruplicate.
FIGURE 11

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>&gt; .05</td>
</tr>
<tr>
<td>1:100</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>1:1000</td>
<td>&lt; .05</td>
</tr>
</tbody>
</table>

- CPM x 10^-3
- NO ANTIGEN
- ANTIGEN DILUTIONS
hamster SV-40 (BTH) cells by a single subcutaneous injection of $1 \times 10^6$ variable cells in 0.1 ml of EMEM without FBS. Fifteen days post inoculation, splenic lymphocytes were obtained and incubated for 72 hours with 1:100 dilution of homologous and heterologous cell-free antigens. Splenic lymphocytes from unimmunized animals were also used. Each culture was pulsed with 1 μCi of $^3$H-thymidine for the final 18 hours prior to harvesting. The results are shown in Table 1. Significant stimulation (Student's t test $p < 0.025$) occurred with homologous but not with heterologous antigens ($p > 0.05$). Reactions with syngeneic hamster lung antigen were not significant ($p > 0.05$). Normal splenic lymphocytes were not significantly stimulated with any antigen ($p > 0.05$). A number of experiments were done with lymphocytes from animals with large tumors, 2 x 2 cm or greater, 30-35 days post tumor cell injection. The results were consistently equivocal. Unstimulated control cultures incorporated high levels of $^3$H-thymidine. As a result, significant stimulation above control level could not be obtained in the cultures with antigen.

**Specific Transformation of Immune Splenic Lymphocytes From Hamsters Receiving Multiple Intradermal Injections**

Splenic lymphocytes from hamsters which had been hyperimmunized to tumor antigens were also investigated for use in specificity experiments. Separate groups of hamsters were immunized with HSV-2 tumor, mouse SV-40 (TCMK-1) and hamster SV-40 (BTH) cells. The animals received three weekly intradermal injections of $3 \times 10^6$ viable tumor cells. Five days after the final injection, splenic lymphocytes were obtained and incubated for 72 hours with 1:100 dilutions of homologous and
# TABLE I

## SPECIFIC TRANSFORMATION OF IMMUNE SPLENIC LYMPHOCYTES WITH CELL-FREE ANTIGENS

<table>
<thead>
<tr>
<th>1 x 10^6 LYMPHOCYTES SENSITIZED TO</th>
<th>STIMULATING ANTIGENS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAMSTER HSV-2 TUMOR</td>
<td>HAMSTER SV-40 CELLS</td>
<td>MOUSE SV-40 CELLS</td>
</tr>
<tr>
<td>HSV-2(^{(a)}) TUMOR CELLS</td>
<td>4046±442(^{(b)}) S.I.(^{(c)})=1.83 P &lt; .025(^{(d)})</td>
<td>1702±142 S.I.(=)0.77 P &lt; .05</td>
<td>ND(^{(e)}) S.I.(=)0.77 P &lt; .05</td>
</tr>
<tr>
<td>HAMSTER SV-40(^{(a)}) (BTH) CELLS</td>
<td>2295±285 S.I.(=)0.83 P &gt; .05</td>
<td>4085±209 S.I.(=)1.49 P &lt; .025(^{(d)})</td>
<td>ND S.I.(=)1.49 P &lt; .025(^{(d)})</td>
</tr>
<tr>
<td>MOUSE(^{(a)}) SV-40 CELLS</td>
<td>2161±234 S.I.(=)0.80 P &gt; .05</td>
<td>ND S.I.(=)1.48 P &lt; .025(^{(d)})</td>
<td>3995±339 S.I.(=)1.48 P &lt; .025(^{(d)})</td>
</tr>
<tr>
<td>UNSENSITIZED</td>
<td>1494±189 S.I.(=)0.95 P &gt; .05</td>
<td>1208±143 S.I.(=)0.76 P &gt; .05</td>
<td>ND S.I.(=)0.76 P &gt; .05</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Animals were sensitized with a single subcutaneous injection of 1 x 10^6 cells 15 days earlier.
\(^{(b)}\) counts per minute \(\pm\) Standard Error
\(^{(c)}\) stimulation Index = \(|\text{CPM Lymphocytes + Antigen} - \text{CPM Lymphocytes + medium}| / \text{CPM Lymphocytes + medium}\)
\(^{(d)}\) significant response student's T test
\(^{(e)}\) ND = not done
heterologous cell-free antigens. Eighteen hours prior to harvesting, 1 
μCi of $^3$H-thymidine was added to each culture. The results are shown in 
Table 2. Splenic lymphocytes immune to HSV-2 tumor cells were 
stimulated by both syngeneic cell-free HSV-2 tumor antigen (p < 0.001) 
and HSV-2 strain 333 infected WI-38 antigen (p < 0.01). These same 
lymphocytes appeared to be stimulated slightly with SV-40 Hamster (BTH) 
and WI-38 cell-free antigens (p < 0.05). However, this low level cross- 
reactivity did not mask the specific recognition of homologous and re- 
lated antigens. Splenic lymphocytes from hamsters immune to SV-40 
transformed cells of either mouse or hamster origin showed two-way 
cross reactivity with cell-free antigens from both mouse and hamster 
SV-40 tumors. See Table 2.

**Effect of Mitomycin-C on Tritiated-Thymidine Uptake**

by Tumor Cells

Various concentrations of mitomycin-C were incubated with BTH 
and HSV-2 tumor cells for 45 minutes at 37°C. The results are shown in 
Figure 12. A concentration of 100 μg of mitomycin-C/ml reduced the 
incorporation of tritiated-thymidine from 232,271 CPM to less than 300 
CPM for HSV-2 tumor cells. Similar findings were obtained for SV-40 
hamster tumor cells (BTH). Although not shown, additional studies in- 
creasing the incubation time from 45 to 60 minutes reduced incorporation 
of $^3$H-thymidine to less than 200 CPM. Therefore, subsequent studies with 
all mitomycin-C treated cells were conducted with an incubation time of 
60 minutes.
### TABLE 2
SPECIFIC TRANSFORMATION OF IMMUNE SPLENIC LYMPHOCYTES FROM HAMSTERS RECEIVING MULTIPLE INTRADERMAL INJECTIONS\(^{(a)}\)

<table>
<thead>
<tr>
<th>1 x 10^6 LYMPHOCYTES SENSITIZED TO</th>
<th>STIMULATING CELL-FREE ANTIGENS</th>
<th>HAMSTER HSV-2 TUMOR CELLS</th>
<th>HAMSTER SV-40 TRANSFORMED CELLS</th>
<th>MOUSE SV-40 TRANSFORMED CELLS</th>
<th>WI-38 CELLS</th>
<th>MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMSTER(^{(a)}) HSV-2 TUMOR CELLS</td>
<td>19,472±52(^{(b)}) S.I(^{(c)})=3.0 P&lt;.001(^{(d)})</td>
<td>14,591±1024 S.I.=2.23 P&lt;.01 (^{(d)})</td>
<td>8,931±143 S.I.=1.35 P&lt;.05 (^{(e)})</td>
<td>7,606±154 S.I.=1.18</td>
<td>9,600±187 S.I.=1.47 P&lt;.05 (^{(e)})</td>
<td>6,514±76</td>
</tr>
<tr>
<td>HAMSTER(^{(a)}) SV-40 TUMOR CELLS</td>
<td>6,285±18 S.I.=0.70 P&lt;.05 Inhibition</td>
<td>7,936±173 S.I.=0.88 P&lt;.05</td>
<td>15,899±254 S.I.=1.76 P&lt;.001(^{(d)})</td>
<td>12,191±718 S.I.=1.46 P&lt;.05 (^{(d)})</td>
<td>6,654±623 S.I.=0.74 P&gt;0.05</td>
<td>9,357±407</td>
</tr>
<tr>
<td>MOUSE(^{(a)}) SV-40 TRANSFORMED CELLS</td>
<td>6,198±494 S.I.=0.66 P&lt;.05 Inhibition</td>
<td>6,367±228 S.I.=0.69 P&lt;.05 Inhibition</td>
<td>13,915±743 S.I.=1.51 P&lt;.05 (^{(d)})</td>
<td>16,554±1435 S.I.=2.01 P&lt;.02 (^{(d)})</td>
<td>981±136 S.I.=1.06 P&gt;0.05</td>
<td>9,468±103</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Animals were sensitized with three weekly intradermal injections of 3 x 10^6 viable tumor cells; animals were sacrificed 5 days after last injection.

\(^{(b)}\) mean counts per minute (CPM) ± Standard Error, based on quadruplicate determinations

\(^{(c)}\) S.I. = stimulation index = \(\frac{(CPM)\text{ Lymphocytes + Antigen}}{(CPM)\text{ Lymphocytes + medium only}}\)

\(^{(d)}\) significant response, student's T test

\(^{(e)}\) significant response over control, but significantly less than responses with HSV-2 tumor cells or HSV-2 infected WI-38 cells, P<.02
FIGURE 12

EFFECT OF MITOMYCIN-C ON INCORPORATION OF $^3$H-THYMIDINE INTO HSV-2 AND SV-40 TUMOR CELLS

Varying concentrations of mitomycin-C were used to treat individual aliquots of $2 \times 10^6$ tumor cells contained in 3 ml of RPMI-1640 medium. After incubation at 37°C for 45 minutes, the tumor cells were washed three times with RPMI-1640 medium and dispensed into Microtest II plates at $1.5 \times 10^4$ cells per well. The test was incubated for 72 hours at 37°C. Each culture was pulsed with 1 μCi of tritiated thymidine for the last 18 hours prior to harvesting. Ten replicate cultures were done for each mitomycin-C concentration. ••• = HSV-2 tumor cells. O--O = SV-40 tumor cells.
Blastogenic Response by Immune Splenic Lymphocytes in the Mixed Lymphocyte-Mitomycin-C Treated Tumor Cell Reaction MLTR

Varying concentrations of lymphocytes and tumor cells were incubated in the MLTR and assayed for the incorporation of $^3$H-thymidine. All tumor cell concentrations resulted in significant stimulation with $1 \times 10^6$ lymphocytes. However, maximal stimulation occurred with 500 to 5000 tumor cells. See Figure 13. The stimulation curve was similar to that obtained with FHA or cell-free antigens. Lymphocytes at a concentration of $5 \times 10^5$ or $2.5 \times 10^5$ were not stimulated by any concentration of tumor cells. Similar results were obtained with mitomycin-C treated SV-40 transformed hamster cells incubated with homologous immune lymphocytes. Maximal stimulation occurred with 5000 tumor cells and $1 \times 10^6$ lymphocytes. Stimulation did not occur with $5 \times 10^5$ or $1 \times 10^5$ lymphocytes. See Figure 14.

Stimulation With Mitomycin-C Treated HSV-2 Tumor Cells According to the Time of Incubation

When $1 \times 10^6$ HSV-2 tumor immune lymphocytes were incubated with 500 mitomycin C treated HSV-2 tumor cells, significant stimulation, Student's t test $p < 0.01$, occurred after 72, 96, and 120 hours (see Figure 15). Normal splenic lymphocytes ($1 \times 10^6$) from non-immunized animals were reacted with 500 HSV-2 tumor cells as part of the above experiment. Significant stimulation was not observed until 120 hours, S.I. of 1.8 (Figure 16). This stimulation was considerably less than that observed for immune lymphocytes at 120 hours, S.I. of 7.5. (See Figure 15).
FIGURE 13

LYMPHOCYTE TRANSFORMATION WITH MITOMYCIN-C TREATED HSV-2 TUMOR CELLS - EFFECT OF VARYING CELL CONCENTRATIONS

Varying numbers of HSV-2 tumor immune splenic lymphocytes were incubated for 72 hours with varying concentrations of mitomycin-C treated HSV-2 tumor cells. Eighteen hours prior to harvesting each culture was pulsed with 1 μCi of tritiated thymidine. All cultures were done in quadruplicate. ∆—∆ = 1 x 10⁶ lymphocytes, ○—○ = 5 x 10⁵ lymphocytes, ×—× = 2.5 x 10⁵ lymphocytes.
FIGURE 13

<table>
<thead>
<tr>
<th>TUMOR CELLS</th>
<th>LYMPHOCYTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>50</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>500</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>5,000</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>50,000</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

Student's T Test, P Value

NUMBER OF MITOMYCIN C TREATED CELLS

CPM x 10^-3

0  50  500  5,000  50,000
Varying numbers of SV-40 tumor immune lymphocytes were incubated for 72 hours with varying concentrations of mitomycin-C treated SV-40 tumor cells. Eighteen hours prior to harvesting, each culture was pulsed with 1 µCi of tritiated thymidine. All cultures were done in quadruplicate. △△ = 1 x 10⁶ lymphocytes, ○○ = 5 x 10⁵ lymphocytes, ×× = 1 x 10⁵ lymphocytes.
FIGURE 14

<table>
<thead>
<tr>
<th>TUMOR CELLS</th>
<th>LYMPHOCYTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>Student's T Test, P Value</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>500</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>5,000</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>50,000</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

![Graph showing CPM x 10^3 vs. NUMBER OF MITOMYCIN C TREATED CELLS](image-url)
FIGURE 15

LYMPHOCYTE TRANSFORMATION WITH MITOMYCIN-C TREATED HSV-2 TUMOR CELLS - EFFECT OF VARYING INCUBATION TIMES

$1 \times 10^6$ HSV-2 tumor immune splenic lymphocytes were incubated with 500 mitomycin-C treated HSV-2 tumor cells. Eighteen hours prior to harvesting, each culture was pulsed with 1 μCi of tritiated thymidine. All cultures were done in quadruplicate. Stimulation Index = Mean (CPM) of lymphocytes incubated with tumor cells/Mean (CPM) of lymphocytes alone + Mean (CPM) tumor cells alone.
FIGURE 16

IN VITRO SENSITIZATION OF NORMAL HAMSTER SPLENIC LYMPHOCYTES
WITH MITOMYCIN-C TREATED SYNGENEIC HSV-2 TUMOR CELLS--
EFFECT OF VARYING INCUBATION TIMES

1 x 10^6 normal splenic lymphocytes were incubated with 500 mitomycin-C
treated HSV-2 tumor cells. For each time interval, 18 hours prior to
harvesting, the cultures were pulsed with 1 μCi of tritiated thymidine.
All cultures were done in quadruplicate. Controls for each time inter-
val were 1 x 10^6 lymphocytes incubated in medium only, and mitomycin-C
treated tumor cells in medium only. Stimulation Index = Mean (CPM)
Lymphocytes incubated with tumor cells/Mean (CPM) Lymphocytes Alone
+ Mean (CPM) Tumor Cells Alone.
STIMULATION INDEX

FIGURE 16

HOURS

72
96
120
144

P > 0.05
P > 0.05
P > 0.05
P < 0.02

P < 0.05
P < 0.05
P < 0.05
Comparison of Tumor Immune Splenic and Blood Lymphocytes for Reactivity to Mitomycin-C Treated HSV-2 Tumor Cells

Immune splenic and peripheral blood lymphocytes from the same animal were stimulated in vitro with varying concentrations of mitomycin-C treated tumor cells. The results are shown in Figure 17. Splenic lymphocytes showed maximal incorporation of $^3$H-thymidine with 500 tumor cells. Peripheral blood lymphocytes were not stimulated with any concentration of tumor cells.

In Vitro Sensitization of Normal Hamster Splenic Lymphocytes With Mitomycin-C Treated Syngeneic HSV-2 Tumor Cells

Our earlier studies showed that normal hamster splenic lymphocytes from unimmunized animals became sensitized in vitro after 120 or 144 hours incubation with mitomycin-C treated HSV-2 tumor cells. The following experiment was done to determine the optimal concentration of tumor cells for in vitro sensitization. Varying concentrations of mitomycin-C treated HSV-2 tumor cells were incubated for 144 hours with $1 \times 10^6$ normal lymphocytes. The results shown in Figure 18 illustrate that the optimal stimulating concentration of HSV-2 tumor cells was $5 \times 10^4$ cells; the results are similar to those obtained with presensitized (in vivo) lymphocytes and homologous antigen. However, the optimal concentration of tumor cells was higher.

Specific Transformation of Immune Splenic Lymphocytes in the Mixed Lymphocyte Mitomycin-C Treated Tumor Cell Reaction

The following experiment was done to determine if splenic lymphocytes from hamsters hyperimmunized to HSV-2 tumor or SV-40 transformed cells were specific in their recognition of mitomycin-C treated tumor cells. Separate groups of hamsters were immunized by three weekly
FIGURE 17

COMPARISON OF IMMUNE SPLENIC AND BLOOD LYMPHOCYTES
FOR REACTIVITY TO MITOMYCIN C TREATED HSV-2 TUMOR CELLS

1 x 10^6 blood and splenic lymphocytes from the same HSV-2 tumor immunized hamsters were incubated for 72 hours with varying concentrations of mitomycin C treated tumor cells. Cultures were pulsed with 1 µCi of tritiated thymidine for the final 18 hours prior to harvesting. All cultures were done in quadruplicate. □ = Splenic lymphocytes, □ = blood lymphocytes. SI = (CPM) lymphocytes + tumor cells/(CPM) lymphocytes alone + (CPM) tumor cells alone.
Figure 17: Stimulation Index of Mitomycin C Treated Cells

Number of Mitomycin C Treated Cells: 50, 500, 5000, 50,000

Stimulation Index:
- 50: P > 0.05
- 500: P < 0.01
- 5000: P < 0.01
- 50,000: P < 0.01
FIGURE 18

IN VITRO SENSITIZATION OF NORMAL HAMSTER LYMPHOCYTES
WITH MITOMYCIN-C TREATED SYNGENEIC HSV-2 TUMOR CELLS--
EFFECTS OF VARYING CELL CONCENTRATIONS

Varying concentrations of tumor cells were incubated for 144 hours with $1 \times 10^6$ normal lymphocytes. Eighteen hours prior to harvesting, each culture was pulsed with 1 µCi of tritiated thymidine. The results are based on quadruplicate cultures for each cell concentration. SI = (CPM) lymphocytes + tumor cells/(CPM) lymphocytes alone + (CPM) tumor cells alone.
Figure 18

Stimulation Index

500  5000  50,000  100,000

Number of Mitomycin C treated tumor cells

P<.05  P<.01  P<.005  P<.01
intradermal injections of $3 \times 10^6$ tumor cells. Five days after the final injection, splenic lymphocytes were obtained and incubated with mitomycin-C treated cells. Freshly excised HSV-2 tumor cells, cultured passage HSV-2 tumor cells, cultured SV-40 hamster tumor cells, and freshly excised hamster lung cells were used as antigens. Lymphocytes were assayed for the incorporation of $^3$H-thymidine at 72 and 120 hours. Similar results were obtained at 72 and 120 hours incubation. The results after 120 hours incubation are shown in Table 3. Significant stimulation occurred only with homologous antigens. Unsensitized normal lymphocytes were not stimulated with any mitomycin-C treated cells. Although not shown, as part of this experiment, immune lymphocytes were exposed to varying concentrations, 50-50,000 mitomycin-C treated cells of each antigen shown in Table 3. Regardless of the concentration of cells, in each case the stimulation remained specific for homologous antigens and immune lymphocytes. In addition, dilutions, 1:10-1:1000, of cell-free antigens of freshly excised HSV-2 tumor and cultured SV-40 transformed hamster cells were reacted with HSV-2 tumor immune lymphocytes. There was no cross reactivity observed, significant stimulation occurred only with HSV-2 cell-free antigens at both 72 and 120 hours.
**TABLE 3**

**SPECIFIC TRANSFORMATION OF IMMUNE SPLENIC LYMPHOCYTES IN THE MIXED LYMPHOCYTE–MITOMYCIN C TREATED TUMOR CELL REACTION**

<table>
<thead>
<tr>
<th></th>
<th>HSV-2 TUMOR CELLS</th>
<th>CULTURED HSV-2 TUMOR CELLS</th>
<th>CULTURED SV-40 (BTH) TUMOR CELLS</th>
<th>NORMAL HAMSTER LUNG CELLS</th>
<th>MEDIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HAMSTER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV-2 TUMOR CELLS</td>
<td>4720±52 (a)</td>
<td>2665±141</td>
<td>1788±138</td>
<td>1526±80</td>
<td>1544±109</td>
</tr>
<tr>
<td></td>
<td>S.I.=3.06 (b)</td>
<td>S.I.=1.72</td>
<td>S.I.=1.16</td>
<td>S.I.=0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P &lt; .001 (c)</td>
<td>P &lt; .005 (c)</td>
<td>P &gt; .05</td>
<td>P &gt; .05</td>
<td></td>
</tr>
<tr>
<td><strong>HAMSTER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV-40 (BTH) TUMOR CELLS</td>
<td>1830±49</td>
<td>1722±148</td>
<td>2770±79</td>
<td>1579±166</td>
<td>1605±90</td>
</tr>
<tr>
<td></td>
<td>S.I.=1.14</td>
<td>S.I.=1.07</td>
<td>S.I.=1.72</td>
<td>S.I.=0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P &gt; .05</td>
<td>P &gt; .05</td>
<td>P &lt; .001 (c)</td>
<td>P &gt; .05</td>
<td></td>
</tr>
<tr>
<td><strong>UNSENSITIZED</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1319±53</td>
<td>1130±110</td>
<td>1309±114</td>
<td>1450±220</td>
<td>1397±124</td>
</tr>
<tr>
<td></td>
<td>S.I.=0.94</td>
<td>S.I.=0.80</td>
<td>S.I.=0.93</td>
<td>S.I.=1.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P &gt; .05</td>
<td>P &gt; .05</td>
<td>P &gt; .05</td>
<td>P &gt; .05</td>
<td></td>
</tr>
</tbody>
</table>

a) counts per minute (CPM) ± SEM

b) stimulation index (SI) = \( \frac{\text{CPM lymphocytes + antigen (mitomycin-c treated cells)}}{\text{CPM lymphocytes alone + mitomycin-c treated cells alone}} \)

c) significant stimulation students T test
DISCUSSION

Several investigators have reported that hamster lymphocytes respond in the lymphocyte transformation assay to non-specific mitogens, soluble antigens, and in MLC. However, reports of reactivity to PHA are conflicting. Fernald and Metzgar (38) using a semimicroculture assay with inbred MHA hamster lymph node cells observed a dose response curve to PHA stimulation similar to that reported for other rodent species. In contrast, Ron, et al. (106) found in a macroculture assay that lymphocytes from inbred Bio RB, Bio 14-6, and outbred Syrian hamsters reacted to PHA concentrations 10-50 times higher than those found optimal for guinea pig, rat, or mouse lymphocytes. In the present study using a microculture assay, we found that inbred LSH hamster splenic and blood lymphocytes responded to PHA stimulation with a dose response curve confirming the observations of Fernald and Metzgar. The disparity in results is perhaps due to differences in culture techniques, or, alternatively, may be gene related and reflect strain differences among inbred Syrian hamsters. Singh and Tevethia (111) found that 10% fetal bovine serum (FBS) was suitable as a medium supplement for inbred MHA hamster lymphocyte cultures, and that after 3 days in culture 60% of the lymphocytes remained viable. Our initial studies showed that lymphocyte cultures supplemented with 10% FBS responded to PHA with statistically significant stimulation. However, splenic lymphocytes exposed to PHA-P reacted differently, Figure 4. The response was influenced by the serum;
cultures supplemented with FBS were either not stimulated with PHA-P or were inhibited. In the same experiment, lymphocytes in medium supplemented with 15% autologous hamster serum were significantly stimulated with PHA-P. Similar observations have been reported by others (38,111).

Our findings with PHA clearly demonstrated the feasibility of studying in vitro lymphocyte transformation in syngeneic LSH hamsters. We applied the lymphocyte transformation assay to investigate the use of splenic lymphocytes from HSV-2 tumor immunized hamsters for recognizing viral associated tumor antigens. Four major observations were made. First, splenic lymphocytes from hamsters with small tumors were reactive to cell-free HSV-2 tumor antigens and mitomycin-C treated HSV-2 tumor cells. The response to these antigens was dose-dependent paralleling the findings with FHA. The reactivity of immune lymphocytes was dependent on the time interval post-tumor inoculation; consistent stimulation was observed with splenic lymphocytes obtained from hamsters receiving 1 x 10^6 tumor cells 15 days earlier. In addition, lymphocytes obtained from hamsters immunized with three weekly intradermal injections of 3 x 10^6 tumor cells were also responsive. However, after approximately 30 days post-tumor cell inoculation when tumors were ≥ 2 cm in size, significant lymphocyte transformation could not be demonstrated. Splenic lymphocytes from animals with large tumors incorporated a greater amount of 3H-thymidine in the unstimulated control, compared to normal lymphocytes (see Figures 2 and 5). Perhaps these immune lymphocytes were already maximally stimulated (saturated) with antigen in vivo, in which case further stimulation could not be induced in vitro. However, lymphocytes from these same tumor-bearing hamsters were responsive to PHA stimulation.
indicating that the hamsters were not immunosuppressed, Figure 5. Similar findings as to loss of activity with increasing tumor size were recently reported by Lausch, et al. (73). These investigators used a spleen cell microcytotoxicity assay to investigate cell-mediated immunity in a Herpes simplex virus type 1 tumor model in LSH hamsters. They also demonstrated cell-mediated immunity to HSV-2 transformed cells using this assay. This latter observation confirmed our preliminary findings in which we reported cell-mediated immunity to HSV-2 tumors in LSH hamsters (52).

Our second observation was that splenic lymphocytes from HSV-2 tumor immune hamsters were not only reactive to cell-free antigens from homologous tumors (Figure 8) but also to cell-free antigens from WI-38 cells abortively infected with HSV-2 (Figure 11). Murasko and Lausch (96) recently reported similar cross reactivity in a spleen cell microcytotoxicity assay between human cytomegalovirus (CMV) transformed hamster cells and human embryonic lung cells productively infected with CMV. Whether the antigen(s) expressed on HSV-2 tumor cells are structural antigens of the virion, non-structural antigens coded for by the virus, or derepressed host cell antigen(s) is not known. However, several investigators have detected both virion and non-virion antigens on the plasma membranes of cells productively infected with HSV types 1 and 2 (15,40,67,87,105,114,124). In addition, viral-specific antigens have been found on the surface of cells transformed by HSV-1 and HSV-2 (33, 35,101). Recently, Lausch et al. (73) reported that LSH hamsters bearing HSV-2 tumors developed cell-mediated immunity which cross reacted in spleen cell microcytotoxicity tests with HSV-1 transformed cells.
However, spleen cells from animals bearing HSV-1 tumors were not cytotoxic to HSV-2 transformed cells. These findings agree with observations of Tarro and Sabin (124) who reported that antiserum to HSV-1 non-virion antigen prepared in guinea pig kidney cells did not cross react with HSV-2 non-virion antigens. However, immune serum for HSV-2 non-virion antigen cross-reacted with HSV-1 non-virion antigen. From observations reported by us and others, it appears likely that HSV-2 transformed cells may contain virion antigens as well as HSV-2 induced non-structural antigens. The expression of HSV-2 virion antigens on these cells could perhaps be confirmed in the lymphocyte transformation assay by reacting HSV-2 tumor immune lymphocytes with HSV-2 virion antigen. Likewise, the question of derepression of host cell embryonic antigens could possibly be answered in the lymphocyte transformation assay with blocking experiments utilizing serum from multiparous hamsters.

Our third observation was that splenic lymphocytes from normal hamsters became sensitized in vitro to syngeneic mitomycin-C treated HSV-2 tumor cells. The response was variable, appearing 120-144 hours after exposure. Several investigators studying cell-mediated immunity in other tumor models also have reported that primary stimulation of spleen cells in vitro can be achieved with mitomycin-C treated tumor cells or tumor membrane extracts (25,97). In our study, immune lymphocytes responded to HSV-2 tumor cells earlier and to a greater degree than did normal splenic lymphocytes, Figures 15 and 16. The initial response of immune cells occurred after 72 hours incubation and may reflect a secondary response of cells already sensitized in vivo. The
increased response occurring after 120 hours incubation may represent specific recruitment of lymphocytes not sensitized \textit{in vivo} or could be an additive effect due to \textit{in vitro} sensitization. However, the observation that reactivity of tumor immune lymphocytes remained specific after 120 hours incubation, Table 3, suggests that specific recruitment of unsensitized lymphocytes may have occurred. This observation also rules out a feeder effect as described by Carrel, \textit{et al.} \cite{19}.

The response of both normal and immune splenic lymphocytes to HSV-2 tumor antigens was dose-dependent. Immune lymphocytes responded optimally with \(1 \times 10^6\) lymphocytes and \(5 \times 10^2\) HSV-2 tumor cells, Figure 13, while normal lymphocytes at \(1 \times 10^6\) reacted optimally with \(5 \times 10^4\) tumor cells, Figure 18. The requirement of fewer tumor cells for stimulation of immune lymphocytes could indicate that the immune lymphocyte receptors had bound tumor antigen \textit{in vivo} hence requiring less antigen for \textit{in vitro} stimulation. Similar findings were obtained with SV-40 tumor immune splenic lymphocytes, Figure 14. Since a similar dose response curve was obtained with PHA, cell-free antigen, or mitomycin-C treated tumor cells, the decrease in lymphocyte responsiveness at higher antigen levels could be a function of protein concentration. In this regard, Poon and Cauchi \cite{97} reported that lymphocyte viability decreased dramatically with increasing amounts of protein in preparations of tumor membrane antigen used to stimulate mouse lymphocyte cultures.

In the present study, variation in the response of immune splenic lymphocytes occurred from test to test and could not be controlled. Since lymphocytes were obtained from a pool of 2-4 hamster spleens, the
fluctuation between tests could perhaps be due to differences in immune status of the animals. Nevertheless, within a given test, variation in incorporation of $^3$H-thymidine was consistently $<10\%$ among replicate cultures. This uniformity of replicate cultures was probably due to dispensing of reagents with microdroppers and mechanical harvesting of cultures with the multiple automated sample harvester (MASH) described by Hartzman, et al. (53).

Our results clearly demonstrate that syngeneic LSH hamsters develop cell-mediated immunity to HSV-2 tumor cells. In our system, the degree of stimulation of immune lymphocytes suggests that HSV-2 induced tumor antigens may be relatively weak. Streilien and Streilien (119) investigated the response of LSH hamsters to weak transplantation antigens from MHA strain hamsters and found that 20% of LSH hamsters were normally tolerant to MHA antigens, as shown by prolonged acceptance of MHA skin allografts. Thus, it is possible that LSH hamsters are inherently weak in cell-mediated response. It is also possible that hamster splenic lymphocytes are weak responders. In this regard, Lausch, et al. (73) found that splenic lymphocytes from HSV-2 tumor-bearing LSH hamsters reacted weakly to HSV-2 tumor cells in a microcytotoxicity assay. Singh and Teyethia (112) compared in MLC the reactivity of lymph node and splenic lymphocytes from CB and MHA strains of inbred Syrian hamsters. The stimulation indexes for spleen cells after 5 days in culture averaged only 3.5, while lymph node cells reacted on the average of 6.1. In our laboratory we did not study lymph node cells. However, we did compare blood and splenic lymphocytes for response to stimulation with HSV-2 tumor antigens and found that blood lymphocytes were unresponsive. The
differences in reactivity observed between blood and splenic lymphocytes could reflect differences in lymphocyte population. Littman, et al. (76) observed similar unresponsiveness of blood lymphocytes in a diethylnitrosamine induced tumor model in syngeneic guinea pigs.

Finally, our fourth observation was that HSV-2 tumor immune splenic lymphocytes were specifically reactive to antigens from HSV-2 tumor and HSV-2 infected WI-38 cells (see Tables 1, 2, 3). In addition, specificity was demonstrated with splenic lymphocytes immune to SV-40 transformed cells; common cross-reactive antigens were detected on both mouse and hamster cells transformed by SV-40. In one experiment (Table 2) minor cross reactivity did occur with HSV-2 tumor immune lymphocytes and heterologous antigens from WI-38 cells and SV-40 transformed hamster cells. However, this response was significantly less than the reactivity with homologous antigens from HSV-2 tumor or HSV-2 infected WI-38 cells. Therefore, the specificity of response was not masked. Similar findings of specificity of lymphocyte transformation in response to homologous tumor antigens were recently reported by Littman, et al. (76) in a diethylnitrosamine induced tumor model in syngeneic guinea pigs. Thus, our studies and reports by others have clearly demonstrated the utility of the lymphocyte transformation assay for specific detection of tumor associated antigens.

Tumor model systems are now available in LSH hamsters for studying immunity to cells transformed by HSV types 1 and 2, and human cytomegalovirus (3,33,35). Recently, a human papovavirus, JC virus, was isolated from the brain tissue of a patient with progressive multifocal leukoencephalopathy (94). This virus has now been shown to induce
malignant gliomas in Syrian hamsters (133). Since cells transformed by a given virus share common virus induced antigens on cells transformed by the homologous virus, these tumor model systems may prove useful for investigating the etiology of certain human neoplasms. The lymphocyte transformation assay should be exploited in the search for such antigens on candidate human neoplasms.
SUMMARY

Cell-mediated immunity in LSH hamsters to isografts of hamster embryo fibroblasts (HEF) transformed by herpes simplex virus type 2 (HSV-2) was investigated with a microculture assay of lymphocyte transformation. The assay was first standardized with a non-specific stimulator of blastogenesis, phytohemagglutinin (PHA). Hamster splenic and blood lymphocytes reacted to PHA with a dose-response curve similar to that reported for other rodent species. It was found that splenic lymphocytes from HSV-2 tumor-bearing hamsters and animals immunized with multiple intradermal injections of tumor cells were responsive to cell-free HSV-2 tumor antigens and mitomycin-C treated tumor cells. The reactions with both antigens were dose-dependent paralleling the findings with PHA. Lymphocytes from hamsters with large tumors ≥ 2 cm in size were unresponsive. However, these lymphocytes were responsive to PHA, indicating that the hamsters were not immunosuppressed. It was also found that normal lymphocytes became sensitized in vitro to HSV-2 tumor cells. The response was variable, occurring after 120-144 hours incubation, and was dose-dependent. Splenic lymphocytes from immune hamsters responded earlier and to a greater degree than normal lymphocytes. The initial response of immune lymphocytes to homologous tumor antigens occurred at 72 hours incubation, and increased after 120 hours. The reactivity remained specific at both intervals. Splenic lymphocytes from HSV-2 tumor immune hamsters were significantly stimulated with
with antigens from HSV-2 tumor cells and HSV-2 infected human embryonic lung (WI-38) cells, but were not stimulated with heterologous antigens from SV-40 transformed mouse or hamster cells. Likewise, lymphocytes from hamsters immune to SV-40 transformed cells reacted with common antigens on both SV-40 transformed mouse and hamster cells, but did not cross-react with heterologous antigens. Thus the utility of the lymphocyte transformation assay for detecting common virus induced antigens on host cells has been demonstrated. This assay has potential for investigating the etiology of certain human neoplasms.
BIBLIOGRAPHY


