VIRAL-INDUCED UNRESPONSIVENESS OF MOUSE LYMPHOCYTES TO PHYTOHEMAGGLUTININ STIMULATION

by

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

[Signature] 24 April 1973
GEORGE B. OLSON
Associate Professor of Microbiology
ACKNOWLEDGMENTS

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ABSTRACT

Spleen cells from Swiss-Webster mice inoculated with viruses were tested for their ability to respond in vitro to phytohemagglutinin-induced DNA synthesis as measured by the amount of tritiated thymidine incorporated into DNA. Application of this response was utilized as a means of detecting a virus infection in vivo.

A preliminary study indicated that splenic lymphocytes obtained from mice infected with Sendai virus, Reovirus, and Mengovirus-L demonstrated a depression of PHA-induced DNA synthesis over a six-day postinfection period.

Mice inoculated with an LD_{50} concentration of Mengovirus-L for a 20-day postinfection period demonstrated an impaired ability of lymphocytes to respond to PHA stimulation, and this coincided with a time when the clinical signs of the disease were apparent. Once signs of hyperactivity and paralysis declined, lymphocyte responsiveness to PHA stimulation returned to normal. These observations suggest that the responsiveness to PHA stimulation may reflect a direct virus-lymphocyte interaction or indicate a more complex physiological relationship in vivo.

Additional examinations of Mengovirus-L infectivity in relation to morphological changes in vivo, such as spleen and body weight changes and altered leukocyte profiles, were not successful in establishing a positive correlation that was consistent with the impaired lymphocyte response.
INTRODUCTION

Early indications of viral-induced unresponsiveness first appeared with von Pirquet's observation (in 1) that tuberculin positive children became negative to tuberculin skin testing a few days prior to the appearance of a measles rash. He further noticed that there was no change in the tuberculin reaction in patients with such diseases as scarlet fever, epidemic meningitis, or typhoid fever. Coincident with von Pirquet's observation, in 1919 Bloomfield and Mateer (2) found that tuberculous patients with a mild, epidemic form of influenza also became negative to the tuberculin reaction during the febrile period of the disease. In both of these early accounts of viral anergy, the depressed response is most apparent during the active phase of the disease but later reverts to a normal tuberculin response once the infection has subsided.

Recently, the effects of a measles-induced anergy in tuberculin sensitivity has been confirmed (3,4). Tuberculous patients infected with a regular measles virus, gamma-globulin modified measles, and measles vaccine, demonstrated a depressed Mantoux test. The depression was greatest using regular measles virus. This was evident during the incubation period and for six weeks after the onset of the rash (5). These observations suggest a viral-induced unresponsiveness of those elements responsible for the characterization of the tuberculin response in vivo.
In order to elucidate this interaction on a cellular basis, a number of investigations were initiated. Normally, peripheral blood lymphocytes from a tuberculin-sensitive individual will transform into blastoid cells after an in vitro exposure to Purified Protein Derivative (PPD) (6). Smithwick and Berkovich (7) found that an in vitro exposure to live measles virus to tuberculin-sensitive lymphocytes would impair the ability of these cells to undergo a blastogenic response following stimulation with PPD.

In summary, two methods are currently available for ascertaining a viral-induced unresponsiveness. One method has been characterized by its ability to show a positive tuberculin skin reaction in vivo (3). The other method involves the stimulation of tuberculin-sensitized lymphocytes with a specific antigen such as PPD and then measure their ability to transform into blastoid cells in vitro (6).

The observations of Nowell (8) in 1960 provided an additional tool for measuring cellular response in vitro. Using phytohemagglutinin (PHA), an extract of the red kidney bean *Phaseolus vulgaris*, it was found that peripheral blood leukocytes of human beings would undergo blast transformation and increased mitotic division. The cells undergoing blastogenesis were later identified as small lymphocytes (9,10).

The treatment of lymphocytes in vitro with PHA results in several distinct morphological and chemical changes. There is an increase in cell size along with a decrease in nuclear to cytoplasm ratio (11). The cytoplasm becomes more basophilic, containing azurophilic granules and small, well-defined vacuoles. One or more prominent nucleoli are
present in the nucleus, which stains with a stippled and fine reticular pattern of chromatin (12-15). Isotope studies have indicated a marked increase in the synthesis of proteins (16), RNA (17), DNA (18), lysosomal enzymes (19), and mitotic division (20).

Several methods are used to measure PHA-induced lymphocyte transformation. One method involves a differential count of blast-like cells per 1000 mononuclear cells (14,21). A second method consists of a differential count of mitotic figures per 1000 mononuclear cells in the preparation (14). A third method is to assay cell cultures for the uptake of radio-isotopes using radioautography (14,22) and liquid scintillation counting procedures (14,23). The latter method represents one of the more quantitative techniques and is dependent upon such isotopes as tritiated thymidine which is incorporated into the DNA of transforming cells (14).

A variety of other agents can be used to induce lymphocyte transformation in vitro. These include pokeweed mitogen (12), staphylococcal filtrate (24), and streptolysin-S, a hemolytic exoprotein produced by streptococcus organisms (25). Phytohemagglutinin and the above agents are classified as general mitogens, since no previous sensitization of lymphocytes is required (26).

A second group of stimulants called specific mitogens, includes tetanus and diphtheria toxoids (27), PPD (28), polio and typhoid vaccines (7), and penicillin (29). Specifically sensitized lymphocytes are required with these mitogens (30) which can transform from 1% to 35% of the small lymphocytes in the cultures (31). Comparatively, a
general mitogen such as PHA is able to transform 90% to 95% of the small lymphocytes in the cell population (30,32,33).

The mitogenic response of lymphocytes in vitro has been found to correlate with lymphocyte function in vivo. In general, lymphocytes from healthy human beings demonstrate a significant in vitro response to PHA and specific mitogen-induced transformation.

Comparatively, it has been observed that in many postoperative situations, the transformation response of lymphocytes to PHA was significantly reduced (34). The use of such chemotherapeutic drugs as 6-mercaptopurine and methotrexate has also indicated a marked inhibition of PHA-induced transformation (35). Lymphocytes from patients with such diseases as Hodgkin's disease (15), sarcoidiosis (36), ataxia-telangiectasia (37), Sjögren's Syndrome (38), chronic lymphocytic leukemia (13,39), and uraemia (40) have all demonstrated an impaired in vitro response to PHA and specific antigenic stimulation.

Many of these diseases reflect a general inadequacy in the expression of such cell-mediated immune responses as skin sensitivity to tuberculin and other antigens, homograft rejection, and susceptibility to infection (41). Although the etiology of these diseases is as yet undetermined, many investigators have suggested a virus lymphocyte interaction which affects cell-mediated immunity and the mitogenic response of lymphocytes in vitro (15,33).

Olson and co-workers (33) found that peripheral blood lymphocytes from babies with congenital rubella virus syndrome demonstrated an impaired in vitro PHA response (21). The maximal depression of
PHA-induced DNA synthesis in lymphocytes was evident during the infectious phase of the disease, and later returned to a normal response once the viremia had disappeared and overt clinical signs of the infection were gone. To confirm the idea that the rubella virus infection was altering the response to PHA, lymphocytes from normal human beings were infected in vitro with rubella virus. Again, an impaired PHA-induced DNA synthesis was evident in addition to a decrease in the synthesis of RNA and structural proteins (33). It was suggested that virus enters the cell, begins synthesizing viral RNA, and subsequently interferes with the cell's ability to synthesize nucleic acids and structural proteins (33).

Additional work by Willems, Melnick, and Rawls (42) has also indicated that in vitro infection of normal peripheral lymphocytes with several noncytopathic RNA and DNA viruses would also alter the response of these cells to PHA stimulation. Ribonucleic acid (RNA) viruses such as Polio I, Echo 11, Influenza, Sendai, and New Castle Disease virus demonstrated varying degrees of impaired PHA response (42). Inhibition by DNA viruses was noted with Herpes simplex virus, Vaccinia, and Wart virus (42).

Experimental evidence has shown that infection of mice with murine leukemia virus can induce an impaired response of lymphocytes to both a general (PHA) and specific (alloantigen) stimulation in vitro. Häyry, Rago, and Defendi in 1970 (43) demonstrated that splenic lymphoid cells from BALB/c mice inoculated intraperitoneally with Rauscher Leukemia virus (RLV) responded minimally or not at all to PHA or
mitomycin-C treated CBA cell stimulation in vitro. This impaired response was evident not only during fully developed leukemia but also during the incubation period when no histological signs were apparent.

The response to both stimuli decreased steadily throughout the 60-day observation period with no indication of a reversion to a normal mitogenic response. Additional experiments using normal BALB/c splenic lymphocytes infected in vitro with RLV confirmed the idea that the depression in the infected animals was the result of a direct effect of the virus on lymphocytes.

Additional evidence obtained from in vitro studies has shown that a nonleukemia producing virus can infect, replicate, and cause an impaired PHA-induced DNA synthesis of mouse lymphocytes. Eustatia and van Der Veen (44) found that mouse nodal lymphocytes stimulated with PHA and infected in vitro with $1.5 \times 10^3$ TCID$_{50}$ of Mengovirus would support viral replication by showing a two-log increase in the TCID$_{50}$ titer. A significant alteration of PHA-induced DNA synthesis of lymphocytes (23% to 47% reduction compared to controls) was also evident. Cell viability, however, was reported as 48% which may have contributed to the reduction in PHA responsiveness of infected cells.

Olson (45) found similar results with Mengovirus using spleen cells. Using a multiplicity of infection of 10:1, a twofold increase in virus titer was noted for 72 hours postinfection. In addition, a significant impairment (30% and 40% reduction compared to noninfected cultures) of PHA-induced DNA synthesis was observed. Cell viability after 72 hours of culture was 90% to 95%, indicating that cytotoxic
effects from the virus were not present. These data provide evidence that Mengovirus can alter the mitogenic response of mouse lymphocytes.

It is evident, then, that the abnormality in lymphocyte response to PHA and antigenic stimulation may reflect a virus alteration of lymphocytes by impairing protein and nucleic acid synthesis and, consequently, affecting the immune competency of these cells.

The purpose of this investigation was to determine if an alteration in PHA-induced DNA synthesis of lymphocytes in vitro could be used to detect a virus lymphocyte interaction in vivo.
MATERIALS AND METHODS

Animals

Random-bred Swiss-Webster male mice, weighing 18 to 28 g, were obtained from the Department of Microbiology and Medical Technology and employed as test animals throughout this study.

Ten animals or less were housed in separate cages and fed Purina mouse chow and tap water ad libitum. Bedding was changed bi-weekly. The environment was controlled in relation to temperature (74 ± 2°F) and relative humidity (50 ± 5%).

Preparation of Reagents

Phytohemagglutinin M (PHA) (Difco Laboratories, Detroit, Mich., lot 532118) was reconstituted to 50.0 ml with saline (0.85%) and used in 0.1 ml amounts per culture.

Preparation of Viral Reagents

Murine strains of Sendai virus \((1 \times 10^6 \text{ EID}_{50}/\text{ml})\) and Reovirus III \((8 \times 10^9 \text{ pfu/ml})\) were obtained from Dr. G. B. Olson, Department of Microbiology and Medical Technology, The University of Arizona.

Mengovirus-L (large plaque variant, \(1.8 \times 10^9 \text{ pfu/ml}\)) was kindly prepared by Dr. C. G. Gauntt, College of Medicine, The University of Arizona. The same stock were used in all experimentations.
Experimental Protocols

Preliminary Study to Determine the In Vitro Response of Lymphocytes to PHA Stimulation following Infection with Various Viruses In Vivo

A preliminary study was undertaken to compare the effect of several murine viruses on PHA-induced DNA synthesis of lymphocytes following infection in vivo. Six each, 40-day-old mice were inoculated intravenously (I.V.) with 0.1-ml volumes of $1 \times 10^4$ EID$_{50}$/ml of Sendai virus, $1 \times 10^7$ pfu/ml of Reovirus, and $1 \times 10^6$ pfu/ml of Mengovirus-L. Six additional noninfected 40-day-old mice were used as controls. On days 2, 4, and 6 following infection, two mice from each group were sacrificed by cervical dislocation; spleens were removed and teased into single cell suspension for in vitro cultivation as explained under a separate heading. Analysis of the in vitro response to PHA stimulation was determined by the amount of tritiated thymidine incorporated into the DNA of lymphocytes.

The percentage of viral-induced inhibition was calculated using the following formula:

$$\text{Percent inhibition} = \frac{(\text{CPM of cells + PHA + virus}) - (\text{CPM of cells + virus})}{(\text{CPM of cells + PHA}) - (\text{CPM of cells only})} \times 100$$

Determination of the LD$_{50}$ of Mengovirus-L

To determine the LD$_{50}$ of Mengovirus-L in Swiss-Webster mice in relation to age and route of injection, stock Mengovirus-L was diluted on a half-log scheme ranging in concentration from $1 \times 10^1$ to $1 \times 10^6$. 

pfu/ml. The diluent used was Dulbecco Phosphate Buffered Saline in 0.1% bovine serum albumin (PBSA) (46,47).

A 0.1-ml volume of each concentration was injected intra-peritoneally (I.P.) into replicates of five mice belonging to four different age groups: 20 days (18 g), 30 days (22 g), and 40 days (28 g). An additional 40-day-old group was inoculated I.V. with the same concentrations of Mengovirus-L.

Mice were observed five times daily for 25 days, and the number of paralytic and dead mice was recorded for each day.

Data were evaluated using the method of Reed and Muench (48) for the calculation of 50% endpoints.

**Determination of the In Vivo Morphological Changes and the In Vitro Response of Lymphocytes to PHA Stimulation following Infection with Mengovirus-L In Vivo**

The reliability of the mouse for the detection of a virus infection in vivo in relation to altered, systemic morphological changes and PHA-induced DNA synthesis of lymphocytes was ascertained as follows: ninety mice, 30 to 35 days old, were inoculated I.V. with $6 \times 10^2$ pfu's (equivalent to LD$_{50}$ value) of Mengovirus-L. All mice were pooled after inoculation and distributed randomly as 10 animals per cage.

At predetermined times of 1, 3, 5, 7, 9, 11, 13, and 20 days following infection, five infected mice were randomly selected each day for investigation. Five additional noninfected mice served as controls for each day.
Prior to sacrifice, each mouse was bled from the reto-orbital-plexus for the determination of total and differential leukocyte profiles. Body weight was also determined at this time.

Each mouse was sacrificed by cervical dislocation, followed by a removal of spleen and brain. Individual spleens were weighed to the nearest 0.001 g, teased into single cell suspension, and cultivated in vitro to measure the response of lymphocytes to PHA stimulation. Cultivation procedures are contained under a separate heading.

The remaining spleen cell suspension and brain were assayed for the presence of Mengovirus-L by standard plaque assay techniques.

Procedure for Culturing Splenic Lymphocytes

At predetermined times following infection, spleens were removed from both infected and noninfected mice and individually teased into single cell suspension with 5 ml of PBSA. The concentration of cells was determined by standard counting techniques (1.5% acetic acid as diluent) and adjusted to $5 \times 10^6$ cells/ml in Roswell Park Memorial Institute medium-1630 (RPMI-1630, Grand Island Biological Co., Grand Island, N.Y.) containing 5% fresh, heat-inactivated (56°C, 30 min) human plasma (author), 100 units/ml of penicillin, 100 μg/ml of streptomycin (Eli Lilly and Co., Indianapolis, Indiana), 100 units/ml of heparin (Rabin Winters, El Segundo, Calif.) and 0.01 M Hepes Buffer (49) (Calbiochem, Los Angeles, Calif.).

Two sets of quadruplicate lymphocyte cultures of each spleen were prepared in 125 x 16 mm, round-bottomed "thro-away" culture tubes.
The final volume for each culture was 2.0 ml. One-tenth ml volumes of PHA were added to one of the culture sets, and both sets were incubated at 37 C at a relative humidity of 95% for 72 hr to provide maximal PHA response (33).

Procedure for Assaying Isotope Incorporation into Splenic Lymphocytes

Twenty-four hr prior to termination of cultures, 0.1 µC of tritiated thymidine ($^3$HTdR, SA 5.0 C/mM, International Chemical and Nuclear Corp., City of Industry, Calif.) was added to 3 tubes of quadruplicate culture sets. At 72 hr, cultures were terminated and placed at 4 C. The fourth culture of each set was assayed for cell viability by dye exclusion using trypan blue (50). The remaining cultures were analyzed for DNA synthesis by liquid scintillation counting, according to a modified procedure described by Olson et al. (33).

The cultures were centrifuged at 677 x g for 10 min; the cell pellets were suspended in 1.5 M NaCl and centrifuged again at 677 x g for 10 min. Nuclear fractions were precipitated with 5.0 ml of cold 5% trichloroacetic acid (TCA) (Baker and Adams, Morristown, N. J.) for 5 min and centrifuged at 677 x g for 10 min. The process was repeated with a second cold TCA wash.

The final TCA precipitate was washed with cold absolute methanol and dissolved with 0.2 ml of Nuclear Chicago Solubilizer (NCS) (Nuclear Chicago Corporation, Chicago, Ill.) and added to 10 ml of scintillation counting fluid (48 g of Packard "pre-mix P," 2000 ml of toluene, 1200 ml
of ethyl glycol monoethyl ether). All samples were counted for 1 min on a Packard Tri-Carb scintillation counter. The amount of radioactivity ($^3$HTdR) incorporated was expressed as counts per minute (CPM) at a 37% machine counting efficiency for tritium.

For comparative purposes, CPM data were averaged for the three culture replicates of PHA-stimulated and nonstimulated lymphocyte cultures of each mouse. A ratio of tritiated thymidine incorporation could then be expressed as mean CPM of PHA-stimulated cultures to mean CPM of nonstimulated cultures. Statistical evaluation is included under a separate heading.

**Procedure for Assaying Mengovirus-L**

Brains and remaining spleen cell suspensions from sacrificed animals were homogenized in 1.0 ml of PBSA and subjected to five freeze-thaw cycles in a dry ice-acetone bath (-65 C).

Cellular extracts were centrifuged 1522 x g for 20 min and the supernate was assayed for Mengovirus-L by a standard plaque assay method using mouse LPA fibroblastic cells obtained from C. G. Gauntt, College of Medicine, The University of Arizona. A concentration of 2 x $10^6$ cells were initiated as monolayers in 60 mm of disposable tissue culture plates (Falcon Plastics, Oxnard, Calif.) and nourished in 5.0 ml of Eagle's Minimum Essential Medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.), 100 units/ml of penicillin, and 100 µg/ml of streptomycin under 5% carbon dioxide ($CO_2$) tension at 37 C for 18 hr.

Tenfold serial dilutions of the cellular extracts were made in PBSA. Monolayers (18 hr old) were washed twice with PBSA, and 0.2 ml
of each diluted extract was absorbed for 1.0 hr (47,51). All tests were performed in duplicate. The preparation was overlayed with 1.0% Bacto-Agar (Difco Laboratories, Detroit, Mich.) dissolved in MEM with 1.0% heat inactivated (56 C, 30 min) fetal calf serum (Flow Laboratories, Rockville, Md.) with 0.08% protamine sulfate (Nutritional Biochemical Corporation, Cleveland, Ohio). After an additional 48 hr of incubation (37 C, 5% CO₂ plates were stained with 0.01% neutral red for enumeration of plaques. Data were expressed as the number of plaque-forming units per ml of inoculum (pfu/ml).

Procedure for Determining Body and Spleen Weights

The body weight of each mouse was measured to the nearest 0.1 g. Mice were sacrificed by cervical dislocation, spleens and brains were removed aseptically and placed into sterile petri dishes. Spleens were weighed (wet) to the nearest 0.001 g. Brains were stored in 1.0 ml of PBSA at -20 C for later analysis.

The values obtained for body weights were combined as an arithmetic mean for each group of five infected and noninfected mice representing a postinfection day.

Spleen weights were similarly combined into their respective groups of five mice and expressed as log₁₀ means. Statistical evaluations are contained under separate heading.
Procedure for Determining Leukocyte Profiles

Prior to sacrifice, each mouse was bled without anesthesia from the reto-orbital-plexus using heparinized microcapillary tubes (52). Total leukocyte counts were made with standard techniques using a 1.5% acetic acid diluent (53). Duplicate blood smears were prepared, stained with Wright-Giemsa, and 100 leukocytes per slide were counted for a differential determination.

The percents of polymorphonuclear cells, lymphocytes, and total leukocyte counts (per mm$^3$) were combined for the infected and non-infected groups of five mice and expressed as group means for each post-infection day.

Statistical Evaluation of Data

Data were subjected to an analysis of variance using a factorial design to assess significant differences of PHA-induced DNA synthesis of lymphocytes obtained from virus-infected and noninfected mice (54). Where applicable, an analysis of variance was used to evaluate in vivo morphological changes such as spleen and body weights and leukocyte profiles.

A regression analysis of all data was also used in an effort to statistically predict the presence of a virus infection in mice.

Statistical analysis and computation were done using programs provided by Dr. L. M. Kelley, Department of Microbiology and Medical Technology, The University of Arizona, and through use of a Control Data Computer, Model 6400.
RESULTS

**Response of Lymphocytes to PHA Stimulation following Virus Infection In Vivo**

Table I summarizes the observations made in a preliminary experiment to examine the in vitro response of mouse lymphocytes to PHA-induced DNA synthesis following infection with three murine viruses in vivo. Spleen cells from noninfected mice cultured in vitro with PHA demonstrated 4.4- to 5.5-fold increases in the amount of tritiated thymidine incorporated into DNA. However, lymphocytes obtained from mice infected with Sendai virus, Reovirus, and Mengovirus-L showed a significant depression of PHA-induced DNA synthesis (P > .05) as compared to the noninfected, PHA-stimulated cultures.

A trend indicating an increasing degree of inhibition of DNA synthesis for each successive postinfection day was also evident (Table I). Spleen cells obtained from Sendai virus-infected mice demonstrated that maximal inhibition of PHA-induced DNA synthesis (95%) occurred on the fourth day following infection. Comparatively, spleen cells from Reovirus and Mengovirus-L infected mice showed 77% and 98% inhibition, respectively, by the sixth day following infection. The decreased response in DNA synthesis following PHA stimulation did not appear to be the result of a virus-induced cytotoxicity, as cell viability of both control and virus-infected lymphocytes ranged from 80% to 95%.
Table I. Suppression of PHA-induced DNA synthesis of mouse lymphocytes following infection with viruses in vivo.

<table>
<thead>
<tr>
<th>Source of spleen cells</th>
<th>2 Days postinfection</th>
<th>4 Days postinfection</th>
<th>6 Days postinfection</th>
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<tr>
<td></td>
<td>CPM of $^3$HtdR labeled DNA</td>
<td>% Viral-induced inhibition</td>
<td>CPM of $^3$HtdR labeled DNA</td>
</tr>
<tr>
<td>Noninfected mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PHA</td>
<td>9,272</td>
<td>-</td>
<td>5,878</td>
</tr>
<tr>
<td>- PHA</td>
<td>40,920</td>
<td></td>
<td>34,735</td>
</tr>
<tr>
<td>Sendai virus infected mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PHA</td>
<td>5,423</td>
<td>30</td>
<td>8,553</td>
</tr>
<tr>
<td>- PHA</td>
<td>27,577</td>
<td></td>
<td>9,936</td>
</tr>
<tr>
<td>Reovirus infected Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PHA</td>
<td>12,778</td>
<td>22</td>
<td>6,840</td>
</tr>
<tr>
<td>- PHA</td>
<td>37,686</td>
<td></td>
<td>16,862</td>
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<tr>
<td>Mengovirus-L infected mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PHA</td>
<td>12,505</td>
<td>32</td>
<td>9,214</td>
</tr>
<tr>
<td>- PHA</td>
<td>34,093</td>
<td></td>
<td>26,456</td>
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</table>

a. Confidence at 95% level. Analysis of variance done using the log of number. $CL_{95\%} = \log N \pm 0.16$. 


The initial experiment also indicated that the alteration of PHA-induced DNA synthesis related to the pathogenesis of the virus infection. Lymphocytes obtained from mice infected with Reovirus and Sendai virus responded defectively to PHA stimulation but did not demonstrate any gross pathological symptoms that could be associated with an exposure to these agents. Comparatively, by the fifth and sixth day following infection with Mengovirus-L, mice were symptomatic showing the initial stages of hyperactivity and paralysis characteristic of this infection (50,55). This time period corresponded to the 98% reduction ($P > .05$) in the amount of PHA-induced DNA synthesis (Table I).

**Virulence Determination of Mengovirus-L in Swiss-Webster Mice**

Since the symptoms of a Mengovirus-L infection in mice were evident at a time that correlated to an alteration of PHA-induced DNA synthesis, we found it necessary to determine $LD_{50}$ levels of infectivity in relation to the age of mice and inoculation route.

The data depicted in Table II indicate that virulence was dependent upon the age of the mice and the size of the inoculum, as shown by the differences in the amount of Mengovirus-L required for an $LD_{50}$. A concentration of 18 pfu's constituted an $LD_{50}$ for 20-day-old mice, as compared to 630 and 1000 pfu's for an $LD_{50}$ in 30- and 40-day-old mice, respectively.

Viremia and paralysis lasted until death. This was also observed in an age-dose related response. Twenty-day-old mice were symptomatic by three days postinfection with the higher concentrations of
Table II. Determination of the virulence of Mengovirus-L for Swiss-Webster mice in relation to age and inoculation route.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Inoculation route</th>
<th>LD$_{50}^a$ pfu/ml</th>
<th>PD$_{50}^b$ pfu/ml</th>
<th>MLDC$^c$ pfu/ml</th>
<th>MPDC$^d$ pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Intraperitoneal</td>
<td>$1.8 \times 10^1$</td>
<td>$1.2 \times 10^1$</td>
<td>$1.0 \times 10^3$</td>
<td>$1.0 \times 10^3$</td>
</tr>
<tr>
<td>30</td>
<td>Intraperitoneal</td>
<td>$6.3 \times 10^2$</td>
<td>$6.1 \times 10^2$</td>
<td>$1.0 \times 10^3$</td>
<td>$1.0 \times 10^3$</td>
</tr>
<tr>
<td>40</td>
<td>Intraperitoneal</td>
<td>$1.0 \times 10^3$</td>
<td>$1.5 \times 10^2$</td>
<td>$1.0 \times 10^5$</td>
<td>$1.0 \times 10^5$</td>
</tr>
<tr>
<td>40</td>
<td>Intravenous</td>
<td>$1.0 \times 10^3$</td>
<td>$1.7 \times 10^2$</td>
<td>$1.0 \times 10^5$</td>
<td>$1.0 \times 10^4$</td>
</tr>
</tbody>
</table>

a. Lethal dose 50%.
b. Paralytic dose 50%
c. Minimum lethal dose
d. Minimum paralytic dose
e. Calculations were determined by method of Reed and Muench (48) on the basis of a 10-day exposure to Mengovirus-L.
Mengovirus-L (5 x 10^3 through 1 x 10^5 pfu/ml) and were dead by the fifth day following infection. Similarly, the 30- and 40-day-old mice showed signs of paralysis by the fourth day and were dead by the seventh and eighth day following infection. In all three age groups used for the LD_{50} determinations, the appearance of paralysis was no longer evident after eight to ten days exposure to Mengovirus-L.

The data did not indicate any difference in virulence for 40-day-old mice after infection by either I.P. or I.V. routes (Table II). In both groups, 1 x 10^3 pfu/ml constituted an LD_{50} and 1 x 10^5 pfu/ml represented the MLD. The values reported for paralytic dose 50% (PD_{50}) and minimum paralytic dose (MPD) are greater than those for the LD_{50} and MLD but were not regarded as significant since paralyzed mice did not recover.

Since the response of 30-day-old mice to Mengovirus-L infection was more homogenous in relation to the appearance and duration of paralysis, this age group was selected for subsequent experimentation in this study.

Detection of a Mengovirus-L Infection In Vivo Based on Lymphocyte Response to PHA Stimulation and In Vivo Morphological Changes

The principal aim in this investigation was to determine if alteration in PHA-induced DNA synthesis of lymphocytes in vitro could be used to detect a virus infection in vivo. To facilitate this, 30- to 35-day-old mice were inoculated I.V. with 600 pfu's of Mengovirus-L (LD_{50}).
The data illustrated in Figure 1 indicate that over a 20-day period following infection with Mengovirus-L, there was no significant difference in the amount of tritiated thymidine incorporated into non-PHA stimulated cell cultures from infected mice compared to an identical treatment for control mice. These data in conjunction with cell viability values ranging from 75% to 95% for both infected and non-infected cultures suggested that cytotoxic effects from Mengovirus-L were not present.

However, lymphocytes from Mengovirus-L infected mice demonstrated a significant inhibition of PHA-induced DNA synthesis (Figure 2). This effect was evident on the third day following infection and corresponded to a time period analogous to the optimal pathogenesis of Mengovirus-L as shown in an earlier study. The data in Figure 2 also indicate a reversion to a normal PHA-induced DNA synthesis by the seventh day following infection, since there was no significant difference in DNA synthesis for lymphocytes obtained from either non-infected or Mengovirus-L infected mice. Again, this effect corresponded to the pathogenicity of Mengovirus-L, since mice no longer demonstrated any signs of the disease by seven and eight days following infection.

Further investigations of in vivo morphological changes did not reveal any significant differences in relation to an altered PHA-induced DNA synthesis of lymphocytes. Figures 3 and 4 illustrate data observed for the body and spleen weight measurements. No difference between Mengovirus-L infected mice and control mice was evident.
Figure 1. The effect of Mengovirus-L infection in vivo on DNA synthesis of non-PHA stimulated lymphocytes.

Vertical lines represent 95% confidence intervals.
Figure 2. The effect of Mengovirus-L infection in vivo on DNA synthesis of lymphocytes following PHA stimulation in vitro.

Vertical lines represent 95% confidence intervals.
Figure 3. Changes in body weight following infection with Mengovirus-L.

Figure 4. Changes in spleen weight following infection with Mengovirus-L.
although each group demonstrated an increase in both body and spleen weight over the 20-day time period.

Similarly, there was no significant difference in total leukocyte counts (Figure 5) or in differential leukocyte patterns (Figure 6). The percentage of lymphocytes in peripheral blood of Mengovirus-L infected mice appeared lower than the controls (Figure 6), but an analysis of variance on these data revealed that this difference was not significant at the 95% confidence level.

The recovery of Mengovirus-L from brains and spleens using a plaque assay technique was not successful. One possible explanation for this lack of virus recovery may be that the samples thawed for various time periods during storage when the freezer malfunctioned.
Figure 5. Total number of leukocytes in peripheral blood following infection with Mengovirus-L.

Figure 6. Percentage of lymphocytes in peripheral blood following infection with Mengovirus-L.
DISCUSSION

The nature and extent of immunological alteration of the immune system following virus infection has only recently gained considerable attention. A survey of the literature has revealed that lymphoid cells from patients with viral diseases and laboratory animals experimentally infected with viruses can demonstrate abnormal lymphocyte responses to a variety of stimuli (3,4,5,21,33,42,43,44,45,56).

To evaluate the effect of virus infections on immune function, many investigators have dictated the necessity of establishing animal models that would provide understanding of these virus host relationships (33,43,56). In this work, the Swiss-Webster mouse was utilized as a means to determine if alteration in PHA-induced DNA synthesis of lymphocytes in vitro could be used to detect a virus infection in vivo.

Mengovirus, a picornavirus (57), was chosen to represent the viral agent for this study since it is a natural pathogen for mice and provides reliable and recognizable paralytic symptoms which are characteristic for the disease (55,57,58,59). A recent study by Colter, Campbell, and Hatch (58) demonstrated that the primary target organs for Mengovirus in mice were the spleen and lymph nodes and that death ensued only after this agent invaded the central nervous system.

The initial experiment in this study has given support to the premise that viral-induced alterations of a normal physiological state can be detectable on the basis of an altered in vitro lymphocyte
response to PHA stimulation. However, if a virus infection has altered the ability of lymphocytes to undergo mitogen-induced DNA synthesis, can this change in responsiveness to PHA be used as a means of ascertaining a virus-lymphocyte interaction in vivo?

Lymphocytes from infected mice, when cultured in the absence of PHA, did not show any significant change in the incorporation of tritiated thymidine as compared to cells from control mice (Figure 1). These data in conjunction with the studies on cell viability indicate that lymphocytes are not inhibited from maintaining a physiological steady state and agree with earlier studies which demonstrated that nonstimulated virus-infected lymphocytes synthesized macromolecular compounds to the same degree as nonstimulated, noninfected lymphocytes (21,33,42,43,45).

Lymphocytes from virus-infected mice, however, when cultured in the presence of PHA demonstrated altered responsiveness to the mitogen (Figure 2). A marked suppression was evident during the time (3 to 5 days postinfection) when the mice showed overt clinical signs of infection. After 7 days postinfection, the mice did not develop further lumbar paralysis and signs of hyperactivity declined. Concomitant with these observations was the finding that lymphocyte responsiveness to PHA stimulation also returned to normal values.

This suggests that the responsiveness of lymphocytes to PHA stimulation may indicate a direct virus-lymphocyte interaction or may reflect a more complex physiological relationship between virus infectivity and lymphocyte responsiveness.
These results, for the most part, are comparable to the response seen in babies with a congenital rubella virus syndrome. The maximal depression of PHA-induced DNA synthesis in lymphocytes was evident during the infectious phase of the disease and later returned to a normal response once the viremia had disappeared and the overt signs of the infection were gone (21,33).

The mechanism for a viral-induced inhibition of the PHA response remains to be elucidated. Olson, South, and Good (21) have suggested two possible mechanisms for a basis of viral-induced impairment of the PHA response: (i) an alteration of receptor membrane sites at the surface of the lymphocytes, or (ii) a change in metabolic activity by an intracellularly located virus. Häyry et al. (43) similarly proposed that the depression may result from a direct interaction of the virus with the recognition sites at the cell surface, thus chemically or physically blocking the reactive sites that PHA requires to induce a cellular response. However, contra-indicating a surface alteration were observations by Olson et al. (33), which indicated that ultraviolet inactivated rubella virus (still capable of reacting with specific membrane sites) failed to impair lymphocyte responsiveness to PHA stimulation. In addition, New Castle Disease virus which has specific membrane receptors differing from those of rubella virus was shown to depress PHA and PWM-induced DNA synthesis (33). It has been suggested that live virus must enter the cell and initiate intracellular metabolic changes that are inhibitory to the PHA stimulation (33).
Additional examination of virus infectivity such as changes in spleen and body weights and altered leukocyte patterns were not conclusive. Such discrepancies in correlation between virus infection and morphological changes have been previously reported. Force and Stewart (60) demonstrated no effect in total leukocyte counts but showed an increase in the percentage of peripheral blood lymphocytes following infection in mice with Columbia-SK virus, a Mengovirus related member of the picornaviruses.

However, suppression of the lymphocyte response to PHA stimulation during the active phase of a Mengovirus infection suggests that this technique may provide some potential advantages for determining the responsiveness of lymphocytes during virus infections in vivo and perhaps in ascertaining the role lymphocytes play during virus infections.
REFERENCES


