

LYMPHOCYTE ACTIVATION IN SUBACUTE SCLEROSING  
PANENCEPHALITIS VIRUS AND CYTOMEGALOVIRUS  
INFECTIONS

IN VITRO STIMULATION IN RESPONSE TO VIRAL-INFECTED CELL LINES\*

BY G. B. THURMAN,† A. AHMED, D. M. STRONG, R. C. KNUDSEN,  
W. R. GRACE, AND K. W. SELL

(From the Experimental Immunology Division, Clinical Medical Sciences Department,  
Naval Medical Research Institute, National Naval Medical Center,  
Bethesda, Maryland 20014)

(Received for publication 19 June 1973)

The cell-mediated immune system plays an important part in combating certain viral infections (1, 2). The failure to respond to "slow" viruses can result in chronic infections that cause serious pathology or even death (3-5).

Conflicting results have been reported regarding the ability of cell-mediated immune systems to respond to viral infection. For instance, patients suffering from subacute sclerosing panencephalitis (SSPE)<sup>1</sup> are known to produce high titers of antibody (especially in the cerebrospinal fluid) (6). These patients can also respond adequately to various antigens other than the measles virus that has been implicated in this neurologic disorder (7, 8). However, lymphocyte responses to measles antigen either in vitro or in vivo have often produced conflicting results (9, 10). Lischner, Sharma, and Grover (10) have suggested that the difficulties encountered might stem from the impurity, variability, or instability of the antigens used by various investigators. They speculated that measles-induced cell-surface antigens may be a more useful antigenic source than viral antigens.

Recent progress by Horta-Barbosa and associates (11, 12) on the isolation and growth of virus from brain and lymph node tissue of patients with SSPE has made it possible to prepare purified SSPE virus and SSPE virus-infected culture cells. Using

\* Supported by the Bureau of Medicine and Surgery Work Unit no. MR011.0001.002.0009. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. The experiments reported herein were conducted according to the principles outlined in the Animal Welfare Act (PL 89-544 as amended) and followed the guidelines prescribed in Department of Health, Education, and Welfare publication no. (National Institutes of Health) 72-23, formerly Public Health Service publication no. 1024, "Guide for Laboratory Animal Facilities and Care."

† Present address: Division of Biochemistry, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Tex. 77550.

<sup>1</sup> Abbreviations used in this paper: CMV, cytomegalovirus; MEM, minimal essential medium; MIF, macrophage migration inhibition factor; MLICC, mixed lymphocyte-infected cell culture; PHA, phytohemagglutinin; SSPE, subacute sclerosing panencephalitis.

these preparations, it was possible to investigate the status of cell-mediated immunity of SSPE patients to the specific antigens of the causative virus. We have recently reported (13) the preliminary finding of responsiveness of SSPE patients' lymphocytes to purified SSPE virus as measured by the production of macrophage migration inhibition factor (MIF) and to SSPE virus-infected cells as measured by lymphocyte transformation. Interestingly, while MIF production could be stimulated by the measles virus, transformation did not occur with the isolated virus but only when virally infected cells were used.

This report confirms our initial findings with SSPE virus-infected cells and extends the experiments to another type of "slow virus" infection caused by cytomegalovirus (CMV). Uncontrolled infection by CMV occurs not infrequently in patients who have been strongly immunosuppressed for renal or bone marrow transplantation (14, 15). The lymphocytes from patients infected with CMV responded in vitro by production of MIF when cultured with purified CMV but were transformed only by CMV-infected WI-38 cell lines.

#### *Materials and Methods*

*Lymphocyte Separation.*—Venous, heparinized human blood was allowed to sediment 2–3 h. The leukocyte-rich plasma was removed by expulsion through the bent needle of the syringe and centrifuged 10 min at 200 *g* to sediment the leukocytes. The platelet-rich plasma was removed and hard spun (2,000 *g*, 20 min) to remove the platelets before it was used to supplement the culture media. The leukocytes were resuspended in culture media supplemented with antibiotics (penicillin, 100 U/ml; streptomycin, 100  $\mu$ g/ml) and fresh L-glutamine (2 mM). This cell suspension was carefully layered on top of an equal volume of fresh filter-sterilized (0.22  $\mu$ m Millipore [Millipore Corp., Bedford, Mass.]) Ficoll-Hypaque gradient (sp gr 1.078) made by mixing four parts of an 8% (wt/vol) Ficoll (Sigma Chemical Co., St. Louis, Mo.) solution with one part of 50% Hypaque (Winthrop Laboratories, New York). The gradient and cell suspension were centrifuged for 40 min at ambient temperature providing 500 *g* at the interface. The band of cells at the media-gradient interface was carefully aspirated with a pipette and then washed once with media. This routinely gave 98–99% lymphocytes with about a 60% recovery.

*Virus-Infected Cultured Cell Lines.*—The SSPE virus-infected HeLa cells and uninfected HeLa cells were provided courtesy of Doctors Horta-Barbosa and Dietzman (National Institute of Mental Health, National Institutes of Health, Bethesda, Md.).

The Wistar-38 (WI-38), 40th passage, cells were obtained from Mr. Richard Grey (Department of Microbiology, Naval Medical Research Institute, Bethesda, Md.) and were grown in minimal essential media (MEM) supplemented with 10% fetal calf serum, 100 U of penicillin/ml, 100  $\mu$ g streptomycin/ml, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids (100 times solution). All were obtained from Grand Island Biological Co. (New York). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Confluent monolayers were infected with cytomegalovirus strain AD169 supplied courtesy of Dr. Nicholas Dunnick (Division of Biological Standards, National Institutes of Health, Bethesda, Md.). Infected cultures were maintained by transferring media from infected cultures to uninfected cultures. Cytomegalovirus-infected cells used in these experiments had shown cytopathogenic effects for approximately 10 days. Infected and noninfected cells were harvested by trypsinization of monolayers. Cells were then washed twice with MEM and resuspended in MEM with supplements.

The cultured HeLa or WI-38 cells were blocked from further incorporation of tritiated

thymidine in each experiment. Blocking was accomplished by incubation with 25  $\mu$ g of mitomycin C (Calbiochem, San Diego, Calif.) per ml at 37°C for 30 min. Blocked cells were washed twice in RPMI 1640 before use in cultures with lymphocytes.

*Mixed Lymphocyte-Infected Cell Culture (MLICC): Culture Preparation and Conditions.*—Cell concentration of lymphocytes and cultured cells was determined in an Autocytometer II (Fisher Scientific Co., Pittsburgh, Pa.). Lymphocyte concentrations were adjusted to provide 200,000 lymphocytes per 0.10 ml, and cultured HeLa or WI-38 cells were diluted to give either 10,000 or 25,000 cells per 0.05 ml. The plasma concentration in RPMI 1640 was adjusted such that 0.05 ml of the dilution would give a final concentration of 10% in the 0.20 ml cultures. Only results with measles or CMV seronegative plasma are reported here.

Triplicate cultures were prepared in Microtest II microtiter plates (Falcon Plastics, Division of BioQuest, Oxnard, Calif.). Rapid dispensing of lymphocytes, cultured cells, and culture additives was accomplished with Hamilton repeating dispensers (Hamilton Co., Reno, Nev.). Cultures were incubated 72 h at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. 18 h before harvesting 0.02 ml of RPMI 1640 containing 1  $\mu$ Ci of [*methyl*-<sup>3</sup>H]thymidine (sp act 1.9 Ci/mM, Schwarz/Mann Div., Becton, Dickinson, and Co., Orangeburg, N.Y.) was added. Harvesting was accomplished using a Multiple Automated Sample Harvester (MASH) as previously described (16), and the tritiated thymidine incorporation was determined by scintillation spectrophotometry. Data from the scintillation counter were recorded on paper tape and analyzed using a Wang 700C Advanced Programming Calculator (Wang Laboratories, Tewksbury, Mass.).

## RESULTS

*SSPE Virus-Infected Patients.*—Purified peripheral blood lymphocytes from a patient (R.S.) with SSPE showed a significant response to SSPE virus-infected HeLa cells (Table I). R.S.'s lymphocytes cultured with noninfected HeLa cells gave 354 ( $\pm$ 20) cpm. When cultured with SSPE virus-infected cells

TABLE I  
*In Vitro Transformation of Lymphocytes from Patients with SSPE by SSPE Virus-Infected HeLa Cells*

| Source of lymphocytes* | Uptake of [ <sup>3</sup> H]thymidine |                                |                              |
|------------------------|--------------------------------------|--------------------------------|------------------------------|
|                        | 0.1% PHA-P                           | Noninfected†<br>HeLa cells     | SSPE-infected§<br>HeLa cells |
|                        |                                      | <i>cpm <math>\pm</math> SE</i> |                              |
| None                   | —                                    | 227 ( $\pm$ 4)                 | 546 ( $\pm$ 34)              |
| Seronegative control   | 50,976 ( $\pm$ 5,127)                | 397 ( $\pm$ 48)                | 444 ( $\pm$ 19)              |
| Seropositive control¶  | 42,975 ( $\pm$ 3,723)                | 287 ( $\pm$ 38)                | 174 ( $\pm$ 19)              |
| SSPE no. 1**           | 43,388 ( $\pm$ 4,481)                | 354 ( $\pm$ 20)                | 4,656 ( $\pm$ 1,197)         |
| SSPE no. 2‡‡           | 65,801 ( $\pm$ 2,032)                | 599 ( $\pm$ 66)                | 6,651 ( $\pm$ 293)           |

\* 200,000 lymphocytes per 0.2 ml culture.

† 10,000 HeLa cells per culture mitomycin C treated.

§ 10,000 HeLa cells infected with SSPE virus, mitomycin C treated.

|| Seronegative control (A.A.) titer <1:10 (CF).

¶ Seropositive control (S.P.) titer 1:128 (CF).

\*\* Patient (R.S.) with SSPE.

‡‡ Patient (O.H.) with SSPE.

(MLICC), they produced 4,656 ( $\pm 1,197$ ) cpm, which gives a stimulation index of 13.9.

In the MIF results on these same individuals (17), the seropositive control as well as the SSPE patient produced MIF when exposed to the purified SSPE virus. However, Table I indicates that in the MLICC, seropositive control lymphocytes did not show a blastogenic response to the SSPE virus-infected HeLa cells, whereas lymphocytes from the SSPE patients did. None of the lymphocytes responded to the noninfected HeLa cells, and the blastogenic responses to 0.1% phytohemagglutinin P (PHA-P) of all three lymphocyte types were within normal range.

The lymphocyte response reported in Table I was at 72 h of incubation. A similar set of cultures incubated 120 h showed a decrease in response compared with the 72-h cultures. All further cultures were therefore harvested at 72 h.

The results were confirmed by a second experiment with another SSPE patient (O.H.). Lymphocytes from O.H. cultured with noninfected HeLa cells gave 599 ( $\pm 66$ ) cpm. When cultured with SSPE virus-infected HeLa cells, they responded with 7,571 ( $\pm 291$ ) cpm, giving a stimulation index of 12.6. No response to noninfected HeLa cells was seen, and the mitogenic responses were within normal range.

*Cytomegalovirus (CMV)-Infected Patients.*—To test if this assay system was effective in determining the reactivity of lymphocytes from other types of viral-infected patients, the response of lymphocytes from CMV-infected patients in the MLICC was measured using CMV-infected WI-38 cells. The results indicate specific responses similar to those seen in the SSPE cases (Table II).

Peripheral blood lymphocytes from CMV-infected patients responded in vitro to CMV-infected WI-38 cells. The response was partially masked if the WI-38 cells were not pretreated with mitomycin C. Lymphocytes from the CMV-infected patient (D.R.) when cultured with noninfected WI-38 cells gave 4,560 ( $\pm 394$ ) cpm, whereas when cultured with CMV-infected WI-38 cells, they gave 15,991 ( $\pm 1,506$ ) cpm. This gives a stimulation index of 3.5, due to the high tritiated thymidine incorporation of the unblocked WI-38 cells. Neither the seronegative nor seropositive control lymphocytes responded to the CMV-infected WI-38 cells. Experiments with lymphocytes from another CMV-infected patient (C.W.) using mitomycin C-blocked CMV-infected WI-38 cells were confirmatory. C.W.'s lymphocytes with noninfected WI-38 cells gave 418 ( $\pm 29$ ) cpm, whereas with the infected WI-38 cells they gave 5,356 ( $\pm 314$ ) cpm. The resulting stimulation index of 12.8 is in the range of the stimulation indices observed in the SSPE cases and when compared with the response to unblocked WI-38 cells indicates the importance of mitomycin C treatment of the stimulating cells.

#### DISCUSSION

The most significant finding to emerge from these experiments is that lymphocytes from SSPE virus or CMV-infected patients can respond in a mixed

TABLE II  
*In Vitro Transformation of Lymphocytes from Patients with Cytomegalovirus Infections Using CMV-Infected WI-38 Cells*

| Source of lymphocytes* | Uptake of [ <sup>3</sup> H]thymidine |                          |                           |
|------------------------|--------------------------------------|--------------------------|---------------------------|
|                        | 0.1% PHA-P                           | Noninfected WI-38 cells† | CMV-infected WI-38 cells§ |
|                        |                                      | <i>cpm ± SE</i>          |                           |
| None                   | —                                    | 356 (± 39)               | 538 (± 73)                |
| Seronegative control   | 65,749 (±4,134)                      | 487 (± 38)               | 456 (± 11)                |
| Seropositive control¶  | 53,611 (±3,120)                      | 339 (± 27)               | 552 (± 49)                |
| CMV patient no. 1**    | 32,774 (±2,027)                      | 4,560 (±394)‡‡           | 15,991 (±1,506)           |
| CMV patient no. 2§§    | 39,649 (±1,986)                      | 418 (± 29)               | 5,356 (± 314)             |

\* 200,000 lymphocytes per 0.2 ml culture.

† 10,000 WI-38 cells per culture mitomycin C treated except for data on CMV patient no.

1.

§ 10,000 WI-38 cells infected with CMV. The infected WI-38 cells were treated with mitomycin C except for data on CMV patient no. 1.

|| Seronegative control (A.A.) titer <1:4 (CF).

¶ Seropositive control (M.L.) titer 1:64 (CF).

\*\* Patient (D.R.) with CMV infection.

‡‡ Response to nonmitomycin C-treated WI-38 cells.

§§ Patient (C.W.) with CMV infection.

culture to tissue culture cells infected with their specific type of virus. Such a response indicates that the etiology of the diseases is not an inability to respond to the virus *in vivo* through a defect in cell-mediated immunity (9, 10). Indications are that the lack of response is due to a factor present in the plasma and cerebrospinal fluids of the patients that blocks the response of immunocompetent lymphocytes (13, 17).

Rosenberg, Farber, and Notkins (18) report 30-fold stimulation of sensitized, rabbit splenic lymphocytes *in vitro* with ultraviolet (UV) light-inactivated herpes simplex virus and vaccinia virus. However, extensive experiments (4,000 cultures) in our laboratories using active and UV-inactivated measles virus, SSPE virus, and CMV strain AD169 cultured *in vitro* with lymphocytes from SSPE or CMV-infected patients and controls failed to show any meaningful stimulation.

Our studies using the macrophage migration inhibition factor (MIF) assay first indicated the reactivity of the patient's lymphocytes and the presence of a blocking factor in the plasma and cerebrospinal fluid of the virus-infected patients (13, 17). However, in the MIF assay, measles seropositive controls also showed MIF production in response to the SSPE virus, and this response could be eliminated by the blocking factor. In the transformation studies reported here, there was no response of the measles seropositive controls to the SSPE virus-infected HeLa cells. The exact meaning of this observation is presently unclear. It may be that different antigens are involved in stimulating the dif-

ferent responses measured, i.e., viral envelope antigens stimulating MIF production, and viral-induced cell surface antigens causing the lymphocyte blastogenesis. The lack of blastogenic response by the positive control to the SSPE virus-infected cells would seem to indicate nonsensitization to the new cell surface antigens induced by the SSPE viral genome. Studies are needed to investigate the responsiveness of controls and SSPE patients to measles virus-infected cell lines and to determine the extent of cross-reactivity between the cell surface antigens from measles and SSPE virus-infected cell lines. The cultured cell lines may only serve to present the viral antigens in a stimulatory form to the responsive lymphocytes, but this does not account for the discrepancy between the positive response of the measles seropositive control in the MIF assay and the negative response in the MLICC assay. The possibility that the SSPE virus may be an altered measles virus or a mutant of measles virus has been suggested (5). In the MIF assay, one is measuring the release of preformed cellular products in response to specific antigen, whereas in blastogenesis, there is an active process whereby presensitized cells undergo DNA synthesis in response to specific antigens. MIF is measured 24 h after the contact of viral antigen with sensitized cells, whereas in blastogenesis the response is measured 72 h after the contact of viral antigen with sensitized cells. It is possible that viruses are neutralized by *in vitro* interferon production by sensitized cells and therefore not available for stimulation of the cells, whereas using virus-infected cells, the antigens are so provided that cells do not respond by producing interferon and therefore a good source of antigen for specific stimulation.

The implications of these findings in the clinical management of patients with slow virus diseases is significant. The fact that the patients' lymphocytes have the capacity to respond to the viral-infected cells argues against the possible therapeutic use of transfer factor or immune lymphocyte transfusions. The role of patients' plasma and spinal fluid in the MLICC assay is currently under investigation. The possibility of such assays utilizing viral-infected cell lines could prove to be a very important tool for assessing the cellular immune function of patients with chronic slow virus diseases.

#### SUMMARY

Efforts to stimulate lymphocytes from measles seropositive and two patients with subacute sclerosing panencephalitis (SSPE) with either commercially available measles virus or virus isolated from a known case of SSPE failed to show any significant data using a microculture assay. Similar results were obtained using lymphocytes from two patients with active cytomegalovirus (CMV) infections and CMV seropositive individuals using CMV suspensions.

On the other hand, lymphocytes from the patients with subacute sclerosing panencephalitis exhibited *in vitro* blastogenesis in culture with SSPE virus-infected HeLa cells. Similarly, lymphocytes from the CMV-infected patients demonstrated blastogenesis when cocultivated with CMV-infected WI-38 cells.

This affords a new method for determining the cell-mediated immune capacity of patients with "slow" virus diseases.

The authors wish to acknowledge the helpful discussions of this research data with Doctors Dietzman, Horta-Barbosa, and Dunnick at the NIH and Doctors J. L. Curry, R. L. Wistar, D. W. Bailey, J. Miller, W. L. Brannon, the staff of the Pediatric Clinic, Neurology Clinic, and the Transplantation Unit of the Naval Hospital, Bethesda, Md. The authors also wish to acknowledge the technical help of Mr. R. Grey, Hospitalman Chief (HMC) Polbos, and HMC Uy, and Miss Kathy Gerstenberg for typing of the manuscript.

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