Blastogenic Response of Human Lymphocytes to Early Antigen(s) of Human Cytomegalovirus

JOSEPH L. WANER,1†† NANCY KONG,1 AND STELLA BIANO2

Department of Tropical Public Health, Harvard School of Public Health,1 and Brigham and Women’s Hospital,2 Boston, Massachusetts 02115

Received 12 October 1982/Accepted 17 May 1983

The lymphocytes of asymptomatic, seropositive donors demonstrated blastogenic responses to early antigens of human cytomegalovirus whether or not antibodies to early antigens were detectable. The lymphocytes of six of nine patients with active cytomegalovirus infections gave stimulation indexes of ≥2.00 with antigens of productively infected cells, whereas only two patients demonstrated comparable stimulation indexes with early antigens. Four patients with stimulation indexes of ≥2.00 to productively infected antigens had stimulation indexes of ≤2.00 to early antigens. Viral polypeptides with molecular weights of 83,000, 72,000, 62,000, 56,000, and 40,000 were identified in early antigen preparations.

Viral polypeptides are produced in cells infected with human cytomegalovirus (CMV) before viral DNA synthesis or in the presence of an inhibitor of DNA synthesis (4, 18, 19). The polypeptides synthesized immediately after the removal of an inhibitor of protein synthesis applied at the time of infection are identified as immediate early polypeptides; other early polypeptides are subsequently synthesized in the presence of an inhibitor of DNA synthesis and appear to require the prior synthesis of the immediate early polypeptides (19).

Immediate early antigens are detected within 1 h of infection by indirect immunofluorescence (5, 11, 15), immunoprecipitation (1, 10, 20), and synthesis in vivo and in vitro (3). Early antigens (EAg) are identified similarly in infected cells incubated for as long as 3 days with an inhibitor of DNA synthesis (22, 23).

This study was conducted to determine the status of lymphocyte blastogenic responses to EAg of normal blood donors and of patients excreting CMV. The blastogenic responses of lymphocytes from patients with active CMV infections to CMV antigens prepared from productively infected (PI) cells are depressed (6, 9, 16, 17). Early antibodies (EAb), i.e., antibodies against EAg, are indicative of current or recent active infections but may be undetectable in the healthy but conventionally seropositive population (7, 22). The concurrent cell-mediated responses to EAg may be important in assessing the immune response to CMV since EAg are the first viral products seen by the host in primary or reactivated infections.

MATERIALS AND METHODS

Cell cultures and virus. Human fibroblast cell cultures derived from fetal lung tissue were grown at 36°C in 1-liter Brockway bottles or roller bottles (285 by 110 mm) and maintained in Eagle minimal essential medium supplemented with 5% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (MEME).

The Davis strain of CMV (passages 80 to 87) was grown and maintained as described previously (24). Cell cultures were free of mycoplasma, as determined by culture techniques and electron microscopy.

Radiochemicals. [3H]Thymidine ([3H]TdR; specific activity, 20 Ci/mmol) and [14C]methionine (specific activity, 1,000 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. 14C-labeled molecular weight standards were the generous gift of Michael Worley, Harvard School of Public Health, Boston, Mass.

Indirect fluorescent-antibody test. The preparation of antigens for the indirect fluorescent-antibody test was by previously described methods (24). Antigens for the determination of antibody to CMV antigens of PI cells consisted of infected cells incubated for 6 days; EAg for determining EAb titers consisted of infected cells incubated for 72 h with 50 μg of cytomeosid (Calbiochem, La Jolla, Calif.) per ml.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (8), employing 5% stacking gels and 7.5% resolving gels of 1.5-mm thickness. Molecular weight standards were electrophoresed in each gel and consisted of phosphorylase b (molecular weight, 92,500), bovine serum albumin (molecular weight, 68,000), ovalbumin (molecular weight, 43,000), and carbonic anhydrase (molecular weight,
30,000). After electrophoresis, gels were fixed in 25% methanol–10% acetic acid for 1 h and processed for fluorography (2). Gels were exposed to Kodak SB-5 film for 1 to 7 days at −70°C.

Preparation of antigens for blastogenic assays and SDS-PAGE analysis. Cell cultures were infected at a multiplicity of infection of 1. After adsorption for 2 h at 36°C on a rotating table, the inoculum was poured off, and the cultures were refed and then incubated for 3 days with maintenance medium containing 50 μg of cytosine arabinoside per ml. CMV and control EAg for blastogenic assays were prepared from infected and uninfected (also incubated with cytosine arabinoside) cell cultures, respectively, by the glycine-buffered saline (GBS; 0.05 M glycine, pH 8.0) extraction procedure (24). Cells were dislodged with a rubber policeman into GBS, washed three times with GBS by consecutive centrifugations at 250 × g for 10 min, and resuspended in GBS to make a 10% suspension. The suspension was disrupted by sonic treatment and centrifuged at 250 × g for 10 min; the resulting supernatant constituted the antigen. CMV and comparable control antigens prepared from PI or uninfected cells for blastogenic assays were prepared similarly except that cytosine arabinoside was not incorporated into the maintenance medium, and incubation was continued for a total of 6 or 7 days.

Radiolabeled CMV and control EAg were prepared as described above, except that after the adsorption period, the cell cultures were incubated in MEME containing 50 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml; 16 h later, the medium was poured off, and the cell culture was washed three times with MEME, each wash of 10-min duration. The cultures were subsequently incubated for a total of 72 h, except that the maintenance medium contained 60% of the normal concentration of methionine and 20 μCi of [35S]methionine per ml. Samples of EAg or comparable control antigens for SDS-PAGE analysis were mixed with an equal volume of 2x solubilizing buffer containing 0.05 M Tris-hydrochloride (pH 6.8), 20% glycerol, and 10% SDS; immediately before electrophoresis, β-mercaptoethanol was added to make a 1% solution, and the mixture was heated at 100°C for 4 min.

Collection of lymphocytes and sera. Approximately 20 ml of venous blood was collected into 50 μg of preservative-free heparin per ml and centrifuged through a Ficoll-Hypaque gradient, as previously described (25), to obtain peripheral blood lymphocytes. A portion of the blood was allowed to clot, and the serum subsequently was obtained after centrifugation at 250 × g.

Blast transformation assay. Cellular viability was determined by the dye exclusion method, using 0.2% trypan blue. Lymphocyte cultures were prepared in plastic tubes (17 by 100 mm) by suspending 10⁶ viable cells in 0.8 ml of RPMI 1640 medium supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml. RPMI 1640 medium was also used as a diluent for the antigens. Each culture received 0.1 ml of homologous plasma (obtained from the top of the Ficoll-Hypaque gradient) and 0.1 ml of EAg, PI antigens, control antigens (EAg or PI), or medium; a previous report showed that the presence or absence of antibody in the plasma does not affect the blastogenic assay (25). Cultures were incubated at 37°C in a 5% CO₂ atmosphere for 7 days. Then, 1 μCi of [3H]ThdR was added to each culture, and 8 h later the cultures were centrifuged at 500 × g for 20 min at an ambient temperature. Supernatants were discarded, the tubes were placed in an ice bath, and the cell pellets were suspended in 1 ml of 0.2 N perchloric acid to obtain the acid-precipitable fraction. Each sample was filtered through a 2.4-cm Whatman GF/CR filter paper disk; each disk was placed in 10 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) scintillation fluid and counted in a scintillation counter. Lymphocyte cultures were prepared in triplicate, and mean counts were determined. Reactions were considered positive if the incorporation of [3H]ThdR by the stimulated cultures was at least twice that of the comparable cultures incubated with control EAg or PI antigens. The stimulation index (SI) was calculated by dividing the number of counts per minute incorporated into CMV antigen cultures minus the counts per minute of background by the number of counts per minute incorporated into control antigen cultures minus the background counts per minute. Cultures incubated only with medium provided the background incorporation values.

The optimum dilution of EAg for use in assays of lymphocyte blastogenesis was determined for each preparation of EAg. Twofold dilutions of EAg and of control EAg were tested in cultures of lymphocytes obtained from a donor who was known to react to EAg. When the optimum dilution of an EAg preparation was determined, that dilution of EAg and of control EAg was used in subsequent determinations.

RESULTS

Blastogenic responses of lymphocytes of asymptomatic donors to CMV EAg. The peripheral blood lymphocytes of healthy male and female adult donors (ages, 22 to 44 years) with antibody to PI antigens showed marked blastogenic activity against EAg (Table 1); the peripheral blood lymphocytes of eight donors seronegative for PI antigens did not respond to EAg (data not shown). Background levels of [3H]ThdR were below 10%. Five of the eight donors also showed

<table>
<thead>
<tr>
<th>Donor</th>
<th>Control EAg</th>
<th>CMV EAg</th>
<th>PIAb</th>
<th>EAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,174 ± 32</td>
<td>6,711 ± 160</td>
<td>5.7</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>3,271 ± 87</td>
<td>60,242 ± 402</td>
<td>18.4</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>2,317 ± 39</td>
<td>26,231 ± 310</td>
<td>11.3</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>2,913 ± 63</td>
<td>65,467 ± 621</td>
<td>22.5</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>2,780 ± 71</td>
<td>104,930 ± 860</td>
<td>37.7</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>2,616 ± 68</td>
<td>46,183 ± 550</td>
<td>17.7</td>
<td>32</td>
</tr>
<tr>
<td>7</td>
<td>3,030 ± 91</td>
<td>38,191 ± 510</td>
<td>12.6</td>
<td>32</td>
</tr>
<tr>
<td>8</td>
<td>5,928 ± 55</td>
<td>50,987 ± 650</td>
<td>8.6</td>
<td>16</td>
</tr>
</tbody>
</table>

a PIAb, Antibody to antigens of PI cells.
low titers of EAb. The peripheral blood lymphocytes of three donors with no detectable EAb (<1:8) did, however, show substantial blastogenic activity against EAg. There was no correlation between PI antibodies or EAb titers and the degree of blastogenic activity to EAg.

**Blastogenic responses of lymphocytes of patients with active CMV infections to CMV EAg and PI antigens.** CMV was isolated in cell cultures of diploid human fibroblasts from the urine of nine patients (Table 2). Patients 6 and 7 were healthy and without clinical indications of the disease. Patients 1 through 5 had clinical findings consistent with the diagnosis. Patients 8 and 9 were 2 and 2.5 months, respectively, beyond receipt of their allografts. All of the patients had detectable PI antibodies (titers ranging from 8 to 256), and eight of nine patients had detectable EAb. Blastogenic responses to PI antigens were depressed (SI < 2.00) in three of the nine patients. Six of the patients had SIs to PI antigens of 2.00 or greater, whereas only two patients demonstrated comparable SIs to EAg. Four patients with SIs of 2.00 or greater to PI antigens had SIs of less than 2.00 to EAg. There was no correlation between antibody titers to PI antigens or EAg and blastogenic responses.

**SDS-PAGE analysis of CMV EAg preparations.** Viral polypeptides were identified by their different rates of electrophoretic mobility and enhanced incorporation of radiolabel relative to polypeptides observed in comparably treated control preparations. Analysis by SDS-PAGE of EAg preparations obtained from cell cultures continuously labeled with [35S]methionine from 16 to 72 h after inoculation revealed five polypeptides (molecular weights, 83,000, 72,000, 62,000, 56,000, and 40,000) not resolved from comparable control preparations (Fig. 1). The bands appearing at molecular weights of 72,000 and 56,000 were consistently the most prominent and reliably observed of 10 EAg preparations analyzed; these were the only polypeptides identified in preparations in which the 16-h cycloheximide block was not employed. The 40,000-molecular-weight band was least prominent and was not observed in all preparations. The experimental protocol employed did not permit characterizing the five polypeptides as to immediate early or early polypeptides.

<table>
<thead>
<tr>
<th>Table 2. Lymphocyte responses and antibody titers to CMV EAg and PIAg in patients with active CMV infections</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1 Hepatitis</td>
</tr>
<tr>
<td>2 Hepatitis</td>
</tr>
<tr>
<td>3 Hepatitis</td>
</tr>
<tr>
<td>4 Heterophile-negative mononucleosis</td>
</tr>
<tr>
<td>5 Heterophile-negative mononucleosis</td>
</tr>
<tr>
<td>6 Heterophile-negative mononucleosis</td>
</tr>
<tr>
<td>7 Mother^c of CID^d baby</td>
</tr>
<tr>
<td>8 Renal transplant</td>
</tr>
<tr>
<td>9 Renal transplant</td>
</tr>
</tbody>
</table>

^a PIAg, Antigen of PI cells.
^b CMV was isolated from the urine of each patient.
^c PIAb, Antibody to antigens of PI cells.
^d Clinically asymptomatic.
^e CID, Congenital cytomegalic inclusion disease.
LYMPHOCYTE RESPONSE TO EAg

DISCUSSION

Healthy donors with antibodies to PI antigens were shown in this study and in a previous study (24) to also have EAb to EAg. The significance of EAb as an indicator of active infection, disease, or both, therefore, requires detection at some threshold value in a single specimen of serum or detection of fourfold or greater rises in paired sera; The et al. identified an EAb titer of 1:40 as significant (22). Numazaki et al. reported geometric mean titers of EAb of 30.4 and 59.0 in infants with hepatitis or hepatosplenomegaly, respectively; healthy infants of the same age group had geometric mean titers of 13.2 (12).

Healthy seropositive (antibodies to PI antigens) donors with relatively low or no detectable EAb showed significant blastogenic responses to EAg (SI ≥ 2.00). Donors without PI antibodies (13, 21, 25) and EAb do not exhibit lymphocyte blastogenesis to either antigen. It appears that a cell-mediated immune response to EAg is maintained in healthy PI antibody-reactive donors in the absence of detectable EAb by the indirect fluorescent-antibody test; a more sensitive serological test, however, might detect EAb. Conserving a cell-mediated immune response to EAg may be important for the host in dealing with a reactivation of a latent infection or a new exposure to an exogenous virus. Cytolytic T-cells (14) which require immune memory could be stimulated to proliferate and become active upon exposure to EAg, the first viral product(s) synthesized in a productively infected cell.

The association of larger titers of EAb with active CMV infection in patients appears to be a consequence of viral replication. High antibody titers to EAg and PI antigens but depressed blastogenic activities in the same patients demonstrate the selective depression of cell-mediated immune responses during active CMV infection. The decreasing levels of EAb in patients recovering from active CMV infections (7, 22) is prognostic of well-being but is not an explanation of cause. A prospective study of cell-mediated immune responses to EAg in recovering CMV patients may provide insight into the effector mechanism and target antigens most pertinent to recovery and immunity.

The number and molecular weights of the early polypeptides identified in this study are consistent with earlier reports (11, 19, 20). Recent studies have shown, however, that there are more early polypeptides synthesized than originally believed. Cameron and Preston have identified 11 immediate early polypeptides synthesized in vitro and in vivo (3). Blanton and Tevethia have reported, by immunoprecipitation techniques, at least 20 polypeptides synthesized before viral DNA synthesis (1). These findings suggest regulatory functions for the early polypeptides. Their role, individually or collectively, in the host immune response to active or latent CMV infections remains to be determined.

ACKNOWLEDGMENTS

We thank Lola Brodfield for excellent technical assistance. This work was supported in part by Public Health Service grant AI-01023 from the National Institute of Allergy and Infectious Diseases and a grant from the United Cerebral Palsy Research and Educational Foundation, Inc.

LITERATURE CITED

1088 WANER, KONG, AND BIANO


