Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 6134–6138, October 1984
Immunology

Cytomegalovirus infects human lymphocytes and monocytes: Virus expression is restricted to immediate-early gene products

(abortive infection)

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Communicated by Gertrude Henle, June 14, 1984

ABSTRACT **In this investigation, we studied the ability of human cytomegalovirus to infect peripheral blood mononuclear cells. With monoclonal antibody technology, we demonstrated that cytomegalovirus could infect human lymphocytes of T- and B-cell lineage, natural killer cells, and monocytes. Furthermore, virus expression was limited to the synthesis of immediate-early cytomegalovirus polypeptides. These peripheral blood mononuclear cells did not produce infectious virus, nor were mature virions visualized by electron microscopy. This abortive infection of mononuclear cells was most convincingly shown with stocks of cytomegalovirus that had been recently isolated from infected patients and passaged minimally in fibroblasts. This argues for an increased lymphotropic effect of some isolates of cytomegalovirus, compared to strains of virus that are extensively adapted to growth in fibroblasts. Furthermore, immunocompetent cells that were shown to be abortively infected with cytomegalovirus lost selected differentiated functions.**

Human cytomegalovirus (CMV) infection is frequently associated with severe immunosuppression, especially in the clinical settings of allograft transplantation (1) and the acquired immunodeficiency syndrome (2). Where the virus is harbored, how the viral genome is expressed, and whether the immunosuppression is mediated directly by the virus or indirectly by another mechanism are important, but as yet unanswered, questions. Former studies with murine CMV in our laboratory suggested that this virus is harbored in a latent state in a subset of B-lymphocytes (3). However, until now, workers in several laboratories failed to demonstrate similar CMV infection of human peripheral blood mononuclear cells (PBM). Infectious CMV has occasionally been found in buffy-coat preparations (4–6) obtained from patients with clinical CMV infection; only rarely has the virus been isolated from healthy donors (7). Neither the exact cell involved in the leukocyte fraction nor the state of the virus in such cells has been established. When transformed lymphoblastoid and erythroleukemia cell lines were studied for the ability to support CMV replication, some researchers found evidence of short-term virus replication (8, 9), but rarely was infectious virus produced (10, 11). There have been occasional reports of expression of CMV antigens on B-lymphoblastoid cell lines (12). Attempts to infect normal PBM from healthy donors with laboratory-adapted strains of CMV have failed (11, 13).

In the cascade regulation of the CMV genome (14), immediate-early protein synthesis precedes early polypeptide synthesis, both of which set the regulatory state for late polypeptide synthesis and production of mature virions. The early class polypeptides appear to have predominantly regulatory functions; the late polypeptides have mainly structural functions. We reasoned that if lymphocytes were abortively infected, techniques such as cocultivation assays and probes for late CMV gene products would be negative. Hence, we looked for CMV expression in peripheral blood lymphocytes (PBL) with monoclonal antibodies specifically produced to detect polypeptides relevant to major epochs in the cascade regulation of the viral genome. Prompted by observations of biological differences between strains of virus recently derived from infected patients and the fibroblast-adapted strains of virus (15), we also studied both forms of this virus.

MATERIALS AND METHODS

Lymphocyte Infection. PBM were removed from the blood of healthy human donors by density gradient centrifugation on Ficoll-Paque and infected at a multiplicity of 0.01–1.0 with CMV recently isolated from patients with various CMV syndromes (Table 1), or with stocks of plaque-purified laboratory-strain AD-169. The recent isolates were propagated in human foreskin fibroblasts (Flow Laboratories) for <12 passages. Because low-passage isolates of human virus are associated predominantly with the cell matrix (15), infected or mock-infected fibroblasts were sonicated, and this material was cultured with PBM for up to 6 days. Thereafter, the PBM were washed 3 times and prepared for immunofluorescence studies by air-drying and fixing the cells with acetone on glass slides. In some experiments, T-lymphocytes were positively selected from the PBM cultures by erythrocyte-rosetting techniques (17) or by sorting on a fluorescence-activated cell sorter (16).

Immunofluorescence Techniques. Monoclonal antibodies were raised against CMV-infected fibroblasts with standard techniques (18). CMV polypeptide specificities were determined by immunofluorescence and standard radioimmunoprecipitation techniques and polyacrylamide gel electrophoresis (19), as outlined in Table 2. After these monoclonal antibodies were allowed to react with the cells, bound antibody was determined by indirect immunofluorescence with fluorescein-labeled pepsin-digested goat antibody to mouse immunoglobulin and by fluorescence microscopy. As specificity controls, we demonstrated that the CMV-specific monoclonal antibodies did not bind either to uninfected fibroblasts or to PBM and that the second antibody did not stain cells by itself. To determine mononuclear cell phenotype, we used standard markers: helper T-cells, OKT4; suppressor/cytotoxic T-cells, OKT8; natural killer (NK) cells, Leu-7; monocytes, Mo2; and B-lymphocytes, antibody to surface immunoglobulin. These techniques have been described (16).

Lymphocyte Function Assays. We tested the ability of infected cells to proliferate in response to phytohemagglutinin (PHA) and a viral antigen, herpes simplex. In these experi-

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Abbreviations: CMV, cytomegalovirus; PBM, peripheral blood mononuclear cell(s); PHA, phytohemagglutinin; NK cell, natural killer cell; ADCC, antibody-dependent cell-mediated cytotoxicity; PBL, peripheral blood lymphocyte(s).

Table 1. CMV polypeptide expression in PBM

CMV isolate*	Source of CMV	Fibroblast passage no.	CMV antigen expression†	
			Immediate-early	Late
I-G	Adult CMV mononucleosis	12	+ + +	Nil
I-R	Congenital CMV	8	+ + +	Nil
I-B	Bone marrow transplantation	8	+ + +	Nil
I-P‡	Bone marrow transplantation	12	Nil	Nil
I-S	Bone marrow transplantation	5	+ + +	Nil
I-M	Congenital CMV	8	+ + +	Nil
I-L	Renal transplantation	3	+ + +	Nil
I-J	Bone marrow transplantation	10	+ + +	Nil
AD-169§		50+	+	Nil

*CMV isolates from patients were provided by M. Hirsch (Massachusetts General Hospital, Boston, MA), T. Merigan (Stanford Medical School, Stanford, CA), S. Plotkin (Children's Hospital, Philadelphia, PA), and G. Quinnan (Bethesda, MD). AD-169 was obtained from the American Tissue Culture Collection in Rockville, MD.

†PBL in these experiments were cultured with sonicates of virus-infected or mock-infected fibroblasts for 1–4 days, and then prepared for immunofluorescence studies. CMV antigen expression was determined with murine monoclonal antibodies L-14, E-3, C-5, F-3, and I-2 (see Table 2, and ref. 16). Bound immunoglobulin was probed with a fluorescein-labeled goat antibody to mouse immunoglobulin and fluorescence microscopy. The number of PBM expressing the immediate-early CMV antigen ranged from 1% to 15%. Fluorescence intensity of infected cells is indicated by the number of "+" signs.

‡I-P failed to induce immediate-early polypeptide synthesis in PBL in 3 of 3 experiments. We are continuing to study the behavior of this isolate after repeated passage in tissue culture.

§Immediate-early antigen expression was seen in only 1 of 10 experiments. In that experiment, <1% of the cells was weakly positive.

ments, PBL were cultured on infected or mock-infected fibroblast monolayers for 4 days, removed from the cultures, washed, and recultured (2×10^5 cells per well) with PHA (GIBCO; 1 μg per well) or viral antigen (10^5 plaque-forming units of heat-inactivated herpes simplex virus, MacIntyre strain), for an additional 4 days. [^{3}H]Thymidine (1 μCi per well; 1 Ci = 37 GBq) incorporation was measured in the final 24 hr. The viability of mononuclear cells was identical in infected and mock-infected cultures. NK cell function was measured against K-562 target cells and antibody-dependent cell-mediated cytotoxicity (ADCC) was indicated by the lysis of antibody-sensitized P-815 cells, in a 6-hr chromium release assay as described (17).

RESULTS AND DISCUSSION

Low-Passage Human Isolate Strains of CMV Infect PBM. Using immunofluorescence techniques, we found immediate-early, but not late gene products in PBM infected with CMV recently isolated from infected patients. The immediate-early gene product was detectable within 24 hr after infection and persisted as long as 6 days. Depending on the isolate of CMV studied, <1%–15% of PBM expressed the major 72-kDa immediate-early protein, the average being ≈3%. The higher the input multiplicity of virus, the greater the number of PBM expressing immediate-early viral antigen. However, even with multiplicities as high as 10, only a small percentage of lymphoid cells could be infected (data not shown). Representative data from several experiments are shown in Table 1. We were able to show immediate-early antigen expression with seven of eight recent isolates of CMV, and for each given isolate, this was a consistent phenomenon upon retesting.

The appearance of peripheral blood T-cells infected with a recent isolate of CMV and probed for CMV antigen expression with the monoclonal antibody L14, is shown in Fig. 1. T-lymphocytes in this example were purified from cultures of infected PBM by two sheep erythrocyte-rosetting procedures and were >99% pure. When probed with the monoclonal antibody L-14, specific to the major 72-kDa immediate-early protein, 15% of the cells exhibited nuclear or perinuclear fluorescence. The same result was obtained with another monoclonal antibody reagent also specific to the major immediate-early gene product (19) but not with monoclonal antibody reagents specific to late gene products. The same phenomenon was demonstrated in T-lymphocytes that had been selected from the cultures by cell-sorter technology. Hence, the infection in PBM and in purified T-lymphocytes appeared to be abortive. In corroboration, we could not detect mature virions in these cells by electron microscopy, nor was infectious virus demonstrable by infectious center assays (data not shown). Einhorn and Ost have recently described a similar restricted infection of human mononuclear cells by CMV (20).

Restricted infection with human CMV has been described in some mouse (21) and rabbit (22) cell lines. Virus transcription in these two models may be blocked in a manner common to both. DeMarchi has provided evidence that productive and nonproductive infection may differ at the level at which some early transcripts associate with polysomes (23). We are currently investigating this possibility in lymphocytes infected with CMV.

CMV infection of PBL was most easily demonstrable with the low-passage human isolates. In only 1 of 10 experiments were we able to show immediate-early antigen expression in

Table 2. Monoclonal antibodies specific to CMV

Monoclonal antibody	Major class of polypeptide recognized	Ig subclass	CMV polypeptide specificity	CMV polypeptide appearance in infected fibroblasts, hr after infection
L-14*	Immediate-early	IgG1	72 kDa	3
E-3†	Immediate-early	IgG1	72 kDa	3
C-5†	Late	IgG1	60 kDa	24
F-3*	Late	IgG1	‡	24
I-2*	Late	IgG1	‡	24

*Monoclonal antibodies developed in our laboratory using a modification of the Kohler–Milstein procedure (18). Immunologic specificity was shown by immunofluorescence and radio-immunoprecipitation techniques and polyacrylamide gel electrophoresis.

†Generously provided by L. Goldstein (19).

‡Not determined.

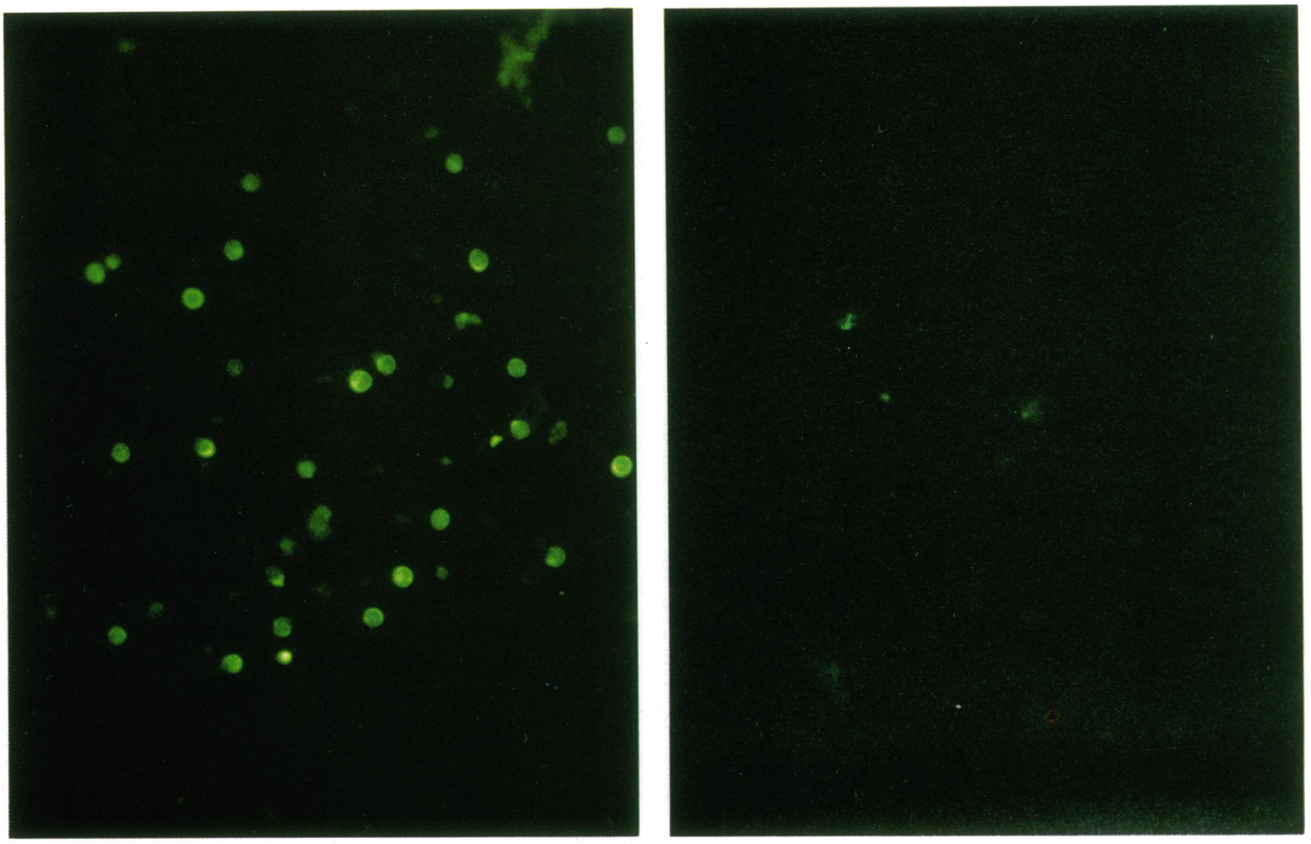

FIG. 1. PBM from a healthy blood donor were cultured 4 days with fibroblasts previously infected with the CMV isolate strain I-G. T-lymphocytes were then isolated from the cultures by two erythrocyte rosettings (19), plated onto glass slides, dried, and fixed for 10 min in acetone. Monocyte and B-lymphocyte contamination was <0.1%, as determined by immunofluorescence. Immediate-early antigen was probed with monoclonal antibody L-14 and indirect immunofluorescence. (*Left*) 15% of the cells exhibited nuclear or perinuclear fluorescence. These T-lymphocytes were not fluorescent when probed for late CMV antigen with the monoclonal antibody C-5 (19) (*Right*).

PBL inoculated with AD169, a strain that has been extensively adapted to growth in fibroblasts. In this instance, <1% of mononuclear cells faintly expressed viral antigen. Contrariwise, we easily demonstrated immediate-early antigen expression with 7 of 8 low-passage human isolates tested (Table 1). In the few opportunities for study, there was a correlation between the passage number of an isolate, and the inability of that isolate to infect mononuclear cells (Table 1, I-P). We are continuing to study the effect of repeated tissue-culture passage on the behavior of these isolates. This dichotomy in the behavior of freshly isolated and laboratory-adapted strains of CMV has recently been corroborated by Einhorn and Ost (20), who also found a differential tropism of mononuclear cells for the different kinds of virus. This may be an important phenomenon among the herpesviruses. Sixbey *et al.* (24) showed that recently isolated, but not laboratory-adapted strains, of Epstein–Barr virus could infect human epithelial cells and, similarly, that viral genomic expression was incomplete in infected cells. Whether our data represent a differential cell tropism among individual CMV strains or an adaptation of laboratory strains to preferential growth in fibroblasts, or both of these factors, remains to be defined.

CMV Can Infect T- and B-Lymphocytes, NK Cells, and Monocytes. To further substantiate our finding that CMV infected lymphocytes, we used a two-color immunofluorescence technique (16). With monoclonal antibodies specific to lymphocyte subclasses and to CMV gene products, we identified the kinds of mononuclear cells that could be infected by CMV. Table 3 lists the proportions of infected mononuclear cells that expressed the major 72-kDa immediate-early polypeptide, as detected by monoclonal antibody L-14. In the experiment in Table 3 (which was representative of three), 3.2% of the total mononuclear cell population expressed a polypeptide detectable by L-14. Similar results were obtained with E-3 (16). As before, late antigens were not detectable in these mononuclear cells. As shown in Table 3, small percentages of T-lymphocytes of helper and suppressor phenotype, B-lymphocytes, and NK cells could be infected by CMV. These data should be interpreted in light of the functional heterogeneity in subsets of mononuclear cells phenotyped by monoclonal antibodies (25–28). Among PBM, monocytes comprised the cell population with the

Table 3. Mononuclear cells expressing the major 72-kDa immediate-early CMV polypeptide*

Mononuclear cell type	Phenotypic cell marker†	Cell marker positive‡	L-14§ positive
Monocyte	Mo2	20	10
NK	HNK-1	16	6.3
B-Lymphocyte	Anti-SIgG	11	1.0
Helper cell	OKT4	49	1.6
Suppressor/cytotoxic	OKT8	40	3.0

*Similar data were obtained in two other experiments.
†The techniques used are described in ref. 16.
‡Monoclonal antibody L-14 detects the major 72-kDa immediate-early protein, with similar reactivity as E-3, obtained from Goldstein *et al.* (19).
§Functional and phenotypic heterogeneity among subsets of mononuclear cells (25, 26) likely explains why the total cell number is >100%.

greatest proportion of cells expressing the immediate-early gene product.

Abortively Infected PBM Lose Some of Their Specialized Functions. In these studies, 15 low passage human isolates of human CMV completely abrogated the mitogenic response of PBM to PHA. Shown in Table 4 (sections 1 and 2) are data from one experiment that compared the immunosuppressive properties of two isolate strains of virus compared to AD-169 and mock-infection. Similarly, the specific proliferative response of immunocompetent cells to herpes simplex virus antigen was aborted by infection of these cells with recent isolate strains of CMV. AD-169 partially suppressed these responses, but even 100-fold greater amounts of AD-169 were significantly less suppressive than the low passage human isolate strains. The suppression of these functions was dependent on the input multiplicity and could be demonstrated with either cell-free or cell-associated virus (unpublished observations).

Similar results were obtained in >20 experiments that examined the effect of 12 isolate strains of CMV on the ability of PBM to act as NK cells. Shown in Table 4 (section 3) are data for three isolates compared to AD-169 and mock-infection. In contrast, strains of CMV did not alter the performance of immunocompetent cells in ADCC (Table 4, section 4). Our observations with this model *in vitro* closely mirror the functional defects in mitogen response and NK-cell activity reported for some patients with severe CMV infection (29).

Table 4. Suppression of some of the differentiated functions of immunocompetent cells by infection with CMV

PBM function	Virus source	[^{3}H]Thymidine incorporation,* cpm	% suppression
1. PHA-induced proliferation†	AD-169	44,478 ± 4,722	0
	I-G	3,081 ± 1,266	89
	I-V	6,129 ± 1,425	79
	Mock	29,158 ± 11,580	‡
2. Antigen-induced proliferation†	AD-169	5,070 ± 286	38
	I-G	3,581 ± 456	56
	I-V	3,801 ± 141	54
	Mock	8,215 ± 3,199	‡
		% specific Cr release§	% suppression
3. NK cell activity¶	AD-169	24	0
	I-G	2	82
	I-R	3	73
	I-P	1	91
	Mock	11	‡
4. ADCC¶	AD-169	60	0
	I-M	58	0
	I-R	71	0
	I-S	72	0
	Mock	60	‡

*[^{3}H]Thymidine incorporation is shown as the mean cpm of triplicate cultures ± 1 SD.

†In these experiments, mononuclear cells were cultured on infected or on mock-infected fibroblast monolayers for 3 days and then removed, washed, and recultured in the presence of PHA or herpes simplex antigen. These data are representative of 15 experiments.

‡Not applicable.

§% specific ^{51}Cr release is determined by the standard formula: (cpm ^{51}Cr release of sample − spontaneous release)/(cpm maximum release − spontaneous release). The mean is shown; the standard error was always <5% of this value.

¶In these experiments, PBM were cultured with infected or with mock-infected fibroblasts and then removed from the cultures, washed, and prepared for cytotoxicity assays against the ^{51}Cr-labeled K-562 target cell, for measurement of NK-cell activity, or against antibody-sensitized P-815 cells, as an indication of ADCC. The effector-to-target cell ratios were 20:1 and the assays were 6 hr long. Suppression of NK-cell function was demonstrated >20 times; failure to alter ADCC was shown in three experiments.

It remains uncertain why such significant immunosuppression occurs *in vitro* when only a small percentage of the mononuclear cells are infected. Both low-passage isolates and the laboratory-adapted strains induce equivalent amounts of interferon from mononuclear cells, making this an unlikely possibility. Since the immunosuppression can be demonstrated with both cell-free and cell-associated virus, it is unlikely that adherence of leukocytes to infected fibroblasts would have significantly decreased cell recovery and, hence, mononuclear cell performance. A more likely explanation is that the suppression of at least some of these functions is mediated through an intermediate cell type. We showed that the monocyte was the mononuclear cell with the greatest propensity for infection by CMV (Table 3) and we have preliminary data from monocyte depletion and reconstitution experiments that show that this cell is integral for the demonstration of CMV-mediated immunosuppression.*

Our results indicate that cells of the immune system, including lymphocytes, can be infected by CMV. This conclusion is based on the demonstration of CMV gene products in immunocompetent cells by using specific monoclonal antibodies and immunofluorescent techniques. This infection is abortive, but the differentiated or specialized functions of immune cells can be perturbed without disturbing their vital, or housekeeping, functions. Furthermore, we have described a restriction in CMV replication, at least after the expression of immediate-early genes. Finally, our results suggest a possible mechanism whereby CMV can be introduced into humans by the transfer of blood products, as has been suggested in some experimental models (30, 31).

*Rice, G. P. A., International Herpes Virus Workshop, July 31–August 5, 1983, Oxford, England, abstr. 85.

The authors thank Carole Shoemaker, Janet Anderson, and Gary McDaniel for technical assistance; Dr. Chen-Ming Chang for electron microscopic studies; and Susan Mattson for preparing the manuscript. Research reported here was supported by U.S. Public Health Service Grants AI-07007 and NS-12428. G.P.A.R. is a recipient of the Centennial Fellowship of the Medical Research Council of Canada and R.D.S. is a postdoctoral fellow of the National Institutes of Health, supported by U.S. Public Health Service Training Grant GM 07437. This is publication no. 3376-IMM from the Department of Immunology, Scripps Clinic and Research Foundation (La Jolla, CA 92037).

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