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Enhancive and Suppressive Effects of Cytomegalovirus on Human Lymphocyte Responses In Vitro

B. WAHREN,[1]* P. LJUNGMAN,[2] T. PAULIN,[3] AND O. RINGDÉN[3,4]

Department of Virology, National Bacteriological Laboratory, S-105 21 Stockholm,[1] and Departments of Medicine,[2] Clinical Immunology,[3] and Transplantation Surgery,[4] Huddinge Hospital, S-141 86 Huddinge,[2] Sweden

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Virus-specific lymphocyte proliferation in the presence of cytomegalovirus (CMV) without and with monocytes was studied in healthy persons. Three categories of lymphocyte response could be distinguished: seropositive low responders, naturally high responders, and lymphocyte populations responding well to CMV antigen in the presence of added CMV-incubated autologous monocytes. This latter category could be identified by preincubating autologous monocytes with CMV. CMV-seronegative persons were nonresponders. Early CMV antigens were produced in monocytes but not in lymphocytes by all CMV isolates. Infection of monocytes as detected by antibody to early viral protein did not appear to abort the antigen-presenting ability. The virus-specific responding lymphocytes were mainly of the $T4^+$ phenotype. In contrast, addition of CMV to polyclonal mitogens significantly suppressed total lymphocyte DNA synthesis. CMV thus may have an enhanced virus-specific stimulatory effect on lymphocytes together with monocytes but a suppressive effect on the total lymphocyte population.

Cytomegalovirus (CMV) infection leads to sustained general immunosuppression of the host, with a decreased $T4^+$-$T8^+$ cell ratio (5). The outcome in individuals who are already immunosuppressed is sometimes lethal, and survival may depend on the patient's cytolytic lymphocyte responses (18, 19). $T4^+$ cells helping the development of immunoglobulin response by B cells have been shown to be dependent on expression of class II major histocompatibility antigens in connection with several viral antigens (7, 15). The lymphocyte DNA stimulation assay with CMV antigen in vitro is virus specific (16a, 16b, 17, 18, 27). This response is probably major histocompatibility restricted, since responses to herpes simplex virus were proven to be so in humans and experimental animals (1, 7, 14). The respective roles of the subsets of T cells efficient in various stages of latent and reactivated herpesvirus infection are, however, not yet clear (16).

The presentation of both foreign and autoantigens to T helper cells appears to be most efficient with monocytes and macrophages (24). It is known that monocytes are primarily infected by CMV and that fresh CMV isolates in particular have this capacity (10, 21).

The apparent dual effect of CMV of stimulation and suppression was investigated in in vitro populations of lymphocytes from healthy persons. The question studied was whether CMV infection of the antigen-presenting monocyte population would lead to suppressed or enhanced activation of lymphocytes. Considerably enhanced DNA synthesis was established in several CMV-seropositive persons by adding autologous CMV-incubated monocytes to the lymphocytes in the antigen-specific reaction. Mitogen-induced activation was suppressed by fresh CMV isolates.

MATERIALS AND METHODS

Cells, virus, and mitogens. Peripheral blood cells from healthy persons were separated on Isopaque-Ficoll (3). The mononuclear cell fraction was further separated into adherent and nonadherent populations by addition to 25-cm^2 plastic culture dishes (Falcon; Becton-Dickinson Labware, Oxnard, Calif.) in Mishell-Dutton medium with 10% heat-inactivated human AB serum at 37°C in 5% CO_2 overnight. Nonadherent cells were removed with the supernatant medium; adherent cells were disengaged by means of a Pasteur pipette at +4°C.

Stimulation of cells was performed with phytohemagglutinin (PHA; 100 μl of a 1:1,000 dilution; Wellcome Research Laboratories, Beckenham, England), pokeweed mitogen (PWM; 100 μl of a 1:3,000 dilution; GIBCO Laboratories, Grand Island, N.Y.), or CMV. The CMV strains were isolates from bone marrow transplant recipients in passages 1 to 6 on human lung (HL) fibroblasts and typed by immunofluorescence (26) and enzyme-linked immunosorbent assay (23). Nine different isolates as well as the laboratory strain CMV Ad.169 were used. The CMV late antigen titers measured with the CMV isolates on HL were 0.5×10^4 to 0.5×10^6 late antigen units/ml (see below); the early antigen (EA) inducing titers were 10^4 to 10^6 EA units/ml (9, 25). After UV irradiation for 30 min, all CMV isolates were rendered noninfectious (27). Control antigen was prepared from uninfected HL cells. CMV nucleocapsid antigen was prepared from infected HL cell nuclei (23).

Proliferation assays. Lymphocytes, monocytes (2×10^5 each), or a mixture of equal parts of both were cultured for 3 or 6 days at 37°C with RPMI 1640 and 10% CMV antibody-negative, AB+ human serum in 96-well microplates (Nunc Immunoplate I, Aarhus, Denmark) in 5% CO_2 in air. In some experiments, the lymphocyte, monocyte, or mixed populations had been preincubated with infectious or UV-treated fresh CMV strains for 2 h at 37°C at multiplicities of infection of 0.1 to 1.0. Twenty-four hours before harvest, 1 μCi of [^{3}H]dT (Amersham International, Amersham, England) was added to each well. When the cells had been harvested (Titertec; Skatron, Lierbyen, Norway), the activity was measured in a scintillation counter. Each test was performed in triplicate, and net counts per minute were calculated as the mean counts per minute of antigen-stimulated cultures minus the mean counts per minute of

* Corresponding author.

TABLE 1. Interaction of mononuclear nonadherent and adherent cells on infection with CMV

CMV category, responsiveness, and individual no.	Net cpm (10^3)[a]					
	Lymphocytes with CMV	Monocytes with CMV	Lymphocytes with CMV and monocytes[b]	Lymphocytes and monocytes with CMV[c]	Ratio of column 4 to column 1	% ACIF-positive monocytes
CMV seropositive						
Low responders						
1	2	0	4	2	1.0	10
2	1	1	1	4	4.0	3
3	8	2	2	2	0.3	2
High responders						
4	60	95	135	110	2.0	2
5	11	11	13	24	2.2	1
6	61	100	108	138	2.3	25
7	21	5	29	29	1.4	4
8	58	30	63	78	1.3	0.5
9	35	ND[d]	105	115	3.3	0.5
Responsive with added monocytes						
10	1	7	3	11	11	2
11	2	2	2	22	11	10
12	2	64	7	48	24	2
13	1	3	0	7	7	4
14	1	38	6	56	56	4
15	2	1	1	10	5	2
CMV seronegative						
16	0	1	1	3	3	3
17	0	0	0	3	3	4
18	0	1	0	3	3	6
19	0	2	0	0	0	6
20	0	0	0	1	1	12

[a] Corrected for the highest mean counts per minute with lymphocytes only, monocytes only, or lymphocytes mixed with monocytes, all 200,000 cells per well.
[b] Lymphocytes were preincubated with CMV; monocytes from the same patient were nonincubated.
[c] Monocytes were preincubated with CMV; lymphocytes were nonincubated.
[d] ND, Not done.

control antigen-stimulated cultures. The standard error in triplicate cultures was 10%. Net counts per minute over 1,000 are considered positive (16a).

Immunofluorescence. (i) CMV antigens. At the time of each experiment, lymphocyte and monocyte populations were separately incubated for 24 h at 37°C with the virus strain. The cells were then spread by cytocentrifugation, fixed for anticomplement immunofluorescence (26) in −20°C ethanol-acetone (1:1), and stained for CMV EA. The serum for CMV EA was a rabbit anti-CMV DNA polymerase (26) together with human complement from a CMV-seronegative donor and anti-human β1C/1A (Hyland Laboratories, Costa Mesa, Calif.). CMV titers were determined by a fluorescence focus assay for late antigen on HL cells 6 to 8 days after infection as well as by induction of EA on HL cells by anticomplement immunofluorescence staining 24 h after infection (26).

(ii) Cellular antigens. After stimulation with CMV antigen for 5 days, [^{3}H]dT was added to lymphocyte-monocyte cultures as described above, and 24 h later the cells were harvested and washed. For characterizaton of T cells, OKT11, OKT4, and OKT8 fluorescein-labeled monoclonal antibodies were used in immunofluorescence (20; Ortho Diagnostics, Raritan, N.J.). The cells were stained with either monoclonal antibody in suspension, spread on slides, and fixed with acetone-methanol (1:1). The slides were coated with Ilford K2 emulsion diluted 1:2 with water at 42°C and exposed for 2 days at 4°C. They were developed in AGFA Rodinol and fixed. Autoradiographic grains and fluorescence were then studied simultaneously in a Zeiss fluorescence microscope (8).

Reactivity by immunofluorescence with the monocyte-reactive monoclonal antibody M1 (4; Ortho) revealed 84 to 90% nonstained cells in the nonadherent fraction (here called the lymphocyte fraction) and 63 to 95% stained cells in the adherent cell fraction (here called monocytes).

RESULTS

Influence of CMV-incubated monocytes on lymphocyte activation. Nine different CMV isolates in early passages were incubated with lymphocytes, monocytes, or both from CMV-seropositive and -seronegative persons (Table 1). With two persons (no. 8 and 9 in Table 1) the infectious CMV was the laboratory strain Ad.169. The percentage of cells infected by CMV in the monocyte population, as judged by anticomplement immunofluorescence staining, ranged from 0.5 to 25%. Of 20 persons, 5 were CMV seronegative, and their lymphocytes also did not respond to stimulation with CMV (Table 1). The lymphocytes of the 15 seropositive persons all had net counts per minute of 1,000 or more over background. Three were low responders; i.e., no increase in net counts per minute was noted when monocytes with or without CMV incubation were added. Lymphocyte populations from six persons (no. 4 to 9) were initially high responders (>10,000 net cpm) to CMV stimulation. The

monocyte population (Table 1), when incubated with CMV, also had increased net counts per minute in most but not all CMV-seropositive persons. Monocyte populations from CMV-seronegative persons showed low but increased net counts per minute in three cases. The monocyte response thus did not seem to be entirely CMV specific, although several cell populations responded exceedingly well. Since the monocyte populations contained various amounts of non-M1-stained cells (see Materials and Methods), it is feasible that these untyped adherent cells contributed to the stimulation seen with the cell populations from CMV-seronegative lymphocyte or monocyte donors. There was, however, no relation between DNA synthesis and percent non-M1-stained cells.

With six further patients (no. 10 to 15), [^{3}H]dT incorporation of lymphocytes increased when CMV-incubated monocytes were added (Table 1). These increases ranged from fivefold to around 50-fold. Such an increased ratio was not obtained when the lymphocytes had been incubated with CMV and uninfected monocytes were added (Table 1). Also, when lymphocyte and monocyte populations were separately exposed to CMV (data not shown), cocultivation did not produce higher figures or ratios than shown in Table 1. Monocytes incubated with CMV and presented to the lymphocyte population thus appeared to give the highest net counts per minute in the majority of individuals. Also, with this procedure a new group of seropositive lymphocyte responders could be identified. Their ratio of stimulation with CMV-incubated monocytes increased to over five times that of lymphocytes with CMV only (Table 1).

The frequency of monocytes with CMV EA bore no apparent relation to stimulatory effects. Both infectious and UV-inactivated CMV gave the same increase of net counts per minute in five patients tested (individuals no. 4, 8, 9, 12, and 14 of Table 1). Inactivated CMV could thus be expressed by monocytes. The two latter findings make it probable that the antigenicity of CMV and not its infectivity was responsible for increased cellular DNA synthesis.

Autoradiography of CMV-activated lymphocytes. The cells found in wells with lymphocytes stimulated with CMV were stained with monoclonal antibodies OKT11, OKT4, and OKT8. T11$^+$ cells were around 90%. From 50 to 97% of the DNA-synthesizing cells appeared to be T4$^+$, and 0 to 2% were T8$^+$ in seropositive persons (Table 2).

Suppression of mitogenic responses by CMV. CMV infection in vivo decreases the responsiveness of peripheral lymphocytes to PHA and other mitogens (16c). In an attempt to study whether CMV has a direct effect on mitogenic lymphocyte responses, CMV was added together with PHA or PWM to lymphocyte populations that were not monocyte depleted (Table 3). The polyclonal lymphocyte DNA synthesis caused by PHA was significantly decreased ($P < 0.001$; Student's t test) by CMV. This was true with lymphocytes from CMV-seronegative as well as CMV-seropositive persons on day 3, which is the optimal day of PHA response. A decrease was still evident on day 6 ($P < 0.01$). With PWM, the reduction was similar ($P < 0.001$ on days 3 and 6). The lymphocyte phenotype found with PHA stimulation consisted of equal proportions of T4 and T8 cells (Table 2).

CMV nucleocapsid antigen was used for stimulation of lymphocytes of a seropositive and a seronegative person in the presence and absence of PHA. Only the seropositive person's PHA response was decreased by CMV antigen.

TABLE 2. Phenotypes of lymphocytes stimulated with CMV or PHA

Stimulation with (individual no.):	CMV antibody status	% Surface fluorescence-positive cells[a]	
		OKT4	OKT8
CMV			
21	+	97	0
22	+	50	2
23	+	91	0
24	−	0	0
PHA			
21	+	46	42

[a] Of cells containing nuclear grains by autoradiography.

TABLE 3. Influence of CMV on mitogen-stimulated lymphocytes

Day	CMV antibody status	No. of individuals	Mitogen response after addition of CMV (% ± SD)[a]		Mean net cpm (range)
			PHA	PWM	
3	+	8	42 ± 25	26 ± 12	
	−	2	15 ± 9	10 ± 26	
6	+	8	64 ± 40	59 ± 18	10,000 (1,400–45,800)
	−	2	49 ± 29	59 ± 16	600 (430–800)

[a] The response of each individual to PHA or PWM was taken as 100%. The figures given were calculated as: $(cpm_{CMV + mitogen}/cpm_{mitogen}) \times 100$.

DISCUSSION

The specific cellular response to CMV, as measured by DNA synthesis in vitro, could be enhanced by subjecting the lymphocytes to monocytes incubated with CMV. In this manner a group of CMV-seropositive persons could be distinguished, whose lymphocyte DNA proliferative response increased considerably. It is probably the superior antigen presentation of the added monocyte population which enhanced the lymphocyte reactivity. We have found (16b) that CMV antigen presented in solid phase is advantageous for specific T-cell proliferation. It cannot be excluded, however, that in some patients suppressor T cells were more strongly activated when the lymphocyte population was preincubated with CMV, leading to decreased total T-cell response.

In several cases, lymphocyte reactivity was already high, and the addition of monocytes with CMV increased it only slightly or not at all. These patients may have had either a larger or more efficient antigen-presenting cell population (B cells can also present antigens; 6) or a more recent subclinical reactivation of their CMV. A few CMV-seropositive persons were poor responders also with monocytes. The reason for this is not clear. It may be technical or due to some of the primary CMV strains used in these experiments being antigenically disparate from the patient's own latent strain (11, 28a).

We used autologous monocytes to obtain maximal responses, since the type of restriction operative with CMV is not known. Mainly T4$^+$ cells were activated by the interaction of lymphocytes with CMV. T4$^+$ cells were previously shown to appear after CMV stimulation in vitro (2). During CMV infection in vivo, T8$^+$ cells increase and T4$^+$ cells decrease (5). This in vivo behavior may be caused by a short survival time of CMV-activated T4$^+$ cells. Monocytes are

abortively infected by CMV (10, 21). This apparently did not affect their role as antigen-presenting cells, since the percentage of cells infected did not influence DNA synthesis. Infectiousness was previously shown to be related to the virus isolate rather than properties of the monocyte donor (10).

The adequate and virus-specific response obtained with Isopaque-Ficoll-prepared mononuclear cells (27; this paper) is almost certainly dependent on the population of endogenous monocytes. With CMV-incubated monocytes, lymphocyte responses were considerably greater than those described by other authors and in our own previous publications (27).

The slightly raised DNA synthesis with monocytes in some CMV-seronegative persons can perhaps be ascribed to the general cell DNA and polyclonal B-cell-stimulating effects described for CMV (12, 13, 22).

CMV had a direct suppressive effect on mitogen stimulation. This occurred in both seropositive and seronegative persons. Such overall suppression may contribute both to the suppression seen in clinical CMV disease and to the lowered mitogenic responses in vitro of lymphocytes from immunosuppressed patients (5, 16c, 28). It is not known whether this suppressive effect also includes specific killing in seropositive individuals. CMV infection or stimulation thus has a dual effect: specific stimulation of primed lymphocytes but also suppressive effects on polyclonal T-cell response. Cytolytic T cells are mainly recruited by the viral antigen presented together with class I antigens (18–20, 29, 30), and it should be relevant to study the kinetics of lymphocytes cytolytic to CMV-infected cells.

The method presented for specifically enhancing DNA synthesis by autologous monocyte addition appears of interest, since cellular reactivity measured this way may indicate the patient's ability to respond to disease at an early stage.

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