INFECTION AND IMMUNITY, Oct. 1980, p. 17-22
0019-9567/80/10-0017/06$02.00/0

Vol. 30, No. 1

Lymphocyte Blastogenesis and Interferon Production in Adult Human Leukocyte Cultures Stimulated with Cytomegalovirus Antigens

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Lymphocyte blastogenesis and interferon production were measured in adult human leukocyte cultures stimulated with purified or crude cytomegalovirus antigens. Leukocytes from seropositive adults underwent blastogenesis when stimulated with purified or crude Towne strain antigen, whereas neither antigen stimulated blastogenesis in cultures from seronegative donors. The concentrations of antigens yielding maximal blastogenesis varied among the individuals tested. When cultures from seropositive individuals were stimulated with antigens prepared from three different CMV strains—AD-169, Towne, and Davis—comparable levels of blastogenesis were detected. Type 1 interferon was detected in supernatants of cultures stimulated with crude antigens regardless of the immune status of the donor. In contrast, when purified antigen was used as the stimulant, only cultures obtained from seropositive individuals produced detectable levels of interferon, which appeared to be predominantly type 2 or immune interferon.

Assays for cytomegalovirus (CMV)-specific lymphocyte blastogenesis and interferon production have been used to measure cellular immune responses in CMV-infected individuals, including congenitally infected children and their mothers, and recipients of cardiac, bone marrow, or renal transplants (3, 5, 6, 11, 13, 14, 15, 18).

In these studies a variety of CMV antigens were used, namely, sonicates or glycine extracts of infected fibroblasts, mitomycin-treated infected fibroblasts, or density gradient-purified virus. Leukocyte cultures were usually stimulated with a single concentration of antigen prepared from only one strain of CMV. The purpose of the present study was to compare results obtained in assays for lymphocyte blastogenesis and interferon production when two different methods were used to prepare antigens and several different strains of CMV were used.

MATERIALS AND METHODS

Study group. Donors consisted of healthy laboratory or hospital personnel 22 to 36 years of age.

Preparation of CMV antigen. For preparation of crude antigen, monolayers of WI-38 cells in 75-cm^2 flasks (Becton, Dickinson and Co., Oxnard, Calif.) were infected with the Towne (10), AD-169, or Davis strains of CMV at a multiplicity of infection of approximately 0.1. When the cultures showed cytopathic effect of 3 to 4+, cells were scraped with a rubber policeman into 2 ml of Eagle minimal essential medium containing 10% fetal calf serum and sonicated for 5 s (Bronson Sonic Power, Danbury, Conn.). For each antigen preparation, sonicates from five flasks were pooled and clarified by low-speed (1,400 × *g*) centrifugation. The resultant pool was assayed for infectivity by plaque formation (20), heat inactivated (56°C for 1 h), and stored at −70°C until use. Before use the pools were diluted to 10^5 plaque-forming units per ml (titer before inactivation). Control antigen was prepared from uninfected WI-38 cells by the same procedure as for infected cells.

For preparation of purified antigen, monolayers of WI-38 cells were infected with Towne strain CMV as described above. When the cultures showed a cytopathic effect of 3 to 4+, the supernatants of 40 infected flasks were harvested, clarified by low-speed centrifugation, and subjected to ultracentrifugation at 50,000 × *g* for 20 min. The resultant virus pellets were pooled in 3 ml of pH 7.5 tris(hydroxymethyl)aminomethane-buffered saline (TBS) and sonicated. The suspension, which contained 1.5 × 10^8 plaque-forming units, was layered onto a discontinuous 10 to 50% sucrose gradient and ultracentrifuged for 2 h at 95,000 × *g* (SW25 rotor, Beckman Instruments, Fullerton, Calif.). A visible band which appeared at the 40-to-50% interface was collected and dialyzed overnight against TBS. The dialyzed preparation was placed on a linear 20 to 50% sucrose gradient and ultracentrifuged for 4 h at 95,000 × *g* (SW25 rotor). A single visible band was collected, dialyzed overnight against TBS, and diluted with TBS to a volume of 10 ml. This preparation, which contained 50 plaque-forming units per ml of live virus, was heat inactivated and then stored at −70°C before use. Purified control antigen was prepared from

uninfected WI-38 cells by the above procedure.

Lymphocyte blastogenesis assay. Mononuclear cells obtained by Ficoll-Hypaque centrifugation of heparinized peripheral blood were suspended at a concentration of 10^6 cells per ml in RPMI-1640 (GIBCO Laboratories, Grand Island, N.Y.) with 15% autologous plasma. Samples of the cell suspension were added to the wells of round-bottomed microtiter plates (Linbro Scientific Co., Hamden, Conn.), 0.2 ml per well. Phytohemagglutinin (1%; Difco Laboratories, Detroit, Mich.), CMV, or control antigens were added to triplicate cultures in a 0.01-ml volume. Mitogen- and antigen-stimulated cultures were incubated for 3 and 6 days, respectively, at 37°C in a 5% CO_2 atmosphere. Tritiated thymidine (6.7 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was added for the final 4 h of incubation. The culture supernatants were collected with a Pasteur pipette, pooled for each set of triplicate cultures, and stored at −70°C until assayed for interferon. The cells were harvested with a multiple sample harvester (MASH II, Microbiological Associates, Walkersville, Md.) and processed for liquid scintillation counting. Results were expressed as net counts, that is, counts per minute (cpm) in CMV-stimulated cultures minus cpm in control cultures.

Interferon assay. Titrations of interferon were performed essentially as described by Paucker et al. (9). Twofold serial dilutions of the supernatants of the leukocyte cultures were dispensed into microtiter wells in 0.1-ml volumes. Each well was then seeded with 0.1 ml of a suspension of 30,000 human foreskin fibroblasts (FS-4 strain), and the plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. On the next day, the cultures were challenged with 0.05 ml of encephalomyocarditis virus at a multiplicity of infection of 0.25 and incubated for an additional day. The plates were then read for inhibition of cytopathic effect, and the titer was expressed as the reciprocal of the dilution protecting 50% of the cell sheet against challenge virus, corrected for a 1-ml volume. Virus, cell, and interferon controls, as well as human leukocyte interferon reference standard GO23-901-527, distributed by the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md., were included in each titration. In this test, approximately eight interferon units correspond to one unit of the reference standard.

Characterization of interferon. Interferons produced in CMV-stimulated leukocyte cultures were tested for neutralization by sheep antibodies to human type 1 interferon prepared by repeated inoculations of a sheep as previously described (8). Sendai virus-induced type 1 human interferon was kindly provided by Kari Cantell, State Serum Institute, Helsinki, Finland. Neutralizing titers of the serum against eight reference units of leukocyte and fibroblast type 1 interferons were 24,000/ml and 3,000/ml, respectively, whereas the antiserum was devoid of activity against eight units of type 2 interferon (neutralizing titer <25), provided by J. Georgiades, University of Texas Medical Branch, Galveston.

The neutralization assay was performed by adding 0.05-ml samples of leukocyte culture supernatants containing approximately eight units of interferon to equal volumes of twofold dilutions of the anti-type 1 serum. The microplates containing the mixtures were incubated for 1 h at 37°C, after which foreskin fibroblasts were added. Subsequent procedures were as described for assays of interferon. The monolayers were challenged with encephalomyocarditis virus after 24 h of incubation, and viral cytopathic effect was read the next day. The reciprocal of highest dilution of antiserum which neutralized 8 U of interferon to the extent that 50% of the cell sheet showed cytopathic effect was considered the neutralizing titer.

Interferons detected in culture supernatants were also tested for pH stability (17). Interferon samples were diluted in minimal essential medium containing 5% heat-inactivated fetal calf serum, to concentrations of 100 U of interferon per 0.1 ml. The untreated samples were stored at 4°C. The treated samples were dialyzed for 24 h at 4°C against pH 2 buffer and then returned to neutrality by dialysis against phosphate-buffered saline (pH 7.0) for 24 h at 4°C. Assays for interferon activity were then carried out as described above.

Complement-fixing antibodies to CMV. Complement-fixing antibody titers to CMV were kindly performed by Harvey Friedman, University of Pennsylvania School of Medicine, Philadelphia, as previously described (1).

RESULTS

Initial experiments were done to establish the doses of purified and crude CMV antigens which stimulated maximal lymphocyte blastogenesis and interferon production in 6-day cultures. When leukocytes from two seropositive individuals were incubated with varying doses of purified Towne antigen, lymphocyte blastogenesis was detected over a broad range of antigen concentration and was highest at final antigen dilutions of 1:80 or 1:320 (Table 1). The highest doses of antigen used stimulated the largest amounts of interferon production. With crude Towne antigen, highest blastogenesis was noted at final antigen concentrations of 1:20 and 1:80 in two donors (Table 2). Interferon production was maximal with a final antigen dilution of 1:20 in the one case tested. Similar results with respect to antigen concentrations were obtained with AD-169 and Davis crude antigens (data not shown).

Since concentrations of antigens required for optimal blastogenesis varied among seropositive individuals, in subsequent experiments multiple dilutions of antigen were used, and the optimal response obtained was selected for analysis. Interferon was assayed only in supernatants of cultures incubated with 1:20 dilutions of antigens. Although other experiments indicated that higher levels of interferon were detected when the final antigen dilution was 1:5, the quantity of antigen required was not practical for routine use.

Experiments were done to determine whether

TABLE 1. *Effect of various concentrations of purified Towne antigen on lymphocyte blastogenesis and interferon production*

Donor[a]	Antigen dilution	Control antigen cpm	CMV antigen cpm	Net cpm[b]	Interferon[c]
1	1:20	98 ± 12.7[d]	15,503 ± 2,155	12,071	300
	1:80	156 ± 31.1	20,141 ± 1,002	19,985	150
	1:320	147 ± 29.4	12,522 ± 1,162	12,375	<10
	1:1,280	99 ± 18.5	5,829 ± 639	5,730	<10
2	1:20	271 ± 76	2,623 ± 348	3,340	200
	1:80	286 ± 30	8,032 ± 1,501	7,746	100
	1:320	224 ± 26	8,555 ± 864	8,331	<10
	1:1,280	176 ± 9	3,450 ± 413	3,274	<10

[a] Complement-fixing titers of donors 1 and 2 were 1:16 and 1:32, respectively.
[b] cpm of Towne antigen minus cpm of control antigen.
[c] Titer in units per milliliter.
[d] Mean ± standard error.

TABLE 2. *Effect of various concentrations of crude Towne antigen on lymphocyte blastogenesis and interferon production*

Donor[a]	Antigen dilution	Control antigen cpm	CMV antigen cpm	Net cpm[b]	Interferon[c]
1	1:20	1,218 ± 75[d]	9,104 ± 566	7,885	800
	1:80	1,276 ± 445	6,528 ± 189	5,306	400
2	1:20	6,571 ± 1,019	13,833 ± 1,730	7,262	ND
	1:80	1,002 ± 344	19,228 ± 1,326	18,226	ND
	1:320	1,033 ± 215	16,230 ± 1,735	15,197	ND
	1:1,280	379 ± 63.5	5,862 ± 251	5,483	ND

[a] Complement-fixing titers of donors 1 and 2 were 1:16 and 1:32, respectively.
[b] cpm of CMV antigen minus cpm of control antigen.
[c] Titer in units per milliliter. ND, Not done.
[d] Mean ± standard error.

lymphocyte blastogenesis and interferon production correlated with donor serological status. For these experiments leukocytes from six seronegative (complement-fixing titer <1:8) and eight seropositive adults were incubated with purified and, in some cases, crude Towne strain antigens as well as control antigens as described above. Lymphocytes from seropositive adults underwent blastogenesis when incubated with either antigen, whereas lymphocytes from seronegative individuals failed to respond (Fig. 1).

Results of assays for interferon in supernatants of crude and purified Towne antigen-stimulated cultures are shown in Fig. 2. In purified antigen-stimulated cultures interferon was detected only when the donor was seropositive, whereas interferon was detected in crude antigen-stimulated cultures regardless of the immune status of the donor. Neither of the cell control antigens stimulated interferon production. The magnitude of purified antigen-stimulated lymphocyte blastogenesis did not correlate with levels of interferon detected in individual cultures by regression analysis (data not shown).

Identification of the type of interferon produced in CMV-stimulated leukocyte cultures was accomplished by neutralization and pH 2 stability tests. The interferon detected in purified antigen-stimulated cultures was not neutralized by the antiserum to type 1 interferon and was completely inactivated by exposure to

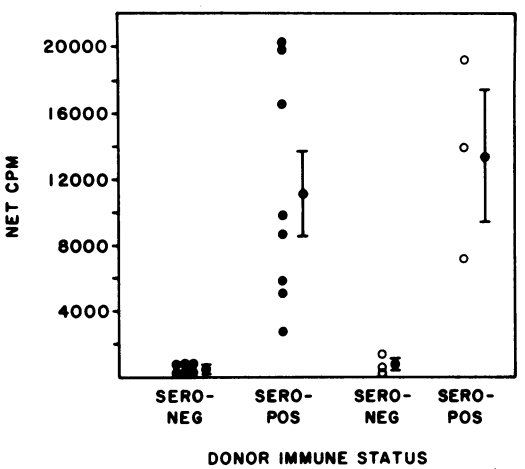

FIG. 1. *Blastogenic responses of adult human leukocytes to CMV antigens. Symbols: ●, purified Towne CMV antigen; ○, crude Towne CMV antigen. The bars represent ±1 standard error of the mean for each group studied.*

low pH (Table 3). Similar results were obtained when the control type 2 interferon was tested. In contrast, the interferon produced in crude antigen-stimulated cultures behaved like the type 1 interferon in that it was neutralized by antiserum to type 1 interferon and was stable at pH 2.

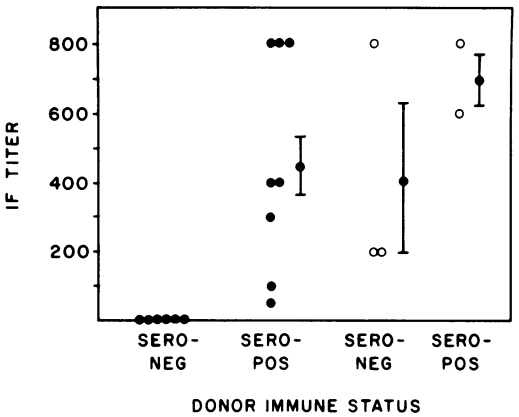

FIG. 2. *Levels of interferon detected in adult human leukocyte cultures stimulated with CMV antigens. Symbols: ●, purified Towne CMV antigen; ○, crude Towne CMV antigen. The bars represent ±1 standard error of the mean for each group studied.*

Additional experiments were done to compare the responses of leukocytes from nine seropositive donors to antigens prepared from different strains of CMV. Interferon was not measured. The maximal blastogenic responses and the dilutions of Towne, AD-169, and Davis crude antigens which yielded such responses are shown in Table 4. As in earlier experiments, the antigen concentrations required for maximal blastogenesis varied and there did not appear to be strain-related antigen dosage requirements. Leuko-

TABLE 4. *Comparative blastogenic responses to three CMV antigens*

Donor	Response to CMV antigen:		
	Towne	AD169	Davis
1	10,446[a] (1:200)[b]	5,636 (1:20)	2,183 (1:20)
2	26,284 (1:20)	26,127 (1:20)	18,398 (1:20)
3	4,120 (1:200)	6,407 (1:200)	4,101 (1:200)
4	4,968 (1:200)	9,225 (1:200)	5,249 (1:200)
5	2,834 (1:20)	5,363 (1:20)	3,332 (1:20)
6	7,051 (1:20)	3,534 (1:20)	4,149 (1:20)
7	8,269 (1:100)	9,938 (1:20)	5,473 (1:100)
8	9,480 (1:100)	7,595 (1:20)	6,760 (1:200)
9	24,664 (1:100)	30,474 (1:20)	19,529 (1:20)
	10,940 ± 2877[c]	11,588 ± 3245	7,686 ± 2177

[a] Net cpm.
[b] Antigen dilution yielding optimal proliferation.
[c] Mean ± standard error.

TABLE 3. *Characterization of CMV-induced lymphocyte interferon*

Donor	Antibody status	CMV antigen	Neutralizing titer anti-human leukocyte type 1 interferon serum[a]	pH 2 stability (interferon units/0.1 ml)		Predominant interferon type
				Untreated	Treated	
1	Seropositive	Purified	≤25	ND[b]	ND	2
2			≤25	ND	ND	2
3			≤150	64	≤4	2
4			ND	24	≤4	2
3	Seropositive	Crude	12,800	64	64	1
4			6,400	128	64	1
5			19,200	64	32	1
6			6,400	ND	ND	1
7			ND	96	64	1
8	Seronegative	Crude	64,000	ND	ND	1
9			16,000	48	32	1
10			32,000	32	32	1
Controls						
A[c]			24,000	96	64	1
			40,000	96	96	1
B[d]			≤25	128	≤16	2
			≤25	128	4	2

[a] Titer per 50 μl.
[b] ND, Not done.
[c] Type 1 leukocyte interferon, Sendai virus induced.
[d] Type 2 immune interferon, produced by immune stimulation of lymphocytes with staphylococcal enterotoxin A.

cytes from all of the individuals tested responded to all three antigens; however, there was some variability in the magnitude of the strain-specific responses. The mean blastogenic responses to Towne and AD-169 antigens were comparable, whereas the mean response to Davis was lower, but the difference was not statistically significant ($P > 0.05$).

DISCUSSION

In most published studies of CMV-specific lymphocyte blastogenesis single concentrations of CMV antigens were used to stimulate leukocyte cultures. However, antigen concentrations yielding maximal blastogenesis are not the same for all individuals, as shown in the present study and previously by Moller-Larsen et al. (7) and Pollard et al. (11) with different CMV antigen preparations. These results indicate that multiple concentrations of antigens should be used in clinical studies so that optimal responses can be obtained.

Whereas lymphocyte blastogenesis was frequently maximal when final antigen dilutions were greater than 1:20, interferon production was enhanced in the presence of high concentrations of antigen. No correlation existed between magnitude of blastogenesis and levels of interferon production in purified antigen-stimulated cultures. Different requirements for antigen and lack of correlation between these two responses suggest that they do not occur in concert with each other. It is indeed possible that different cell populations undergo blastogenesis and produce interferon, as has been recently described for herpes simplex virus-stimulated leukocyte cultures by Rasmussen and Merigan (12).

The lymphocyte blastogenesis assay described in the present study appeared to be specific since blastogenesis was detected only in leukocyte cultures from seropositive donors. Studies in which blastogenic responses to herpesvirus antigens including CMV correlated with the results of serological testing of the donors further document the specificity of blastogenesis assays in which herpesvirus antigens are used (7, 16, 21).

Important differences were noted in interferon production elicited with purified and crude CMV antigens. Crude antigen stimulated type 1 interferon production in leukocyte cultures from both seronegative and seropositive donors as previously reported by Pollard et al. (11). In contrast, interferon was detected in purified antigen-stimulated cultures only if the donor was seropositive, and the interferon detected appeared to be predominantly type 2 or immune interferon, although the possibility of the presence of small amounts of type 1 interferon cannot be completely excluded. Levin et al. reported that interferon produced in leukocyte cultures stimulated with glycine-extracted CMV antigen appeared to be a mixture of type 1 and type 2 interferon (5). Taken together, these results suggest that the type of interferon produced in CMV-stimulated leukocyte cultures may depend on the nature of the CMV antigen used. It was previously shown that the antigenic composition of interferon elicited in human diploid cells can be profoundly influenced by the nature of the inducing agent (4).

Evidence for antigenic heterogeneity among CMV strains has been obtained in neutralization assays most recently by Waner and Weller, who studied the kinetic neutralization of Towne, AD-169, and Davis strains of CMV using sera raised in rabbits (19). Few data have appeared on the strain specificity of cell-mediated immune responses to CMV. In the present study all seropositive individuals responded to the three CMV antigens used, and the magnitudes of the mean responses were not significantly different. We have also shown that recipients of Towne strain CMV vaccine develop blastogenic responses to these same CMV antigens (Starr et al., unpublished observation). These results contrast with those of Beutner et al., who studied CMV-specific blastogenesis in children (2). Among seropositive children only 52, 62, and 19% responded to AD-169, ADH-1-41, and Davis antigen, respectively. Differences in results might be attributed to methods of preparation of antigen, concentrations of antigen used in assays, or to the ages of the individuals studied.

ACKNOWLEDGMENTS

This work was supported by Biomedical Research support grant RR005506-15, and by Public Health Service grants AI 13649 and A1-12151 from the National Institute of Allergy and Infectious Diseases.

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