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Cytomegalovirus-Specific Lymphocyte Transformations in Subjects of Different Ages with Primary Immunodeficiency

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The role of specific cell-mediated immune responses in human cytomegalovirus (CMV) infections was studied by an in vitro lymphocyte transformation test, using a whole blood culture. CMV-induced in vitro lymphocyte proliferation was mainly dependent on the presence of sensitized T-cells. Three of five seronegative patients with B-cell deficiency showed positive lymphocyte responses to CMV antigen. In contrast, a patient with ataxia-telangiectasia who was shedding the virus in urine did not show a cellular response to either CMV or phytohemagglutinin. Age-related differences were found in the CMV lymphocyte transformation test. The responses were generally low ($P < 0.025$) in infants and higher in older children and adults. CMV lymphocyte transformation responses were lower in 15 children who were excreting CMV in urine when tested than in 17 seropositive children who were not excreting the virus.

A number of clinical and experimental studies have suggested that cellular immune mechanisms play an important role in immunity to cytomegalovirus (CMV) infection (2, 4, 12, 15, 18). Several in vitro assays for cell-mediated immunity to CMV, including the lymphocyte transformation (LTF) test, are currently being developed (5, 11, 14, 19, 20). Investigators have reported that CMV antigens stimulate peripheral blood lymphocytes obtained from CMV antibody-positive individuals. The in vitro LTF technique made it possible to look for selective defects in cell-mediated immunity to CMV antigen in specific disease states, such as congenital CMV infection or an immunocompromised host with CMV infection, and to study the relationship between the cell-mediated and humoral immune responses in CMV infection. The purpose of the present study was to characterize the transformation of lymphocytes obtained from CMV-seropositive healthy subjects in different age groups. In addition, the response to CMV antigen was studied in both a T-cell-enriched fraction and a depleted fraction. Also six patients with primary immunodeficiency were examined to determine lymphocyte subpopulation response to CMV antigen.

MATERIALS AND METHODS

Study population. Sera and peripheral blood lymphocytes were obtained from 50 unselected CMV-seropositive children (ages, 1 month to 15 years) and adults (ages, 20 to 45 years), 20 CMV-seronegative subjects, blood from six umbilical cords, and six patients with primary immunodeficiency.

Serological study. Sera were tested for CMV and varicella zoster virus (VZV) antibodies by complement fixation tests (17), using an initial serum dilution of 1:4.

Virological study. CMV isolation was carried out by inoculations of fresh urine samples into human embryonic fibroblast cultures.

Antigen preparation. CMV strain AD-169 grown in human embryonic fibroblasts was used as an antigen and was prepared by extraction of cell-associated antigen with alkaline buffers (6). VZV (Kawaguchi strain) antigen was prepared in the same way. A control antigen was prepared from uninfected cells treated in exactly the same way. Antigens were inactivated by ultraviolet irradiation with a 10-W germicidal lamp at a distance of 10 cm for 60 min. The highest response from the three dilutions of antigen used (1:4, 1:16, and 1:64) was applied to experimental data.

Mitogen preparation. Lymphocyte responses to mitogen stimulation were tested by using phytohemagglutinin at a concentration of 100 μg/ml.

Lymphocyte culture and harvest. Lymphocytes and appropriate antigens were incubated together by using a whole blood culture technique (7, 13). Blood, which was collected in preservative-free heparin (10 U/ml), was diluted with 15 and 10 volumes of RPMI 1640 culture medium for children and adults, respectively. The final lymphocyte concentration was adjusted to 2×10^5 cells per ml. Triplicate cultures (0.5 ml/tube) were set up and contained 0.05 ml of antigen per tube. The cultures were maintained at 37°C in air with 5% CO_2 for 6, 7, and 8 days. At 24 h before harvest, 10 μCi of [^{3}H]thymidine per ml was added to each culture. The cells were harvested and processed for scintillation counting as previously described (7, 13).

The arithmetic mean and standard deviation of triplicate cultures were calculated. Virus-induced lymphocyte stimulation, expressed as stimulation index (SI), was calculated as follows: SI = (mean counts per minute in stimulated cultures)/(mean counts per min-

ute in unstimulated cultures)(controls). Statistical significance was determined by Student's t test.

Preparation of lymphocyte subpopulations. T-cell-enriched fractions and T-cell-depleted fractions were obtained by application of the rosette-forming assay (8, 9, 16). Erythrocyte-antibody-complement-binding and surface immunoglobulin-bearing lymphocytes were detected by methods described previously (8, 16). In this culture system human blood type AB serum without CMV antibody was used.

RESULTS

Phytohemagglutinin LTF response. Phytohemagglutinin generally induced good lymphocyte proliferation in subjects except for the patients with primary immunodeficiency. The following SIs were obtained in the different age groups; <1 year, 91.0 ± 34.3; 1 to 5 years, 97.1 ± 34.2; 6 to 15 years, 135.5 ± 47.7; adults, 135.5 ± 55.2.

Antigen specificity. To assess the specificity of the CMV LTF response, lymphocytes from seven healthy individuals were tested (Table 1) with both CMV and another herpes group virus, VZV. Heterotypic LTF was not observed. For example, cases 3 through 5 (with humoral antibody to CMV but no humoral antibody to VZV) had significant lymphoproliferative responses to only CMV. Case 6 had humoral antibody to VZV but no antibody to CMV and a positive LTF response to only VZV.

CMV LTF responses in the different age groups. The CMV-specific LTF test was performed in the different age groups to compare the differences in responsiveness to CMV antigen (Fig. 1). Positive lymphocyte proliferation to CMV was observed in all but one of the seropositive subjects. The SIs were all more than 2.0. Significant lymphoproliferative activity to CMV was not observed in the seronegative subjects and cord blood (SI, <2.0). Thus, there was a correlation between the results of our in vitro LTF test and humoral antibody to CMV.

The age-related difference was demonstrated in the CMV LTF test. The lymphocyte responses to CMV were generally low (P <0.025) in infants (SI, 5.7 ± 3.0) and higher in older children (SI, 36.7 ± 33.1) and adults (SI, 52.6 ± 55.4). In the adult subjects, the SIs were scattered from extremely high values to relatively low ones.

CMV LTF responses were lower in 15 children who were excreting CMV in urine when tested (SI, 7.3 ± 5.4) than in 17 seropositive children who were not excreting the virus (SI, 19.8 ± 27.7), but this difference was not statistically significant.

CMV LTF tests on lymphocyte subpopulations. CMV LTF tests were performed on two CMV-seropositive individuals to determine the lymphocyte subpopulations responding to CMV antigen. As Table 2 shows, a T-cell-enriched fraction responded very well to CMV antigen, whereas a T-cell-depleted lymphocyte fraction showed poor response. The highest stimulation was obtained in the whole blood cultures.

CMV LTF test in primary immunodeficiency. CMV LTF tests were carried out in six patients with primary immunodeficiency, five with B-cell deficiency (cases 1, 2, 3, and 4 had agammaglobulinemia; case 5 had hypogammaglobulinemia with hyper-immunoglobulin M) and case 6 with ataxia-telangiectasia. These six patients had no humoral antibody to CMV. Table 3 shows the responses of lymphocytes to CMV antigen in these immunodeficient patients. CMV was isolated from the urine of case 1 when the CMV LTF test was performed. Cases 4 and 5 had histories of CMV infection which were confirmed by virus isolations from urine 2 years ago. These three of five seronegative patients

TABLE 1. *Specificity of in vitro LTF with CMV and VZV antigens in healthy adults and children*[a]

Case no.	Age	Sex	Antibody titer[b]		Lymphoproliferative activity				
			CMV	VZV	Control antigen (cpm)	CMV		VZV	
						Activity (cpm)	SI	Activity (cpm)	SI
1	28 yr	M	8	8	127 ± 14[c]	366 ± 23	2.9	3,576 ± 861	28.2
2	3 yr	F	16	16	242 ± 67	650 ± 25	2.7	971 ± 234	4.0
3	9 mo	M	32	<4	852 ± 52	4,077 ± 687	4.8	760 ± 127	0.9
4	1 yr	M	8	<4	190 ± 2	1,113 ± 308	5.9	260 ± 40	1.4
5	2 yr	F	16	<4	319 ± 18	2,347 ± 497	7.4	294 ± 71	0.8
6	1 yr	F	<4	64	554 ± 81	778 ± 138	1.4	196 ± 32[d]	4.1
7	10 mo	F	<4	<4	77 ± 6	90 ± 20	1.2	88 ± 8	1.1

[a] Cases 1 and 2 were immune against CMV and VZV, cases 3 through 5 were immune against only CMV, case 6 was immune against only VZV, and case 7 was not immune against either CMV or VZV.

[b] Complement-fixing antibody titer.

[c] Mean ± standard deviation.

[d] Done by micromethod; the lymphoproliferative activity with control antigen was 48 ± 7 cpm.

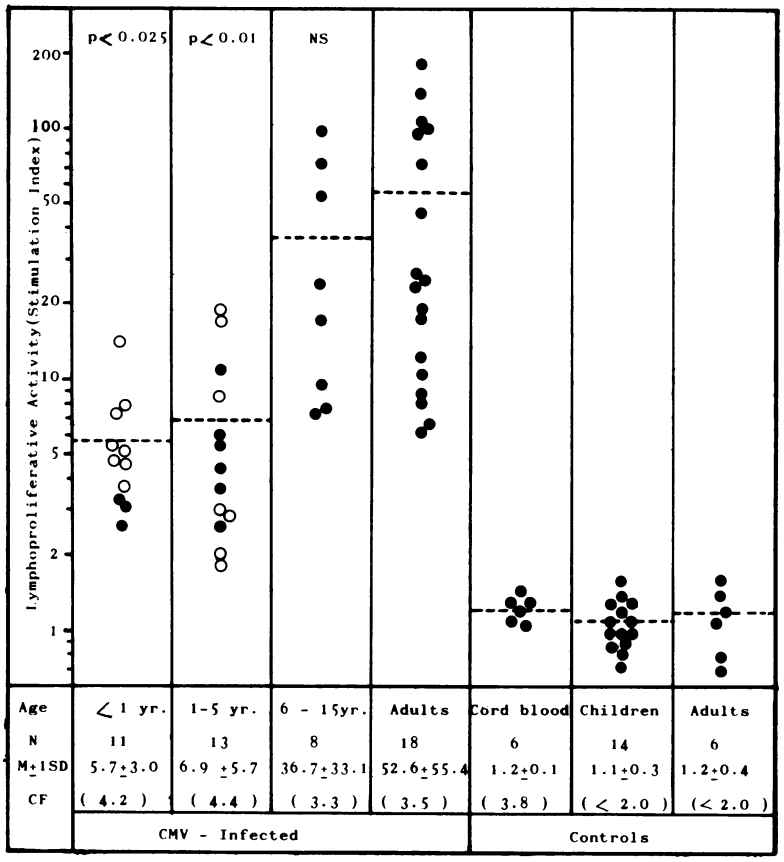

FIG. 1. *CMV-specific LTF in different age groups. CF, Geometric mean of complement-fixing antibody titer (2^n). Each point represents the mean of triplicate samples of lymphocytes from a single subject. Symbols: ○, CMV excreters; ●, nonexcreters. Dotted lines represent mean values. Virus isolation was not attempted from adults. Statistical significance was assessed by comparing the responses of lymphocytes from the children with CMV infections with those of lymphocytes from healthy seropositive adults (as determined by Student's t test). NS, Not significant; M, mean; SD, standard deviation.*

with B-cell deficiency showed positive lymphocyte responses to CMV. A patient with ataxia-telangiectasia who had been continuously shedding the virus in his urine for 1 year showed no cellular response to CMV antigen or phytohemagglutinin. The results were reproduced repeatedly with these patients.

DISCUSSION

T-cells are of key importance in the recognition and management of viral infections. As far as has been determined in murine systems, in vitro lymphocyte reactivity to CMV antigen is dependent on the presence of sensitized T-cells (2, 4, 15, 18). A similar finding was also obtained in the present studies on primary immunodeficiency. Lymphocytes from CMV-infected patients with B-cell deficiencies who were normal in T-cell function showed good responses to CMV antigen. Thus, LTF response could be used as an index of prior exposure to CMV in patients with B-cell deficiencies. LTF tests using separated lymphocytes also indicated that the response of T-cell-enriched fractions from seropositive individuals was more dominant than that of T-cell-depleted fractions. When the whole blood culture technique was used, however, CMV LTF responses were enhanced. It was speculated that other humoral or cellular elements might play supplementary roles in the whole blood culture.

Strain specificity in LTF responses was reported recently by Beutner et al. (1). In the present study, however, only one seropositive infant failed to respond to the AD169 strain.

Age-related differences in CMV LTF responses may be a characteristic feature in CMV infections and be closely related to the mode of infection. The finding that adults and school children have higher lymphoproliferative re-

TABLE 2. *LTF with CMV antigen in a T-cell-enriched fraction and a T-cell-depleted fraction*

Age of patient (yr)	Sex	Fraction	Lymphocyte proliferation					
			CMV			Phytohemagglutinin		
			Control antigen activity (cpm)	Activity (cpm)	SI	Control antigen activity (cpm)	Activity (cpm)	SI
42	M	Whole blood	409 ± 29[a]	16,282 ± 1,755	39.8	395 ± 59	39,520 ± 9,039	100.1
		T-cell enriched	309 ± 42	7,870 ± 608	25.6	594 ± 16	125,472 ± 14,510	211.1
		T-cell depleted	728 ± 137	3,647 ± 506	5.0	649 ± 210	12,700 ± 1,015	18.3
32	M	Whole blood	451 ± 94	11,059 ± 578	24.5	471 ± 127	40,703 ± 8,672	86.4
		T-cell enriched	280 ± 36	4,443 ± 393	15.9	234 ± 95	28,388 ± 4,153	121.3
		T-cell depleted	146 ± 20	367 ± 26	2.5	226 ± 74	2,998 ± 154	13.3

[a] Mean ± standard deviation.

TABLE 3. *In vitro LTF test with CMV antigen in six patients with primary immunodeficiency*[a]

Case	Age (yr)	Sex	Lymphoproliferative activity						% of lymphocyte cell markers		
			CMV			Phytohemagglutinin			E rosettes	EAC rosettes	Surface immunoglobulin
			Control antigen activity (cpm)	Activity (cpm)	SI	Control activity (cpm)	PHA	SI			
1[b]	1	M	362 ± 40[c]	9,646 ± 519	26.6	387 ± 26[c]	41,289 ± 6,326	106.3	90.2	2.0	0
2	7	M	304 ± 27	217 ± 25	0.7	467 ± 32	34,165 ± 6,134	73.2	80.5	8.2	0
3	5	M	268 ± 44	391 ± 75	1.5	283 ± 38	17,355 ± 1,165	61.3	87.6	3.7	1.8
4	3	M	305 ± 41	2,427 ± 91	8.0	403 ± 43	30,694 ± 5,725	76.2	88.6	2.4	0.4
5	3	M	325 ± 6	1,327 ± 143	4.2	383 ± 84	48,097 ± 2,143	125.6	83.1	4.6	4.8
6	4	M	190 ± 42	253 ± 11	1.3	386 ± 69	7,238 ± 189	18.8	39.1	32.2	3.5

[a] Values for age-matched normal controls: SI for phytohemagglutinin, 97.1 ± 34.2(n = 17); SI for CMV, 7.7 ± 5.9(n = 15); E rosettes, 62.4 ± 4.5%; erythrocyte-antibody-complement (EAC) rosettes, 19.8 ± 3.8%; surface immunoglobulin, 7.8 ± 5.9%(n = 18). Cases 1 to 4 had agammaglobulinemia, case 5 had hypogammaglobulinemia with hyper-immunoglobulin M, and case 6 had ataxia-telangiectasia.

[b] CMV infection was demonstrated by viruria in these patients.

[c] Mean ± standard deviation.

sponses in CMV LTF tests than infants and younger children perhaps reflects the reactivation of latent infections. A group of adults showed extremely high SIs. From the fact that enhancement of CMV infection (3, 10) is recognized during pregnancy, blood transfusions, graft versus host reactions, immunosuppressive therapy, and other viral infections, it is speculated that the adults who showed extremely high SIs had some experiences which accelerated reactivation of latent CMV infection. Other explanations might be that the differences in the ages of the subjects when they had primary infections were responsible or that a genetically determined factor was responsible for the degree of LTF response.

It is likely that the low SIs in younger children might have been due to the presence of an active CMV infection, since more children than adults were excreters of the virus. CMV LTF responses were generally lower in the virus-excreting children than in the nonexcreting ones, although the differences were not statistically significant. It was suspected that CMV infection may suppress cell-mediated immunity. Prospective studies on CMV excreters will be needed to clarify the significance of the degree of LTF activity in the persistence or cessation of viruria.

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