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Alteration of lymphocyte subpopulations with cytomegalovirus infection in infancy

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SUMMARY

The distribution of immunoglobulin bearing (Ig^+), T, and null lymphocyte subpopulations and the lymphocyte response to phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) were determined in three infants with cytomegalovirus (CMV) infection. These infants had significantly decreased percentages of T cells (13%, 29% and 40%) compared to age-matched controls ($61 \pm 2\%$). Compensatory increases in the percentages of Ig^+ and null cells occurred. Decreased lymphocyte reactivity to PHA and PWM occurred in two patients. Purified T cells from these patients had normal reactivity indicating a disproportion of T cells in the peripheral blood. These abnormalities may result from CMV infection of lymphocytes and could be responsible for prolonged CMV viruria.

INTRODUCTION

Immunologic disorders including dysgammaglobulinaemia and decreased lymphocyte reactivity to mitogens and antigens occur in some patients with congenital rubella (Plotkin, Klaus, & Whiteley, 1966; Olson, South, & Good, 1967; White *et al.*, 1968). Dysgammaglobulinaemia also occurs in patients with congenital cytomegalovirus (CMV) infection (McCracken & Shinefield, 1965). Although other immunologic abnormalities have not been documented in these patients, their existence has been anticipated (Schwartz, Daniels, & Klintworth, 1975; South, Montgomery, & Rawls, 1975). Groshong *et al.* (1975) reported an infant with chronic CMV infection and immunodeficiency, and suggested that the infection might have caused the immunodeficiency. We present data demonstrating alterations in lymphocyte subpopulations in three infants with CMV infection and discuss the relationship of these abnormalities to infection.

MATERIALS AND METHODS

Rosettes. The number and percentage of immunoglobulin bearing (Ig^+), T, and null cells in the peripheral blood of patients and controls were determined. The T cells in lymphocytes separated on Ficoll–Hypaque gradients were enumerated by indirect rosetting, first by incubation with rabbit antiserum to human T cells and then by incubation with red blood cells coated with purified anti-rabbit light chain antibody as described (Strelkauskas, Teodorescu, & Dray, 1975; Strelkauskas *et al.*, 1976). E rosettes were determined using a modified technique (Strelkauskas *et al.*, 1975). Ig^+ cells were enumerated by direct rosetting with red blood cells coated with purified rabbit anti-human light chain antibody (Strelkauskas *et al.*, 1975). Lymphocytes lacking Ig and T-cell markers are designated null cells. Mean values between patient and control groups were compared using the Student's *t*-test.

Preparation of purified T cells. T cells were isolated from Ficoll–Hypaque purified lymphocytes by negative selection (Strelkauskas *et al.*, 1975). Lymphocytes were mixed with anti-human light chain antibody coated human erythrocytes at a

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ratio of twenty erythrocytes to one lymphocyte. This mixture was centrifuged at 1700 *g* for 5 min at 4°C. The pellet was vigorously resuspended and 2 ml of the rosetted mixture was layered onto 2 ml of cold Ficoll–Hypaque. The gradients were then centrifuged at 800 *g* for 15 min at 4°C and the cells at the interface were collected and washed with Eagle's Minimal Essential Medium (MEM, Grand Island Biologicals, Grand Island, New York). T-cell preparations were tested for purity by rosetting with anti-light chain antibody-coated erythrocytes and found to contain an average of only one percent Ig^+ cells. When these preparations were tested with anti-T cell antiserum for indirect rosettes, 90–95% of the cells were T cells.

Lymphocyte cultures. Each patient was tested with at least one simultaneous control. Lymphocyte cultures prepared from Ficoll–Hypaque gradients and purified T-cell cultures were adjusted to 1×10^6 cells/ml in RPMI 1640 medium (Grand Island Biologicals) supplemented with 10% foetal calf serum (FCS) (Grand Island Biologicals). Cells were placed in quadruplicate cultures at a cell density of 2×10^5/0·2 ml in Micro culture plates (Cooke Laboratory Products, Alexandria, Virginia). They were incubated at 37°C for 3 days without mitogen or with phytohaemagglutinin (PHA-M) (Difco batch 608682, Detroit, Michigan) at a concentration of 0·01 ml/ml, or pokeweed mitogen (PWM) (Grand Island Biologicals, batch R051102) at a concentration of 0·01 ml/ml. During the last 24 hr of incubation, 0·2 μCi of [^{3}H]thymidine (specific activity 2 Ci/mM Amersham/Searle, Arlington Heights, Illinois) was added to each culture. Cells were collected with the Mash III multiple harvesting device (Microbiological Associates, Bethesda, Maryland) and measured for the amount of [^{3}H]thymidine incorporation. The mean ct/min incorporated and standard error were calculated for replicate cultures.

Patients. Patient 1. A 6-month-old white female was evaluated for delayed developmental maturation. She had been treated several times for purulent conjunctivitis and chronic dacryocystitis. She was born at approximately 36-weeks gestation after an uneventful pregnancy. Birth weight and head circumference were both third percentile. On admission, physical examination revealed a hypotonic infant with retarded growth and development. Her weight, height, and head circumference were at the thirtieth percentile. Head shape was brachycephalic. The anterior fontanelle was 5 × 5 cm. Transillumination of the head was increased. Fundoscopic examination was normal. Lungs, heart, abdomen and genitalia were normal. Lymph nodes were palpable. Complete blood count, thyroxine level, urinalysis, chest roentgenogram, electrocardiogram and electroencephalogram were normal. Skull roentgenogram showed a wide fontenelle with no calcifications, severe platybasia and a wide sella turcica. A carotid angiogram revealed findings compatible with hydrocephalus and interruption and deviation of the superior sagittal sinus and atrophy of the right hemisphere. Pneumoventriculogram was consistent with hydrocephalus without block and cerebral atrophy. During the following year she was seen as an outpatient for four upper respiratory tract infections, two episodes of otitis media, herpes stomatitis, five episodes of gastroenteritis, and an infected ear lobe. She was re-admitted at age 8 months with septic arthritis of the left elbow. Culture of the joint aspirate showed mixed growth with *Haemophilus influenza*, type B, micrococci, genus Bacillus, *Clostridia inocum*, and *Bacteroides melaninogenicus*. She was treated with i.v. ampicillin and recovery was uneventful.

Patient 2. A 3-day-old infant was evaluated for jaundice which was noted 13 hr after birth. Physical examination also revealed purpuric lesions over the extremities. His head circumference, height and weight were in the twenty-fifth percentile. Heart and lungs were normal. Liver and spleen were both palpable 3 cm below the costal margins. His neurological examination was normal. Laboratory investigations included: white blood cell count, 5500 cells/mm^3, with 35% neutrophils, 2% band forms, 53% lymphocytes, and 10% monocytes; haemoglobin 13·5% mg; haematocrit 40%; platelets 31,000. Total bilirubin was 20·8 mg%, direct 12·6 mg%. Skull roentgenograms revealed no calcifications. The jaundice subsided over several weeks. By 6 months of age growth and neurological development were apparently normal.

Patient 3. A 1400 g boy was born after 30-weeks gestation. Weight, height (42 cm), and head circumference (29·5 cm) were appropriate for the gestational age. At 3 months he was evaluated because of poor gr owth and possible sepsis. A diagnosis of CMV infection was made because of positive urine cytology. E rosettes were 55% and immunoglobulin-bearing cells (B cells) were 8% of total lymphocytes. Subsequently he was seen in the outpatient department six times because of fever and upper respiratory tract infection and four times because of otitis media. At 16 months growth and development were retarded.

Controls. White blood count, percent lymphocytes, and the number and distribution of lymphocyte classes were determined in six age-matched children. These children were tested as part of evaluation for a variety of illness. None had stigmata of CMV infection. None had immunodeficiency.

RESULTS

Virus studies

CMV was isolated from the urine of patient 1 at age 6 months and ten months and from the urine of patient 2 at age 1 week. Viral isolates were identified as CMV by their characteristic cytopathic effect in WI 38 cells with the presence of intranuclear inclusions in haematoxylin-eosin-stained preparations. Urine cytology demonstrated intranuclear inclusions typical for CMV in patient 3 at age 2 months.

Humoral immunity

Immunoglobulin concentrations and CMV antibody titres are shown in Table 1. IgM was elevated in patient 2 at 1 week of age, but was normal in patient 1 at 10 months. Patients 1 and 2 had comple-

TABLE 1. Humoral immunity in CMV patients

Patient	Age	Immunoglobulins (mg/dl)			CMV antibodies*		
						IFA	
		IgG	IgM	IgA	CF	IgM	IgG
1	6 months				64	160	10
	10 months	1300	148	51			
2	1 week	1080	102	0	64†	320	320
	3 weeks				64	160	320

CF = complement fixation; IFA = immunofluorescent antibody.

* Reciprocal of titre.

† Mother's value 64.

ment fixing (CF) antibodies and IgG and IgM CMV antibodies demonstrable by immunofluorescence (IFA). The titre of CF antibodies in patient 2 equalled that of his mother.

Leucocytes

The white blood cell count and percentage of lymphocytes were apparently increased in children with CMV infection when compared to controls; however, these differences were not statistically significant (Table 2). Transient granulocytopenia was noted in patient 1 at age 17 months (950 granulocytes/mm^3) and in patient 2 at age 1 week (935 and 650 granulocytes/mm^3).

Cellular immunity

The average percentage of T cells in patients was 27%, a significant reduction from the control average of 61% ($P<0{\cdot}02$, Table 2). A compensatory increase in the Ig^+ and null cell populations occurred. The average percentage of Ig^+ cells in patients was 36% compared to 27% in controls ($P<0{\cdot}1$). The average percentage of null cells in patients was 37% compared to 12% in controls ($P<0{\cdot}005$).

TABLE 2. Number and distribution of T, B and null lymphocytes in CMV infection

Donor	Age (months)	WBC	Lympho-cytes (%)	E*		T		B		Null	
				Cells/mm^3		Cells/mm^3		Cells/mm^3		cells/mm^3	
1	15	17,000	78	1193	(9)	1723	(13)	4773	(36)	6762	(51)
	17	8700	80			1322	(19)	2645	(38)	2923	(42)
2	5	6300	86	1679	(31)	1571	(29)	2276	(42)	1571	(29)
3	16	10,300	74			3049	(40)	2287	(30)	2287	(30)
Mean±s.e.		11,200±3822†	79±4†			2114±469	27±8‡	3112±830	36±3	3540±1624	37±7¶
Controls											
Mean±s.e.	3–14	7076±1327	57±14			2434±757	61±2	1130±264	27±2	627±301	12±3

Figures in parentheses are percentages.

* E rosettes

† Only first value from patient 1 included.

‡ $P<0{\cdot}02$ compared to control.

¶ $P<0{\cdot}005$ compared to control.

TABLE 3. Lymphocyte reactivity to mitogens in patients and controls

Patient	Age	Cells	No mitogen	PHA*	PWM†
			ct/min±s.e.	ct/min±s.e.	ct/min±s.e.
1	17 months	Total	495±69	18,898±1532	4,743±444
		T	1160±214	31,591±1580	9,322±1505
2	5 months	Total	930±409	26,664±4144	6,637±921
		T	1743±429	37,078±1150	10,835±758
3	12 months	Total	1326±70	43,527±731	13,096±1740
Control					
1	30 years	Total	638±146	40,986±2293	12,618±1818
		T	1001±247	42,329±4043	10,320±275
2	8 years	Total	1192±94	43,048±1134	16,119±674
3	14 months	Total	744±60	52,075±809	14,156±612
4	3 months	Total	546±73	52,114±2125	13,520±668
Mean		Total		47,056±3398	14,103±858

* PHA = Phytohaemagglutinin.
† PWM = Pokeweed mitogen.

The absolute numbers of Ig^+, T and null cells did not differ significantly between patients and controls. In two cases indirect and E rosettes were both measured and values were comparable.

Replicate lymphocyte cultures were incubated with PHA, PWM, or no mitogens and labelled with [^{3}H]thymidine (Table 3). Spontaneous label incorporation was comparable in patients and controls. PHA-stimulated label incorporation was reduced in patient 1 to 40% and in patient 2 to 57% of the average value of the controls. PWM activity was reduced in patient 1 to 34% and in patient 2 to 47% of the average value of the controls. Lymphocyte cultures of purified T cells from patients 1 and 2 had reactivity equal to control values with PWM and were only slightly decreased with PHA. Patient 3 had normal reactivity to mitogens.

All infants had received live oral polio vaccine prior to the demonstration of immunologic abnormalities. None developed vaccine-related complications.

DISCUSSION

CMV infection was definitely congenital in patient 2 since viruria was demonstrated in the 1st week of life. Characteristic clinical findings of jaundice, hepatosplenomegaly, and thrombocytopenia were evident. As patients with immunodeficiency are at risk for acquired CMV infection, it is remotely possible that the immunologic abnormalities in patient 1 and 3 antedated CMV infection. Since patient 1 was not evaluated until age 6 months, it cannot be proven that she had congenital infection. The delayed growth and development, hydrocephalus, and cerebral atrophy in this child are most consistent with, but not pathognomonic for, congenital CMV infection (Hanshaw, 1970; Hanshaw, 1971). Since patient 3 was evaluated only by cytologic study at 3 months of age, we cannot be certain infection was congenital.

The elevated cord IgM and the presence of IgM CMV antibodies in patient 2 are characteristic serologic findings in congenital CMV infection. The presence of IgM CMV antibodies in patient 1 does not prove recent infection since these antibodies may persist for the 1st year after congenital infection (Hanshaw, 1971).

Transient granulocytopenia occurred in patients 1 and 2. Thrombocytopenia and anaemia occur commonly in congenital CMV infection and at least the former may result from decreased marrow production (Oski & Naiman, 1972). This mechanism could explain the granulocytopenia as well. Bone-marrow examination would be helpful in elucidating the basis for granulocytopenia.

The alteration in distribution of lymphocytes occurred without significant changes in the absolute

numbers of Ig^+, T, and null cells. The decreased percentage of T cells in patients was offset by the increases in percentage of total lymphocytes and the white blood cell count. Since increments occurred in both Ig^+ and null-cell percentages, gains in total lymphocytes were divided between these groups and individual increases were not great enough to achieve statistical significance.

Dysgammaglobulinaemias and serologic abnormalities have been observed in congenital CMV infection and in post perfusion CMV mononucleosis (McCracken & Shinefield, 1965; Kantor *et al.*, 1970). Cellular immunologic abnormalities occurred in an infant with CMV infection but it was unknown whether the CMV infection was congenital or acquired (Groshong *et al.*, 1975). Decreased proportions of T cells in congenital CMV infection have not been reported previously.

In infectious mononucleosis, which results from infection by the related herpes virus, Epstein–Barr virus (EBV), B-cell proliferation usually produced a reversal of the ratio of T to B cells during the first week of the disease (Mangi *et al.*, 1974). Decreased percentages of T cells occur in malnourished children including infants without congenital infection who are small for gestational age (Ferguson *et al.*, 1974). Similar changes have also been reported in connective tissue diseases including juvenile rheumatoid arthritis (JRA) (Schauf, Strelkauskas, & Dray, 1975).

PHA response was reported to be normal in congenital rubella and CMV infection (Dudgeon, Marshall, & Soothill, 1969; Marshall, Cope, Soothill, 1970). However, Olson *et al.* (1967 and 1968) have shown that PHA responsiveness is decreased in the 'escalated' rubella syndrome and returns to normal when the infection subsides. Transient PHA unresponsiveness may explain the conflicting reports in the rubella literature as well as the disagreement between our data and previously reported cases of CMV infection (Dudgeon *et al.*, 1969; Marshall *et al.*, 1970). The decreased response to PHA and PWM at a single dose in lymphocyte cultures in patients 1 and 2 compared to controls is apparently due to a relative deficiency of T cells since purified T-cell cultures from the same patients had near normal or normal reactivity. PHA response is also diminished in association with imbalance of lymphocyte subpopulations in EBV mononucleosis (Mangi *et al.*, 1974) and in JRA (Schauf *et al.*, 1975).

Imbalance of lymphocyte subpopulations and decreased responsiveness to mitogens in CMV infection may result either from congenital immunologic abnormalities induced by intrauterine infection, or alternatively, directly from viral infection of the lymphocytes. Decreased mitogenic response has been attributed to viral infection of lymphocytes in murine CMV infection (Selgrade *et al.*, 1976) and is also likely in rubella (Olson *et al.*, 1968). Since human CMV is also carried in leucocytes, this mechanism should be explored as an explanation for our observations.

The immunologic abnormalities described in these infants may explain the characteristic prolonged viruria that occurs in congenital CMV infection. Future observations should be directed to the question whether congenital CMV infection frequently induces immunologic abnormalities, and the nature and clinical significance of these abnormalities.

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