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Strain-Specific Local and Systemic Cell-Mediated Immune Responses to Cytomegalovirus in Humans

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Employing the techniques of complement fixation, immunofluorescence, and in vitro lymphocyte transformation assay, the antibody and cell-mediated immunity to cytomegalovirus (CMV) were studied in the serum, peripheral blood lymphocytes, tonsillar lymphocytes, and cord blood lymphocytes. The study population consisted of 32 children undergoing tonsillectomy and adenoidectomy. In the lymphocyte transformation assay, three strains of CMV (AD-169, ADH-1-41, and Davis), herpes simplex type 1, and phytohemagglutinin were employed as antigens. Sixty-five percent of the subjects were found to have CMV-specific antibody activity. The lymphocyte transformation response to phytohemagglutinin was similar in all subjects. No CMV-specific lymphocyte transformation activity was detected in cultures of cord blood lymphocytes. Significant cell-mediated immunity was observed in the tonsillar lymphocytes of 30% (3/10) of the seronegative individuals and in the peripheral blood lymphocytes obtained from one such subject. Over 75% (16/21) of the seropositive subjects demonstrated cell-mediated immunity against one or more strains of CMV in the peripheral blood lymphocytes and tonsillar lymphocytes. In the lymphocyte transformation assay, no cross-reactivity was apparent between CMV and herpes simplex type 1. These studies demonstrate the presence of strain-specific systemic and mucosal cell-mediated immune responses to CMV in humans. The frequency and distribution of lymphocyte transformation responses to the three CMV strains suggest antigenic heterogeneity of CMV.

Although the antibody response to cytomegalovirus (CMV) infection has been extensively studied (8, 10), little or no information is available as to the nature of the cell-mediated immune (CMI) response to CMV. The CMI response to CMV may effect the outcome of primary CMV infections or reactivation of latent CMV infections (10). In addition, little information is available about the local mucosal immune response to CMV. The immunocompetent lymphoid tissue of mucosal surfaces is in constant contact with the external environment. These surfaces constitute a site of initial exposure to ingested or inhaled antigens. We initiated the present study to examine the local and systemic CMI response to CMV. For these studies, three strains of CMV were employed to investigate the possibility of detecting CMV antigenic heterogeneity based on in vitro lymphoproliferative reactions of sensitized lymphocytes.

MATERIALS AND METHODS

Study population. Thirty-two children and young adults, ranging in age from 3 to 18 years and of whom 15 were males and 17 females, participated in the study. All subjects were admitted to Buffalo Children's Hospital and underwent tonsillectomy and adenoidectomy (T&A) as a result of recurrent acute or chronic tonsillitis, otitis media, or other respiratory tract infections. None of the subjects was under immunosuppressive therapy during or before surgery.

Specimens. Heparinized blood specimens were obtained from all T&A patients on the day of surgery. Plasma for serological studies was obtained by centrifugation and stored at −20°C. Lymphocytes were obtained from blood specimens as described below and utilized for the lymphocyte transformation (LTF) assay. Tonsils were obtained within 1 h after surgery and were immediately processed as described below. Heparinized cord blood samples from randomly selected healthy newborn infants were processed in parallel with blood specimens obtained from T&A patients.

Stimulants for LTF assay. Three strains of CMV (AD-169, ADH-1-41, and Davis), herpes simplex virus type 1 (HSV-1), and phytohemagglutinin (PHA) were employed as stimulants in the LTF assay. A continuous line of human diploid fibroblasts derived from fetal tonsil cells was employed for the cultivation of the three CMV strains. The virus stock was diluted with RPMI-1640 to a final concentration of $10^{4.5}$ 50%

tissue culture infective doses per ml and inactivated by exposure to ultraviolet light for 30 min at room temperature. The inactivated virus preparations were aliquoted and stored at −20°C until use. Uninfected fetal tonsil cells grown and processed in a similar manner served as an antigen control. HSV-1 was propagated in a continuous line of African green monkey kidney cells (BGM). For use as a stimulant in the LTF assay, HSV-1 was diluted in RPMI-1640 to a final concentration of 10^4 plaque-forming units per ml, inactivated by exposure to ultraviolet light for 30 min at room temperature, aliquoted, and stored at −20°C until use. Uninfected BGM cells processed in a similar manner served as an antigen control. PHA-M (General Biochemicals, Cleveland, Ohio), diluted 1:10 with RPMI-1640, aliquoted, and stored at −20°C, was employed as a mitogen.

Collection and processing of lymphocytes. Peripheral blood lymphocytes (PBL) and cord blood lymphocytes (CBL) were obtained from heparinized blood samples by Ficoll-Hypaque centrifugation as previously described (1). Tonsillar lymphocytes (TL) were obtained by gentle grinding of small pieces of tonsillar tissue over a sterile wire mesh and washing with Hanks balanced salt solution as previously described (7). The cells were washed by centrifugation three times with Hanks balanced salt solution. More than 95% of PBL and TL were shown to be viable by trypan blue dye exclusion. PBL, CBL, and TL were suspended to a final concentration of 10^6/ml in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and 100 U of penicillin G and 100 μg of streptomycin per ml.

LTF assay. The LTF assay was performed as previously described (7). Briefly, 1-ml samples of PBL, TL, or CBL cell suspensions were dispensed into sterile glass screw-cap tubes (16 by 125 mm). To triplicate cultures of each cell type, 0.1 ml of each of the following was added: RPMI-1640, PHA, one of the three inactivated CMV strains, and uninfected fetal tonsil cells. In a few selected experiments, 0.1 ml each of inactivated HSV-1 antigen and uninfected BGM cells were added to triplicate cultures. Thus the final concentration of CMV antigens and HSV-1 antigen was equivalent to $10^{3.5}$ 50% tissue culture infective doses per ml and 10^3 plaque-forming units per ml, respectively.

After the addition of stimulants, the cell cultures were incubated at 37°C. On day 5 of incubation, 1 μCi of [^{3}H]thymidine (Schwartz/Mann, Orangeburg, N.Y.) in 0.1 ml of RPMI-1640 was added to each culture. At 18 h after labeling, the cell cultures were harvested and processed for liquid scintillation counting as previously described (7).

The results of the LTF assay were expressed as stimulation indexes, which were calculated as follows: SI = mean counts per minute of cell culture containing stimulant/mean counts per minute of cell culture without stimulant. A stimulation index greater than or equal to 3.0 was considered indicative of a positive response.

CMV antibody determination. CMV antibody activity was determined by complement fixation (CF) or indirect immunofluorescence assay, as previously described (3, 4, 6, 9). For these studies, CMV AD-169 was employed as the antigen source. The majority of samples were evaluated by CF. Samples found to be anti-complementary were subsequently tested by indirect immunofluorescence assay.

RESULTS

CMV serological status. Serological evaluation of the study population demonstrated that 21 (65%) of the 32 subjects were seropositive for CMV-specific antibody activity. An equal percentage of males and females was found to be seropositive. The median age of seropositive subjects was 7 years, with a range of 2 to 17 years. The median age of seronegative subjects was 8 years, with a range of 4 to 18 years.

PHA response of PBL and TL. The in vitro response to PBL and TL to the mitogen PHA are presented in Fig. 1. Significant responses were observed in all cell cultures. Although there was variability within each group, there was no significant difference ($P > 0.1$) between the responses observed in lymphocyte cultures from seronegative and seropositive subjects.

LTF response of seropositive subjects. The in vitro responses to CMV antigens of the 21 seropositive subjects are presented in Table 1. Although 18 of 21 (86%) seropositive subjects manifested LTF responses to at least one strain of CMV, the LTF response to different CMV strains manifested considerable variability in individual subjects. The PBL and TL of 14 subjects (groups A, B, and C) demonstrated lymphoproliferative activity to at least one strain of

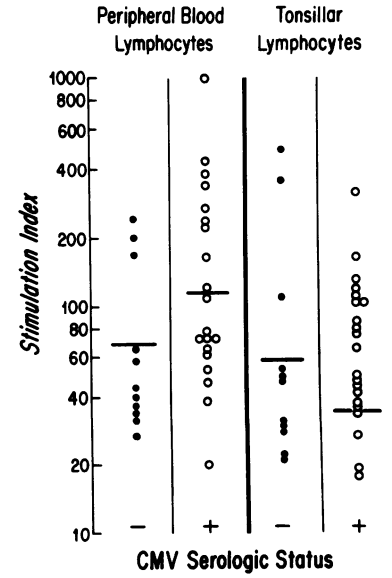

FIG. 1. *Stimulation indexes of PBL and TL to PHA in CMV seronegative (●) and seropositive (○) subjects. Solid bars indicate geometric mean stimulation indexes.*

CMV. The TL but not the PBL cultures of two patients (group D) responded to CMV antigens. PBL but not TL cultures from two subjects (group E) responded to CMV antigens. No lymphoproliferative activity was observed in three subjects (group F).

PBL and TL cultures from the same individual frequently differed in reactivity to the three strains of CMV. Considering only the 14 subjects whose PBL and TL cultures responded to CMV antigens (groups A, B, and C), PBL and TL cultures from 5 of these patients (group A) reacted with the same strain or strains of CMV. In five other patients (group B), TL manifested responses to strains of CMV which failed to stimulate the subjects' PBL cultures. Conversely, in four patients (group C), PBL cultures were stimulated by CMV strains which failed to elicit a response in TL cultures.

The intensity of the lymphoproliferative response observed in an individual's PBL and TL cultures to the same strains often differed in magnitude. For example, the response of TL cultures obtained from patients 90, 91, and 94 to the ADH-1-41 strain was three- to fourfold greater than the response to this antigen in the PBL cultures from these patients.

The magnitude of the lymphoproliferative response to CMV antigens was not proportional to the CMV-specific CF antibody titer. This is illustrated by patients 65, 95, 98, 106, and 116, who demonstrated serum antibody titers of 8 or less. The LTF activities of these patients' lymphocyte cultures were comparable to the responses observed in patients with two- to eightfold-higher antibody titers.

LTF response of seronegative patients. The LTF responses of PBL and TL cultures of patients seronegative for CMV-specific serum antibody are presented in Table 2. The PBL cultures of patient 120 manifested a minimal response to strain AD-169. The TL cultures from three seronegative subjects (114, 119, and 124) demonstrated lymphoproliferative activity to CMV antigens in the absence of such activity in PBL cultures.

LTF response of CBL. Due to technical difficulties in obtaining sufficient numbers of

TABLE 1. *Individual serum CMV-CF antibody titers and LTF responses to CMV antigens of seropositive subjects*

Group	Patient no.	CMV-CF titer	Stimulation index					
			PBL			TL		
			AD-169	ADH-1-41	Davis	AD-169	ADH-1-41	Davis
A	91	32	**5.0**[a]	**6.5**	**16.0**	**7.9**	**30.3**	**4.5**
	92	16	**4.4**	**7.0**	1.8	**3.7**	**3.3**	**2.4**
	94	16	**5.4**	**7.5**	1.7	**10.1**	**21.8**	0.2
	95	4	**6.8**	**6.3**	2.2	**3.4**	**10.1**	1.7
	121	32	**5.7**	1.0	1.8	**4.3**	1.0	1.6
B	83	32	**3.1**	**7.3**	0.4	**4.7**	**11.1**	**9.2**
	90	16	1.5	**5.3**	1.2	1.3	**28.0**	**4.3**
	108	16	0.5	**21.4**	0.4	1.0	**3.4**	**3.9**
	118	32	1.3	**6.1**	1.0	1.5	**3.1**	**4.2**
	123	16	**3.4**	1.4	1.1	**5.2**	**8.8**	**6.9**
C	98	8	**4.4**	**5.5**	0.3	2.3	**5.2**	0.9
	101	16	**3.9**	**3.1**	**4.9**	**3.1**	2.6	2.1
	106	4	1.3	**4.0**	**7.7**	1.5	**27.1**	2.8
	107	16	0.6	**23.1**	**3.4**	1.0	**12.0**	0.6
D	65	4		2.0		**3.1**	**3.9**	
	116	4	0.6	1.4	1.2	2.2	**3.4**	1.9
E	100	AC[b]	**5.3**	**3.2**	2.3	2.4	2.1	2.2
	117	32	**4.7**	0.8	1.7	1.1	1.2	1.4
F	82	16	2.4	0.5	1.8	0.4	0.9	2.7
	86	8	0.9	0.2	1.1	1.7	1.9	0.5
	115	4	1.5	0.7	1.4	2.5	2.7	0.9

[a] Significant responses are shown in bold-face type.
[b] AC, Anticomplementary by CF but seropositive by immunofluorescence.

TABLE 2. *Individual serum CMV-CF antibody titers and lymphoproliferative responses to CMV of seronegative subjects*

Patient no.	CMV-CF titer	Stimulation index[a]					
		PBL			TL		
		AD-169	ADH-1-41	Davis	AD-169	ADH-1-41	Davis
72	AC[b]	0.4	1.2				
93	AC	0.9	0.2	1.1	0.7	1.3	0.4
109	<4	0.3	0.6	0.5	1.3	1.0	1.7
110	<4	0.9	1.1	2.0	2.2	2.3	2.9
112	<4	0.9	0.8	0.9	0.8	2.7	1.6
113	<4	2.7	2.6	2.6	1.6	**3.4**	0.4
114	<4	1.4	1.3	1.3	2.9	**7.9**	**7.3**
119	<4	0.6	1.1	0.9	0.8	0.5	1.1
120	<4	**3.2**	1.9	2.9	2.1	1.5	1.6
122	AC	1.1	1.9	1.2	1.5	1.7	1.2
124	<4	0.9	1.6	1.0	2.2	**6.8**	2.8

[a] Significant responses are shown in bold-face type.
[b] AC, Anticomplementary by CF test but seronegative by immunofluorescence.

TABLE 3. *Individual lymphoproliferative responses of CBL to PHA and two CMV strains*

Sample no.	Stimulation index		
	PHA	AD-169	ADH-1-41
CBL5	18.0	2.8	2.1
CBL6	24.0		1.7
CBL7	28.0	1.4	1.2
CBL8	31.8	1.5	2.0
CBL9	54.0	0.6	0.4
CBL10	57.0	1.8	0.6
CBL11	61.0	2.0	1.3

lymphocytes, CBL were tested only with CMV AD-169 and ADH-1-41. All CBL cultures demonstrated significant lymphoproliferative activity to PHA (Table 3). No significant stimulatory activity was observed in CBL cultured with CMV antigens.

LTF response to HSV-1. To investigate the specificity of the CMI response to CMV antigens, PBL and TL of five subjects were also cultured with HSV-1 antigen (Table 4). No apparent cross-reactivity was observed between the CMI response to CMV antigens and the response to HSV-1. PBL and TL of patients 106 and 108 manifested lymphoproliferative activity in response to CMV antigens but no response to HSV-1 antigen. TL cultures from patient 109 were stimulated by HSV-1 antigen but did not manifest lymphoproliferative activity in response to CMV antigens.

CMV heterogeneity. An analysis of the frequency and distribution of all significant LTF responses to the three strains of CMV is shown in Fig. 2. A certain degree of heterogeneity in the CMI response to CMV was observed upon simultaneous testing with the three strains of CMV. The lymphoproliferative responses to AD-169, ADH-1-41, and Davis strains were observed in 21, 30, and 11 subjects, respectively. Five lymphocyte cultures responded to all three CMV strains. Davis strain only stimulated cells that were also reactive with ADH-1-41 strain. However, not all cultures stimulated by ADH-1-41 responded to Davis strain. Six responses were observed to AD-169 alone and nine responses were to ADH-1-41 alone.

DISCUSSION

Serological surveys have established CMV as a very ubiquitous agent. Clinical entities that have been associated with post-natal CMV infections include CMV mononucleosis, hepatitis, interstitial pneumonitis, myocarditis, pericarditis, and peripheral neuropathies (8, 11). The incidence of infection with clinical manifestations is much lower than the incidence of subclinical CMV infection, as indicated by serological study.

The incidence of CMV-specific antibody activity reported here in T&A patients (65%) is notably greater than would be expected in children of this age group. In a study of additional T&A patients and age-, sex- and socioeconomic-matched non-T&A controls, we have observed a fourfold or greater incidence of CMV-specific antibody activity in the T&A patients (K. R. Beutner, A. Morag, B. Morag, R. Diebel, and P. L. Ogra, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, E19, p. 66) over the non-T&A controls. These observations suggest that T&A patients may have an increased susceptibility to CMV infection or that recurrent tonsillitis may be due to CMV infections.

The LTF assay is generally accepted as an in

TABLE 4. *Individual values of serum CMV and HSV-1 CF antibody titers and in vitro lymphoproliferative responses to three strains of CMV and HSV-1*

Patient no.	Serum CF titer		Stimulation index[a]							
			PBL				TL			
			CMV			HSV1	CMV			HSV-1
	CMV	HSV-1	AD-169	ADH-1-41	Davis		AD-169	ADH-1-41	Davis	
106	4	<4	1.3	**4.0**	**7.7**	**2.8**	1.5	**27.1**	2.8	1.8
107	16	8	0.6	**23.1**	**3.4**	**6.4**	1.0	**12.0**	0.6	0.5
108	16	4	0.5	**21.4**	0.4	0.8	1.0	**3.4**	**3.9**	1.3
109	<4	<4	0.3	0.6	0.5	0.7	1.3	1.0	1.7	**3.1**
123	16	16	**3.4**	1.4	1.1	**3.5**	**5.2**	**8.8**	**6.9**	**6.0**

[a] Significant responses are shown in bold-face type.

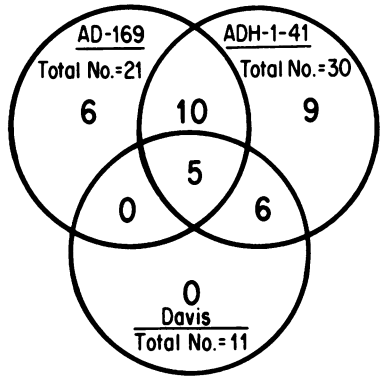

FIG. 2. *Frequency and distribution of significant LTF responses (stimulation index ≥3) in PBL and TL to AD-169, ADH-1-41, and Davis strains of CMV.*

vitro correlate of the CMI response. Although the induction of lymphoproliferative activity by mitogens has been shown to involve both T and B cells, the initial triggering of the proliferative response to bacterial (purified protein derivative) and viral (mumps and rubella) antigens has been demonstrated to be primarily a T-cell response (2, 7). The specificity of the CMI response to CMV was confirmed by experiments with HSV-1 and CBL. The CMI responses to HSV-1 and three strains of CMV appeared to be distinct. The possibility that the lymphoproliferative responses to CMV antigens observed were due to mitogenic properties of these preparations was eliminated by the observation that CBL manifested no response when cultured with CMV antigen preparations.

The present study has demonstrated LTF activity in both PBL and TL. The important characteristics of the CMI response to CMV include the presence of LTF activity in cultured TL of some seronegative subjects and in the majority of TL and PBL cultures obtained from seropositive subjects. The LTF response of CMV-seronegative subjects may be a manifestation of the variability of CMV-CF antibody titers. In a longitudinal study of blood donors, Waner et al. (10) have reported that CMV-CF antibody titers can fluctuate between significant and undetectable levels in the same individual over a period of time. TL and PBL from the same individuals frequently manifested different patterns of reactivity to the three strains of CMV. Analysis of the frequency and distribution of CMI response to the three strains demonstrated an apparent heterogeneity in the CMI response to different CMV strains. These strain-specific responses suggest that the systemic and local mucosal CMI responses to CMV are independent. The apparent heterogeneity in the CMI response to the three strains of CMV and the frequent discrepancies between the reactivity of PBL and TL from the same individual are of particular interest. These observations suggest that some individuals may experience repeated CMV infections by different strains of CMV, and these infections may at times be limited to the local mucosal tissue.

A number of serological studies by other investigators have demonstrated antigenic differences between different strains of CMV (5, 11). Our observation of heterogeneity in the specificity of the CMI response to three CMV strains provides additional evidence for antigenic heterogeneity of CMV. If the antigenic structure of CMV that stimulates lymphoproliferative activity were strain independent, all lymphocyte cultures would manifest comparable activity to all three strains. This was not observed in the present study; no one strain demonstrated the ability to stimulate all responding lymphocyte cultures. The analysis of the frequency and distribution of significant LTF response to CMV strains (Fig. 2) suggests that ADH-1-41 and Davis strains of CMV have similar but not identical antigens capable of stimulating sensitized lymphocytes.

The similarity is demonstrated by the ability of ADH-1-41 strain to stimulate all lymphocyte cultures that also responded to Davis strain. A difference between ADH-1-41 and Davis strains is indicated by the observation that some lymphocyte cultures were stimulated by ADH-1-41 but not by the Davis strain. The antigenic structure of strain AD-169 responsible for the in vitro lymphoproliferative response appears to be distinct from the antigens of both Davis and ADH-1-41 strains. AD-169 strain stimulated lymphocyte cultures which failed to respond to either ADH-1-41 or Davis strains.

This study has demonstrated the presence of local and systemic strain-specific CMI to CMV, and antigenic heterogeneity of CMV strains as defined by the LTF assay. Based on these preliminary studies, there appear to be at least two distinct strain-specific CMV antigens capable of stimulating in vitro lymphoproliferation.

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