

## Cytomegalovirus-Specific Cell-Mediated Immunity in Lower-Socioeconomic-Class Adolescent Women with Local Cytomegalovirus Infections

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The factors that regulate cytomegalovirus (CMV) excretion from the genitourinary tract are poorly understood. To assess the role of cell-mediated immunity in such excretion, a CMV-specific mononuclear blastogenesis assay was used to study a predominantly lower-socioeconomic-status population of 92 healthy nonpregnant adolescent women who also had CMV complement-fixing antibody titers and viral cultures of cervix, urine, saliva, and blood performed. Eighteen were studied more than once. No blood cultures were positive and no seroconversions were noted. There was no significant difference for frequency or degree of systemic CMV-specific blastogenesis between the 20 who were culture positive and the 41 who were seropositive but culture negative, although 40% of the culture-positive group and 27% of the seropositive, culture-negative group lacked CMV-specific blastogenesis. One of 31 seronegative subjects displayed CMV-specific blastogenesis. No systematic deficits were noted in any groups or individuals for E rosette number or mitogen response, though some isolated significant differences among groups for mitogen responses existed. Local CMV excretion in the study population was not related to systemic CMV-specific mononuclear blastogenesis.

Carriage of cytomegalovirus (CMV) in the genitourinary tract is common in healthy, nonpregnant, young women (7, 8, 15, 23). Such local (i.e., mucosal) infections may represent a significant reservoir for congenital infection of subsequent offspring and transmission of infection to susceptible individuals in the community. The factors that regulate local viral excretion in these women are poorly understood. Previous investigations have demonstrated that local CMV infection may occur despite specific local and systemic antibody (18, 21). The possible importance of cell-mediated immunity as a modulator of human CMV infection has been supported by observations of depressed CMV-specific cell-mediated immunity in congenitally and natively CMV-infected infants, their mothers, and renal transplant recipients (4, 10, 11, 13, 17). Studies of human subjects with CMV mononucleosis suggest that CMV infection may itself attenuate general and CMV-specific cell-mediated immunity (9, 12, 19, 20), which might then facilitate

persistence, extension, and clinical exacerbation of the infection. Although these studies suggest a substantial interaction of clinically significant CMV infections with cell-mediated immunity, the role of systemic CMV-specific cell-mediated immunity in asymptomatic local CMV infections of otherwise healthy immunocompetent subjects is unclear. Deficient CMV-specific cell-mediated immunity in such infections might help to explain viral reactivation and persistence; conversely, intact specific cell-mediated immunity might help explain the local asymptomatic nature of such infections. To explore the role of systemic CMV-specific cell-mediated immunity in local CMV infections, we used a CMV-specific mononuclear blastogenesis assay to study a predominantly lower-socioeconomic-class population of 92 sexually active, healthy, nonpregnant, adolescent women whose virological and serological statuses were also determined.

### MATERIALS AND METHODS

**Subjects.** One hundred five adolescent women attending a public health adolescent family planning clinic were enrolled. This population was chosen because a previous study of a very similar, although pregnant, population in the same community had

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found a high incidence of CMV-positive genitourinary cultures (D. J. Lang, S. I. Welt, J. F. Kummer, A. O'Quinn, and R. J. Thompson, Jr., abstr. 10, *Pediatr. Res.* 12:365, 1978). Informed consent was obtained from each subject and, if under 18 years of age, from her parents or guardians as well. Twelve subjects were excluded because appropriate specimens were not obtained, and one was excluded because she was found to be pregnant. Ninety-two women (mean age, 16.3 years; range, 13 to 19 years) were thus included. Demographic, sexual activity, birth control, and general health information was obtained for each patient from the clinic chart and a standard questionnaire. None of the patients were receiving immunosuppressive medications, and none reported hepatitis, mononucleosis, or blood transfusion within the preceding 5 years. Eighty-five of the women were unmarried. Forty-nine had previously been pregnant. Seventy-eight were determined to be of lower socioeconomic status as judged by level of parental education and inability to pay for medical care.

Specimens of clean-catch urine, saliva, cervical fluid (aspirate and swab), and heparinized blood were obtained for CMV culture. Clotted blood was obtained for complement-fixing CMV (CF-CMV) antibody determinations. All CF-CMV antibody-negative sera (titer of <1:8) were tested for indirect immunofluorescent CMV (IIF-CMV) antibody. Heparinized blood was obtained for E rosette enumeration, mitogen stimulation with phytohemagglutinin-M (PHA), concanavalin A (ConA), pokeweed mitogen (PWM), and CMV-specific mononuclear blastogenesis.

Three mutually exclusive groups were identified on the basis of virological and serological data: (i) 20 women who were seropositive (CF-CMV or IIF-CMV titer of 1:8 or greater) and culture positive for CMV from at least one site (V+ group); (ii) 41 women who were seropositive but culture negative (S+ group); and (iii) 31 women who were culture negative and seronegative (S- group). Eighteen women were studied on more than one occasion (5 V+, 8 S+, 5 S-). There were no significant differences among the groups for incidence of prior pregnancy, pregnancy within 1 year, oral contraceptive use, intrauterine device use, smoking, alcohol use, frequency of sexual activity, number of sexual partners, past or present gonorrhea, past or present urinary tract infection, or past or present vaginitis. No acute illness or symptoms attributable to CMV were present in any of the subjects.

**Virology.** Specimens were inoculated onto duplicate confluent monolayers of locally prepared human embryonic fibroblasts which were observed for the development of the characteristic cytopathic effect of CMV. Tubes were examined at least twice weekly for at least 4 weeks before being discarded. Urine was filtered through 0.45- $\mu$ m Millipore filters (Bedford, Mass.) after pH correction to 7.0 with sodium bicarbonate, and both filtered and unfiltered specimens were inoculated. Leukocytes for viral culture were prepared by allowing 10 ml of heparinized whole blood to stand at 37°C for 30 min; the leukocyte-enriched plasma was then aspirated, washed with phosphate-buffered saline (PBS) at 1,000 rpm for 10 min, resuspended to 1.0 ml in PBS, and inoculated.

**Serology.** CF antibodies to CMV were assayed by the previously described microtiter technique of Hanshaw (6). CF antigen was prepared from the AD 169

strain of CMV by the glycine extraction technique of Waner et al. (22). Titers were expressed as the reciprocal of serum dilutions. All sera from one subject were tested at the same time.

For the IIF-CMV antibody determinations, confluent human fibroblast monolayers grown on cover slips were infected with the Davis strain of CMV. Approximately 48 to 72 h after inoculation, the slides were washed with PBS, air dried, and fixed with acetone. Test sera were serially diluted with PBS, individually applied dropwise to the cover slip, and incubated at 37°C for 30 min in a moist chamber. The preparations were washed three times and overlaid with a drop of fluorescein-conjugated anti-human polyvalent immunoglobulin (Cappel Laboratories, Cochranville, Pa.) for 30 min at 37°C. The conjugate was then washed off, the cells were rewashed twice, and the preparation was covered with Elvanol (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Cover slips of uninfected human fibroblast monolayers were also prepared to control for nonspecific fluorescence. Positive and negative serum controls were used in each test. Specific nuclear fluorescence was assessed by microscopic examination with UV illumination. All readings were done by the same person, who was unaware of the CF-CMV antibody results. IIF-CMV antibody-positive sera were assessed for rheumatoid factor by latex agglutination (Rapi/tex-RF; Calbiochem-Behring Corp., San Diego, Calif.).

**General cellular immunity.** Mononuclear cells were separated from heparinized whole blood by Ficoll-Hypaque centrifugation at 1,500 rpm for 20 min at room temperature. After harvest from the interface, mononuclear cells were washed twice in PBS and once in RPMI 1640, and cell concentration was adjusted to  $2 \times 10^6$ /ml for E rosette enumeration and CMV-specific mononuclear blastogenesis and to  $10^6$ /ml for mitogen stimulation in RPMI 1640 with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (GIBCO, Grand Island, N.Y.) and 100 U of penicillin G, 100  $\mu$ g of streptomycin sulfate, 2  $\mu$ g of amphotericin B, 0.29 mg of L-glutamine, and 5% pooled normal human serum (Bio-Bee, Boston, Mass.) per ml of RPMI 1640.

E rosettes were prepared in the standard fashion by incubating washed mononuclear cells with sheep erythrocytes overnight at 4°C. E rosette number was determined by multiplying the E rosette percentage times the absolute lymphocyte count.

For mitogen stimulation, 100  $\mu$ l of cell suspension wells of a 96-well, flat-bottomed tissue culture plate (Falcon Plastics, Oxnard, Calif.). Two concentrations of each mitogen were used: 1:10 and 1:100 dilutions of PHA and PWM (GIBCO) and 20 and 10  $\mu$ g of ConA (Sigma Chemical Co., St. Louis, Mo.) per ml. All dilutions were made with the RPMI 1640-antibiotic-serum mixture described above. All tests were set up in triplicate and incubated at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>. Each well was inoculated with 1  $\mu$ Ci of [*methyl*-<sup>3</sup>H]thymidine (ICN Radioisotopes, Irvine, Calif.) 18 h before harvest. Wells were harvested with a semiautomatic harvester (Otto Hiller, Madison, Wis.) onto fiber glass filter paper. After drying, appropriate circles of filter paper were punched into mini-scintillation vials, and scintillation fluid was added. Scintillation counting was performed in an LS-150 counter (Beckman Instruments, Inc., Fullerton, Calif.)

for 1 min. Stimulated and control wells were harvested on day 4 for PHA, on day 5 for ConA, and on day 6 for PWM. Mean counts per minute for mitogen- and control stimulated cells were determined, and the higher result from the two mitogen concentrations was used for analysis. Results were expressed both as stimulation index (SI) (the ratio of counts per minute incorporated by mitogen-stimulated cells to counts per minute incorporated by unstimulated cells) and net counts per minute (the difference between the two).

**CMV-specific mononuclear blastogenesis.** Purified CMV antigen for the CMV-specific mononuclear blastogenesis assay was prepared by a modification of the method of Sarov and Abady (14). Roller bottles of confluent monolayers of human fibroblasts were each inoculated with approximately  $10^6$  PFU of CMV AD 169. One of every six roller bottles was pulsed with 100  $\mu$ Ci of [*methyl*- $^3$ H]thymidine 24 h after infection. Subsequently, the products of these radiolabeled bottles were processed in parallel, but in separate vessels, with those of the unlabeled bottles. After 90 to 100% cytopathic effect was observed, the supernatants were harvested and spun at 1,500 rpm for 20 min at 4°C. This supernatant was spun at 7,000 rpm for 20 min at 0°C, and the resultant supernatants were spun at 18,000 rpm for 60 min at 0°C. The pellets were suspended in Tris buffer, and after sonication (1.0 A for 1 min; Raytheon Dismembrator, Waltham, Mass.), they were layered onto continuous 10 to 50% sucrose density gradients. These were spun at 25,000 rpm for 90 min at 0°C. Fractions (1 ml) were harvested, and fractions enriched in viral DNA were identified by scintillation counting of fraction samples from the gradient that contained radiolabeled supernatant. Appropriate fractions were then pooled, diluted at least 1:1 with Tris buffer, layered atop cesium chloride density gradients (specific gravity, 1.18 to 1.37), and spun at 25,000 rpm for 90 min at 0°C. Fractionation and identification of viral DNA-enriched fractions were again performed. Appropriate fractions were again pooled and dialyzed overnight at 4°C against Tris-buffer. The dialysate was then spun at 25,000 rpm for 90 min at 0°C, and the resultant pellet was sonically resuspended in 0.5 ml of Tris buffer. Protein concentration was then determined spectrophotometrically by UV absorbance at 280 nm. Sham antigen was prepared in an identical fashion, except that the roller bottles were not inoculated with virus. Appropriate stock dilutions were made and stored at -70°C until use. Stock preparations were heat inactivated at 56°C for 30 min, and the final concentration was adjusted with RPMI 1640, antibiotics, and 5% pooled human serum before use in the assay.

Optimal concentrations of antigen (0.15 and 0.075 mg of protein per ml) and sham antigen as well as optimal duration of incubation (6 and 7 days) with such stimuli were determined for the blastogenesis assay by studies on 10 known CMV-seropositive and 5 known CMV-seronegative normal subjects (ages, 26 to 44 years). The assay was performed by incubating  $2 \times 10^5$  cells in 100  $\mu$ l of medium in triplicate wells with two concentrations of antigen and corresponding dilutions of sham antigen, with duplicate sets to allow for harvest on days 6 and 7. Each well was inoculated with 1  $\mu$ Ci of [*methyl*- $^3$ H]thymidine 18 h before harvest. Harvest and scintillation counting were performed as described above for mitogen stimulation. Results were

expressed as SI (ratio of counts per minute incorporated by antigen-stimulated cells to counts per minute incorporated by sham antigen-stimulated cells) and net counts per minute (the difference between the two). Since each assay produced four values of SI and net counts per minute, the highest SI and net counts per minute values were selected for analysis. Sensitivity and specificity of the assay were assessed with 42 nonpregnant healthy adult subjects (ages, 23 to 48 years) whose serological status was unknown at the time the assay was performed. All but one seropositive subject (24 of 25) had SIs greater than 3 and net counts per minute greater than 10,000; all seronegative subjects (17) had SIs less than 3 or net counts per minute less than 10,000 or both. An assay was therefore considered reactive if the SI was greater than 3 and the net counts per minute were greater than 10,000.

**Statistical analysis.** Comparisons of discrete variables were performed by the chi-square test with Yates' correction or by Fisher's exact test. Comparisons of continuous variables were performed by the two-tailed Student *t* test. CMV-specific mononuclear blastogenesis data conformed to a log-normal distribution, but since statistical analysis produced similar results whether or not the data were log transformed, results without transformation are presented.

## RESULTS

**Virology.** Twenty of the 92 subjects (21.7%) were culture positive for CMV from at least one site when first studied. Eight were positive only from the cervix, three only from urine, one only from saliva, five from cervix and urine, two from cervix and saliva, and one from urine and saliva. None had positive leukocyte cultures. Among the 18 women who were studied more than once, 13 were culture negative on each occasion (mean, 16.3 weeks after the first study; range, 2 to 35 weeks). Among the five restudied who were culture positive at least once (Table 1), three were culture negative on the second occasion (5, 23, and 28 weeks after the initial study) and two were culture positive at the same sites (3 and 4 weeks after the initial study). One of these latter two was culture positive from urine and cervix on the initial study but only from the cervix on the second.

**Serology.** The geometric mean titer of CF-CMV antibodies for the V+ group was  $74.0 \pm 16.9$  and for the S+ group was  $35.5 \pm 6.2$ . This difference was statistically significant ( $P < 0.02$ ). There were no seroconversions or four-fold titer changes among those studied more than once. There were only two subjects who were negative for CF-CMV antibody but positive by the IIF technique (titer, 1:16 in both) and both were in the S+ group. Neither subject had rheumatoid factor.

**General cellular immunity.** There were no significant differences among the three groups for E rosette percentage or number (Table 2). The mean net counts per minute for PHA of S-

TABLE 1. Results of cultures, serology, and CMV-specific mononuclear blastogenesis among V+ subjects studied more than once

Subject no.	Interval since first study (wk)	CF-CMV titer	Culture results <sup>a</sup>	CMV-specific mononuclear blastogenesis	
				Mean SI	Mean net cpm
10	4	32	+ U, Cx	4.54	4,079
		16	+ Cx	6.32	13,758
27	5	64	+ Sa, Cx	9.49	13,593
		32		15.9	15,984
33	28	128	+ U, Cx	6.60	19,129
		128		3.46	9,568
34	23	8	+ U	3.40	6,540
		16		13.9	45,282
84	3	16	+ Cx	9.27	9,892
		16	+ Cx	15.1	27,743

<sup>a</sup> U, Urine; Cx, cervix; Sa, saliva.

subjects was significantly greater than that of V+ subjects ( $P < 0.05$ ), although the mean SI was not. The mean SI and mean net counts per minute for ConA of S- subjects was significantly greater than that of S+ subjects. Despite these statistically significant differences in mitogen responses, each group and all individuals within each group displayed notable responses to all mitogens. None approached response levels seen in patients with cell-mediated immunodeficiencies. Data from repeat studies were not included in Table 2, but inclusion did not alter the differences.

**CMV-specific mononuclear blastogenesis.** The CMV-specific mononuclear blastogenesis assay was reactive for the V+ group in 12 of 20 subjects (60.0%), for the S+ group in 30 of 41 subjects (73.2%), and for the S- group in 1 of 31 subjects (3.2%). The frequency of reactivity was significantly greater for both the V+ and S+ groups ( $P < 0.001$ ) than for the S- group, but there was no significant difference between the V+ and S+ groups ( $P > 0.40$ ). Inclusion of data from repeat studies did not alter these findings. It should be noted (see Table 1) that among the five V+ patients who were restudied, two who were initially nonreactive became reactive, two who were initially reactive became nonreactive, and one was reactive on both occasions. Of eight S+ subjects who were restudied, two who were reactive became nonreactive, five were reactive on all occasions (including one who was studied three times), and one was nonreactive on both occasions. All five S- subjects who were restudied (including one who was studied three times) were nonreactive on all occasions. Both S+ subjects who were CF seronegative but IIF seropositive were reactive. There was no significant difference in CF-CMV geometric mean titer

between those with reactive and nonreactive mononuclear cells within the V+ or S+ groups.

To assess the degree of CMV-specific mononuclear blastogenesis, mean stimulation index and mean net counts per minute for each group was compared. For mean SI and mean net counts per minute, the V+ and S+ groups both had significantly greater values than the S- group ( $P < 0.001$ ). The differences between the V+ and S+ groups were not significant ( $P > 0.50$  for mean net counts per minute and  $P > 0.40$  for mean SI). Inclusion of data from repeat studies did not significantly alter the means or the results of the statistical analysis.

## DISCUSSION

The results of this study fail to demonstrate a strong relationship between deficient systemic CMV-specific cell-mediated immunity and asymptomatic local CMV infections. The results of the CMV-specific mononuclear blastogenesis assay in the group actively excreting virus were very similar to those of the S+ group. There were a number of subjects who lacked appropriate blastogenic responses to purified CMV antigen in both groups. All three groups were comparable for E rosette number and percentage. There were isolated significant differences between groups for mitogen stimulation, but all groups did demonstrate notable responses to all mitogens.

The lack of appropriate blastogenic response to CMV in substantial fractions of both the V+ and S+ groups is noteworthy. It is possible that the cells of some subjects may require different concentrations of antigen than used in this study to elicit reactivity, as suggested by the findings of Starr et al. (16). In some cases, non-reactivity

TABLE 2. Correlation of virological and serological status for CMV with studies of cell-mediated immunity<sup>a</sup>

Group	E rosette		PHA		ConA		PWM		CMV	
	%	No./mm <sup>3</sup>	SI	Net cpm	SI	Net cpm	SI	Net cpm	SI	Net cpm
V+	66.7 ± 1.0	2,437 ± 355	148.3 ± 15.5	163,687 ± 8,868	49.9 ± 7.6	55,014 ± 5,476	78.6 ± 9.6	93,551 ± 10,659	7.02 ± 0.76 <sup>b</sup>	16,547 ± 2,702 <sup>b</sup>
S+	65.9 ± 0.8	2,670 ± 599	155.0 ± 12.5	176,687 ± 5,929	41.1 ± 3.6	52,249 ± 3,422	80.1 ± 9.4 <sup>c</sup>	97,288 ± 8,234	8.21 ± 0.76 <sup>b</sup>	17,473 ± 1,590 <sup>b</sup>
S-	66.3 ± 0.9	2,522 ± 488	172.7 ± 14.0	191,540 ± 7,382 <sup>c</sup>	54.3 ± 6.3 <sup>d</sup>	64,215 ± 5,613 <sup>d</sup>	88.6 ± 11.7	111,944 ± 10,088	2.35 ± 0.19	4,246 ± 847

<sup>a</sup> All data are presented as the mean ± standard error of the mean.

<sup>b</sup> Significantly greater than for S- ( $P < 0.001$ ).

<sup>c</sup> Significantly greater than for V+ ( $P < 0.05$ ).

<sup>d</sup> Significantly greater than for S+ ( $P < 0.05$ ).

may represent antigenic heterogeneity between CMV AD 169 and the wild-type strain(s) with which the V+ or S+ subject had prior immunological experience. The appearance of CMV-specific reactivity after initial non-reactivity in several restudied V+ and S+ patients indicates that this is not the total explanation. Pregnancy has been reported to be associated with attenuation of CMV-specific mononuclear blastogenesis even in the absence of active infection (3), but pregnant adolescents were specifically excluded from this study. Since 34 of 35 of the normal seropositive adults originally used to proof test and standardize the assay were reactive, it seems unlikely that assay insensitivity is responsible. Cells from 10 of these seropositive adults were assayed on 14 later occasions in parallel with cells from the study patients, and reactivity of the adults was consistently demonstrated, although the degree of reactivity did fluctuate. The striking frequency of non-reactivity in this study population suggests a relation to the high incidence of active infection in this population, but the lack of difference for frequency and degree of reactivity between the V+ and S+ groups within the population suggests that this is not the critical factor. The mechanism for CMV-specific mononuclear non-reactivity in these study patients is obscure.

Patients with CMV mononucleosis, a systemic disease usually resulting from primary infection, have been observed to have prolonged depression of responsiveness to mitogens and other herpesvirus antigens as well as prolongation of the time required to develop the expected normal mononuclear proliferative response to CMV (9, 12, 19, 20). Evidence thus exists that CMV may be immunosuppressive. In this study of healthy subjects, the lack of depression of CMV-specific mononuclear blastogenesis in the V+ group compared with the S+ group suggests that no such suppression of CMV-specific mononuclear blastogenesis occurs with asymptomatic local infection. It is possible that the lack of significant difference between the two groups may be due to lack of local antigenic contact with systemic immunocytes. This has been suggested by several studies which failed to document significant rises in antibody titer among patients with local CMV infections (5, 15). The significantly greater mean CF-CMV titer of V+ subjects compared with that of S+ subjects in this study suggests that locally replicating virus was being presented to systemic immunocytes, although no significant titer rise was noted in those V+ subjects who were studied on more than one occasion.

Although we were unable to demonstrate a correlation of deficient systemic CMV-specific mononuclear blastogenesis with local CMV in-

fection, this does not rule out the possibility that cell-mediated immunity may play a role in regulation of such infections. The blastogenesis assay tests the effector arm of cellular immunity; it is possible that studies of the effector arm (e.g., cytotoxicity, lymphokine production) may produce different results. Although systemic cellular immunity may not prevent local CMV infection, it may significantly modulate the course of the infection. Corey et al. (2) have shown in a longitudinal study of genital herpes simplex virus infection that the SI in a herpes simplex virus-specific blastogenesis assay correlated inversely with the duration of genital herpes simplex virus infection. Similar longitudinal studies of cell-mediated immunity in local CMV infections may yield similar conclusions. Intact systemic CMV-specific cell-mediated immunity may not prevent local CMV infection but may be an important factor in preventing dissemination of such infections. Local immunity may be the critical determinant for local CMV infections. Although Waner et al. (21) have demonstrated human cervical CMV infection in the presence of specific local antibody, it is possible that local cell-mediated immunity may be a critical factor in the acquisition, reactivation, or persistence (or all three) of local CMV infection. Beutner et al. (1) have demonstrated that local CMV-specific cell-mediated immunity may not reflect systemic CMV-specific cell-mediated immunity. Further study is needed to identify regulatory factors in this epidemiologically significant form of CMV infection.

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