INFECTION AND IMMUNITY, Dec. 1978, p. 833-841
0019-9567/78/0022-0833$02.00/0

Vol. 22, No. 3
Printed in U.S.A.

Immunoglobulin G to Virus-Specific Early Antigens in Congenital, Primary, and Reactivated Human Cytomegalovirus Infections

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Received for publication 28 September 1978

Immunoglobulin G antibody to human cytomegalovirus (CMV)-specific early antigens (EA-Ab) was determined by the immunoperoxidase antibody technique in several cases of congenital, primary, and reactivated CMV infections. Mothers of congenitally infected infants and a group of leukemic children and pregnant women were also studied. In 11 cases of congenital infection, CMV EA-Ab was always associated with CMV excretion whether immunoglobulin M antibody was present or not. Nine mothers of congenitally infected infants had CMV EA-Ab for several months after delivery, but association with CMV elimination was not established when urine and/or saliva were tested for virus isolation. In all nine cases of primary CMV infection, CMV EA-Ab was present, and in five its detection was associated with CMV isolation. In one case, disappearance of EA-Ab occurred when virus excretion ceased. In five cases of reactivated CMV infections, a consistent association between CMV EA-Ab and virus isolation was found. Six of 31 leukemia children had CMV EA-Ab, and virus was isolated from 3 of these. Four of 28 pregnant women showed EA-Ab in their serum, but tests for isolation were not done. These data suggest that CMV EA-Ab is not a marker of a current primary CMV infection, as previously reported, but a marker of an active CMV replication which can take place in primary as well as in congenital and reactivated CMV infections.

Determination of antibody to early antigens of Epstein-Barr virus in human sera has been reported and its significance defined (9). Using the indirect fluorescent antibody (IFA) technique, The et al. (19) were the first to study the behavior of antibodies to human cytomegalovirus (CMV) early antigens (EA-Ab) in cases of primary CMV infections (most of which followed the administration of several fresh blood units). The presence of CMV EA-Ab was then reported in individuals receiving CMV experimental vaccine (15) and in women excreting CMV in cervical secretions (21). However, the real meaning and practical importance of this type of antibody have not been clarified. In the present report, we used the indirect immunoperoxidase antibody technique (IPA) to study the behavior of CMV EA-Ab in several cases of congenital, primary, and reactivated CMV infections. Furthermore, since leukemia (2, 18) and pregnancy (13, 14, 16) are both known as possible sources of latent CMV infection reactivation, CMV EA-Ab was searched for in a group of children suffering from acute lymphocytic leukemia and in a group of pregnant women. Results show that CMV EA-Ab is consistently detectable in patients from whom CMV is isolated. In a minor number of cases the presence of EA-Ab is not associated with virus isolation, but active CMV replication and excretion cannot be excluded.

MATERIALS AND METHODS

Virus strain and cell cultures. Reference CMV strain AD-169 was initially obtained from the American Type Culture Collection (Rockville, Md.). MA-184 (Microbiological Associates, Bethesda, Md.) cell cultures were subcultured in our laboratory from early-passage material, and passage 21 to 25 subcultures were used for testing. Tissue culture flasks were inoculated with CMV at a multiplicity of infection of 0.1. After virus adsorption for 60 min at 37°C, maintenance medium (Eagle minimum essential medium with 2% fetal calf serum) was added for 2 to 4 h. Cell cultures were then trypsinized and resuspended in growth medium (Eagle minimum essential medium with 10% fetal calf serum) at a ratio of 1:1 with uninfected MA-184 cells. The final suspension contained 10^5 cells per ml; 0.05 ml of the suspension was dropped into each well of tissue culture microplates (Falcon Plastics, Oxnard, Calif.), or 0.4 ml was inoculated into each of eight wells of tissue culture chamber slides (Lab-Tek, Naperville, Ill.), depending on the tissue system se-

lected. Microplates and chamber slides were then incubated in a CO_2 incubator and observed twice a day. When cytopathic effect was considered optimal (about 50% of cell monolayer), cells were fixed with absolute alcohol and stored at −80°C. In parallel, uninfected cell monolayers were grown and fixed. Fixation was usually done after 96 h of incubation.

Preparation of cell cultures containing CMV EA-Ab. MA-184 cell cultures containing CMV EA-Ab were prepared by following the same procedure reported above. However, both maintenance and growth medium were supplemented with cytosine arabinoside at a concentration of 40 μg/ml. Fixation was accomplished 72 h after infection. The absence of mature viral particles in cytosine arabinoside-treated CMV-infected cell cultures was shown by lack of CMV reisolation as well as by electron microscopy.

Determination of CMV EA-Ab by the IPA technique. Goat anti-human immunoglobulin G (IgG) (gamma-chain-specific) serum, commercially obtained (Electro-Nucleonics, Bethesda, Md.), was purified and coupled to horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.) according to a reported procedure (6). The application of sera and conjugate for CMV EA-Ab determination followed the same steps previously described for IgG detection (5).

Reference sera. Sera drawn from two patients with CMV mononucleosis 3 to 4 weeks after the onset of the infection were used as CMV EA-Ab-positive serum control. Sera from two healthy blood donors were used as EA-Ab-negative IgG-positive serum control.

IHA test. Indirect hemagglutination (IHA) reagent preparation and test were performed according to the procedure of Bernstein and Stewart (1) for detection of broad-spectrum CMV antibody (ΣAb). MA-184 cell cultures were used for antigen preparation.

IgM determination on serum fractions. Serum fractionation was performed by sucrose density gradient centrifugation following the modification recommended by the Center for Disease Control (Atlanta, Ga.) of a previous procedure (20). On the same day that serum fractions were collected, unfractionated sera as well as fractions of each serum were tested by IHA for IgM and IgG detection. Simultaneously, fractions were tested for the presence of IgM, IgG, and IgA using radial immunodiffusion plates (Behring-Werke AG, Marburg/Lahn, W. Germany). Furthermore, when specific antibody activity was found in fractions 2 to 4, tests for low levels of IgG were performed by radial immunodiffusion.

CMV isolation and identification. CMV isolation was carried out on MRC-5 (Flow Laboratories, Irvine, Scotland) and MA-184 cell cultures, and identification was achieved using an IPA technique as previously reported (6).

Subjects and patients examined. The following groups of subjects and patients were examined for determination of CMV IHA antibody titer, IPA-IgG, IHA-IgM on serum fractions, and EA-Ab: (i) 9 cases of primary CMV infection; (ii) 11 infants with congenital infection; (iii) 5 cases of reactivated infection in people admitted to the hospital with different clinical symptoms; and (iv) a group of 20 healthy people of different ages (children and adults) seropositive (10 with a CMV IHA ΣAb titer of ≥1:1,024 and 7 with a titer of ≤1:256) or seronegative for CMV antibody, a group of 31 leukemic children, and a group of 28 pregnant women.

Diagnosis of congenital infection was based upon the presence of some typical clinical symptoms of congenital infection and either CMV isolation or IgM antibody detection or both. Symptoms were essential for the diagnosis of congenital infection when infants were observed for the first time several weeks after birth. A primary (recent) infection was suggested by seroconversion and/or appearance of an IgM antibody response at a titer of 1:8 or greater. Reactivation (or persistence) of a CMV infection was suggested by CMV isolation in patients with CMV antibody titer and no IgM antibody. In most cases, specimens for virus isolation (urine, saliva, cervical secretions) were obtained; in a minor number of cases and in the pregnant women's group, specimens for isolation were not available.

RESULTS

Patterns of IPA staining of cells containing CMV EA-Ab with reference sera. Control serum drawn during the convalescent phase of MCV mononucleosis (CMV EA-Ab- and IgG-positive) produced a strong fine granular staining of the entire nucleus of cytosine arabinoside-treated CMV-infected cells, whereas the intensity of the cytoplasmic staining ranged from absent to faint. Selected CMV-positive and CMV-negative sera from blood donors (EA-Ab negative) did not stain either the nucleus or the cytoplasm of the cytosine arabinoside-treated cells (Fig. 1). Staining patterns of CMV-infected cells used as a substrate for CMV IgG and IgM determination by the IPA technique have been previously described (4).

Primary infections. Of nine cases of primary CMV infection, six were CMV mononucleosis and one was associated with myopericarditis, one with cerebral atrophy, and one with respiratory symptoms (Fig. 2 and Table 1). In all cases, CMV-specific IgM antibody and EA-Ab were detected in serum. In five cases CMV EA-Ab was associated with virus isolation; in three cases CMV was not recovered from urine and/or saliva; and in one, specimens for isolation were not available. However, in the last four cases CMV EA-Ab was also found at a high titer. Figure 2 reports titers of three cases followed for several weeks. In the first case (G.S.), EA-Ab disappeared when virus excretion ceased, but reappeared with reactivation of CMV infection. In the second case (a patient suffering from myopericarditis, D.D.P.D.), a clear tendency of EA-Ab to decrease in titer 11 weeks after the onset of infection (even in the presence of continuous CMV excretion) was followed by a new peak in parallel with a relapse of the cardiac infection. During the entire 6-month period of

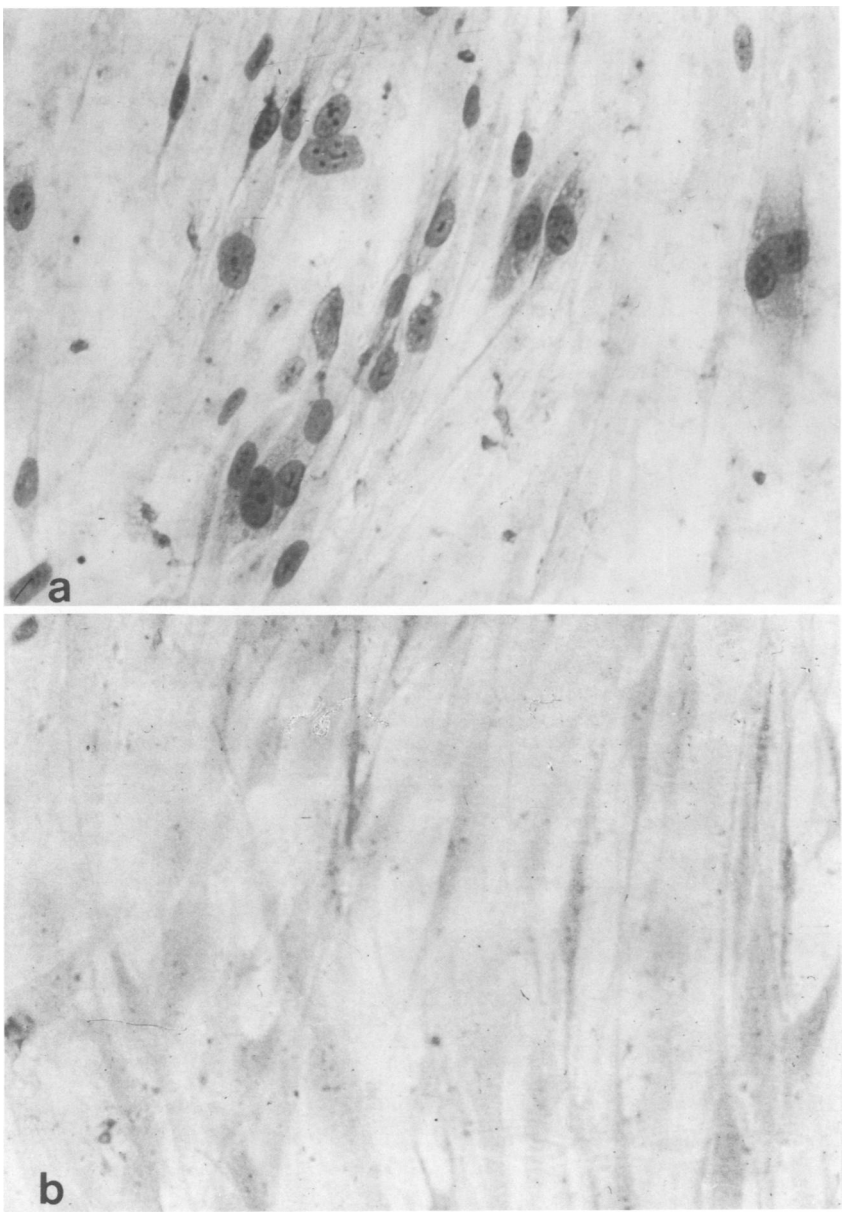

FIG. 1. *Patterns of staining of cytosine arabinoside-treated CMV-infected cells using (a) a convalescent serum from a case of CMV mononucleosis and (b) a CMV-positive serum from a blood donor.*

observation, CMV IgM antibody titer was consistently high. In the third case (M.M.), CMV EA-Ab appeared within the first week after the onset of infection and became undetectable 18 weeks later. CMV-specific IgM antibody showed a similar curve. However, in this case the antibody response was somewhat influenced by the treatment of acute lymphocytic leukemia during the follow-up period.

Congenital infections. Of 11 cases of congenital CMV infection, 6 were first examined between weeks 1 and 13 after birth (Fig. 3 and Table 2). All infants were excreting virus during the follow-up period, whereas CMV-specific IgM antibody was detected no longer than 16 weeks after birth. CMV EA-Ab was present in sera of all infants during the period of observation. A great range of changes in titer was not usually observed in the same patient. A gross parallelism between the ΣAb titer and the level of CMV

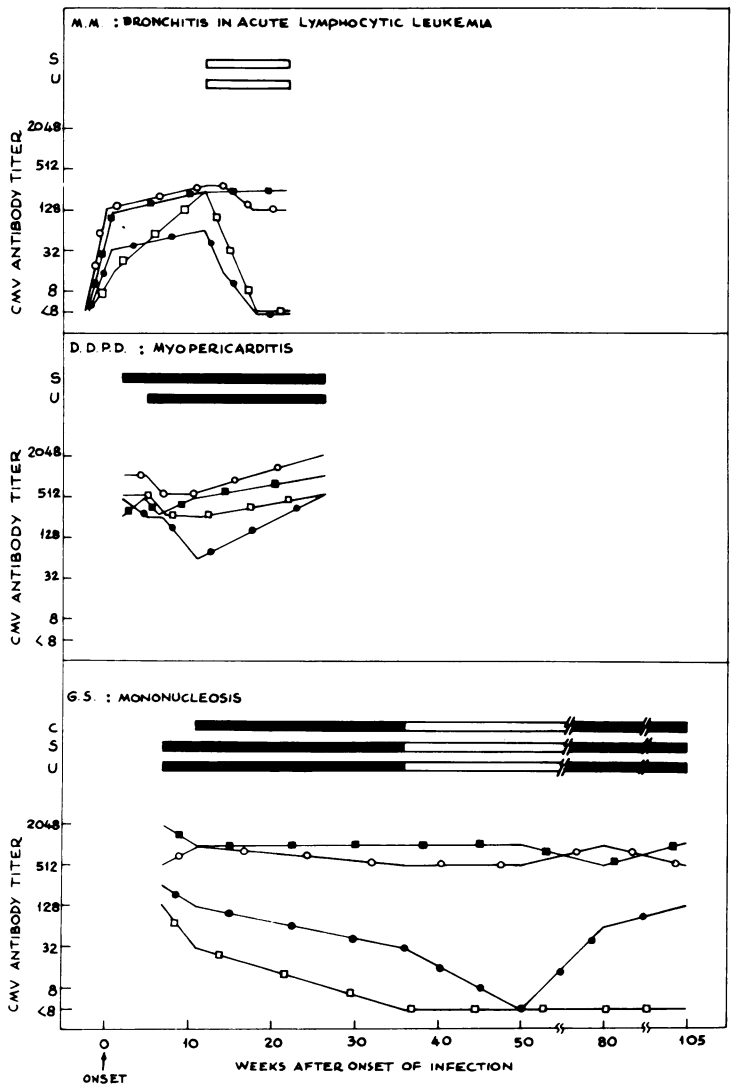

FIG. 2. *CMV excretion and different types of specific antibody response in three cases of primary infection. Symbols:* (●) *EA-Ab;* (□) *IgM;* (○) *ΣAb;* (■) *IgG. CMV (solid bars) presence and (open bars) absence in (U) urine, (S) saliva, and (C) cervix.*

EA-Ab titer was observed. However, EA-Ab titers were usually lower than ΣAb titers. In nine cases, mother's serum samples were available for testing, and in all nine CMV EA-Ab was detected even more than 10 weeks after delivery. However, in a restricted number of cases, where mother's specimens were submitted for isolation, CMV was not recovered from urine and/or saliva. In the three cases where multiple sequential mother's serum samples were available, a slow fall in CMV EA-Ab titer was observed (F.A., A.G., and P.S., mothers; Fig. 3).

Reactivated infections. When CMV was isolated from patients with an associated disease and CMV-specific IgM antibody was not detectable, CMV infection was considered to be a reactivation of a latent infection (Table 3). In all cases, presence of CMV in urine and/or saliva was associated with positivity for CMV EA-Ab in serum.

Healthy people, leukemic children, and pregnant women. Of 20 healthy people tested (17 positive for CMV antibody and 3 negative), EA-Ab was present in 3 of the 10 cases with a ΣAb titer of ≥1:1,024. In one of these cases CMV could be isolated from urine the same day the

TABLE 1. *CMV antibody responses in six cases of primary postnatal infection*[a]

Patient and age (years)	Clinial diagnosis	Weeks after onset of infection	CMV isolation	CMV antibody titer			
				ΣAb	IgG	IgM	EA-Ab
T.L., 17	Mononucleosis	2		256	256	64	128
		3		256	256	64	128
		9		512	512	<4	64
T.J., 55	Mononucleosis	2	U+, S+	64	32	64	1,024
		3		128	128	128	1,024
D.A., 17	Mononucleosis	2	U+, S+	64	64	64	256
M.L., 4	Mononucleosis	7	U+, S+	512	512	32	128
		10	U+, S+	512	512	<4	64
		15	U+, S+	512	512	<4	32
M.P., 21	Mononucleosis	9		1,024	1,024	16	256
		23	U−, S−	2,048	1,024	<4	256
P.C., 16	Endocrine disorder, cerebral atrophy	?	U−, S−	32,768	32,768	64	1,024

[a] Abbreviations and symbols: U, urine; S, saliva; C, cervix; +, positive isolation; −, negative isolation.

serum sample was drawn. In the other two cases, CMV was not isolated, but specimens for isolation were available only a few months later.

In the group of 31 leukemic children, EA-Ab was present in 6 cases and CMV was isolated from 3 of these. In the group of 28 pregnant women, CMV EA-Ab was detected in 4 (Table 4). All four were in the second or third trimester of pregnancy. Unfortunately, in this group, specimens for isolation were not available.

DISCUSSION

The first report studying the behavior of CMV EA-Ab in patients with CMV infection stated that the presence of this type of antibody in an acute-phase serum at a titer of 1:80 can be taken as suggestive of a current primary infection (19). However, this study did not establish any relationship between the presence of CMV EA-Ab and CMV excretion and between EA-Ab and CMV infections other than primary (i.e., congenital or reactivated). The data reported in the present paper appear to emphasize a close correlation between the presence of CMV EA-Ab and an active virus replication, and show that CMV EA-Ab is present in primary as well as in congenital and reactivated CMV infections, whether IgM antibody is detectable or lacking. The suggested relationship between different types of CMV infection and different types of CMV antibody response (ΣAb, IgM, and EA-Ab) is reported in Table 5.

The data of The et al. (19) in primary infections are here confirmed, but the presence of CMV EA-Ab is also related to CMV excretion and CMV-specific IgM antibody. The presence of EA-Ab does not correlate with the detection of IgM antibody but appears to correlate well with an active CMV replication and excretion. This seems to be confirmed by case G.S. (Fig. 2), where EA-bA disappeared when virus excretion stopped and reappeared when virus elimination started again. The absolute titer of CMV EA-Ab alone does not seem to be significant as far as diagnosis of a current primary CMV infection is concerned. Titers of ≥1:64 were found in primary as well as in congenital and reactivated CMV infections. The time of appearance of CMV EA-Ab seems to be within the first week after the onset of symptoms (case M.M., Fig. 2). In a study of varicella-zoster EA-Ab during the acute phase of varicella, we found that this antibody appears 2 to 3 days later than IgG antibody, but always within a week after the onset of the exanthem (manuscript in preparation).

In congenitally infected infants the association between the presence of CMV EA-Ab and CMV excretion was observed in all cases studied, even when tests were performed 11 years after birth. In these cases, CMV IgM antibody was never detected more than 16 weeks after birth. In the group of mothers of infants with congenital CMV infection, EA-Ab was, in all cases, detectable for at least several months after delivery. However, in a few instances where specimens for virus isolation were available (urine and/or saliva), virus was not recovered. CMV isolation from urine and cervical secretions of mothers of congenitally infected infants has been reported for several months or a few years after delivery,

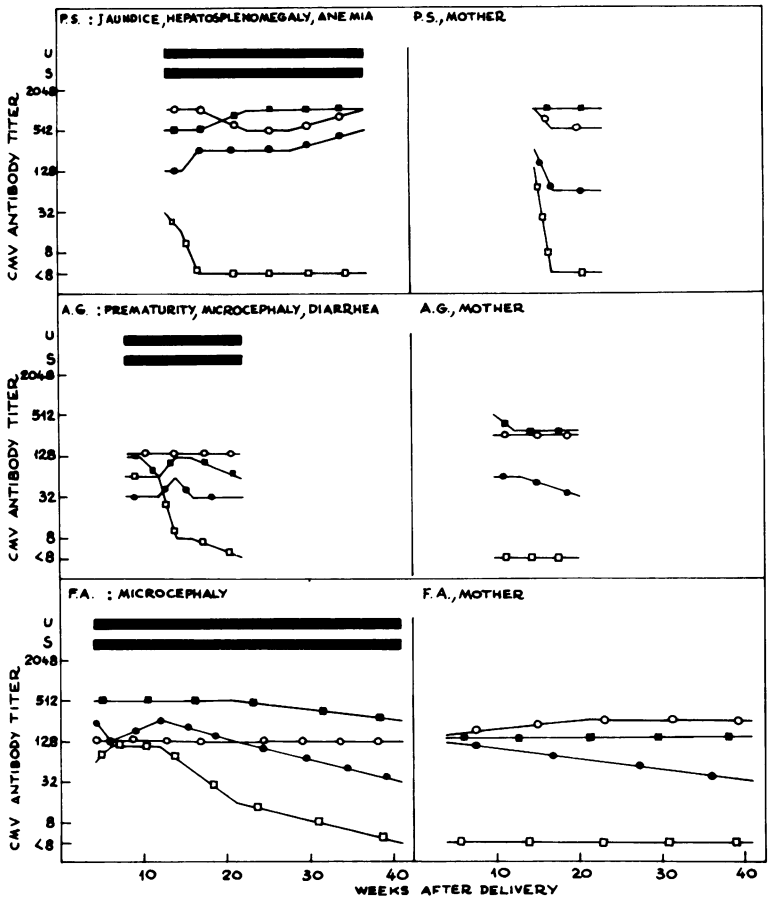

FIG. 3. *CMV excretion and different types of specific antibody response in three congenitally infected infants and their mothers. Symbols:* (●) *EA-Ab;* (□) *IgM;* (○) *ΣAb;* (■) *IgG. CMV (solid bars) presence and (open bars) absence in (U) urine and (S) saliva.*

and the persistence of virus excretion has been shown to be associated with a consistent specific impairment of cell-mediated immunity to CMV (17). The consistent finding of CMV-specific EA-Ab in mothers of infants with congenital CMV infection lends further evidence to the possibility that active CMV replication takes place for a long time after delivery in these women. However, in three of our cases, where mothers were followed for several months, EA-Ab showed a progressive fall in titer.

In the group of five reactivated CMV infections, CMV elimination was always associated with detection of EA-Ab at different titers. In the three groups of subjects (healthy people, leukemic children, and pregnant women) tested for presence of CMV EA-Ab, the prevalence of this antibody was 6 of 31 leukemic children, 4 of 28 pregnant women, and 3 of 17 healthy people. CMV was isolated from 4 of these 13 EA-Ab-positive individuals, but only 9 were tested for virus isolation. It may be of some interest that all three healthy subjects positive for CMV EA-Ab had a CMV antibody titer of ≥1:1,024. Whether, in healthy people, an elevated CMV antibody titer could be associated with a higher prevalence of CMV EA-Ab remains to be investigated in further studies. We believe that the finding of CMV EA-Ab is not unusual in people considered healthy and is possibly a marker of an active CMV replication. Thus, our data do not add evidence to the finding that acute lymphocytic leukemia (2, 18) and pregnancy (13, 14, 16) may increase the CMV infection reactivation rate.

The appearance of CMV EA-Ab in two of four CMV-seronegative subjects receiving a live attenuated CMV vaccine has been reported, but virus excretion was never detected (15). Only three out of eight women eliminating virus with

TABLE 2. *CMV antibody responses in eight cases of congenital infection*[a]

Patient	Clinical symptoms	Age (weeks)	CMV isolation	CMV antibody titer			
				ΣAb	IgG	IgM	EA-Ab
G.S.	Jaundice, biliary duct atresia	13	U+, S+	128	128	64	64
		14		256	128	64	64
		15	U+, S+				
G.S., mother		14		512	512	<4	128
		16	U−, S−				
M.E.	Microcephaly, microphthalmia; psychomotor and mental retardation	32	U+	256	128	<4	64
		34	U+, S+	256	128	<4	64
		39	U+, S+	256	128	<4	64
M.E., mother		35		1,024	1,024	<4	64
		39		512	1,024	<4	512
F.C.	Microcephaly	30	U+, S+	4,096	2,048	<4	256
F.C., mother		30	S−, C−	2,048	2,048	<4	1,024
S.A.	Microcephaly	40	U+, S+	4,096	4,096	<4	512
		43	S+	4,096	4,096	<4	1,024
S.M.	None	43	U+, S+	2,048	2,048	<4	256
S.A. and S.M., mother		41	U−, S−	512	256	<4	128
N.M.	Jaundice, hepatosplenomegaly, anemia, pneumonia	6		512	2,048	<4	64
		8	U+				
N.M., mother		10		1,024	1,024	<4	128
		23		512	1,024	<4	64
I.C.	Spastic diplegia, psychomotor retardation	4 years	U+, S+	64	64	<4	32
P.A.	Jaundice, hepatosplenomegaly	11 years	U+	512	256	<4	128
S.O.	Microcephaly, cataracts	12	U+, S+	128	256	64	128

[a] Abbreviations and symbols as in Table 1.

TABLE 3. *CMV antibody responses in five cases of reactivated infection*[a]

Patient and age (years)	Associated disease	CMV isolation	CMV antibody titer			
			ΣAb	IgG	IgM	EA-Ab
L.C., 17	Myocarditis	U+	256	256	<4	32
N.L., 19	Myocarditis	S+	2,048	1,024	4	256
C.D., 5	Measles	S+	256	256	<4	256
F.F., 3	Measles	U+, S−	256	256	<4	64
D.G.G., 3	Laryngeal papilloma	U+, S+	64	32	<4	32

[a] Abbreviations and symbols as in Table 1.

cervical secretions showed EA-Ab in their serum in association with the presence of CMV-specific cervical secretory IgA (21). These discrepancies with our results might be due to several factors: different antigenicity of the attenuated CMV strain, qualitative instead of quantitative EA-Ab determination with possible interference of prozone phenomenon (often observed by us in the present study), different technique used (IPA in place of IFA), or different type of fixation. These factors might also be involved in the complete seronegativity for EA-Ab found in 70 patients

TABLE 4. *Summary of results of CMV antibody titer determination in a group of leukemic children and pregnant women*

Patients	Clinical stage	No. tested	No. with CMV antibody			CMV isolation (positive[a]/tested)
			ΣAb	IgM	EA-Ab	
Leukemic children	Induction	10	7	0	1	1/9
	Remission	10	10	0	2	1/5
	Relapse	11	10	0	3	1/8
Pregnant women	1st trimester	10	10	0	0	ND
	2nd trimester	9	9	0	3	ND
	3rd trimester	9	9	0	1	ND

[a] All positive cases showed presence of EA-Ab. ND, Not done.

TABLE 5. *Suggested relationship between different types of CMV infection and respective laboratory viral parameters*[a]

Type of CMV infection	Virus isolation		Presence of CMV antibody		
			ΣAb	IgM	EA-Ab
Congenital	+	+	+	+	
Primary	+	+	+	+	
Recurrent	+	+	−	+	
Remote	−	+	−	−	
Absent	−	−	−	−	

[a] Symbols: +, positive virus isolation, or presence of indicated type of antibody; −, negative virus isolation, or absence of indicated type of antibody.

not excreting virus (21) and in 57 sera from healthy people or from patients with infections of different etiology (19).

In our study, all patients excreting CMV showed the presence of EA-Ab in serum, whereas in some subjects the presence of EA-Ab was not associated with virus excretion. In these cases the lack of virus isolation might be due to several reasons: (i) CMV replication could occur without detectable virus excretion; (ii) CMV excretion is intermittent (7); or (iii) CMV excretion can occur from one site but not from another; thus virus excretion with semen (11, 12), tears (3), breast milk (8), and cervical secretions (13, 14, 16) could occur in people with EA-Ab who are not eliminating virus with urine and saliva (specimens routinely tested). Although we cannot conclusively rule out that EA-Ab may be detectable also in the absence of CMV excretion, we believe that CMV EA-Ab determination might be useful for the detection of an active CMV infection.

The IPA technique used in the present study for CMV EA-Ab determination presents several well-known advantages (10) over the IFA technique used by others (15, 19, 21).

ACKNOWLEDGMENTS

The technical assistance of Maria Torsellini Gerna and Olga Bonazza and the photographic expertise of Roberto Genova are gratefully acknowledged.

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