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Immunoperoxidase Technique for Detection of Antibodies to Human Cytomegalovirus

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The indirect immunoperoxidase antibody technique (IPA) has been applied to determine immunoglobulin (Ig)G to humans cytomegalovirus (CMV) antibodies in 114 blood donor sera, four cases of congenital cytomegalic inclusion disease, and four cases of acquired CMV infection. The results have been compared with those obtained with the CMV complement fixation (CF) test and indirect fluorescent antibody technique (IFA) for broad spectrum CMV antibody (ΣAb) detection. IgG antibody has been detected by both CF and IPA. In healthy adult people IPA titers are usually higher than CF titers. In addition, IFA ΣAb titers are generally higher than CF titers. Some sera negative by CF and IPA are positive at low dilutions by IFA ΣAb antibody determination, due to the detection of small amounts of IgA or noncomplement-fixing IgG. Nonspecific results seem unlikely, since only nuclear inclusion fluorescence was interpreted as specific, as demonstrated by blocking tests. In acute CMV infection, the IFA ΣAb and IPA IgG titers are essentially the same, except during the first weeks of infection, when IFA titers are higher and IgM is detectable. No cross-reactivity with other herpes group viruses, herpes simplex and varicella-zoster, was observed. Although some problems of nonspecific staining of cytoplasmic inclusions are shared by both methods, the IPA technique seems to possess the same degree of sensitivity and specificity as the IFA technique, but interpretation is easier and various procedural steps can be delayed without the technical problems associated with fluorescence microscopy.

Human cytomegalovirus (CMV) is an increasingly important etiological agent in clinical virology. A rapid, easily performed, sensitive, and specific test for the recognition of the immune response to the virus is vital to an effective diagnostic effort. Classical procedures do not completely meet these criteria.

The most commonly used method for detecting antibody to CMV is the complement fixation (CF) test (17). However, the CF test has several deficiencies with regard to antibody detection in sera from individuals with clinical illness. Some subclasses of immunoglobulin (Ig)G do not fix complement (12) as well as other members of the class (20), and IgM antibody is not believed to be reactive in the CMV CF test (15, 21).

The neutralization test (18) is the most strain specific and is capable of detecting both IgG and IgM (3, 6), but it is a cumbersome method and its interpretation is hampered by the slow growth of CMV isolates.

Indirect hemagglutination test has been recently shown to be much more sensitive than CF and able to detect both IgG and IgM (3, 6), but the antigen coating sheep erythrocytes is only a soluble antigen, whereas the antigen reacting in the CF test is a combination of both viral and soluble components (2).

The indirect fluorescent antibody technique (IFA) has been very useful for IgG and IgM detection in acute CMV infections, showing a greater sensitivity than CF for detecting small amounts of antibody when correct antigen can be ascertained (5). However, problems related to the commercial availability of good quality fluorescein-conjugated antisera and tissue culture slides of CMV-infected WI-38 cells and the difficulty in reading the IFA results hinder its application for studying the epidemiology and natural history of an infection widespread in human populations.

The present report describes the development and our own experience with rapid indirect immunoperoxidase antibody technique (IPA) for detecting CMV IgG antibody in a normal population (blood donors) and in some cases of congenital and postnatal cytomegalic inclusion disease (CID). Comparative data on the sensitivity and specificity of the IPA, CF, and IFA meth-

ods indicate that the IPA is more sensitive than the CF test, equal in sensitivity and specificity to IFA, but is more easily performed.

MATERIALS AND METHODS

Cell cultures. Each one of eight wells of chambered tissue culture slides (Lab-Tek, Naperville, Ill.) was inoculated with 0.4 ml of a CMV-infected WI-38 cell suspension in minimal essential medium with 10% fetal calf serum at a concentration of 10^5 cells/ml. After incubation in a CO_2 incubator at 37 C for 4 days, the slides were washed with phosphate-buffered saline, fixed in cold acetone, and then air-dried and stored at −70 C. The same procedure was used for the preparation of varicella-zoster (V-Z)- and herpes simplex virus (HSV)-infected cell culture slides.

Virus strains. Reference CMV strain AD-169 (10^5 50% tissue culture infective dose/ml) was used for cell infection at an input multiplicity of 1. Three high-titer CMV isolates from newborns with congenital CID were also employed. Reference V-Z (P 8-6-27-71, $10^{3.5}$ plaque-forming units/ml) and HSV (HF-490, $10^{6.5}$ 50% tissue culture infective dose/ml) strains were similarly used for WI-38 cell infection.

Sera and peroxidase-labeled antibody preparation. After testing several sera by CF, a positive CMV serum with a titer of 1:128 by CF and 1:2,048 by IFA but free of complement-fixing antibody at a 1:2 dilution against V-Z and HSV was found. Similarly, a positive serum against V-Z (1:256) by CF but negative for HSV and CMV and a positive serum against HSV (1:1,024) by CF but negative against V-Z and CMV were found.

Goat anti-human IgG antibody was purified from serum obtained commercially (Electro-Nucleonics, Bethesda, Md.) by precipitation using 30% and then one washing with 45% saturated ammonium sulfate (11). The immunoglobulins were then coupled to horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.) according to the Avrameas technique (1). The conjugate was then absorbed with WI-38 cells to remove nonspecific anti-human antibody.

Application of peroxidase-labeled antibody. Stored slides were thawed, washed three times with phosphate-buffered saline, and covered with test serum or control sera. After incubation at 37 C for 30 min followed by two washings, slides were incubated for an additional 30 min with anti-human peroxidase-labeled antibodies. After washing, enzymatic activity was detected using the method of Graham and Karnovsky (8).

Controls for specificity and reproducibility. Uninfected as well as CMV-, V-Z- and HSV-infected WI-38 cells were treated with CMV (1:16 through 1:16,384), V-Z- and HSV-positive, and CMV-negative serum (<1:16) to test for cross-reactivity between different herpes group viruses (Table 1). Moreover, CMV-infected cell slides were treated with: (i) CMV-positive serum, unlabeled and then labeled anti-human IgG goat antibody (blocking test); (ii) labeled anti-human IgG only; and (iii) phosphate-buffered saline only in both steps, for control of endogenous peroxidase cell activity.

After determination of the optimal conjugate dilution (working dilution) in the checkerboard titration, for the preliminary tests we chose as routine test controls a CMV-negative (<1:16) and a low- (1:32-1:64) and a high- (1:256) titer CMV-positive serum.

CF test. CF test was performed following the Laboratory Branch Complement Fixation procedure applied to the Microtiter system (22) and employing antigen from AD-169-infected WI-38 cells (Flow Laboratories, Inc., Rockville, Md.).

IFA technique. Fluorescein-conjugated goat anti-human globulin antibody as well as CMV-(AD-169)-infected and -uninfected cell slides were supplied by the same source (Electro-Nucleonics, Bethesda, Md.). A known CMV-positive (1:64) and -negative (<1:16) serum were routinely included in each test. Moreover, uninfected cell controls were performed on each serum to test for the presence of antinuclear antibody. The reading of CMV-positive sera titers is sometimes hindered by antinuclear antibody in human sera.

In a few cases of primary acute CMV infection it has been possible to determine IgM titers, using fluorescein-labeled goat anti-human IgM antibody obtained from the same supplier. In this test the incubation times were doubled as compared with the incubation times in IFA summary antibody (ΣAb) test. Similarly, in some cases a specific IgG titer was determined by IFA for comparison with the IPA IgG titer.

Blood donors and patient sera. One-hundred fourteen single sera obtained from blood donors (presumed healthy people) and serial sera from four newborns with congenital CID (and from three of their mothers), three leukemic patients and one case of CMV mononucleosis were tested by the three different methods (CF, IFA, and IPA). The starting dilution was 1:8 for CF and 1:16 for IPA and IFA tests.

CMV isolation. CMV isolation was routinely attempted from urine and/or saliva from clinical cases. Inoculated WI-38 cell cultures were observed for 60 days for evidence of cytopathic effect.

RESULTS

Conjugate titration, specificity, and result reproducibility. Preliminary experiments were carried out on CMV-infected cells using the high-titered CMV serum either unabsorbed or absorbed with CMV-infected cells at 37 C for 1 h and at 4 C overnight.

Afterwards, it was decided that only intranuclear inclusion body (INI) staining, which was usually associated with diffuse cytoplasmic stain, was specific and that intracytoplasmic inclusion body (ICI) staining, corresponding to the Golgi area, was insufficient as a positive (Fig. 1).

Nonspecific staining of both INI and ICI was observed with 1:5 and 1:10 dilutions of the conjugate (Table 1). Nonspecific staining of ICI was

TABLE 1. *Block titration of peroxidase-labeled coat anti-human IgG antibody*[a]

Serum (dilutions)	Conjugate dilutions (reciprocal) on CMV-infected WI-38 cells									Conjugate dilution 1:20 on WI-38 cells		
	5	10	20	40	80	160	320	640	1,280	V-Z-infected	HSV-infected	Uninfected
CMV-positive human serum												
1:16	++	++	++	++	++	+	+	+	−	−	−	−
1:32	++	++	++	++	++	+	+	+	−	−	−	−
1:64	++	++	++	++	+	+	+	+	−	−	−	−
1:128	++	++	++	++	+	+	+		−	−	−	−
1:256	++	++	++	++	+	+	+	+	−	−	−	−
1:512	++	++	++	++	+	+	+	−	−	−	−	−
1:1,024	++	++	++	++	+	+	+	−	−	−	−	−
1:2,048	++	++	++	++	+	+	−	−	−	−	−	−
1:4,096	++	++	++	++	+	+	−	−	−	−	−	−
1:8,192	++	++	G	G	−	−	−	−	−	−	−	−
1:16,384	++	++	G	G	−	−	−	−	−	−	−	−
CMV-, V-Z- and HSV-negative human serum (1:16)	++	++	G	G	G	−	−	−	−	−	−	−
V-Z-positive human serum (1:16)	++	++	G	G	G	−	−	−	−	+	−	−
HSV-positive human serum (1:16)	++	++	G	G	G	−	−	−	−	−	+	−
Phosphate-buffered saline	+	+	−	−	−	−	−	−	−	−	−	−

[a] Symbols: ++, Intranuclear (INI) and intracytoplasmic (ICI) inclusion bodies dark-brown stained, positive reaction plus nonspecific staining (NSS); +, INI dark-brown stained, positive reaction; −, INI not stained, negative reaction; G, just Golgi area (ICI) was stained, negative reaction plus NSS.

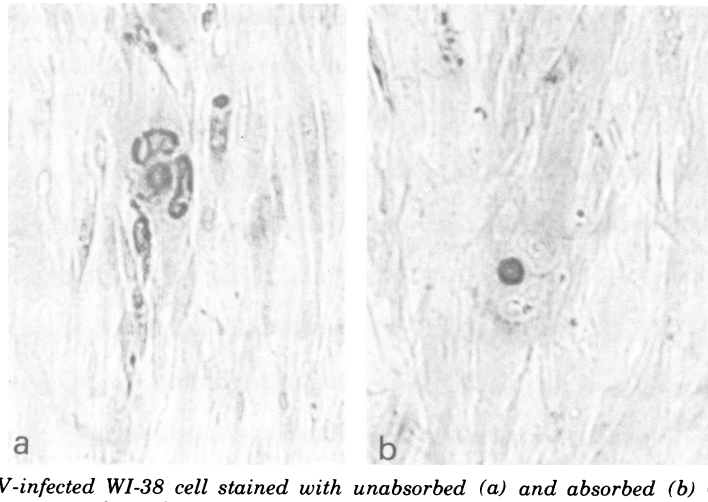

FIG. 1. *A CMV-infected WI-38 cell stained with unabsorbed (a) and absorbed (b) CMV high-titered serum. Absorption was performed using CMV-infected WI-38 cells. Only the intracytoplasmic inclusion is stained with absorbed serum; outlines of the unstained intranuclear inclusions can be clearly seen in the background.*

observed through a 1:80 dilution of the conjugate for the lowest dilutions of CMV-positive, CMV-negative, and V-Z- and HSV-positive sera. The treatment with conjugate alone showed nonspecific staining of INI through a 1:10 dilution. No endogenous peroxidase was detectable by the specific histochemical reaction, unless previously treated with the labeled or unlabeled sera, either in infected or uninfected cells.

In the block titration the optimal dilution of the conjugate giving INI but not ICI staining with the highest dilution of the positive CMV serum was 1:160. This was the working dilution in performing the tests.

The specificity was demonstrated by: (i) the inability of CMV-positive human serum to stain V-Z- and HSV-infected and uninfected WI-38 cells, using a 1:20 or greater dilution of conjugate; (ii) the inability of V-Z- and HSV-positive human serum to stain the INI of WI-38 cells infected with a heterologous virus, when used at a 1:16 dilution against a dilution of the conjugate $\geq$1:20; (iii) the inability of CMV-negative human serum to stain INI with a conjugate dilution $\geq$1:20; (iv) disappearance of specific INI stain in all infected cells following previous treatment with unlabeled anti-human IgG goat serum (blocking test). A negative CMV serum was well as low- and high-titer CMV-positive sera were routinely included as controls. The reproducibility of results was ascertained, and no difference in titer was observed in repeated testing of the same sera over a 3-month period.

Similarly, no difference was observed when a group of 10 sera was tested using different batches of slides: (i) freshly prepared or 3 months old (storage at -70 C); (ii) infected with reference CMV strain AD-169 or with three different high-titer CMV strains isolated from three newborns with congenital CID.

The morphological features of a negative, a weak, and a strong positive reaction by IPA are demonstrated in Fig. 2. For comparison a strong and a weak positive by IFA are shown in Fig. 3.

Nonspecific staining of ICI. In the block titration, nonspecific staining of the ICI (Golgi area) was almost completely eliminated using a conjugate dilution of 1:160. However, in performing tests, many CMV-positive sera as well as negative sera showed ICI staining in the highest dilution.

The same problem was encountered with IFA (ΣAb and IgG), but not usually in IgM titration. Moreover, the IFA test interpretation of positive sera in a few cases was difficult due to the presence of antinuclear antibodies, as shown by fluorescence of uninfected WI-38 nuclei.

Blood donors. In a group of 114 single sera from healthy blood donors, 48 were found negative by CF, 36 by IPA, and only 21 by IFA. Among 48 sera negative by CF, 18 were negative by all three methods, 8 were positive by both IPA and IFA, 4 were positive at the starting dilution (1:16) by IPA only and 18 by IFA only with titers of 1:16 to 1:64.

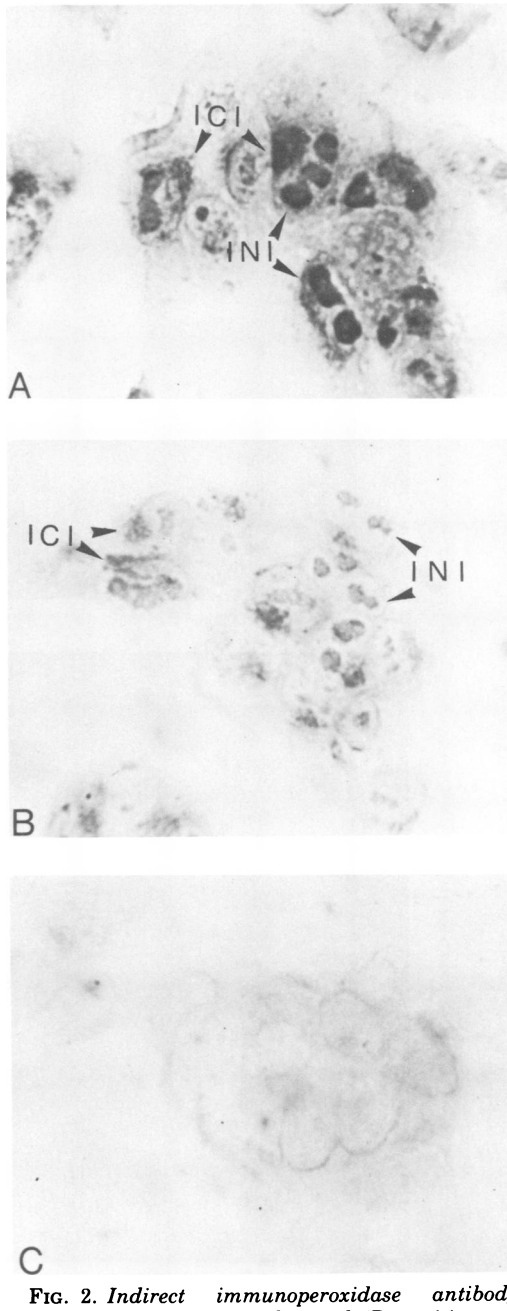

FIG. 2. *Indirect immunoperoxidase antibody technique. A strong (A) and a weak (B) positive and a negative (C) reaction is shown. Intranuclear (INI) as well as intracytoplasmic (ICI) inclusion bodies are indicated.*

In comparison to CF, IPA IgG titers were the same or presented a twofold dilution difference in 49 cases (36 negative and 13 positive) and a fourfold dilution difference or greater in the other 63 cases; whereas IFA ΣAb titers were

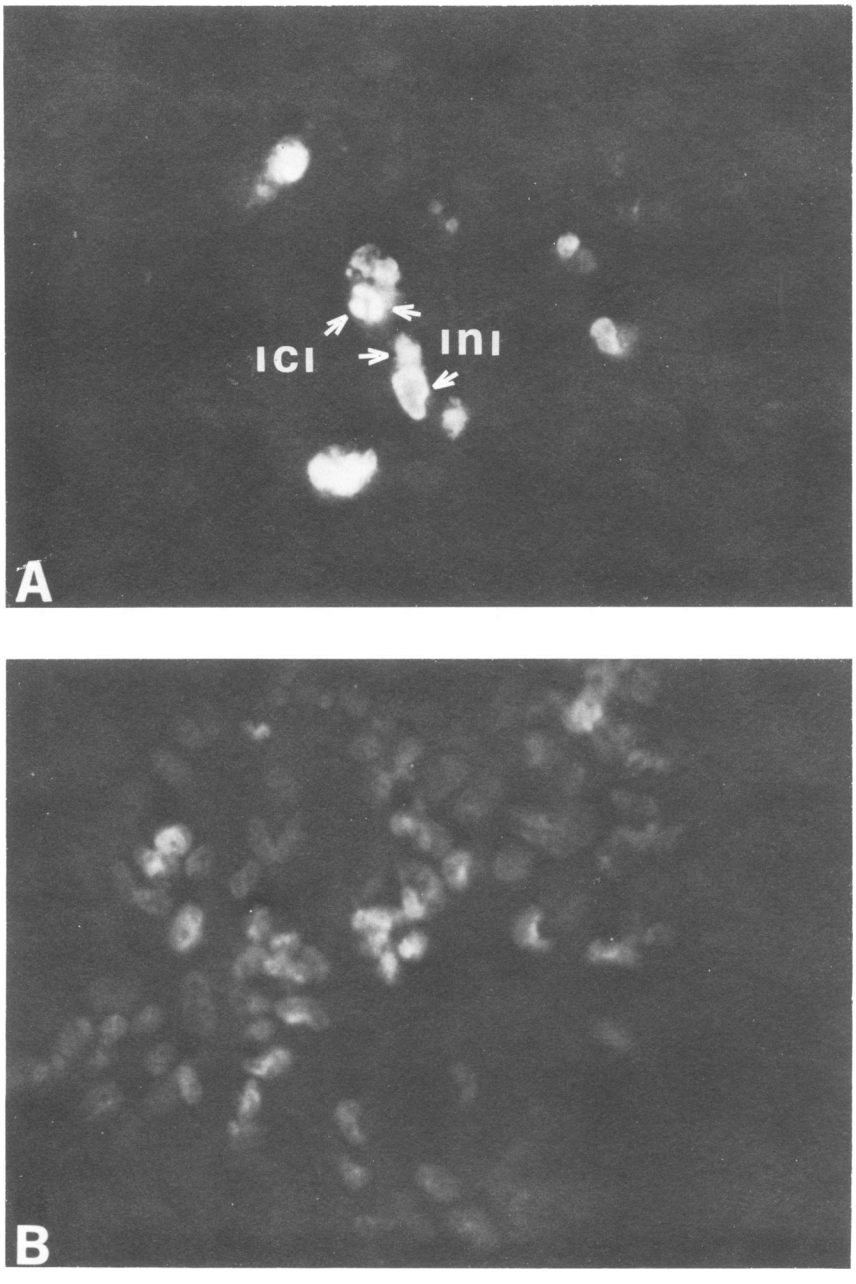

FIG. 3. *Indirect fluorescent antibody technique. A strong (A) and a weak (B) positive reaction are shown. Intranuclear (INI) and intracytoplasmic (ICI) inclusion bodies are indicated.*

comparable in 54 sera (21 negative and 33 positive) and presented a fourfold difference or greater in 56 sera (one serum had a lower titer than CF, but the CF titer was unreliable due to the presence of high anticomplementary activity) (Fig. 4). Three sera were not titratable (two by CF because of anticomplementary activity and one by IFA because of antinuclear antibody).

Comparison of IPA IgG and IFA ΣAb titers demonstrated similar results in 71 cases (62.8%). In 20 cases the IPA titers were higher than IFA ΣAb titers, and four cases were positive only by the IPA test. Results also showed

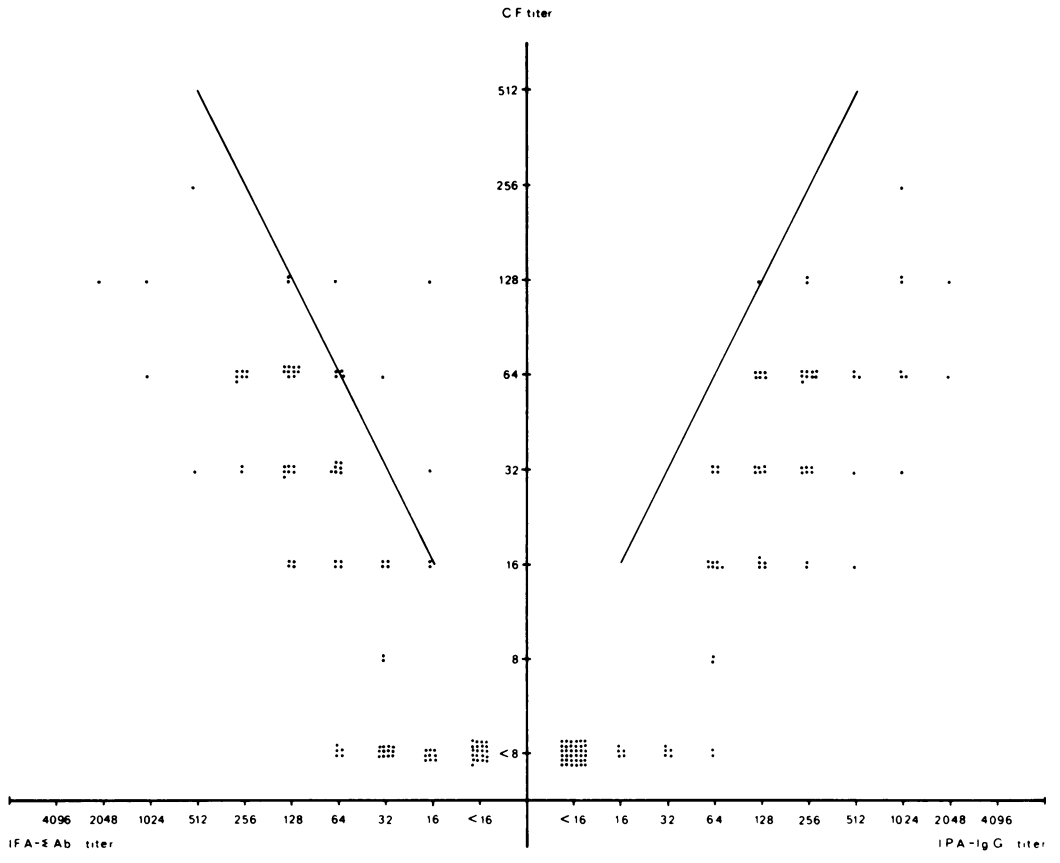

FIG. 4. *Comparison of CF versus IPA IgG and IFA ΣAb titers in 114 blood donors. The continuous line represents the identity of titers between two tests.*

18 cases positive only by the IFA ΣAb test plus one case with a higher IFA ΣAb titer due to a probable recent primary infection (Table 2).

Patients with acute CMV infection. In the group of newborns with congenital CID (Table 3), two were followed starting 3 months after delivery. The first case (C.W.) suffered at delivery from microcephaly and blindness. This patient has been followed to this point for 16 months and found to have persistently elevated CMV titers, as determined by all three methods, in the presence of persistent virus excretion. The mother surprisingly was neither excreting CMV nor did she have antibody for a period of at least 7 months following delivery.

The second infant (N.C.) presented with microcephaly and mental retardation, excreted CMV in the urine and had high antibody titers, as did his mother, indicating a recent CMV infection during pregnancy.

The last two cases of congenital CID were followed starting 1 week after delivery. One newborn (S.N.) had hepatosplenomegaly, hepatitis, and a CMV-IPA IgG titer greater than the IFA ΣAb titers concomitant with CMV excretion in the urine. The mother had CMV antibody but not of such a degree as to suggest a recent infection. The other one (B.B.R.) excre-

TABLE 2. *Comparison of IPA IgG versus IFA ΣAb titers in 114 blood donors*[a]

IFA ΣAb titers	IPA IgG titers								
	<16	16	32	64	128	256	512	1,024	2,048
<16	*18*	4							
16	8	*1*		1	3				
32	7	1	*4*	4	2	2			
64	3		1	*8*	5	3		2	
128				3	*6*	9	3		2
256					1	*4*	2	2	
512						1	*0*	1	
1,024								*2*	
2,048						1			

[a] A fourfold difference was considered significant. Titers are expressed as the reciprocal of serum dilutions.

TABLE 3. *Clinical cases with CF, IFA ΣAb, and IPA IgG titers (Congenital CID)*

Patient	Interval following delivery (mo)	Virus isolation	Days needed for isolation	Titers			
				CF	IFA ΣAb	IPA IgG	IFA IgM
C.W.	3	ND	–	256	1.024	512	ND
	4	U+	27	512	512	1.024	ND
	5	U+	36	512	ND	1.024	ND
	6	U+	9	ND	ND	ND	ND
	7	ND	–	64	ND	ND	ND
	8	U+	8	ND	ND	ND	ND
	12	U+	7	ND	ND	ND	ND
	19	U+	8	64	256	512	ND
Mother of C.W.	4	ND	–	<8	<16	<16	ND
	6	U–	–	<8	<16	<16	ND
	7	ND	–	<8	<16	<16	ND
	19	ND	–	64	1.024	512	ND
N.C.	3	ND	–	256	2.048	1.024	ND
	5	U+	10	ND	ND	ND	ND
	6	U+	8	128	1,024	1.024	ND
Mother of N.C.	3	ND	–	256	1,024	2,048	ND
	5	U+	20	ND	ND	ND	ND
	6	ND	–	256	2,048	2,048	ND
S.N.	1 week	ND	–	ND	32	128	ND
	2	U+	3	32	128	512	ND
	3	U+	4	ND	ND	ND	ND
	4	U contaminated	–	64	256	2,048	ND
Mother of S.N.	1 week	ND	–	ND	64	64	ND
	2	ND	–	16	16	128	ND
B.B.R.	1 week	U+	1	1,024	4,096	2,048	16
	1 week	N-P+	8				
	1 week	O-P+	13				

[a] Abbreviations: ND, Not done; U, urine; N-P, naso-pharynx; O-P, oro-pharynx. Symbols: +, Positive isolation; –, negative isolation.

ted large quantities of CMV in the urine and oro- and naso-pharyngeal secretions. This patient already had a high CMV antibody titer 1 week after delivery. Microphthalmy was present and IgM titer by IFA was 1:16. The mother's serum was unavailable for testing.

In two leukemic patients excreting CMV in the urine (D.H. and H.M.), IPA IgG titers were not different from IFA ΣAb titers (Table 4).

In two cases of adult postnatal CMV infection, one leukemic patient and one CMV mononucleosis, each associated with virus excretion, antibody titers as determined by all three methods are reported (Table 4). In CMV mononucleosis the initial high IgM antibody titer rapidly decreased and was as one would expect replaced by a very high specific IgG titer.

DISCUSSION

The IPA technique has significant advantages over the other two methods (CF, IFA) with which it has been compared. It is a rapid test, since it takes 90 min to perform, whereas the CF test requires overnight incubation. As compared with the IFA technique, the IPA technique does not require immediate interpretation, since it is dependent on a very stable histochemical reaction. Finally, only a light microscope is required.

Results of the present study support the view that IPA is more sensitive than CF and presents about equal sensitivity and specificity as IFA (different titers on the same sera are most likely due to different immunoglobulin specificity of the reagents). Moreover, since the IPA technique is easy to perform, it can be used as a screening procedure. In the group of blood donor sera the IPA IgG technique failed to detect approximately 15% of positive sera which were detected by IFA ΣAb. These results are probably due to the presence in some sera of a different antibody class than IgG. IgM antibody spe-

TABLE 4. *Clinical cases with CF, IFA ΣAb, and IPA IgG titers (postnatal CMV infection)*[a]

Patient	Age	Specimen date	CMV isolation	Days needed for isolation	Titers				
					CF	IFA ΣAb	IPA IgG	IFA IgM	IFA IgG
D.H.	33	7/31/1974	U+	26	32	64	64	ND	ND
		9/25/1974	U+	12	16	64	32	ND	ND
H.M.	29	7/31/1974	U+	21	256	512	512	ND	ND
		8/12/1974	ND	–	AC	512	256	ND	ND
J.R.	24	8/30/1973	ND	–	<8	<16	<16	ND	ND
		9/4/1973	ND	–	<8	<16	<16	ND	ND
		8/27/1974	ND	–	<8	<16	<16	ND	ND
		9/12/1974	U–	–	≥1,024	1,024	1,024	ND	ND
		9/19/1974	S–	–	ND	ND	ND	ND	ND
		9/30/1974	S+ U–	31	ND	ND	ND	ND	ND
		10/7/1974	S+ U–	14	128	512	256	ND	ND
		10/15/1974	S+ U–	6	ND	ND	ND	ND	ND
		10/21/1974	U–	–	ND	ND	ND	ND	ND
		11/15/1974	lung biopsy +	13	64	≥1,024	ND	ND	ND
S.G.	25	3/5/1974	ND		128	16,384	4,096	128	1,024
		4/1/1974	U+	10	512	32,768	ND	64	1,024
		4/18/1974	S+	15	>128	8,192	ND	16	8,192
		6/5/1974	U+	20	256	2,048	8,192	<4	ND
		6/17/1974	C+	30	128	2,048	4,096	<4	ND
		9/3/1974	U contaminated	–	256	512	1,024	<4	ND

[a] D.H., H.M., and J.R. were leukemic patients; patient S.G. had CMV mononucleosis. Abbreviations: U, Urine; ND, not done; AC, anticomplementary activity; S, saliva; C, cervix. Symbols: +, Positive isolation; –, negative isolation.

cific to CMV usually does not persist more than 6 to 8 months after the onset of infection, but it seems that IgA can last longer (20). Sometimes CMV IgM antibody with a titer no greater than 1:8 has been found in healthy adults (16). Therefore, it seems more convenient in epidemiological surveys to use the IPA ΣAb over the IPA IgG test.

As compared with ΣAb titers determined by IFA, IgG titers detected by IPA were not different in about 60% of healthy adults. These results indicate that IgG antibody is the largest component of specific CMV immunoglobulins in adults. A higher anti-human IgG antibody titer of peroxidase-labeled antibody, as compared to that of fluorescein-labeled anti-human antibody, may account for the higher IPA-IgG titers found in about 20% of cases.

The presence of IgG antibody may explain the finding that in the sera obtained from clinical cases of acute (congenital and postnatal) CMV infection, the IPA detected as many positive sera as CF and IFA. In addition, the high IgG titers we have found in the first three cases of CID in the period of 4 to 6 months after delivery confirm the preeminent value of the CF test for diagnosing CID in the period of age from 5 to 12 months (9). The very high IgG titer found in the last case (B.B.R.) 1 week after delivery lends support to the concept that IgG synthesis occurs in infected infants in utero or at least immediately after birth (13).

No difference in IPA IgG titers was observed when comparing identical sera on WI-38 cells infected with the reference CMV strain (AD-169) or with three high-titer CMV isolates. In IPA as well as in IFA testing the antibody evaluated is bound to CMV nucleocapsids inside the intranuclear inclusion (19). On this basis one can assume that CF, IPA, and IFA tests do not present very much difference as far as the antigens are concerned. The two main strains of CMV so far identified (AD-169 and Davis) by homologous neutralization test (23) were not differentiated by IFA (5). Also in the neutralization test (4) and in the IFA IgM test (10) AD-169 strain appeared to be broadly reacting when compared with a low-passage strain recently isolated. These data suggest that WI-38 cells infected with AD-169 strain are probably the most suitable substrate for determination of CMV antibody titer with IPA and IFA techniques.

The almost complete disappearance of nonspecific staining, localized in the Golgi area (ICI) in IPA as well as in IFA test for IgG or ΣAb titration during IgM or IgA antibody determination, suggests that antibody of the IgG class may be the source of this staining (20). However, the presence of this type of antibody

in CMV-negative sera possibly directed against some new cell antigens (7), or in positive sera up to high dilutions does not resolve the issue. Immunoelectronmicroscopic studies with the IPA technique reveal lysosome-like structures and a great number of enveloped and unenveloped virus particles in the cell area showing nonspecific staining (14). Therefore, in CMV-positive sera it is likely that a specific stain is associated with the nonspecific staining in infected cells, but cannot be differentiated.

In conclusion, the IPA technique appears to be a rapid, easily performed, and specific test for CMV antibody detection. It can be usefully employed both for the determination of the immune status and the follow-up of the immune response in acute CMV infections. The serological approach to the diagnosis of CMV infections would be significantly improved by the routine application of the IPA technique.

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