

# Micro Indirect Hemagglutination Test for Cytomegalovirus

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In an effort to obtain the flexibility and ease of performance of a rapid, serological test for detection of cytomegalovirus antibody, the indirect hemagglutination (IHA) technique was investigated by using a microserological system. Antigens were prepared from tissue cultures of infected human fibroblasts. The specificity of the cytomegalovirus antibody response detected by the IHA test correlated well with the standard neutralization test. The IHA method was more sensitive than the complement fixation test in detecting antibody in congenitally infected newborns. There appeared to be some heterologous antibody response with *Herpesvirus hominis* or varicella virus infections. The IHA test pattern was found to be very stable with excellent persistence of agglutination.

Serological methods available for diagnosing cytomegalovirus (CMV) infection have been limited in their usefulness. CMV complement fixation (CF) tests with sera from congenitally infected infants may remain negative in the presence of high titers of neutralizing antibody and despite continued virus excretion (4, 9). Also, with the CF test there are cross-reactions with herpesvirus. Neutralization tests have been found to be relatively strain-specific and require a lengthy incubation time before results can be obtained.

The present report describes the development of and our experience with a rapid, micro indirect hemagglutination (IHA) test for detecting CMV antibody.

## MATERIALS AND METHODS

**Virus strains.** The CMV AD-169 strain (passage 351), supplied by Wallace P. Rowe, National Institutes of Health, was used for the neutralization tests and for the production of viral antigens used in the IHA method. Another strain, P.N. (passage 3), was isolated in our laboratory from an infant with congenital cytomegalic inclusion disease and was also used as a seed virus in the neutralization test (1). The *Herpesvirus hominis* strains VR<sub>2</sub> (type I) and M.S. (type II) were used for the microneutralization test. These virus strains were obtained from Andre J. Nahmias, Emory University, Atlanta, Ga., at passage level 25 and carried an additional 10 passages in our laboratory.

**CF test.** The CMV CF antigen (AD-169 strain) and the herpes CF antigen (McIntyre strain), both cell pack antigens, were prepared by Gabriel A.

Castellano, under contract at Microbiological Associates, Bethesda, Md. The methods used for the preparation of these antigens have been reported previously (7). The CMV and herpes CF tests were performed by utilizing the microtechnique (6). Titrations were performed with 2 exact units of complement and 4 units of antigen.

**Tissue culture.** Both strains of CMV, the AD-169 and P.N., were propagated in MA-184 (passage level 25), a new human fibroblastic cell line derived from the foreskin of an 8-day-old baby (Microbiological Associates). The growth medium was Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 0.1 mM Eagle's nonessential amino acids, and sodium pyruvate at a concentration of 1.0 mM. Penicillin (100 units/ml) and streptomycin (100 µg/ml) were added to the medium. For maintenance, the medium was the same as above except the fetal bovine serum was reduced to a 2% concentration.

**Neutralization tests.** The CMV serum neutralization tests were performed in MA-184 tissue with 10 to 30 TCID<sub>50</sub> per ml of virus. The test was read after 4 weeks by using methods previously described (5). Primary rabbit kidney cell cultures were used for the *H. hominis* microneutralization tests with 100 TCID<sub>50</sub> per ml of virus and performed as previously reported (2).

**Sera.** Nineteen sera from grade school children who were actively excreting CMV were tested to determine the sensitivity of the IHA test for detecting CMV antibody. Three sera from congenitally infected infants were also tested for this purpose. Fourteen pairs of human sera demonstrating seroconversion for *H. hominis* with the standard CF test were coded and tested with the IHA test for CMV to determine its specificity. The initial dilution of sera tested was 1:8 with the IHA method, since lower dilutions oc-

casionaly demonstrated nonspecific reactions. All sera were inactivated (56 C, 30 min) and were adsorbed with 0.025 ml of packed tannic acid-treated red cells (4 C, 30 min) to remove nonspecific agglutinins. Heat-inactivated normal rabbit serum diluted to a 2% solution in phosphate-buffered saline (PBS) (pH 7.2) was used as diluent for the IHA test.

**Antigen production for the CMV IHA test.** The MA-184 cell cultures were grown to confluency in 32-oz (ca-900 ml) prescription-type culture flasks. After removal of growth medium, the cell cultures were inoculated with 1 ml of AD-169 CMV with a titer of  $10^{-5}$  TCID<sub>50</sub> per ml. Virus absorption was allowed to occur for 30 min at room temperature, and then maintenance medium (30 ml per bottle) was added and the bottles were incubated at 37 C. Cultures were observed until almost complete cytopathic effect was obtained which occurred usually within 12 to 14 days. Cells were then scraped from the bottle with a rubber spatula, and the suspension was repetitively frozen at -90 C and thawed three times. After the third thaw, cellular debris was removed by centrifugation at  $600 \times g$  for 10 min at 4 C, and the supernatant fluid was used as the antigen preparation. Control antigens were prepared similarly from uninoculated tissue cultures.

**IHA test.** Sheep erythrocytes (3% suspension) were tanned and sensitized as previously described (3). Briefly, tannic acid (C<sub>7</sub>H<sub>5</sub>O<sub>4</sub>) diluted 1:80,000 in PBS was used to tan the red cells. Suspension of cells were then sensitized with a previously determined dilution of antigen in saline (pH 6.4).

The dilution of antigen-sensitized cells used in the test was determined by chessboard titration by using twofold dilutions of antigen against an immune serum previously tested by CF and neutralization tests. Antigens prepared as described above were satisfactory when employed at a dilution of 1:8. This dilution was selected since further increase in antigen dilution did not appreciably change the titer of the control immune serum. Control antigens were used at the same dilution as employed in the test.

Testing was performed with the micromethod utilizing spiral loops and V-bottom, soft, plastic plates (Cooke Engineering Co., Alexandria, Va.) as previously described (3, 6).

Standard negative and positive antisera were included in all tests. Controls consisted of (i) serum diluent with sensitized red cells, (ii) serum diluent with antigen-free tanned red cells, (iii) 1:8 dilution of serum with tanned cells. Titers were taken as the highest dilution of serum which gave a 3+ agglutination on a scale of 0 to 4+. A control antigen from each lot of test antigen produced was prescreened for any non-specific reactions with a series of human sera.

## RESULTS

The MA-184 cell cultures proved to be the best tissue for antigen production; however, WI-38 tissue has also been used with some success. Cell pack antigens were also tested but did not demonstrate sufficient antigen.

Both the cells and the medium of infected cul-

tures had to be used to produce satisfactory antigens. This may indicate that a soluble antigen is involved with the hemagglutinating activity. Numerous tests with various lots of control antigens have not demonstrated any nonspecific reactions with tissue or media components. The antigen is very stable at -90 C. After 3 months of storage, various lots of antigen have not lost their original titers.

The sensitivity of the CMV IHA test for the serological detection of CMV antibody was studied with the use of sera from 19 grade school children who were actively excreting CMV virus. These sera were tested by the CF, neutralization, and IHA techniques and the results were compared. With the CMV CF test, 8 of 19 (42%) of the patients had detectable antibody. The CMV neutralization test was performed on 18 sera (quantity not sufficient on one serum), 14 of 18 (78%) having detectable antibody. With the IHA test, 17 of 19 (89%) of the patients excreting virus were found to have antibody.

An additional study of sensitivity and specificity was done with serum specimens from three congenitally infected children (Table 1). Neutralizing antibody titers were determined by using the AD-169 strain and the virus from patient 1 (strain P.N.).

At the ages shown, all patients developed neutralizing antibody to AD-169 but only patient 1 developed antibody to strain P.N., his own virus strain. CF antibody was assayed for all serum samples. The results show that patients 1 and 3 had detectable CF antibody, but patient 2 had no CF antibody until 1 year of age despite continued virus excretion. With the IHA test, all three patients had antibody at a titer of 128. Herpes CF antibody was detected in the serum of patient 3, but the other two patients were negative, indicating that the IHA results were not caused by a heterologous reaction with the closely related herpesvirus antigen.

TABLE 1. *Patients with cytomegalic inclusion disease: neutralizing, complement fixation (CF), and indirect hemagglutination (IHA) antibodies*

Patient and age when sera collected	Neutralizing antibody		CMV CF antibody (AD-169)	CMV IHA antibody (AD-169)	Herpes CF antibody
	AD-169 antigen	PN <sup>a</sup> antigen			
1 (8 months)	16	32	32	128	<8
2 (7 months)	16	<4	<8	128	<8
3 (5.5 months)	16	<4	8	128	64

<sup>a</sup> CMV isolate from patient 1 used as antigen.

To determine further the specificity of the IHA test, acute and convalescent sera from 14 patients with CF seroconversions to *H. hominis* were tested with the herpes microneutralization test and the IHA technique for CMV (Fig. 1). Of the 14 patients tested, 13 had a serological response with the herpes neutralization test and one had no detectable antibody to this antigen. With CMV using the IHA test, four patients were found to have a fourfold or greater increase in antibody with the IHA technique. Of the remaining 10 patients without increases in titer to CMV from the acute to convalescent specimen, 9 had antibody and 1 had no detectable antibody to CMV with the IHA test. The CMV antibody increases found by the IHA method were confirmed by the CMV neutralization test, except for the sera of one patient which did not demonstrate an increase in titer.

Human sera with and without CF antibody for varicella-zoster and *H. hominis* and with no antibody to CMV as tested by CF or neutralization were used to detect possible cross-reactions with these viruses. No cross-reactions were found.

Rabbit CMV pre- and postimmunization sera were obtained from Lidia M. Martos, National Cancer Institute. Rabbits were immunized with the AD-169 strain, and the hyperimmune serum had a titer of 128 with the plaque reduction test. With the IHA CMV test, the preimmunization specimen was negative and the postimmunization specimen had a titer of 64.

14 Patients with HVH		Comparison tests for antibody response		
HVH CF	HVH neutralization	CMV IHA	CMV neutralization	
14 (SC)	9 (SC)	1 (SC)	1 (SC)	
		2 (4X)	1 (4X)	
			1 (NC)	
		6 (NC)	6 (NC)	
	4 (4X)	1 (4X)	1 (SC)	
		2 (NC)	2 (NC)	
		1 (0)	1 (0)	
	1 (0)	1 (NC)	1 (NC)	

FIG. 1. Patients with *Herpesvirus hominis* (HVH) complement fixation (CF) seroconversions tested for HVH neutralization, cytomegalovirus indirect hemagglutination (CMV IHA), and CMV neutralization antibody. Abbreviations: SC, seroconversion; NC, no change or decrease in titer; 4X, fourfold or greater increase in titer; 0, no detectable antibody in acute or convalescent specimen.

## DISCUSSION

The data presented in this report indicate that the CMV antibody response detected by the IHA test correlates well with infection as detected by virus excretion. Only two sera from 19 patients excreting virus failed to show an antibody with the IHA method. Paired sera were not available from these patients, thus the possibility exists that these may be primary infections and sera were obtained before the appearance of antibody. Also, the CMV strains from these patients may be sufficiently distinct from the AD-169 strain, which is used as antigen, that no cross-reactivity would be detected with the IHA test.

Investigations with the sera from infants congenitally infected with CMV also demonstrated the increased sensitivity of the IHA test as compared to the CMV CF test. The apparent lack of sensitivity of the CMV CF test to gamma M (19S) antibody has been previously reported (8). The present findings would suggest that the IHA test is capable of detecting this type of antibody.

The results of the serological studies to determine specificity of the IHA test indicated that there was little cross-reactivity with the closely related herpes antigens. Three of the four increases in CMV antibody titers which were detected among patients with herpes seroconversions were also found with the CMV neutralization test.

Repeat serum titrations, with different lots of antigen, were reproducible within twofold of the original determination. The relatively simple procedure developed for preparation of antigen demonstrates an added potential usefulness of applying the method to distinguishing CMV strains. Preliminary studies also indicate that the technique will be useful for producing hemagglutinating antigens to monkey CMV strains.

The IHA test was found to be very stable with excellent persistence of agglutination. Readings can be made after 1.5 hr at room temperature or overnight at 4 C. The ease of performance should make this technique a valuable tool for clinical and epidemiological studies of CMV infections.

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