

Indirect Fluorescent-Antibody Test for Human Cytomegalovirus Infection in the Absence of Interfering Immunoglobulin G Receptors

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The presence of immunoglobulin G receptors in human fibroblasts infected with human cytomegalovirus (CMV) resulted in a nonspecific cytoplasmic reaction in the indirect fluorescent-antibody test. Both CMV antibody-positive and antibody-negative sera from human or other animal species produced the cytoplasmic reaction. The substitution of a simian CMV strain for the human virus successfully eliminated this cytoplasmic reaction and, thus, allowed for the observation of virus-induced fluorescent intranuclear inclusions. With the latter system, CMV antibody titers in human sera were equivalent to those obtained by using the human virus and, in addition, allowed for the detection of relatively low-titered serum samples in which antibody measurement was difficult when human CMV-infected cells were used in the indirect fluorescent-antibody test.

Fluorescent-antibody procedures have become widely accepted for the study of the immunological response to human cytomegalovirus (CMV) infections. The immunofluorescent focus technique for the quantitative determination of virus (8) has been employed for the study of CMV replication (7) and for the detection and measurement of specific antibody (4, 9). In these studies, human CMV-infected cell cultures were observed for the presence of fluorescent cells, and no distinction was made between cytoplasmic and/or perinuclear fluorescence and fluorescence due to virus-induced intranuclear inclusions, particularly when this procedure was used in the measurement of antibody. Recently it has been reported that nonspecific immunoglobulin G (IgG) receptors unrelated to specific antibody are induced in cell cultures infected with human CMV (3, 5, 6). These IgG receptors react in both direct and indirect fluorescent-antibody (IFA) procedures, appearing as diffuse cytoplasmic or perinuclear fluorescence, and may be a source of error in the detection and measurement of human CMV antibody in the IFA test. During the course of the study of simian CMV infections in our laboratory, it was observed that these interfering reactions did not occur in human fibroblasts infected with the simian virus strain. In addition, the more rapid growth of the simian CMV strain, together with the fact that CMV antibody in human sera cross-react with the simian virus strain in the IFA test, suggested that this system might be applicable for the measure-

ment of human CMV antibody titers without the complications of the nonspecific cytoplasmic reactions occurring when human CMV strains are used.

MATERIALS AND METHODS

Virus strains. Viruses used in this study were the AD-169 strain of human CMV obtained from the American Type Culture Collection and the CSG simian CMV strain originally isolated from an African green monkey (2). Both virus strains were grown in monolayer cell cultures of human diploid cell strain WI-38 (HDGS-WI-38) in Eagle basal medium supplemented with 10 or 5% fetal bovine serum for growth or maintenance, respectively.

Immunofluorescent staining. When virus-specific cytopathic effect occurred in 50% of the monolayers, both infected and control cover slip cultures, grown in Leighton tubes, were washed with phosphate-buffered saline, air-dried, fixed in cold acetone for 20 min, and stored at -20°C until use. The IFA procedure was used for the measurement of CMV antibody. Briefly, cover slips infected with either the AD-169 or CSG CMV strain were flooded with diluted sera and incubated at 35°C for 20 min. After washing in phosphate-buffered saline, the cell preparations were flooded for 20 min with goat anti-human gamma globulin conjugate labeled with fluorescein isothiocyanate (BBL, Cockeysville, Md.) or fluorescein-conjugated anti-human IgG (Hyland Laboratories, Costa Mesa, Calif.). After additional washing, the cover slips were mounted in buffered glycerol (90% glycerol and 10% phosphate-buffered saline) and examined with a Leitz microscope equipped with an HB 220 mercury lamp and a dry-dark-field condenser. The highest serum dilution demonstrating the presence of fluorescent virus-in-

duced intranuclear inclusions was considered the endpoint.

Human and simian sera studied. Human sera for study were obtained from the Clinical Immunology Laboratory, Yale-New Haven Hospital. Serum samples were selected on the basis of the complement fixation test for human CMV with antigen purchased from Flow Laboratories, Rockville, Md. A complement fixation CMV antibody titer equal to or greater than 1:4 was considered to be positive.

The neutralization test was used to select simian CMV (CSG strain) antibody-positive simian sera. Serum samples were obtained from naturally infected African green and rhesus monkeys and from rhesus monkeys experimentally infected with the CSG strain of CMV. In the neutralization test, each serum dilution was mixed with 100 50% tissue culture infective doses of the CSG virus strain. After 30 min at room temperature, 0.2 ml of each mixture was inoculated into replicate cultures of HDCS-WI-38. The highest serum dilution completely preventing the appearance of cytopathic effect characteristic for CMV was considered the endpoint.

RESULTS

Fluorescent-antibody reactions with human and simian CMV strains. The differences

in fluorescent-antibody reactions in human or simian CMV-infected cells are illustrated in Fig. 1. Figure 1a illustrates the typical cytoplasmic fluorescence obtained with cell cultures infected with the human CMV strain. Typically, intranuclear inclusions were not clearly discernible because of the presence of interfering cytoplasmic fluorescence, whereas cells infected with the simian strain showed only intranuclear inclusions (Fig. 1b). Examples of the different types of fluorescent-antibody reactions obtained with the human and simian CMV strains are shown in Table 1. The cytoplasmic fluorescent reaction, using a known CMV antibody-positive human serum, was obtained with the human virus strain at all serum dilutions tested. Even at a dilution of 1:2,560, 6% of the cells examined still showed the presence of fluorescence. Visualization of fluorescent intranuclear inclusions with the human CMV strain was particularly difficult at high serum dilutions because of the background of nonspecific cytoplasmic fluorescence. When simian CMV-infected cells were used in the same procedure, however, clearly outlined intranuclear inclu-

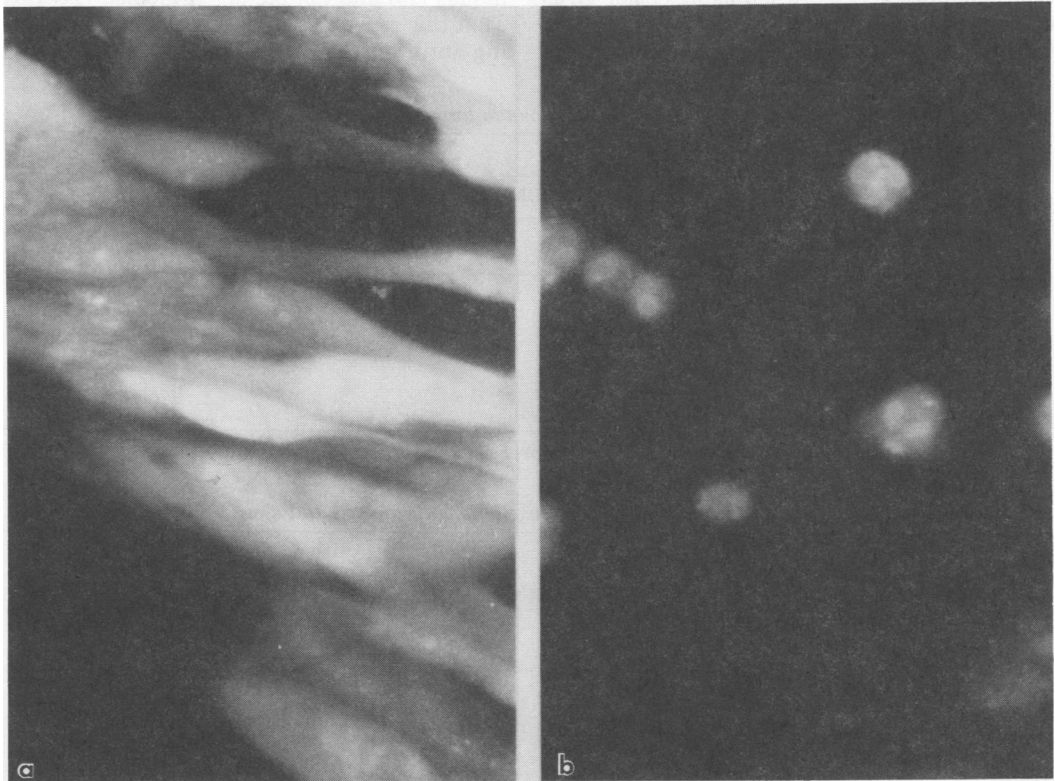


FIG. 1. Indirect fluorescent-antibody reactions of human diploid cell strain WI-38 infected with CMV. (a), Widespread fluorescence in cytoplasm and intranuclear inclusions of cells infected with human CMV strain. (b), Specific fluorescence in nuclear inclusions infected with simian CMV strain.

sions were observed, thus eliminating the difficulties in determining the CMV antibody titer.

Specificity of the cytoplasmic and intranuclear reactions. Both human sera, previously shown to be complement fixation antibody positive to the AD-169 CMV strain, and monkey sera, shown to have neutralizing antibody titers to the CSG strain, were found to react with both virus strains with the IFA procedure (Table 2). The cytoplasmic fluorescence observed with human, simian, or bovine sera in human CMV-infected cells was absent when the same sera were added to simian CMV-infected cells. While both simian and human sera produced fluorescent intranuclear inclusions with both viruses, the intense cytoplasmic reaction in the human CMV-infected cells masked the presence of fluorescent intranuclear inclusions in a number of samples.

With human sera previously shown to be positive for complement-fixing antibody, IFA titers to the simian CMV strain were compared with IFA titers to the human CMV virus. Analysis of these titers by the least-squares line of best fit (coefficient of correlation = 0.88) showed that serum titers of the same magnitude could be expected when using either virus strain (Fig. 2). Sera having low antibody titers were more easily measured with the simian

virus-infected cells, since in this range (1:5 to 1:10) the high background of nonspecific cytoplasmic fluorescence occurring in cells infected with the human CMV virus often obscured the observation of virus-specific intranuclear inclusions.

A similar relationship was observed when the complement-fixing CMV antibody titers were compared with IFA titers measured on the same human sera (Fig. 3). The groups of serum samples having high complement fixation antibody titers also had the higher IFA titers. A comparison of these titers showed a linear relationship (coefficient of correlation, 0.94). The response of the sera to immunoglobulins other than IgG was not studied.

DISCUSSION

The discovery and characterization of the IgG receptor induced in cell cultures infected with human CMV have been reported by a number of investigators (3, 5, 6). This capacity has also been observed in cells infected with another member of the herpesvirus group, herpes simplex virus (HSV). Induction of IgG receptors by HSV has been demonstrated by rosetting techniques (10) and uptake of ¹²⁵I-labeled IgG (11). That this reaction can be a problem in measuring antibodies to HSV in the IFA test has been

TABLE 1. *Fluorescent-antibody reactions to human and simian CMV strains*

Serum dilution	Complement fixation reaction	Fluorescent reaction			
		Human CMV (AD-169)		Simian CMV (CSG)	
		Cytoplasmic (%)	Intranuclear	Cytoplasmic (%)	Intranuclear (%)
1:5	+	52	+ ^a	0	31
1:20	+	32	+	0	30
1:80	+	28	+	0	28
1:320	-	13	-	0	0.25
1:1280	-	5	-	0	0
1:2560	-	6	-	0	0

^a Enumeration of intranuclear inclusions was impractical due to cytoplasmic fluorescence.

TABLE 2. *Specificity of immunofluorescent reactions with human and simian CMV strains*

Test serum	Fluorescein-labeled antiserum	CMV antibody status ^a	No. tested ^b	Immunofluorescent reaction			
				Human CMV		Simian CMV	
				Intranuclear	Cytoplasmic	Intranuclear	Cytoplasmic
Human	Anti-human IgG	pos	20	17	20	20	0
		neg	18	0	17	0	0
Simian	Anti-simian IgG	pos	21	19	21	21	0
		neg	20	0	20	0	0
Bovine	Anti-bovine globulin	neg	10	0	10	0	0

^a Sera pretested by complement fixation (human) and neutralization (simian) tests.

^b All sera tested at a 1:5 dilution.

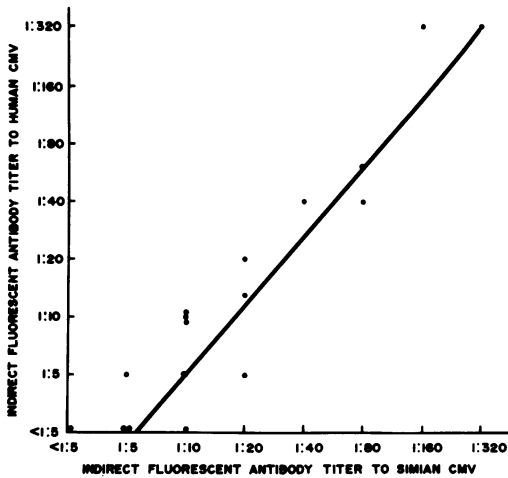


FIG. 2. Comparison of CMV immunofluorescent-antibody titers in human sera to human and simian virus strains.

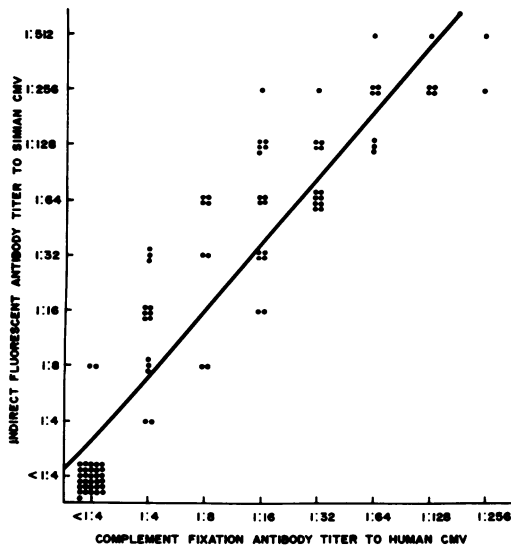


FIG. 3. Comparison of complement fixation antibody titers to human CMV and immunofluorescent-antibody titers to simian CMV in human sera.

recently reported (R. N. Feorino, S. L. Shore, and C. B. Reimer, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1976, S262, p. 248) in that both HSV antibody-positive and -negative sera had titers of 1:320 to 1:640, using fluorescein-labeled antisera to human IgG as determined by cytoplasmic fluorescence. In our laboratories using HSV-infected primary rabbit kidney cells, antibody titers in excess of 1:10,000 were obtained by the IFA test with cytoplasmic fluorescence as a positive reaction (unpublished data).

The present study demonstrated that human fibroblast cultures infected with the CSG strain

of simian CMV could serve as a satisfactory antigen in the IFA test for the measurement of CMV antibody in human sera. With this system, the interference caused by the presence of nonspecific cytoplasmic fluorescence was avoided and, thus, allowed for the clear visualization of fluorescent virus-induced intranuclear inclusions. Furthermore, it has been our experience that the growth characteristics of the simian CMV strain in cell culture are more rapid and reproducible than those of human AD-169 CMV strain, providing a more convenient and relatively simple test.

The nonspecificity of the IgG receptor activity induced by human CMV is demonstrated because the receptors combined with the IgG of human, simian, and bovine species (Table 2). Sera from other species have also been shown to combine with these receptors such as rabbit, guinea pig, and hamster (3) as well as rat sera, although the relative reactivities of these sera were not equal (5). Keller et al. suggested that the ability to synthesize this receptor was coded in the viral genome, since it was found in Vero cells, derived from monkey kidney, after infection with human CMV (5).

Waner and Budnick have shown that the use of a fluorescent-antibody test provided results equivalent to the neutralization test, using a plaque assay, and offered the added advantage of speed and ready availability of reagents (9). However, the use of the human virus strain requires very careful examination for the presence of fluorescent virus-specific intranuclear inclusions, a procedure which was found in our laboratory to be tedious and uncertain because of the background of intense cytoplasmic fluorescence. Since IFA titers of human and simian CMV-infected cells showed similar values (see Fig. 2), the latter system would provide a more sensitive and accurate test for human CMV antibody studies.

When compared with the complement fixation test the IFA procedure appeared to be slightly more sensitive. Furthermore, the IFA procedure has been of great value in our laboratory in the testing of sera having anti-complementary activity. However the relationship between the IFA titer and the complement fixation titer still requires considerable study. Betts et al. showed that the complement fixation procedure compares very favorably with IFA methods, provided the antigen in the former procedure is prepared properly (1). In the present study, IFA titers were distributed over a wide range within groups of sera having the same complement fixation antibody titers similar to the studies reported by Furukawa et al. (3). The use of different lots of commercially

prepared human CMV antigens might account, in part, for the variation in the complement fixation antibody titers obtained in the present study.

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