Use of Isolated Nuclei in the Indirect Fluorescent-Antibody Test for Human Cytomegalovirus Infection: Comparison with Microneutralization, Anticomplement, and Conventional Indirect Fluorescent-Antibody Assays

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Use of an antigen consisting of purified isolated nuclei from a mixture of human cytomegalovirus-infected and uninfected fibroblasts in a 2:1 ratio is a simple and reliable method for eliminating nonspecific fluorescence associated with the presence of Fc-immunoglobulin G receptors in the cytoplasm of infected cells. The specificity obtained with this antigen on 100 normal human sera was 99, 100, and 98% when compared with microneutralization, anticomplement immunofluorescence, and conventional indirect fluorescent-antibody assays, respectively. Also, 95% of the antibody titers obtained with the nuclear antigen had a perfect correlation with or were within a fourfold-dilution difference of the antibody levels obtained by anticomplement immunofluorescence and the conventional indirect fluorescent-antibody test.

The indirect immunofluorescent-antibody (IFA) assay has become a routine laboratory method to assess antibody responses to human cytomegalovirus (HCMV) infection. The attributes that have contributed to the acceptance of this serological test are its high sensitivity and rapid execution and the ability to define antibody titers in sera having anti-complementary activity.

Recently, however, questions have been raised with respect to the practical value of this assay because CMV-infected human fibroblasts, which constitute the antigen for the IFA test, develop Fc-immunoglobulin G (IgG) receptors in their cytoplasm (3, 6, 11). Upon staining by either the direct or the IFA procedure, these receptors give diffuse cytoplasmic and perinuclear fluorescence. This background of nonspecific staining, which may occur with certain sera even at high dilutions, interferes with the visualization of the fluorescent virus-induced intranuclear inclusions. Under these circumstances microscopic examination of slides becomes tedious and many times unreliable to the extent that false positive results become a potential hazard (1, 3).

The observation that human fibroblasts infected with the CSG strain of simian CMV failed to synthesize Fc receptors led Swack et al. to test and subsequently propose the use of this antigen in the IFA assay (11). Their results showed that antibody titers obtained with HCMV- and simian CMV-infected human fibro-

blasts were similar. In addition, the interference caused by the presence of nonspecific cytoplasmic fluorescence could be avoided, thus allowing for a better visualization of virus-specific intranuclear fluorescence.

The fluorescence located in the nucleus of the HCMV-infected human fibroblasts is the most reliable criterion for the correct interpretation of the IFA (4, 5). It seemed reasonable, therefore, to use an antigen consisting of purified nuclei as a means of eliminating nonspecific cytoplasmic fluorescence associated with the Fc-IgG receptor. In this report, we compare the sensitivity and specificity of the IFA using this nuclear antigen (IFA-NA) with the specificity and sensitivity of the microneutralization, anticomplement immunofluorescence (ACIF), and conventional IFA assays on sera collected from 100 normal adults.

MATERIALS AND METHODS

Sera. Sera were obtained from 100 normal pregnant females and stored at -20°C until tested. Samples were heat inactivated for 30 min at 56°C , and fourfold dilutions were prepared in phosphate-buffered saline.

Nuclear antigen preparation. Confluent monolayers of human fibroblasts grown in 75-cm^2 plastic flasks (Falcon Plastics, Oxnard, Calif.) were infected with the AD-169 strain of HCMV (cell-free virus) at a multiplicity of infectivity of approximately 1. After 1 h of adsorption at 37°C , the inoculum was replaced by 25 ml of maintenance medium (medium 199 in Earle balanced salt solution with 5% calf serum and $50\text{ }\mu\text{g}$ of

gentamicin per ml). On day 6 postinfection, cells were trypsin dispersed and mixed with similarly prepared uninfected cells at a ratio of 2:1. After two washes in phosphate-buffered saline, cells were resuspended 1/10 in hypotonic buffer [50 mM NaCl-20 mM tris-(hydroxymethyl)aminomethane-hydrochloride (pH 7.4)-4 mM MgCl₂-1 mM trisodium ethylenediamine-tetraacetate] and allowed to swell in an ice bath for 30 min (7). Cells were then disrupted in a Dounce homogenizer (the number of strokes in the homogenizer was determined by periodic microscopic observation). Thereafter, nuclei were diluted to a concentration of approximately 100×10^6 /ml with cold isotonic phosphate-buffered saline and separated from cellular debris by centrifugation at $400 \times g$ for 15 min through a cushion of 22.5% Ficoll 400 in isotonic buffer at 4°C (7). The pellet of partially purified nuclei was resuspended in phosphate-buffered saline, and 1 drop (0.025 ml) of this suspension, containing approximately 5×10^3 nuclei, was placed on each of eight 5-mm wells on preprinted slides (Carl Scientific, Matterson, Ill.). Preparations were air dried, fixed for 10 min in chilled acetone, and stored at -20°C until used.

Antigen preparation for ACIF and conventional IFA. Confluent monolayers of human fibroblasts were infected as above. On day 6 postinfection, cells were dispersed by trypsinization (0.25%), washed three times in phosphate-buffered saline, and mixed with uninfected fibroblasts at a ratio of 2:1. Approximately 2×10^4 cells were delivered into each of the eight 5-mm wells of the preprinted slides. They were fixed and stored as described above.

Serological procedures. All three indirect immunofluorescent assays were performed as previously described by us and others (5, 8-10). Fluorescein-conjugated antiserum to human IgG was used for the conventional IFA and IFA-NA, whereas goat anti-human C3/C3c conjugate was used for the ACIF test. Known positive and negative sera plus the appropriate controls for specificity were included in each run (9, 10). The highest serum dilution demonstrating clear nuclear staining was considered the end point in all three assays. Titers equal to or above 1/8 in the IFA-NA and ACIF tests and 1/16 in the conventional IFA were judged to be positive. All microscopic observations were made on a single-blind basis.

A slight modification of the microneutralization test described by Chiba et al. (2) was used for the measurement of CMV-neutralizing antibodies. Confluent monolayers of human foreskin fibroblasts grown in plastic tissue culture trays (Linbro Co., New Haven, Conn.) were used as the indicator system. A 0.2-ml amount of virus suspended in minimal essential medium with a final concentration of 10% guinea pig complement (Colorado Serum Co., Denver, Colo.) was mixed with an equal volume of serial dilutions of heat-inactivated (56°C for 30 min) test sera beginning at a 1/2 dilution. Infectivity of the viral inocula ranged between 1,000 and 2,000 plaque-forming units/0.2 ml. After a 1-h incubation at 37°C, 0.05 ml of the mixture was assayed for residual infectivity in duplicate wells. Overlay medium consisted of minimal essential medium with 2% methyl cellulose. Plaques were enumerated on day 10 postinoculation after Formalin fixation and methylene blue staining. A 60% reduction in

plaque-forming units as compared with control wells was judged as the end point titer.

RESULTS

To determine the presence of specific antibodies to CMV, 100 sera were screened at a 1/4 dilution by the microneutralization test. Sixty-six samples (66%) contained neutralizing antibodies, and, for the purpose of comparing specificity and sensitivity with the other serological assays, these sera were considered positive.

Specificity. The correlation of positive results between the microneutralization test and the IFA-NA, ACIF, and conventional IFA assays reached 99, 100, and 98%, respectively (Table 1). The discrepancy between the microneutralization and the IFA-NA assay was due to a single sample that reacted at a 1/8 dilution in the IFA-NA assay and was found to be negative with the other three serological methods used. Consequently, this may be considered as a false positive result with the IFA-NA test. There were two sera with discrepant negative results in the conventional IFA test. These samples had titers of 1/8 in the ACIF and 1/16 and 1/64, respectively, in the IFA-NA assay (Fig. 1).

Sensitivity. The sensitivity of the IFA-NA test compared well with that of the ACIF and the conventional IFA assay (Fig. 1). At least 95% of the antibody titers obtained with these three indirect immunofluorescent methods had either a perfect correlation or differed by no more than a $\pm$ fourfold dilution.

Demonstration of the pattern of nuclear fluorescence. Nuclei were recognized by their homogeneous size and ovoid or reniform shape (Fig. 2). The fluorescence obtained in the IFA-NA test appeared as bright nuclear staining with maximum intensity within the skeinlike nuclear inclusion. The fluorescent staining of the background was faint and amorphous. Very few cells still maintained some cytoplasm around the nucleus.

DISCUSSION

The results of this study indicate that use of an antigen consisting of nuclei isolated from

TABLE 1. *Correlation of results between the microneutralization test and IFA-NA, ACIF, and conventional IFA assays in 100 human sera*

Micro-neu- tralization	IFA-NA		ACIF		IFA	
	Posi- tive	Nega- tive	Posi- tive	Nega- tive	Posi- tive	Nega- tive
Positive	66	0	66	0	64	2
Negative	1	33	0	34	0	34
Percent cor- relation	99		100		98

FIG. 1. Comparison of antibody titers to HCMV as determined by IFA-NA, ACIF, and conventional IFA assays in 100 human sera. Each number represents the number of sera reactive at the given antibody titer.

HCMV-infected fibroblasts is a simple and reliable means for eliminating the nonspecific fluorescence associated with the development of Fc-IgG receptors in the cytoplasm of these cells. This significant technical improvement was accomplished without diminishing the specificity or the sensitivity of the IFA assay. In fact, an almost perfect correlation of positive results and antibody titers was obtained between the IFA-NA method and the conventional IFA, ACIF, and microneutralization assays. In addition, the test could be read more quickly, and results were highly reproducible.

The induction of an Fc-IgG receptor on human fibroblasts after infection with HCMV may interfere with the interpretation of specific fluorescence in the IFA (1, 3, 6, 11). This is particularly true with antigens prepared from resuspended, fixed fibroblasts because the modification of the shape of these cells hampers the distinction between specific nuclear fluorescence and nonspecific cytoplasmic staining. However, if the antigen consists of intact monolayers fixed 72 to 96 h postinfection, the difference between specific and nonspecific staining can be easily established; the latter is restricted to the cytoplasm and appears as dim diffuse and bright perinuclear fluorescence, whereas specific staining always involves the nucleus. On occasion, making the distinction may be difficult, particularly if the background and cytoplasmic fluorescence is intense. The report of Swack et al. (11) demonstrates that this interference can be avoided by using an antigen consisting of fixed monolayers of human fibroblasts infected with the CSG strain of simian CMV. In this particular heterologous system, Fc-IgG receptors are not induced. Use of the ACIF also avoids staining of Fc-IgG receptors.

Regardless of the origin of fibroblasts and viral strains used to prepare the antigen, the need for fixed, intact monolayers grown on cover slips or tissue culture slides is a technical limitation when large numbers of sera are to be analyzed

in routine diagnostic serology. In this study a large quantity of antigen slides were prepared from a single batch of resuspended HCMV-in-

FIG. 2. (Top) Photomicrograph of positive nuclear fluorescence using an antigen consisting of isolated nuclei obtained from fibroblasts on day 6 postinfection with HCMV. Note that maximum fluorescence appears as bright nuclear staining. $\times 100$ magnification, Ektachrome 160 ASA. (Bottom) Same as (top) but at $\times 200$ magnification. Note that maximum fluorescence occurs within the nuclear inclusion.

fected human fibroblasts. This must be viewed as an additional advantage of the IFA-NA assay.

The presence of antinuclear antibodies in some human sera can lead to misinterpretation of nuclear fluorescence and consequently to false positive results in all IFA assays. Although not encountered in this study, we have detected antinuclear antibodies in approximately 1 out of 150 normal adult sera. The false positive results produced by the antinuclear antibodies can be best recognized by mixing infected with uninfected fibroblasts for preparation of the antigen. Under these circumstances, unless the test serum contains antinuclear antibodies, a certain proportion of nuclei will always remain unstained.

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LITERATURE CITED

1. **Benyesh-Melnich, M.** 1974. Human cytomegalovirus, p. 762-772. In E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), *Manual of clinical microbiology*, 2nd ed. American Society for Microbiology, Washington, D.C.
2. **Chiba, S., R. L. Striker, and M. Benyesh-Melnick.** 1972. Microculture plaque assay for human and simian cytomegalovirus. *Appl. Microbiol.* **23**:780-783.
3. **Furukawa, T., E. Hornberger, S. Sakuma, and S. A. Plotkin.** 1975. Demonstration of immunoglobulin G receptors induced by human cytomegalovirus. *J. Clin. Microbiol.* **2**:332-336.
4. **Geder, L.** 1976. Evidence for early nuclear antigens in cytomegalovirus-infected cells. *J. Gen. Virol.* **32**:315-319.
5. **Giraldo, G., E. Beth, U. Hammerling, G. Tarro, and F. M. Kourilsky.** 1977. Detection of early antigens in nuclei of cells infected with cytomegalovirus or herpes simplex virus type 1 and 2 by anti-complement immunofluorescence, and use of a blocking assay to demonstrate their specificity. *Int. J. Cancer* **19**:107-116.
6. **Keller, R., R. Peitchel, J. N. Goldman, and M. Goldman.** 1976. An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts. *J. Immunol.* **116**:772-777.
7. **Luka, J., W. Siegert, and G. Klein.** 1977. Solubilization of the Epstein-Barr virus-determined nuclear antigen and its characterization as a DNA-binding protein. *J. Virol.* **22**:1-8.
8. **Reedman, B. M., J. Hilgers, F. Hilgers, and G. Klein.** 1975. Immunofluorescence and anti-complement immunofluorescence absorption tests for quantitation of Epstein-Barr virus-associated antigens. *Int. J. Cancer* **15**:566-571.
9. **Stagno, S., D. W. Reynolds, A. Tsiantos, D. A. Fucillo, W. Long, and C. A. Alford.** 1975. Comparative serial virologic and serologic studies of symptomatic and subclinical congenitally and natively acquired cytomegalovirus infections. *J. Infect. Dis.* **132**:568-577.
10. **Stagno, S., J. E. Volanakis, D. W. Reynolds, R. Stroud, and C. A. Alford.** 1977. Immune complexes in congenital and natal cytomegalovirus infections of man. *J. Clin. Invest.* **60**:838-845.
11. **Swack, N. S., F. J. Michalski, A. Baumgarten, and G. D. Hsiung.** 1977. Indirect fluorescent-antibody test for human cytomegalovirus infection in the absence of interfering immunoglobulin G receptors. *Infect. Immun.* **16**:522-526.