Simultaneous Detection of Antibodies to Cytomegalovirus and Herpes Simplex Virus by Using Flow Cytometry and a Microsphere-Based Fluorescence Immunoassay

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A sensitive assay for the simultaneous detection of anti-cytomegalovirus and anti-herpes simplex virus antibodies was developed. Two different sizes of polystyrene microspheres were coated with purified viral antigens. Human antiviral antibodies were detected with a biotin-streptavidin amplification procedure with phycoerythrin as the fluorescent label. Microsphere-associated fluorescence was quantitated with a flow cytometer. Sixteen percent of samples initially scored as seronegative for cytomegalovirus and 35% of samples initially scored as seronegative for herpes simplex virus by conventional assays were clearly found positive by the microsphere technique. This flow cytometric assay can simultaneously detect several specific antibodies at levels which are below the sensitivity of standard assays. The dynamic range of this assay is at least sixfold greater than that of enzyme immunoassays. This technique is amenable to numerous serologic assays and could greatly expand the clinical laboratory applications of flow cytometry.

The detection of antibodies to cytomegalovirus (CMV) and herpes simplex virus (HSV) is used to determine past exposure and as an aid in documenting acute infection. Additionally, blood products and organs from CMV-seronegative donors are essential for the treatment of CMV-seronegative recipients (1). Thus, it is imperative to have accurate viral serologic results for both the donor and the recipient. The presence of HSV antibody has been used in kidney transplant recipients to predict serious HSV infection (4) and in documenting HSV encephalitis (9).

Numerous methods exist for the detection of specific antibodies to CMV and HSV. Assays vary in the volume of specimen required, the principle and reagents used, and analytic sensitivity. Previous reports have demonstrated differences in the performance of the currently available procedures and also false-negative rates for some serologic assays (6, 10, 13). In this report we describe an assay which allows for simultaneous detection and quantitation of antibodies to CMV and HSV. The assay uses polystyrene microspheres of two different diameters as a solid phase to which are adsorbed purified viral antigens. Human antibodies which react with the antigens on coated microspheres are detected with a biotin-streptavidin technique with phycoerythrin (PE) as the fluorescent probe. Microsphere-associated fluorescence is quantitatively measured by flow cytometry, which allows rapid analysis of thousands of microspheres (15). Maximal excitation of PE yields increased sensitivity, owing to increased fluorescence emission from PE (11, 14). Analysis of fluorescence associated with each size of microsphere without interference from other sizes of microspheres allows for multiple assays to be performed simultaneously in the same reaction mixture.

MATERIALS AND METHODS

Serum specimens. Seventy-five serum specimens were analyzed. All serum specimens were tested with commercial

assays for antibodies to CMV and HSV (see below) and were divided into the following three groups: 20 of the serum specimens were negative for antibodies to both CMV and HSV, 11 were positive for antibodies to HSV but negative for antibodies to CMV, and the remaining 44 were positive for antibodies to both CMV and HSV.

Viral antigens. CMV and HSV were grown separately in human fibroblasts in Eagle minimal essential medium with 50 µg of gentamicin and 2.5 µg of amphotericin B per ml and 10% fetal calf serum. When 80 to 90% of the cells showed cytopathic effects, the supernatant was collected and centrifuged at $600 \times g$ for 15 min at 4°C. The supernatant was centrifuged at $113,000 \times g$ for 1 h at 4°C, and the pellet was suspended in 0.1 M glycine-NaOH buffer (pH 8.8). This suspension was centrifuged at $113,000 \times g$ for 1 h at 4°C, and the pellet was suspended as described above. Sodium deoxycholate was added to the dissolved antigen preparation to a final concentration of 0.15%. The antigen mixture was stirred for 30 min at 4°C. This mixture was centrifuged at $76,000 \times g$ for 1 h at 4°C, and the final pellet was suspended in phosphate-buffered saline-0.01% sodium azide. Serial dilutions of the final antigen mixture were coated onto microspheres and reacted with negative and positive sera. The dilution of antigen which produced the greatest difference in fluorescence between the negative and positive sera was used as the optimal antigen dilution for the remainder of the study. The supernatant from uninfected fibroblasts was processed similarly and used to coat microspheres to monitor background fluorescence with each serum sample.

MIA. For the microsphere immunoassay (MIA), viral antigens, diluted in 0.1 M Na₂CO₃ buffer (pH 9.5), were incubated with washed microspheres (Seradyn, Indianapolis, Ind., and Polysciences, Warrington, Pa.) for 3 h at 37°C and then for 18 h at 4°C. Excess antigen was removed by washing in phosphate-buffered saline-0.5% bovine serum albumin-0.1% Tween 20 (PBS-BSA-Tw). The microspheres were incubated for 1 h at 37°C with PBS-BSA-Tw to block unoccupied polystyrene sites. The CMV antigen was ad-

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sorbed to the 7.0- μ m microspheres, the HSV antigen was adsorbed to the 5.0- μ m microspheres, and the two microsphere populations were mixed in equal proportions. A 1:20 dilution of serum (100 μ l) was added to 10^5 microspheres and incubated for 2 h at 37°C. The microspheres were washed three times with PBS-BSA-Tw, and the pellet was suspended in 100 μ l of a 1:30 dilution of biotinylated goat F(ab')₂ antihuman immunoglobulin G (TAGO Inc., Burlingame, Calif.). The microspheres were incubated for 1 h at 37°C and washed as described above, and the pellet was suspended in 100 μ l of a 1:5 dilution of streptavidin-PE (provided by N. Warner, Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) and incubated for 30 min at 22°C. The microspheres were washed as described above, and the pellet was suspended in 200 μ l of phosphate-buffered saline. A separate sample of microspheres of each diameter was coated with the supernatant from uninfected fibroblasts and used with each serum sample to control for background fluorescence.

Flow cytometric analysis. A total of 10^4 microspheres were analyzed on a FACS Analyzer (Becton Dickinson) at 500 microspheres per s. The two microsphere populations were discriminated on the basis of size by using electrical impedance. PE was excited maximally at 546 nm, and the fluorescent light emitted at greater than 575 nm was measured. The resulting fluorescence was displayed as profile histograms, and the peak modal channel (PMC) from the logarithmic display of fluorescence was used as the unit of antiviral antibody concentration. Three standard deviations above the mean of the background fluorescence from all 75 specimens with the control supernatant-coated microspheres was used as the cutoff for determining a positive signal with the antigen-coated microspheres. Both nonfluorescent and PE-conjugated microspheres (Becton Dickinson) were used daily as fluorescence controls for the flow cytometer.

Standard CMV and HSV immunoassays. Antibody to CMV was detected by latex agglutination (LA) as previously described (13). Briefly, the sample was mixed with CMV antigen-coated latex particles (CMV Scan; Hynson, Westcott and Dunning, Baltimore, Md.) for 8 min at 22°C, and agglutination was used as an indication of the presence of anti-CMV antibody. All sera were tested undiluted and, if positive, at serial twofold dilutions. Antibody to HSV was detected by an indirect immunofluorescence assay (IFA; Microbiological Research Corp., Bountiful, Utah) as previously described (5). Briefly, the sample was added to multiwell slides containing HSV-infected fibroblasts. Typical nuclear fluorescence in 50% of the cells was used as an indication of anti-HSV antibody. All sera were tested at 1:10 and, if positive, at serial twofold dilutions. The reciprocal of the highest dilution showing agglutination or fluorescence for anti-CMV or anti-HSV antibody, respectively, was designated as the antibody titer.

RESULTS

Dilution studies with five known negative and positive serum samples demonstrated that a 1:20 dilution of serum provided the least background with the control microspheres and the strongest fluorescence signal with the antigen-coated microspheres. Thus, the remainder of the analyses were done with a 1:20 dilution of serum.

Figure 1 shows both the volume profile histogram of the two microsphere populations and the fluorescence profile histograms obtained with a representative positive serum sample. The control microspheres of both diameters had

FIG. 1. Profile histograms of volume and fluorescence in the MIA. The ordinate is the relative number of events, and the abscissa is the increasing volume or fluorescence. (A) Volume histogram showing the two microsphere populations of 5 and 7 μ m. (B) Fluorescence histogram of the 5- μ m microspheres coated with HSV (solid line) or control antigen (broken line) and reacted with a 1:20 dilution of an HSV-seropositive sample, reactive at 1:10 in the IFA. (C) Fluorescence histogram of the 7- μ m microspheres coated with CMV (solid line) or control antigen (broken line) and reacted with a 1:20 dilution of a CMV-seropositive sample, reactive at 1:32 in LA.

similar fluorescence signals, with the 7.0- μ m microspheres having a slightly higher background signal. The reaction seen with the CMV- and HSV-coated microspheres occurred well above the cutoff for a positive signal. The fluorescence signal was quantitated and expressed as the PMC on a scale of 1 through 1,000. The fluorescence signals obtained were the same when the serum sample was incubated with the two microsphere populations mixed and when the serum sample was incubated with each microsphere population separately.

The flow cytometer was calibrated daily by using standard fluorescent microspheres. The separation between fluorescent and nonfluorescent microspheres was recorded before each experiment, and the flow cytometer was set so that the PE-treated microspheres were in the same fluorescence channel each day.

For determination of the fluorescence cutoff to distinguish positive and negative serum samples, all 75 serum samples were diluted to 1:20 and incubated with both sizes of microspheres coated with the control antigen. Three standard deviations above the mean fluorescence signal from all 75 samples was expressed as the PMC and used as the cut-off for positivity with the antigen-coated microspheres. With the antigen-coated microspheres, any serum sample producing fluorescence greater than a PMC of 7.1 for CMV and a PMC of 4.2 for HSV was considered positive for antibody to the respective antigen.

TABLE 1. Comparison of anti-CMV and anti-HSV antibody levels by the MIA, LA, and the IFA

CMV				HSV			
No. of samples	Titer by LA	MIA result		No. of samples	Titer by IFA	MIA result	
		Mean PMC (range)	% of samples above cutoff ^a			Mean PMC (range)	% of samples above cutoff ^a
31	Negative	4 (1-18)	16	20	<1:10	6 (1-20)	35
9	<1:4	23 (10-38)	100	27	1:10-1:20	90 (31-163)	100
15	1:8-1:16	84 (15-140)	100	17	1:40-1:80	189 (149-823)	100
10	1:32-1:64	304 (103-502)	100	10	1:160-1:320	888 (516-1,510)	100
5	1:128-1:256	617 (514-704)	100	1	>1:640	3,230	
5	1:512-1:1,024	3,003 (1,870-4,535)	100				

^a >7.1 is positive for anti-CMV, and >4.2 is positive for anti-HSV.

Table 1 lists the anti-CMV and anti-HSV antibody results with the MIA as compared with the antibody titers determined by LA or the IFA, respectively. There was general agreement between the MIA value and the titer determined by standard techniques. However, there were specimens which were negative in LA or the IFA which were clearly positive in the MIA. Of 31 samples negative for anti-CMV antibody in LA, 5 (16%) were positive in the MIA. Of 20 samples negative for anti-HSV antibody in the IFA, 7 (35%) were positive in the MIA. Samples which were positive in LA for anti-CMV at titers equal to or greater than 512 and those which were positive in the IFA for anti-HSV at titers equal to or greater than 320 had to be diluted 1:100 in the MIA, and the resulting PMC had to be multiplied by 5. The 1:20 dilution of these serum samples yielded a PMC greater than 1,000. The average antigen fluorescence signal from the samples scored as seropositive in the MIA was 100 times greater than the fluorescence signal obtained with the control microspheres.

To investigate the apparent increased ability to detect low levels of specific antibody by using the MIA, we serially diluted positive serum samples into known negative serum samples and analyzed them by the MIA, LA, and the IFA. Table 2 shows a representative dilution experiment with a specimen seropositive for CMV and HSV assayed by all three methods. More than one known negative serum sample was used to ensure that the negative serum sample was not interfering with the detection of specific antibody. The MIA

detected specific antibody at higher serum dilutions than did the conventional methods. The MIA was able to detect specific antibody at three to four doubling dilutions beyond the point at which LA or the IFA could detect antiviral antibody.

In an attempt to verify that the samples which were positive in the MIA and negative in the standard assays were true-positives, lymphocytes from four cases were cultured in vitro with CMV antigen. Two samples negative for anti-CMV antibodies in the MIA and LA showed no lymphocyte proliferation against CMV (data not shown). Two other samples positive for anti-CMV antibodies in the MIA and negative in LA showed lymphocyte proliferation in vitro against soluble CMV antigen (data not shown).

To use the MIA as a quantitative assay, we studied acute- and convalescent-phase serum pairs from patients with acute CMV infections. Both the acute- and convalescent-phase serum samples were tested at a 1:20 dilution in the same run. Table 3 shows the MIA results for each serum pair for each of the four patients tested. The convalescent-phase specimen in each case had a significantly increased level of specific anti-CMV antibody as compared with the acute-phase specimen. Included in Table 3 are the values from the LA assay used to originally identify these serum pairs. Also listed are the anti-HSV antibody levels, determined by the MIA, which did not increase during acute CMV infections.

The run-to-run variability of the MIA was evaluated by a serum sample negative for both CMV and HSV antibodies and a serum sample positive for both antibodies. Each sample was assayed in 10 separate experiments. The coeffi-

TABLE 2. Dilution study of a CMV- and HSV-seropositive sample assayed by the MIA, LA, and the IFA

Dilution of the positive sample ^a	Result for:			
	CMV		HSV	
	MIA PMC ^b	LA	MIA PMC ^b	IFA
1:40	487	+	613	+
1:80	269	+	290	+
1:160	190	+	174	+
1:320	94	+	88	+
1:640	65	+/- ^c	63	-
1:1,280	41	-	51	-
1:2,560	20	-	21	-
1:5,120	9	-	8	-
1:10,240	5	-	3	-
1:20,480	2	-	2	-

^a The positive sample was diluted into a serum sample negative for antibodies to CMV and HSV.

^b A final serum dilution of 1:20 was used; >7.1 is positive for anti-CMV, and >4.2 is positive for anti-HSV.

^c Weakly positive.

TABLE 3. Acute- and convalescent-phase serum pairs from patients with acute CMV infections

Sample	Phase of infection ^a	CMV MIA value ^b	CMV LA value ^c	HSV MIA value ^b
1	A	3	Negative	58
	C	532	256	67
2	A	2	Negative	163
	C	381	64	107
3	A	1	Negative	94
	C	599	128	108
4	A	1	Negative	190
	C	275	64	201

^a A, Acute; C, convalescent.

^b >7.1 is positive for anti-CMV, and >4.2 is positive for anti-HSV.

^c Reciprocal of the highest positive serum dilution.

cient of variation for the negative serum sample was 18.0%, while that for the positive serum sample was 5.0%.

DISCUSSION

Polystyrene microspheres were used as supports for viral antigen attachment and eventual simultaneous anti-CMV and anti-HSV antibody detection. The two different sizes of microspheres used were sharply discriminated by the flow cytometer. These microspheres could then be analyzed by focusing on one size of microsphere without interference from the other size of microsphere. This allowed for the separate immunofluorescence analysis of two different antibodies, since each microsphere population was coated with a different antigen mixture. Human antibodies bound to the antigen-coated microspheres were detected with a biotinylated antihuman immunoglobulin G reagent, followed by streptavidin-PE. Microsphere-associated fluorescence was quantitated and used as the unit of antiviral antibody level in each sample. The fluorescence signal correlated well with the antiviral antibody titer determined by standard assays. However, some samples were scored as seronegative by standard assays but positive by the MIA. The MIA was able to detect specific antibody at dilutions below the sensitivity of standard assays. The assay also provided clear serological evidence of acute CMV infection in paired serum samples.

Very little background fluorescence resulted with the control microspheres, indicating efficient blocking with bovine serum albumin of unoccupied binding sites on the microspheres and little reactivity of these serum samples with the control antigen from the uninfected fibroblasts. This low level of nonspecific or non-viral antibody labeling of the antigen-coated microspheres allowed for the unequivocal demonstration of positive reactions. With the MIA, the average increase in positive fluorescence over background fluorescence was greater than 100-fold. The maximum increase obtainable with the MIA is 250, assuming a background of 4 and a positive signal at 1,000. This dynamic range of the MIA is at least six times that of conventional methods, such as the enzyme immunoassay, and therefore allows for the quantitation of antibody levels in most samples with only one serum dilution.

The increased fluorescence signal was obtained by using a streptavidin-biotin technique. PE conjugated to the streptavidin was used as the fluorochrome, since PE provides a high fluorescence signal with a very low background (11). The fluorochrome PE has been used in previous studies and has demonstrated greatly increased fluorescence emission when excited at 546 nm (14). This combination of streptavidin-PE and biotin produced a very sensitive assay which was able to detect antibodies at levels which were undetectable by conventional methods. This assay also provided for complete distinction between positive and negative signals, an important consideration when the antibody levels are low. This assay used 546-nm excitation light for PE from a mercury-cadmium lamp in the flow cytometer. We have recently evaluated a 15-mW argon-ion laser for use with this assay and have found similar results, although with slightly decreased signal intensities.

Many studies have described the varied sensitivity of assays for antiviral antibodies. Gilljam et al. (6) found that approximately 19% of patients seronegative for HSV had antibodies to HSV when tested in an assay with increased sensitivity. We found that 16% of patients classified as negative for CMV were positive when tested with the MIA. In our patients found to be negative for HSV by the IFA,

35% were positive when tested with the MIA. This striking incidence of low-level positivity, missed by conventional assays, is probably due to four major characteristics of the MIA: (i) the streptavidin-biotin amplification step, (ii) the use of PE, which emits increased amounts of fluorescence, (iii) the detection of the fluorescence signal with a flow cytometer, which has the capability to measure very small amounts of fluorescence, and (iv) the use of microspheres as the solid phase to capture antibodies. Alternatively, low-level positivity could be due to the detection of antibodies to other herpesviruses, such as Epstein-Barr virus. While HSV types 1 and 2 are known to have extensive homology and cross-reacting antibodies, a recent study has demonstrated that cross-reacting antibodies can be seen with CMV, HSV, and Epstein-Barr virus (3). However, the detection of increased CMV antibody in patients with documented acute CMV infections without an increase in HSV antibody in the acute- and convalescent-phase serum pairs suggests that our assay was probably not detecting cross-reacting antibodies. In addition, we showed that two individuals who were seropositive for CMV antibodies only in the MIA exhibited *in vitro* lymphocyte proliferation against CMV. The lymphocytes from two CMV-seronegative individuals did not proliferate *in vitro* against CMV. This is strong evidence that the samples with low-level CMV antibodies, as detected by the MIA, are true-positives (12).

Sensitive detection of specific viral antibodies is often clinically useful. In cases of transfusion or organ transplantation into a CMV-seronegative recipient, the donor should probably be CMV seronegative to avoid the transmission of CMV (1). In bone marrow transplantation into a CMV-seropositive recipient, the administration of marrow from a CMV-seropositive donor has been suggested as a possible benefit in protecting the recipient from leukemic relapse (8). This situation occurs because patients with chronic graft-versus-host disease may also have graft-versus-leukemia, and patients seropositive for CMV have a higher incidence of graft-versus-host disease (8). In kidney transplant recipients, the pretransplant levels of HSV antibody have been used as a predictive marker for HSV excretion and serious infection posttransplant (4). It has also been suggested that some kidney transplant patients have an acute viral infection without increases in total antiviral antibody (16). Through electrophoresis of viral lysates and immunoblotting, changing levels of antibodies to individual antigens may provide serologic evidence of a current viral infection when total antiviral antibody remains unchanged (16). In these situations, when the antibody response to selected antigens is of value, purified, synthetic, or recombinant antigens could be attached to microspheres of different sizes and analyzed separately. MIA would offer increased sensitivity and the ability to quantitate antibody levels, as compared with immunoblotting. Furthermore, in vaccine trials antibody responses to specific antigens must be demonstrated to distinguish these responses from antibody responses to wild-type virus (2). The MIA may be of great value in such situations because of its increased sensitivity and ability to analyze for multiple antibodies simultaneously.

Horan and Wheelless (7) first described the potential for using microspheres as supports for flow cytometric immunoassays. Subsequently, Saunders et al. (17) described a microsphere-based flow cytometric immunoassay for the sensitive detection of horseradish peroxidase. The first publication of a clinical application for these microspheres with flow cytometry was a study of immune complexes (15). Recently, Wilson and Wotherspoon (18) described a tech-

nique that uses microspheres to capture serum or cell proteins and screen hybridomas for monoclonal antibody reactivity to these captured antigens by flow cytometry. However, to our knowledge this is the first study in which multiple-sizes of microspheres were used in simultaneous flow cytometric immunoassays. Although we used two sizes of microspheres as solid supports, it is conceivable that 5 to 10 different sizes of microspheres could be used, providing 5 to 10 simultaneous, yet discrete, assays. Evaluations of other applications of the MIA are under way.

ACKNOWLEDGMENTS

We thank Noel Warner for providing the flow cytometer used in this study and Lawrence Drew for providing the acute- and convalescent-phase CMV serum pairs.

This work was supported in part by Public Health Service grants AI-24286 and AI-23778 from the National Institutes of Health and the California State Universitywide Task Force on AIDS.

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