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Simultaneous Detection of Antibodies to Six Nonhuman-Primate Viruses by Multiplex Microbead Immunoassay

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To maintain healthy nonhuman primates for use in biomedical research, animals are routinely screened for several infectious agents at most facilities. Commonly, monkey serum samples are tested by conventional immunoassays, such as enzyme-linked immunosorbent assays (ELISAs) or Western blotting, for antibodies to specific infectious agents. For testing for antibodies against multiple agents in each sample, conventional immunoassays are laborious and time-consuming. More efficient immunoassays are needed. Accordingly, we have developed a novel multiplex serodiagnostic system based on individually identifiable, fluorescent microbead sets, where each bead set is coated with antigens from a purified preparation of a specific virus. The coated bead sets are mixed to enable the detection of antibodies to multiple viruses in one serum or plasma sample. These viruses include four agents that are routinely tested for maintenance of specific-pathogen-free monkeys, namely, simian immunodeficiency virus, simian type D retrovirus, simian T-cell lymphotropic virus, and herpes B virus, as well as simian foamy virus and rhesus cytomegalovirus, both of which are commonly found in nonhuman primates. This multiplex microbead immunoassay (MMIA) enabled the simultaneous detection of antibodies to all six viruses in single serum samples as small as 1 microliter. The results obtained by MMIA analysis correlated with results of conventional ELISAs, which detect antibodies to single agents. Thus, this multiplex microbead detection system is an efficient diagnostic modality for serosurveillance of nonhuman primates.

Nonhuman primates provide an excellent animal model for biomedical research. Most nonhuman primate housing and breeding facilities maintain a dedicated health monitoring program to provide a steady supply of healthy animals for research and preclinical studies. Animals exposed to or infected with various infectious agents may confound the results of a scientific study. Routine screening for specific-pathogen-free status is a time-consuming and tedious task. Most protocols use enzyme-linked immunosorbent assays (ELISAs), indirect immunofluorescent antibody assays (IFAs), Western blot analysis, or various combinations of these immunoassays. Although these conventional immunoassays provide important information on the exposure history of the animals to various infectious agents, the limitations include significant requirements of labor, sample volume, and time. Assay throughput is an additional limitation. With an increasing demand for nonhuman primates in research, there is a need to develop more efficient assays for screening colonies of these animals.

The multiplex system designated multiple analyte profiling (Luminex Corp., Austin, TX) enables simultaneous detection of multiple analytes in a small amount of sample (1, 4, 5, 7, 11, 13, 16). Up to 100 analytes can be measured in a single reaction. The multiplexing capabilities of multiple analyte profiling are based on individually identifiable, fluorescently coded sets of polystyrene microbeads (5.6-μm diameter) (5, 16). A specific fluorescent signature is imparted to each bead set by labeling with a specific ratio of orange and red fluorophores embedded within the matrix of each microbead set (5, 16). Uniquely labeled microbead sets are conjugated to known biomolecules and mixed. A mixture of coated bead sets is added to the test sample. Analytes in the sample react with biomolecules coating the microbeads. Interactions of sample analytes with each bead set are detected by a common reporter fluorochrome (e.g., phycoerythrin) conjugated to a secondary detection reagent. Thus, the multiplex microbead assay enables the simultaneous detection of antibodies to several infectious agents in one reaction container, resulting in a more efficient immunoassay than conventional methods such as ELISA and IFA. In addition, by virtue of its design, multiplex technology is more easily adaptable for high-throughput formats. Because several hundred microbeads coated with a particular reagent can be scanned within a few seconds, the technique allows for rapid analysis of a large number of replicates; this is an advantage over ELISA, where there are typically a small number of replicates.

The multiplex microbead immunoassay (MMIA) has been used for the detection of serum antibodies to multiple peptide epitopes (8), auto-antigens (3), bacterial antigens (14, 15), and viral antigens (12). We previously reported the development of multiplex microbead immunoassays for serodetection of *Helicobacter* species (2) and for 10 highly prevalent infectious agents in mice (6). This study describes the development of a multiplex microbead immunoassay for the detection of antibodies to six simian viruses in sera from nonhuman primates.

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MATERIALS AND METHODS

Viruses. All viruses, including simian immunodeficiency virus (SIV), simian type D retrovirus 5 (SRV-5), simian foamy virus (SFV), rhesus cytomegalovirus (RhCMV), herpesvirus papio 2 (HVP-2), and human T-cell lymphotropic virus 1 (HTLV-1), were purified by sucrose density centrifugation (Advanced Biotechnologies Inc., Columbia, MD). Purified preparations contained a total protein concentration of 1 mg/ml. HTLV-1 and HVP-2 are serologically cross-reactive to simian T-cell lymphotropic virus 1 (STLV-1) and herpes B virus (B virus), respectively (12, 17).

Antigen preparation for immunoassays. Viral antigens for coating microbeads and testing by ELISA were prepared by 1:1 mixing of the purified virus preparation with 0.5% Triton X-100 (Fisher Scientific, Fairlawn, NJ) and protease inhibitor cocktail (Roche) in phosphate-buffered saline (PBS; pH 7.2). All antigen preparations were stored frozen at −80°C. Prior to use, preparations were thawed and centrifuged in a microcentrifuge at 10,000 × *g* for 10 min to remove debris.

Monkey sera. A serum sample known to be positive only for SIV antibodies from a SIV-infected infant rhesus macaque (*Macaca mulatta*) was provided by Marta Marthas (California National Primate Research Center [CNPRC], University of California, Davis). Serum samples that were known to be positive for antibodies to RhCMV and herpes B virus were identified by ELISAs, using cells infected with RhCMV or HVP-2 as target antigens, according to published protocols (10, 12). Serum/plasma samples known to be positive for SRV, STLV, and SFV were provided from the frozen, banked archives of the Pathogen Detection Laboratory at the CNPRC. In addition, 133 blinded serum/plasma samples to be tested in parallel by the multiplex microbead immunoassay and ELISA were also provided from the frozen, banked archives of the Pathogen Detection Laboratory at the CNPRC. The archived sera were obtained from animals that were experimentally infected, immunized, or naturally infected with various viruses. Sera used as positive standards for the detection of antibodies to different viruses, including the two sera positive for antibodies to SIV and RhCMV, were confirmed to be positive for antibodies by ELISA and Western blotting. In addition, sera from three monkeys confirmed to be negative for antibodies to all six viruses by ELISA, Western blotting, and a multiplex assay were used as negative controls. The negative control sera were included in all ELISAs and multiplex assays. The majority of the animals were rhesus macaques. Other species are indicated when present.

ELISA. Several different ELISA systems were used, depending on need and availability. One system consisted of commercial ELISA kits used for SIV and STLV serodiagnostics at CNPRC. For SIV, human immunodeficiency virus type 1 and 2 synthetic peptide enzyme immunoassay kits were purchased from Bio-Rad Laboratories (Hercules, CA). For STLV, HTLV-1 and -2 enzyme immunoassay kits were obtained from bioMerieux (Durham, NC). Both of these kits were previously validated to cross-react with antibodies against SIV and STLV, respectively (9). These kits were used according to the manufacturers' instructions. For SFV and SRV antibody detection in routine serodiagnostics performed at CNPRC, purified whole viral antigens obtained from ABI (Columbia, MD) were used. Briefly, ELISA plates were prepared as follows. Immulon II microtiter plate wells were coated with viral lysates of SFV and SRV-5 at optimal protein concentrations of 2.0 μg/ml and 1.5 μg/ml, respectively. Viral antigens were diluted in coating buffer, 0.1 M sodium bicarbonate (pH 9.6). Diluted antigens were added to ELISA plate wells and incubated overnight at 37°C. After being coated, the plates were washed and blocked. For antibody detection, sera were diluted, 1:100 for SFV and 1:50 for SRV, in Tris-buffered saline (pH 7.2) containing 1% bovine serum albumin (BSA) and added to the plates. Plates were incubated for 1 hour at 37°C. Wells were washed, and the secondary antibody, a goat anti-monkey antibody conjugated to horseradish peroxidase (ICN Biomedical, Irvine, CA), was added at a dilution of 1:1,000. Plates were incubated for 30 min at 37°C. After the washing step, ABTS [2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid)] chromogen (Kirkegaard & Perry, Gaithersburg, MD) was added and incubated for 10 min at room temperature. The reaction was stopped by adding an equal volume of 1% sodium dodecyl sulfate. Color development was read at 405 nm, measured as optical density (OD) (Sunrise absorbance reader; Tecan, Research Triangle Park, NC).

ELISAs for the detection of antibodies to RhCMV and B virus were performed according to previously published protocols (10, 12). Briefly, Immulon 4 plates were coated with 1 μg of protein/well of extracts of cells that exhibited 100% cytopathic effect following infection with RhCMV strain 68-1 or HVP-2, respectively. Serum/plasma samples were analyzed at a 1:100 dilution for 2 hours. Following six washes in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), a secondary antibody, horseradish peroxidase-conjugated goat anti-monkey immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), was added for 1 hour. The optimal secondary concentration was empirically determined for each antigen and each lot of secondary antibody. After washing of the plates with PBS-T, the 3,3′,5,5′-tetramethylbenzidine substrate (Sigma Chemicals, St. Louis, MO) was added for 30 min, and the reaction was terminated by the addition of 50 μl of 1 M sulfuric acid. The absorbance at 450 nm was measured in a microplate reader (Bio-Rad).

To test the specificity of antigens used to coat microbeads in the multiplex immunoassay, ELISA was performed on all viral antigens, as previously described (2, 6). Briefly, Immulon 4 HBX flat-bottomed microtiter plates (Thermo Labsystems, Franklin, MA) were coated with viral lysates at the following optimal protein concentrations: SIV, 0.10 μg/ml; HTLV-1, 0.10 μg/ml; SRV, 0.50 μg/ml; HVP-2, 0.30 μg/ml; SFV, 0.30 μg/ml; and RhCMV, 0.30 μg/ml. Monkey sera positive for the relevant antibodies and the negative control sera were used to test these antigens. After being blocked, the plates were washed, and 100 μl of serum diluted 1:200 in 5% nonfat milk prepared in PBS-T was added. The detection antibody, biotinylated goat anti-human IgG (Kirkegaard & Perry), was diluted 1:10,000 in BLOTTO and added to ELISA plates (100 μl/well). Plates were incubated with 100 μl/well of Vectastain ABC detection reagent containing horseradish peroxidase H (Vector Laboratories, Burlingame, CA), washed, and developed with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine color development reagent (Sigma Chemicals, St. Louis, MO).

The above ELISA methods are qualitative assays for determination of the presence or absence of antibodies against certain viruses in serum or plasma. In Tables 1 and 4, the ELISA data are shown as arbitrary numerical values based on the relative strength of OD values, as described in the tables. The data are not meant to represent quantitative antibody levels. The numbers merely reflect the relative OD produced by each sample in the individual ELISA tests.

Coupling proteins to Luminex microbeads. Microbeads were purchased from Luminex Corp. (Austin, TX). Various antigen preparations were chemically cross-linked to the microbeads according to the manufacturer's instructions. The bead stock was resuspended by vortexing and treatment in a sonicator bath (15 to 30 s) (model 1510; Branson, Danbury, CT). An aliquot of 2.5×10^6 beads was removed and centrifuged at 21,000 × *g* for 2 min. Beads were resuspended in 80 μl of activation buffer (100 mM monobasic sodium phosphate; pH 6.3) by vortexing and sonication (15 to 30 s).

To activate the beads for cross-linking to proteins, 10 μl of 50-mg/ml sulfo-*N*-hydroxysulfosuccinamide (NHS; Pierce, Rockford, IL) was added, and the beads were mixed by vortexing. Next, 10 μl of 50-mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC; Pierce, Rockford, IL) was added, and the beads were mixed again by vortexing. All incubations of beads were performed in the dark. The bead mixture was shaken on a rotary shaker at room temperature for 20 min and then centrifuged at 21,000 × *g* for 2 min. Beads were washed twice with 250 μl of 50 mM morpholineethanesulfonic acid (pH 6.0) buffer. To coat the beads with antigens, pelleted beads were resuspended in the relevant antigen preparation diluted in 50 mM morpholineethanesulfonic acid (pH 6.0) buffer. Optimization of the viral antigen concentration was performed by coating different microbead sets with a range of protein concentrations, from 10 to 200 μg/ml, of each antigen. The microbead sets were then used to test standard monkey sera which were positive for antibodies to the relevant infectious agents. The microbead set that provided the strongest specific signal for each antigen against the positive monkey sera was selected. The following total protein concentrations for purified viruses for coating microbeads were used: 25 μg/ml for SFV; 100 μg/ml for SIV, HVP-2, and RhCMV; and 250 μg/ml for SRV and HTLV.

Bead sets were also coated with biotin-conjugated goat IgG (100 μg/ml), a positive control protein for reaction with streptavidin conjugated to R-phycoerythrin, and with BSA (100 μg/ml) as a negative control protein (Pierce, Rockford, IL). For coupling, mixtures of activated beads and proteins were incubated by shaking on a rocker for 2 h at room temperature. After being coated with proteins, beads were washed twice with 250 μl wash buffer (0.1% Tween 20 in PBS [pH 7.4]), resuspended in 250 μl of blocking buffer (1% BSA, 0.1% Tween 20 in PBS, pH 7.4, 0.05% sodium azide), and shaken on a rocker at room temperature for 30 min. Microbeads coated with viral proteins were resuspended in 1 ml blocking buffer and stored at 4°C for up to a week. For long-term storage, beads were kept frozen at −70°C for several months.

Multiplex detection of antibodies in monkey serum. Immunoreactions were performed in 96-well, filter-bottomed plates designed for high-throughput separations (1.2-μm MultiScreen; Millipore Corporation, Bedford, MA). Typically, 2,000 beads from each individual bead set, coated with either specific viral antigens or a control protein, were added per well. For example, for an eight-plex assay, 2,000 microbeads from each protein-coated bead set were mixed to provide a total of 16,000 beads per well. Monkey serum was diluted 1:200 in 5% BLOTTO (Pierce, Rockford, IL), and 50 μl of this diluted serum was mixed with

TABLE 1. Antiviral antibody detection in monkey sera by ELISA

Sample[a]	Dilution	ELISA signal for virus[b]					
		SIV	SRV	HTLV	SFV	RhCMV	HVP-2
SIV$^+$ serum	1:10	5					
SRV$^+$ serum*	1:50		2		1		
STLV$^+$ serum	1:5			3			
SFV$^+$ serum*	1:100				3		
RhCMV$^+$ serum	1:100					5	
B virus$^+$ serum	1:100				3	2	5

[a] *, serum samples from *Macaca fascicularis*. All other serum samples were from *M. mulatta*.

[b] Results do not represent quantitative antibody levels. The numbers reflect relative signals in individual ELISA assays and are arbitrarily designated as follows: 1, OD units up to 0.2 above background; 2, 0.2 to 0.4 above background; 3, 0.4 to 0.8 above background; 4, 0.8 to 1.6 above background; and 5, >1.6 above background.

the six-plex bead mixture per well. The mixture of beads and serum was then incubated on a shaker for 1 h at room temperature. After incubation, liquid was drained from the bottom of the plate in a vacuum manifold designed to hold 96-well plates (Millipore Corporation, Bedford, MA). The beads were washed two times by adding 100 μl of wash buffer per well and draining under vacuum. For detection of monkey IgG, phycoerythrin-conjugated goat anti-human IgG (CalTag, Burlingame, CA) was diluted 1:1,000 in wash buffer, and 100 μl was added to each well. Beads were mixed as described above and incubated at room temperature for 30 min. Beads were washed once with wash buffer, resuspended in 100 μl of wash buffer per well, and analyzed in a Luminex-100 instrument equipped with an XY-Platform for automatic reading of 96-well plates.

Luminex-100 cytometer operation and data analysis. The Luminex-100 instrument was manufactured by Luminex Corp. and purchased from Upstate USA (Lake Placid, NY). The instrument was used at default settings, set by the manufacturer for routine applications, as directed by the user's manual. Data were acquired by the Luminex data collection software (version 1.7). This software package allows routine operation of the instrument and data acquisition. Calibration beads supplied by the manufacturer were used to adjust the instrument settings for bead set identification and for the detection of the reporter (phycoerythrin). Events were gated to exclude doublets and other aggregates. A minimum of 100 independent, gated events were acquired for each bead set. The ratio of the median fluorescence intensity (MFI) of antigen-coated bead sets to the MFI of BSA-coated beads in each reaction was calculated. This ratio, designated the signal, was used as a measure of antibody detection. After acquisition by Luminex software, the data were further processed by Microsoft Excel software. The background, or normal serum reactivity, for each antigen-coated bead set was determined as follows. For each bead set, the average of the signals from duplicate wells of three normal monkey sera was calculated, and three times the standard deviation value was added to the average. For routine analysis, each experiment also contained a positive control serum for all of the infectious agents. Samples were analyzed in duplicate, and a minimum of two separate multiplex immunoassay experiments were performed. Sera were designated positive for antibodies to an infectious agent if the signal from the relevant antigen-coated bead set was greater than the background.

RESULTS

Antibody analysis of monkey sera by ELISA. To obtain animal samples positive for antibodies against only one infectious agent, sera from approximately 200 monkeys were analyzed. Animal sera that were confirmed by ELISA to be positive for antibodies to a single virus (SIV, STLV, SFV, or RhCMV) were selected (Table 1). One serum sample which was positive for antibodies to SRV but contained low levels of antibodies to SFV was selected as an SRV-positive serum (Table 1). However, a serum sample(s) positive for antibodies to B virus only or with low levels of antibodies to another virus could not be obtained. Therefore, a serum sample that was positive for antibodies to B virus but also positive for antibodies to SFV and RhCMV was selected (Table 1) for the multiplex serodetection assay described below.

Antibody analysis of singly positive monkey sera by multiplex microbead immunoassay. Data from the multiplex microbead analysis of serum samples selected on the basis of ELISA analysis are presented in Table 2; the data are formatted to show interassay variation (i.e., standard deviations). MMIA was performed by incubating each sample with a mixture of six bead sets in one reaction well. Each of the six bead sets was coated with a specific viral antigen preparation (SIV, SRV, STLV, RhCMV, B virus, or SFV) for the simultaneous detection of antibodies to all six viruses. The data demonstrate that bead sets coated with various antigens were able to detect the relevant antibodies in these sera. Importantly, the bead sets displayed high degrees of specificity in their reactivities; sera positive for antibodies to SIV, STLV, SFV, and RhCMV displayed reactivities only to the relevant bead sets. The serum sample positive for antibodies to SRV also displayed low-level reactivity to beads coated with SFV. Because this serum sample was also found to be positive for antibodies to SFV by ELISA (Table 1), results from the multiplex microbead assay do not indicate that SFV-coated beads were cross-reactive to SRV antibodies. As expected from the results of ELISA analysis (Table 1), the serum sample positive for antibodies to B virus reacted strongly to beads coated with SFV and RhCMV. Again, because this serum was shown by ELISA analysis to contain strong antibodies to SFV and RhCMV, the results of the multiplex microbead assay do not indicate that there is a cross-reactivity of SFV- and RhCMV-coated bead sets to B virus antibodies.

TABLE 2. Antiviral antibody detection in monkey sera by multiplex microbead immunoassay

Sample[a]	Dilution	MFI ratio of antigen-coated beads to BSA-coated beads[b]					
		SIV	SRV	HTLV	SFV	RhCMV	HVP-2
Background		0.34	0.56	0.11	0.16	1.46	0.21
SIV	1:200	333 ± 45	0	0	0	1	0
SRV*	1:200	0	9 ± 2	0	1	0	0
STLV	1:200	0	0	57 ± 3	0	0	0
SFV*	1:200	0	1	0	44 ± 2	0	1
RhCMV	1:200	0	0	0	0	230 ± 38	0
B virus	1:200	0	0	0	121 ± 6	35 ± 4	196 ± 19

[a] *, serum samples from *M. fascicularis*; all other serum samples were from *M. mulatta.*

[b] Data are medians ± standard deviations. The MFI ratios do not represent quantitative antibody levels; they reflect relative signals obtained for serum sample reactions to the individual antigen-coated bead sets.

TABLE 3. Multiplex microbead immunoassay of five mixed monkey sera positive for antibodies to multiple viruses

Sample	Dilution[a]	MFI ratio of antigen-coated beads to BSA-coated beads[b]				
		SIV	SRV	HTLV	SFV	RhCMV
Background		0.34	0.56	0.11	0.16	1.46
All sera mixed	1:200	215 ± 22	4 ± 1	21 ± 5	18 ± 4	102 ± 17
	1:1,000	303 ± 12	2 ± 0	10 ± 1	10 ± 1	60 ± 4
Minus SIV-positive serum	1:200	0	8 ± 0	50 ± 1	41 ± 2	182 ± 0
	1:1,000	0	3 ± 0	19 ± 2	20 ± 2	91 ± 7
Minus SRV-positive serum	1:200	222 ± 20	0	21 ± 3	18 ± 3	104 ± 13
	1:1,000	304 ± 9	0	10 ± 0	11 ± 1	58 ± 2
Minus STLV-positive serum	1:200	268 ± 21	5 ± 1	0	23 ± 1	136 ± 9
	1:1,000	302 ± 0	2 ± 0	0	11 ± 0	60 ± 0
Minus SFV-positive serum	1:200	252 ± 11	4 ± 0	26 ± 3	1 ± 0	122 ± 11
	1:1,000	299 ± 17	2 ± 0	9 ± 1	0	58 ± 5
Minus RhCMV-positive serum	1:200	263 ± 9	5 ± 0	29 ± 2	22 ± 1	0
	1:1,000	325 ± 1	3 ± 0	12 ± 1	13 ± 0	1 ± 0

[a] Serum dilutions reflect the final dilution of each serum in the mixture.
[b] Results are presented as described in Table 2.

Analysis of mixed singly positive monkey sera by multiplex microbead immunoassay. To further test the specificity and sensitivity of the multiplex microbead assay, singly positive monkey sera, as described in Tables 1 and 2, were mixed in equal proportions. Serum mixtures were prepared so that one positive serum was omitted from each mixture, as shown in Table 3; the data are formatted to show interassay variation (i.e., standard deviations). The serum positive for B virus was not used in this analysis because this serum sample also contained antibodies to SFV and RhCMV. Thus, a total of five serum mixtures were prepared. The mixed sera were tested in two dilutions such that the final dilution of each individual serum in the mixture was either 1:200 or 1:1,000. MMIA was performed to simultaneously detect antibodies to all six viruses in one reaction well, as described above. No reaction to the specific antigen-coated beads was observed in serum mixtures missing the relevant antibodies. For example, SIV-coated beads showed no reaction to "serum mixture 1," which was missing the SIV-positive serum. Yet serum mixture 1 reacted positively to all other bead sets. These results show that the antigen-coated microbeads are specific to the relevant antibodies, even when antibodies against multiple viruses are present in the same sample. In general, the specific antibody signals in the multiplex immunoassay were lower (up to 50%) for all the sera in various mixtures than for single sera, despite the fact that the final dilutions of each serum in the two experiments were the same (compare Table 2 to Table 3). This observation suggests that there may be negative interference with specific reactions when sera from several animals are mixed. However, the specific signal for each virus was clearly detectable, even at a 1:1,000 dilution of serum samples.

Comparison of multiplex microbead immunoassay and ELISA for detection of antibodies in monkey sera. Blinded serum/plasma samples from 133 monkeys were tested in parallel by the multiplex immunoassay and ELISA. Sera were diluted as described in Tables 1 and 2. MMIA was performed to simultaneously detect antibodies to all six viruses in one reaction well, as described above. ELISA and MMIA results for serodetection of the six viruses were identical for 109 of 133 serum samples (Table 4). The discordant results were all positive for antibody detection by the multiplex microbead assay and negative by ELISA, suggesting that MMIA may be a more sensitive immunoassay format than ELISA. For 23 of 24 samples where results from the two methods did not agree, the disparity in each sample was for antibodies to only one of the six viruses. In most of these disparate cases (10 samples), MMIA detected SRV antibodies while ELISA did not. This apparently higher sensitivity displayed by MMIA than that by ELISA is probably due to greater accessibility of the epitopes on the microbeads (see Discussion).

DISCUSSION

This report describes a multiplex microbead assay where uniquely fluorescent beads coated with different purified antigens (viruses) are mixed together in a single container and incubated with animal sera to detect antibodies to six simian viruses simultaneously. To test the performance of conventional immunoassays such as ELISA, Western blotting, and IFA, it is not important to use sera that are positive for antibodies to only a single infectious agent. Serum samples positive for antibodies to multiple agents can be used because they are tested for one specific agent at a time. In contrast, to establish the specificity and sensitivity of a multiplex assay, the availability of individual serum samples positive for antibodies against one agent alone is critical. However, such singly positive monkey sera are not commonly available. B virus, RhCMV, and SFV are endemic in most breeding colonies, so animals are usually infected with these viruses. Consequently, for this study approximately 200 animal sera were screened by ELISA to select monkeys that were positive for antibodies to a single virus (data not shown). Sera that were positive only for SIV, STLV, SFV, or RhCMV were identified. One serum sample that was positive for SRV but contained low-level antibodies against SFV was also obtained. However, serum samples positive for antibodies to B virus only could not be found. Accordingly, one serum sample which was strongly positive for B virus and positive for the least number of other viruses (i.e., SFV and RhCMV) was identified for use in the analysis (Table 1).

All viral antigens used to coat the microbeads were initially tested by ELISA. Testing of antigen preparations by ELISA was performed using known positive monkey sera containing

TABLE 4. Detection of serum antibodies by ELISA and MMIA

Sample no.[a]	Test signal for indicated virus[b]											
	SIV		SRV		STLV		RhCMV		B virus		SFV	
	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA
2L27093			1	2	1	2	4	4		Ind	2	3
2L27094		3	Ind	1		Ind	4	4		1	2	4
2L27095							4	2			2	2
2L27096			1	2		Ind	4	4	5	2	1	2
2L27097				Ind	1	4	4	4	5	4	2	4
2L27098	5	4					4	4	3	1	1	2
2L27100			1	1		Ind	4	3	4	2	1	3
2L27101		1					4	3	4	2	1	2
2L27102							4	4	4	2	2	4
2L27103							4	5	1	1	2	4
2L27104							4	4	4	2	2	4
2L27105					Ind		4	2			2	2
2L27107						1	3	3	4	2	2	4
2L27108				Ind		1	4	4	5	2	2	4
2L27109			1	1	2	3	4	5	5	3	2	4
2L27110				1			3	2	5	3	2	3
2L27111							3	3	5	3	1	3
2L27112						5	4	3	5	3	2	3
2L27113					1	1	4	4	4	2	2	4
2L27114				1			4	3	5	3	2	4
2L27115					2	5	4	4	5	3	2	4
2L27116							4	4	5	3	2	4
2L27118							4	4	4	2	2	3
2L27119					3	4	4	4	5	3	2	3
2L27120			1	1			4	4	4	2	2	3
2L27121		Ind		1			4	4	5	3	2	4
2L27122							4	4				
2L27123							4	4			2	3
2L27124												
2L27125							4	4	4	2	2	4
2L27126						Ind	4	2			2	4
2L27127							3	3			2	3
2L27128			3	1			3	3		1	2	4
2L27131						Ind	3	3		1	2	3
2L27132				1			4	4			2	3
2L27133							4	4	Ind		2	3
2L27134			Ind				4	4			2	3
2L27135				1			3	3			2	4
2L27136							4	4			Ind	2
2L27137							4	4				1
2L27138							4	3				
2L27139							4	4			1	2
2L27140							4	3				1
2L28222												
2L28223												
2L28224												
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2L28226												
2L28227												
2L28228			1									
2L28229												
2L28230												
2L28231												
2L28232												
2L28233												
2L28234			Ind									
2L28235												
2L28236												
2L28237												
2L28238												
2L28239												
2L28240												
2L28241												
2L28242			Ind									
2L28243												
2L28244												
2L28245												
2L28278			1	2	3	4	4	3	4	2	1	2
2L28279			Ind				3	3	5	3	2	3
2L28280							3	2	3	2	2	4
2L28281							4	3			1	3
2L28282							4	3	1		1	3
2L28283					2	4	4	3	4	2	2	3

Continued on following page

TABLE 4—*Continued*

Sample no.[a]	Test signal for indicated virus[b]											
	SIV		SRV		STLV		RhCMV		B virus		SFV	
	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA	ELISA	MMIA
2L28284			Ind		Ind	1	4	4	5	3	2	3
2L28285			Ind				4	3	4	3	2	3
2L28286					1	2	4	3	4	2	2	3
2L28287							3	2	5	3	1	3
2L28288							4	4	5	1	2	3
2L28289					3	3	4	2	4	2	2	2
2L28290					Ind	4	4	4			1	2
2L28291				1			4	2	4	2	2	3
2L28292					2	3	4	3	4	2	2	3
2L28293					1	3	4	2	4	2	2	3
2L28294							4	3	4	1	2	2
2L28295							3	3	4	2	2	3
2L28296							4	2	4	3	2	3
2L28297												
2L28298							4	3	4	3	2	3
2L28299					1	4	4	3	4	2	2	2
2L28300							4	3	5	2	2	3
2L28301							4	3	4	2	2	3
2L28302							4	3	4	2	2	3
2L28303							4	3	5	2	2	3
2L28304					2	4	4	3	4	2	2	2
2L28305				1			3	1	4	2	2	3
2L28306					1	2	4	3	4	2	2	3
2L28307							4	3	5	3	2	3
2L28308							3	2	4	2	2	3
2L28548							4	5			2	3
2L28549							4	4				
2L28550							4	3			1	3
2L28551							4	3				
2L28552							4	4		1		2
2L28553							4	4				
2L28554							4	4				
2L28555							3	3			1	3
2L28556							3	3				
2L28557							4	4		1		
2L28558							4	4			1	3
2L28559							4	4			1	2
2L28560							4	4				
2L28561							4	3			2	2
2L28562							4	4				
2L28563							4	4				
2L28564				1			4	4				
2L28565							4	3				
2L28566							4	3				
2L28567							4	4				
2L28568							4	4			1	2
2L28569							4	4				
2L28570							3	4				
2L28571							4	4				
2L28572							4	4				
2L28573							3	2				
2L28574							4	3			1	3
2L28575				1			4	4			1	3
2L28576							3	4				
2L28577							4	3			1	2
2L28578							4	3				
2L28579							4	4				
2L28580							4	4				
2L28581				1			3	3				
2L28582							4	3				

[a] Animal samples 2L28548 to 2L28582 were from *M. fascicularis*. All other samples were from *M. mulatta*.

[b] For ELISA, serum samples were diluted as described in Materials and Methods. Results do not represent quantitative antibody levels. The numbers reflect relative signals in individual ELISAs and are arbitrarily designated as follows: Ind, 0.025 OD unit above background; 1, up to 0.2 unit above background; 2, 0.2 to 0.4 unit above background; 3, 0.4 to 0.8 unit above background; 4, 0.8 to 1.6 units above background; 5, >1.6 units above background. For MMIA, all sera were diluted 1:200 as described in Materials and Methods. Results do not represent quantitative antibody levels. The numbers reflect relative signals in the multiplex microbead immunoassay and are arbitrarily designated as follows: Ind, up to 1 value above background; 1, 1 to 5 values above background; 2, 5 to 25 values above background; 3, 25 to 100 values above background; 4, 100 to 400 values above background; 5, >400 values above background.

antibodies to different viruses as well as sera that were negative for antibodies to all six viruses. Purified viruses that performed well as antigens in ELISAs also performed well in the multiplex microbead assay. However, crude infected cell lysates of B virus and RhCMV performed variably in these two methods. Both infected cell lysates performed well in ELISAs but produced very weak signals in multiplex microbead immunoassays using monkey sera strongly positive for the relevant antibodies

(data not shown). We have previously shown that crude infected cell extracts that work well in ELISA do not perform well in a multiplex microbead immunoassay for the detection of *Helicobacter bilis* antibodies in mouse serum (10). Similar results were also obtained with crude infected cell lysates of several mouse viruses (unpublished data). These findings suggest that the presentation of antigenic epitopes on microbeads is different from that in ELISA. Due to the larger available surface area in ELISA wells, the concentration of antigenic molecules may not be limiting. However, due to the smaller surface area of microbeads, the availability of the relevant antigenic molecules to react with serum antibodies may be limiting. Accordingly, all the viral antigens selected for use in this study were obtained by sucrose density gradient purification of whole viruses. The use of purified viruses may have additionally contributed to the specificity of the multiplex microbead assay.

For the detection of antibodies to STLV and B virus, immunologically cross-reactive viruses, i.e., HTLV and HPV-2, respectively, were used (12). HTLV is readily available. HPV-2, unlike B virus, is nonpathogenic to humans and therefore safer to handle under standard laboratory conditions. The specificity of HTLV for the detection of anti-STLV antibodies was demonstrated by the use of singly positive sera (Tables 2 and 3). Because sera positive for antibodies against B virus only were not available, the data presented in this study do not demonstrate the specificity of HVP-2-coated beads as clearly as that for the other five viral antigen-coated bead sets. Nevertheless, specificity could be inferred from two observations: (i) there was a lack of reaction of the B virus bead set to sera positive for antibodies to SIV, SRV, or STLV, and (ii) the B virus antibody-positive serum used for the data in Tables 1 and 2 was confirmed to be positive for antibodies to both SFV and RhCMV, suggesting that the reaction of SFV- and RhCMV-coated beads to this serum was specific and not due to cross-reactivity.

It is noteworthy that the multiplex microbead assay was not only specific for the detection of antiviral antibodies but was also highly sensitive. We have previously shown that the multiplex microbead format is at least as sensitive as ELISA for the detection of antibodies to *H. bilis* and *Helicobacter hepaticus* (2) and to 10 highly prevalent mouse infectious agents (6) in mouse sera. In this study, the multiplex microbead format readily detected antibodies to all six viruses at a 1:200 dilution (Table 2). Moreover, the relevant antibodies in the mixed monkey sera were detected up to a dilution of 1:1,000 (Table 3). This high level of sensitivity of the multiplex microbead immunoassay was achieved despite the observed negative interference. This interference occurred after mixing of sera from multiple animals. As shown in Table 3, the presence of SIV antibody-positive serum in the serum mixture appears to have contributed the most interference. However, the data obtained in this study do not explain whether such interference is related to the presence of SIV antibodies or if interference is related to the particular monkey serum. We have previously reported a similar negative interference in mixed, singly positive mouse sera where antibodies to 10 infectious agents were detected simultaneously by a multiplex microbead immunoassay (6). Nevertheless, the multiplex assay for the serodetection of mouse infectious agents was highly sensitive. Thus, the multiplex microbead assay is well suited to detect antibodies to several infectious agents in a highly sensitive and specific manner.

TABLE 5. Comparison of serodetection of viruses in monkey sera by ELISA and MMIA

Virus	ELISA result		MMIA result	
	No. of positive samples/total	(% Positive)	No. of positive samples/total	(% Positive)
SIV	1/133	0.75	3/133	2.2
SRV	8/133	6.0	18/133	13.5
STLV	15/133	11.3	20/133	15.0
SFV	77/133	57.9	81/133	60.9
RhCMV	107/133	80.5	107/133	80.5
B virus	51/133	38.3	55/133	41.3

A summary of the serodetection results for 133 monkey serum samples is presented in Table 5 to show a comparison between ELISA and the multiplex microbead immunoassay. The multiplex microbead immunoassay displayed positive results in a few more samples than did ELISA. The exception was the detection of antibodies to RhCMV, where the same number of sera were found to be antibody positive by the two serodetection methods. The microbead immunoassay may be more sensitive than ELISA; this could be due to differences in the attachment of viral proteins to microbeads (chemical linkage) compared to that in ELISA wells (adsorption). When attached to microbeads, epitopes are likely to be more readily accessible to serum or plasma antibodies, as they are probably presented in a three-dimensional form. In contrast, in ELISA wells, epitopes may be occluded due to adsorption of antigens. Additional studies with large numbers of field serum samples are needed for full validation of the multiplex immunoassay for routine serosurveillance of monkeys. Such studies will also be important for developing an efficient high-throughput format as well as evaluating recombinant viral antigens and synthetic viral peptides. Validation studies will involve analyses of several hundred field serum samples, further optimization of antigen attachment and assay conditions, and correlation with other immunoassay formats. We propose that the multiplex microbead immunoassay be designated by the acronym MMIA.

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