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Detection of Semliki Forest Virus in Cell Culture by Use of an Enzyme Immunoassay with Peroxidase-Labeled Monoclonal Antibodies Specific for Glycoproteins E1 and E2

FRANK H. VAN TIEL,* WIL A. M. BOERE, JAN VINJÉ, THEO HARMSEN, BARRY J. BENAISSA-TROUW, CORNELIS A. KRAAIJEVELD, AND HARM SNIPPE

Department of Virology, Laboratory of Microbiology, State University of Utrecht, 3511 GG Utrecht, The Netherlands

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Four noncompeting monoclonal antibodies (MA) directed against either the E1 (UM 8.64 and 8.139) or E2 (UM 8.55 and 8.73) glycoprotein of Semliki Forest virus were purified and labeled with horseradish peroxidase. Each enzyme-labeled MA was tested alone and in combination with others for its sensitivity to detect virus-infected cells. Semliki Forest virus-infected L cells seeded as monolayers in 96-well plates were screened for the virus after incubation with enzyme-labeled MA and a substrate. In this system single enzyme-labeled MA even at high dilution ($10^{3.0}$ to $10^{4.5}$) were able to detect virus-infected cells. The sensitivity of the test could be enhanced by combining two noncompeting MA ($10^{4.5}$ to $10^{5.0}$). Combinations of three and four MA were less effective, due to high absorbance values for noninfected cells. The threshold of virus detection was between 10^5 and 10^6 PFU/ml. This test is sensitive and specific and therefore may be useful for diagnostic purposes.

Monoclonal antibodies (MA) have great promise as reagents in the diagnostic laboratory (13, 16). One application of MA is in the detection or identification of specific viral antigens in clinical specimens, directly or after sufficient replication in permissive cells (4). In most studies so far, MA served as primary antibodies in indirect immunofluorescence staining or immunoassay with heterologous anti-mouse immunoglobulin G (IgG) labeled with fluorescent molecules or with enzymes, e.g., alkaline phosphatase or peroxidase (5, 12). A less versatile, but basically very convenient, test is the direct detection of viral antigens with virus-specific enzyme-labeled MA (9, 14). In this study we have explored some aspects of this test with horseradish peroxidase (HRPO)-labeled MA directed to either the E1 or E2 glycoprotein of Semliki Forest virus (SFV), belonging to the alphavirus group of togaviridae. In other reports (2; W. A. M. Boere, T. Harmsen, J. Vinjé, B. J. Benaissa-Trouw, C. A. Kraaijeveld, and H. Snippe, submitted for publication) we described the production, purification, and enzyme labeling of these MA, with which we defined several epitopes on the surface of SFV. This enabled us to select more rationally noncompeting HRPO-labeled MA and their mixtures to improve the sensitivity of viral antigen detection.

MATERIALS AND METHODS

Virus strain. The avirulent SFV strain MRSMP192/7 (6) was received from K. G. Oei (Royal Tropical Institute, Amsterdam, The Netherlands). SFV was inoculated at a multiplicity of infection (MOI) of about 1 on monolayers of L cells in Roux flasks. After 20 h at 37°C the fluid was collected and centrifuged at low speed. The virus in the supernatant fluid had a titer of 2×10^9 PFU/ml and was stored in small portions at −20°C. Plaque titration and other general virological methods have been described previously (8).

Cells and media. L cells, a continuous line of mouse fibroblasts, grown in Yamane medium with 0.01 M *N*-2-hydroxyethyl-piperazine-*N'*-ethanesulfonic acid buffer supplemented with 10% calf serum and antibiotics, were used throughout this study. The complete growth medium was also used in diluting infectious virus or cells to the required concentrations.

MA. The production, purification, and further characterization (isotype, biological activities) of MA against the E1 and E2 glycoproteins of SFV are described in other papers (2; Boere et al., submitted for publication). In brief, myeloma cells, P3-X63-Ag8 (P3), were fused with spleen cells from BALB/c mice immunized with avirulent SFV. Clones secreting SFV antibodies were injected into pristane-primed BALB/c mice. MA from mouse ascitic fluids were purified with protein A-Sepharose columns (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The purified MA were all concentrated by filtration (XM 50; nominal molecular weight cutoff, 50,000; Amicon Corp., Danvers, Mass.) to 4 mg of protein per ml.

Conjugation of MA with HRPO. HRPO was conjugated with the anti-E1 and anti-E2 MA used in this study by the periodate method (11). All undiluted, conjugated MA contained the same amount of protein.

Enzyme immunoassay with HRPO-conjugated MA against purified SFV. SFV-specific conjugates were tested for their reactivity against purified UV-inactivated SFV in an enzyme immunoassay. Wells were coated with virus (0.2 to 0.5 μg per well) overnight at 4°C. After washing with phosphate-buffered saline containing 0.05% Tween 20, the plates were incubated with HRPO-conjugated MA for 1 h at 37°C. After washing, the amount of bound HRPO was visualized by incubating the wells with 100 μl of a solution of 3',3',5',5'-tetramethylbenzidine (Sigma Chemical Co., St. Louis, Mo.; 60 mg dissolved in 10 ml of dimethyl sulfoxide) and 100 μl of ureumperoxide (Organon Teknika, Boxtel, The Netherlands; 1 tablet of ureumperoxide dissolved in 7.5 ml of distilled water) in 0.11 M sodium acetate adjusted to pH 5.5 with saturated citric acid. After 10 min, the enzyme reaction was terminated by adding 100 μl of 2 M H_2SO_4 per well, and peroxidase activity was quantified by measuring the optical density at 450 nm with a Titertek Multiskan (Flow Laboratories, Scotland).

* Corresponding author.

Detection of SFV antigens in cell culture with HRPO-conjugated E1- and E2-specific MA. Avirulent SFV was diluted in growth medium and added in 0.025-ml volumes to wells of a flat-bottomed 96-well plate (catalog no. 3596; Costar Plastics, Cambridge, Mass.). In control wells only the dilution fluid was pipetted. To each well 5×10^4 L cells in 0.05-ml volumes were added to form monolayers. The plates with infected and control cells were incubated at 37°C. After selected time intervals the supernatant fluids were discarded, and the cells were fixed by the addition of 0.05 ml of 0.05% glutaraldehyde (E. Merck AG, Darmstadt, Federal Republic of Germany) and incubated for 10 min at 37°C. The plates were washed with tap water, rinsed with phosphate-buffered saline (pH 7.2), and finally shaken dry. HRPO-conjugated MA diluted in phosphate-buffered saline containing 4% calf serum and 0.5% Tween 20 were added in 0.05-ml volumes and incubated for 1 h at 37°C. The plates were washed three times with phosphate-buffered saline and shaken dry. The substrate for HRPO detection (tetramethylbenzidine-ureumperoxide) was added in 0.05-ml quantities and incubated for 30 min at room temperature. The reaction was stopped with 0.05 ml of 2 M H_2SO_4. Absorbance values were read at 450 nm in a Flow Laboratories Titertek Multiscan photometer.

Positive/negative (P/N) ratios were obtained by dividing the extinction value for infected cells by the corresponding extinction value measured for noninfected (control) cells. If the extinction value for control cells was below 0.100, the P/N ratio was arbitrarily calculated by division with an extinction value of 0.100. A P/N ratio of 2.0 or more was considered to be indicative of the presence of specific antigen. All extinction values shown are the means of duplicates.

RESULTS

Comparison of various HRPO-labeled MA as primary antibodies in enzyme immunoassay. Twelve HRPO-conjugated MA, directed to either the E1 or E2 glycoprotein of SFV, were tested for reactivity in enzyme immunoassays with both purified SFV and SFV-infected L cells. The results (Table 1) revealed that MA UM 8.4.2, 8.20, 8.77, 8.55.2, and 8.73.3 resulted in similar absorbance values when tested against purified SFV and against SFV-infected L cells. As for MA UM 8.115, 8.139, 8.64, 5.1, and 4.2, absorbance values for purified SFV surpassed those for SFV-infected L cells. Low absorbance values were measured for noninfected control cells and were reflected in the calculated P/N ratios. Low ratios indicate low avidity and high ratios indicate high avidity of the investigated conjugate. On the basis of their high P/N ratios, the following four conjugates were selected for further study: UM 8.139 and 8.64, directed against the E1 glycoprotein of SFV; UM 8.55.2 and 8.73.3, directed against the E2 glycoprotein. In another study we showed that the selected MA were not competing for epitopes on purified SFV (Boere et al., submitted for publication).

TABLE 1. Comparison of reactivity in enzyme immunoassays of various HRPO-labeled MA with specificity for either the E1 or E2 glycoprotein against both purified SFV and SFV-infected L cells

Clone no.	Class of antibody	Specificity	Reactivity (absorbance at 450 nm) against the following:			P/N ratio
			Purified SFV[a]	Noninfected L cells	SFV-infected L cells[b]	
8.4.2	IgM	E1	0.020	0.028	0.210	2.1
8.115	IgG2a	E1	1.125	0.146	0.436	3.0
8.20	IgG2a	E1	0.440	0.036	0.447	4.5
8.47	IgG2a	E1	ND[c]	0.041	0.455	4.5
8.28.3	IgG2a	E1	ND	0.116	0.620	5.3
8.139	IgG2a	E1	1.500	0.052	0.731	7.3
8.64	IgG1	E1	1.320	0.016	0.955	9.5
8.77	IgG2b	E2	0.140	0.015	0.160	1.6
5.1	IgG2a	E2	0.920	0.132	0.530	4.0
8.55.2	IgG2a	E2	1.360	0.144	1.029	7.1
4.2	IgG2a	E2	1.380	0.108	0.806	7.5
8.73.3	IgG1	E2	1.390	0.122	1.135	9.3

[a] Purified SFV was diluted 1/400, and the HRPO-labeled MA were diluted 1/1,000.

[b] L cells were infected with a MOI of 40. After 6.5 h, infected and control cells were fixed with 0.05% glutaraldehyde and incubated with conjugate.

[c] ND, Not determined.

Reactivity of single and combinations of conjugates in an enzyme immunoassay. Selected conjugates of UM 8.139, 8.64, 8.55.2, and 8.73.3 were tested in duplicate against SFV-infected L cells (MOI, 40) versus noninfected cells as a control. A time interval of 6.5 h was chosen for the duration of infection. The conjugates were used in the following $-\log_{10}$ dilutions: 3, 3.5, 4, 4.5, and 5. In one single experiment the reactivities of the four individual conjugates were compared as well as the six combinations of two conjugates, the four combinations of three, and the only combination of four. Reactivities of these conjugates and their combinations were compared by using similar amounts of protein at each dilution. That means that the conjugates were diluted to 1:2, 1:3, and 1:4, respectively, in the above combinations.

The measured absorbance values and the derived mean P/N ratios are presented in Fig. 1 and 2. The results presented indicate that at high dilutions (dilution $10^{4.5}$ and 10^5) the combinations of two or more conjugates were superior to single conjugates in detecting viral antigen. At the lower dilutions (10^3 and $10^{3.5}$) however, combining more than two conjugates resulted in poor discriminative quality. The low P/N ratios obtained at these dilutions were accounted for by the relatively high absorbance value of the noninfected cells, possibly due to nonspecific binding (Fig. 1c). Therefore the combination of two conjugates was most suitable for detecting viral antigens, giving acceptable absorbance values for noninfected cells, except at the lowest dilution (Fig. 2).

Detection of viral antigen on infected cells with HRPO-labeled MA in the enzyme immunoassay. L cells in suspension were infected with avirulent SFV at MOIs of 0.0004, 0.004, 0.04, 0.4, 4, and 40, respectively, and seeded in 96-well plates to form monolayers. The monolayers of L cells were microscopically surveyed for the presence of virus-induced cytopathological changes, and each plate was fixed with 0.05% glutaraldehyde after a chosen period of infection. The monolayers were tested with either a mixture of two anti-E1 HRPO-labeled MA (UM 8.64 [1/3,000] and 8.139 [1/1,000]) or a mixture of two anti-E2 HRPO-labeled MA (UM 8.55.2 [1/3,000] and 8.73.3 [1/3,000]). Before the substrate reaction was terminated with H_2SO_4, groups of blue cells could be seen microscopically between the normal transparent cells of the monolayer. Moreover, these cells could be seen macroscopically as blue foci. The mean extinction values of duplicate determinations are presented in Fig. 3. It is evident from this figure that both mixtures of HRPO-labeled MA detect viral antigen of SFV in cell culture. Rising extinction values (indicating viral replication) were not observed until 4 h after inoculation at high MOIs (40 and 4), until 7 h at an MOI of 0.4, and until 17 h at an MOI of 0.0004. At the high MOIs the conjugates detected viral antigen before viral replication could have occurred. Thus, the

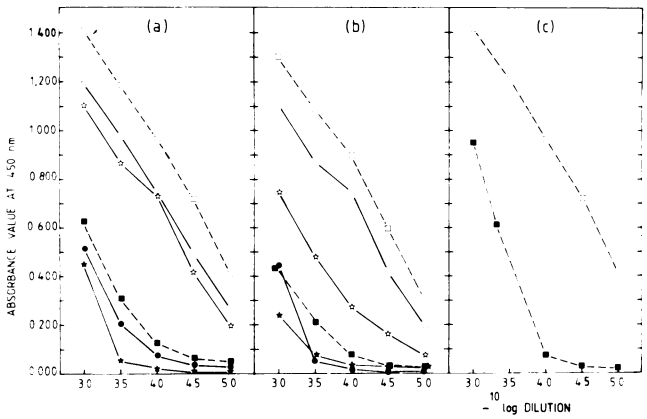

FIG. 1. Titration of HRPO-labeled specific MA against SFV in cell culture. Suspensions of L cells were infected with avirulent SFV (MOI, 40) and seeded in 96-well plates. Control cells were not infected. At 6.5 h after inoculation the cells were fixed, washed, and incubated with HRPO-labeled MA. Absorbance values are given at the following graded dilutions. (a) ○, Anti-E2 UM 8.73.3; ☆, anti-E2 UM 8.55.2; □, mixture of UM 8.73.3 and 8.55.2. (b) ○, Anti-E1 UM 8.139; ☆, anti-E1 UM 8.64; □, mixture of equal volumes of UM 8.139 and 8.64. (c) □, Mixture of equal volumes of UM 8.73.3, 8.55.2, 8.139, and 8.64. The reactions against noninfected cells are presented by closed symbols. Comparisons are based on similar amounts of protein in each dilution.

starting points of the steep slopes of extinction values are correlated with the MOI, which is also reflected in the simultaneous appearance of cytopathological changes (rounding of cells; indicated by arrows in Fig. 3a).

DISCUSSION

In preliminary experiments we used a commercial peroxidase-labeled anti-mouse immunoglobulin to detect viral antigen in a sandwich immunoassay using virus specific MA. A disadvantage of this method was poor reproducibility and frequently high background activities (i.e., high absorbance values for noninfected cells). This is in contrast to the results of other authors, who claim to have obtained quite satisfactory results with MA as primary antibodies in either indirect

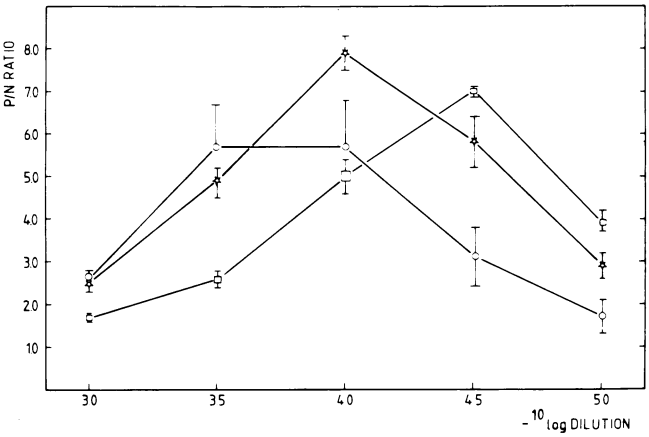

FIG. 2. Comparison of individual HRPO-conjugated MA and their combinations in sensitivity for SFV detection in cell culture. The mean P/N ratios were calculated from the data shown in Fig. 1 and are presented for four individual conjugates (○), six combinations of two conjugates (☆), and the five combinations of three and four conjugates (□). Vertical bars represent the standard errors of the mean.

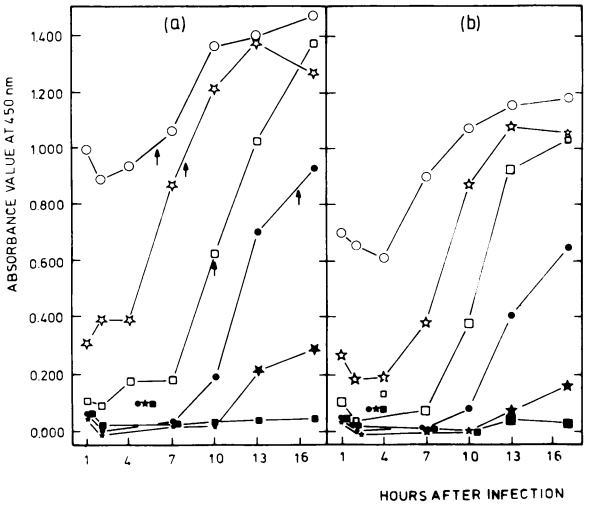

FIG. 3. Detection of viral antigen with HRPO-labeled specific MA. Suspensions of L cells were infected with SFV with different MOIs and seeded in 96-well plates. At various time intervals after infection (1, 2, 4, 7, 10, 13, and 17 h) monolayers of L cells were fixed. The monolayers were incubated with either a mixture of two HRPO-labeled anti-E1 MA (a) or a mixture of two anti-E2 HRPO-labeled MA (b). Absorption values are presented for (a) anti-E1 UM 8.139 (1/3,000) and 8.64 (1/1,000) and (b) anti-E2 UM 8.55.2 (1/3,000) and 8.73.3 (1/3,000), with MOIs of 40 (○), 4 (☆), 0.4 (□), 0.04 (●), 0.004 (★), and 0.0004 (■).

immunofluorescence tests or indirect enzyme immunoassays (5, 12).

In our study we obtained promising results in a direct immunoassay with peroxidase-labeled MA, directed against either the E1 or E2 glycoprotein of SFV. Four HRPO-conjugated MA were selected on the basis of their reactivity with purified SFV as well as with SFV-infected L cells (Table 1); 5 of the 10 HRPO-conjugated MA showed a stronger reactivity with purified SFV than with SFV-infected L cells. Viral antigen on the mature virus particle differs in some cases from the viral antigen on the infected cell. Possibly certain antigenic determinants are less fully exposed during membrane maturation; thus the reactivity of infected cells with conjugated MA would be less than that of purified virus (15). Viral antigens could be detected in cell culture by HRPO-labeled MA, even at high dilutions ($10^{4.5}$ and 10^5), with negligible reactions against noninfected cells. Moreover, antigen detection could be improved considerably by combining two or more noncompeting MA at high dilutions ($10^{4.5}$ and 10^5). At lower dilutions (10^3 and $10^{3.5}$), however, mixtures of three and four HRPO-labeled MA caused enhanced nonspecific reactions, possibly due to aggregate formation (10) (Fig. 1 and 2).

The test as described above was applied to detect the appearance of viral antigen in cell culture. Two anti-E1 MA and two anti-E2 MA were used (Fig. 3). Viral input at high MOIs (40, 4, and 0.4) could be detected as early as 1 h of infection, before multiplication of virus could possibly have occurred. The threshold of detection, defined as the lowest virus titer of inoculum that resulted in absorbance values higher than 0.200 and a P/N ratio of 2 or greater, lay between 10^5 and 10^6 PFU/ml, corresponding to MOIs of 0.4 to 4.

The earliest rise in extinction, indicating viral replication, started 4 h after inoculation of L cells with SFV at high MOIs (40 and 4). As could be expected, the rise of extinction values occurred later at low viral inputs. The expression of

E1 and E2 glycoproteins as detected by the two combinations of MA tested was found to be concordant. The results obtained were in agreement with the observations of other authors who used different methods to study the replication of togaviridae (1, 3, 7).

Viral antigen detection by direct immunoassay using HRPO-labeled MA proved to be highly sensitive and specific and may avoid the need for previously used practices in serology for purification of conventional antisera by absorption techniques with erythrocytes, liver powder, etc. Preliminary experiments with detection assays for mumps virus with conjugated MA yielded promising results. Therefore, we expect that enzyme-labeled MA may prove to be a useful tool for detecting pathogens in diagnostic laboratories.

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