Detection of Alphaviruses in a Genus-Specific Antigen Capture Enzyme Immunoassay Using Monoclonal Antibodies

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A genus-specific antigen capture assay using similar combinations of monoclonal antibodies for capture and detection of 24 alphaviruses belonging to the seven serocomplexes was developed. The sensitivity of the test ranged from $10^{3.4}$ 50% tissue culture infective doses/ml for o'nyong-nyong virus to $10^{6.1}$ 50% tissue culture infective doses/ml for Middelburg virus. The antigen capture test uses a combination of cross-reacting monoclonal antibodies directed against the nucleocapsid protein and envelope glycoprotein E1 of Semliki Forest virus.

The present classification of alphaviruses into seven complexes is based on antigenic relationships among the individual members of the genus (25, 30). The serological grouping of the viruses was determined by hemagglutination inhibition, complement fixation, and cross-neutralization (6-9, 17). Currently, surveys for the presence of alphaviruses are performed by virus isolation from arthropods or vertebrates and cultivation on cells, followed by serological identification of particular alphaviruses. These methods are time-consuming and require adequate facilities. The availability of monoclonal antibodies and their use in enzyme-linked-immunosorbent assays (ELISA) may provide an inexpensive, fast, and sensitive alternative for alphavirus surveillance (14, 15, 29).

Monoclonal antibodies to glycoproteins E1 and E2 of alphaviruses show relatively broad cross-reactivity in binding assays such as ELISA (26). In contrast, the neutralization activity of the E2 glycoprotein-specific monoclonal antibodies has been found to be restricted to the homologous and some closely related viruses only (24, 27, 31). The monoclonal antibodies with the broadest reaction spectrum are those directed against the nucleocapsid protein (13, 26).

In the study reported here, we constructed a genus-specific antigen capture test that detects alphaviruses from all antigenic complexes by using similar combinations of cross-reacting monoclonal antibodies against glycoprotein E1 and the nucleocapsid protein of Semliki Forest (SF) virus for antigen capture and detection of the bound antigen. The 24 alphaviruses tested were detected with sensitivities ranging from $10^{3.4}$ to $10^{6.1}$ 50% tissue culture infective doses (TCID₅₀)/ml.

MATERIALS AND METHODS

Cells and viruses. The alphaviruses used were from stocks maintained at the Yale Arbovirus Research Unit, New Haven, Conn. They were grown on C6/36 *Aedes albopictus* mosquito or BHK-21 cells. Titrations were performed on either mosquito cells or BHK-21 monolayers in microtiter plates. For antigen identification on mosquito cells, the microtiter plates were fixed at 48 h postinoculation overnight

with 5% neutral formaldehyde. Subsequently, an ELISA with polyclonal mouse immune ascitic fluid specific for each virus, rabbit anti-mouse immunoglobulin (peroxidase [PO] labeled), and 2,2'-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (Kirkegaard and Perry) was performed. Optical densities were read at 404 nm in an ELISA reader (11). Virus titrations on BHK-21 cells were evaluated by reading cytopathic effects caused by the virus. In both cell systems, virus titers were calculated by the method of Kärber (18).

Virus purification. Sindbis (SIN) and SF viruses were grown in BHK-21 cells. Virus was purified by ultracentrifugation of cell-free supernatants of SIN or SF virus-infected BHK-21 cells at $90,000 \times g$ for 2 h at 4°C. Resuspended virus pellets were spun for 4 h in linear 15 to 60% (wt/wt) sucrose gradients. The visible virus band at a density of about 1.17 g/cm³ was collected, diluted with phosphate-buffered saline (PBS), and sedimented for 2 h at $90,000 \times g$, 4°C. The virus was suspended in PBS and stored in aliquots at -70°C. Viral preparations were shown to be electrophoretically uniform.

Monoclonal antibodies. Monoclonal antibodies against the nucleocapsid protein and glycoprotein E1 were used (13). In immunoblots under nonreducing conditions (3), the glycoprotein-specific antibodies reacted with the faster-migrating protein of SIN virus. This protein has been identified by pulse-chase labeling of viral proteins as E1 (10) and not—as originally reported—E2. For establishment of the hybridomas (13), BALB/c mice were immunized intraperitoneally six times at weekly intervals with 0.2 ml of a 10% suspension ($10^{8.9}$ PFU/ml) of brain tissue from SF virus-infected baby mice. Spleen cells of immunized mice were fused with P3-NS1/1-Ag-4-1 (NS1) myeloma cells (19). Positive hybridomas were selected in an ELISA using the heterologous SIN virus as described below.

Production and PO labeling of monoclonal antibodies. Cloned antibody-producing hybridoma cells were collected by centrifugation ($1,000 \times g$, 10 min, 4°C) and adjusted to 5×10^5 to 1×10^6 cells per ml in 1 liter of the Dulbecco modification of Eagle medium without addition of serum. The cells were incubated for 3 to 4 days at 37°C. After removal of cell debris, supernatant fluids were titrated by ELISA. Thereafter, they were concentrated by two cycles of ammonium sulfate precipitation at 50% saturation for 60 min at 4°C. Precipitates were recovered by centrifugation ($5,000$

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× g for 25 min). The last pellet was suspended in 2.5 ml of PBS. Residual ammonium sulfate was removed by desalting over PD-10 gel filtration chromatography (Pharmacia). Further purification of antibodies was achieved by affinity chromatography using protein A-Sepharose (Affi-Gel; Bio-Rad). Protein determinations (20) showed that concentrated antibody preparations contained between 0.2 (SFV/C8) and 1 (SFV/C12) mg of protein per ml. Aliquots (1 mg) of concentrated monoclonal antibodies were labeled with horseradish PO (Sigma) (4). The protein/enzyme ratio was 3:2.

ELISA for titration of monoclonal antibodies. Antibody activities were assayed by solid-phase ELISA (2) using urea-treated, purified viral antigen and PO-labeled, affinity-purified goat anti-mouse immunoglobulin G (Sigma). To disrupt and solubilize the gradient-purified virus particles (SIN or SF virus), 4 M urea was added. After 15 min of incubation at room temperature, the antigen was directly (without removing the urea) diluted to 5 µg/ml in 0.05 M carbonate buffer, pH 9.5. Wells of microtiter plates (Immuno Plates I; NUNC) were coated overnight at 4°C with 100 µl of antigen per well. Nonspecific binding sites were blocked by incubation with 1% horse serum in PBS for 30 min. All washing steps between incubations were performed with PBS containing 0.05% Tween 20 (Sigma). Incubations with monoclonal antibodies and PO-labeled, affinity-purified goat anti-mouse immunoglobulin G were performed at room temperature for 1 h each. *o*-Phenylenediamine (Sigma) at 1.85 mM in 50 mM disodium phosphate–25 mM citric acid buffer, pH 5, containing 0.012% H₂O₂ was used as the substrate for PO. Enzymatic reactions were stopped after 10 min by adding 1 volume of 2.5 M H₂SO₄. The optical density at 492 nm (OD₄₉₂) was determined in an SLT 210 ELISA reader (SLT-Labinstruments). Background values (mean absorbance of two wells on each microtiter plate when the antigen was incubated with the PO substrate only; OD₄₉₂, ≤0.1) were subtracted. All samples giving an absorbance value above the threshold value were considered positive signals. The threshold value was determined by calculating the mean absorbance of 50 wells when the ELISA was performed with an unrelated monoclonal antibody (mean OD₄₉₂, about 0.1).

Alternatively, monoclonal antibody reactivity was tested in an ELISA on formaldehyde-fixed, virus-infected mosquito cells. Incubations were performed as described above. 2,2'-Azinobis(3-ethylbenzthiazoline)-6-sulfonic acid was used as the substrate for PO (11).

Antigen capture tests. (i) Preparation of virus antigen. For antigen capture tests, tissue culture supernatants from virus-infected C6/36 mosquito or BHK-21 cells were used. Supernatants were harvested from infected mosquito cells 3 to 6 days postinoculation and from infected BHK-21 after the cells were destroyed by the cytopathic effect. After centrifugation (10,000 × g, 4°C, 30 min) to remove cell debris, a sample was used to determine the virus titer. For virus inactivation, the antigen was incubated for 3 days at 4°C with 7.5% (vol/vol) β-propiolactone. Thereafter, it was aliquoted and stored at –20°C until use. As negative controls, supernatants from uninfected cells were prepared by the same method. Several antigens (eastern equine encephalitis [EEE], western equine encephalitis [WEE], SIN, Mayaro, and Middelburg viruses) were prepared from viruses grown in BHK-21 or mosquito cells. Although different titers were obtained with the different cell types, the detection limits in the antigen capture tests were identical. Therefore, specific

host cells for the viruses were not indicated. The antigens used and their titers are listed in Table 1.

(ii) Preparation of microtiter plates. Microtiter plates were coated with purified capture monoclonal antibody or antibody mixtures diluted in PBS, pH 7.4, overnight at 4°C. To find the optimal protein concentrations of the antibodies, microtiter plates were coated with solutions containing 2, 4, 8, and 16 µg of protein per ml of PBS at 100 µl per cup, as indicated in Results. Blocking of nonspecific binding sites was performed as indicated above.

(iii) Antigen capture ELISA. The antigens (100 µl per well) were incubated in twofold serial dilutions, starting at a 1:4 dilution of the antigens listed in Table 1, with gentle shaking on a rocker plate (IKA-Vibrax VXR). After incubation, the plates were washed three times with PBS-Tween 20 and the PO-conjugated monoclonal antibodies and the *o*-phenylenediamine substrate were added. All incubations were performed on the rocker plate. The incubation conditions were optimized after each step and are described in Results. Background values (mean absorbance of two wells on each microtiter plate when the bound antibodies were incubated with the PO substrate only; OD₄₉₂, ≤0.05) were subtracted. All samples giving an absorbance value above the threshold value (mean OD₄₉₂ as indicated for each experiment) were considered positive. The threshold value was determined by calculating the mean absorbance of 50 wells when the test was performed with supernatant from uninfected mosquito or BHK-21 supernatant, the PO-conjugated monoclonal antibodies, and the substrate. The results given in the tables are mean values from four experiments.

RESULTS

Selection of monoclonal antibodies with broad reactivity.

Three monoclonal antibodies specific for SIN and SF viral glycoproteins were screened for cross-reactivity with a total of 24 alphaviruses. An indirect ELISA on formaldehyde-fixed, infected mosquito cells was used. The hybridoma supernatants from SFV/C3 and SFV/C8 reacted with all of the viruses tested. Antibody SFV/C5 did not recognize Ross River virus (data not shown).

Because of their broad cross-reactivity with alphaviruses from all complexes except Venezuelan equine encephalitis (VEE) and Barmah Forest (BF) viruses in the indirect ELISA (13), nucleocapsid protein-specific antibodies SFV/C2, SFV/C12, and SFV/C42, together with E1 glycoprotein-specific antibodies SFV/C3 and SFV/C8, were selected for further experiments.

Relative avidities of monoclonal antibodies for SIN and SF viral antigens. The relative avidities of the monoclonal antibodies were estimated by the slopes of their titration curves and by the amounts of antibody needed to saturate the antigens. Thus, binding of purified antibody to gradient-purified, urea-treated SIN and SF viruses was determined in an indirect ELISA (Fig. 1). Steep slopes in the region below antigen saturation of the titration curves from nucleocapsid protein-specific antibodies SFV/C2, SFV/C12, and SFV/C42 indicated that they had high avidities for their epitopes on both viral antigens (Fig. 1A and B). About 15 to 30 ng of each of the antibodies was needed to saturate the antigen. Glycoprotein E1-specific SFV/C3 had high affinity for SIN but relatively low affinity for the homologous SF virus (Fig. 1C and D). While the SIN virus antigen was saturated with 30 ng of antibody, more than 10³ ng of antibodies was needed to reach antigen saturation with SF. In contrast, SFV/C8 had

TABLE 1. Viral antigens and their reactivities in the antigen capture test using single monoclonal antibodies for antigen capture and the homologous PO conjugates for detection

Virus	Titer (log TCID ₅₀ /ml)	Detection limit ^a with:				
		SFV/C2	SFV/C12	SFV/C42	SFV/C3	SFV/C8
EEE	8.5	6.4	6.1	7.9	6.7	6.7
VEE complex						
VEE	7.1	NR ^b	NR	NR	5.0	4.7
Everglades	8.2	NR	NR	NR	7.6	7.6
Mucambo	7.4	NR	NR	NR	5.0	5.0
Pixuna	7.0	NR	NR	NR	NR	NR
Bijou Bridge	6.9	NR	NR	NR	4.8	5.4
WEE complex						
WEE	8.2	4.9	4.6	7.0	6.7	7.6
Y62-33	7.2	5.1	5.1	NR	6.3	NR
Fort Morgan	7.6	5.5	5.2	6.0	6.4	NR
SIN	7.6	5.2	4.9	6.4	6.1	7.0
Whataroa	5.2	4.3	4.0	NR	4.3	NR
Kyzylagach	6.5	3.8	3.8	4.7	4.4	4.4
SF complex						
SF	8.6	6.5	6.3	6.5	7.7	8.0
Chikungunya	6.4	NR	5.8	5.8	NR	NR
O'nyong nyong	6.4	3.7	3.7	4.3	4.0	4.0
Getah	8.0	6.2	5.9	6.8	6.8	6.8
Sagiyama	8.3	6.5	6.5	6.8	NR	NR
Bebaru	7.5	5.7	5.7	6.0	NR	6.6
Ross River	7.9	6.4	6.1	6.4	7.0	7.0
Mayaro	7.9	6.1	5.8	6.1	6.4	6.1
Una	8.2	7.0	6.4	7.0	6.4	7.3
Ndumu	7.4	5.6	5.3	5.9	7.1	NR
Middelburg	7.9	6.4	6.1	6.4	NR	6.7
BF	6.6	NR	NR	NR	NR	NR

^a The detection limit is the number of TCID₅₀ per milliliter required for a detectable reaction.

^b NR, No reaction in the antigen capture test starting with a 1:4 dilution of antigens.

low affinities for its epitope on both antigens and 250 ng of antibody was not enough to saturate the antigens.

Determination of optimal incubation conditions for the antigen capture test. To determine the optimal time and temperature of incubation for antigen capture and detection, microtiter plates were coated with 100 μ l of diluted monoclonal antibody SFV/C12 per ml (4 μ g/ml) overnight at 4°C. Then, antigen capture tests were performed with twofold dilutions of β -propiolactone-inactivated tissue culture supernatant from BHK-21 cells infected with SIN virus (starting dilution, 10^{6.7} TCID₅₀/ml). Test mixtures were incubated for 1, 2, 4, and 18 h at 4°C (data not shown), at 22°C (Fig. 2A), and at 37°C (data not shown). Thereafter, test mixtures were washed and for detection of bound antigen, they were incubated with PO-labeled SFV/C12 (SFV/C12-PO) for 1, 2, 3, and 4 h on the rocker plate. The best results were achieved after 18 h of incubation at 22°C or after 4 h at 37°C with the antigen. For detection of bound antigen with the PO-labeled antibody, an incubation time of 4 h at 22°C (Fig. 2B) or 2 h at 37°C produced the most sensitivity. With the *o*-phenylenediamine substrate, incubation times of more than 10 min raised the background without improving the sensitivity of the test (data not shown). Although the incubation times could be reduced when the test mixtures were incubated at 37°C, at this temperature unspecific reactions were observed in the outer rows of cups of the microtiter plates. This effect could not be overcome by using damp chambers in addition to gentle shaking and reduced the capacity of the plates by

about 40%. Therefore, all further experiments were performed at 22°C.

Determination of optimal antibody concentrations for the antigen capture test. For determination of optimal concentrations of capture antibodies, twofold dilutions of monoclonal antibodies in PBS, starting at 16 μ g/ml, were coated onto the wells of microtiter plates (100 μ l per well). Nonabsorbed free antibody was titrated in the indirect ELISA by using purified SF virus as the antigen. After coating of less than 4 μ g/ml to the plastic, no free antibody was detected. Coating overnight at 4°C or for 2 h at room temperature yielded the same results.

Microtiter plates coated with different concentrations (2 to 16 μ g/ml) of capture antibodies were used for antigen capture tests with SF virus. These experiments showed that high antibody concentrations increased the background noise (obtained when antibody-coated wells were incubated with the PO conjugate and the substrate only), thus lowering the sensitivity of the assay. For monoclonal antibody SFV/C42 (plotted in Fig. 3 without subtraction of the background noise), the highest sensitivity and best signal-to-noise ratio were achieved with an antibody concentration of 2 to 4 μ g/ml (background noise OD₄₉₂, <0.05). Antibody concentrations of 8 and 16 μ g/ml drastically raised the background noise to OD₄₉₂ values of about 0.3 and 0.6, respectively. Essentially the same effect was seen when the concentration of the PO-labeled detection antibody was raised (data not shown). The best signal-to-noise ratios were achieved with conjugate

FIG. 1. ELISA titration curves of the monoclonal antibodies on SIN (A and C) and SF (B and D) virus antigens. The antibodies used were SFV/C2 (□), SFV/C12 (+), and SFV/C42 (▲) (A and B) and SFV/C3 (×), and SFV/C8 (·) (C and D). The starting antibody concentration was 2.5 µg/ml. Background noise was subtracted.

dilutions determined to be in the log phase of the titration curve (yielding an OD_{492} of 0.5 to 1.0) generated in a direct ELISA using purified urea-treated SIN virus. This means that for use in the antigen capture test the labeled antibody should not be in excess over the antigen. Under these conditions, background OD_{492} values of ≤ 0.05 were obtained with almost all antibody combinations.

Reactivities of individual monoclonal antibodies with alphaviruses in the antigen capture test. In a first series of experiments, the same individual monoclonal antibodies for capture and for detection of bound antigen were assayed for cross-reactivity with 24 alphaviruses in an antigen capture test (Table 1). Nucleocapsid protein-specific antibodies SFV/C2, SFV/C12, and SFV/C42 reacted with neither viruses of the VEE complex nor BF virus. Among these, antibody SFV/C12 showed the broadest cross-reactivity with the rest of the alphaviruses. Detection levels ranged from $10^{3.8}$ TCID₅₀/ml for Kyzylagach virus to $10^{6.4}$ TCID₅₀/ml for Una virus (Table 1). Antigen detection limits were comparable to those found for SFV/C2, except that this antibody did not detect chikungunya virus. Antibody SFV/C42 displayed lower sensitivities, ranging from $10^{4.3}$ TCID₅₀/ml for o'nyong nyong virus to $10^{7.9}$ TCID₅₀/ml for EEE virus. Whataroa and Y62-33 viruses were not detected.

The two anti-E1 glycoprotein antibodies showed different reactivity patterns (Table 1). Monoclonal antibody SFV/C3 reacted with four of the five VEE complex viruses tested but not with BF, Middelburg, Bebaru, Sagiyama, and chikungunya viruses. Antibody SFV/C8 reacted with the same viruses of the VEE complex but with neither BF nor some viruses from other complexes. Both antibodies reacted only poorly in this assay with SF and SIN viruses.

Reactivities of combinations of monoclonal antibodies in the antigen capture test with alphaviruses. Different combina-

tions of two or three of the nucleocapsid protein-specific antibodies for capture (total protein concentration, 4 µg/ml) and/or for detection (dilutions determined in checkerboard titrations) did not affect the sensitivity of the test (data not shown). The best results were obtained when SFV/C12 alone was used as both the capture (protein concentration, 4 µg/ml) and detection antibody.

When a combination of envelope-specific antibodies SFV/C3 and SFV/C8 (total protein concentration, 4 µg/ml) was used for antigen capture, the background noise increased (OD_{492} , about 0.3), thus reducing the sensitivity of antigen detection. This effect was independent of the concentration or the identity of the PO conjugates used. Even when a PO conjugate of unrelated nucleocapsid protein-specific antibody SFV/C12 was used, high background noise resulted (data not shown).

For construction of an antigen capture test with a wide reactivity range, different combinations of the three nucleocapsid protein- and the two E1 glycoprotein-specific antibodies were analyzed (Table 2). No combination of nucleocapsid protein- or E1 glycoprotein-specific antibodies alone was able to detect Pixuna or BF virus. This was possible only when both types of antibodies were combined for capture as well as detection. The most efficient combinations of capture antibodies were equal amounts of SFV/C12 and SFV/C3 (4 µg of total protein per ml) or, alternatively, SFV/C2, SFV/C12, SFV/C42, and SFV/C3 (4 µg of total protein per ml).

Detection was most sensitive with a mixture of all five PO-labeled monoclonal antibodies on SFV/C12 and SFV/C3 as capture antibodies. The results are summarized in Table 2. With the above-described combination, the 24 alphaviruses tested were detectable and the sensitivity for each of the viruses was higher than with other antibody combinations.

FIG. 2. Determination of optimal incubation conditions for the antigen capture test. SFV/C12 (4 μ g/ml) was used as the capture antibody. Titrations were performed with twofold serial dilutions of SIN virus, starting at $10^{6.7}$ TCID₅₀/ml, and for detection of bound antigen, SFV/C12-PO was used. Background noise was subtracted. (A) Incubation times with antigen at 22°C were for 18 (■), 4 (Δ), 2 (+), and 1 (□) h. (B) Incubation times with PO-labeled conjugate SFV/C12-PO were 4 (■), 3 (Δ), 2 (+), and 1 (□) h (22°C).

FIG. 3. Determination of the optimal concentration of capture antibody SFV/C42. Serial twofold dilutions of SF virus were detected with a fixed concentration of SFV/C42-PO. The capture antibody concentrations were 16 (□), 8 (+), 4 (■), and 2 (Δ) μ g/ml. Background noise was not subtracted.

TABLE 2. Reactivities of combinations of monoclonal antibodies with alphaviruses in antigen capture tests

Virus antigen	Detection limit ^a with ^b :		
	Mixture 1	Mixture 2	Mixture 3
EEE	6.4	5.8	6.1
VEE complex			
VEE	5.3	5.0	5.3
Everglades	5.4	5.7	5.4
Mucambo	5.9	5.0	5.6
Pixuna	4.8	5.4	4.8
Bijou Bridge	5.1	4.8	5.4
WEE complex			
WEE	5.5	5.5	5.8
Y62-33	5.4	4.8	5.1
Fort Morgan	6.1	5.2	5.5
SIN	5.5	5.2	5.8
Whataroa	3.7	3.7	3.7
Kyzylagach	4.4	4.1	4.1
SF complex			
SF	5.9	5.6	5.6
Chikungunya	5.8	5.2	5.5
O'nyong nyong	3.7	3.1	3.4
Getah	6.2	5.9	5.9
Sagiyama	5.9	5.6	5.6
Bebaru	5.7	5.4	5.4
Ross River	6.4	5.8	5.8
Mayaro	5.8	5.5	5.8
Una	6.1	5.8	5.8
Ndumu	5.3	5.0	5.3
Middelburg	6.1	5.5	6.1
BF	5.1	5.1	5.1

^a Number of TCID₅₀ per milliliter required for a detectable reaction.

^b The following combinations of antibodies were used: mixture 1, SFV/C12 and SFV/C3 for capture and SFV/C12-PO and SFV/C3-PO for detection; mixture 2, SFV/C12 and SFV/C3 for capture and SFV/C2-PO, SFV/C12-PO, SFV/C42-PO, SFV/C3-PO, and SFV/C8-PO for detection; mixture 3, SFV/C2, SFV/C12, SFV/C42, and SFV/C3 for capture and SFV/C2-PO, SFV/C12-PO, SFV/C42-PO, SFV/C3-PO, and SFV/C8-PO for detection.

DISCUSSION

The morphological and serological relationships among alphaviruses have been well documented by using a variety of experimental approaches (7–9, 17). Data on the molecular level indicate that alphaviruses originated from a common ancestor virus (1). This provided the rationale to identify conserved epitopic regions on viral structural proteins with monoclonal antibodies and use the antibodies as broadly reacting diagnostic tools.

Two sets of monoclonal antibodies against SF virus were developed. They were selected on the basis of cross-reactivity with SIN virus. One group of 14 monoclonal antibodies was directed against a cluster of conserved epitopes on the nucleocapsid protein. These antibodies reacted with all of the alphaviruses except VEE and BF viruses (13).

The second set of three monoclonal antibodies was directed against the E1 envelope glycoprotein. They had no neutralizing activity. However, when assayed in the hemagglutination inhibition test, they reacted with viruses from four different serocomplexes (WEE, VEE, SF, and EEE; 5a). By using the indirect ELISA on formaldehyde-fixed, virus-infected mosquito cells, we showed that two of these antibodies (SFV/C3 and SFV/C8) detected conserved

epitopes on all 24 of the alphaviruses tested. They were selected for establishment of the antigen capture test.

In the antigen capture test, the reactivities of SFV/C3 and SFV/C8 turned out to be more restricted. Only 18 (SFV/C3) and 16 (SFV/C8) of the 24 viruses tested were detected. This failure to detect all of the viruses in the antigen capture test might reflect differences in epitope conformation on fixed cells and mature virus particles (28). Furthermore, the affinities of the antibodies for the same epitope on each individual virus may be different. Thus, only viruses which express epitopes to which the antibodies bind with high affinity were detected. It is unclear why these antibodies did not react in the antigen capture test with BF and Pixuna viruses unless they were mixed with nucleocapsid protein-specific antibody SFV/C12.

The sensitivity of the antigen capture test was not impaired by use of almost identical combinations of monoclonal antibodies for antigen capture and detection. This indicated that competition between capture and detection antibodies for the same epitopes did not affect the test. The antigens consisted of β -propiolactone-inactivated culture supernatants from virus-infected mosquito or BHK-21 cells. They were not subjected to any treatment for disruption of the virus other than natural decay that occurred during storage. In addition, two cycles of freeze-thawing or storage for 1 week at 15°C in a refrigerator did not affect the sensitivity of the test (data not shown). This and the fact that all incubations were performed in the presence of detergent (0.05% Tween 20)—which solubilizes the proteins—makes it conceivable that major aggregation of whole virus particles did not occur. As we can almost exclude the possibility that the nucleocapsid protein and the E1 glycoproteins contain several copies of the same epitope on one molecule, successful performance of the test probably depends on stable complexes formed between the glycoproteins and the nucleocapsid protein. Although it was shown that the virus spikes consist of heterodimers of E1 and E2 (12), it was also suggested that there may be an interaction between E2 and a component of the nucleocapsid (5).

The sensitivity of the antigen capture test ranged from $10^{3.4}$ TCID₅₀/ml for o'nyong-nyong virus to $10^{6.1}$ TCID₅₀/ml for Middelburg virus. In comparison, an antigen capture test using purified antibodies from hyperimmune mouse ascites and from rabbit sera in combination with monoclonal antibodies to detect a single species of virus detected $10^{3.4}$ TCID₅₀ of EEE virus per ml (29). An improved antigen capture test should detect all alphaviruses with comparable sensitivities. This will be achieved only if new monoclonal antibodies—generated against the viruses detected with low sensitivities—are included in the test.

Enzyme immunoassays for detection of single species of arboviruses have been successfully developed for flaviviruses (21, 22) and La Crosse (16), Rift Valley fever (23), EEE (14, 15, 29), and Highlands J (14, 15) viruses. We have developed a genus-specific antigen capture assay using monoclonal antibodies detecting 24 alphaviruses belonging to the seven serocomplexes. Three different combinations of three anti-capsid protein and two anti-E1 glycoprotein monoclonal antibodies were found to be most effective for antigen detection. Our data support the contention that an alphavirus genus-specific assay, in combination with the serotype-specific assays, may be a useful addition to current surveillance techniques if an alphavirus outbreak is suspected. In several regions of the world, more than one alphavirus occurs. The genus-specific antigen capture test could be used as a rapid screening method to detect the

antigen in arthropods and tissues from human patients or vertebrate animals. Specific identification could then be performed in a second test.

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