Method for Detection of Simian Immunodeficiency Virus Neutralizing Antibodies Using a Noncommercial Antigen Capture Enzyme-Linked Immunosorbent Assay

MARY J. WELCH¹ AND MARGARET E. THOULESS^{1,2*}

University of Washington Regional Primate Research Center¹ and Department of Pathobiology,² University of Washington, Seattle, Washington 98195

Received 12 November 1991/Accepted 2 March 1992

A neutralization test (NT) using a noncommercial antigen capture enzyme-linked immunosorbent assay (ELISA) to detect simian immunodeficiency virus (SIV) growth in vitro was developed. The capture antibody was a mixture of purified macaque anti-SIV immunoglobulin G (IgG) and a monoclonal antibody to SIV p27. Captured antigens were detected by using purified macaque anti-SIV IgG conjugated to horseradish peroxidase. The NT reliably and sensitively detected differences when various amounts of SIV were used with positive and negative control macaque sera. Dilutions of sequential sera from a macaque (*Macaca nemestrina*) that had been experimentally infected with SIV were tested for neutralizing antibody with 300 50% tissue culture infective doses of SIV. In this macaque, neutralizing activity and anti-SIV IgG levels in serum (detected by ELISA) increased with time after SIV inoculation, and high IgG titers were required in serum before neutralization occurred in vitro. This simple NT, which detects the presence of SIV serum neutralizing antibodies at a low cost, will be useful for investigating the role of neutralizing antibodies in the SIV-infected macaque model for AIDS.

The simian immunodeficiency virus (SIV)-infected macaque model for human immunodeficiency virus infection is used to gain understanding of host immune responses and for vaccine development (3, 4, 14). The role of neutralizing antibodies in delaying the progression of AIDS is not well understood (24, 26), but because they prevent initial infection and reduce the spread of virus in vitro, it is an important aspect of vaccine research (5). There is an increased interest in the peptides and polypeptides that may induce a protective neutralizing immune response in vaccinated animals (7, 10). To detect the SIV- or human immunodeficiency virus-neutralizing activity of serum samples in a neutralization test (NT), different methods have been used to measure virus growth (23). These include visual scoring of the cytopathic effect or syncytium formation (13, 16), determination of reverse transcriptase activity (6, 17) and [³H] thymidine incorporation (8), and colorimetric determination of incorporation of vital dyes such as tetrazolium salt (12, 21), as well as antigen capture (7). There is a need for a low-hazard, inexpensive, but reliable serum NT that is not cumbersome to perform. Here we report a new SIV serum NT that uses 174×CEM cells and a noncommercial antigen capture enzyme-linked immunosorbent assay (ELISA). The neutralization results obtained with different SIV doses and sequential serum samples from experimentally SIV-infected macaques are presented.

MATERIALS AND METHODS

Experimental monkey sera. A male macaque (*Macaca nemestrina*) was shown to be clinically healthy by examination and negative for SIV and simian type D retroviruses by immunoglobulin G (IgG) ELISA and cocultivation of peripheral blood lymphocytes as described previously (1, 20). The

animal was bled regularly after intrarectal inoculation with 1,000 50% tissue culture infectious doses (TCID₅₀s) of SIV_{E11S} (a biological clone of SIV_{MNE}) (2). The monkey developed lymphadenopathy at week 8 and died at week 43 postinoculation. Serum samples collected between weeks 0 and 39 were tested in this study.

Serum IgG ELISA. All macaque serum samples were preabsorbed by diluting them 1/10 in a mixture of bovine fetal and calf sera to prevent nonspecific binding and were titrated across 96-well vinyl assay plates (Costar, Pleasanton, Calif.) precoated with SIV antigen (strain E11S clone 5, B1419, donated by R. E. Benveniste, National Cancer Institute). Binding of anti-SIV IgG was detected with anti-human IgG conjugated to horseradish peroxidase (Sigma, St. Louis, Mo.). *o*-Phenylenediamine was used for the color reaction, and the plates were read at a wavelength of 492 nm. The optical densities (ODs) were blanked on wells that were not coated with antigen but that had the first serum dilution, conjugate, and substrate. Titers were calculated as the reciprocal dilution at which an OD of 0.60 was reached. This is an arbitrary value on the linear portion of the plot of serum dilution-versus-OD at 492 nm.

Cell culture. The 174×CEM cells were obtained from Peter Cresswell, AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (19). The cells are a fusion product of the human B-cell line 721.174 and human T-cell line CEM. They were grown in growth medium (RPMI 1640, 10% inactivated fetal bovine serum, 1 mM glutamine, 1 mM pyruvate, 1 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 100 U of potassium penicillin G per ml, 100 µg of streptomycin sulfate per ml, 2.5 µg of amphotericin B per ml).

SIV TCID₅₀s. Polyethylene glycol-concentrated SIV, which had been frozen only once, was titrated across sterile 96-well tissue culture plates in 50-µl volumes of growth medium. The 174×CEM cells (100 µl, 3 × 10⁵/ml) were

* Corresponding author.

added to each well. After a 7-day incubation in 5% CO₂, the well contents were assayed for SIV growth by using the antigen capture procedure described below. Wells that received SIV doses of 10³ TCID₅₀s or larger produced OD results greater than 2.00, while uninfected wells consistently gave OD results of between 0.01 and 0.06. Wells infected with 1 TCID₅₀ gave OD values of about 0.40. The TCID₅₀ for each virus titration was calculated by using the formula of Reed and Muench (18).

Serum NT. All sera were inactivated at 56°C for 30 min. Doubling serum dilutions were made (in quadruplicate) across 96-well sterile tissue culture plates in growth medium. The first two wells in each row had identical serum dilutions. The first wells received 50 µl of growth medium only, as a control for cell growth. The remaining wells received 50 µl of medium containing 30, 300, or 600 TCID₅₀s of SIV_{E11S}. Neutralization was allowed to occur for 1 h at room temperature before 100 µl of 174×CEM cells (3 × 10⁵/ml in growth medium) was added. The plates were then incubated in 5% CO₂ at 37°C for 7 days before evaluation of viral growth was performed by the SIV antigen capture ELISA described below.

Antibodies for SIV antigen capture. A male macaque (*M. nemestrina*) shown to be SIV and simian type D retrovirus negative was experimentally infected with SIV_{E11S}. Blood was collected at the time of death, and the serum was shown by ELISA to contain anti-SIV IgG. The serum IgG was purified by using a protein A column, and the total IgG was measured (9). IgG (5 mg) was conjugated to horseradish peroxidase (Sigma) by a previously described method (15). The conjugate was used for detecting the SIV antigens captured on the plate.

The hybridoma FA₂ (obtained from Suganto Sutjipto and Preston Marx through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases) produces a monoclonal antibody to SIV p27 core antigen (21). IgG was concentrated and purified from the hybridoma culture supernatants by using a protein A column and was quantified as described above.

Antigen capture assay. A total of 50 µl of 2% sodium deoxycholate in phosphate-buffered saline (PBS) was added to each well of neutralization or virus titration plates. They were held at room temperature for 1 h to inactivate the virus. Immulon 1 ELISA plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 3 µg of FA₂ monoclonal antibody per ml and 3 µg of purified macaque anti-SIV IgG per ml in 0.1 M sodium carbonate buffer (pH 9.6). After washing with PBS-0.1% Tween 20, 100 µl of the contents of each well of the inactivated NT plates was transferred to the corresponding well in the coated ELISA plate. The plates were incubated overnight at room temperature. After washing, macaque anti-SIV IgG conjugated to horseradish peroxidase, diluted to 1/500 in PBS-0.1% Tween 20 containing 0.5% fetal bovine serum and 0.5% bovine calf serum, was applied to all wells and the plates were reincubated for 2 h at room temperature. After a final wash, *o*-phenylenediamine substrate solution was applied. After 30 min, the reaction was stopped with 0.1 M sulfuric acid. The plates were read at 492 nm and blanked on the control wells. Serum neutralization titers are given as the reciprocal of the serum dilution which gave 50 or 90% reduction of virus growth in comparison with virus growth in the preimmune serum.

FIG. 1. SIV growth determined by antigen capture ELISA in the presence of positive (solid symbols and lines) and negative (open symbols and broken lines) control sera that was diluted and challenged with one of the following three SIV doses: 30 TCID₅₀s (◇), 300 TCID₅₀s (△, ▲), and 600 TCID₅₀s (□, ■).

RESULTS

Neutralization using three SIV doses. The results obtained when the NT was performed on IgG ELISA-positive and -negative macaque sera at three different SIV doses (30, 300, and 600 TCID₅₀s per well) are shown in Fig. 1. The ELISA-negative serum allowed SIV growth at all three virus doses, while the ELISA-positive serum neutralized all three virus doses. The 30-TCID₅₀ dose yielded unreliable results for both the ELISA-positive and -negative sera; virus at this quantity did not appear to inoculate all wells uniformly. The ELISA-positive serum sample demonstrated a smaller neutralizing activity with 600 TCID₅₀s and a greater activity with 300 TCID₅₀s. Three hundred TCID₅₀s of SIV was completely neutralized by the ELISA-positive serum sample up to a dilution of 1/80.

NT on sequential SIV-infected monkey sera. NT results (using 300 TCID₅₀s of SIV per well) for four sequential sera from a macaque experimentally infected with SIV_{E11S} are given in Table 1. The serum IgG ELISA and the NT titers (by all three means of measurement) increased with time after SIV inoculation. The 50% endpoint NT titers were all greater than the 90% endpoint titers. The titers obtained by visual observation for cytopathic effect fell between the corresponding titers obtained by the antigen capture method described above. The 50% antigen capture titers were within 2 doubling dilutions above those obtained by scoring the cytopathic effect. While high IgG titers were present in

TABLE 1. Neutralization titers obtained for sequential serum samples from an SIV_{E11S}-inoculated macaque by the non-commercial antigen capture assay and inhibition of syncytium formation

Wk postinoculation	ELISA titer dilution (log ₁₀)	SIV NT titer ^a		CPE ^b
		50%	90%	
0	<1.5	<20	<20	<5
8	3.5	33	<20	10
17	4.8	340	100	160
39	5.6	1,600	240	>320

^a Reciprocal of serum dilution at 50 or 90% reduction in the OD of SIV antigen capture ELISA.

^b CPE, cytopathic effect; reciprocal of last serum dilution at which there was total inhibition of syncytium formation.

FIG. 2. Neutralization titers (50% virus inhibition by noncommercial antigen capture) in sequential serum samples from five macaques (*M. nemestrina*) experimentally infected with SIV.

postinoculation serum samples, the neutralizing titers were lower, the 50% endpoint titers being approximately 100-fold less than the corresponding IgG ELISA titer.

Sequential serum samples from additional *M. nemestrina* macaques were also tested for neutralizing antibodies to SIV. Figure 2 shows the change in titers with time for five of the animals. In all cases there was an increase in titer, but there was considerable variation in the immune response between individual animals.

DISCUSSION

The test for detecting neutralizing antibodies to SIV in macaque sera described here has been shown to work effectively and reliably. It confirmed the observation made by others that a high anti-SIV IgG level in serum is required before neutralizing activity occurs (5). This effect may be due in part to the large dose of virus which must be used in order to obtain reliable NT results. The dose of virus used by others in SIV and HIV NTs varies from 50 to 1,000 TCID₅₀s (10, 11, 13, 16, 22). The actual calculated dose of virus depends on the method of virus titration used; a particular virus stock may contain different TCID₅₀s for different cell lines. SIV has also been found to be about 100-fold more infectious in macaques than in tissue culture (4). Observation of cytopathic effect appeared to be a less sensitive method for detecting SIV growth than our antigen capture assay was; the titers that we obtained by the cytopathic effect scoring method were lower. But the increase in neutralization titer with time that was seen by the antigen capture ELISA was also seen by the cytopathic effect scoring method.

To discover the correct dose of virus that should be used in the neutralization assay described here, a simple check-board of virus dose against positive and negative serum dilutions was made (25). For this NT the 300-TCID₅₀ dose was best because negative sera allowed maximum virus growth and good positive sera totally neutralized the virus at a dilution of 1/80; larger doses of virus reduced the sensitivity of the test and smaller doses gave unreliable results.

Known positive sera, when tested on different occasions, gave titers within a range of 1 doubling dilution. The NT was sensitive enough to show differences in the sequential serum samples from the experimentally infected macaques, thus fulfilling one requirement of a good NT.

An ELISA that uses purified whole macaque anti-SIV IgG to capture and detect the SIV antigens would not be a suitable system for diagnostic purposes, because the whole macaque IgG contains antibodies to other viruses in addition to antibodies to SIV. However, in a controlled assay, with SIV known to be the only virus added to the system, the test works well. The monoclonal antibody produced by the hybridoma FA₂ did not work effectively as an antigen capture antibody or as a detection antibody in a conjugated form, but it enhanced the capture of SIV antigens when it was combined with the monkey anti-SIV IgG on the ELISA plates, which was seen as an increase in OD (25).

Although the anti-SIV reagents used in the NT described here were all purified in our laboratory, other laboratories could perform this test themselves. The hybridoma FA₂ is available from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and the macaque polyclonal anti-SIV IgG can be purified from any serum sample that has a high ELISA titer. Purification of the anti-SIV reagents in the laboratory makes this antigen capture ELISA inexpensive. Therefore, testing of sequential serum samples at many dilutions to study changes in the anti-SIV humoral response over time is now practical.

In conclusion, the NT described here is relatively inexpensive and involves no radioactive labels or extensive manipulation of infected cells, and a trained observer is not needed for the tiresome task of scoring individual wells by observation through a microscope. Therefore, potential hazards, errors, and subjectivity are reduced.

ACKNOWLEDGMENTS

This work was supported by grant NIH/NIAID A126503 from the National Cooperative Vaccine Development Group for AIDS and by University of Washington Regional Primate Research Center grant NIH RR00166.

Special thanks go to William Morton for support and LaRene Kuller for assistance.

REFERENCES

1. Benveniste, R. E., L. O. Arthur, C.-C. Tsai, R. Sowder, T. D. Copeland, L. E. Henderson, and S. Oroszlan. 1986. Isolation of a lentivirus from a macaque with lymphoma: comparison with HTLV-III/LAV and other lentiviruses. *J. Virol.* **60**:483-490.
2. Benveniste, R. E., R. W. Hill, L. J. Eron, U. M. Csaikl, W. B. Knott, L. E. Henderson, R. C. Sowder, K. Nagashima, and M. A. Gonda. 1990. Characterization of clones of HIV-1 infected HuT78 cells defective in *gag* gene processing and of SIV clones producing large amounts of envelope glycoprotein. *J. Med. Primatol.* **19**:351-366.
3. Daniel, M. D., P. K. Sehgal, T. Kodama, M. S. Wyand, D. J. Ringler, N. W. King, D. K. Schmidt, C. D. Troup, and R. C. Desrosiers. 1990. Use of simian immunodeficiency virus for vaccine research. *J. Med. Primatol.* **19**:395-399.
4. Desrosiers, R. C., M. S. Wyand, T. Kodama, D. J. Ringler, L. O. Arthur, P. K. Sehgal, N. L. Letvin, N. W. King, and M. D. Daniel. 1989. Vaccine protection against simian immunodeficiency virus infection. *Proc. Natl. Acad. Sci. USA* **86**:6353-6357.
5. Emini, E. A., P. L. Nara, W. A. Schleif, J. A. Lewis, J. P. Davide, D. R. Lee, J. Kessler, S. Conley, S. Matsushita, S. D.

- Putney, R. J. Gerety, and J. W. Eichberg. 1990. Antibody-mediated in vitro neutralization of human immunodeficiency virus type 1 abolishes infectivity for chimpanzees. *J. Virol.* **64**:3674–3678.
6. Groopman, J. E., P. M. Benz, R. Ferriani, K. Mayer, J. D. Allan, and L. A. Weymouth. 1987. Characterization of serum neutralization response to the human immunodeficiency virus (HIV). *AIDS Res. Hum. Retroviruses* **3**:71–85.
7. Haigwood, N. L., J. R. Shuster, G. K. Moore, H. Lee, P. V. Skiles, K. W. Higgins, P. J. Barr, C. George-Nascimento, and K. S. Steimer. 1990. Importance of hypervariable regions of HIV-1 gp120 in the generation of virus neutralizing antibodies. *AIDS Res. Hum. Retroviruses* **6**:855–869.
8. Harada, S., D. T. Purtilo, Y. Koyanagi, J. Sonnabend, and N. Yamamoto. 1986. Sensitive assay for neutralizing antibodies against AIDS-related viruses (HTLV-III/LAV). *J. Immunol. Methods* **92**:177–181.
9. Harlow, E., and D. Lane (ed.). 1988. *In* Antibodies, a laboratory manual, p. 310 and 670. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. Ho, D. D., M. G. Sarngadharan, M. S. Hirsch, R. T. Schooley, T. R. Rota, R. C. Kennedy, T. C. Chanh, and V. L. Sato. 1987. Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. *J. Virol.* **61**:2024–2028.
11. Kodama, T., D. P. W. Burns, D. P. Silva, F. D. Veronese, and R. C. Desrosiers. 1991. Strain-specific neutralizing determinant in the transmembrane protein of simian immunodeficiency virus. *J. Virol.* **65**:2010–2018.
12. Marthas, M. L., S. Sutjipto, J. Higgins, B. Lohman, J. Torten, P. A. Luciw, P. A. Marx, and N. C. Pedersen. 1990. Immunization with a live, attenuated simian immunodeficiency virus (SIV) prevents early disease but not infection in rhesus macaques challenged with pathogenic SIV. *J. Virol.* **64**:3694–3700.
13. McKeating, J. A., A. McKnight, K. McIntosh, P. R. Clapham, C. Mulder, and R. A. Weiss. 1989. Evaluation of human and simian immunodeficiency virus plaques and neutralization assays. *J. Gen. Virol.* **70**:3327–3333.
14. Murphey-Corb, M., L. N. Martin, B. Davison-Fairburn, R. C. Montelaro, M. Miller, M. West, S. Ohkawa, G. B. Baskin, J.-Y. Zhang, S. D. Putney, A. C. Allison, and D. A. Eppstein. 1989. A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* **246**:1293–1297.
15. Nakane, P. K., and A. Kawaoi. 1974. Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* **22**:1084–1091.
16. Nara, P. L., W. C. Hatch, N. M. Dunlop, W. G. Robey, L. O. Arthur, M. A. Gonda, and P. J. Fischinger. 1987. Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. *AIDS Res. Hum. Retroviruses* **3**:283–302.
17. Rasheed, S., G. L. Norman, P. S. Gill, P. R. Meyer, L. Cheng, and A. M. Levine. 1986. Virus-neutralizing activity, serologic heterogeneity, and retrovirus isolation from homosexual men in the Los Angeles area. *Virology* **150**:1–9.
18. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**:493–497.
19. Salter, R. D., D. N. Howell, and P. Cresswell. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* **21**:235–246.
20. Stromberg, K., R. E. Benveniste, L. O. Arthur, H. Rabin, W. E. Giddens, Jr., H. D. Ochs, R. W. Morton, and C.-C. Tsai. 1984. Characterization of exogenous type D retrovirus from a fibroma of a macaque with simian AIDS and fibromatosis. *Science* **224**:289–291.
21. Sutjipto, S., T. Kodama, J. Yee, A. Gettie, M. Jennings, R. C. Desrosiers, and P. A. Marx. 1990. Characterization of monoclonal antibodies that distinguish simian immunodeficiency virus isolates from each other and human immunodeficiency virus types 1 and 2. *J. Gen. Virol.* **71**:247–249.
22. Sutjipto, S., N. C. Pedersen, C. J. Miller, M. B. Gardner, C. V. Hanson, A. Gettie, M. Jennings, J. Higgins, and P. A. Marx. 1990. Inactivated simian immunodeficiency virus vaccine failed to protect rhesus macaques from intravenous or genital mucosal infection but delayed disease in intravenously exposed animals. *J. Virol.* **64**:2290–2297.
23. Vujcic, L., D. Katzenstein, M. Martin, G. Quinnan, and Collaborating Laboratories. 1990. International collaborative study to compare assays for antibodies that neutralize human immunodeficiency virus. *AIDS Res. Hum. Retroviruses* **6**:847–853.
24. Weber, J., and G. Schild. 1989. Meeting report, World Health Organization workshop on the measurement and significance of neutralizing antibody to HIV and SIV. *AIDS* **3**:109–111.
25. Welch, M. J. (University of Washington). 1991. Personal communication.
26. World Health Organization Global Programme on AIDS. 1990. Report of a WHO workshop on the measurement and significance of neutralizing antibody to HIV and SIV, London, 3–5 October, 1988. *AIDS* **4**:269–275.