

Passive Hemagglutination Test for Detection of Antibodies to Human Immunodeficiency Virus Type 1 and Comparison of the Test with Enzyme-Linked Immunosorbent Assay and Western Blot (Immunoblot) Analysis

M. B. VASUDEVACHARI,¹ K. W. UFFELMAN,¹ T. C. MAST,¹ R. L. DEWAR,¹ V. NATARAJAN,¹
H. C. LANE,² AND N. P. SALZMAN^{1*}

*Division of Molecular Virology and Immunology, Georgetown University School of Medicine, Washington, D.C. 20007,¹
and Laboratory of Immunoregulation, National Institutes of Health, Bethesda, Maryland 20892²*

Received 17 June 1988/Accepted 29 September 1988

A passive hemagglutination test (PHA) was developed for detecting antibodies to human immunodeficiency virus type 1 (HIV-1) utilizing sheep erythrocytes cross-linked with purified envelope glycoprotein (gp160) of HIV-1. In an analysis of 216 human serum samples, 100% correlation was observed in 86 reactive and 124 nonreactive serum samples between PHA and commercial enzyme-linked immunosorbent assays and Western blot (immunoblot) analysis. Serum samples from gp160-immunized chimpanzees also reacted equally well in PHA. The test is simple, rapid, and inexpensive, thus providing an alternate, quick method of detecting HIV antibodies. These advantages and the thermal stability of the reagents that are used make this an attractive alternative for detecting prior exposure of individuals to HIV-1.

During the last 6 years, acquired immunodeficiency syndrome, which is caused by human immunodeficiency virus type 1 (HIV-1) (1, 14, 17), has drawn worldwide attention. The virus is transmitted through transfusion of blood and blood products (5) as well as through sexual contact (10, 20). Serological tests such as enzyme immunoassays (3, 13, 15, 23), Western blots (immunoblots) (13, 21), and radioimmuno-precipitation assays (12) to detect the presence of viral antigens or virus-specific antibodies or both have been useful in the diagnosis of HIV-1 infection. Most of these tests offer the advantage of high sensitivity but have disadvantages such as requiring expensive kits and long periods of time for running tests and the need for types of instrumentation which may not be available in some laboratories. Recently, agglutination tests with either latex beads (18) or gelatin particles (4, 8, 24) have been described which overcome some of these problems. However, the passive hemagglutination test (PHA) described here not only eliminates these problems but is specific for HIV-1 and has the added advantages of being rapid, simple, highly sensitive, and easy to read. PHA is especially useful in situations in which the time and technology are not available for more sophisticated tests, but it should be stressed that the PHA gives data which are as reliable as those from widely used commercial tests.

Sheep erythrocytes (RBC) were cross-linked to HIV-1 envelope glycoprotein as described by Petchclai and co-workers (16). In brief, sheep RBC collected in Alsever solution (Environmental Diagnostics, Burlington, N.C.) were washed three times in phosphate-buffered saline (pH 7.4) (PBS) and stabilized by adding 1.2 ml of PBS and 0.25 ml of 0.6% glutaraldehyde (grade I; Sigma Chemical Co., St. Louis, Mo.) in water for every 0.1 ml of packed RBC and rotating the mixture at room temperature for 2 h. After the RBC were washed in PBS, a 2.5% suspension was made in PBS, mixed with an equal volume of a 1:20,000 solution of tannic acid in PBS, incubated for 30 min at 37°C, and washed

three times in PBS. A 2.5% tanned RBC suspension in PBS (pH 6.4) was cross-linked to purified HIV-1 envelope glycoprotein (gp160) obtained from recombinant baculovirus vector-infected cells (MicroGeneSys, Inc., West Haven, Conn.) by incubating the two together for 30 min at 37°C and washing the mixture three times in PBS to remove uncoupled antigen. The RBC were finally suspended in PBS containing 0.5% bovine serum albumin (recrystallized; ICN Pharmaceuticals, Inc., Irvine, Calif.). The optimum concentration of gp160 was determined by checkerboard titration, and we found that 20 µg/ml gave a good hemagglutination pattern. The PHA was done in U-bottom microdilution plates (Immulon-1; Dynatech Laboratories, Inc., Alexandria, Va.) with PBS containing 0.5% bovine serum albumin and 0.1% sodium azide as the serum diluent. With the initial titration of several serum samples, an optimal dilution of 1:2,000 was determined. Subsequently, all the samples showing a clear (100%) agglutination pattern at 1:2,000 dilution were scored positive. Bovine serum albumin-coupled RBC served as negative controls. The PHA reaction was carried out at room temperature for 1 h. A typical PHA titration of four samples is shown in Fig. 1.

Enzyme-linked immunosorbent assays (ELISAs; Du Pont Co., Wilmington, Del.; Abbott Laboratories, North Chicago, Ill.) and Western blot assays (Bio-Rad Laboratories, Richmond, Calif.) were done as specified in the instructions of the manufacturers, and the cutoff values for ELISAs were determined as described in their instructions to score positive samples.

A total of 216 serum samples were used in this study to compare the PHA with commercial ELISAs and Western blot analysis. The results are presented in Table 1. It was observed that there was a 100% correlation in 86 HIV-1-reactive samples when comparing PHA, Dupont ELISA, and Abbott ELISA. When these 86 samples were characterized by Western blots, 80 reacted with almost all the readily identifiable HIV-1 proteins (gp160, gp120, p65, p55, p51, gp41, p32, p24, and p18) and the remaining 6 showed strong

* Corresponding author.

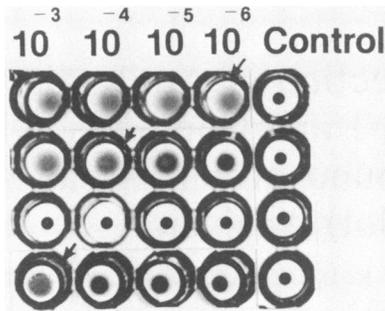


FIG. 1. Representative titration pattern of sera by PHA. Arrows indicate the highest dilution of serum giving a positive agglutination pattern. The sample in row 3 (from the top) is a negative serum. The last well in each row (control) contains a 10^{-3} dilution of the respective serum to which RBC coupled with bovine serum albumin are added.

reactivity to envelope proteins (gp160, gp120, and gp41) and very weak reactivity to core and reverse transcriptase proteins. The Dupont ELISA reacted with an additional two (0.9%) serum samples which contained antibodies only to either p24 or p18 as demonstrated in Western blots. Four (1.8%) additional serum samples were positive by Abbott ELISA exclusively. None of these four serum samples reacted with any viral proteins in Western blots, suggesting that they were indeed false-positives. In addition, 12 (5.6%) serum samples which were negative by the PHA and Dupont and Abbott ELISAs showed a very weak reactivity to either p24 (9 serum samples) or p18 (3 serum samples) protein in Western blots. The presence of antibodies to p24 and p18 has been seen in serum samples from individuals with other viral diseases and may relate to cross-reacting epitopes (2, 19). The remaining 112 samples were negative by all the tests.

A comparison was made to analyze the sensitivity of antibody detection in these tests. Thirty-two positive serum samples were serially 10-fold diluted before being tested for antibodies. The results shown in Fig. 2 indicate that the PHA was highly sensitive and was able to detect antibodies in more serum samples at a 10^{-5} serum dilution than the Abbott and Dupont ELISAs. The Dupont ELISA was the least sensitive of the three tests.

To test the reproducibility of the PHA, a set of 40 coded serum samples were independently assayed for antibodies by the PHA by three investigators. There was a 100% correlation between the results obtained. Freezing and thawing of serum samples for four cycles did not change the results of the PHA. The addition of 0.5% Nonidet P-40, a common detergent used to inactivate HIV-1, did not alter the readability or the titer of the PHA (data not shown). A

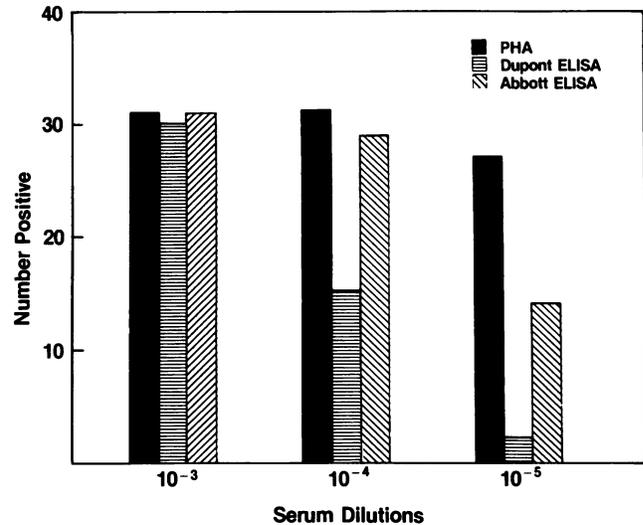


FIG. 2. Comparison of sensitivities of PHA, Dupont ELISA, and Abbott ELISA. Serum samples that remain positive by each test at 10^{-3} , 10^{-4} , and 10^{-5} dilution are compared.

prozone effect is frequently encountered at lower dilutions of serum samples in the PHA (9); however, it was easily eliminated by diluting the samples to 1:2,000.

Currently, purified envelope proteins are being tested as vaccines against HIV-1 infection in humans. In our present study, the PHA was successfully used to demonstrate the immune response in chimpanzees which had been immunized with the envelope proteins that are currently being tested in human vaccine trials. Eighty serum samples periodically collected over a 10-month period from chimpanzees immunized with gp160 (one placebo-inoculated and two gp160-inoculated animals) were tested for antibodies by the PHA, Dupont and Abbott ELISAs, and Western blots. Of the 80 samples, 28 were found to be positive in the PHA and each reacted with envelope proteins in Western blots, whereas only 4 of the 80 and 16 of the 80 were tested positive by the Dupont and Abbott ELISAs, respectively. The initial selection of HIV-1 gp160 as the antigen in our PHA was based on observations in previous studies (6, 7, 11, 22) in which it was demonstrated that the antibodies to envelope proteins (particularly to gp41) are more prevalent and persist for longer periods than do those to core proteins.

The present PHA is specific, and its sensitivity is comparable to or higher than that of two widely used commercial ELISAs. The procedure for the PHA is much simpler than that for the ELISA and involves a one-step reaction of antigen and antibody and requires no washing steps, thereby

TABLE 1. Comparison of PHA with ELISAs and Western blot assay

PHA	No. of samples positive		Total no. of samples	Western blot assay comments
	Dupont ELISA	Abbott ELISA		
80	80	80	80	Showed specific reactivity to most of the envelope core and reverse transcriptase proteins.
6	6	6	6	Showed strong reactivity to envelope and very weak reactivity (if any) to core and reverse transcriptase proteins.
0	1	0	1	Strong reactivity only to p24.
0	1	0	1	Strong reactivity specific to p18.
0	0	4	4	No reactivity to any HIV proteins.
0	0	0	12	Weak reactivity to either p24 or p18 protein.
0	0	0	112	No reactivity to any HIV-1 proteins.

requiring much shorter times as compared with the other available tests. Coupled RBC can be stored frozen or lyophilized for prolonged periods. Thus, the PHA should be suitable for mass screening of sera, particularly in developing countries.

We thank Gale Smith, MicroGeneSys, Inc., for providing recombinant HIV-1 gp160 and Amy Mannarelli for excellent assistance in the preparation of the manuscript.

LITERATURE CITED

1. Barre-Sinoussi, F., F. C. Chermann, F. Rey, M. T. Nugeyre, S. Charnaret, J. Gruest, C. Duguet, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphocyte retrovirus from a patient at risk for acquired immunodeficiency syndrome (AIDS). *Science* **220**:868-871.
2. Biberfeld, G., U. Bredberg-Raden, B. Bottiger, P. Putkonen, J. Blomberg, P. Juto, and G. Wadell. 1986. Blood donor sera with false-positive Western blot reactions to human immunodeficiency virus. *Lancet* **ii**:289-290.
3. Cao, Y., F. Valentine, S. Hojvat, J.-P. Allain, P. Rubinstein, M. Mirabile, S. Czelusniak, M. Leuther, L. Baker, and A. E. Friedman-Kien. 1987. Detection of HIV antigen and specific antibodies to HIV core and envelope proteins in sera of patients with HIV infection. *Blood* **70**:575-578.
4. Crofts, J. N., W. J. Maskill, D. S. Healy, and I. D. Gust. 1987. Particle agglutination assay for anti-HIV. *Lancet* **ii**:797-798.
5. Feorino, P. M., V. S. Kalyanaraman, H. W. Haverkos, C. D. Cabradilla, D. T. Warfield, H. W. Jaffe, A. K. Harrison, M. S. Gottlieb, D. Goldfinger, J. C. Cherman, F. Barre-Sinoussi, T. T. Spira, J. S. McDougal, J. W. Curran, L. Montagnier, F. A. Murphy, and D. P. Francis. 1984. Lymphadenopathy associated virus infection of a blood donor-recipient pair with acquired immunodeficiency syndrome. *Science* **225**:69-72.
6. Gnann, J. W., Jr., P. L. Schwimmbeck, J. A. Nelson, A. B. Truax, and M. B. A. Oldstone. 1987. Diagnosis of AIDS by using a 12-amino acid peptide representing an immunodominant epitope of the human immunodeficiency virus. *J. Infect. Dis.* **156**:261-267.
7. Hofbauer, J. M., T. F. Schultz, P. Hengster, C. Larcher, R. Zangerle, H. Kofler, P. Fritsch, H. Wachter, and M. P. Dierich. 1988. Comparison of Western blot (immunoblot) based on recombinant-derived p41 with conventional tests for serodiagnosis of human immunodeficiency virus infections. *J. Clin. Microbiol.* **26**:116-120.
8. Ikeda, M., R. Funjino, T. Matsui, T. Yoshida, H. Komoda, and J. Imai. 1984. A new agglutination test for serum antibodies to adult T-cell leukemia virus. *Jpn. J. Cancer Res.* **75**:845-848.
9. Imrie, A., S. Best, P. Ronald, and D. A. Cooper. 1987. False-negative ELISA results for HIV antibody due to an apparent prozone phenomenon. *Med. J. Aust.* **147**:42.
10. Kalyanaraman, V. S., C. D. Cabradilla, J. P. Getschell, R. Narayan, E. H. Braff, J. C. Chermann, F. Barre-Sinoussi, L. Montagnier, T. J. S. Kaplan, D. F. H. W. Jaffe, and D. P. Francis. 1984. Antibodies to the core protein of lymphadenopathy-associated virus (LAV) in patients with AIDS. *Science* **225**:321-323.
11. Kenealy, W., D. Reed, R. Cybulski, D. Tribe, P. Taylor, C. Stevens, T. Matthews, and S. Petteway. 1987. Analysis of human serum antibodies to human immunodeficiency virus (HIV) using recombinant ENV and GAG antigens. *AIDS Res. Hum. Retroviruses* **3**:95-105.
12. Kitchen, L. W., F. Barin, J. L. Sullivan, M. F. McLane, D. B. Brettler, P. H. Levine, and M. Essex. 1984. Aetiology of AIDS-antibodies to human T-cell leukemia virus (type III) in hemophiliacs. *Nature (London)* **312**:367-369.
13. Lelie, P. N., H. W. Reesink, and J. G. Huisman. 1987. Earlier detection of HIV and second-generation antibody assays. *Lancet* **ii**:343.
14. Levy, J. A., A. D. Hoffmann, S. M. Dramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytotropic retroviruses from San Francisco patients with AIDS. *Science* **225**:840-842.
15. Mortimer, P. P., J. V. Parry, and J. Y. Mortimer. 1985. Which anti-HTLV-III assays for screening and confirmatory testing? *Lancet* **ii**:873-877.
16. Petchclai, B., K. Khupulsup, C. W. Wasi, and N. Bhamarapravati. 1986. Passive hemagglutination test for antibody to hepatitis B core antigen. *J. Clin. Microbiol.* **24**:642-643.
17. Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo. 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**:497-500.
18. Riggan, C. H., G. A. Beltz, C. H. Hung, R. M. Thorn, and D. J. Marciani. 1987. Detection of antibodies to human immunodeficiency virus by latex agglutination with recombinant antigen. *J. Clin. Microbiol.* **25**:1772-1773.
19. Saag, M. S., and J. Britz. 1986. Asymptomatic blood donor with a false positive HTLV-III Western blot. *N. Engl. J. Med.* **314**:118.
20. Sarngadharan, M. G., M. Popovic, L. Bruch, J. Schüpbach, and R. C. Gallo. 1984. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* **224**:506-508.
21. Schüpbach, J., M. Popovic, R. V. Gilden, M. A. Gonda, M. G. Sarngadharan, and R. C. Gallo. 1984. Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* **224**:503-505.
22. Thorn, R. M., G. A. Beltz, C.-H. Hung, B. F. Fallis, S. Winkle, K.-L. Cheng, and D. J. Marciani. 1987. Enzyme immunoassay using a novel recombinant polypeptide to detect human immunodeficiency virus *env* antibody. *J. Clin. Microbiol.* **25**:1207-1212.
23. Viscidi, R., H. Farzadegan, F. Leister, M. L. Francisco, and R. Yolken. 1988. Enzyme immunoassay for detection of human immunodeficiency virus antigens in cell cultures. *J. Clin. Microbiol.* **26**:453-458.
24. Yoshida, T., T. Matsui, S. Kobayashi, S. Harada, T. Kurimura, Y. Hinuma, and N. Yamamoto. 1986. A novel agglutination test for the human immunodeficiency virus antibody: a comparative study with enzyme-linked immunosorbent assay and immunofluorescence. *Jpn. J. Cancer Res.* **77**:1211-1213.

Letter to the Editor

Passive Hemagglutination Test for Detection of Antibodies to Human Immunodeficiency Virus Type 1 in Developing Countries

The report on the passive hemagglutination test for antibodies to human immunodeficiency virus type 1 (HIV-1) using sheep erythrocytes cross-linked to purified HIV-1 envelope glycoprotein gp160 (3) is encouraging, with a single-step reaction not involving any washing. Nevertheless, a 100% correlation in 86 HIV-1-reactive and 124 non-reactive serum specimens with enzyme-linked immunosorbent assay and immunoblot analysis on Western blotting should not per se lead to universal acceptance of the passive hemagglutination test for mass screening for HIV-1 antibodies in vast developing areas in Africa, Asia, and Latin America, where the performance of the otherwise efficient vaccines has continued to be dismal. In an appraisal of widespread outbreaks of poliomyelitis in 1986 in Senegal and The Gambia, the overall efficacies of the live and high-potency inactivated polio vaccines were 74 and 69%, respectively (2), although in developed countries both have been nearly 100% efficient for almost three decades. Ambient temperatures far exceeding 40°C, marked diurnal variation in sunlight, drought, and humidity are the rule rather than the exception in such areas and have been incriminated in the continuing failures of vaccines (1). Similar mishaps with a promising diagnostic test, such as passive hemagglutination, for HIV-1 (3) in such areas can be prevented by deliberately subjecting the cross-linked lyophilized erythrocytes to rough and careless handling, mimicking every conceivable rigor of the field. Only the maintenance of the excellent sensitivity and specificity of the cross-linked erythrocytes (3) during such rough and rigorous handling could be expected to prevent any unpredicted disasters during their use in blood donor screening for HIV-1 in developing countries.

Diagnostic reagents for HIV-1 or HIV-2 meant for use in areas with extremely inadequate storage and transportation facilities should as well be carried to different remote locations with the most unsatisfactory environments. Retrieval of reagents for reassessment of their sensitivity and specificity could be expected to help prevent, by appropriate

technological modifications, any unexpected failures in diagnosis of the presence of HIV infection in blood banks, hospitals, and public health laboratories located in areas with the least possible facilities for storage and technical assistance.

LITERATURE CITED

1. **Arya, S. C.** 1988. Can the new polio vaccines take the heat? *Nature* (London) **332**:789.
2. **Third International Seminar on Immunization in Africa.** 1987. Report. *World Immunization News* **3**:7-10.
3. **Vasudevachari, M. B., K. W. Uffelman, T. C. Mast, R. L. Dewar, V. Natarajan, H. C. Lane, and N. P. Salzman.** 1989. Passive hemagglutination test for detection of antibodies to human immunodeficiency virus type 1 and comparison of the test with enzyme-linked immunosorbent assay and Western blot (immunoblot) analysis. *J. Clin. Microbiol.* **27**:179-181.

Subhash C. Arya
Ved P. Pathak
Syed K. Ashraf
King Fahad Central Hospital
Gizan, Saudi Arabia

Author's Reply

We agree with Dr. Arya's comments and plan to evaluate the stability of all of the components in the test that we have described. This is essential to assure that the same reliability that we observed in the laboratory is maintained under field conditions. We have established that the stability of the reagents described in our article is greater than that of the two commercial tests with which it was compared. Recently, we have been able to demonstrate that whole blood and saliva, as well as serum, can be tested for human immunodeficiency virus antibodies by passive hemagglutination, significantly increasing the scope and value of the test.

Norman P. Salzman
Division of Molecular Virology and Immunology
Georgetown University School of Medicine
Washington, D.C. 20007