JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1992, p. 1005–1007
0095-1137/92/041005-03$02.00/0

Vol. 30, No. 4

Highly Sensitive Enhanced Chemiluminescence Immunodetection Method for Herpes Simplex Virus Type 2 Western Immunoblot

JULIE DALESSIO AND RHODA ASHLEY*

Virology Division, ZC-10, Department of Laboratory Medicine, University of Washington, Seattle, Washington 98195

Received 16 August 1991/Accepted 30 December 1991

Three Western blot (immunoblot) methods for detecting antibodies to herpes simplex virus type 2 were compared: (i) nitrocellulose blots with 4-chloro-1-naphthol immunostaining (4CN-WB); (ii) polyvinylidene difluoride (PVDF) blots with 3,3′,5,5′-tetramethylbenzidene immunostaining (TMB-WB); and (iii) PVDF blots with enhanced chemiluminescence (ECL-WB). TMB-WB was 10-fold more sensitive than 4CN-WB, while ECL-WB was as much as 500-fold more sensitive.

Western blotting (immunoblotting) is a sensitive and specific method to detect and subtype antibodies to herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) in humans (3, 7, 9, 13, 15) and in animal models (8). Western blotting is particularly useful for identifying HSV-2 antibodies in patients with prior antibodies to HSV-1 (1, 2, 10). However, patients with culture-proven primary HSV infections may remain seronegative by Western blot for several weeks after onset of symptoms (7, 17). Antiviral chemotherapy may prolong detectable HSV seroconversion when administered during first episodes (4, 6, 11) and can diminish levels of antibodies when used for chronic suppression of recurrent genital herpes (14). To increase the sensitivity of HSV Western blot serology, we developed two alternative blotting and immunodetection techniques and compared them with our standard assay.

Western blot methods. The standard Western blot system (4CN-WB) was used as described previously (5, 7). It employed 75 μl of denatured proteins from HSV-2-infected human diploid fibroblasts per gel. Separated proteins were transferred to nitrocellulose (NC; Schleicher & Schuell, Keene, N.H.). NC strips were reacted with human serum diluted 1:50 in BLOTTO (5% nonfat milk in phosphate-buffered saline [PBS]) and, after being washed, were reacted with goat anti-human immunoglobulin G(γ)-peroxidase (diluted 1:1,000; Boehringer Mannheim, Indianapolis, Ind.). 4-Chloro-1-naphthol (4CN) substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used as directed by the manufacturer for immunodetection.

A second test system, TMB-WB, used 15 μl of HSV-2-infected cell proteins per gel. Separated proteins were transferred to polyvinylidene difluoride (PVDF; Immobilon P, Millipore, Bedford, Mass.) rather than to NC. PVDF strips were moistened with methanol and then blocked with 0.5% Tween 20 (Sigma, St. Louis, Mo.) in PBS for 1 h. Human serum was diluted 1:1,000 in 4% goat serum (in PBS) and reacted as in the 4CN-WB method; secondary antibody was then added at a 1:10,000 dilution. The 3,3′,5,5′-tetramethylbenzidene (TMB) peroxidase substrate system was prepared and applied to blots as directed by the manufacturer (Kirkegaard & Perry).

The third system, enhanced chemiluminescence Western blot (ECL-WB), was like TMB-WB except that after incubation with the secondary antibody, the ECL Western blotting detection system (Amersham, Arlington Heights, Ill.) was added to the blots as directed by the manufacturer and reacted for 1 min. Strips were then covered with plastic wrap and exposed to X-ray film (Hyperfilm-ECL; Amersham) for 30 s or as specified below. Film was developed in a Kodak X-Omat processor.

NC or PVDF control blots with proteins from mock-infected cells were prepared along with blots containing proteins from HSV-2-infected cells. Control blots were reacted in parallel in each experiment to determine background staining with each method. Mouse monoclonal antibodies to gG-2 (kindly provided by N. Balachandran, University of Kansas, Kansas City) and VP16 (kindly provided by A. Minson, University of Cambridge, Cambridge, United Kingdom) were used to identify reactive bands.

Sensitivity of 4CN-WB, TMB-WB, and ECL-WB. A pool of human serum samples from patients with culture-documented HSV-2 infections was diluted by factors of 500 to 200,000 in the appropriate diluents for the three Western blot systems. 4CN-WB, TMB-WB, and ECL-WB were then performed on the diluted preparations. The resulting profiles were scored by using previously established diagnostic criteria for HSV-2 seropositivity, which include the presence of a band corresponding to gG-2 and at least three other bands which appear on the HSV-2 Western blot but not on the blot with uninfected cell proteins (5). These criteria were met by a 1:1,000 dilution with 4CN-WB and 1:10,000 dilutions with TMB-WB and ECL-WB (Fig. 1). Little or no reactivity against the blots with mock-infected cell proteins was observed at any dilution. Furthermore, little or no reactivity with sera from patients without antibodies to HSV was observed at these dilutions (data not shown).

The sensitivity of ECL-WB can be increased by exposing the strips to X-ray film for longer periods. To illustrate this point, a 1:500,000 dilution of the HSV-2-positive serum pool was subjected to ECL-WB, and the strips were exposed for increasing times: 30 s, 5 min, 15 min, 30 min, and 1 h (Fig. 2). While antibodies were not detected with serum dilutions of ≥1:200,000 after a 30-s exposure (Fig. 1), a full profile of antibodies could be detected in the 1:500,000 dilution after a 1-h exposure. Antibodies to major immunogens were detected after only 15 min.

Detection of seroconversion to HSV-2. Serum samples from 27 patients who were treated with acyclovir for culture-documented first-episode primary HSV-2 genital infections

* Corresponding author.

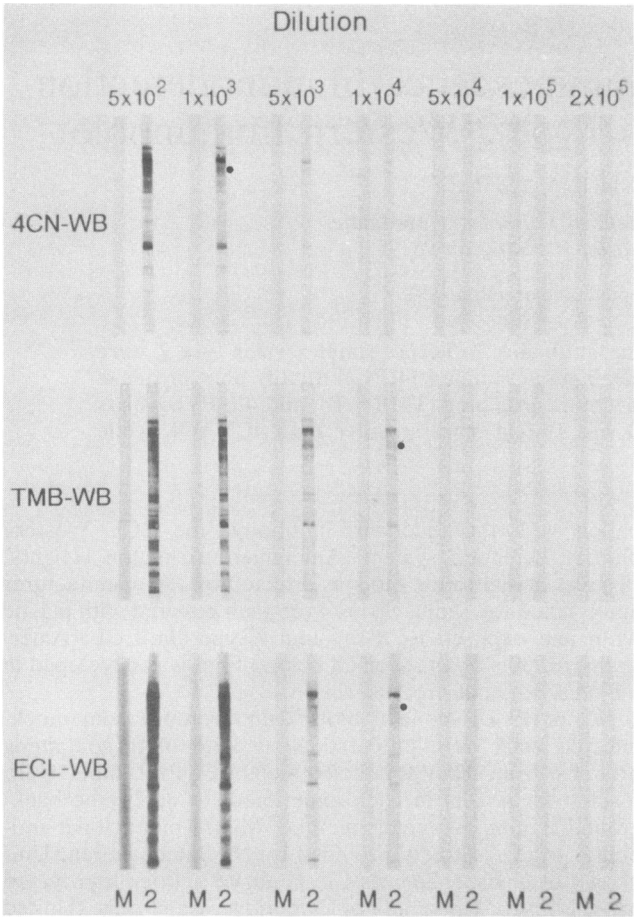

FIG. 1. Sensitivity of three Western blot systems. A human serum pool containing antibodies to HSV-2 was diluted as indicated and tested by 4CN-WB, TMB-WB, or ECL-WB against mock-infected (lanes M) or HSV-2-infected (lanes 2) cell proteins. Bands identified as gG-2 are indicated at the endpoint dilutions with a closed circle.

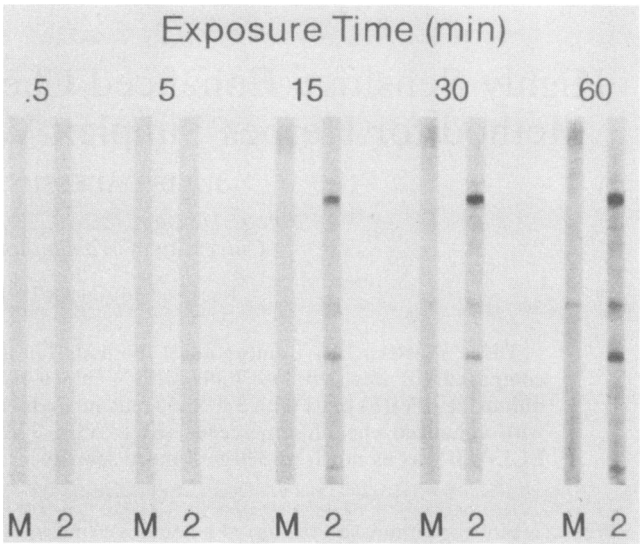

FIG. 2. Increased sensitivity of ECL-WB with increased film exposure time. A human serum pool containing antibodies to HSV-2 was diluted 1:500,000 and tested by ECL-WB against mock-infected (lanes M) or HSV-2-infected (lanes 2) cell proteins. Resulting blots were exposed for the times indicated.

were collected at 0 to 7 days (acute phase) and at 20 to 29 days (convalescent phase) after onset. All sera were tested in parallel by the three methods. Antibody profiles were interpreted by using standard diagnostic criteria (5) without knowledge of the clinical status or duration of infection of any of the patients. 4CN-WB detected antibodies diagnostic for HSV-2 in 5 (19%) of 27 acute-phase serum samples and in 26 (96%) of 27 convalescent-phase serum samples. TMB-WB detected antibodies diagnostic for HSV-2 in 9 (33%) of 27 acute-phase serum samples and in 25 (93%) of 27 convalescent-phase serum samples. By chi-square analysis, 4CN-WB and TMB-WB did not differ significantly in sensitivity with either acute- or convalescent-phase sera. ECL-WB was positive for 17 (63%) of 27 acute-phase serum samples and for 27 (100%) of 27 convalescent-phase serum samples. ECL-WB sensitivity for acute-phase sera was significantly greater than that of either 4CN-WB ($P < 0.01$) or TMB-WB ($P < 0.05$). No significant differences in sensitivity of the three tests were noted when convalescent-phase sera were considered.

This comparison study showed that TMB-WB and ECL-WB were substantially more sensitive than 4CN-WB in detecting antibodies to HSV-2 in diluted, pooled sera. In dilution studies, TMB-WB and ECL-WB detected antibody at 10-fold-lower concentrations than did 4CN-WB. With prolonged exposure time, the ECL-WB could detect antibodies in serum that had been diluted 500,000-fold, a 500-fold increase in sensitivity over 4CN-WB. ECL-WB was significantly more sensitive than either 4CN-WB or TMB-WB in detecting antibodies within the first week after onset of HSV-2 infection in patients undergoing seroconversion.

Although Western blotting is not in wide use for diagnostic serology, the 4CN-WB method has been an important tool for serotyping HSV antibodies in large-scale seroepidemiology studies and vaccine trials (12, 16, 18). TMB-WB would be particularly useful for these types of studies because the enhanced binding ability of PVDF over NC and the improved staining sensitivity of TMB over 4CN result in lower reagent costs and reduced preparation time for large-scale antigen lots. TMB-WB requires 5-fold less antigen, 20-fold less serum, and 10-fold less secondary antibody than 4CN-WB.

ECL-WB produces a durable X-ray film image which does not fade, discolor, or break with storage. ECL-WB is clearly more sensitive than either 4CN-WB or TMB-WB; full antibody profiles were demonstrated with sera diluted by as much as 500,000-fold. However, ECL-WB may not be practical for large-scale screening applications because of the relatively high cost of the ECL reagent and the need for X-ray film, dark room, and developing equipment. Best results are obtained by ECL-WB when antigen concentration, serum dilution, and exposure time are adjusted for individual samples. The optimal exposure time for clear and readable profiles depends on the extent of antigen-antibody reaction on the Western blot which, in turn, is affected by the relative concentrations of the reactants. We recommend that sera initially be tested under the conditions specified here (15 μl of antigen preparation per gel and a sample dilution of 1:1,000) and that film of the ECL reaction be exposed for three different lengths of time; 5, 10, and 30 s. If

sensitivity is too high at all exposure times and bands are not resolved for readable profiles, the specimen and/or antigen concentrations should be increased and the ECL development time can also be extended. Under these conditions, ECL-WB provides a powerful analytic tool for identifying low concentrations of both antibodies and proteins. Application of this technique to HSV and other viruses should be useful for the study of both immunogenic proteins and the related immune responses.

This work was supported by grant NIH AI 20381.

REFERENCES

1. **Ashley, R., and J. Militoni.** 1987. Use of densitometric analysis for interpreting HSV serologies based on Western Blot. J. Virol. Methods **18**:159–168.
2. **Ashley, R. L.** 1989. Genital herpes infections. Clin. Lab. Med. **9**:405–420.
3. **Ashley, R. L., A. Cent, V. Maggs, and L. Corey.** 1991. Inability of a commercial enzyme immunoassay to accurately identify herpes simplex types 1 or 2 infection. Ann. Intern. Med. **115**:520–526.
4. **Ashley, R. L., and L. Corey.** 1984. Effect of acyclovir treatment of primary genital herpes on the antibody response to herpes simplex virus. J. Clin. Invest. **73**:681–688.
5. **Ashley, R. L., and L. Corey.** 1989. Herpes simplex viruses and B virus, 6th edition, p. 265–317. *In* N. J. Schmidt and R. Emmons (ed.), Diagnostic procedures for viral, rickettsial, and chlamydial infections. American Public Health Association, Washington, D.C.
6. **Ashley, R. L., K. Mack, M. Shurtleff, C. Critchlow, and L. Corey.** 1988. Differential effect of systemic acyclovir treatment of genital HSV-2 infections on antibody responses to individual HSV-2 proteins. J. Med. Virol. **24**:309–320.
7. **Ashley, R. L., J. Militoni, F. Lee, A. Nahmias, and L. Corey.** 1988. Comparison of Western blot (immunoblot) and glycoprotein G-specific immunodot enzyme assay for detecting antibodies to herpes simplex virus types 1 and 2 in human sera. J. Clin. Microbiol. **26**:662–667.
8. **Bernstein, D. I., R. L. Ashley, L. Stanberry, and M. Myers.** 1990. Detection of asymptomatic initial HSV infections in animals previously immunized with subunit herpes simplex virus glycoprotein vaccines. J. Clin. Microbiol. **28**:11–15.
9. **Bernstein, D. I., Y. Bryson, and M. Lovett.** 1985. Antibody response to type-common and type-unique epitopes of herpes simplex virus polypeptides. J. Med. Virol. **15**:251–263.
10. **Bernstein, D. I., M. A. Lovett, and Y. J. Bryson.** 1984. Serologic analysis of first episode nonprimary genital herpes simplex virus infection. Am. J. Med. **77**:1055–1060.
11. **Bernstein, D. I., M. A. Lovett, and Y. J. Bryson.** 1984. The effects of acyclovir on antibody response to herpes simplex virus in primary genital herpetic infections. J. Infect. Dis. **150**:7–13.
12. **Brown, Z. A., J. Benedetti, R. Ashley, S. Burchett, S. Selka, S. Berry, L. A. Vontver, and L. Corey.** 1991. Neonatal herpes simplex virus infection in relation to asymptomatic maternal infection at the time of labor. N. Engl. J. Med. **324**:1247–1252.
13. **Eberle, R., and R. J. Courtney.** 1981. Assay of type-specific and type-common antibodies to herpes simplex virus types 1 and 2 in human sera. Infect. Immun. **31**:1062–1070.
14. **Gold, D., R. L. Ashley, G. Solberg, H. Abbo, and L. Corey.** 1988. Chronic dose acyclovir for suppression of frequently recurring genital herpes simplex virus infection: effect on antibody response to HSV-2 proteins. J. Infect. Dis. **158**:1227–1234.
15. **Kahlon, J., F. D. Lakeman, M. Ackermann, and R. J. Whitley.** 1986. Human antibody response to herpes simplex virus-specified polypeptides following primary and recurrent infection. J. Clin. Microbiol. **23**:725–730.
16. **Koutsky, L., R. L. Ashley, and K. Holmes.** 1990. The frequency of unrecognized type 2 herpes simplex virus infection among women: implications for the control of genital herpes. Sex. Transm. Dis. **17**:90–94.
17. **Lopez, C., A. Arvin, and R. L. Ashley.** Immunity to herpesvirus infection in man. *In* B. Roizman and C. Lopez (ed.), The herpesviruses, in press.
18. **Mertz, G., R. Ashley, R. Burke, J. Benedetti, C. Critchlow, C. Jones, and L. Corey.** 1990. Double-blind, placebo-controlled trial of a herpes simplex virus type-2 glycoprotein vaccine in persons at high risk for genital herpes infection. J. Infect. Dis. **161**:653–660.