

Sequential Detection of Different Antigens Induced by Epstein-Barr Virus and Herpes Simplex Virus in the Same Western Blot by Using Dual Antibody Probes

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A dual antibody probing technique that permitted a color-coded identification of polypeptides representing different classes of Epstein-Barr virus (EBV) antigens as well as differentiation of the polypeptides induced by different herpesviruses in the same Western blot was developed. When the nitrocellulose sheet was probed first with monoclonal antibody against EBV early antigen diffuse component (EA-D) and then stained with 4-chloro-1-naphthol, four polypeptides specific for EA-D were identified by purple bands. Subsequently, the same nitrocellulose sheet was reprobed with human serum containing antibodies against EBV early antigen, viral capsid antigen, and nuclear antigen and stained with 3,3'-diaminobenzidine. Several brown bands corresponding to early, viral capsid, and nuclear antigen polypeptides were detected. The dual antibody probing technique was used in an analysis to differentiate polypeptides resulting from either EBV or herpes simplex virus infection, either in cells infected by individual virus or in a cell line dually infected by both viruses. On the basis of different colored bands in different lanes of the same gel, 20 polypeptides with molecular weights ranging from 31,000 to 165,000 were identified as herpes simplex virus-specific proteins. These results suggested that the dual antibody probing technique may be applicable in clinical diagnosis for detecting antigens and antibodies derived from different pathogens.

Recently we demonstrated the feasibility and usefulness of a Western blotting enzyme-linked immunosorbent technique in the qualitative and quantitative analyses of polypeptides induced by Epstein-Barr virus (EBV) (1). We developed a rapid immunochemical method that allows sequential analysis and detection of different antigens induced by EBV and herpes simplex virus (HSV) by using dual antibody probes. An advantage of this method is that it can identify the polypeptides representing different classes of EBV antigens and also differentiate the antigens resulting from EBV or HSV infection.

The propagation of human lymphoblastoid cell lines and superinfection of Raji cells were described elsewhere (2, 3). Preparation of whole-cell extracts and Western blotting of the separated proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis were described previously (1).

To identify the polypeptides representing different classes of antigen in EBV-infected cells, the nitrocellulose sheet of the Western blot was sequentially probed by reacting with different antibodies. When the sheet was first reacted with monoclonal antibody to early antigen diffuse component (EA-D) by using 4-chloro-1-naphthol as a substrate, polypeptides representing EA-D were identified by purple bands. As reported before (1), a family of four polypeptides with molecular sizes of 46,000, 49,000, 52,000, and 55,000 daltons were expressed in superinfected Raji cells (Fig. 1, lane C, purple bands) but not in mock-infected Raji cells (Fig. 1, lane B), in BJAB (an EBV-negative cell line; Fig. 1, lane A), or in four different cord blood lymphocyte cell lines (Fig. 1, lanes D, E, F, and G) transformed by EBV derived from throat washings of patients with infectious mononucleosis.

Since Raji cells and transformed cord blood lymphocytes contain EBV DNA and express EBV nuclear antigen (EBNA), the same nitrocellulose sheet was reprobed by human early antigen-positive (EA⁺), viral capsid antigen-positive (VCA⁺), EBNA⁺ serum (titers, 1,200:2,500:640) and subsequently stained with 3,3'-diaminobenzidine, which developed brown bands (Fig. 1). Both mock-infected Raji cells (Fig. 1, lane B) and superinfected Raji cells (Fig. 1, lane C) expressed a 68,000-molecular-weight EBNA polypeptide (68K polypeptide). In contrast, different cord blood lymphoblastoid cell lines expressed a variety of EBNA species with a predominant molecular weight of 78,000 (Fig. 1, lanes D, F, and G) and a 76K and 80K doublet (Fig. 1, lane E). In addition, some minor bands around 72K were detectable in lanes E, F, and G. No EBNA polypeptide was detected in BJAB cells (Fig. 1, lane A) or in untransformed cord blood lymphocytes (Fig. 1, lane H). Additional polypeptides (200K, 135K, 110K, 90K, 78K, and 63K) were detected in superinfected Raji cells (Fig. 1, lane C).

The same procedures were used to differentiate antigens induced by different herpesvirus infections. For instance, cell extracts derived from HSV-infected Vero cells and from the EBV-producing cell line P3HR-1 were subjected to electrophoresis in polyacrylamide gels and were Western blotted onto a nitrocellulose sheet. The sheet was first reacted with human serum with EA-VCA-EBNA antibody titers and then incubated with a peroxidase-conjugated second antibody, and the antigenic bands were revealed by staining with 4-chloro-1-naphthol (purple color). Several purple bands specific for EBV-induced polypeptides were observed (Fig. 2, lanes C and D). Two polypeptides with molecular weights above 135,000 were partially inhibited by acyclovir (Fig. 2, lane D), as reported previously (3).

Only a single EBNA polypeptide (68K) was detected in the hybrid cell line D98/Raji (Fig. 2, lane A). The same

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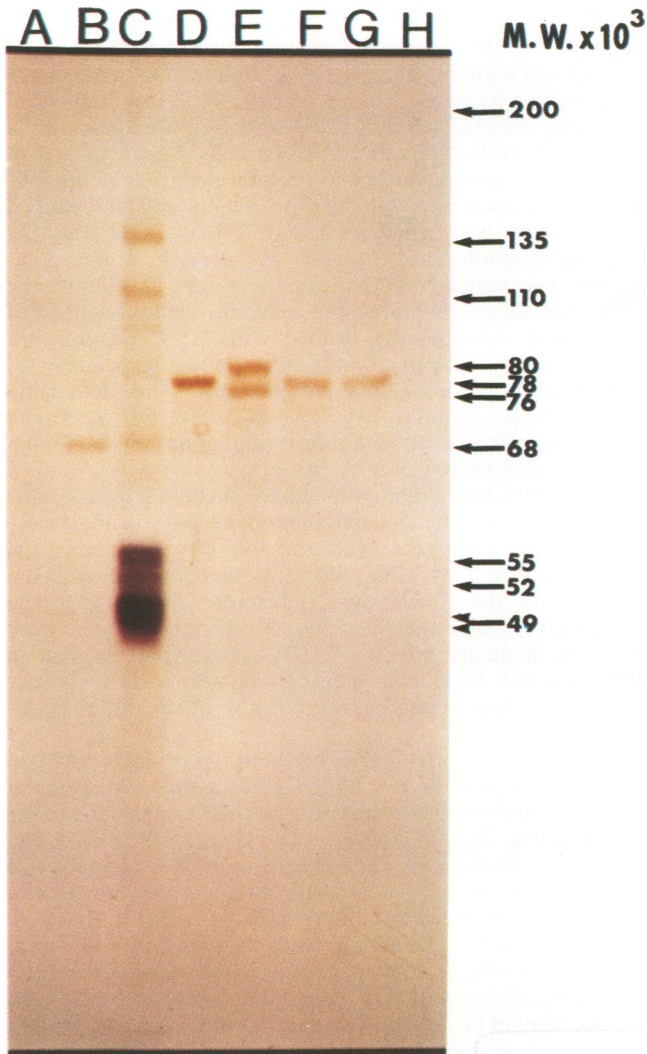


FIG. 1. Simultaneous detection of different classes of EBV-induced polypeptides by dual antibody probes. The nitrocellulose sheet was saturated with protein by incubating for 1 h at 37°C in blocking solution containing phosphate-buffered saline solution (pH 7.4), 3% bovine serum albumin, and 10% heat-inactivated calf serum. The sheet was then incubated for 1 h at 37°C with monoclonal EBV EA-D antiserum, followed by washing in phosphate-buffered saline four times, 15 min each time. The final incubation was for 2 h at room temperature in rabbit anti-mouse immunoglobulin G conjugated with peroxidase (Miles Laboratories, Inc., Elkhart, Ind.). After the sheet was again washed as described above, antigenic bands were stained in 4-chloro-1-naphthol (60 mg in 20 ml of ice-cold methanol), 0.015% H₂O₂, and 100 ml of TBS buffer (20 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl). Purple bands appeared within 20 min after incubation of the mixture at room temperature in the dark. The same nitrocellulose sheet was reprobbed with human serum with antibody titers against EA, VCA, and EBNA. The sheet was first soaked in buffer A, containing 10 mM Tris hydrochloride (pH 7.0), 50 mM NaCl, 2 mM sodium EDTA, 4 M urea, and 0.1 mM dithiothreitol, for 30 min at room temperature. The sheet was then treated with blocking solution for 1 h, followed by reaction with human serum containing EA, VCA, and EBNA antibodies. The sheet was then washed with phosphate-buffered saline as described above. The sheet was then incubated for 2 h at room temperature with goat anti-human immunoglobulin G conjugated with peroxidase. Antigenic bands were then revealed by staining with a different substrate, 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) (1 mg/ml in dimethyl sulfoxide), in 50 mM Tris hydrochloride (pH 7.5) containing 0.015% H₂O₂. Brown

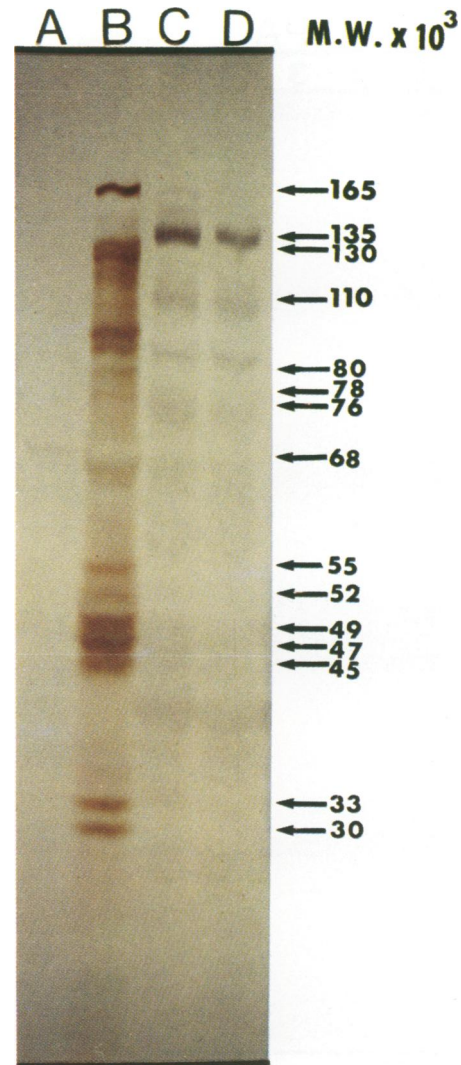


FIG. 2. Identification of EBV- and HSV-induced polypeptides by dual antibody probes. The nitrocellulose sheet of the Western blot was first probed by HSV-specific antibody and stained with brown color substrate. Brown bands represent HSV-specific polypeptides (lane B). Subsequently, the same nitrocellulose sheet was probed by EBV-specific antibody, and the antigenic bands were stained with purple color substrate. Lane A, D98/Raji, a somatic hybrid cell line; lane B, HSV-1 infected Vero cells; lanes C and D, P3HR-1 in the absence and presence of the antiviral drug acyclovir, respectively.

nitrocellulose sheet, after soaking in buffer A (10 mM Tris hydrochloride [pH 7.0], 50 mM NaCl, 2 mM sodium EDTA, 4 M urea, 0.1 mM dithiothreitol) for 30 min, was reprobbed with HSV type 1-specific antiserum (DAKO Corporation, Santa Barbara, Calif.), followed by peroxidase-conjugated second antibody, and was stained with 3,3'-diamino-

bands developed after a few minutes. Lanes: A, BJAB, an EBV-negative cell line; B, mock-infected Raji cells; C, superinfected Raji cells; D, E, F, and G, four different EBV-transformed cord blood cell lines; H, untransformed cord blood lymphocytes. Antiserum had EA, VCA, and EBNA titers of 1,200, 2,500, and 640, respectively; a 10-fold dilution of the serum was used.

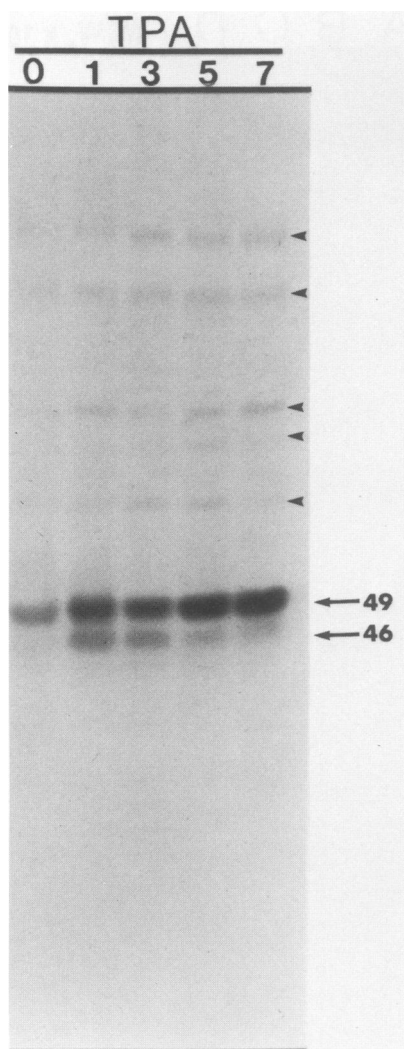


FIG. 3. Differentiation of EBV and HSV polypeptides expressed in dually infected P3HR-1 cells. HSV-induced polypeptides (indicated by arrowheads; brown in original) were identified by probing with HSV-specific antiserum, whereas 49K and 46K polypeptides (indicated by arrows; purple in original) were EBV-induced EA-D components identified by probing with monoclonal antibody against EA-D. Days after 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induction are shown above lanes.

benzidine (brown color) as described above. At least 20 polypeptides with molecular weights ranging from 31,000 to 165,000 were detectable in HSV-infected Vero cells by using HSV-specific antiserum (Fig. 2, lane B, brown bands). These HSV-induced polypeptides were not detected in the EBV-producing cell line (P3HR-1) grown in the absence (Fig. 2, lane C) and presence (Fig. 2, lane D) of the antiviral drug acyclovir or in a somatic cell hybrid line, D98/Raji (Fig. 2, lane A). These results clearly indicate that there was no cross-reactivity between HSV- and EBV-induced polypeptides in the antisera used.

In a separate experiment, we intended to differentiate EBV and HSV polypeptides expressed in a human lymphoblastoid cell line, P3HR-1, dually infected with both EBV and HSV (4). Expression of resident EBV genomes in the dually infected P3HR-1 cells was induced by 12-*O*-tetradecanoyl-phorbol-13-acetate. The kinetics of EA-D synthesis in this cell line after 12-*O*-tetradecanoyl-phorbol-13-

acetate induction are shown in Fig. 3. The nitrocellulose sheet of the Western blot was first probed with an EBV monoclonal antibody against EA-D, and the antigenic bands were located by purple-colored substrate. Before induction, the dually infected P3HR-1 cells expressed predominantly a 49K polypeptide (zero time). When cells were exposed to 12-*O*-tetradecanoyl-phorbol-13-acetate, synthesis of the 49K polypeptide increased with the time of induction and reached its maximum between days 5 and 7. In parallel with the increase in synthesis of the 49K polypeptide, a 46K polypeptide was induced on day 1 and thereafter. To identify the HSV-induced polypeptides, the same nitrocellulose sheet of Fig. 3 was subsequently probed with HSV-specific antiserum, and the antigenic bands were stained brown (Fig. 3, arrowheads). At least five polypeptides were detectable under these conditions. The expression of these polypeptides increased with time of induction; the maximum synthesis was on day 5.

It should be noted that in the dually infected P3HR-1 cells, less than 1% of the cell population became productively infected (4), compared with the majority of Vero cells lytically infected by HSV. Besides, the presence of EBV genomes in these cells may interfere with HSV gene expression. Thus, the fact that fewer polypeptides were detected in dually infected P3HR-1 cells (Fig. 3) than in HSV-infected Vero cells (Fig. 2) is not because of the limitation of the technique, but because of the different cell systems.

The dual antibody probe method has a high level of specificity and sensitivity, evidenced by a clear distinction of EBV- and HSV-induced polypeptides in the same lane of the same gel (Fig. 3) or in different lanes (Fig. 2, lanes B, C, and D). The technology presented here combines convenience and simplicity in the rapid visualization and identification of polypeptides induced by different herpesviruses. Since simultaneous active infection with two or three different herpesviruses in the same patient is not unusual in immunocompromised hosts, this technique has practical utility. The method should be valuable for the routine medical diagnosis of states of viral infection and for detection of antibodies in pathological human sera and can yield information regarding the type specificity of the antibody. By using different antibody probes simultaneously with different combinations of peroxidase substrates, it should be possible to detect the presence of several pathogens in patients with acute infections expeditiously.

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