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Western and Dot Immunoblotting Analysis of Viral Antigens and Antibodies: Application to Murine Hepatitis Virus

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Viral proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred quantitatively to nitrocellulose by electroblotting in SDS-containing buffer. Monoclonal antibodies directed against previously defined epitopes on the viral proteins were used as probes to detect viral protein synthesis and processing, as well as expression in animal tissues. Circulating polyclonal antibodies were also probed and characterized for their polypeptide specificities. Under appropriate conditions, this Western immunoblotting technique was quantitative. Finally, a highly sensitive dot immunoblotting assay was used to analyze the sensitivity to denaturation of various epitopes on the viral proteins. This assay detected picogram quantities of viral antigens and antibodies.

Key words: *immunoblotting - dot blot - nitrocellulose replica - antigenic analysis - antibodies - virus*

Introduction

The detection and characterization of trace amounts of specific proteins in biological materials has been facilitated by the use of specific polyclonal antisera and recently monoclonal hybridoma antibodies. Immunoprecipitation of *in vitro* radiolabeled cell extracts has been the method of choice for such detection and characterization. However, coprecipitation of unrelated proteins and proteolytic degradation of antigens during incubation can frequently lead to erroneous conclu-

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MHV-4, JHM strain of murine hepatitis virus-4; DBM, diazobenzyloxymethyl; N, nucleocapsid; M_r , relative molecular weight; CPE, cytopathic effect; PBS, Dulbecco's phosphate-buffered saline, pH 7.4; MAb, monoclonal antibodies; PFU, plaque-forming units; LD₅₀, lethal dose for 50% of animals; BSA, bovine serum albumin; m.o.i., multiplicity of infection; Ig, immunoglobulin; cpm, counts per min; NGS, normal goat serum; NP-40, Nonidet P40; EDTA, ethylene diamine tetraacetic acid; PMSF, phenyl methyl sulfonyl fluoride.

sions. Moreover, this method cannot be used effectively with non-precipitating antibodies or with poorly soluble antigens (Braun et al., 1983). To circumvent these problems, techniques similar in principle to the nucleic acid blotting methods pioneered by Southern (1975), Alwine et al. (1977), and Thomas (1980) were recently adapted to proteins. Initial separation by SDS-PAGE is followed by electrophoretic transfer of the proteins from the gel matrix onto a nitrocellulose or DBM-derivatized filter paper, producing a replica or blot of the original gel. Fractionated proteins blotted in this manner are accessible to specific antibodies.

Renart et al. (1979) first reported the transfer of proteins by diffusion from polyacrylamide-agarose composite gels and their covalent coupling to DBM paper. Bowen et al. (1980) demonstrated that more convenient nitrocellulose supports could be used effectively with this passive diffusion technique. Rapid electrophoretic transfer to nitrocellulose or DBM paper was described by Towbin et al. (1979) and Symington et al. (1981). Transfer of proteins from SDS-PAGE gels was quantitative after long transfer times of 20–22 h at 6–8 V/cm (Burnette, 1981) or in the presence of SDS in the transfer buffer (Erickson et al., 1982). Transfer was a function of molecular weight; the largest proteins were transferred more slowly. Blots were shown to be re-usable after removal of the bound antibody probes (Renart et al., 1979; Legocki and Verma, 1981; Symington et al., 1981; Erickson et al., 1982).

The murine hepatitis viruses are coronaviruses which are causative agents of such diverse diseases as upper respiratory infection, gastroenteritis, hepatitis, encephalomyelitis and demyelination in colonized mice (Siddell et al., 1983). The MHV-4 (JHM strain) isolate is of interest because it induces central nervous system demyelinating disease (Weiner, 1973; Knobler et al., 1981, 1982; Stohlman and Weiner, 1981; Buchmeier et al., 1984).

The virion contains a single-stranded RNA genome of positive polarity complexed with a nucleocapsid (N) protein of M_r 56,000 (56 K) and 2 envelope glycoproteins E1 and E2 (M_r = 23 K and 25 K for E1; 100 K and around 200 K for E2) (Siddell et al., 1982; Sturman and Holmes, 1983). We have produced monoclonal antibodies in this laboratory against the MHV-4 structural proteins and used these in structural and biological studies of the virus (Collins et al., 1982; Buchmeier et al., 1984; Talbot et al., 1984). In the course of these studies, we have explored the sensitivity of the Western immunoblotting technique for detection of viral antigens. We now report application of this method for the detection and characterization of viral antigens and antibodies in vitro and in vivo. We describe conditions under which it was possible to use this immunoblotting technique quantitatively. Moreover, we describe the use of a highly sensitive dot immunoblotting assay for analysis of the stability to denaturation of various epitopes recognized in the viral proteins by our panel of monoclonal antibodies.

Materials and Methods

Virus, cell culture and antigen preparation

Murine hepatitis virus, strain JHM (MHV-4) is routinely propagated and assayed in our laboratory as previously described (Collins et al., 1982). To serve as a source

of viral antigen for Western immunoblotting assays, cells were infected with MHV-4 at an m.o.i. of 1 until extensive CPE was observed (14–15 h) or for various incubation times as indicated. Infected and uninfected control cultures of 2×10^7 cells were washed with rinse buffer (20 mM Tris-HCl, pH 9.0; 137 mM NaCl; 1 mM CaCl_2 and 0.5 mM MgCl_2) and solubilized for 20 min in 3 ml ice-cold lysis buffer (rinse buffer with 1% (v/v) NP-40, 10% (v/v) glycerol and 1% (v/v) aprotinin. Cell debris was pelleted at $1000 \times g$ for 10 min and cytosols obtained after centrifugation at $100,000 \times g$ for 60 min.

Antigen preparations for dot immunoblotting assays consisted of a microsomal fraction from MHV-4 infected or uninfected control cells, as described previously (Talbot et al., 1984). Native antigen was used as a suspension in PBS or after treatment at 37°C for 20 min with a final concentration of 2% (v/v) NP-40 in 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 0.5 M KCl, 2 mM EDTA, 1 mM PMSF and 1% (v/v) aprotinin. Denatured antigen was prepared by boiling for 2 min in an SDS-containing sample buffer for SDS-PAGE (Laemmli, 1970). Protein concentrations were determined (Lowry et al., 1951) using BSA as a standard.

Mice sera and brain homogenates

Four- to 5-week-old BALB/c mice were obtained from our institutional breeding colony. Sera were obtained by bleeding from the retroorbital plexus of ether-anesthetized animals. Acutely infected mice were obtained after intracerebral inoculation of 3 PFU ($10\text{--}30 \text{ LD}_{50}$) of MHV-4. Three days after infection, when virus titers in the brain were high ($1\text{--}2 \times 10^5$ PFU per gram of brain), mice were sacrificed, their brains removed and a 10% (w/v) homogenate prepared in PBS. Homogenates were clarified by centrifugation at $700 \times g$ for 10 min.

Monoclonal antibodies to MHV-4

A library of monoclonal antibodies to the structural proteins of MHV-4 was raised and characterized as described elsewhere (Collins et al., 1982; Buchmeier et al., 1984; Talbot et al., 1984).

Electrophoresis

Proteins obtained from cytosol extracts of infected and control L-24 cells, or from infected and control mouse brain homogenates were boiled in the presence of 2% (v/v) 2-mercaptoethanol for 2 min in SDS-PAGE sample buffer and separated on 10.5% acrylamide separating gels (Laemmli, 1970). Each separating gel lane ($6 \times 80 \times 1.5$ mm) received either 8.5–51 μg of total cytosol proteins or the equivalent of 750 μg wet weight of brain tissue. Gels were electrophoresed for 2–3 h at 150 V.

Western immunoblotting

Gel replicas were prepared by electrophoretic transfer to nitrocellulose sheets (0.45 μm pore size, Schleicher and Schuell, Keene, NH) using a Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA). The electrophoretic transfer method of Towbin et al. (1979) was used with the following modifications. SDS (0.1% (w/v)) was added to the transfer buffer in order to achieve quantitative protein transfer

(Erickson et al., 1982). We found that a 3 h transfer at 5 V/cm was sufficient for quantitative transfer of the MHV-4 N protein ($M_r = 56$ K), whereas 6 h were required for complete transfer of proteins of M_r higher than about 100–150 K, such as the E2 glycoprotein. Proteins transferred to nitrocellulose sheets were stained as described (Towbin et al., 1979), then destained in distilled water. If necessary, the nitrocellulose sheets were stored overnight at 4°C in 3% (w/v) BSA in PBS with 1% (v/v) NGS.

Nitrocellulose strips in plastic 15 ml conical tubes were saturated by incubation with constant shaking for 60 min at 37°C with 8 ml of blocking buffer (3% (w/v) BSA in PBS containing 0.5% (v/v) NP-40, 0.1% (w/v) sodium deoxycholate and 1% (v/v) NGS). To probe for viral antigen, strips were then incubated for 3 h at 37°C with either 400-fold dilution of mouse sera or hybridoma cell culture supernatants diluted 2–4-fold with blocking buffer. In 1 experiment, various amounts (5–500-fold dilutions) of supernatants containing MAb 4B6.2 were added (46 µg–4.6 ng antibody, as determined by radial immunodiffusion against sheep anti-mouse IgG). Strips were washed (3 times, 10 min) in PBS with 0.05% (v/v) Tween 20 and bound antibodies were detected by incubation for 1–1.5 h at 37°C with ^{125}I -labeled, affinity purified goat antibody to mouse Ig (Buchmeier et al., 1984; specific activity 3–5 µCi/µg; $0.5\text{--}1 \times 10^6$ cpm per gel lane) diluted in 8 ml of blocking buffer. The strips were again washed, extensively dried at room temperature and autoradiographed for 6–48 h at –70°C with an intensifying screen. In 1 experiment, bound radioiodinated antibodies were quantitated by counting portions of nitrocellulose strips containing N protein and scanning corresponding exposed X-ray films at 550 nm in a spectrophotometer. We routinely use radioiodinated, affinity purified goat anti-mouse Ig for our immunoblotting studies because we find this probe to be much more sensitive for detecting mouse Ig than radiolabeled protein A.

Dot immunoblotting

Either native or denatured viral or control antigen (19–2400 ng total protein) prepared as described above was spotted onto nitrocellulose strips in a volume of 3.5 µl. The strips were dried under a heat lamp for 10 min, saturated in blocking buffer and probed with 2–400-fold dilutions of various hybridoma supernatants, as described for Western immunoblotting, except that incubations with antibody were reduced to 1 h each. In 1 experiment, various amounts (2–48,600-fold dilutions) of supernatants containing MAb 5B188.2 were added (40 µg to 1.6 ng antibody, as determined by radial immunodiffusion against sheep anti-mouse IgG).

Results and Discussion

Detection of viral antigens

(1) Antigenic analysis of viral epitopes

Immunoprecipitation of glycoproteins from solution may yield ambiguous results due to coprecipitation, or poor antigen solubility. These shortcomings and ambiguities were largely eliminated by the immunoblotting technique (Talbot et al., 1984).

Polypeptide specificity of different MAb can be clearly determined, providing the antibody can still recognize its target epitope after protein denaturation. All 5 of our MAb to the N protein and more than half (7 out of 13) of the antibodies to the viral glycoproteins bound well to the denatured protein (Talbot et al., 1984). Other MAb appeared to recognize a conformation-dependent structure which was lost upon antigen preparation for Western immunoblotting. For these antibodies, immunoprecipitation had to be utilized. In order to further analyze this postulated sensitivity to denaturing agents such as SDS of some viral epitopes, we used a convenient and highly sensitive dot immunoblotting assay (Hawkes et al., 1982; Herbrink et al., 1982).

As shown in Fig. 1, all 18 of our monoclonal antibodies to the MHV-4 structural proteins specifically recognized the native viral antigen even when treated with the non-ionic detergent NP-40. However, upon denaturation by boiling in SDS and 2-mercaptoethanol, some of our MAb to the viral glycoproteins E2 and E1 lost reactivities (Fig. 1, lanes 3, 4, 8, 9, 11, 13: note absence of virus-specific binding) as predicted from their absence of binding on Western immunoblots (Talbot et al., 1984). It is likely that the epitopes recognized by such antibodies are dependent on the secondary and tertiary structure of the protein. Antibody binding to such conformation-dependent structures thus requires preservation of the native structure of the protein, which can be achieved with the non-ionic detergent NP-40. It is unclear why 3 MAb to the N protein lost their reactivities on the SDS-treated antigen (Fig. 1, lanes 15, 16, 17) by dot immunoblotting but reacted on Western immunoblots (Talbot et al., 1984).

The exquisite sensitivity of the dot immunoblotting assay is evident in Fig. 2. In panel A, we show that using an excess of antibody, as little as 38 ng of membrane-

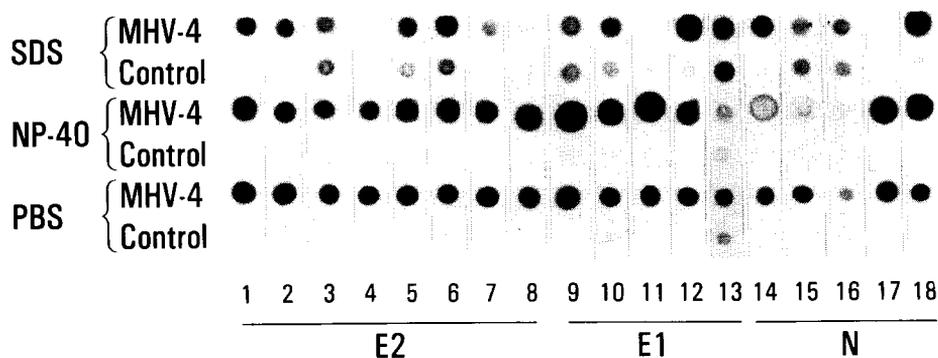


Fig. 1. Dot immunoblotting analysis of binding of monoclonal antibodies to MHV-4 structural proteins (E2, E1, N) in their native (PBS, NP-40) or denatured (SDS) state. Microsomal proteins ($2.4 \mu\text{g}$ in $3.5 \mu\text{l}$) from infected (MHV-4) or uninfected (control) cells were applied as a dot to nitrocellulose strips and probed with a previously determined optimal amount of hybridoma cell culture supernatant (lanes 1, 2, 7, 18: $10 \mu\text{l}$; lane 4: $20 \mu\text{l}$; lanes 5, 6, 10, 12: 1 ml ; lanes 3, 8, 9, 11, 14, 17: 2 ml ; lanes 13, 15, 16: 3 ml , in a total of 4 ml blocking buffer) and radioiodinated antibodies to mouse Ig, as described in the text. The hybridoma designations are as follows: 1, 5B19.2; 2, 5B170.3; 3, 5A13.5; 4, 4B11.6; 5, 5B21.5; 6, 5B93.7; 7, 5B207.3; 8, 5B216.8; 9, 5B11.5; 10, 5A5.2; 11, 5B119.6; 12, 5B128.3; 13, 5B39.1; 14, 4B6.2; 15, 5B145.5; 16, 5B175.5; 17, 5B86.2; 18, 5B188.2.

enriched fraction from infected cells is sufficient for the detection of the MHV-4 N protein. Coomassie blue staining of SDS-PAGE gels of such antigen preparations showed that they contained approximately 1% of N protein (data not shown). Therefore, 380 pg of N protein can be detected in such an assay, which thus compares favorably with radioimmunoassays (RIA) or enzyme immunoassays (EIA). Fig. 2B describes the sensitivity of this technique for the detection of specific viral antibodies. Using an excess of viral antigen, as little as 1.6 ng of MAb to the N protein can be detected, the equivalent of 82 nl of hybridoma cell supernatant. Specifically, a 48,600-fold dilution of this antibody still gave a specific signal; this compares advantageously to its end-point titer determined by EIA of 18,000 (data not shown).

This dot immunoblotting assay should prove valuable in the screening of hybridomas for the secretion of virus-specific antibodies. In addition, the sensitivity of target epitopes to denaturation can be rapidly determined in order to predict which antibodies are likely to perform well on Western immunoblotting analyses. Other applications include the detection of viral antigens in cell or tissue extracts, as well as a simple monitoring assay during the purification of viral proteins. We are currently using the dot immunoblotting assay to monitor the effluent from immunoaffinity columns.

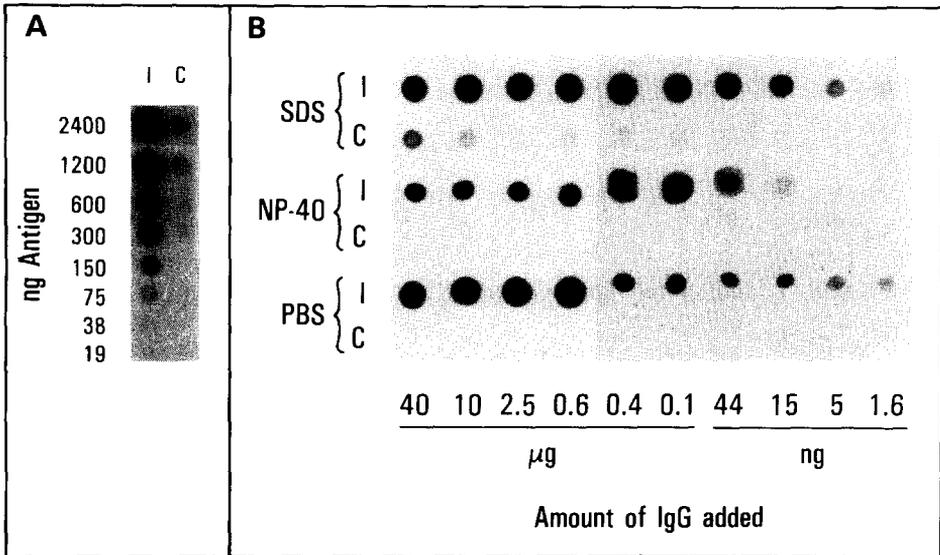


Fig. 2. Determination of the sensitivity of the dot immunoblotting assay for antigen (A) or antibody (B). Increasing amounts (19 ng–2.4 μg) of NP-40 extracted microsomal proteins from infected (I) or uninfected control (C) cells were applied as 3.5 μl dots on a nitrocellulose strip which was then probed with an excess of MAb 5B188.2 to the MHV-4 nucleocapsid protein (40 μg of IgG or a 4-fold dilution in a total of 8 ml blocking buffer) and radioiodinated antibodies to mouse Ig, as described in the text (A). Alternatively, antigen was applied as described in the legend of Fig. 1 and probed with decreasing amounts of MAb 5B188.2 (2–48,600-fold dilutions in 4 ml, with the corresponding amounts of IgG shown) and radioiodinated antibodies to mouse Ig, as described in the text (B).

Fig. 2B also illustrates a common problem encountered in Western immunoblotting: non-specific binding of antibody to control antigen. When native antigen was used for dot immunoblotting, this problem was negligible. However, denatured antigen showed significant background binding in conditions of excess antibody (0.1–40 μg). The background binding was eliminated when a limiting amount of antibody was used (less than 0.1 μg in this case). Therefore, background problems on Western immunoblots should likely be significantly reduced by optimizing the immunobinding conditions of the assay.

(2) Quantitation of antigens

In the course of our studies, we were interested to determine if the Western immunoblotting technique could be used for quantitation of viral antigens in infected cells or tissues. Using MAb 4B6.2 against the N protein as a probe, we performed the experiment illustrated in Fig. 3. In this experiment, a linear dose response of antibody binding was observed when an excess of antibody was used to detect limiting amounts of viral antigen in infected cell lysates which contained approximately 1% of N protein as shown by Coomassie blue staining of SDS-PAGE gels of such lysates (Fig. 3A). It was also possible to demonstrate saturation of viral antigen with increasing amounts of antibody (Fig. 3B). Thus, within these limits we have defined the technique could be used in a quantitative manner for studies on the expression of viral antigens without the need for radiolabeling.

(3) Time course of protein synthesis and processing

Another application of this immunoblotting procedure was the analysis of viral protein synthesis and processing, as illustrated in Figs. 4 and 5. The intracellular

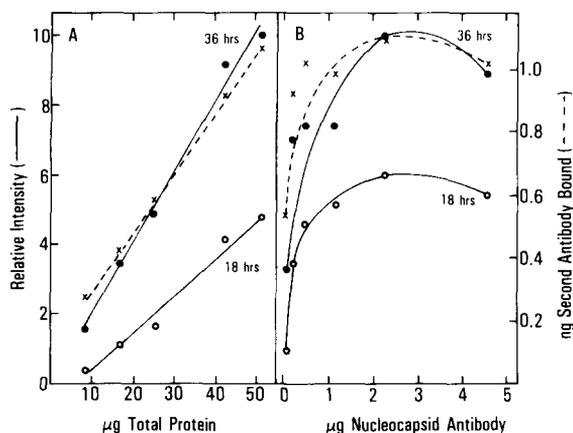


Fig. 3. Quantitative detection of MHV-4 nucleocapsid protein by Western immunoblotting. Different amounts (A) or a constant amount of 51 μg (B) of cytosol proteins from infected cells were separated by SDS-PAGE, transferred to nitrocellulose and probed with increasing amounts (B) or an excess (A) of MAb 4B6.2 against the N protein, as described in the text. The binding of radioiodinated antibodies against mouse Ig to MAb 4B6.2 was quantitated by cutting out the areas containing bands and gamma-counting of nitrocellulose (-----) or scanning of corresponding darkened X-ray film (——) exposed for 18 (O) or 36 (●) h at -70°C with an intensifying screen.

accumulation of viral proteins in L-24 cells infected with MHV-4 at an m.o.i. of 1 was detected by Western immunoblotting.

As shown in Fig. 4, the MHV-4 N protein was detected in infected cells as early as 8 h after infection, 2 h prior to release of progeny virions (data not shown). It was synthesized in the form of a 56 K polypeptide which accumulated up to 14 h after infection. Thereafter, the total amount of this 56 K protein gradually decreased, concomitant with the appearance of a smaller 50 K polypeptide. Since the 2 polypeptides were recognized by the same MAb and appear in a well-defined temporal order, they likely constitute 2 forms of the N protein, the 50 K molecule being likely derived by proteolytic cleavage from the 56 K, as was suggested previously (Anderson et al., 1979). Progeny virions appear to contain exclusively the 56 K polypeptide (data not shown), which makes the significance of the intracellular 50 K protein unclear.

In a similar manner, the time course of appearance of the viral glycoproteins could be analyzed as shown in Fig. 5. The glycoprotein E2 was first detected at 10–12 h after infection (Fig. 5A) as high molecular weight aggregates which did not penetrate separating gels. Thereafter, there was a gradual increase in a polypeptide of M_r approximately 200 K. Cleavage of this 200 K molecule into two 100 K subunits has been associated with acquisition of cell fusion activity (Sturman and Holmes, 1984). In addition, an approximately 150 K intracellular precursor to the

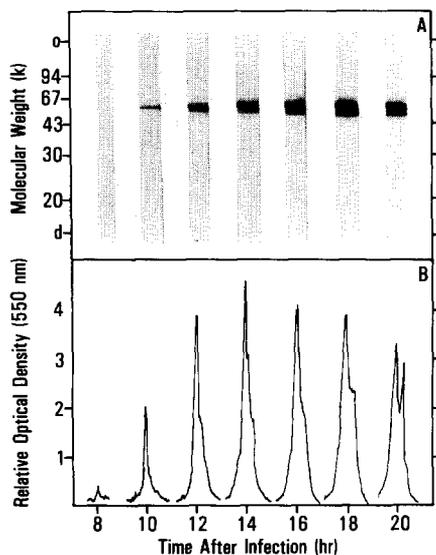


Fig. 4. Time course of appearance of MHV-4 nucleocapsid polypeptides detected by Western immunoblotting. L-24 cells were infected with MHV-4 at an m.o.i. of 1 for various times, and polypeptides reacting with MAb 4B6.2 to N protein detected by Western immunoblotting, as described in the text. Blots shown in A were scanned at 550 nm from the origin (o) to the dye front (d) to quantitate the appearance of the 56 and 50 K polypeptides, as illustrated in B. The migration of molecular weight markers is shown on the left of A.

structural glycoprotein E2 (Siddell et al., 1981; Talbot et al., 1984) was transiently detected at 14 and 16 h after infection, but not later on (Fig. 5A, arrow), suggesting a precursor-product relationship. In our hands, the 100 K monomeric form of E2 was not detected in this experiment even at late times after infection when extensive cell-cell fusion was evident.

The small glycoprotein E1 was initially detected at around 10 h after infection in an unglycosylated 23 K form (Fig. 5B). Thereafter, a gradual increase in the glycosylated 25 K form of the molecule was apparent. Both forms of the protein are incorporated into progeny virions (Wege et al., 1979; data not shown).

These studies emphasize the usefulness of this immunoblotting technique as a complement to pulse and pulse-chase radiolabeling. Although it is aimed at the detection of accumulated protein precursors and their processed products, our work clearly demonstrates that it can identify precursor-product relationships and that the use of MAb minimizes ambiguities. Moreover, this technique eliminates the need for radiolabeling the proteins of interest. It should prove especially invaluable in studies when these proteins constitute only a very small proportion of the total protein content of the sample, such as the synthesis of viral protein when cellular protein synthesis is only inefficiently or not at all turned off by the infection, or the detection of viral proteins in infected tissues.

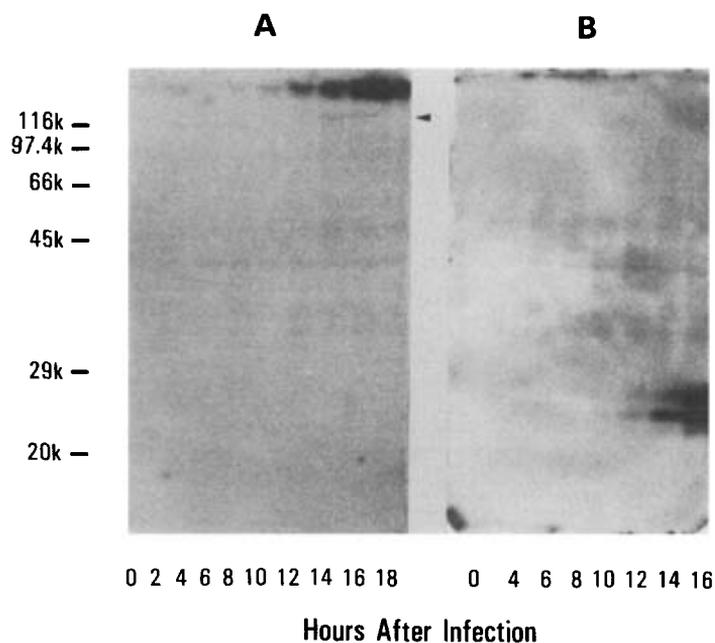


Fig. 5. Time course of appearance of MHV-4 glycoproteins detected by Western immunoblotting. L-24 cells were infected with MHV-4 at an m.o.i. of 1 for various times, and polypeptides reacting with either MAb 5B19.2 to the E2 glycoprotein (A) or 5A5.2 to the E1 glycoprotein (B) detected by Western immunoblotting, as described in the text. The migration of molecular weight markers is shown on the left.

(4) Detection of viral antigens in animal tissues

The Western immunoblotting technique has been used successfully to detect the expression of viral antigens in the brains of infected animals (Fig. 6). We prepared homogenates of brain tissue from MHV-4 infected mice and using MAb 4B6.2, we could detect both the 56 K and 50 K forms of the N protein (Fig. 6, lane 3), which constituted a minor proportion of total protein. By comparison with a standard curve such as shown in Fig. 3A, we estimated that the brain sample analyzed in such a way contained approximately 150 ng of the 56 K polypeptide and 250 ng of the 50 K molecule (in 0.75 mg wet weight of brain tissue). These values represent 0.02 and 0.03% of total brain weight, respectively.

We have failed so far to detect the E1 glycoprotein in acutely infected brain tissue (Fig. 6, lane 2). On the other hand, the E2 glycoprotein was detected as a faint 200 K band (Fig. 6, arrow).

Detection of antiviral antibodies

In addition to the use of Western immunoblotting to identify and quantitate viral antigens in cells and tissues, we have also found this technique to be a sensitive means of detecting antiviral antibodies in animals and determining their polypeptide specificity. Murine coronaviruses are frequently enzootic in colonized mice. In this study, mice were screened prior to experimental infection for pre-existing antibodies

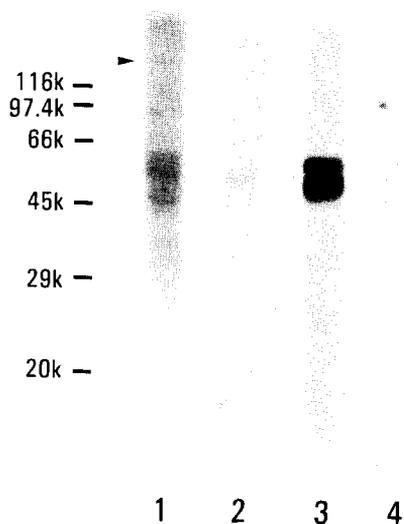


Fig. 6. Detection of MHV-4 polypeptides in mouse brain by Western immunoblotting. Mice were infected with MHV-4 and brain homogenates prepared 3 days later as described in the text. Nitrocellulose replicas of SDS-PAGE separated tissue proteins were probed with MAb 5B19.2 to the viral E2 glycoprotein (lane 1), 5A5.2 to the E1 glycoprotein (lane 2) or 4B6.2 to the N protein (lanes 3 and 4). Lane 4 contains tissue proteins from uninfected, MHV-4 seronegative mice. Note the presence of a 200 K polypeptide in lane 1 (arrow) and both 56 and 50 K polypeptides in lane 3. The migration of molecular weight markers is shown on the left.

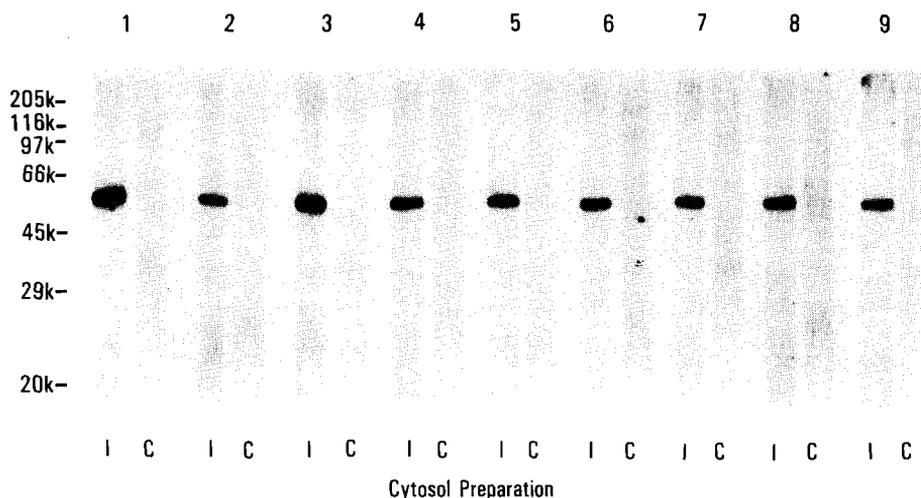


Fig. 7. Detection of antibody to MHV-4 in the serum of BALB/c mice by Western immunoblotting. Nine individual mice were eye-bled and 400-fold dilutions of their sera reacted with SDS-PAGE separated proteins from cytosol extracts of MHV-4 infected (I) or control uninfected (C) L-24 cells, as described in the text. The migration of molecular weight markers is shown on the left. Note the binding to a 56 K viral polypeptide.

to an antigenically related murine coronavirus. As shown in Fig. 7, we frequently found antibodies to a viral protein of approximately 56 K, presumably the N protein. Antibodies to MHV glycoproteins were not detected, either because of a low titer or failure to cross-react with the MHV-4 strain used as antigen. This technique offers the advantage of simultaneous determination of both presence and polypeptide specificity of antibodies.

Concluding Remarks

Western immunoblotting is a convenient, sensitive and specific technique for the detection of antigens and antibodies. It has been used mainly for the determination of polypeptide specificity of monoclonal antibodies. In the present study, we have applied this technique to the analysis of viral protein synthesis and processing, expression of viral proteins in animal tissues and characterization of circulating antiviral antibodies. Under appropriate conditions, quantitative analyses could be performed. We have also shown that a dot immunoblotting assay can be a very sensitive means for the detection of viral antigens and antibodies, as well as the antigenic analysis of viral proteins.

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