The polyclonal immunoglobulin response to HSV-1 viral proteins: determination of immunogenic proteins and relative antibody titres to individual polypeptides by immunoblotting

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Summary. We developed an immunoblotting procedure to characterise the polyclonal immunoglobulin response to the proteins of herpes simplex virus (HSV)-1. We found that 8–20% gradient polyacrylamide gels provided no advantage over fixed 8.5% gels for preparing Western blots for use in immunoblotting. The amount of protein loaded on the gels markedly influenced which proteins were detected by immune serum. The presence of Triton-X-100 0.5% in washes and buffers improved band clarity on immunoblots. In optimal conditions, immune mouse serum reacted with up to 33 HSV-1 lysate proteins. Six bands or regions appeared to be of major immunogenic reactivity, including the (122–130)×10^3-mol. wt region, a 75×10^3-mol. wt protein, the gD region of approximately (56–64)×10^3-mol. wt and two non-glycosylated bands at mol. wts(10^3) 42 and 44. Minor proteins, more weakly reactive, were detected at 27 other areas. The relative antibody titres in immune mouse serum to the different major regions showed antibody titre to gD > gB/gC > (42/44)×10^3 >> P75 >> VP154. Most human sera reacted with all of the major and many of the minor immunogenic proteins but individual sera varied markedly in the proteins recognised. We conclude that immunoblotting is valuable for evaluating immunoglobulin responses to major and minor immunogenic proteins of HSV-1.

Introduction

The humoral immune response to herpes simplex virus (HSV) has been studied by numerous techniques, including complement fixation, neutralisation, ELISA, radioimmunoassay, immunoprecipitation and immunoblotting. The latter two techniques have the advantage of enabling the determination of the individual viral polypeptides recognised by the polyclonal immunoglobulin. More than 50 proteins have been described in HSV-1 infected cell lysates. Immunoprecipitation studies of sera from patients with primary or recurrent herpes simplex have shown that significantly fewer proteins can be demonstrated in precipitates; 7–18 proteins have been demonstrated in various immunoprecipitation studies (Gilman et al., 1981; Zweerink and Stanton, 1981; Zweerink and Corey, 1982). Several factors may limit the sensitivity of the immunoprecipitation assay. Some minor polypeptides are present at low concentrations in the cell lysate. Isotope incorporation depends on many factors which result in differing specific activities between different polypeptides. Some viral proteins are insoluble and so unavailable in the cell lysate as usually prepared.

Immunoblotting, on the other hand, with unlabelled polypeptides electrophoretically separated and then transferred to nitrocellulose (NC) paper reduces these problems and, furthermore, offers the advantage of amplification through the use of secondary or tertiary antibody layers. Despite the expected loss of some antigenic sites by denaturation, immunoblotting has been shown to be a very sensitive technique for detection of antigens in many systems. Norrild et al. (1981) in the first HSV-1 immunoblotting studies showed that immune rabbit serum recognised 17 different proteins in an immunoblotting procedure employing 125I-protein A. In human sera studies by Eberle and Mou (1983), reactivity with 10–28 HSV-1 proteins was seen. Finally, 4–12 HSV-1 or HSV-2 polypep-
tides were recognised by human sera in the studies by Lehtinen et al. (1985).

Our studies had three purposes: to develop an immunoblotting procedure which could detect as many minor as well as major immunogenic proteins of HSV-1 as possible; to identify the major immunogenic proteins recognised by human and mouse immune serum; and to determine the relative titres of antibody in immune mouse serum to each major protein.

Materials and methods

Virus

HSV-1 (Heitzaan) was originally isolated from a patient with pharyngitis. The isolate was typed as HSV-1 by neutralisation kinetics and SDS-polyacrylamide polypeptide pattern with isotopically labelled proteins.

Preparation of immune mouse serum

BALB/c male mice, 6 weeks old, were given $10^5$ pfu of HSV-1 by injection into the footpad. Two weeks later a second dose of $10^6$ pfu was given intraperitoneally (ip). After a resting period of one month or more, mice received a third dose of $10^7$ pfu ip. Serum was then collected and pooled.

Human sera

Human serum samples were obtained from a bank of sera from laboratory workers. They were selected from the bank at random without regard to past history of HSV exposure or HSV recurrences.

Incorporation of radio labelled amino acids into infected cell-lysate proteins

Human diploid lung fibroblasts, MRC-5, were grown to confluency and infected with HSV-1 at a multiplicity of 10 pfu/cell. One h after infection the residual inoculum was washed away, the cell sheet was incubated for 1 h in PBS to deplete amino acid stores and then $^{35}S$-methionine was added at a final concentration of 80 $\mu$Ci/ml. In some experiments $^{14}C$-labelled amino acids were added at a final concentration of 15 $\mu$Ci/ml or $^{14}C$-labelled glucosamine was added at a final concentration of 5 $\mu$Ci/ml. The labelling was terminated at 24-28 h. The infected cell sheets were washed exhaustively with cold PBS and scraped with a rubber policeman. The infected cells were pelleted at 400 $g$ at 4°C and then resuspended in lysate buffer containing SDS 0.1% w/v, Triton-X-100 1% v/v and deoxycholate 1% w/v, pH 6.8, 0.5 M NaCl. Nuclei were pelleted at 10 000 $g$ and the labelled proteins in the supernate harvested and frozen at $-70°C$ until use. The total protein content of lysates was 4.5 mg/ml and the specific activity was 0.01-0.1 $\mu$Ci/$\mu$g total protein, depending on the radioreagent used in the lysate preparation.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed according to the standard procedure of Laemmli (1970). An 8–5% w/v acrylamide resolving gel cross linked with bisacrylamide or DATD and a stacking gel of 3.5% w/v bisacrylamide were employed. In some experiments 8–20% linear gradient polyacrylamide was used. HSV-1 infected cell-lysate proteins were loaded at various concentrations (see Results). Electrophoresis was performed at 15 mA constant current on gels of 0.75 ml thickness by 14 cm length. Gels were dried by heat under vacuum and autoradiography was performed with Kodak XAR film.

Western blotting of HSV-1 infected cell polypeptides

Gels prepared as above were assembled against NC paper (Biorad Co., Richmond, CA, USA). The gel and NC were loaded into the transfer cassette. Proteins were transferred to the NC paper electrophoretically by use of Tris-glycine buffer with methanol 20% v/v as described by Towbin et al. (1979), with a Transphor apparatus (Hoefer Co, San Francisco, CA, USA) at 25 V, 0.2 A overnight. Time and amperage were adjusted in different experiments to determine the most sensitive conditions.

Detection of antibody to HSV-1 polypeptides by immunoblotting

Western blots containing HSV-1 polypeptides were cut into strips 2-mm wide and put into plexiglass troughs. Strips were initially wetted with PBS and then blocked for 1 h with bovine serum albumin (BSA) 3% w/v and Triton-X-100 0.5% v/v in PBS. After washing with wash buffer containing BSA 1% w/v and Triton-X-100 0.5% v/v in PBS the strips were overlayed for 1 h with primary antiserum diluted in 50 to 1 in 100 000 in immunoblot buffer containing Triton-X-100 0.5% v/v and BSA 3% w/v in PBS. Affinity purified $^{125}I$-labelled sheep antimouse or anti-human Ig (Amersham, Arlington Heights, IL, USA) (specific activity 17–20 $\mu$Ci/$\mu$g) was used at 0.045 $\mu$Ci/ml as a second antibody in immunoblot buffer with an incubation period of 1 h at room temperature. After extensive washing, the strips were dried in air, mounted on filter paper and exposed for 24 h to 17 days to Kodak XAR film for autoradiography.

Results

Detection of major immunogenic HSV-1 proteins immobilised on nitrocellulose (NC) paper by probing with immune mouse serum and $^{125}I$-labelled second antibody

To determine which HSV-1 proteins were recognised by antibody in immune mouse serum, $^{35}S$-methionine or $^{14}C$-glucosamine-labelled lysate pro-
Fig. 1. Autoradiographs of major immunogenic HSV-1 protein bands detected by immunoblotting (a) on Western blots from 8.5% bisacrylamide SDS-PAGE gels and (b) on Western blots of proteins separated by SDS-PAGE with an 8–20% bisacrylamide gradient gel. Strips of NC paper containing unlabelled HSV-1 infected cell proteins were probed with immune mouse serum (lane A) or normal mouse serum (lane B) followed by 125I-labelled sheep anti-mouse Ig. 35S-methionine labelled (a, lane D; b, lane C) or 14C-glucosamine labelled (a, lane C; b, lane D) HSV-1-infected cell proteins were used as markers to identify the major regions detected on the immunoblot strips. Mol. wts(10^3) and designations of bands are shown.

From HSV-1-infected cells were used as marker proteins on SDS-polyacrylamide gels which were then electrophoretically blotted on to NC paper. In an adjacent lane, unlabelled HSV-1 cell-lysate proteins were co-electrophoresed with the labelled proteins and blotted. The NC papers containing the unlabelled proteins were cut into strips and used in an immunoblotting procedure employing immune mouse serum as primary antibody followed by 125I-labelled sheep anti-mouse Ig. Fig. 1a shows the relationship of the bands detected by immunoblotting to the bands on the Western blot. Mol. wt markers (not shown), run in an adjacent lane, allowed assignment of mol. wts to the major bands. The major regions detected (lane A) include the (122–130) × 10^3 region corresponding to the high mol. wt glycoproteins gB and gC, a protein of mol. wt 75 × 10^3, a region at (56–64) × 10^3 mol. wt which approximates to the location of gD in the 14C-glucosamine labelled lane, and a region containing bands of mol. wt (10^3) 42 and 44 which are not glycosylated.

In an attempt to select a gel separation system which would maximise detection of both high and low mol. wt proteins, we probed Western blots of proteins separated on 8–20% bisacrylamide linear gradient gel. Fig. 1b shows that no additional major bands of low mol. wt were detected although two bands of mol. wt (10^3) c. 47 and 49 were seen on the gradient gel.
**Determination of optimal protein load on Western blots of HSV-1 cell-lysate proteins**

To determine the protein concentration needed to detect the major immunogenic proteins on western blots, lanes of 4 mm width were loaded with 1.4–22 μg of HSV-1-infected cell-lysate proteins. Fig. 2 shows that 11 μg or more per lane was required to visualise the (42/44) × 10^3-mol. wt proteins but that as little as 1.4 μg was sufficient to detect gC. Silver staining of the gel after blotting to nitrocellulose indicated a significant level of untransferred proteins (data not shown). We did not observe any evidence for impaired transfer of high mol. wt proteins compared to low mol. wt proteins. For our procedure, 11 or 22 μg total protein/4 mm lane appeared to be optimal to produce high quality Western blots.

![Fig. 2. Autoradiograph of an immunoblot detecting HSV-1 proteins loaded in different concentrations. Lanes of an 8.5% SDS–PAGE gel were loaded with 22 μg(A), 11 μg(B), 5.5 μg(C), 2.8 μg(D) or 1.4 μg(E) of HSV-1 infected cell lysate protein and were blotted to NC paper. The Western blot was then probed with immune mouse serum followed by 125I-labelled sheep anti-mouse Ig.](image)

**Detection of minor immunogenic HSV-1 proteins**

To detect immunoblot bands due to minor proteins recognised by immune mouse serum we used a high concentration of immune mouse serum (1 in 20 dilution), and exposures of 2, 5 and 17 days. Fig. 3 shows several bands between the major immunogenic protein regions. Above the major gB and gC region (130 × 10^3 mol. wt), five proteins of high mol. wt were detected including the major capsid protein at 154 × 10^3 mol. wt and a protein of (175–180) × 10^3 mol. wt which is most likely ICP4. Between the major regions of mol. wts (10^3) 130 and 75 there are regions of mol. wt (10^3) 101–93 and 90–81. Below the 75 × 10^3-mol. wt protein there
are five minor bands detected with mol. wts \((10^3)\) 72–70, 67, 66, 62, and 59. The long radiograph exposure obscures the clarity of some of the bands in this region but different photographic exposures used in lanes A, B and C compensate for this somewhat. Below the major gD region (mol. wt \(56 \times 10^3\)) there are three minor bands with apparent mol. wts \((10^3)\) 52, 49 and 48, and below the major region of \((42/44) \times 10^3\) mol. wt there are 12 minor bands of mol. wts \((10^3)\) 41, 40, 39–5, 38–5, 36, 35, 34–5, 33, 32, 31, 30 and 29. In all, 27 minor bands were recognised by the immune serum but not by non-immune mouse serum. Three faint bands were noted in the non-immune mouse serum (lane D): the capsid protein VP154, a \(73 \times 10^3\)-mol. wt band and a \(45 \times 10^3\)-mol. wt band. With blots containing uninfected cell-lysat proteins, it was shown that none of the immune serum bands was the result of cross-reactivity with cell proteins (data not shown).

**Effect of Triton-X-100 on sensitivity of immunoblot detection of antibody to HSV-1 polypeptides**

Although we had little difficulty detecting antibody to many different HSV-1 polypeptides, some reports had suggested that the denaturing conditions of SDS-PAGE destroyed some antigenic epitopes of blotted proteins and that incubation of NC strips with Triton-X-100 during immunoblotting procedures enhanced antibody binding to HSV-1 proteins by allowing renaturation of some epitopes (Braun et al., 1983). It was, therefore, of interest to examine the effect of Triton-X-100 on band appearance in immunoblotting. Fig. 4 compares strips processed with Triton-X-100 0.5% to strips processed without Triton-X-100. Strips treated with Triton-X-100 for 30 min (lane C) before being probed with primary antibody showed bands which appeared sharper in most of the major immunogenic regions than strips processed without Triton-X-100 (lane A). Furthermore, most major regions appeared darker, suggesting that more antigen binding sites were available. In particular there was enhanced detection of the \(75 \times 10^3\)-mol.wt protein which appeared much sharper. In the \((56-64)\) \(\times 10^3\)-mol. wt region, however, the enhanced darkness obscured two bands (short arrows) which were clearer without Triton-X-100.

To determine if the presence of Triton-X-100 in the primary and secondary antibodies would reduce antibody binding, we processed strips with Triton-X-100 0.5% present in all solutions including the washes. No reduction in specific antibody binding was detected in strips, including those probed with immune serum (lane E). A significant reduction in nonspecific background was, however, noted in all strips, including those probed with normal mouse serum. Thus, the presence of Triton-X-100 reduced non-specific binding and enhanced specific binding of some bands but other bands may be obscured in these conditions.
Determination of antibody titres to individual HSV-1 polypeptides by immunoblotting

To determine the relative antibody titres to individual HSV-1 polypeptides, immunoblotting was performed in the presence of Triton-X-100 with primary hyperimmune mouse antisera dilutions from 1 in 50 to 1 in 100 000. Fig. 5 is a composite of strip autoradiographs of different exposures and shows that the titre of anti-gD antibody was >100 000, whereas antibody to gB/gC and the (42/44) x 10^3-mol. wt region was barely detectable at the 1 in 100 000 dilution. The anti-VP154 titre was <3000 and the anti-p75 was >20 000 < 100 000.

Human serum antibodies to individual HSV-1 polypeptides

Human sera were surveyed and their reactivity compared with the polypeptide reactivity observed with the immune mouse serum; 16 random sera were screened by immunoblotting and 11 had detectable bands. Fig. 6 shows the positive ones (lanes A–K) and one negative serum as a control (lane L). Overall, human and mouse sera recognised the same major immunogenic proteins, except for the 75 x 10^3-mol wt protein. Sera C and D gave results very similar to those obtained with the immune mouse serum. Serum H showed greater reactivity with the gD and (42/44) x 10^3-mol. wt regions and notably less reactivity with the gB/gC region. Conversely, serum I showed greatest reactivity with the gB/gC region and somewhat less with the gD and (42/44) x 10^3-mol. wt regions. Serum G had an unusual pattern of faint reactivity with (42/44) x 10^3-mol. wt and gD regions and greater reactivity with unidentified proteins in the (65–70) x 10^3-mol. wt range. Thus the individual human specimens show widely different patterns, although most specimens displayed some degree of reactivity with the gB/gC, gD and the (42/44) x 10^3-mol. wt regions.

Discussion

By immunoblotting we have shown that immunological reactivity of 33 major and minor HSV-1 proteins can be detected despite any denaturation resulting from the electrophoretic methods. The sensitivity shown here exceeds that achieved in earlier immunoblot studies (Norrild et al., 1981), probably as a result of greater gel resolution, more complete transfer of protein to paper by electrophoretic methods, and careful determination of optimal protein concentrations on the SDS-polyacrylamide gels. Eberle and Mou (1983) used a sensitive immunoblot system and one of their human sera, HS-3, recognised 28 HSV-1 polypeptides. Sera of other patients in their study reacted with only 10 proteins. We found similar variation between human sera in our studies. Thus, differences in the
various immunoblotting studies are also due to the antisera studied. Furthermore, some studies have used peroxidase-conjugated second antibodies (Braun et al., 1983) rather than $^{125}$I-staphylococcal protein A or $^{125}$I-anti-IgG (Norrid et al., 1981; Braun et al., 1983; this study). In our hands, with peroxidase-conjugated second antibody (Vector Labs, Burlingame, CA, USA), the immune serum could be diluted only to 1 in 5000 before all bands became undetectable (McKendall, unpublished observation), whereas some major bands were detectable at a 1 in 100 000 dilution of the same immune mouse serum when $^{125}$I-goat anti-mouse Ig was used (Fig. 5). Therefore, immunoblotting with $^{125}$I-labelled reagents, at least those we employed, is more sensitive.

We compared our findings of antibody reactivity to individual HSV-1 polypeptides with published results obtained by immunoprecipitation, the only other technique capable of providing comparable data. One study of sera from seropositive patients with and without facial herpetic recurrences found that 18–22 HSV-1 polypeptides were precipitated (Zweerink and Stanton, 1981) whereas another study of 231 patients found that individual sera recognised, at most, seven polypeptides, although 31 different proteins were recognised by the entire bank of 231 sera (Gilman et al., 1981). Serum from

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**Fig. 6.** Human serum antibodies to HSV-1 polypeptides studied by immunoblotting. Individual specimens (lanes A–L) were diluted 1 in 50 for study. $^{125}$I-labelled sheep anti-human Ig was used as the second antibody.
one patient with herpes encephalitis showed seven bands detectable by immunoprecipitation (Mann and Hilty, 1982). A fourth immunoprecipitation study of HSV-polypeptide reactivity with 50 human sera showed that 12–14 HSV-1 or HSV-2 peptides were precipitated (Norrild, 1985). However, 23 polypeptides were recognised by the same sera when studied in an immunoblotting assay with an 125I-protein A probe (Norrild, 1985). Other immunoprecipitation studies with HSV-2 antigens have shown about 18 precipitated bands with serum from patients with primary genital herpes (Zweerink and Corey, 1982; Ashley and Corey, 1984). While some of the variability in results is due to differences in the immune status of patients and experimental animals, it appears that immunoprecipitation can enable recognition of 7–22 bands whereas immunoblotting allows recognition of up to 33 bands.

The major proteins detected in our immunoblots were gC, gB, 75 × 10^3, gD (56 × 10^3), 44 × 10^3 and 42 × 10^3. The highest titres of antibody were directed at gC, gB, gD and the (44/42) × 10^3-mol. wt proteins. High titres of antibody to gD and gB are also seen in quantitative immunoelectrophoretic studies on human sera (Vestergaard, 1979). There is generally good agreement between our results and those of Eberle and Mou (1983) except for the 75 × 10^3-mol. wt protein which was not a major protein in their studies. Correlation with immunoprecipitation studies shows that (115–130) × 10^3-mol. wt proteins (probably gB and gC) and gD are the most commonly precipitated HSV-1 proteins (Gilman et al., 1981; Zweerink and Stanton, 1981; Mann and Hilty, 1982). It is of note that the proteins of mol. wts. (10^3) 44 and 42 were not precipitated in these studies, or were only minor proteins in immunoprecipitates. This disparity has previously been recognised by Eberle and Mou (1983). These proteins are probably related to the p40 protein purified from HSV nucleocapsids by Heilman et al. (1981) because monoclonal aniseru to p40 (Zweig et al., 1980) bind to a series of proteins ranging in mol. wt (10^3) from 34 to 49 (Eberle et al., 1984, 1985). It is also possible that antibody to viral thymidine kinase (mol. wt 45 × 10^3) is responsible for some of the reactivity.

Our finding of a major band of mol. wt 75 × 10^3 is of interest in that it has not been recognised as a major band before. The identity of this protein is unknown. It is evidently not a cell protein because it is absent from blots prepared with uninfected cell lysates and because it is present in serum from mice with a primary infection (McKendall, unpublished observations). Therefore the 75 × 10^3-mol. wt protein is not likely to be a cell protein recognised as a result of the immunisation procedure. Norrild (1985) detected a 77 × 10^3-mol. wt protein and Gilman et al. (1981) detected proteins of mol. wts (10^3) 73 and 75-5 which reacted with human immune serum but neither report considered the protein to be of major immunogenic importance. The 75 × 10^3-mol. wt band was not a major band in the panel of human specimens we studied and so may be conspicuous in some species only. The 75 × 10^3-mol. wt protein in our mouse studies could represent a precursor form of other major immunogenic proteins. A non-glycosylated form of gC obtained by endo-β-N-acetylglucosaminidase H treatment of a high mannose intermediate precursor of gC yields a 75 × 10^3-mol. wt protein (Wenske et al., 1982). Non-glycosylated gC synthesised in vitro has a mol. wt of 75 × 10^3 (Lee et al., 1982). Also, gE purified by affinity chromatography utilising its Fc binding properties shows three forms of gE ranging in mol. wt (10^3) from 65 to 80 (Baucke and Spear, 1979). However it is unlikely that our 75 × 10^3-mol. wt protein is a form of gE because gE is a minor protein in infected cell lysates and the appearance of the band in our blots is unlike the diffuse bands of gE1 or gE2 (Baucke and Spear, 1979).

We have shown that immunoblotting, as used by us, is a highly sensitive method of detecting and characterising the HSV-1 polypeptides recognised by immune serum. Great variability in quantitative and qualitative serum antibody responses to HSV-1 proteins is apparent in both immunoblotting and immunoprecipitation studies. Further work will be required to identify all the proteins to which antibody is made and to define the conditions which lead to production of antibody to individual polypeptides in one serum and not in another.

REFERENCES


