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IMMUNOBLOTTING ANALYSIS OF CLASS-SPECIFIC ANTIBODY RESPONSE IN PATIENTS WITH PRIMARY HSV INFECTION

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Summary. — Class-specific immune response in acute herpes simplex virus (HSV) infections to individual virus specified polypeptide antigens was analysed by immunoblotting. The HSV specified glycoproteins B (gB), C (gC), and D (gD) were detected. IgG-antibody response was shown to develop to various other virus specific polypeptides as well. The IgM- and IgA-antibody responses remained restricted to only a few HSV specific proteins, namely VP 13 (80kD), VP 20 (52kD), and VP 23 (40kD) and to the low molecular weight polypeptides.

Key words: *herpes simplex virus*; *immunoblotting*; *immunoglobulin classes G, A, M*

Introduction

The application of HSV specified protein antigens in immunoblotting studies have shown that IgG-antibody response occurs to a wide variety of viral proteins in virtually all HSV infected patients (Lehtinen *et al.* 1985, 1985b, Norrild 1985, Teglbjaerg *et al.* 1986). Vaccines consisting of a mixture of lectin purified HSV glycoproteins or cloned gD have been shown to elicit neutralizing anti-glycoprotein antibodies both in animal models and man (Mertz *et al.* 1984, Watson *et al.* 1984, Muniu *et al.* 1987). IgG antibody response following vaccination with a DNA-free vaccine is directed against the HSV glycoproteins (Muniu *et al.* 1987). HSV specified glycoproteins play a central role also in the antibody dependent cellular cytotoxicity (ADCC) reaction, which has been shown to be rapid and effective both *in vitro* and *in vivo* (Kohl *et al.* 1982, Glorioso *et al.* 1984). Cytotoxic T-cells and NK-cells have been shown to recognize HSV glycoproteins on the surface of the infected cell (Carter *et al.* 1981, Bishop *et al.* 1986).

Local IgA-response has been shown to have an inverse relationship to the duration of virus shedding in genital herpes (Merri man *et al.* 1984). However, little is known on the nature of the viral antigens, which elicit the secretory immunity. Recently, IgM-specific antibody response was shown to be directed against viral nucleocapsid proteins but not against the HSV specified

glycoproteins (Kühn *et al.* 1987). We have evaluated the development of class-specific antibody response to individual virus proteins in primary HSV infections.

Materials and Methods

Cells and viruses. Early passages of HSV-1 (strain Turku) were used for the infection of African green monkey kidney (VERO) cells as described previously (Lehtinen *et al.* 1985*a*).

Chemicals and antisera. Phenyl methyl sulphonyl fluoride (PMSF) and 3,3-diaminobenzidine-tetrahydrochloride (DAB) were from Sigma Co. (U.S.A.) and Fluka AG (F.R.G.), respectively NP-40 was purchased from Shell Co. (Netherlands). Protein-A linked to Sepharose 4B (Pharmacia, Sweden) was used. The rabbit anti-HSV-1 and anti-HSV-2 antisera and peroxidase conjugated anti-human IgG, IgM, IgA and antirabbit immunoglobulins (Ig) were from Dakopatts. a/s (Denmark).

Infection and labelling of the infected cell specified polypeptides. Nearly confluent monolayers were infected at a multiplicity of infection of 5 PFU/cell. In the labelling experiments the medium was poured off and the cells (5×10^7) were washed twice with Hanks' balanced salt solution (HBSS) and covered with 10 ml of labelling medium containing four parts of HBSS and one part of MEM, supplemented with 2 % dialyzed newborn calf serum. We used 74 kBq/ml |^{14}C| glucosamine hydrochloride (Amersham) for the labelling of glycosylated proteins. The |^{14}C|-glucosamine labelling took place between 4-18 hr p.i. Labelling of late HSV-1 specified polypeptides was done with 185 kBq/l |^{14}C|-amino acid hydrolysate (The Radiochemical Centre Amersham, England) added to the HSV-1 infected cells 16-18 h p.i.
The labelling experiments were stopped by pouring off the medium.

Preparation of an infected cell lysate. In experiments designated for the immunoblotting analyses 5×10^8 cells were used unlabelled. The cells were extracted into ice-cold PBS containing 0.5 mmol/l PMSF, centrifugated (500 g for 10 min) and suspended in 10 ml (1 ml for the labelled material) of PBS with 1 % Nonidet P-40 (Shell Co., Netherlands) and 0.5 mmol/l PMSF. After extraction in an ice-bath for 15 min the nuclei were pelleted at 300 g for 5 min. The supernatant was sonicated, and cleared by centrifugation at 2000 g for 10 min. The remaining supernatant was divided into 100 µl aliquots and stored at -70 °C until used for fluorographic analysis (labelled material) or immunoblotting (unlabelled material).

Polyacrylamide gel electrophoresis and immunoblotting. Analysis of electrophoretically separated proteins was by fluorography (labelled material) or immunoblotting (unlabelled material). Polyacrylamide gel electrophoresis, and silver staining were performed as described previously (Lehtinen *et al.* 1985*a*, 1985*b*). Briefly, we used 10 % polyacrylamide slab gels with N′, N′-methylene bisacrylamide as a cross-linker, and a discontinuous buffer system to separate the lysate polypeptides. The infected cell lysate was mixed in a SDS-sample buffer at a final concentration 2 mg of protein/ml.

Analysis of the HSV antibodies by the immunoblotting method was as described earlier for typing of HSV infected patients sera (Lehtinen *et al.*, 1985). The transfer was at 40 V for 8 hr. Serum samples (preadsorbed with protein-A Sepharose in the IgM and IgA analyses for the removal of IgG, 14) and rabbit anti-HSV-1 or anti-HSV-2 antisera were diluted 1/50 in a dilution buffer (PBS + 10 % of foetal calf serum). They were allowed to react for 2 hr with the paper, which had been cut into strips. The strips were thereafter washed carefully for 60 minutes. Fixed antibodies were detected with peroxidaseconjugated anti-human or anti-rabbit immunoglobulins diluted 1/100 (anti-human IgG and anti-rabbit IgG) or 1/50 (anti-IgM and anti-IgA). After washing, the bands were visualized with DAB.

Serum samples. Serial serum samples of alltogether 9 patients suffering from primary HSV infection were available. The type of the infecting virus was determinated by using a commercial Mikrotrak^r kit (Syva, U.S.A.).

Results

Identification of HSV specified proteins

Glucosamine labelling revealed three major glycosylated polypeptide bands with a molecular mass (M_r) of 125 kD, 118 kD and 58 kD. These poly-

Table 1. Immunoblotting and ELISA analysis of class-specific antibodies in the convalescent phase sera of HSV infected patients

Patients	Age/ sex	Type* of isolated virus	ELISA Ab level**			IMMUNOBLOTTING											
						125 kD (+/−)			118 kD (+/−)			52 kD (+/−)			LMW*** (+/−)		
			IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
1.	5, M	HSV-1	45	25	20	+	−	−	+	+	−	+	+	+	+	−	+
2.	19, F	HSV-1	65	65	35	+	−	−	+	−	−	+	+	+	+	+	−
3.	20, M	HSV-1	200	115	30	+	−	+	+	−	−	+	+	−	+	−	−
4.	58, M	not done	145	35	40	+	−	−	+	−	+	+	+	+	+	+	+
5.	28, F	HSV-1	35	65	35	−	−	−	+	−	−	+	+	+	+	+	+
6.	22, M	HSV-2	110	50	55	+	−	−	+	−	−	+	−	−	+	+	+
7.	29, F	HSV-2	100	125	85	−	−	−	+	−	−	+	+	+	+	+	+
8.	34, M	HSV-2	70	120	50	−	−	−	+	−	−	+	+	−	+	+	+
9.	46, F	HSV-2	115	80	20	+	−	−	+	+	+	+	+	+	+	+	+

Cases 1—4 had an acute primary cutaneous (1, 2), mucocutaneous (3) or ocular (4) herpetic infection, cases 5—9 had genital herpes. Cases 1—5 and 7 and 8 were initially HSV seronegative.

* Typing of the isolated virus was performed by using a commercial Microtrak^{R} kit (Syva, U.S.A.).

** Antibody levels in convalescent phase sera. The ELISA results are expressed on Enzyme-Immuno Unitsobtained by comparison to known positive and negative reference sera (Lehtinen *et al.*, 1985).

*** Low molecular weight proteins (35 kD, 30 kD, 25 kD).

peptides were readily detectable by pulse labelling 16 to 18 hr p.i. (Fig. 1). In the unlabelled material, polypeptides with a correspondig M_r were found by using both rabbit HSV-1 and HSV-2 antisera. Also low molecular weight (LMW) polypeptides with approximate M_r of 25 kD, 30 kD and 35 kD were seen in the immunoblotting analyses with human serum IgG. In the following, these proteins will be dealt as the glycoprotein C (gC, 125kD), glycoprotein B (gB, 118 kD), and glycoprotein D (gD, 58 kD) and LMW proteins.

Development of IgG-class antibody response to the HSV proteins

Serial serum samples (0, 12, 47 and 71 days after onset of a primary herpetic stomatitis) were obtained from a 5-year old boy of whom HSV type 1 was isolated. In the acute phase serum sample taken 7 days after the onset of symptoms an antibody response to only a few HSV polypeptides namely, 52 kD and the LMW proteins was noted (Fig. 1). Later on, a clearcut IgG-antibody response to many other HSV protein antigens; polypeptides migrating like the gC, gB and gD was seen. In addition, 110 kD, 80 kD, 70 kD, 67 kD and 40 kD polypeptides were detected by the convalescent phase sera taken 47 and 71 days later.

Development of IgM-, IgA-, and IgG-class antibody response to the HSV proteins

When looking for the HSV infected patient's sera for the class specific response to the individual HSV specified polypeptides we noted that IgM- and IgA-antibody response was restricted to only a few polypeptides. Reactivity of serial serum samples of two female genital herpes patients (HSV-1 and HSV-2 isolation positive cases) were analyzed sample by sample.

In the HSV-1 case three polypeptide bands: the 110 kD, 40 kD and 35 kD polypeptides were detected by the IgM-antibodies of the convalescent and late convalescent phase serum samples (Fig. 2). The IgA-antibodies could detect only one protein band (the 40 kD polypeptide). The HSV-2 case also showed only a few IgM and IgA reactive polypeptides. Specifically, the 80 kD, 52 kD and 40 kD polypeptides were readily detectable by IgM-antibodies while IgA-antibodies detected only two polypeptides: 80 kD and 40 kD. The polypeptide migrating like gB also showed a faint band (Fig. 3). In the immunoblotting analyses similar IgG antibody responses were noted in both patients with the exception that the HSV-2 infected patient lacked antibodies to the 125 kD polypeptide (Figs. 2 and 3).

A majority of our 9 patients with a primary HSV-1 or HSV-2 infection had IgM- or IgA-antibodies to the HSV specified 52 kD and LMW proteins in their convalescent phase sera (Table 1). Antibody responses to the 125 kD and 118 kD polypeptides were also seen in one and two cases, respectively.

Discussion

Only a few HSV specified polypeptides showed reactivity with IgM- or IgA- antibodies in the immunoblotting analyses. Our results are in accordance with the previously described predominance of certain HSV specified

proteins as immunogens in the acute phase of the infection (Eberle and Courtney 1981, Eberle and Mour 1983, Teglbjaerg *et al.* 1986, Kühn *et al.* 1987). Comparable results have been obtained with *Toxoplasma gondii* (Partanen *et al.* 1984) and Coxackie B virus (Dörries and ter Meulen 1983). It is possible that the restriction of both IgM- and IgA-antibody responses to only a few protein antigens represents a more general property of the immune system.

Two HSV specified glycoproteins, the 118 kD protein and the 58 kD protein migrated in the SDS-gels like the viral glycoproteins gB and gD (Palfreyman *et al.* 1983, Zezulak and Spear 1984). In the immunoblotting analyses corresponding M_r polypeptides were only occationally detected by IgM- or IgA-class antibodies. A 125 kD protein migrating like the HSV-1 glycoprotein gC was detected by anti-HSV-1 and anti-HSV-2 rabbit antisera but not with human sera drawn from patients with primary HSV-2 infection. This is in agreement with the partially type-specific nature of glycoprotein gC (Zweig *et al.* 1983, Lehtinen 1985, Lehtinen *et al.* 1985). The 118 kD and 58 kD proteins migrating like glycoproteins gB and gD were detected by both HSV-1 and HSV-2 infected patients sera. The characteristic reactivity of the 125 kD, 118 kD and 58 kD polypeptides in the immunoblotting analyses further supports their identity.

The predominant IgM and IgA reactive 80kD, 52kD and 40kD polypeptides most likely correspond to viral nucleocapsid proteins VP 13, VP 20 and VP 23 described by Heine *et al.* (1979) and used by Kühn *et al.* (1987). These may be among the first HSV antigens detected by the host immunity. It is possible that also early viral proteins play an important role early in the infection (Lehtinen *et al.* 1985*b*, Kühn *et al.* 1987). An analogous situation has been described in the induction of cell-mediated immunity by early viral proteins of murine cytomegalovirus (Reddehase and Koszinowski, 1984).

The apparent lack of IgM and IgA antibody formation against viral glycoproteins in most cases confirms the previous findings of Kühn *et al.* (1987) on HSV specific IgM. Corresponding defective IgG antibody response in acyclovir treated patients (Bernstein *et al.* 1984) was not reported to cause prolongation of symptoms. In fact, high titres of neutralizing antibodies to HSV (viral glycoprotein antibodies most likely included) have been found to correlate with an increased tendency of recurrences (Reeves *et al.* 1981). Thus, the rationale of using virus glycoproteins as vaccines against HSV warrants further investigation.

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Legends for Figures (Plates I–III):

Fig. 1. Fluorographic, silver staining, and immunoblotting analysis of HSV-1 specified polypeptides. HSV-1 specified proteins labelled between 16 to 18 hr post-infection (p.i.) by ^{14}C amino acid hydrolysate (S) or 4–18 hr p.i. by ^{14}C glucosamine (G) are marked (●). All proteins in the gel were made visible by silver staining (S). Immunoblotting analysis of the HSV antigen was done by rabbit anti-HSV-1 (I) and anti-HSV-2 (II) antisera (Dakopatts a/s, Denmark) and a preimmune rabbit serum (0). Serial serum samples of a 5-year old boy with primary HSV-1 infection were also available. Reactivity of IgG-antibodies in serum samples taken 0 (0), 12 (1), 47 (2), and 71 (3) days after the onset of symptoms is shown. Standard molecular weights are given on the left.

Fig. 2. Immunoblotting analysis of HSV antigen antibodies in a 28-year old woman with an acute genital herpes infection. The patient had HSV-1 isolated and acute phase, early convalescent phase and convalescent phase serum samples were taken 0, 10, and 30 days after the virus isolation. The glycoproteins migrating like the HSV-1 specified gC, gB or gD are marked (●). G = IgG, M = IgM, A = IgA.

Fig. 3. Immunoblotting analysis of HSV antigen antibodies in a 29-year old woman with an acute genital herpes infection. The patient had HSV-2 isolated and acute phase, early convalescent phase and convalescent phase serum samples were taken 0, 14 and 45 days after the virus isolation. The glycoproteins migrating like the HSV-1 specified gB, or gD are marked (●). G = IgG, M = IgM, A = IgA.