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SEROLOGICAL RESPONSES TO HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) ANALYSED WITH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND WESTERN BLOT (WB)

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Summary. - Serological response of 56 patients to primary and recurrent herpes simplex virus type 1 (HSV-1) infection were studied by enzyme-linked immunosorbent assay (ELISA) and by Western blot analysis (WB). ELISA test showed a high sensitivity in detecting IgG, IgM, and IgA antibodies. From 27 patients with recurrent infection, 13 (48%) had IgM antibody. The percentage of patients positive for IgA was similar among those with primary (72.4%) or recurrent (81%) infection. The band pattern in WB alone did not allow to distinguish between patients with primary or recurrent infections. Furthermore, no correlation between the particular viral proteins and clinical manifestation could be determined. The kinetics of antibody response could be followed with both methods.

Key words: herpes simplex virus; antibody response; Western blot; ELISA

Introduction

Infection with herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) may be symptomatic or asymptomatic. In the former case, the lesions usually involve the epithelium only. After initial replication the virus enters a latent state in the ganglia of the peripheral nervous system (Stevens and Cook, 1971), from which it may be periodically reactivated. Severe forms of primary or secondary HSV infection include encephalitis, *eczema herpeticum*, and keratitis (Nahmias and Norrlid, 1979). The pathology, latency, and epidemiology of HSV have been repeatedly discussed in several reviews (Rawls, 1973; Nahmias and Roizman, 1973; Klein, 1982; Braun and Reiser, 1987).

Serological response to HSV infection has been intensively studied (Jordan and Rytel, 1981; Kühn *et al.*, 1986). However, several aspects remain unclear, for example, the immune status of patients with recurrent infection and the contribution of the recently introduced Western blot procedure (WB). Therefore, in the present study we analysed the serum IgM and IgA in patients with

recurrent HSV-1 infection and the significance of WB with regard to a) distinction between primary and recurrent HSV infection, b) the correlation between various clinical manifestations and antibody production to different viral proteins, and c) the kinetics of antibody response following infection.

Materials and Methods

Human sera. Sera and, in one case, cerebrospinal fluid (CSF) coming from 56 patients were taken at different stages of HSV infection in clinical diagnosis of *stomatitis aphthosa* (32 patients), *eczema herpeticum* (17 patients), encephalitis (3 patients), meningitis (1 patient), and *herpes genitalis* (3 patients) (HSV-2). All sera were stored at -20 ^{0}C until use. One HSV-negative and one HSV-positive commercially available sera (Behringwerke AG, Marburg, Germany), as well as one serum from a patient with no history or symptoms of HSV infection, were included as controls. The CSF specimens were obtained from one patient with HSV encephalitis. All sera were routinely tested for the presence of antibodies against HSV by complement fixation test (CFT), neutralization test (NT), indirect immunofluorescence test (IFT), enzyme-linked immunosorbent assay (ELISA), and Western blot (WB). Only ELISA and WB data are shown here.

Enzyme-linked immunosorbent assay (ELISA). Detection of IgG, IgM, and IgA class-specific antibodies to HSV was made by the microtitre system (Enzygnost, Behringwerke AG, Marburg, Germany). HSV antigen-coated microtitre plates were used, the antibodies were detected by alkaline phosphatase (AP) - conjugated rabbit anti-human-IgG (γ-chain-specific), IgM (μ-chain-specific), as well as IgA (α-chain-specific) antibody (DAKOPATTS, Glostrup, Denmark). For determination of IgM antibodies all sera were pretreated with rheumatoid factor (RF adsorbents, Behringwerke AG, Marburg, Germany). Positive results were defined by a titre of 1/44 for IgG and IgA, and 1/42 for IgM. The absorbance was determined by spectrophotometry (Microelisa System, Dynatech, Denkendorf, Germany), using a control buffer as blank. An absorbance difference (Δ A 405) of 0.200 was regarded as cut-off.

Western blot. African Green monkey cells RC-37 provided by Braun and Kühn, Heidelberg, (1988) were grown in Basal Medium Eagle (BME) supplemented with 10% inactivated foetal calf serum (FCS) the cells were infected with the HSV-1 strain MacIntyre (ATCC Nr. VR 539) at a multiplicity of approximately 5 PFU/cell and incubated for 1 hr at 37 ^{0}C. After washing the monolayers were supplemented with fresh BME containing 5% inactivated FCS. The cells were incubated for exactly 36 hr. The supernatants from infected cultures were collected and centrifuged at low speed (1,500 x g) for 20 min. Subsequently, virions from the supernatant were pelleted by high speed centrifugation (100,00 x g) for 60 min in a SW 28 rotor (Beckman Instruments, Munich, Germany) and resuspended in 1.5 ml Tris-buffered saline (TBS) with 1 mmol/l phenylmethylsulphonyl flouride (PMSF) from Sigma (Munich, Germany). HSV proteins were purified according to the method of Kühn *et al.*, (1987). Purified HSV-1 proteins were separated by PAGE as described (Thomas and Kornberg, 1975) and then transferred to nitrocellulose sheets (Towbin *et al.*, 1979) as later modified by Braun and Reiser (1987). Following incubation with patient's serum, the reaction was detected with horseradish peroxidase (HRPO)-conjugated affinity purified goat anti-human IgG (H+L) (Bio-Rad, Munich, Germany) and visualized using 4-chloro-l-naphthol (Sigma, Munich, Germany) as substrate. The general procedure was similar as described by Kühn *et al.* (1987), except that all steps were performed at room temperature.

Results

ELISA showed a high sensitivity in detecting IgM, IgG, and IgA antibodies.

Table 1. Summary of patients with primary and recurrent HSV-1 infection

Symptoms	Primary infection (n = 29)	Recurrent infection (n = 27)
Stomatitis aphthosa	23	9
Eczema herpeticum	6	11
Encephalitis	-	3
Meningitis	-	1
Herpes genitalis	-	3

The IgG including positive controls ranged between 1/44 - 1/180, 224, the IgM titres between 1/42 - 1/5, 376, while the IgA antibody response was qualitatively determined using a patient serum in dilution 1/44. Both negative controls were negative. Based on ELISA results on other serological tests (CFT, IFT, NT) as well as the clinical history (data not shown), 29 patients could be identified as having a primary HSV infection while 27 had recurrent infection (Table 1).

The IgM antibody response could be detected as early as on day 3 and lasted

Table 2. IgM positive patients with recurrent HSV-1 infection

Patient Number (n=13)	Age (YR)	Days after onset of symptoms	Reciprocal of the titer IgM	Clinical manifestation
3	46	4	84	*Stomatitis aphthosa*
4	31	11	168	*Stomatitis aphthosa*
9	39	7	42	*Stomatitis aphthosa*
13	23	4	168	*Eczema herpeticum*
		15	168	
15	20	18	168	*Eczema herpeticum*
16	23	3	42	*Eczema herpeticum*
12	21	11	84	*Eczema herpeticum*
10	20	11	672	*Eczema herpeticum*
20	17	20	42	*Eczema herpeticum*
21	19	11	336	Encephalitis
		14	1344	
23	62	27	42	Encephalitis
		22 CSF	2688	
24	30	31	168	*Herpes genitalis*
26	19	19	42	*Herpes genitalis*

CSF = Cerebrospinal fluid

at least up to 4 weeks since the onset of symptoms. All patients with primary infection were positive for IgM. From the 27 patients with recurrent infection, 13 (48%) had IgM antibody (Table 2). On the other hand, the percentage of patients positive for IgA was similar for those with primary (21/29 = 72.4%) or recurrent (22/27 = 81%) infection (data not shown). The IgG antibody response as determined by ELISA appeared in the serum at the same time as IgM or may be followed within a few days by the production of HSV-IgM antibody. Thirty-two patients had significant rises of specific IgG titre, the highest being 1/11,264 in primary infection and 1/90,112 in recurrent infection.

The IgG antibody response from 51 of the 56 patients was followed by WB. Antibodies to viral proteins VP1/2, VP4/5, gC, gB, pgB, gE, gD, VP20, VP22a, and VP23 were detected in the tested sera during various stages of clinical manifestation (Tables 3 and 4). No bands were observed with the negative control serum obtained from a patient who had no history and symptoms of HSV infection. The band pattern alone did not provide a criterion for distinguishing patients with primary and recurrent infection the IgG response in general showed a stronger and broader reactivity pattern as compared to the response in primary infection (Fig. 1). Out of 27 patients with primary infection

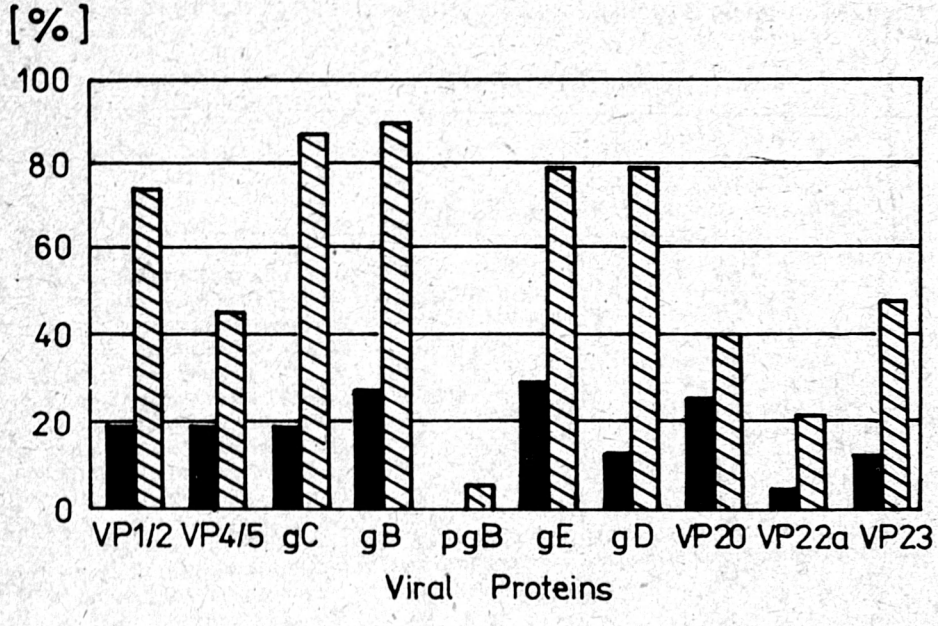

Fig. 1
Reactivity of sera from 27 patients with primary (empty columns) and 24 patients with recurrent HSV-1 infection (hatched columns) against various HSV-1 viral proteins as tested in WB

Table 3. Detection of IgG antibody response to HSV-1 using Western blot in 29 patients with primary infection

Patient Number (n = 29)	Days after onset of symptoms	VIRAL PROTEINS VP 1/2	VP 4/5	gC	gB	pgB	gE	gD	VP 20	VP 22a	VP 23	Clinical manifestation
1	1	neg.										*Stomatitis aphthosa*
	6			X					X			
2	4	neg.										*Stomatitis aphthosa*
	7		X		X				X			
3	7	X		X					X			*Stomatitis aphthosa*
4	8	neg.										*Stomatitis aphthosa*
	16						X					
5	4								X			*Eczema herpeticum*
	9			X	X				X		X	
	17			X	X				X		X	
6	7	neg.										*Stomatitis aphthosa*
	12				X		X					
7	2				X						X	*Eczema herpeticum*
	17			X	X		X		X			
8	5	neg.										*Stomatitis aphthosa*
	12				X		X		X		X	
9	8	neg.										*Eczema herpeticum*
	13								X			
10	5	N.d.										*Stomatitis aphthosa*
	10		X				X					
11	8	N.d.										*Stomatitis aphthosa*
12	8	N.d.										*Stomatitis aphthosa*
13	5	neg.										*Eczema herpeticum*
	14	N.d.										
14	21	neg.										*Stomatitis aphthosa*
	27	X					X					
15	4	neg.										*Stomatitis aphthosa*
	12	X	X		X		X					
16	11				X							*Stomatitis aphthosa*
	16				X							
17	6	neg.										*Stomatitis aphthosa*
	12	X					X					
18	1		X									*Eczema herpeticum*
	6		X									
	10		X									
19	2	neg.										*Stomatitis aphthosa*
	8		X					X		X		
20	4	N.d.										*Stomatitis aphthosa*
	11						X	X			X	
21	4	neg.										*Stomatitis aphthosa*
	14						X	X		X		
22	5		X	X	X				X		X	
23	7	neg.										*Eczema herpeticum*
	15	X	X		X		X	X				
24	5	neg.										*Stomatitis aphthosa*

Table 3 continued

	11	X	X		X						
25	3	N.d.									*Stomatitis aphthosa*
	9	X				X					
26	3	neg.									*Stomatitis aphthosa*
	19	N.d.									
27	4	X			X						
28	3	neg.									*Stomatitis aphthosa*
	11		X				X				
29	3	neg.									*Stomatitis aphthosa*
	n =	8	9	8	12	-	13	5	11	2	6

neg. = negative, N.d. = not done.

tested, 3 were negative, 10 were positive, and 14 were negative in the first serum but positive in the second (Table 3). Patients with primary infection responded in average to 2.7 bands. Patient's sera reacted most frequently to gE (28.2%) followed by gB (26%), VP20 (23.9%), VP4/5 (19.5%), gC and VP1/2 (both 17.3%), gD and VP23 (both 10.8%), and VP22a (4.3%); pgB was not detected in the tested sera.

Out of 24 patients with recurrent infection tested in WB, 2 were negative and 21 positive. In contrast to patients with primary infection, only one patient's first serum was negative and became positive in the second sample. (Table 4). Patients with secondary infection responded on the average to 5.8 bands. The highest reactivity was found to be against gB (87.5%), followed by gC (85%), gD (82.5%), gE (75%), VP1/2 (72.5%), VP23 (47.5%), VP4/5 (42.5%), VP20 (40%), VP22a (20%), and pgB (5%) (Fig. 1). Reactivity against pgB was detected only in sera from patients with recurrent infection.

Patients with both primary and secondary infections could be classified according to a variety of clinical manifestations (Table 1). It was of interest to determine whether particular clinical symptoms could be correlated with the presence of antibody to particular viral proteins. From WB results obtained with these patients, however (Tables 3 and 4), no such correlation could be observed. Out of 56 patients in this study, 3 had clinically confirmed herpes genitalis (HSV-2). Two of these patients (patients No. 24 and 25) were tested in WB and responded to the HSV-1 viral proteins gC, gB, and gE, and either VP1/2 or gD (Table 4).

Both methods, ELISA and WB, allowed to follow the kinetics of antibody response. Sera from two patients (Table 5) showed an increase in titre paralleling the increase in number of bands from first to the second, and in one case, to the third serum. In patient No. 5 (Table 5) the titre of IgG antibody in the second serum was 4 times higher, in the third serum 8 times higher than in the first serum. In addition, the IgM antibody titre in the third serum was twice of that seen in the first and the second sera. No change was seen in the IgA

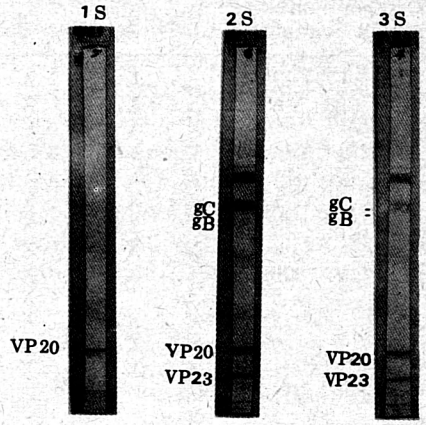

Fig. 2
Recognition of HSV-1 viral proteins in WB by IgG antibodies in three serum samples (1S, 2S, 3S) from Patient No. 5 with primary *eczema herpeticum*

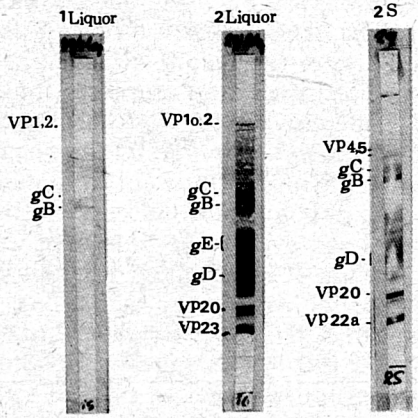

Fig. 3
Recognition of HSV-1 viral proteins in WB by IgG in two cerebrospinal fluids (1 and 2 CSF) in patient No. 23 with encephalitis after reactivation

Table 4. Detection of IgG antibody response HSV-1 using western blot in 27 patients with recurrent infection

Patient Number (n= 29)	Days after onset of symptoms	VIRAL PROTEINS VP 1/2	VP 4/5	gC	gB	pgB	gE	gD	VP 20	VP 22a	VP 23	Clinical manifestation
1	7		X		X		X	X		X		*Stomatitis aphthosa*
2	6	X	X	X	X		X	X	X		X	*Stomatitis aphthosa*
3	2	X	X	X	X		X	X				*Stomatitis aphthosa*
	4	X	X	X	X		X	X				
	11	X	X	X	X		X	X	X			
4	1	X	X	X	X		X	X	X		X	*Stomatitis aphthosa*
	11	X	X	X	X		X	X	X		X	
5	3	X	X	X	X		X	X				*Stomatitis aphthosa*
	20	X	X	X	X		X	X				
6	5	X		X	X			X		X		*Stomatitis aphthosa*
7	3	X	X	X	X		X	X		X	X	*Stomatitis aphthosa*
	9	X	X	X	X		X	X			X	
	11	X	X	X	X	X	X	X	X	X		
8	732	N.d.										*Stomatitis aphthosa*
9	7	X		X								*Stomatitis aphthosa*
10	11	X		X	X		X	X	X		X	*Eczema herpeticum*
11	5	N.d.										*Eczema herpeticum*
12	1	X	X	X	X		X	X	X			*Eczema herpeticum*
	11	X	X	X	X		X	X	X			
13	4		X	X	X			X			X	*Eczema herpeticum*
	15		X	X	X	X	X	X			X	
14	11	neg.										*Eczema herpeticum*
15	7	X		X	X		X					*Eczema herpeticum*
	18	X	X	X	X		X	X			X	
16	3	X		X	X		X	X		X	X	*Eczema herpeticum*
17	5	neg.										*Eczema herpeticum*
	13	X		X	X		X	X	X		X	
18	4	X		X	X		X	X	X		X	*Eczema herpeticum*
	15	X		X	X		X	X	X		X	
19	3	X		X	X		X	X	X		X	*Eczema herpeticum*
20	20	X			X		X	X				*Eczema herpeticum*
21	4						X	X		X		Encephalitis
	11			X	X		X	X			X	
	14			X	X		X	X		X	X	
22	3	neg.										Encephalitis
	16	N.d.										
23	4	N.d.										Encephalitis
	27		X	X	X			X	X	X		
	4	CSF X		X	X							
	22	CSF X		X	X		X	X	X		X	
24	31			X	X		X					*Herpes genitalis*
25	165	X		X	X		X	X				*Herpes genitalis*
26	19	N.d.										*Herpes genitalis*
	24	N.d.										
27	18			X	X			X	X		X	Meningitis
	34	X		X	X			X	X		X	
	n =	28	18	34	35	2	30	33	16	8	19	

response. In WB the first serum of this patient reacted only to one protein (VP20), while the second and third sera reacted to 4 proteins (Fig. 2). Furthermore, the IgG antibody titre in the second serum of patient No. 23 (with HSV-encephalitis) was 4 times higher than in the first serum. The IgM antibody was negative in the first serum but positive in the second, while the IgA response remained positive. The first serum was no longer available for testing in WB, but the second serum was found to react with 6 viral proteins (Fig. 3). In addition to serum, CSF from the latter patient was tested in ELISA and WB. The antibody response in the first CSF in ELISA was negative but became positive for all immunoglobulin classes in the second sample (Table 5). On the other hand, antibodies could be detected in the first CSF by WB (3 bands); the bands became more intensive in the second CSF sample (7 bands) (Fig. 3). Of interest was the much higher IgM response in the second CSF (1/2,688) as compared to the second serum (1/42).

Discussion

In this work sera of 56 patients were tested for antibodies to HSV-1 in ELISA and WB. Approximately the half of patients (29) were found to have primary HSV infection, in which IgG and IgM antibodies either appeared in the serum at the same time, or IgG antibodies were followed within a few days by the production of HSV IgM, as observed by Kurtz (1974). The IgM antibody persisted for at least 4 weeks (Enders, 1984; van Loon *et al.*, 1985). Our finding that HSV-IgM antibodies were present in patients with recurrent HSV infections as well has been previously reported (Kurtz, 1974; Kalimo *et al.*, 1977; van Loon *et al.*, 1985; Juto and Settergren, 1988). They suggested that the presence of HSV-IgM may be related to the severity of the infection. IgM antibodies have also been detected in patients with recurrent infections by other herpes viruses, such as VZV (Cradock-Watson *et al.*, 1979) or CMV (Cappel *et al.*, 1978; van Loon *et al.*, 1981). On the other hand, Kimmel *et al.* (1982) found no HSV-IgM in their patients with recurrent infection. In both, their and our studies, all sera were pretreated with RF-adsorbent to avoid false-positive IgM results. However, Kimmel *et al.* (1982) tested only 20 patients, 6 of whom had recurrent infection, compared to the 56 patients (27 with recurrent infection) tested in this study.

The presence and function of HSV-specific IgA in serum remains unclear. As such antibodies have been reported to be present in patients with both primary or recurrent HSV-infection, a distinction between these two groups based on IgA response alone may not be possible (El Falaky *et al.*, 1977). We found no difference in IgA response of patients with primary or secondary infection. On the other hand, Friedman and Kimmel (1982) found IgA antibodies only in patients with primary infection, whereas Doerr and Rentschler

Table 5. Kinetics of antibody response to HSV-1 in 2 patients as detected by ELISA and WB

Patient Number	Specimen	Days after onset of symptoms	ELISA Reciprocal of the titers IgC	IgM	IgA	Western blot Viral proteins
5	1S	4	1 408	672	44	VP20
	2S	9	5 632	672	44	gC, gB, VP20, VP23
	3S	17	11 264	1 344	44	gC, gB, VP20, VP23
23	1S	4	1 408	neg.	44	N.d.
	2S	27	5 632	42	44	VP4/5, gC, gB, gD, VP20, VP22a
	1CSF	4	neg.	neg.	neg.	VP1/2, gC, gB
	2CSF	22	2 816	2 688	44	VP1/2, gC, gB, gE, gD, VP20, VP23

S – Serum, CSF – Cereprospinal fluid, neg. – negative, N. d. – not. done.
Patient No. 5 – primary infection
Patient No. 23 – recurrent infection

(1987) and Juto and Settergren (1988) only in patients with secondary infection. Furthermore, in some cases of primary or severe recurrent HSV infections with specific IgM antibody, a significant rise of HSV IgG and IgA antibodies has been observed (Kalimo *et al.*, 1977; Kimmel *et al.*, 1982; Hadar and Sarov, 1984). We could not confirm the latter, as IgA was determined only qualitatively in this study.

IgG antibodies directed against various viral structural proteins could be demonstrated by WB soon after the onset of clinical symptoms. The proteins recognized by IgG antibodies (gC, gB, pgB, gD, gE, VP1/2, VP4/5, VP20, VP22a, and VP23) were also described (Kühn *et al.*, 1987). As compared to primary HSV-1 infections, the IgG response in recurrent infections showed a stronger and broader spectrum of reactivity. The pattern of glycoprotein bands in recurrent infections revealed a strong reactivity with nearly all viral proteins. On the other hand, no correlation could be observed in HSV-1 infected patients between clinical manifestations and particular viral proteins. The latter has not been studied elsewhere in the literature. Various data have been published on the appearance of antibodies against gC and gE. Antibodies against gC could be demonstrated in the majority of sera tested by Zweerink and Stanton (1981) using WB, as well as by Ashley *et al.* (1985) using radioimmunoprecipitation followed by polycarylamide gel electrophoresis (RIPA-PAGE). Our data, particularly from patients with recurrent infection, confirm this finding. On the other hand, Lehtinen *et al.* (1985) found only minimal immune response to gC using WB. Similarly, Kühn *et al.* (1987) reported antibodies to gC in only a limited number of cases tested, apparently due to the presence on gC of epitopes sensitive to denaturation during WB. This is, however, unlikely to be the only explanation, as our viral proteins were also denatured prior to electrophoretic separation.

Antibodies to gE were found by Ashley *et al.* (1985) but only seldom in sera tested by Eberle *et al.* (1984) or by Kühn *et al.* (1987) using both WB and RIPA-PAGE. The low response to gE in the latter two studies could be explained by an extensive blocking with calf serum and the use of peroxidase-conjugated $F(ab)_2$-fragments in WB. While we also used calf serum for blocking, for detection we used peroxidase-conjugated, affinity-purified goat anti-human IgG (H+L). The majority of our patients was positive for antibodies to gE. The demonstration of antibody reactivity directed against gC is of diagnostic and pathogenic interest, since gC is a predominately type-specific protein (Spear, 1976; Kühn *et al.*, 1987), which would allow serotyping of the infection by WB. In our study antibodies to gC were detected, but their presence was not informative as only HSV-1 proteins were tested. Similarly, antibodies to gG can be useful for serotyping of HSV infection (Braun and Reiser, 1987). Antibodies to gG, however, were not identified in this study, nor were they reported by Buckmaster *et al.* (1984) and Richman *et al. (1986).*

In addition to WB it is possible to distinguish between HSV-1 and HSV-2 infection by immunoprecipitation assay. The latter test detects a variable

response to gC, as well as another 4 glycoproteins of lower molecular weight with HSV-1 as with HSV-2 antigen (Eberle and Courting, 1981; Bernstein *et al.*, 1985).

The kinetics of antibody response was studied with both WB and ELISA. An increase in titre in ELISA was paralleled by an increase in number of bands in WB in sequential serum probes. Occasionally WB detected antibodies to HSV-1 proteins prior to their detection in ELISA. This tributes to the higher sensitivity of WB compared to ELISA. In analysing the kinetics of antibody response in the serum and CSF of the one adult patient with HSV-encephalitis, it was found that the IgM titre was much higher in the second CSF as compared to the second serum. Lerner *et al.* (1972) also found IgM antibody in the CSF of a neonate with encephalitis, not in the adult. In contrast, Connolly *et al.* (1971) and Kurtz *et al.* (1974) found no IgM in CSF in their patients with subacute sclerosing panencephalitis and HSV encephalitis, respectively. When present, IgM antibody in the CSF may be due to local antibody production, as IgM is incapable of penetrating the blood brain barrier (Kurtz, 1974; Jordan and Rytel, 1981). Detection of such antibodies in CSF could be of diagnostic value for HSV encephalitis.

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