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Evaluation of Solubilized Herpes Simplex Virus Membrane Antigen by Enzyme-Linked Immunosorbent Assay

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An antigen prepared by solubilization of membranes from herpes simplex virus (HSV)-infected cells with deoxycholate was evaluated by enzyme-linked immunosorbent assay. The deoxycholate-solubilized antigen, previously shown to contain all major HSV glycoproteins, was noninfectious and adsorbed easily and reproducibly to a polystyrene surface at pH 9.6. The deoxycholate-solubilized antigen provided an enzyme-linked immunosorbent assay of high sensitivity and reproducibility with complete correlation with complement fixation for the diagnosis of acute HSV infection. The correlation with neutralization and immunofluorescence for the presence or absence of anti-HSV activity was very good. Comparison with an HSV envelope preparation yielded results slightly in favor of the deoxycholate-solubilized antigen. The assay seems to be useful for demonstration of intrathecal production of antibody activity in HSV encephalitis.

In herpes simplex virus (HSV) infections, the antigenically altered cell membrane seems to be a major antigenic stimulus. HSV antigens in the membranes of infected cells are targets for defence mechanisms of the host (10, 11), and antibodies against HSV-induced glycoproteins can neutralize the virus (19) and will also kill virus-infected cells (11, 16). In recurrent infections, immunoglobulin G (IgG) response is primarily directed against cell membrane-associated HSV glycoproteins (7). Membrane-bound antigens may be of particular importance in serodiagnosis, and many assays for measurement of antibodies to surface antigens of virus or virus-infected cells have been developed. Radioimmunoassay has been used for determination of class-specific antibodies against HSV antigens in the HSV envelope fraction (7). Enzyme-linked immunosorbent assay (ELISA) has the same potential as radioimmunoassay for routine serology. However, the use of defined, purified, and preferably soluble antigens is a prerequisite for a specific, sensitive, and reliable assay. In the present communication, a solubilized membrane-associated HSV antigen for the determination of HSV antibodies by ELISA is described.

MATERIALS AND METHODS

Cells and virus. BHK-21 clone 13 hamster kidney cells were grown in 1-liter roller bottles for use in antigen production (5). For use in immunofluorescence (IF) and neutralization (NT) tests, GMK-AH1 cells were grown as previously described (5). The HSV type 1 (HSV-1) F strain was obtained from B. Roizman, University of Chicago, Chicago, Ill.

Antigen production. The preparation and solubilization of membranes from packed HSV-infected BHK-21 cells have been described by Jeansson et al. (6). Cells were mixed with 0.025 M Tris-hydrochloride (pH 8.0), homogenized with a Dounce homogenizer, and centrifuged at 1,500 × *g* for 15 min at 4°C. The membranes remaining in the supernatant were pelleted by centrifugation at 160,000 × *g* for 1 h, washed by suspension in 0.10 M glycine-sodium hydroxide buffer (pH 8.8), and centrifuged at 160,000 × *g* for 1 h. Four milliliters of washed membranes was suspended in 32 ml of cold 0.10 M glycine-sodium hydroxide buffer (pH 8.8), and 1.1 ml of 10% (wt/vol) sodium deoxycholate (DOC) (E. Merck AG, Darmstadt, Federal Republic of Germany) was added with stirring. The mixture was homogenized with 40 strokes of a Dounce homogenizer, incubated for 10 min on ice, and centrifuged at 160,000 × *g* for 1 h. The supernatant solution constituting the HSV antigen and the negative control antigen from uninfected membranes were stored at −70°C (Fig. 1).

Preparation of HSV envelope antigen. The procedure described by Martin et al. (9) and modified by Kalimo et al. (7) was followed. Briefly, infected cells were broken by ultrasonic treatment and subjected to differential centrifugation, including centrifugation at 100,000 × *g* for 1 h. The virion pellet was suspended in distilled water and treated with an equal volume of diethyl ether. Ether was removed, and the treated virus preparation was dialyzed. Envelopes were separated from capsids by centrifugation at 100,000 × *g* in a sucrose gradient.

Quantitation of HSV antigen and protein. The amount of precipitating HSV antigen present in the antigen preparations was determined by testing serial fourfold dilutions of the preparation against homologous HSV-1 antisera in countercurrent electrophoresis

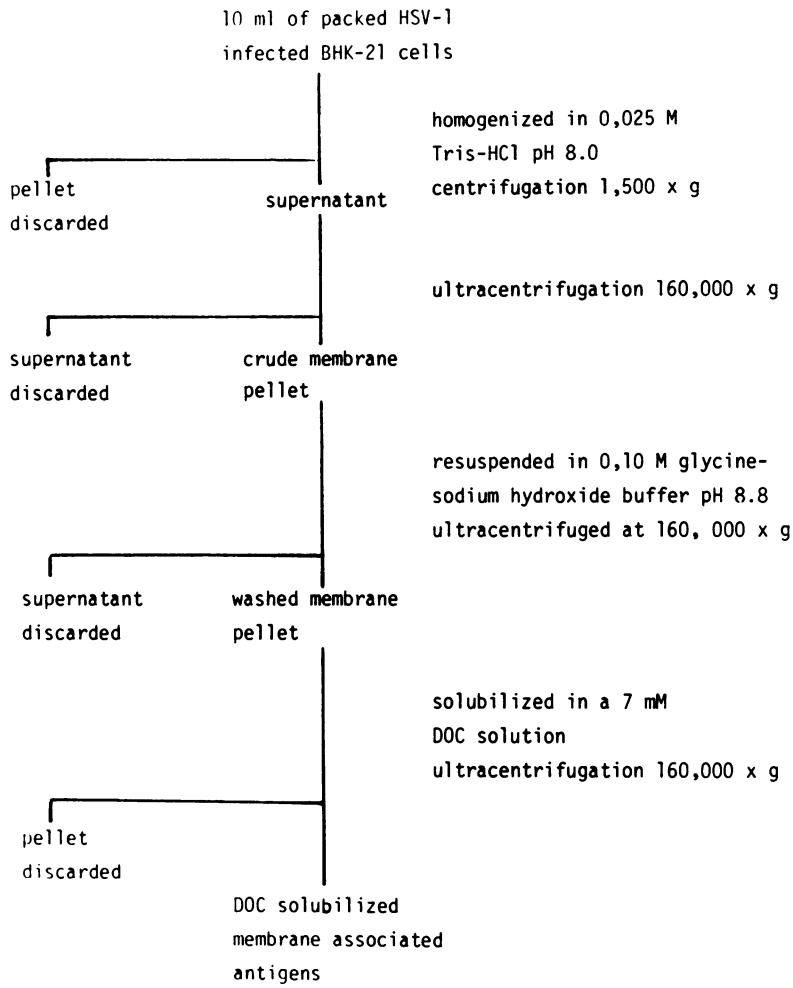

FIG. 1. Scheme for fractionation and solubilization of membrane-associated HSV-1 antigens from packed infected cells.

(4). Protein concentration was determined as the differences in UV absorbance at 280 and 310 nm.

Sera. Antisera against HSV-1 were prepared in rabbits by infection as previously described (5). Two panels of 50 and 94 blood donor serum samples were used for evaluation of the ELISA, as were paired serum samples from 37 patients with diseases compatible with primary type 1 or 2 infections (e.g., stomatogingivitis or herpes genitalis-meningitis; and paired serum samples from two immunosuppressed patients with recurrent type 1 infection with significant rise in complement fixation [CF] against HSV antigen). Paired serum samples from patients infected with varicella-zoster virus (eight patients), measles (seven patients), cytomegalovirus (two patients), Epstein-Barr virus (one patient), and tick-borne encephalitis (two patients) were also tested. Samples of cerebrospinal fluid (CSF) and serum taken on the same day were obtained from three patients with verified or probable HSV encephalitis.

Serological tests. Indirect IF was performed as previously described (18). HSV-1-infected GMK-AH1 cells were used as antigen in IF. CF was performed by the method of Sever (14) with minor modifications. Heat-inactivated, tissue-culture-derived crude HSV-1 CF antigen was used (5). NT tests were performed on GMK-AH1 cells in microtiter plates with 100 median tissue culture infective doses of HSV-1. The technique used was that of Pauls and Dowdle (12), modified as described by Grillner and Blomberg (3). Serum samples were tested in twofold dilutions. A titer of ≥ 2 was considered positive for both techniques.

The ELISA was performed in microtiter polystyrene plates from Dynatech AG (Nurtingen, Federal Republic of Germany) Greiner 129B irradiated plates. For comparison, Nunc (Roskilde, Denmark) A/S irradiated immunoplates and Nunc nonirradiated plates were used.

Antigens were used at optimal dilutions as determined by titration against known positive and negative serum samples. For coating, 200 μl of viral or control antigen diluted in 0.05 M sodium carbonate buffer, pH 9.6, was added to each well, and the plates were stored at 4°C for at least a week. Before use, the plates were

washed three times with phosphate-buffered saline containing Tween 20 (0.05%, wt/vol). Dilutions of serum or CSF in phosphate-buffered saline–Tween with 1% bovine albumin were reacted with the antigen in a volume of 200 μl. Standard dilutions of known positive and negative serum samples and blanks were included in each experiment. After three washings, alkaline phosphatase-labeled anti-human IgG was added at optimal dilutions in phosphate-buffered saline–Tween with 1% bovine albumin (1:2,000 Dako anti-IgG, conjugated by the method of Engvall and Perlman [1], and 1:400 Orion anti-IgG). Samples and conjugate were incubated for 2 h at 35°C. Phosphatase substrate:*p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) at 1 mg/ml in diethanolamine buffer, pH 9.8, was added. The reaction was stopped by the addition of 100 μl of 3 M NaOH, and the color reaction was read at 405 nm (Multiscan; Flow Laboratories, McLean, Va.).

RESULTS

Protein content and antigenic composition of HSV-1 antigens. After solubilization of crude membranes with DOC, the 160,000 × *g* supernatant had a protein content of 10 mg/ml (measured by UV absorbance at 280 to 310 nm); 10 ml of packed cells yielded 40 ml of antigen. For estimation of the amount of antigen, the DOC-solubilized antigen was titrated by countercurrent electrophoresis. Four precipitin lines were detected with HSV-1 antisera; 10 μl diluted 1:256 gave just visible precipitates after Coomassie brilliant blue staining. The HSV envelope antigen had a protein content of 3 mg/ml as determined by UV absorbance. One precipitin line was detected in countercurrent electrophoresis, and the antigen titer was 16.

The two antigens were reproducibly adsorbed to the polystyrene surface by incubation in sodium carbonate buffer, pH 9.6, at 4°C. After only 1 day, the coated plates could be utilized. However, further incubation up to 3 to 4 days yielded a more sensitive assay, with the endpoint titers of a positive reference serum becoming twofold higher. Further incubation did not increase the sensitivity.

Prolonged storage of the plates with the antigen in sodium carbonate buffer at 4°C did not result in any substantial loss of antigen: identical serum activities were recorded in a comparison between plates incubated for 1 week and for 2 to 3 months ($n = 7$; endpoint titer, 40,000; the mean absorbance with positive reference serum diluted 10,000 to 20,000 was 1.404 to 0.852 after incubation for 1 week and 1.350 to 0.753 after incubation for 2 to 3 months). A few plates incubated with the DOC-solubilized antigen in sodium carbonate buffer for more than a year yielded comparable results. In the following studies, plates incubated for 1 week to some months were used. Identical serum activities were reached when serum samples were tested in irradiated (Nunc immunoplate I, Greiner 129B) or nonirradiated (Nunc) polystyrene plates (data not shown). The majority of experiments were performed with Greiner 129B plates.

Determination of optimal dilution of antigens is shown in Fig. 2. Twofold dilution steps of positive antigens and corresponding negative antigens were reacted with a positive reference serum diluted 1:1,000 and a negative reference serum diluted 1:100. Results were recorded as optical density (OD) values at 405 nm. OD values reached somewhat higher levels with the DOC-solubilized antigen than with the HSV envelope antigen. The DOC-solubilized antigen was used at a dilution of 1:5,000, and the HSV envelope antigen was used at a dilution of 1:1,500. With negative control antigens diluted 1:200, only low activity was recorded (0.1 ODU).

Background OD values were somewhat lower for the DOC-solubilized antigen (the arithmetic mean for 11 negative serum samples was 0.050) than for HSV envelope antigen (arithmetic mean, 0.250) for the same serum samples under identical experimental conditions.

The assay was equally reproducible with either antigen. In replicate tests of a single serum dilution incubated for 16 h ($n = 48$), the coefficient of variation was 5.3% for the HSV envelope antigen (mean absorbance, 1.388) and 5.0%

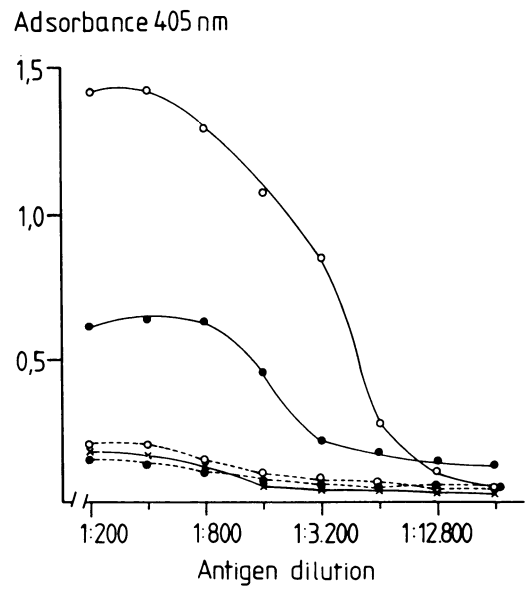

FIG. 2. Titration of DOC-solubilized antigen (○), HSV envelope antigen (●), and control antigens (×) in ELISA with use of standard concentrations of conjugate and antibody. ——, known positive antisera; – – – known negative antisera.

for the DOC-solubilized antigen (mean absorbance, 1.128). Alterations of parameters such as quality of polystyrene plates or time for incubation of serum samples (1 to 16 h) did not affect the reproducibility of the assay. The variation within each assay was further assessed by duplicate analyses of standard dilutions of two serum samples. In the absorbance interval from 0.5 to 1.8, the mean differences were 0.054 ± 0.047 (n = 207) and 0.046 ± 0.048 (n = 137) ODU, respectively. Added to this background, an absorbance value of 0.200 ODU was considered to be the cutoff level for positive readings. Such an absorbance difference represents less than a twofold titer difference. Endpoint titers of serum samples titrated in two- to fourfold dilution steps were recorded as the highest twofold dilutions yielding an absorbance difference between positive and negative antigen exceeding 0.200.

Owing to uncontrollable variations in temperature, polystyrene, technique, and timing, the variation between assays was greater. With the DOC-solubilized antigen with three serum samples, the coefficient of variation was 16.4% at an absorbance level of 1.306 (n = 122), 17% at 0.820 (n = 50), and 23% at 0.760 (n = 62). The resulting variation of the assigned endpoint may reach twofold.

Correlation with other serological tests. Agreement between NT and ELISA results with regard to positive and negative reactions was found in 93 of 94 serum samples (Table 1). One serum sample negative by NT testing was found positive at a moderate level by the ELISA with both types of antigen.

The titer range of NT tests was 2 to 32, and that of ELISA was 500 to 50,000. Absorbance values were somewhat higher with the DOC-solubilized antigen than with the HSV envelope antigen. With another panel of serum samples from 50 blood donors, ELISA activity was compared with IF antibody activity (Fig. 3). On the average, ELISA titers to DOC-solubilized antigen were 40-fold higher than IF titers and about 10-fold higher than ELISA titers obtained with HSV envelope antigen. A complete agreement between negative ELISA and IF results was found, and there was a clear correlation between the titers of serum samples containing antibody with the DOC-solubilized antigen (r = 0.76). The titers against HSV envelope antigen did not show the same linear correlation (r = 0.50) with IF.

TABLE 1. Correlation between neutralizing activity and ELISA anti-HSV IgG in serum samples from 94 blood donors

NT titer	ELISA titer							
	DOC-solubilized antigen				HSV envelope antigen			
	<50	50	500	≥5,000	<50	50	500	≥5,000
64				1				1
32				10				10
16				26				26
8			1	17			8	10
4			1	8			5	4
2			1	2		1	1	1
<2	26		1		26		1	

With the ELISA, a significant (4- to 100-fold) rise in titers to the DOC-solubilized antigen was found in all serum pairs from 39 patients with disease compatible with primary or activated HSV-1 or HSV-2 infections and for whom the diagnosis was confirmed by isolation of HSV or a rise in CF activity to HSV (Table 2). The first serum samples were taken on days 1 to 11 (mean, 5.7) and the second were taken on days 10 to 20 (mean, 16.4). The shortest interval between samples was 3 days. Significant rises in titers to the HSV envelope antigen were demonstrable in 37 of 39 serum pairs. In varicella infections, concomitant titer rises to HSV antigen were demonstrable in three patients by CF and in two of these three by ELISA with both types of antigen. Six varicella patients who were initially HSV negative remained negative in tests of their second serum samples. In 12 patients with viral infections other than HSV and varicella (10 seropositive to HSV), no titer rises were demonstrable. Intrathecal production of HSV antibodies was demonstrable with both antigens in three patients with verified or probable HSV encephalitis (Table 3); antimeasles activity, used as reference antibody, remained stationary.

DISCUSSION

In the ELISA, both the DOC-solubilized antigen and the HSV envelope antigen could be used at high dilutions (1:5,000 and 1:1,500, respectively), corresponding to protein concentrations of about 2 μg/ml. Similar findings have been reported by Leinikki and Passila (8) for highly purified mumps antigen in ELISA. Leinikki and Passila found that the antigen could be used at a high dilution (1:2,000) and a low protein concentration (0.5 μg/ml) for adsorption to the solid phase. For diagnostic purposes, a multicomponent antigen preparation is desirable because of individual variations in the antibody response to infection. The DOC-solubilized antigen, in which all major HSV glycoproteins were represented (6), proved very useful in the ELISA for diagnostic purposes and compared well with an HSV envelope preparation, which was previously demonstrated to be superior to crude virion, capsid, or excreted antigen preparations (7). We found that the ELISA titers obtained with the DOC-solubilized antigen were higher than those with HSV envelope antigen and that the correla-

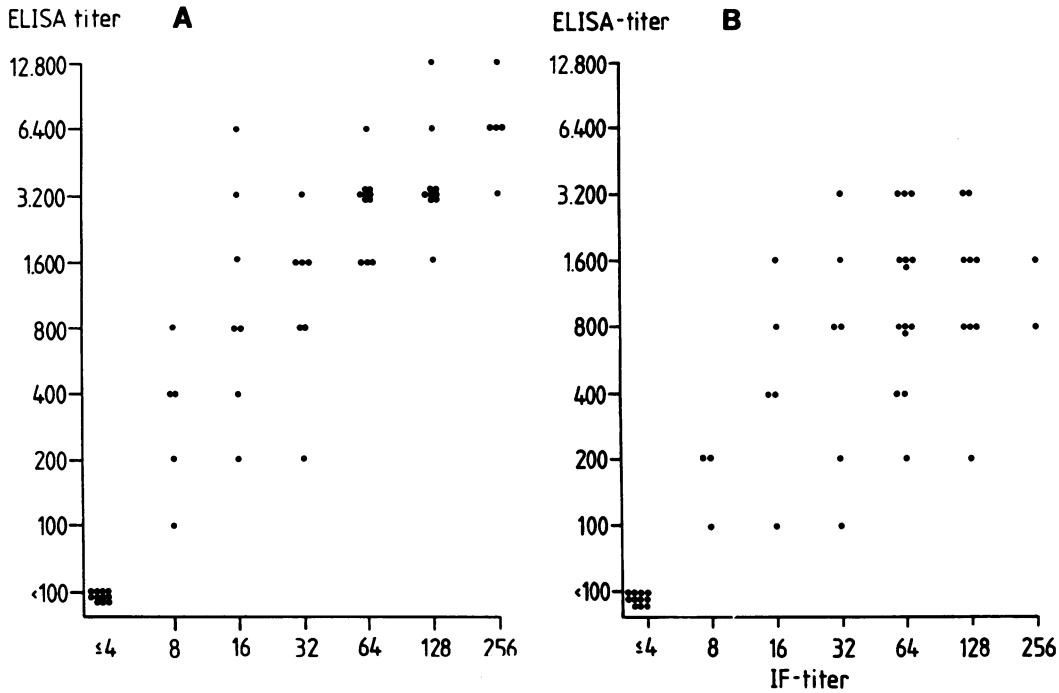

FIG. 3. Relationship between results obtained from analysis of blood donor serum samples by IF and ELISA, with use of DOC-solubilized antigen (A) and HSV envelope antigen (B).

tion with IF results was better. In the panel of acute- and convalescent-phase serum samples showing rises in CF antibody titers to HSV, significant rises were also shown for all patients by ELISA with DOC-solubilized antigen and for 37 of 39 patients by ELISA with the HSV envelope antigen. Neither antigen distinguished between HSV-1 and HSV-2 antibodies.

The relationship between HSV and other herpesviruses, especially varicella-zoster virus (13, 15, 17), could be expected to cause cross-reactions in ELISA with the two antigens. With both antigens, a rise of HSV antibodies was found in two of three initially HSV-positive varicella patients, whereas HSV-negative varicella patients remained negative. The cross-reaction seemingly occurs only in patients with prior HSV infection. The nearly complete correlation with seronegativity by NT tests in a panel of blood donor serum samples with high prevalence of varicella-positive individuals further supports the anamnestic nature of the cross-reaction. In the limited number of serum samples tested from patients with other virus infections, no cross-reactions could be detected. Experience in routine serology in parallel with CF tests for 2 years confirms this observation. The DOC-solubilized antigen has excellent adsorption properties and adheres well to the solid phase, and elution of adsorbed antigen during incubation and washing has not been a serious problem. The antigen has yielded highly reproducible results. If desired, the variation between assays may be reduced by recording the endpoint titer not as an absolute endpoint titer, but as a value related to an internal standard. The incubation time of sample and conjugate may be shortened to 60 min or even less, resulting in a rapid but still highly sensitive test. Prolongation of serum or conjugate incubation, on the other hand, yields a slightly more sensitive test (two-

TABLE 2. ELISA with DOC-solubilized HSV antigen and HSV envelope antigen on paired serum samples from patients with HSV and other virus infections

Type of infection	No. of patients			
	Total	With CF	With significant titer rise to HSV in ELISA	
			DOC-solubilized antigen	HSV envelope antigen
Primary HSV	37	37	37	35
Secondary HSV	2	2	2	2
Varicella-zoster virus	8	3	2	2
Other[a]	12	0	0	0

[a] Cytomegalovirus, Epstein-Barr virus, mumps, measles, and tick-borne encephalitis.

TABLE 3. ELISA IgG antibody activity against HSV and measles virus in CSF and serum from patients with herpes encephalitis

Patient		Time after onset	Titer					
			DOC-solubilized antigen		HSV envelope antigen		Measles	
Age (yr)	Sex		CSF[a]	Serum[b]	CSF	Serum	CSF	Serum
77[c]	Male	3 days	200	25,000	ND[d]	ND	200	25,000
		10 days	1,600	25,000	ND	ND	400	25,000
62	Female	5 days	200	12,800	100	6,400	200	6,400
		11 days	800	12,800	200	6,400	50	6,400
		15 days	12,800		6,400		50	6,400
		100 days	50,000	200,000	50,000	100,000	50	12,800
34	Female	7 yr	25,000	100,000	12,800	50,000	400	50,000

[a] Tested in fourfold dilutions from 1:12.5; incubation time, 16 h.
[b] Tested in fourfold dilutions from 1:50; incubation time, 16 h.
[c] HSV recovered from the brain at autopsy.
[d] ND, Not done.

to fourfold); more background activity will be encountered at low serum dilutions, but it may be advantageous to prolong incubation if IgM activity is analyzed in parallel.

The ELISA with the DOC-solubilized antigen is under evaluation for serological diagnosis of herpes encephalitis. HSV activity in CSF and serum is compared with reference antibody activity (generally measles) to correct for increased leakage over the blood-brain barrier. Intrathecal production of HSV antibody activity has been demonstrable to date in 12 of 13 biopsy- or autopsy-proven cases surviving ≥11 days after the onset of illness. In the CSF-negative case, chemotherapy was already started at the day of the onset of disease. In this case, antibody production to other types of HSV antigen (envelope, capsid, or crude nuclear extract antigen) could not be demonstrated. In central nervous system infections caused by bacteria or viruses other than HSV, no intrathecal HSV antibody production has been observed (15 cases observed hitherto). However, further observations are needed since production of anti-HSV IgG activity has occasionally been observed in other diseases, e.g., mumps infections (2). The cross-reactions with varicella virus must also be kept in mind in interpretation of the serological CSF data.

The antigen has been found in preliminary studies to be suitable for determination of IgM and IgA antibodies. Evaluation of parameters for IgM analysis is still in progress.

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