Passive Hemagglutinating Antibodies in Cerebrospinal Fluids in Herpesvirus Hominis Encephalitis[1] (36696)

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Along with other workers, we are studying the incidence and sequelae of herpesvirus hominis encephalitis (1). Under-reporting and failure to diagnose many patients with herpes simplex virus encephalitis persist. It is estimated that several thousand persons in this country are seriously afflicted yearly (2–4). On account of diagnostic difficulties the real prevalence of this encephalitis remains unknown.

Investigations of antiviral drugs make prompt diagnosis imperative (5). Diagnoses dependent upon biopsies of brain and subsequent isolations of virus or demonstration of intranuclear inclusions may miss a significant number of cases because the encephalitis, at least early in the disease, is a patchy lesion (6). Exposures to this common virus make rises in conventional neutralizing (N) or complement-fixing (CF) antibodies in blood either unreliable or of little prospective use. A ratio of four or more of complement-requiring (CRN) to conventional neutralizing antibodies of herpesvirus hominis, type 1 in a single acute phase serum indicates a concurrent antigenic stimulus. However, like other tests of antibodies in serum, humoral responses do not prove that an encephalitic process is due to herpesvirus hominis (6, 7).

We report initial studies of passive hemagglutinating antibodies (PHA)[3] to herpesvirus hominis, types 1 or 2 in cerebrospinal fluids of seven patients with encephalitis.

Methods. Patients. Cerebrospinal fluids and sera from patients with presumed herpesvirus encephalitis, collected by us from 1967–1971, were used. Two of the cases are perinatal infections. Herpesvirus hominis was isolated from the brain at necropsy in four cases. In the remaining cases diagnoses were based upon clinical courses and serological evidence of recent exposure to this herpesvirus as outline elsewhere (1). Fourfold rises in bloods of N, CF, or PHA are considered significant, as is a single sample with CRN/N of four or more (6).

For comparison with the cases with encephalitis, paired bloods and spinal fluids from patients who required lumbar punctures in their care were tested. These controls included 17 patients with benign virus meningitis, four patients with bacterial or fungal meningitis, and 14 others with varied diagnoses (cerebrovascular accidents, Parkinson's disease, cancers, epilepsy). We cannot justify spinal punctures in patients with herpes labialis. Although of interest, cerebrospinal fluids from this group were not studied. Earlier, we measured antiherpesvirus antibodies in serum from a number of patients in this latter group (6).

Virological methods. Isolation, passage, and quantitation of herpesviruses; immunotyping of strains; and measures of conventional and complement-requiring neutralizing antibodies are described elsewhere (1, 5, 6).

Passive hemagglutinating antibodies. The method was developed for herpes simplex viruses by Felton and Scott in 1958 (8). It was modified and adapted as a microprocedure by Fuccillo *et al.* (9). The latter workers

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[3] PHA is used to mean passive hemagglutinating antibody(ies) or passive hemagglutinating antibody titer(s).

reported results of tests for 14 patients with rises in complement-fixing antibodies. They noted that PHA do not cross react with cytomegalovirus or varicella-zoster virus. We have further modified the procedure and studied these antibodies in rabbits and man. When specimens were sufficient, sera and spinal fluids were subjected to disulfide bond cleavage with 2-mercaptoethanol, followed by alkylation with iodoacetamide.[4]

A. Preparation of antigen. 1. Inoculate twelve to fifteen 32 oz bottles of MA-196 cells with 1 ml of seed virus titering approximately $10^{6-6.5}$ TCD_{50}/ml. Ket (type 1) and Brod. (type 2) strains were used. Potent antigen can also be prepared using primary human foreskin cultures. Foreskin tissue cultures are now used routinely by us.

2. Add 50–75 ml of Eagle's medium containing 2% fetal calf serum; incubate at 35°.

3. Change medium 24 hr later.

4. After 3 to 4 days, cytopathic effects are complete. Scrape cells from bottles with a rubber policeman, and centrifuge at 2000 rpm (4°) for 5 min. Discard supernate.

5. Wash cells once with sterile phosphate buffered saline (PBS), pH 7.2.

6. Make a 10% suspension of the infected cells in PBS, and rapidly freeze–thaw three times using acetone and dry ice.

7. Centrifuge at 2000 rpm for 30 min.

8. Remove supernate and store in 1 ml samples at —70°. Discard cellular debris.

9. Culture prepared antigen (thioglycolate broth), and titer virus in BHK-21 (baby hamster kidney) tube cultures. Good antigens titer about $10^{7.5}$ TCD_{50}/ml.

B. Preparation of tanned sheep erythrocytes [TSRBC]. 1. Wash sheep blood three times in PBS (pH 7.2) and make a 3% suspension.

2. Add this suspension to an equal volume of freshly prepared tannic acid, diluted 1:40,000 in PBS.

3. Incubate in a water bath (37°) for 15 min, shaking gently at 5-min intervals.

4. Centrifuge at 2000 rpm for 5 min, and wash once with PBS. Cells are usually slightly hemolyzed following treatment with tannic acid.

5. Dilute the packed cells in saline (pH 6.4) to make a 3% suspension.

C. Sensitization of the tanned sheep RBC (SSRBC). 1. Dilute antigen in saline (pH 6.4) as determined under "standardization of antigen." Add an equal volume of TSRBC. Currently, we dilute antigen in saline containing merthiolate 1:10,000. Incubate 23° for 4 hrs. The herpesvirus used in the final test is inactive.

2. Allow the mixture to stand at room temperature for 15 min, agitating at 5-min intervals.

3. Centrifuge at 2000 rpm for 5 min and wash twice in PBS containing 1% pooled normal rabbit serum (PNRS).

4. Resuspend the SSRBCs in PBS with 1% PNRS to a concentration of 1%.

D. Micro-passive hemagglutination test. 1. Using V-bottom plastic plates (Microbiological Associates), add 0.025 ml of diluent (PBS + 1% PNRS) to each well.

2. Make serial twofold dilutions of heat inactivated test serum from 1:5 to 1:10,240. If serum controls (e, f, below) are positive, absorb and test the serum diluted 1:5 in PBS with TSRBC for 1 hr at 23°.

3. Add 0.025 ml diluent to each well and shake gently.

4. Add 0.025 ml of the 1% SSRBC to each well and shake.

5. Seal plates with cellophane tape, and incubate at 23° for 1.5 hr.

6. Record highest dilution exhibiting hemagglutination.

7. Controls. a. a known positive standard antiserum with a predetermined passive hemagglutinating antibody titer (PHA) (6);

b. washed SSRBC plus diluent;

c. TSRBC plus diluent;

d. SSRBC plus diluent;

e. lowest dilution (1:5) of test serum plus washed SRBC;

f. lowest dilution (1.5) of test serum plus TSRBC.

E. Standardization of herpesvirus antigen. 1. Dilute antigen 1:2, 1:4, 1:8, 1:16 in saline (pH 6.4) and add an equal volume of TSRBC (3%) to each dilution.

2. Allow the mixture to stand at room

[4] Lerner, A. M., and Shippey, M. J., unpublished data.

TABLE I. PHA in CSF and Serum of Patients with Herpesvirus Hominis Encephalitis.

Sex	Age	Herpesvirus hominis type	Date of specimen	Antibodies: PHA[a] Type 1 CSF	PHA[a] Type 1 Serum	PHA[a] Type 2 CSF	PHA[a] Type 2 Serum	Type 1; se N	Type 1; se CRN
rus isolated from brain									
F	27 yr	1	2/18/71	4[b]	160(160)[c]	—[d]	5(40)	8	16
			2/20/71	16	320	8	40	—	—
			2/24/71	—	320	—	40	—	—
			2/27/71	—	640(640)	—	40(80)	8	64
F	62 yr	1	7/29/68	8	160(320)	4	40(40)	64	128
F	7 days	2	8/20/71	2(4)	40(10)	256(<2)	5120(<2)	—	—
	11 days		8/24/71	4(<2)	40(10)	1024(<2)	5120(<5)	—	—
F	7 days	2	12/16/69	32(<2)	—	1024(<2)	—	—	—
rus not isolated from brain									
F	48 yr		5/4/66	128[b](128)[c]	1280(160)	64(32)	1280(160)	32	>512
			7/15/66	—	1280(160)	—	320(40)	>256	>512
F	22 yr		12/22/67	16(16)	80(320)	8(4)	<5(80)	16	128
			1/9/68	—	1280(5120)	—	20(320)	32	64
			5/29/68	—	160(640)	—	<5(80)	128	512
			7/22/68	—	160(640)	—	<5(160)	16	1024
F	61 yr		1/5/71	—	320(160)[a]	—	20(20)	64	128
			1/19/71	32(8)	—	4(8)	—	—	—
			2/2/71	256(512)	—	32(16)	—	—	—

A, passive hemagglutinating antibodies; N, conventional neutralizing antibodies; CRN, complement-requiring neutralizing anti
nent-fixing antibodies; CSF, cerebrospinal fluid.
ılts of tests are expressed as reciprocal of the highest positive dilution.
r in parenthesis represents result after reduction (2-mercaptoethanol) and alkylation (iodoacetamide) of specimen (10).
test not done.

temperature for 15 min, shaking gently at 5-min intervals.

3. Centrifuge at 2000 rpm for 5 min and wash twice in PBS plus 1% PNRS.

4. Resuspend in PBS plus 1% PNRS to a concentration of 1%.

5. Run these four preparations of antigen in the passive microindirect hemagglutination test using a known positive antiserum determined by conventional neutralization test (4).

6. In later testing, use the highest dilution of antigen that gives the highest titer of PHA against the standard antiserum (usually 1:4).

F. Reagents. 1. PBS (pH 7.2): Solution A: NaCl, 8 g; KCl, 0.2 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.2 g. Add distilled water to a volume of 800 ml. Solution B: $CaCl_2$, 0.1 g. Add distilled water to a volume of 200 ml. Mix solutions A and B. Adjust pH to 7.2.

2. Sheep red blood cells preserved in Alsevier's solution are purchased from Colorado Serum Co., Denver, CO. Sheep cells are not used for longer than 15 days. They are stored in the dark (4°).

3. Tannic acid (Fisher Scientific Co., Detroit, MI) is diluted 1:100 in saline. It is stable for 30 days when stored in the dark (4°).

4. Saline (0.15 *N* in distilled water), pH 6.4.

5. Pooled rabbit serum. Fresh pools or commercial sources may be used. Inactivate (56°, 1 hr) and absorb overnight (23°) with TSRBC (0.1 ml packed TSRBC for each milliliter of serum); store (−20°).

Results of tests for antibodies are expressed as the reciprocal of the highest dilution showing hemagglutination.

Results. Comparisons of PHA in cerebrospinal fluids and sera with N, CRN, and CF antibodies in sera of four fatal cases of encephalitis from which strains of herpesvirus hominis were isolated from brains at autopsy are shown in Table IA. These patients are female, two adult and two neonates. Herpesvirus hominis, type 1 was isolated from the two adults, and type 2 viruses were recovered from the infants. In patient 1, initial PHA to type 1 virus in CSF and sera are 4 and 160, respectively. The PHA in serum to type 2 herpesvirus hominis is 5. Neutralizing antibodies (N, 8 and CRN, 16) are found in this same serum, but CF antibodies are not noted. In this specimen of serum CRN/N is 2, and does not differentiate a prior from a current antigenic exposure. Two days later a significant fourfold rise in CSF-PHA[5] to 16 to type 1 herpesvirus hominis is noted at a time when similar serum antibodies rise only twofold. In this second specimen of CSF the PHA to herpesvirus hominis, type 2 is 8, indicating a heterotypic antibody rise in the CSF. In the single CSF available in patient 2, PHA to herpesviruses types 1 and 2 are 8 and 4, respectively.

Sera and spinal fluids from the two perinatal cases of herpesvirus hominis infections have PHA to type 2 viruses which greatly exceed those to type 1 (Table IA). In patient 3 homotypic PHA in CSF and sera are 256 and 5120 on the seventh day of life, respectively. Four days later the titer in serum is constant, but antibodies in CSF multiply by four. In patient 4 PHA to herpesvirus hominis, type 2 in CSF is 1024 on the seventh day of life. After treatment of CSF of these infants with 2-mercaptoethanol antibodies of type 2 herpesvirus disappear, indicating that the involved immunoglobulins are IgM, and produced by the infants (10). In patients 1 and 3 in whom two specimens of spinal fluid and blood are available, significant rises in antibodies in the central nervous system occur when similar increases are not yet present in sera. In this regard, concurrent experiments in rabbits indicate that early after infection with type 1 herpesvirus hominis tests for PHA are 3 to 14 times more sensitive than similar values measuring N antibodies. Likewise, PHA are 5 to 22 times more sensitive than N antibodies for type 2 virus.[4]

Data from three additional patients (all women) in whom diagnoses of herpesvirus hominis encephalitis (type 1) are based upon clinical courses, microbiolgic studies, and analyses of tests for antibodies in serum as

[5] CSF-PHA is a single designation used to indicate cerebrospinal fluid–passive hemagglutinating antibody titer(s).

TABLE II. PHA to Herpesvirus Hominis in CSF During Various Conditions.

Disease	No. of cases	No. of cases with PHA ≧ 16 (type 1 herpesvirus)	Mean PHA	
			Type 1	Type 2
Virus meningitis, benign (enterovirus, mumps, herpesvirus hominis?, ...)	17	1	3	5
Various (cerebrovascular accidents, Parkinson's disease, cancers, epilepsy, osteoarthritis, ...)	14	0	6	10
Meningitis (bacterial of fungal)	4	0	4	5

indicated earlier are also shown in Table IB (1). Attempts at isolation of herpesviruses by biopsy of the brain were not made. PHA to type 1 herpesvirus hominis in CSF are 128 (patient 5), 16 (patient 6), and 32 and 256 (patient 7). Lesser CSF responses to type 2, herpesvirus hominis are also noted. Mercaptoethanol reductions of the fluids suggest that most, but not all, of these immunoglobulins are IgG.

On the other hand, PHA are remarkably absent in CSF among 35 control patients with various conditions (Table II). Only one of this group had a CSF-PHA (type 1, herpesvirus) of 16. This patient had benign virus meningitis of unknown cause. It is conjectural whether this disease may have been etiologically related to herpesvirus hominis, type 1. Mean CSF-PHA to herpesvirus hominis, types 1 and 2 in 17 patients with benign virus meningitides, 14 in various states, and four with bacterial or fungal meningitis are 3, and 5; 6 and 10; and 4 and 5, respectively (Table II). The absence of CSF-PHA in the controls is of especial interest since most adults in Michigan have N antibodies to herpes simplex virus in their sera (6).

Cells, protein, and glucose are shown in the 9 spinal fluids of patients with herpesvirus encephalitis (Table III). PHA are 256 (patient 4), and 128 (patient 5), and 256 (patient 7) when there are 8 white blood cells, 12 cells, and 15 cells/mm^3, respectively, in CSF. In these patients with high CSF-PHA

TABLE III. PHA and Other Findings in CSF of Patients with Herpesvirus Hominis Encephalitis.

Type of herpesvirus hominis	Patient[a]	CSF-PHA	Other findings			
			White blood cells/mm^3	Red blood cells/mm^3	Protein (mg/100 ml)	Sugar (mg/100 ml)
1	1	4	0	20	110	—[b]
		16	20(1P, 19L)[c]	140	128	—
1	2	8	54(12P, 42L)	0	70	82
2	3	1024	83(13P, 70L)	Many	288	41
2	4	256	8(—)	156	64	48
1	5	128	12(1P, 11L)	0	95	40
1	6	16	200(200L)	25	214	54
1	7	32	130(—)	35,000	280	120
		256	15(15L)	770	—	—

[a] Number of case is the same as that used in Table I.

[b] —, test was not done.

[c] P, polymorphonuclear leukocytes; L, lymphocytes or monocytes.

there are 156 (patient 4), 0 (patient 5) and 770 (patient 7) red blood cells/mm^3 in fluids. Proteins are 64 mg/100 ml (patient 4) and 95 mg/100 ml (patient 5). Cells and proteins in spinal fluids roughly measure the degree of injury to the membranes comprising the blood–brain barrier. Greater damage to the pia-arachnoidal membranes theoretically allows easier access to spinal fluid of antiherpesvirus immunoglobulins from blood. Nevertheless, there is no apparent relation between parameters of injury to the meninges and CSF-PHA. Glucose in CSF is somewhat depressed in 4 of 6 specimens.

Discussion. Antibodies in CSF to rubeola virus have been found in patients with subacute sclerosing panencephalitis (11, 12). This test is very helpful in the diagnosis of this condition. Several years ago, we attempted unsuccessfully to find neutralizing antibodies in cerebrospinal fluids of patients with herpesvirus encephalitis (6). We were stimulated again by the preliminary findings of Nahmias *et al.* (13) who, using immunofluorescent techniques, demonstrated IgM antiherpesvirus immunoglobulins in spinal fluids of several patients with neonatal infection. Increased sensitivity for antiherpesvirus antibodies of the micro indirect passive hemagglutination procedure allows the present results.

PHA are virtually absent in 35 spinal fluids from patients with various virus, fungal, or bacterial meningitis, strokes, Parkinsonism, cancers of the central nervous system, vertebral osteoarthritis, or convulsive disorders. Antiherpesvirus PHA in the central nervous system do not correlate with the degree of injury to the meninges, at least as measured by pleocytoses or content of protein. We have remarked that severe herpesvirus encephalitis may occur without increases in cells or protein in spinal fluid (1). The disparity in kinetics of the earlier rises in PHA in CSF as compared with serum suggests that the IgM or IgG antiherpesvirus immunoglobulins may be synthesized in lymphocytes comprising inflammatory exudates within the brain. We note that initial titers of PHA in CSF in the two patients with herpesvirus hominis encephalitis from whom strains of type 1 virus were isolated at brain biopsy are low (Table IA). However, 2 days later, in patient 1 the CSF-PHA is 16.

It is noteworthy that PHA in spinal fluids remain free; that is, not bound to herpesviruses in antigen–antibody complexes. However, in herpesvirus hominis encephalitis, like poliovirus encephalitis, virus is rarely present in spinal fluid (14, 15). CSF γ-globulins are normally derived from serum, but in patients with inflammatory or demyelinating diseases of the central nervous system, a significant portion of the γ-globulin is locally produced. Such local production has already been shown in multiple sclerosis, neurosyphilis, and subacute sclerosing panencephalitis (16, 17). We now propose herpesvirus hominis encephalitis to this list.

These preliminary data suggest that measure of passive antiherpesvirus hemagglutinating antibodies in spinal fluids of patients with encephalitis may be useful in diagnosis. Results are available within hours after lumbar puncture and might be used to initiate or temper antiviral chemotherapy.

Summary. Passive hemagglutinating antibodies (PHA) to herpesvirus hominis were assayed in spinal fluids obtained during illnesses in seven patients with herpesvirus encephalitis. There were five cases in adults due to type 1 virus, and two perinatal type 2 infections. CSF-PHA were present in each case. Antibodies in CSF were IgM in the neonatal cases, but predominantly IgG in the rest. PHA in spinal fluid do not correlate with degree of meningeal injury as indicated by pleocytoses or protein. Kinetics of rises in PHA in spinal fluids and sera suggest that the antiherpesvirus antibodies in the central nervous system may be locally produced. PHA to herpesviruses were virtually absent in spinal fluids of 35 control patients with various conditions requiring lumbar puncture.

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