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Improvements in Obtaining and Characterizing Mouse Cerebrospinal Fluid

Application to Mouse Hepatitis Virus-induced Encephalomyelitis

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Summary

This report describes advances in techniques for analyzing cellular and humoral immune components in the cerebrospinal fluid (CSF) of the mouse that are applicable to other laboratory animals. CSF studies undertaken during experimental infection of mice with JHM strain virus (JHMV) of mouse hepatitis virus are presented. A critical pitfall which can lead to erroneous or invalid results is contamination of the CSF by even minute quantities of blood. Means of avoiding this contamination are attention to anatomical reference points, the use of a micropipet, and prior intracardiac perfusion of animals with phosphate-buffered saline. Cells in the CSF were typed as either B, T, polymorphonuclear, or mononuclear cells by the combination of a microcytotoxicity assay and histologic stains. A radioimmunoassay (RIA) allowed quantification of antibodies to JHMV in the CSF and indicated the presence of intrathecal synthesis of antibody in chronically infected mice. The combined use of these sensitive methods makes possible CSF analysis in individual mice rather than in pooled groups.

Key words: *Cerebrospinal fluid – Encephalomyelitis – JHM strain virus – Mouse hepatitis virus*

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Introduction

The central nervous system (CNS) has been referred to as an immunologically privileged site because of the relative isolation of brain and spinal cord from the systemic vascular and immune systems (Medawar 1948). By contrast, the cerebrospinal fluid (CSF) is in intimate contact with the CNS parenchyma and is considered an excellent indicator of metabolic (Cutler 1980), neoplastic (Wertlake et al. 1972), and immune (Lisak and Zweiman 1977; Trotter and Brooks 1980) processes occuring within the CNS itself. Thus, examination of the CSF is often of considerable clinical and theoretical importance in assessing neurological diseases, especially those with immunological features, such as multiple sclerosis or neurosyphilis.

CSF analysis is also of value in the study of experimental neurological disease. With large animals such as sheep or primates, essentially the same techniques used in human CSF examination may be applied (Nathanson et al. 1979). However, in small laboratory animals, obtaining and characterizing CSF is often fraught with technical difficulties. Because mice are commonly employed in animal models of disease, it would be useful to have a reliable means of assessing CSF in this species. Carp et al. (1971) first introduced a method of withdrawing CSF from mice by means of cisterna magna puncture. Zinkernagel and Doherty (1973) adapted this method to the study of sensitized lymphocytes in the CSF during lymphocytic choriomeningitis virus encephalitis. Gerhard et al. (1978) analyzed antiviral antibodies in the CSF following infection with Parainfluenza type 1 (6/94) virus, and Griffin (1981) has studied the entrance of immunoglobulin classes into the CSF of mice during acute viral encephalitis. More recently, Doherty and Gerhard (1981) have used antiviral monoclonal antibodies as probes in the analysis of the blood-brain barrier.

The present paper draws upon and extends these studies. JHM virus (JHMV), a neurotropic member of the mouse hepatitis (MHV) group of coronaviruses, has been extensively used to study viral-induced demyelination in mice (Weiner 1973; Robb and Bond 1979; Stohlman and Weiner 1981). As part of an on-going study of pathogenesis in this model, CSF was obtained during several experiments. A modification of the method of Carp et al. (1971) for obtaining CSF and a specially designed micropipet were utilized. A microcytotoxicity assay, together with a non-specific esterase stain, allowed enumeration of all major cellular types within the CSF. Determination of CSF antiviral antibody by RIA was performed. The means of avoiding contamination of CSF with blood or tissue, as well as the importance of non-contamination of CSF, are stressed in the techniques presented. Using these methods, CSF from individual mice may be characterized.

Materials and Methods

Mice

Animals used in these experiments were 6–8 week-old C57BL/6 (B6) and A/J mice obtained from Jackson Laboratories, Bar Harbor, ME. Mice were spot-checked by the solid phase RIA described below for the presence of antibody to JHMV

before use in these studies. Chimeric mice were constructed by the method of von Boehmer et al. (1975). Bone marrow recipients, 6 months of age, were irradiated with 950 rads. Femur marrow cells were removed from donors, treated with Anti Thy-1 plus complement as previously described (Stohlman et al. 1980) to remove mature T lymphocytes, and then injected into recipients at 2×10^7 cells/animal. Each animal was checked for chimerization by tissue typing its splenic cells using the microcytotoxicity assay described in Methods.

Viruses

The JHM strain of mouse hepatits virus (JHMV) was originally isolated from a mouse with hind leg paralysis and demyelination (Cheever et al. 1949). The DS strain, a small-plaque variant of JHMV, was used in this study. This virus was plaque-purified and propagated in DBT cells as described previously (Stohlman et al. 1982). For studies of acute viral infection, mice were given 1000 plaque-forming units (pfu) intracerebrally (i.c.) (chimeric mice) or 3600 pfu intranasally (i.n.) (B6). For studies of chronic viral infection with JHMV, B6 mice were given 1000 pfu i.c., and CSF was taken 12 months later. Control viruses used in the RIA were influenza virus type A, mouse-adapted, kindly supplied by Dr. Peter Brayton, and herpes simplex virus type 1 kindly supplied by Dr. Dru Willey, both of the University of Southern California, School of Medicine.

Collection of CSF

Mice were first anesthestized with methyoxyflurane inhalation (Metafane, Pitman-Moore, Washington Crossing, NJ) or intraperitoneal pentobarbitol, and a thoracotomy was performed with the animals supine on a dissection rack. The right atrium was opened and the mice perfused through the left ventricle by hand or by a pressure apparatus set at 3 p.s.i. with approximately 30 ml of phosphate-buffered saline pH 7.2 (PBS) via a 26-gauge needle. Adequate perfusion was signaled by blanching of the liver.

The skin over the posterior neck was removed by two incisions, the first in the midline from low cervical area to the anterior cranium and a second across the craniocervical junction, just below the level of the ears. Subcutaneous tissue and nuchal muscles were exposed, sectioned along the rim of the occipital bone, and then removed laterally unveiling the glistening clear arachnoid membrane overlying the cisterna magna (Fig. 1). By utilizing a stereo dissecting microscope, a micropipet was guided into the cisterna magna as shown in Fig. 1. The micropipet was constructed by cutting a 30-gauge needle with scissors (Yale Incorporated, Rutherford, NJ) to approximately 6–7 mm; a thin guide wire was placed through the needle when cutting it, so as to avoid collapsing the lumen. The needle was then inserted 4–5 mm into the lumen of a 10- or 20-μl micropipet (Van-Lab micropipets, VWR Scientific Inc., Los Angeles,CA). The junction was sealed with a drop of epoxy resin (Foxy Poxy, Krazy Glue, New York, NY). The needle–pipet assembly was then attached to the mouth suction apparatus supplied with the micropipets. An in-line trap provided with the micropipet prevented aspiration of CSF.

Care was taken not to puncture the fourth ventricle across the inferior medullary

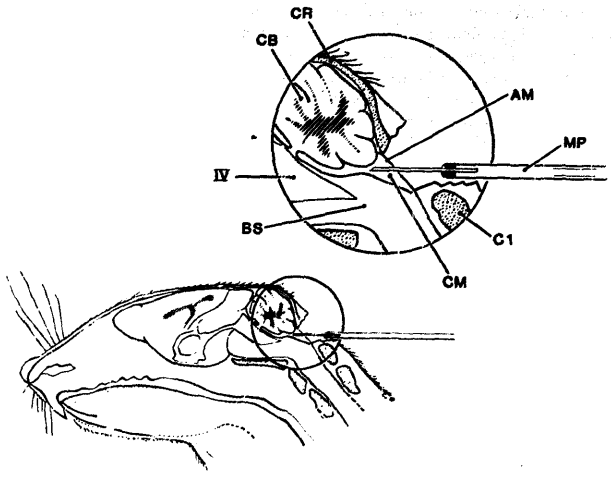

Fig. 1. Method of cisterna magna puncture. Midsaggital section. AM, arachnoid membrane; BS, brain stem; C1, first cervical vertebra; CB, cerebellum; CM, cisterna magna; CR, cranium; MP, micropipet; and IV, 4th ventricle.

velum. Although the inferior medullary velum has only 2 cell layers at its most inferior aspect, it is a much thicker tissue as it rises to join the cerebellum, and at this level it contains ependyma, glial cells, myelinated axons, and capillaries (Williams and Warwick 1975). Puncture of this structure thus risks blood and tissue contamination of CSF. CSF was aspirated by gentle mouth suction, and volumes were estimated by comparison with the markings on the pipet. For critical experiments, the volume may be determined by weighing tubes prior to and after addition of fluid. Usually 5–15 μl of clear CSF were obtained. In most instances, CSF was immediately diluted in tissue culture media for cell analysis or PBS (1:5) for antibody determination (see below).

Analysis of CSF cell types

A combination of Wright's stain (Diff-Quik, Harleco, Gibbstown, NJ), nonspecific esterase stain for monocytes (Yam et al. 1971), and antibody-mediated, complement-dependent microcytotoxicity assay (Frelinger et al. 1974) were used. For the stains, cells were mixed with a drop of fetal calf serum diluted 1:5 in PBS, smeared on a glass slide, and then air-dried. Alternatively, they were prepared by cytocentrifugation. The CSF sample, 10–15 μl, was added to 0.3 ml of tissue culture

media with 10% fetal calf serum, vortexed, and spun onto a clean slide at 800 rpm for 5 min with a Shandon–Southern Cytospin (Sewickley, PA) It was found that the latter method gave superior morphology. For the microcytotoxicity assay, cells were immediately counted and resuspended at approximately 10^6/ml. Two μl of antibody were incubated with 2 μl of cells for 15 min at room temperature in a Terasaki tray. The antibodies used were anti-μ (Cappel Laboratories, West Chester, PA) and anti-Thy 1.2, (PL/J × A.AKR)F_1 anti-A.AL, kindly provided by Dr. Jeffrey Frelinger, (University of Southern California, School of Medicine) for B cells and T cells, respectively. Both antisera were tested on cells from spleen and thymus and were found to be appropriately specific. Following incubation, the antibody was removed and then replaced with 2 μl of rabbit complement. After 30 min incubation at 37°C, the cells were fixed with formaldehyde and viability determined by phase contrast microscopy. The microcytotoxity assay is ideal for CSF analysis since so few cells are required.

Radioimmunoassay for antibody to JHMV

The solid phase radioimmunoassay (RIA) developed in our laboratory is a modification of the methods of Rosenthal et al. (1973) and Griffin (1981). Serum-free JHMV, typically 10^4–10^5 pfu/ml, was used as an immunoadsorbant. Polyvinylchloride flexible microtitration 96-well plates (Dynatech Laboratories, Alexandria, VA) were coated with 100 μl per well of virus incubation overnight at 4°C. The plates were next incubated for 1 h at room temperature (RT) with each well containing 100 μl of 0.5% bovine serum albumin (BSA) in PBS pH 7.2 with

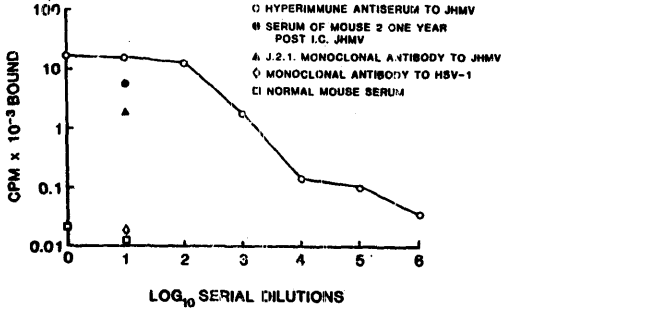

Fig. 2. Solid phase radioimmunoassay for antibody to JHM virus. Symbol denotes reference hyperimmune antisera to JHMV, ●, experimental mouse serum 12 mo after JHMV i.c. challenge; ▲, monoclonal antibody to JHMV, □, normal mouse serum; ◇, monoclonal antibody to herpes simplex virus, type 1. Substituting herpes simplex virus, type 1, or influenza virus, type A, for JHMV as immunoadsorbants did not result in significant counts when JHMV reference sera (hyperimmune and monoclonal) were used. (data not shown).

0.2% Tween-40 (polyoxyethylene sorbitan monopalmitate, Sigma Chemical Company, St. Louis, MO) (RIA diluent). Sodium azide, 0.08% was added to all steps requiring incubations of 1 h or greater. Plates were washed twice in RIA diluent and 25 μl of antibody added per well. Following incubation for 1 h at RT, plates were washed 5 × with RIA diluent, and [^{125}I]protein A was added in 100 μl of RIA diluent at a concentration of 2–4 × 10^4 cpm/well. Purified Protein A (Pharmacia, Uppsala, Sweden) was radioiodinated with ^{125}I by the lactoperoxidase method (Enzymobeads, Bio-Rad, Richmond, CA) to achieve a specific activity of approximately 20 μCi/μg. Although a 2-h incubation with [^{125}I]protein A was adequate, overnight incubation gave optimal results. Following incubation, plates were washed 5 × with RIA diluent, 5 × with PBS, and 5 × with distilled water. Individual wells were cut out and counted in a gamma counter. A representative assay is shown in Fig. 2.

Results

Perfusion studies

The interpretation and validity of results obtained from CSF examination is critically dependent on the absence of blood contamination. As a preliminary effort to minimize contamination, the effect of cardiac perfusion by PBS was examined using erythrocytes (rbc) as markers of CSF : blood mixing. During initial studies, CSF was obtained from mice both with and without prior perfusion. Gross contamination of CSF by rbc was apparent by visual inspection in only 2 of 11 mice not perfused and in none of 13 mice perfused. Nevertheless, as shown in Table I, microscopic analysis showed contamination in 82% and 15% of the non-perfused groups, respectively. Furthermore, the magnitude of contamination in the non-perfused group was significant (1 part in 1000), even when the 2 grossly bloody samples were excluded from consideration. The perfused group, by contrast, showed insignificant ($< 1 : 10^5$ parts) contamination by microscopic criteria. Therefore, in the subsequent studies described all mice were perfused prior to CSF sampling.

TABLE I

EFFECT OF PERFUSION ON CONTAMINATION OF CSF BY BLOOD

Treatment	Percent of samples contaminated	CSF rbc/mm^3 [a]	Contamination ratio [b]
None (n = 11)	82	$8.9 \times 10^3 \pm 7.5 \times 10^3$	1 part in 1000
Perfused (n = 13)	15	21 ± 58	< 1 part in 10^5

[a] Mean and standard deviation of macroscopically clear samples only.

[b] Approximate ratio of CSF : blood rbc, assuming 9.6×10^6 rbc/μl blood (Russell et al. 1951).

TABLE 2

KINETICS OF CELLULAR INFILTRATION INTO THE CSF OF INDIVIDUAL MICE DURING ACUTE VIRAL ENCEPHALITIS

Days post infection	Clinical[a] signs	wbc/μl	Percentage of cell types			
			T cells	B cells	Monocytes	PMN
4	–	0				
	–	0				
	–	0				
7	+	4000	27	21	15	5
	+	1500	59	19	NT	0
	–	0				
10	+	2000	37	26	12	1
	–	0				
	–	0				
	–	0				
12	+	1200	35	23	NT	0
	±	700	31	31	33	1
	±	10	NT	NT	NT	NT
21	+	1000	NT	NT	NT	1
	–	0				

NT = Not tested.

[a] – (mice appeared normal); + (ruffled fur, seizures, paralysis, obtundation); ± (equivocal signs).

Acute viral infection

B6 mice were first infected using the intranasal route of infection, which, while less efficient than the intracerebral route at causing disease, does not result in direct trauma to the blood–brain barrier. Twenty-three of 79 mice inoculated developed

TABLE 3

H-2 TYPE OF THE CELLS RECRUITED INTO THE CSF DURING ACUTE VIRAL ENCEPHALOMYELITIS IN CHIMERIC MICE

Donor	Recipient	Animal[a] No.	H-2 type of CSF cells	
			Donor	Recipient
$(A/J \times B6)F_1$	B6	1	< 95%	< 5%
		2	< 98%	0
A/J	$(A/J \times B6)F_1$	3	< 99%	0
		4	< 95%	0

[a] 3 d post-inoculation.

signs of clinical disease (seizures, paralysis, ruffled fur, myoclonus, obtundation), and of these 23 there was a mortality rate of 60%. The results of examining the CSF in 15 individuals for the relative percentage of T cells, B cells, monocytes, and PMN's are shown in Table 2. Mice with evidence of abnormal neurological status showed varying degrees of pleocytosis. Cells were first noted at day 7 p.i. and persisted in ill mice until at least day 21. Although a limited number of mice were examined in this pilot study, it is clear that the percentage of T and B cells did not fluctuate greatly over the course of the infection. However, the B cell fraction (mean, 24%) may be slightly enriched over that normally seen in peripheral blood (Greaves

TABLE 4

SERUM AND CSF ANTIBODY TO JHMV IN INDIVIDUAL MICE 12 MONTHS POST-INFECTION [a]

Mouse [a]	cpm		CSF/serum ratio [b]
	CSF (1:5)	Serum (1:5)	
Assay I			
C-1	0	0	–
C-2	0	0	–
C-3	0	0	–
C-4	0	0	–
M-1	309	4500	1:25
M-2	59	11000	>1:300
M-3	0	950	>1:300
M-4	0	450	>1:300
Assay II			
M-5	38	420	>1:300
M-6	12	405	1:200
M-7	112	740	1:42
Assay III			
M-8	9	4100	>1:300
M-9	82	4810	>1:300
M-10	0	1655	>1:300
M-11	0	295	>1:300

[a] Control mice (C-1, C-2, etc) were 6-week-old B6 mice analyzed within 24 h of receipt from a laboratory known to be free of JHMV. Experimental mice (M-1, M-2, etc) were 6-week-old B6 animals given 1000 pfu of JHMV i.c. and subject to CSF analyses 1 year later.

[b] Ratios were calculated by the following method: CSF counts were determined at 1:5 dilution. Serum samples were measured at several dilutions, typically undiluted, 1:5, 1:10, 1:100, 1:1000. A standard curve was constructed for each serum sample. These plots were linear on semilog paper in the relevant ranges. The dilution of serum which would give the same cpm as the CSF was found and the titer expressed as a ratio of the two dilutions. For example, if a CSF sample yielded 100 cpm at 1:5 dilution and the corresponding serum gave 100 cpm at a 1:500 dilution, the CSF:serum ratio was expressed as 1:100.

et al. 1974). Mice without clinical signs had no detectable cells in the CSF. Histologic examination of all mice infected by the intranasal route showed evidence of interstitial pneumonitis; sham-infected animals showed only minimal pulmonary changes. Examination of the brains of these groups of mice showed that only mice with clinical signs of illness (see Table 2) had histologic evidence of encephalitis, correlating with the finding of pleocytosis in the CSF.

To determine the origin of cells found in CSF, chimeric mice were infected with JHMV and the cells entering the CSF examined for H-2 histocompatibility type. Since donor and recipient have different H-2 types, the origin of the cells found in the CSF can be determined. In the following experiment, 2 sets of chimeric mice were tested: (A/J × B6) F_1 recipients transplanted with B6 cells, and B6 recipients transplanted with (A/J × B6) F_1 cells. Both groups were inoculated i.c. with JHMV, and CSF was removed at day 3 post inoculation (p.i.). Table 3 shows the lymphocytes in the CSF were exclusively of donor H-2 type, confirming that the cellular infiltrate during acute viral infection is originally from a hematogenous source.

Chronic infection

It has been previously shown that approximately 60% of B6 mice surviving JHMV infection show evidence of chronic CNS infection (Stohlman and Weiner 1981). We used our method for obtaining contamination-free CSF to examine a group of these chronically infected mice. Table 4 shows that all the mice tested one year post infection had significant serum antibody titers to JHMV. In 5 of the 11 mice tested antibody to JHMV was detected in the CSF. In at least 2 of these mice the ratio of the serum titer to CSF titer was less than that expected by diffusion, suggesting the possibility of specific antiviral antibody synthesis within the CSF. Control mice did not show detectable anti-JHMV antibody in serum or CSF.

Discussion

Obtaining and analyzing CSF often are important in the study of clinical and experimental neurological disease. A critical problem in this procedure is contamination of CSF by blood. In normal humans and most other species studied, there are CSF to blood ratios of approximately 1/350, 1/2000, and $> 1/10^6$ for immunoglobulins, leukocytes, and erythrocytes, respectively (Davson 1967; Tourtellotte 1970; Fishman 1980). In view of the relatively low concentrations of CSF components, small degrees of contamination by blood may lead to inaccurate or invalid results. For example, mixing 1 part blood in 350 parts CSF may falsely double the value obtained for immunoglobulin concentration. In this regard, the presence of rbc's serve as a sensitive marker of blood mixing with CSF; thus, as little as 8–9 rbc's/μl of CSF indicate contamination on the order of one part in a million.

In the past, CSF has been obtained from mice by first puncturing the arachnoid membrane and then removing the CSF that flows onto its surface. In our view, this procedure entails a high risk of CSF contamination with blood or tissue components remaining on the arachnoid after dissection. The original report of this method

(Carp et al. 1971) stated that more than 70% of samples were virtually free of blood as judged by macroscopic appearance and hemoglobin electrophoresis. This implies that approximately 30% of samples were not free of gross contamination and that a lesser degree of mixing may have been present in other samples. When a similar technique was applied to neonatal rats, it was reported that CSF was rarely grossly bloody but most samples did contain small numbers of rbc's (range 0–1 000/μl) (Moxon and Ostrow 1977). This degree of contamination may be unacceptable for many experiments, such as those dealing with the integrity of the blood–brain and blood–CSF barrier systems. Contamination of CSF by blood can be reduced by first exsanguinating mice; however, as Gerhard et al. (1978) have shown, there remains a residual mean mixing of blood and CSF on the order of 1/500 parts.

In the procedure employed in the present experiments, mice were exsanguinated and then perfused with PBS (Lipton and Gonzales-Scarano 1978) prior to removal of the CSF by direct arachnoid puncture (Griffin 1981). As shown in Table 1, blood/CSF contamination was reduced to less than a mean of 1 part in 10^5. In non-perfused mice, CSF was usually clear on visual inspection; however, microscopic analysis showed unacceptably high rbc contamination in these samples. Thus, our results confirm the usefulness of the exsanguination–perfusion procedure and point out the unreliability of macroscopic inspection as the only guide to contamination.

This procedure was used in conjunction with other microtechniques to analyze CSF during acute and chronic viral encephalomyelitis. To avoid trauma to the blood–brain barrier, mice were initially infected via the intranasal route and the relative numbers of T cells, B cells, PMN, and monocytes were monitored. Experiments with chimeric mice, infected i.c., showed that this methodology could demonstrate that the cells recruited into the CSF were of hematogenous origin. Finally, antiviral antibody was measured in the blood and CSF of chronically infected mice. Five of 11 mice had detectable CSF anti-JHMV antibody, and in at least 2 of these mice CSF : blood ratios implied local synthesis of antibody, a phenomenon noted in other experimental infections (Nathanson 1979), as well as human diseases such as subacute sclerosing panencephalitis and multiple sclerosis (Tourtellotte 1970). Ultrastructural examination of the chronically infected mice used in this study has shown plasma cells in their subarachnoid space (Erlich, personal communication).

The methods employed in this study are applicable to many other studies. The microcytotoxicity assay may be used to enumerate any cell type for which cytolytic antibody exists. The RIA may be utilized to determine other immunoglobulin classes and specificities (Griffin 1981). Finally, the microtechniques described have been shown to be sensitive enough to analyze CSF from individual mice, rather than pooled samples. In some experiments, such as those shown in Tables 2 and 4, pooling CSF would have obscured important individual differences.

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