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Spinal Fluid Differences in Experimental Allergic Encephalomyelitis and Multiple Sclerosis

Abstract. *The spinal fluid of sheep with experimental allergic encephalomyelitis contains myelin basic protein (6 to 18 nanograms per milliliter) bound to antibody as well as excess free antibody. This bound myelin basic protein appeared concurrently with the onset of the disease and remained elevated until death. In contrast, in active multiple sclerosis, the spinal fluid contains free myelin basic protein and there are no detectable levels of antibody. The results indicate that the antibodies enter the spinal fluid from the serum by passive diffusion. This mechanism may also explain the presence of viral antibodies in the spinal fluid of multiple sclerosis patients.*

Experimental allergic encephalomyelitis (EAE) has been used as an animal model for multiple sclerosis (MS) (*1*). EAE is an autoimmune demyelinating disease induced in susceptible animals by immunizing them with tissue from the central nervous system or the basic protein of myelin, the antigen responsible for the immune reactions of the disease (*2*). We have examined myelin basic protein in the cerebrospinal fluid (CSF) of MS patients and have shown that, during episodes of acute exacerbation, myelin basic protein is released into the spinal fluid (*3*). These observations stimulated our interest in the dynamics of the release of myelin basic protein into spinal fluid during a defined demyelinating episode. The limitations in obtaining frequent serial samples of spinal fluid in humans and the unpredictability of the course of MS led us to study an animal model of demyelination.

Experimental allergic encephalomyelitis has been well established in sheep. The clinical sign of ataxia and paresis appear suddenly and are easily detectable (*4*). Animals usually develop EAE within 2 weeks after injection of a mixture of complete Freund's adjuvant emulsified with brain homogenate. Spinal fluid was obtained through an indwelling cannula over the cisterna magna (*5*). After implantation of the cannula, EAE was induced (*6*) and spinal fluid was withdrawn at frequent intervals from all animals. The spinal fluid was examined for cell counts, total protein, gamma globulin, basic protein (*7*), and antibodies to basic protein (*8*).

Eight animals were studied. Although all the animals were of the same breed and were treated in the same way, only half developed clinical evidence of EAE 8 to 20 days after immunization; the other half remained asymptomatic. This is similar to the results found by Panitch *et al.* (*4*) for this breed of sheep, which are outbred, and, as demonstrated by experiments with rats (*9*), the necessary determinants for EAE susceptibility are probably genetically controlled.

Studies of antibody to myelin basic protein in the spinal fluid from four animals with postmortem histological evidence of EAE are shown in Fig. 1A. In this group, antibody appeared in the CSF within 2 days of the onset of the disease. In contrast to MS, the spinal fluid contained high levels of antibodies to myelin basic protein which rose in parallel with serum antibody. The serum (not shown) contained 100 to 200 times more myelin basic protein antibody than the CSF (as measured by the reciprocal of the dilution that gave 50 percent binding of 1 ng of ^{125}I-labeled basic protein). These levels of CSF antibody could not be accounted for by leakage of blood into the spinal fluid as determined by cell counts of the CSF.

Surprisingly, the spinal fluid of the four animals without signs of the disease also contained increased amounts of antibody to myelin basic protein (Fig. 1B). All these animals were without postmortem histological evidence of EAE. The time of appearance and the amounts of antibodies in the spinal fluid were virtually undistinguishable from those in the group that did show EAE. These animals also had serum myelin basic protein antibodies in titers similar to those in the sheep with EAE.

We were unable to detect any free

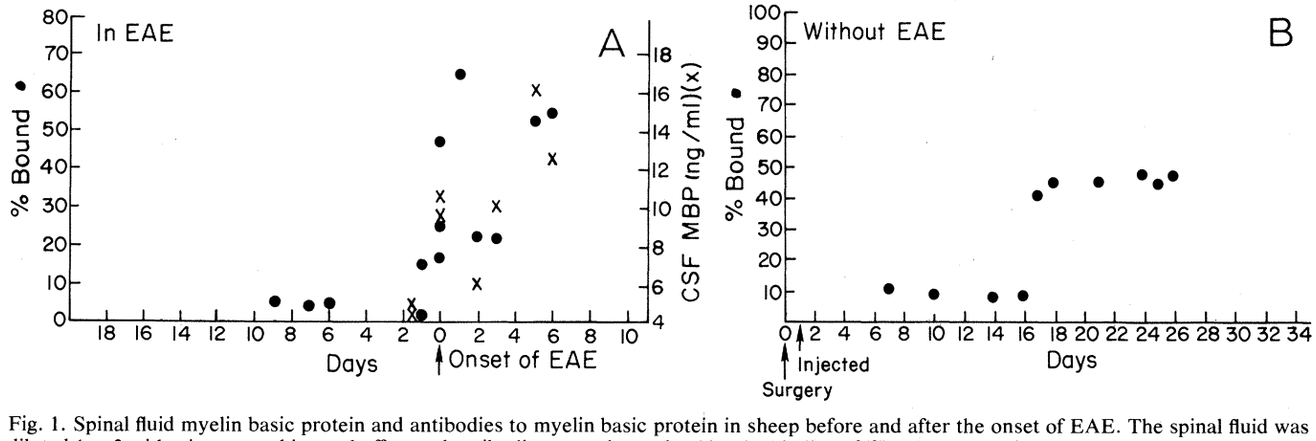

Fig. 1. Spinal fluid myelin basic protein and antibodies to myelin basic protein in sheep before and after the onset of EAE. The spinal fluid was diluted 1 to 3 with tris-acetate histone buffer, and antibodies were determined by the binding of ^{125}I-labeled myelin basic protein (*8*). Myelin basic protein was determined by radioimmunoassay (*7*). Data are expressed as percent of ^{125}I-labeled basic protein bound by the spinal fluid sample (•) and nanograms of myelin basic protein per milliliter of spinal fluid (x). Each point is the average of two values for each spinal fluid sample. (A) Levels of antibody from four animals and levels of myelin basic protein from three of these animals with EAE. In this group, cannulas were implanted between days 10 and 20, before the onset of EAE. They were immunized with human brain–complete Freund's adjuvant the following day. (B) Data from one animal representative of the group of four without EAE. This animal had a cannula implanted on day zero and was immunized on day 1.

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Table 1. Presence of myelin basic protein and its antibody in cerebrospinal fluid in EAE and MS.

Category	Samples tested for:			
	Myelin basic protein		Antibody	
	Positive	Total	Positive	Total
Sheep				
Before immunization	0	6	0	6
Without EAE	0	4	4	4
With EAE	3*	3	4	4
Patients				
Nondemyelinative neurological diseases	3†	600	0	600
Inactive MS	0	30	0	30
Active MS	42‡	42	0	42

*Present bound to antibody; equivalent to 6 to 18 ng/ml. †These three patients had very extensive brain damage; two had severe strokes near the surface of the brain, and the third had necrosis due to radiation overdose. ‡Values of 12 to 75 ng/ml were obtained.

myelin basic protein in the untreated spinal fluid of any animal. The possibility that myelin basic protein was present but that it was bound to antibody, and therefore not detectable, was investigated. Portions of spinal fluid (3 to 5 ml) were treated with 1 percent mercaptoethanol and heated to boiling to destroy antibodies. The solution was allowed to cool, after which it was dialyzed for 2 days against 1 liter of 0.9 percent NaCl. The spinal fluid was then assayed for myelin basic protein as described above. Treatment in this manner resulted in the release of 6 to 18 ng/ml of previously bound myelin basic protein in each of three animals with signs of EAE (Fig. 1A). The bound myelin basic protein appeared within 1 day of signs of the disease and remained elevated until death. Furthermore, in two animals there was no myelin basic protein in the spinal fluid 1 day before onset of the disease. In contrast, the spinal fluid from all four sheep without clinical or histological EAE contained spinal fluid antibody but did not have any detectable free or bound myelin basic protein.

These studies agree with the findings of others that the serum antibodies to myelin basic protein found in EAE are not responsible for induction of EAE. The disease can be produced in guinea pigs without appreciable levels of antibody to myelin basic protein (*10*), and chicks made agammaglobulinemic by neonatal bursectomy and irradiation develop EAE (*11*). Many attempts at passive transfer of the disease with serum from sensitized animals have been unsuccessful, even when the blood-brain barrier is artificially compromised by heat lesions (*12*). The appearance of antibodies to myelin basic protein in the spinal fluid correlates with the onset of EAE in those animals destined to develop the disease. However, it is not possible to predict the onset or the course of EAE based on the appearance of spinal fluid antibody because some animals that have this spinal fluid antibody do not exhibit signs of EAE.

There is little information available on the origin of the spinal fluid immunoglobulin in EAE. The possibilities include (i) simple passive diffusion from serum to spinal fluid, which is supported by evidence of passage of labeled immunoglobulin G (IgG) and other macromolecules from blood into the spinal fluid (*13, 14*); (ii) selective transport of IgG from the serum into the spinal fluid, which is unlikely, based on isotope studies by different groups of investigators (*13*); and (iii) de novo synthesis within the central nervous system. Tourtelotte, Cutler, and others have demonstrated that components of the central nervous system have the capacity to produce antibodies in MS and subacute sclerosing panencephalitis (*13, 15*).

Our studies indicate that simple passive transfer of proteins from serum to spinal fluid is most likely. The ratio of albumin to globulin in control animals was 4:1 in spinal fluid and 2:1 in serum. As EAE developed, the albumin-to-globulin ratios in serum and spinal fluid became nearly identical, suggesting that antibodies to myelin basic protein could undergo passive transfer from the serum into spinal fluid. We therefore immunized two animals with bovine serum albumin in complete Freund's adjuvant emulsion and, at the same time, induced EAE with brain tissue as described above. Antibodies to myelin basic protein and bovine serum albumin appeared concomitantly in serum and spinal fluid. Moreover, the ratios for each antibody in serum to that in spinal fluid were very similar. Thus, it seems likely that an alteration in vascular permeability allows the exudation of gamma globulins from the serum into the spinal fluid.

These studies were initiated to determine the amount of basic protein in the spinal fluid of sheep with EAE and to compare these values with those found in patients with MS (Table 1). It is well known, and we also confirmed, that increases of total protein and gamma globulin in spinal fluid are found in both conditions. The spinal fluid of patients during an acute attack of MS contains high levels of free myelin basic protein, but no antibodies to this protein are detectable. In contrast, the spinal fluid of sheep with EAE contains low levels of antibody-bound myelin basic protein, as well as excess free antibody to myelin basic protein. Moreover, the free antibody is present whether or not the animals develop EAE.

These findings relate to human demyelinating diseases in two ways. First, although EAE and MS are both demyelinative diseases of the central nervous system, the observations of basic protein and its antibody in spinal fluid from the two diseases are qualitatively different. Therefore the use of EAE as a model for MS should be carefully questioned. Secondly, during the inflammatory disease EAE, antibodies appear in the CSF by passive transfer from the serum. This may explain the mechanism by which viral antibodies appear in the spinal fluid of MS patients.

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5. Eight male and female Hampshire sheep (6 to 10 months old) were obtained from a local breeder. We have used a modification of the technique described by Pappenheimer to implant Teflon cannulas over the cisterna magna [J. R. Pappenheimer, S. R. Helsey, E. F. Jordan, J. deC. Downer, *Am. J. Physiol.* **203**, 763 (1962)]. The animals were intubated and anesthetized with halothane gas. By means of a sterile technique, the occipital bone overlying the cisterna was exposed and a portable dental drill was used to bore through the cortex and medullary cavity of the bone until the dura overlying the cisterna magna was reached. A 14-gauge Teflon angiocath (Vicra Division, Travenol Laboratories, Dallas, Texas) cut to an appropriate length was then placed in this position, and two stainless steel anchoring screws were inserted into the bone next to the guide tube. It was permanently fixed in place by covering the entire apparatus with sterile nonantigenic acrylic resin cement (Lange Dental Manufacturing Co., Chicago, Illinois). The animals tolerated the procedure well and did not require any special care except for a prophylactic 3-day course of 600,000 units of procaine penicillin and 750 mg of dihydrostreptomycin (Med-Tech, Inc., St. Joseph, Missouri).

6. The day after surgery the animals were immunized with a 20 percent homogenate of human brain emulsified with an equal volume of complete Freund's adjuvant fortified with heat-killed tuberculosis cells (0.1 mg/ml) (Lilly Laboratories, Indianapolis, Indiana). A total of 4 ml of the emulsion was given to each animal; 1-ml portions were injected into the large shoulder and thigh muscles of the fore and hind limbs, respectively. The animals were observed daily for clinical signs of the disease. When the animals died or were killed, autopsies were performed and tissue was obtained for microscopic examination.
7. The assay for myelin basic protein has been described (2). Briefly, 0.05-ml portions of a tenfold concentrated assay buffer (2*M* tris-acetate, *p*H 7.5, containing 10 mg of histone per milliliter) and antiserum at the appropriate concentration were added directly to 0.5 ml of spinal fluid. This mixture was incubated for 1 hour at 37°C; ^{125}I-labeled basic protein (15,000 count/min; specific activity, 3 to 7 μc/μg) were added, and the mixture was incubated for an additional 16 to 24 hours at 4°C. The antibody-basic protein complex was then precipitated with cold ethanol, the pellet and supernatant fraction were separated by centrifugation, and each was assayed for radioactivity. The percentage of ^{125}I-labeled basic protein bound was then determined.
8. Antibody to myelin basic protein was determined by mixing 500 μl of assay buffer (0.2*M* tris-acetate, *p*H 7.5, containing 1 mg per milliliter of histone, and 1 percent fetal calf serum), serum or spinal fluid of the appropriate dilutions, and 10 μl (15,000 count/min) of ^{125}I-labeled basic protein (specific activity, 3 to 7 μc/μg). The mixture was incubated for 24 hours at room temperature; cold ethanol was added, the pellet and supernatant fraction were separated by centrifugation, and each was assayed for radioactivity. The percentage of ^{125}I-labeled basic protein bound was then determined for each dilution.
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Nicotine in Breast Fluid of Nonlactating Women

Abstract. *Using a combination of gas chromatography, mass spectrometry, and selected ion recording techniques, we have identified nicotine and its major metabolite, cotinine, in the breast fluid of nonlactating women smokers. As little as 25 picograms could be measured by using the deuterated variants, [5′,5′-^{2}H]nicotine and [3,3-^{2}H]cotinine, both as internal standards and as carriers in an inverse isotope dilution method.*

It is generally known that human milk may contain a broad spectrum of extraneous chemical substances to which the mother is exposed (*1*). It is less well-known that during the major portion of adult life the nonlactating breast glands of women secrete and reabsorb breast fluid (*2*). Because of this secretory mechanism, the accumulation of potentially harmful exogenous substances in breast tissue and its secretions could pose a serious hazard to the breast epithelium.

Specimens obtained by a standardized nipple aspiration technique enable us to study the physiology, biochemistry, and cytology of secretions from the nipples of nonlactating women (*3*). We have examined nipple aspirates of breast fluid for the presence of foreign compounds to which women may be exposed and have chosen the tobacco alkaloid nicotine for our initial study because both "exposed" and control populations are readily available.

The unambiguous identification of trace amounts of small molecules in the complex milieu of body fluids can be difficult. The concentration of nicotine to be expected is probably similar to that found in plasma (*4*), that is, approximately 10 ng/ml, and the amount of breast fluid obtainable per subject is limited to approximately 50 μl. It follows that the amount of nicotine present in the total sample, if one allows for a 50 percent recovery and a ± 10 percent accuracy, would require the measurement of approximately 250 pg with an accuracy of ± 25 pg.

Table 1. The GC-MS-SIR analysis of standard solutions of [^{2}H]nicotine enriched with unlabeled nicotine. Each injection contained a total of 25 ng of nicotine.

Ratio of weight of nicotine to weight of [^{2}H]nicotine (%)	Observed ion current ratios* (%)	
	Before extraction†	After extraction‡
0.0	2.7	4.3
0.0	2.9	4.5
5.0	7.2	8.4
5.0	7.4	
10.0	11.5	13.3
10.0	11.6	13.9
20.0	20.0	21.9
20.0	20.3	22.1

*Ratio of the ion current at *m/e* 84 to that at *m/e* 86. †Calculation of a regression line: $y = 2.88 + 0.865x$ with $r^2 = 1.000$. ‡Calculation of a regression line: $y = 4.41 + 0.885x$ with $r^2 = 0.998$.

We achieved this degree of sensitivity, specificity, and accuracy by means of an analytical method in which [5′,5′-^{2}H] nicotine (*5*) is used as an internal standard for quantitation and as a carrier in an "inverse isotope dilution" technique.

By using a combination of gas chromatography, mass spectrometry, and selected ion recording (GC-MS-SIR) techniques (*6*), we can, with good precision, measure the ratio of the ions of mass to charge ratios, *m/e*, 84 (the base peak of the mass spectrum of natural nicotine) and of *m/e* 86 (the base peak of the internal standard [^{2}H]nicotine). In this way quantities of nicotine as small as 25 pg can be measured.

Previously, nicotine in body fluids has been assessed by thin-layer chromatography used in conjunction with radioactive isotopes (*7*), gas-liquid chromatography (GLC) (*4*), and atmospheric pressure ionization (API) mass spectrometry (*8*).

Breast fluid samples were obtained from nonlactating volunteers with a history of smoking, by a standardized nipple aspiration technique with a breast pump (*3*) 15 minutes after a single cigarette had been smoked. Nonsmokers were used as controls. Each sample of breast fluid was placed in a 1-ml reaction vial and treated with 5.0 μl of [^{2}H]nicotine standard solution (9.1 ng/μl) in methanol, 100 μl of 1*N* NaOH solution, and 200 μl of dichloromethane (spectroscopic grade, purified by washing 100 ml of solvent two times with 50 ml of 1*N* HCl). All aqueous reagents were made from doubly distilled deionized water purified by washing 100 ml of water twice with 40 ml of dichloromethane.

After being agitated on a vortex mixer for 5 minutes, the vial was centrifuged for 10 minutes and the organic layer was removed to a second tube. This extraction was repeated with another 200-μl portion of dichloromethane; the combined dichloromethane extracts were then extracted once with 200 μl of 1*N* HCl. The aqueous layer was removed, brought to a *p*H of at least 10 with 150 μl of aqueous 2*N* NaOH solution, and the resulting solution extracted with two 300-μl portions of dichloromethane. The combined organic extracts were dried for 1 hour with 5 mg of anhydrous potassium carbonate, and the solution then decanted in portions to a 0.3-ml reaction vial and carefully reduced in volume to about 15 μl under a stream of nitrogen. Absolute ethanol (10 μl) was added, the total volume reduced to about 5 μl, and the entire sample analyzed on the GC-MS-SIR system in a single run.

A gas chromatograph (Infotronics

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