Study of Protein Characteristics That Influence Entry into the Cerebrospinal Fluid of Normal Mice and Mice with Encephalitis

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A B S T R A C T Entry of proteins into the cerebrospinal fluid (CSF) from the blood is partially determined by the size of the protein. To determine whether other characteristics of proteins influence CSF entry, proteins or protein fragments were iodinated, inoculated intravenously, and serum and CSF were sampled at later times. The Fc fragment of immunoglobulin G (IgG) did not enter the CSF significantly better than the Fab fragment suggesting that choroidal Fc receptors are not of importance for selective immunoglobulin entry. To determine the role of protein charge on entry, bovine serum albumin [isoelectric point (pI) = 3.9] was chemically altered to provide an albumin with an average pI of 6 (A-6) and another with a pI of 8.5 (A-8). All albumins were of the same size on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A-8 entered the CSF ~10-fold better than the native albumin. A-6 was intermediate, entering approximately twofold better. At the time of increased CSF protein concentration during an acute viral encephalitis these differences were narrowed but not eliminated. It is concluded that charge is an important determinant of protein entry into the CSF.

INTRODUCTION

In normal animals, proteins are relatively excluded from the central nervous system (CNS)[1] by an anatomic barrier of tight junctions connecting cerebral capillary endothelial cells, arachnoid cells, and choroid plexus epithelial cells. In contrast, proteins appear to move freely between the CNS extracellular fluid and the cerebrospinal fluid (CSF) because similar junctions do not exist between ventricular ependymal cells (1–4).

The proteins present in the CSF are derived primarily from serum (5–8). The passage of these proteins across the blood-CSF barrier is inversely related to size, resulting in relatively greater amounts of small proteins than large proteins in the CSF compared with amounts present in the serum (9). However, during the study of immunoglobulin entry into the CSF of mice with viral encephalitis, differences among IgG isotopes of the same size were noted in entry into the CSF of normal mice before infection (10). A major site of CSF production is the choroid plexus, and current knowledge of the functional anatomy of this organ suggested two possible explanations for these differences in IgG entry. First, Fc receptors have been described on the normal choroid plexus (11, 12). Transfer of immunoglobulins across the choroid plexus could be analogous to the Fc-dependent transfer of IgG from mother to fetus across the placenta (13, 14). Second, the basement membrane of the choroid plexus is similar to the glomerular basement membrane of the kidney (15). Because of the polyanionic character of the glomerular basement membrane (16, 17), passage of proteins from blood to urine is dependent on the charge as well as on the size of the protein (18). In this report, these possibilities have been investigated by comparing the passage first of Fab and Fc fragments of IgG and second of albumins chemically altered to provide proteins of the same size and different charge. Evidence is presented that charge is an important determinant in protein passage from blood to CSF.

METHODS

Mice. 6–12-wk-old BALB/c AnNrIBR mice (Charles River Breeding Laboratories, Wilmington, MA) were used for studies in normal animals. For studies of mice with encephalitis, 4-wk-old animals were used.

Proteins. Murine serum albumin was obtained from Miles Laboratories Inc., Research Products Div., (Elkhardt, IN). Bovine serum albumin (BSA), crystallized, lyophilized, and essentially gammaglobulin free, was obtained from Sigma Chemical Co. (St. Louis, MO). The following murine myeloma proteins were obtained from Litton Bionetics (Kensington, MD): MOPC 21 (IgG1$_k$), UPC-10 (IgG2A$_k$), RPC 5 (IgG2A$_k$), MOPC 195 (IgG2B$_k$), MOPC 141 (IgG2B$_k$), J606 (IgG3$_k$), TEPC 15 (IgA$_k$), and MOPC 315 (IgAλ_2).

Murine and rabbit IgG were obtained by passaging normal mouse or rabbit serum over a staphylococcal protein A (SPA)-Sepharose affinity column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ). Fab and Fc pieces of rabbit IgG were prepared by papain (Sigma Chemical Co.) digestion for 6 h at 37°C using an enzyme/protein ratio of 1:100. Fab was separated from Fc on SPA-Sepharose and the fragments were checked for size homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for pI by horizontal gel isoelectric focusing and for antigenic integrity and purity using immunoprecipitation in agarose with antisera against rabbit IgG and Fc piece (Miles Laboratories Inc.). Fab fragments were 40,000 daltons and had an isoelectric point (pI) of 5.7. Fc fragments were 45,000 daltons and had pI of 6.8 and 8.5.

Isoelectric focusing. Isoelectric points (pI) of proteins were determined either using Ampholine PAG-plates pH 3.5–9.5 (LKB Instruments, Inc., Rockville, MD) or 1% IEF agarose (Marine Colloids Div., FMC Corp., Rockland, ME) mixed with ampholines (Pharmacia Fine Chemicals) with a pH range of 3 to 10. Each gel was run with a set of proteins of known pI (Pharmacia Fine Chemicals).

Albumin derivations. Albumins of varying pI were prepared after the method of Hoare and Koshland (19). BSA was dissolved in distilled water (25% wt/vol) and dialyzed extensively against deionized distilled water (ddH$_2$O) at 4°C. To 1 8-ml (2 g) aliquot of BSA was added 0.565 g of N-ethyl,N' (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma Chemical Co.); 0.725 g of EDC was added to another 8-ml aliquot. Each mixture was then reacted with a solution of 66.8 ml ethylene diamine, (Sigma Chemical Co.) in 500 ml ddH$_2$O that had previously been adjusted to pH 4.75 with ~350 ml of 6 N HCl and cooled to 25°C. The reaction was allowed to proceed at room temperature for 2 h while periodically readjusting the pH to 4.75. The derivation reactions were quenched by adding 3 ml of 4 M acetate buffer pH 4.75 and stored at 4°C. Each mixture, plus an unaltered sample of BSA, was lyophilized, reconstituted with ddH$_2$O, and then extensively dialyzed against ddH$_2$O. The yield was nearly quantitative. The pI of unaltered BSA was 3.9 (A), of the most altered albumin was 8.5 (A-8). The partially altered albumin represented a broad range of intermediate pI with the largest amount of protein focusing around a pI of 6 (A-6). The albumins migrated with identical R$_f$ values on SDS-PAGE demonstrating that significant aggregation had not occurred during the derivation procedure and that the size of the three proteins was essentially the same.

Iodination. Immunoglobulins were iodinated using the lactoperoxidase (Sigma Chemical Co.) method (20). Albumins and immunoglobulin fragments were iodinated either with the Bolton-Hunter reagent (Amersham Corp., Arlington Heights, IL) or with iodogen (Pierce Chemical Co., Rockford, IL) (21). For all proteins 1–2 mCi ^{125}I (Amersham Corp.) was used per milligram protein. Free iodine was removed by passage through a Sephadex G-50 (Pharmacia Fine Chemicals) column. Specific activities of the iodinated proteins were 2–10 × 10^5 cpm/μg protein. Radioactivity was

95% precipitable by cold 10% trichloroacetic acid. Before inoculation, protein concentrations were adjusted so all preparations to be compared had the same specific activity.

Animal manipulations. 1–5 × 10^7 cpm of the iodinated protein to be studied was inoculated intravenously into a lateral tail vein. Blood was obtained by severing the axillary artery. CSF was obtained by cisternal puncture performed as a terminal procedure in methoxyfluorane-anesthetized, exsanguinated mice (10).

Virus. Sindbis virus, strain AR339 (American Type Culture Collection, Rockville, MD) was passaged twice in chick embryo fibroblast cells and stock virus was prepared in BHK-21 cells. Stock virus assayed 10^6 plaque forming units (PFU) per milliliter. 4-wk-old BALB/c mice were inoculated intracerebrally with 1,000 PFU in 0.03 ml. Protein transfer was tested 4 d after infection, the time of maximal blood brain barrier dysfunction (10).

RESULTS

Study of Fab and Fc fragments of IgG. To determine the functional importance of the previously reported choroid plexus Fc receptors (11, 12) for immunoglobulin transfer from blood to CSF, Fab and Fc fragments were prepared from normal rabbit IgG. Rabbit IgG (CSF:serum = 0.00191±0.00019) crosses from blood into CSF of the normal mouse as well as murine IgG (CSF:serum = 0.00114±0.00009). Mice were inoculated intravenously with iodinated Fab and Fc fragments and serum and CSF were sampled 5 h after intravenous inoculation, a time previously shown to be sufficient for equilibration (10). Both Fab and Fc fragments entered CSF more readily than IgG from either species, consistent with their smaller size (Table I). CSF to serum ratios of Fab and Fc fragments were not significantly different ($P > 0.2$). There is therefore no evidence that binding of Fc piece to a choroid plexus Fc receptor facilitates entry of immunoglobulin molecules into the CSF.

Study of albumins of different pI. To determine whether charge is an important determinant for entry into the CSF from the blood, two derivatives of BSA were prepared having average pI of 6 (A-6) and 8 (A-8) compared with native BSA (A) with a pI of 3.9. Before comparing these proteins, entry of BSA into the CSF (CSF:serum = 0.0033±0.0006) was compared to that of normal murine serum albumin (CSF:serum

TABLE I
Entry of Iodinated Rabbit Fab and Fc Fragments into the CSF of Normal BALB/c Mice

Protein	No. of mice	$\dfrac{\text{cpm CSF}^*}{\text{cpm serum}}$
Fab	11	0.0069±0.00060‡
Fc	11	0.0079±0.00103

* Samples taken 5 h after injection of iodinated protein.
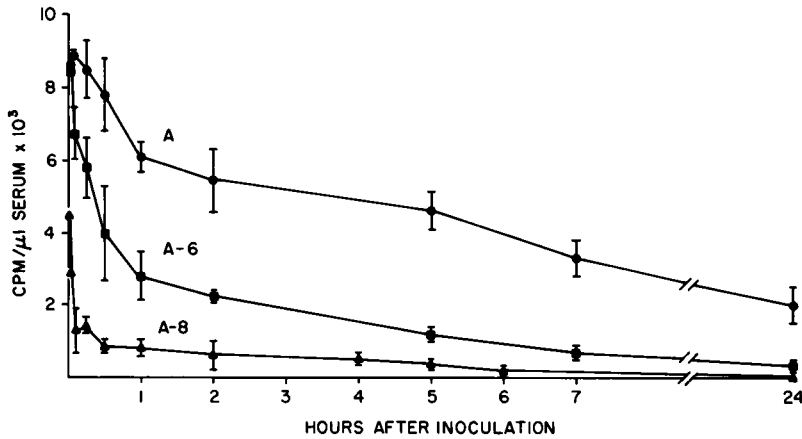‡ SEM.

FIGURE 1 Clearance from the blood of 5×10^7 cpm of iodinated albumin (A, ●), albumin pI 6 (A-6, ■), and albumin, pI 8.5 (A-8, ▲) after intravenous inoculation. Each point represents the mean±SD of three to six measurements. Using Student's t test the clearance of A6 and A8 is significantly different ($P < 0.05$) from A at all sampling points after 1 min.

= 0.00404±0.0005) 24 h after inoculation. The two native albumins entered the CSF comparably ($P > 0.2$). Initial studies of mice injected with 5×10^7 cpm of iodinated A, A-6, or A-8 revealed that A-6 and A-8 were cleared from blood more rapidly than the native albumin (Fig. 1). In fact, clearance of A-8 was already evident 1 min after inoculation, the earliest sampling point (Fig. 1). Because of this rapid clearance of A-6 and A-8 from the blood it was necessary to sample CSF and serum at multiple early intervals after intravenous inoculation to characterize the entry into CSF (Fig. 2). 2–10 mice were sampled per point. The equilibration of the iodinated proteins between serum and CSF appeared biphasic and was complete within 4 h. A-8 entered the CSF much better than A-6, which entered better than A. These relative relationships were maintained throughout the period of time that sufficient amounts of A-8 remained in circulation to permit measurement in both locations (6–7 h after injection). Thus, altering the pI of albumin from 3.9 to 8.5 increased its entry into the CSF from blood ~10-fold.

Study of myeloma proteins. To determine whether charge may also explain the original observation of a differential entry of immunoglobulins into the CSF in normal mice (10), several myeloma proteins were iodinated, inoculated intravenously, and serum and CSF sampled at 24 h. The pI of these proteins were also determined (Table II). Within the IgG myeloma proteins there was a positive but statistically insignificant correlation of CSF entry with pI ($r = 0.75$, $P > 0.1$). For the two isotypes for which two proteins were studied (IgA and IgG2B) in each pair the protein with the higher pI (MOPC 141 and MOPC 315) entered the CSF better than the protein with the lower pI (MOPC

195 and TEPC 15), suggesting that correlations within isotypes where the proteins have fewer variables may be better than between all immunoglobulins.

Characterization of the CSF proteins. To be certain that the radioactivity measured in the CSF was still associated with the protein inoculated and did not represent a breakdown product or minor contaminant,
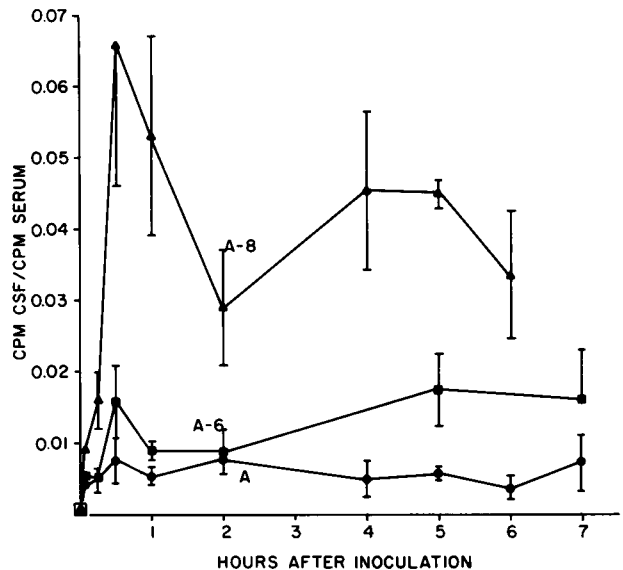

FIGURE 2 Amounts of iodinated albumin (A, ●), albumin, pI 6 (A-6, ■), and albumin, pI 8.5 (A-8, ▲) appearing in cerebrospinal fluid compared with amounts in the serum at various times after intravenous inoculation. Each point represents the mean±SEM of 2–10 measurements. Using Student's t test the entry ratios of A8 are significantly different ($P < 0.05$) from A at all sampling points after 5 min. A-6 is different from A at 1 h, and 5 h.

TABLE II

Correlation of CSF Entry of Myeloma Proteins with the
Isoelectric Point of the Protein

Myeloma protein		No. mice	$\dfrac{\text{cpm CSF}}{\text{cpm serum}}$	pI
IgG1,	MOPC 21	12	0.00169±0.00014*	6.6
IgG2A,	UPC 10	12	0.00153±0.00038	6.6
IgG2B,	MOPC 141	18	0.00207±0.00022	7.1
	MOPC 195	13	0.00250±0.0002	7.7
IgG3,	J606	9	0.00106±0.00014	6.9
IgA,	MOPC 315	8	0.00156±0.00042	5.7
	TEPC 15	13	0.00201±0.00024	5.9

* SEM.

CSF and serum from mice inoculated 4 h earlier with A, A8, and MOPC 21 were precipitated with 10% cold trichloroacetic acid. Counts in serum were >90% precipitable, and in CSF >95% precipitable, indicating that the radioactivity was associated high molecular weight substances. In addition, CSF taken from mice inoculated 24 h before sampling either with A or with RPC-5 were analyzed on SDS-PAGE along with a sample of the protein inoculated and examined by fluorography (Fig. 3). The iodinated protein component in the CSF comigrated with the inoculated protein, confirming that the protein inoculated was the one measured in the CSF.

Changes during meningoencephalitis. To determine what effect the breakdown in blood brain barrier function that occurs during viral meningoencephalitis would have on the comparative entry of A-8 and A into CSF, the kinetics of entry were studied 4 d after intracerebral inoculation of Sindbis virus (Fig. 4). This time point had previously been shown to be the time of maximal CSF protein concentration and blood brain
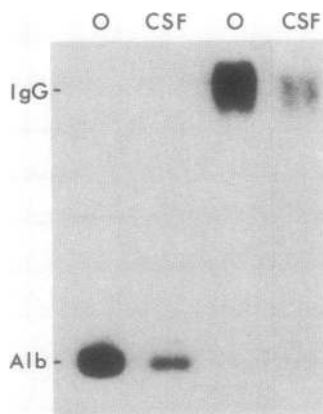

FIGURE 3 Fluorography of an SDS-PAGE of the originally inoculated albumin or myeloma protein (O) and the pooled CSF from inoculated mice.
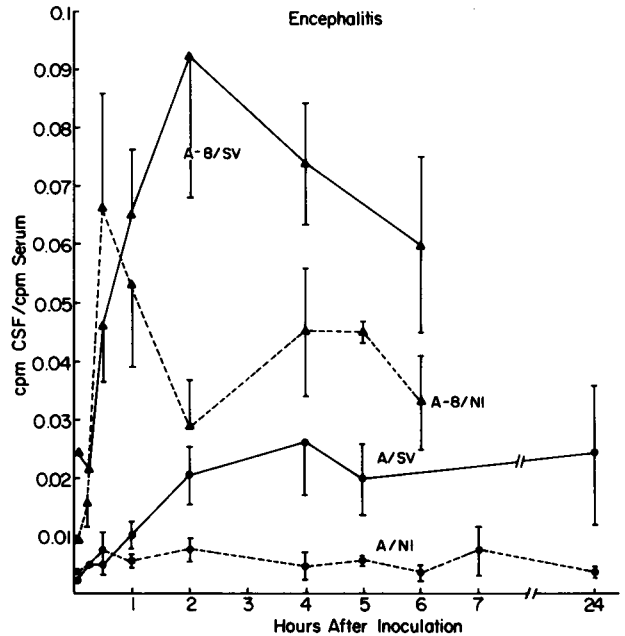

FIGURE 4 Amounts of iodinated albumin (A, ●) and albumin, pI 8.5 (A-8, ▲) appearing in cerebrospinal fluid compared with serum at various times after intravenous inoculation into mice with Sindbis virus (SV) encephalitis. Comparable curves for normal (Nl) mice (– – –) included for comparison. Each point represents the mean±SEM of two to four measurements. Using Student's t test A/SV is significantly different ($P < 0.05$) from A/Nl at all sampling points after 30 min while A8/SV is different from A8/Nl at 2 and 4 h.

barrier dysfunction during this infection (10). Two to four infected mice were sampled at each time point. Increased entry of the proteins into the CSF of infected mice was not evident at the earliest time points, but showed a gradual increase over the next 2–3 h, eliminating the biphasic character of the curve seen in normal mice. Unaltered albumin was increased in CSF approximately fivefold, while A-8 was increased approximately twofold. Although the differences between the albumins were narrowed, A-8 continued to be present in CSF in higher concentrations relative to blood concentrations than A even during this inflammatory process.

DISCUSSION

CSF is a body fluid that normally has a very low protein content. At least two factors govern the relative amounts of the serum proteins present in the CSF. Molecular size has been recognized to be a major determining factor (9). These studies have demonstrated that molecular charge is also an important determining factor.

Approximately 60–70% of the CSF is produced by the choroid plexus with the remainder rising from secretion of interstitial fluid either across the ependymal lining of the ventricles or into the subarachnoid space (22, 23). At least two potential sites of protein entry into the CSF must then be considered: the cerebral vasculature and the choroid plexus. Cerebral capillary endothelial cells are linked by tight junctions that probably control entry of proteins into the brain parenchyma and interstitial space (1, 2). The choroid plexus is composed of fenestrated capillaries separated from the choroid plexus epithelial cells by a loose stromal matrix and the choroidal basement membrane (24). Choroidal epithelial cells are linked by tight junctions (25). Ultrastructural studies of the entry of horseradish peroxidase (mol wt 40,000, pI = 7.2) into the CSF have shown passage of the protein across the fenestrated capillaries, binding to the surface of the choroid epithelial cell, followed by pinocytosis and transport to the ventricular surface (26, 27). It has, however, been postulated that the majority of proteins "leak" into the CSF passively through occasional pores present in the choroid plexus epithelium (28). These pores may be present within the somewhat leaky tight junctions between the epithelial cells (25) because tight junctions have been shown to control paracellular diffusion in other epithelia (29). The biphasic character of the albumin equilibration curves in normal mice suggests that two sites of protein entry may exist: one with a rapid equilibration (possibly choroid plexus) and another with a slower equilibration (possibly cerebral capillaries).

The original concept of the blood brain barrier was based on the observation that dyes, such as trypan blue, now known to bind rapidly to albumin, stained most tissues of the body but not the brain and spinal cord. It is now recognized that the cerebral capillaries represent the anatomic site of this blood brain barrier to proteins (3, 4). Cerebral capillary endothelial cells are linked by tight junctions impermeable to horseradish peroxidase, and the cells exhibit very limited pinocytosis (3), except in a few anatomic areas (30). Some small molecules cross this endothelial barrier by means of carrier-mediated transport and others by means of lipid solubility (31). In Ehrlich's original dye studies (32) it was observed that if the dye tested was one of a group of basic aniline dyes, such as methylene blue or bismark brown rather than an acidic dye, CNS tissue was stained as well as other tissues. In fact, on the basis of these observations and an analysis of the neurotoxicity of certain toxins and the neurotropism of certain viruses Friedeman (32) concluded that the nature of the blood brain barrier was primarily electrostatic. Charge, however, was not considered to be of importance for entry of substances into the CSF.

Our observation that charge alters passage from blood to CSF is consistent with any of these proposed sites of entry. Increased binding to a negatively charged endothelial or choroidal cell surface would increase the likelihood of pinocytosis; likewise if an intercellular tight junction pore carriers a net negative charge, molecules, uncharged or positively charged at physiologic pH, could be expected to pass through more easily. Whether charge can account for differences in CSF entry of various immunoglobulin isotypes is less clearcut than for the albumin derivatives. There is a positive correlation between IgG entry and pI. IgA, however, lies outside this correlation. Isoelectric point, therefore, can only partially account for the differences noted. There is no evidence that Fc receptors facilitate transport to any important degree. Carbohydrate composition appears to be important for uptake efficiency of horseradish peroxidase isozymes by nerve terminals of the visual system (34). Although of very similar molecular weight the immunoglobulin isotypes differ in shape and in glycosylation, which may account for differences in entry between subclasses while differences within a subclass may be on the basis of charge.

These studies also provide further evidence for similarities between the choroid plexus and the renal glomerulus. Both organs relatively exclude blood proteins providing an ultrafiltrate in the normal individual with a low protein concentration. The proteins that are present in the ultrafiltrate come from the serum and relative amounts are determined partly by size. By analysis of the proteins present in urine and CSF compared with serum Felgenhauer (35) has concluded that both are highly "restrictive" (low fluid protein to serum protein ratio) but that the renal glomerulus is more "selective" of proteins on the basis of size than the choroid plexus. Studies of chemically altered albumins like the ones used for this study (36) as well as dextrans (37) and peroxidases (38) of different charge have demonstrated the additional importance of charge in restricting protein entry into urine. The basement membranes from the renal glomerulus and choroid plexus have been shown to be similar chemically (15), immunologically (39), and anatomically (27). Both sites may be involved in certain pathologic processes such as immune complex disease (40) and Goodpasture's syndrome (41).

Under normal conditions this basement membrane, which is rich in carboxyl groups (dicarboxylic amino acids) and sialic acid, carries a negative charge that functions to repel negatively charged proteins and limit their exit from the blood (42). In the presence of conditions that decrease this negative charge, such as altered glycosylation during diabetes mellitus (43), aminonucleoside nephrosis (44), immune complex ne-

phropathy (45), or nephrotoxic serum nephritis (46) proteinuria occurs (18). We would postulate that a similar mechanism operating at the choroid plexus basement membrane may account for the increased CSF protein found in immune complex disease (47) or long-standing diabetes mellitus (48).

Nephrotoxic serum nephritis (49, 37) and aminonucleoside nephropathy (50) have been studied in detail to determine the cause of proteinuria. Studies of filtration of dextrans of different size and charge during these induced proteinuric states have revealed that size restriction is preserved while the charge differential is eliminated. It has been concluded that pore size and number are unchanged but rather that the electrostatic barrier has been altered to allow increased passage of polyanions. In our studies of protein entry into the CSF of mice with acute meningoencephalitis the differences in CSF entry between albumins of different charge were narrowed but not eliminated. Furthermore, the time course of the protein entry was changed from that seen in the normal mouse. The normal biphasic equilibrium curve became monophasic and the time course approximated the later rather than the earlier phases of the normal curve. This suggests that leakage of protein from blood to CSF during blood brain barrier dysfunction in viral meningoencephalitis occurs primarily either at anatomic sites where protein transfer does not normally occur or at only one of the normal sites. Studies of protein leakage in a variety of traumatic and toxic conditions of the CNS have suggested that protein extravasation occurs between cerebral capillary endothelial cells (3). In acute experimental allergic encephalomyelitis protein leakage from vessels has been observed only in areas with perivascular inflammation (51–53). It is possible that in a normal animal most of the CSF protein enters by way of the choroid plexus with small amounts from the cerebral capillaries; however during certain CNS inflammatory diseases such as experimental allergic encephalomyelitis or viral encephalitis, there is a substantial amount of protein added by passage across the cerebral capillaries from the blood. It may be concluded that protein transfer from blood to CSF, particularly during pathologic conditions, is probably more complex than transfer from blood to urine, and may well occur at multiple anatomic sites.

ACKNOWLEDGMENTS

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