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CEREBROSPINAL FLUID BETA-GLUCURONIDASE ACTIVITY IN RABBITS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

P. Y. PATERSON AND FRANK M. STEELE

Samuel J. Sackett Research Laboratories and the Infectious Diseases-Hypersensitivity Section, Department of Medicine, Northwestern University Medical Center, Chicago, Illinois

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SUMMARY

Using a microassay, cerebrospinal fluid (CSF) β-glucuronidase activity increased precipitously in rabbits developing paralytic signs and characteristic lesions of experimental allergic encephalomyelitis (EAE). CSF protein concentration also increased in animals with EAE, but striking dissociation between protein and enzyme values in occasional rabbits suggests that each parameter represents an independent response to nervous tissue injury.

INTRODUCTION

Measurement of enzymes released from injured tissues has found wide application in clinical medicine (Innerfield, 1960). Recent focus has been on hydrolytic lysosomal enzymes, e.g. proteases, because of their potentially harmful effects on living cells (reviewed by Weissmann, 1967). Hirschhorn *et al.* (1965, 1968) have reported release of lysosomal enzymes by peripheral blood lymphocytes when cultured *in vitro* with specific antigen. Because these cells are key factors in immunologic tissue damage, considerable interest has developed in the possible role of lysosomal enzymes in autoimmune diseases (Weissmann, 1964).

Experimental allergic encephalomyelitis (EAE) has been widely employed for the study of neurological disease (Paterson, 1966). EAE is believed to have an autoimmune basis, the critical event being an interaction of sensitized lymphocytes with myelinated nerve fibres and glial cells in the neuraxis (Paterson, 1968; Paterson 1969a, b). β-glucuronidase, a prototype lysosomal enzyme, is present in central nervous system tissue (Koenig *et al.*, 1964; Waltimo & Talanti, 1965; Robins, Hirsch & Emmons, 1968; Robins & Hirsch, 1968). EAE, thus, provides an excellent opportunity for studying the relationships between lysosomal enzyme release, as reflected by β-glucuronidase, and development of autoimmune nervous tissue injury.

This paper describes a microassay for β-glucuronidase in very small samples of rabbit

Correspondence to: Dr Philip Y. Paterson, Department of Medicine, Northwestern University, Medical Center, Chicago, Illinois 60611, U.S.A.

CSF and patterns of CSF β-glucuronidase activity in rabbits with EAE. CSF β-glucuronidase activity rose precipitously with the onset of clinical signs of EAE and represents an additional method of monitoring the course of the disease in this host.

MATERIALS AND METHODS

Animals

White New Zealand male rabbits (Blue Spruce Farms, Inc., Altamont, N.Y., 12009), weighing 3–4 kg, were housed in stainless steel cages and provided daily with commercial food pellets and water.

Sensitizing inoculum for EAE

Spinal cord and brain stem were removed aseptically from normal rabbits, the meninges trimmed away, and the nervous tissue stored at 4–6°C for 24 hr to several weeks before use. Weighed portions of spinal cord and brain were worked into a 33% (weight/volume) homogenate using 0·25% phenol in distilled water and a TenBroeck grinder. Equal volumes of the nervous tissue homogenate were added drop wise to Freund's complete adjuvant (H37Rv strain of mycobacteria, 4 mg/ml). Under ether anaesthesia, 0·15 ml of the emulsion was injected intradermally into each foot pad of each rabbit. Control rabbits were sensitized in a similar way with 0·15 ml of emulsion consisting only of phenol-water plus Freund's adjuvant.

Cerebrospinal fluid (CSF) specimens

CSF samples were withdrawn from the cisternal space using a 5/8 in. 25 gauge needle on a tuberculin syringe while rabbits were anaesthetized with ether and maintained in a left decubitus position with maximum flexion of the head. Approximately 0·8 ml of spinal fluid could be removed with relative ease. The number of red blood cells and white blood cells in each sample of spinal fluid was determined by direct count using a haemocytometer and standard methods. Each sample of fluid was promptly centrifuged at 1500 rev/min for 7 min. One aliquot of the supernatant was used immediately for β-glucuronidase assay. The remaining supernatant was frozen at −20°C for later protein determination (Daughaday, Lowry, Rosebrough & Fields, 1952).

CSF β-glucuronidase assay and normal values

The basic procedure of Fishman, Springer & Brunetti (1948) and Fishman & Bernfield (1955) was modified to permit use of semi-micro methods because of the small samples of rabbit CSF available for study. A suitable assay which was sensitive and reproducible was developed as follows.

100 λ rabbit CSF were incubated with 50 λ of 0·1 M phenolphthalein β-monoglucuronide (Sigma Chemical Company, St. Louis, Mo.) and 200 λ of 0·4 M acetic acid–sodium acetate buffer at a pH of 4·5 at 38°C. After 24 hr incubation, 400 λ of 1 M glycine buffer were added to bring the pH to 10·2 and the free phenolphthalein previously released from the substrate was measured by recording absorbance at 555 mμ in a Beckman DU in a 2 ml Pyrocell semi-micro cuvette. Samples were run in duplicate in every case and usually in triplicate if sufficient spinal fluid was available. Substrate was added to the blanks after incubation. Standard phenolphthalein curves were constructed using 150 λ of phenolphthalein solution to replace the volume of CSF in the assay and substrate. The other reagents

were added as described above. Final β-glucuronidase activity was expressed as micrograms phenolphthalein released/hr/100 ml CSF.

The reproducibility of this micro-assay was assessed by simultaneously testing thirty aliquots of a pool of human CSF. Mean value for β-glucuronidase was 71 μg of phenolphthalein/hr/100 ml with a range of plus or minus twice the standard error of the mean of 66·5–75·5.

From assay of CSF samples from twenty-five normal rabbits, mean CSF β-glucuronidase activity was 47 μg phenolphthalein/hr/100 ml with a range of 26–74. Twenty-three samples of CSF collected serially from five additional normal rabbits had a mean enzyme value of 44 μg phenolphthalein/hr/100 ml with a comparable range of activity, indicating that multiple cisternal punctures did not influence the enzyme activity pattern in normal animals.

Serum β-glucuronidase was determined using the method of Fishman, Kato, Anstiss & Green (1967).

Criteria for EAE

Sensitized rabbits were observed 6 or 7 days per week and were considered to have clinical neurological signs of EAE when they exhibited weakness of one or more legs resulting in an unsteady gait or frank paralysis of both hind legs with dragging of hind legs and hind quarters. The brain and spinal cord were removed from each rabbit at the termination of experiments and were fixed in 10% formaldehyde in tap water. Blocks of the cerebrum, mid-brain, cerebellum-pons, and four levels of spinal cord were fixed in paraffin, sectioned at 5–7 μ and stained with haematoxylin and eosin. An animal was classified as 'positive' for lesions of EAE if one or more characteristic perivascular mononuclear cell infiltrates was found in any of the seven sections of brain-cord routinely examined.

RESULTS

Contamination of CSF with blood

While erythrocytes contain only trace amounts of β-glucuronidase, enzyme derived from leucocytes or present in the plasma fraction of contaminating blood might cause spuriously high CSF enzyme values. It was necessary, therefore, to determine how much blood-contamination of CSF was permissible, as judged by erythrocyte counts.

Data bearing on this question are shown in Table 1. Approximately two-thirds of the

TABLE 1. Influence of blood contamination of rabbit cerebrospinal fluid (CSF) samples on β-glucuronidase activity

CSF erythrocyte counts (per mm³)	No. CSF samples	CSF β-glucuronidase activity*	
		Mean	Range
0–500	20	42	21–74
500–5000	22	43	21–69
5000–10,000	11	45	26–69
10,000–20,000	9	40	23–69
20,000–150,000	6	62	20–108

* Activity expressed as μg phenolphthalein/hr/100 ml CSF.

CSF samples had 5000 or fewer erythrocytes/mm^3. These samples as well as others containing up to 20,000 erythrocytes/mm^3 had enzyme activities within the normal range for rabbit CSF (mean value 47; range 26–74). In only six samples did erythrocyte counts exceed 20,000/mm^3. In three of these with counts of 100,000–150,000 elevated β-glucuronidase activity was detected, viz. 75, 83 and 108 μg phenolphthalein/hr/100 ml CSF. These observations and the results of other experiments where predetermined amounts of heparinized whole rabbit blood were added to aliquots of erythrocyte-free pooled CSF, led us to exclude CSF samples with erythrocyte counts above 60,000 or 70,000/mm^3.

β-glucuronidase in CSF of rabbits with EAE

As shown in Table 2, of twenty-one rabbits sensitized to spinal cord-adjuvant, the mean CSF β-glucuronidase activity was elevated only in those animals that developed clinical signs

TABLE 2. β-glucuronidase activity and protein levels in cerebrospinal fluid (CSF) samples of rabbits before and after sensitization to spinal cord-adjuvant

Days post-sens.*	Clinical status	No. of rabbit CSF samples	CSF β-glucuronidase†		CSF protein (mg%)‡	
			Mean (range)	SD§	Mean (range)	SD
Pre-sens.	Well	19	48 (26–74)	13·5	40 (25–80)	13·7
7–10	Well	14	41 (23–69)	13·4	47 (18–110)	22·8
11–20	Paralysed	9	117 (65–161)	37·1	162 (34–>200)	57·5
11–20	Well	12	36 (20–64)	14·8	42 (25–67)	16·9
21–27	Paralysed	10	152 (38–250)	53·6	164 (30–>200)	76·9
21–27	Well	8	52 (28–84)	20·3	68 (30–110)	32·4
28–35	Paralysed	3	205 (125–250)	69·2	104 (96–112)	11·3
28–35	Well	9	68 (39–120)	31·8	81 (46–175)	48·7

* Total of twenty-one rabbits sensitized to allogeneic spinal cord-adjuvant.

† Activity expressed as μg phenolphthalein/hr/100 ml CSF.

‡ Protein concentration data for the following groups of rabbits derived from the indicated number of CSF samples: 7–10 days (clinically well)—13; 21–27 days (paralysed)—8; > 28 days (paralysed)—2. Where protein concentration > 200 mg% (five samples), 200 mg% was used to calculate mean concentration.

§ SD ± one standard deviation.

of EAE. By 13–19 days after sensitization, nine rabbits exhibited paralysis and mean CSF β-glucuronidase activity in these animals was greater than 100 mg phenolphthalein/hr/100 ml CSF. By 24 days, two additional rabbits had now developed EAE; mean CSF enzyme activity in these two animals and eight of the nine still paralysed rabbits was above 150. Subsequently, mean enzyme activity exceeded a value of 200 in three animals still paralysed and alive 28 days or longer after sensitization. While CSF enzyme activity tended to rise sharply at about the time paralytic signs of EAE occurred and to increase progressively with continued paralysis, occasionally deviations from this pattern were observed. For example, CSF from one animal (rabbit 803) collected 1 week before onset of clinical neurologic signs had increased enzyme activity.

Rabbits sensitized to spinal cord-adjuvant and which appeared clinically well throughout

the 28–34 day study period consistently had normal β-glucuronidase CSF activity. In addition, six rabbits simultaneously sensitized to adjuvant only and observed for comparable periods showed no elevation in CSF enzyme activity above the normal range.

All eleven paralysed rabbits had typical focal lesions of EAE throughout the brain and spinal cord. Of the remaining ten rabbits sensitized to spinal cord-adjuvant and which remained well, lesions of EAE were found in only one animal (a total of six minimal focal areas of vasculitis). All six rabbits sensitized to adjuvant only were free of EAE lesions. Thus, elevations in CSF β-glucuronidase activity were restricted to rabbits that developed both clinical and histologic evidence of EAE.

No relationship was apparent between CSF β-glucuronidase activity and CSF pleocytosis which at no time exceeded 200 white blood cells/mm^3 CSF. Several rabbits had high enzyme activity despite no demonstrable CSF pleocytosis.

Conceivably, increased blood levels of β-glucuronidase might result from spinal cord-adjuvant sensitization and lead to increased CSF activity, especially in rabbits developing EAE with an increase in permeability of the blood–brain and blood–CSF barriers. To investigate this possibility, β-glucuronidase activity in serial serum specimens from six paralysed rabbits were measured. Care was taken to collect the serum samples on the same days when CSF was collected (or as close to these days as possible). None of the serum samples from any of the six rabbits had appreciable increases in β-glucuronidase activity. Mean enzyme activity of sera collected before or 7 days after sensitization (before appearance of neurologic signs) was 1200 (range 900–1400) units/hr/100 ml serum. Mean enzyme activities at 2 weeks and 3 or 4 weeks post-sensitization were 1128 (range 970–1300) and 1350 (range

TABLE 3. Representative β-glucuronidase activity of simultaneously collected cerebrospinal fluid (CSF) and serum samples from rabbits before and after sensitization

Rabbit No.	Days post-sens.*	β-glucuronidase†		Clinical status
		CSF	Serum	
M5	(-) 5	54	1400	Well
	10	48	970	Well
	12	83	630	Ataxia
	14	123	710	Paraplegia
	19	231	970	Paraplegia
	26	120	1100	Paraplegia
	34	250	490	Paraplegia
M7	(-) 5	41	1300	Well
	10	40	1100	Well
	13	113	560	Ataxia
	15	161	1200	Paraplegia
	20	180	860	Paraplegia

* Rabbits sensitized to allogeneic spinal cord-adjuvant; hyphen inside parentheses indicates samples collected 5 days before sensitization; other figures represent days after sensitization.

† Activity expressed as μg phenolphthalein/hr/100 ml CSF or units/hr/100 ml serum, respectively.

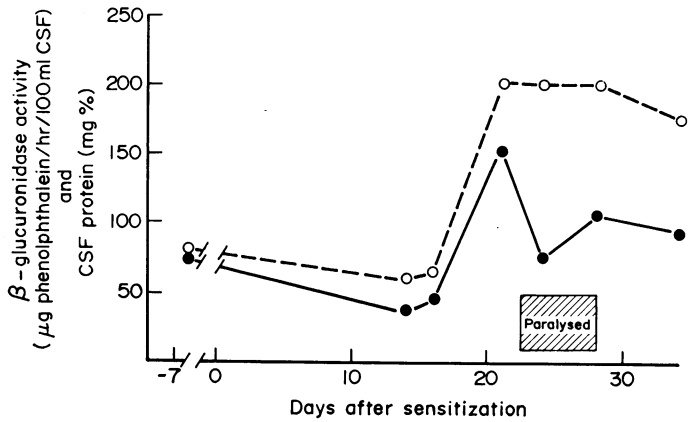

FIG. 1. β-glucuronidase activity (●) and protein concentration (○) of serial cerebrospinal fluid (CSF) specimens from rabbit M-1.

970–2100) units/hr/100 ml serum. Representative comparative serum and CSF β-glucuronidase data for two rabbits are shown in Table 3. These data together with that of the other rabbits clearly indicated no direct relationship between onset of paralysis, serum enzyme and CSF enzyme activity.

CSF protein in rabbits with EAE

A parallel relationship was observed between CSF β-glucuronidase activity and protein concentration in some rabbits, as may be seen in Table 2. However, this was not true for all rabbits. For example, rabbit M-1 (Fig. 1) showed a marked increase in enzyme activity and protein concentration between the 16th and 20th day after sensitization. During this period the rabbit remained clinically well. Paralysis appeared abruptly on the 23rd day. Thereafter,

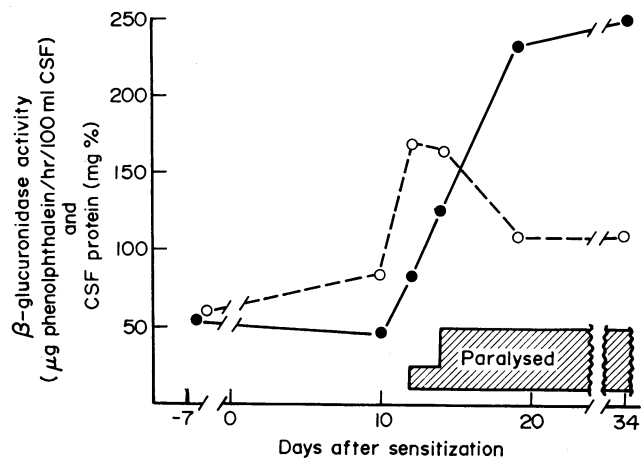

FIG. 2. β-glucuronidase activity (●) and protein concentration (○) of serial cerebrospinal fluid (CSF) specimens from rabbit M-5.

protein concentration remained elevated but β-glucuronidase activity fell toward pre-paralytic levels as paralytic signs receded. This rabbit was the only one of the eleven animals to recover from paralysis and it is of considerable interest that appearance of clinical recovery was accompanied by fall in enzyme activity but not in protein concentration. In rabbit M-5 (Fig. 2), β-glucuronidase activity lagged behind rising protein concentrations as paralysis appeared. Enzyme activity continued to rise as paralysis progressed, whereas protein concentration tended to fall and plateau. Similar dissociations between CSF enzyme activity and protein concentration were observed in two other paralysed rabbits and, again, suggested that β-glucuronidase CSF activity might be a more sensitive reflection of paralytic status than CSF protein.

DISCUSSION

Using the micro CSF β-glucuronidase assay here described, a sharp increase in enzyme activity has been consistently observed in rabbits developing paralytic and histologic manifestations of EAE (Table 2). Increased CSF β-glucuronidase activity occurs a few days before or at the same time as clinical neurologic signs of the disease (Figs 1 and 2). Further increases in enzyme activity may parallel continued or progressive neurologic clinical abnormalities (Fig. 2 and Table 2). In only one of the eleven paralysed rabbits was a departure from this CSF enzyme-EAE pattern observed. In this rabbit, β-glucuronidase activity in serial CSF samples remained within the normal range, despite appearance of otherwise typical hindleg weakness approximately 2 weeks after sensitization, which progressed over a few days to paraplegia. In another rabbit, a rapid fall in enzyme activity was observed some 36 hr after onset of paralysis and preceded rapid improvement in neurologic status and return to clinical well being (Fig. 1). Return of elevated β-glucuronidase activity toward the normal range, thus, may herald clinical remission of EAE.

Elevated CSF β-glucuronidase activity paralleled increased CSF protein levels and proved no more sensitive in signalling impending or frank occurrences of paralytic EAE (Table 2). Study of sequential enzyme-protein patterns in serial CSF samples from individual paralysed rabbits, however, revealed striking dissociations in some animals, e.g. fall in protein concentration with further increases in enzyme activity (Fig. 2). The comparatively greater increases in enzyme activity as compared to protein concentration in rabbits with progressive paralytic disease (Table 2), the occasional striking dissociation between enzyme and protein values in a given rabbit (Fig. 2) and the rapid fall in CSF activity before recovery from paralysis (Fig. 1), together point to β-glucuronidase reflecting more directly the march of pathogenetic events causing injury to the neuraxis of rabbits with EAE.

The precise origin of increased CSF β-glucuronidase is not clear. One source, obviously, is release from injured central nervous system tissue. In rabbit nervous tissue, Robins, Hirsch & Emmons (1968) found an inverse relationship between enzyme activity and myelinated nerve fibres, and localized β-glucuronidase activity to nerve cell bodies and their mitochrondrial-lysosomal granule fractions by density gradient centrifugation. Studies of the distribution of β-glucuronidase enzyme in rat brain revealed high concentrations in the choroid plexus network (Waltimo & Talanti, 1965), a site of predilection for EAE lesions in this species of animal. Other workers have reported elevated β-glucuronidase activity of central nervous system tissue of chicks following experimental encephalomalacia (Jibril &

McCoy, 1965). Arnaki & Weissbarth (1964) reported increased β-glucuronidase activity within the spinal cord, but not the brain, of rabbits with paralytic signs of EAE. β-glucuronidase activity of CSF was not studied. The results of this study are difficult to interpret since no control rabbits (sensitized to adjuvant only) were employed, the method of inducing EAE is not completely clear and an expected parallel increase in acid phosphatase (another lysosomal hydrolytic enzyme) was not observed.

Another source of β-glucuronidase is the dense perivascular collections of lymphocytes and histiocytes around small vessels, i.e. the hallmark of EAE. While polymorphonuclear leucocytes are present in fair numbers in very early lesions in some rabbits and might release their high content of enzyme (Rossiter & Wong, 1950), the primary inflammatory event consists of perivascular accumulations of lymphocytes and histiocytes. Human peripheral blood lymphocytes contain β-glucuronidase, along with a number of other hydrolytic enzymes which are localized to the lysosomal granules of these cells (Brittinger *et al.*, 1968). These cells undergo mitotic division and transform into 'blast' cells, along with release of these enzymes into cell sap and extracellular environment, when they are cultured *in vitro* in the presence of non-specific mitogenic stimuli (e.g. phytohaemagglutinin) or specific antigens to which the lymphocyte donor is sensitized (Hirschhorn *et al.*, 1965, 1968). Studies in progress in our laboratories indicate that the same situation obtains in rabbit peripheral blood lymphocytes.

One can visualize specific interaction of invading small lymphocytes sensitized to nervous tissue with myelin encephalitogenic antigen which results in mitotic division and blast transformation of the cells in question. These cellular responses might well result in release of intracellular lysosomal enzymes, including β-glucuronidase, into tissue extracellular spaces and CSF. The outpouring of hydrolytic enzymes or other injurious cell products, e.g. lymphotoxin, might be an important factor not only in initiating but perpetuating damage to glial cells responsible for myelin synthesis and other nervous system elements.

In earlier reports of CSF β-glucuronidase activity in neurologic disease of man, Anlyan & Starr (1952), Allen & Reagan (1964) and Plum (1967) indicated that increased CSF activity was associated with those diseases characterized by rapid cellular mobilization or proliferation or tissue necrosis e.g. acute inflammatory conditions (excluding bacterial meningitis), glioblastoma multiforme and tumour meningeal implants. β-glucuronidase is present in high concentration in polymorphonuclear cells of both man (Fishman, Springer & Brunetti, 1948) and rabbit (Rossiter & Wong, 1950). The prominent role of these cells in acute inflammation and necrotic processes suggests they might well release considerable enzyme into the CSF when such conditions obtain. In demyelinating disease of man, e.g. multiple sclerosis, only minimal or moderate increases in CSF β-glucuronidase activity have been observed with the highest activity again paralleling more acute and active phases of the disease (Allen & Reagan, 1964).

One major focus of our study in rabbits has been the use of the β-glucuronidase system as a probe for better understanding of cellular and subcellular events of paramount importance in the pathogenesis of EAE. In this light, the report of Wajda, Lee & Neidle (1969) of increased transglutaminase activity in brain tissue of EAE-susceptible BSVS mice with development of paralysis, in the face of normal serum enzyme levels, has pointed meaning. The reports of Riekkinen & Rinne (1967) and Rinne & Riekkinen (1968) of increased esterase, peptidase and proteinase activity of CSF from patients with multiple sclerosis, especially in relapse, are of added interest and further suggest that biochemical-enzyme

markers of one type or another may hold promise in continued study of neurologic disease of animals and man.

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