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IMMUNOCHEMICAL ANALYSIS OF SOME PROTEINS IN CEREBROSPINAL FLUID AND SERUM OF PATIENTS WITH ISCHEMIC STROKES

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Immunochemical studies of $\gamma\gamma$-neuron specific enolase (NSE), parvalbumin (PV), S-100 protein (S-100) and acidic fibrillary glial protein (GFAP) were studied in the cerebrospinal fluid and blood serum in 7 patients with ischemic cerebral stroke, aged 57 to 81 years. Cerebrospinal fluid and the first blood sample were taken on the first or second day of the disease. Further blood samples were taken once a week till the end of patients hospitalization, ending by patients discharge or death. Immunochemical identification of proteins under study were performed with Western-blotting technique. It was found that all proteins studied were present in both cerebrospinal fluid and blood serum on the first two days of the disease in small quantities. The blood content of both NSE and PV increased significantly during the first week of the disease. Both proteins disappeared from the blood serum between the second and fourth disease weeks. S-100 protein and GFAP contents in the blood reached significantly high level within the time interval between second and fifth disease weeks, and remained at a relatively high level till patients' death. In all cases computed tomography study and/or brain autopsy revealed extensive ischemic foci localized within areas supplied by the middle cerebral artery. No clear-cut correlation between extensiveness of the ischemic cerebral damage and the content of the proteins studied in both cerebrospinal fluid and blood serum was found. However, our data indicate that serial studies of the above proteins in patients with ischemic stroke may be useful in monitoring the progress of the disease, and occasionally in the prognosis at least in some cases.

Key words: *ischemic stroke, cerebral proteins, cerebrospinal fluid, blood serum*

Despite remarkable progress in modern diagnostic procedures, such as computed tomography, nuclear magnetic resonance imaging and PET-scanning their value in evaluating the extent of ischemic damage of the brain is still limited. Therefore, some other methods are also needed for quantitative information on the size of ischemic lesions of the brain and the prediction of possible outcome of the disease.

A large number of proteins may be released into the cerebrospinal fluid and serum as a consequence of brain injury. They can serve as potential markers of the extent of the pathologic changes. Neuron-specific enolase (NSE) and S-100 protein in cerebrospinal fluid (CSF) have already been determined in cerebral infarction in human (Sindic et al. 1982; Mokuno et al. 1983; Royds et al. 1983; Hay et al. 1984; Persson et al. 1987), and in experimental brain ischemia (Steinberg et al. 1984; Härdemark et al. 1988, 1989; Kotwica et al. 1989a, b). Glial fibrillary acidic protein (GFAP) has been studied in CSF in one case of cerebral infarction (Noppe et al. 1986). All these studies stressed that the presence of NSE and S-100 protein, at least in CSF is a reliable

marker of brain damage and that changes in their concentration could have a prognostic value.

In this paper we describe the results of studies on NSE, S-100 protein, GFAP and parvalbumin (PV) concentration in CSF, as well as in serial blood serum samples taken till the outcome in patients with completed ischemic strokes, and assess their potential usefulness in clinical applications.

Material and methods

The studies were performed on seven clinical cases of completed ischemia stroke. The patients' age ranged from 57 to 81 years (Table 1). CSF and blood samples were taken on the day of patients' admission to the hospital (first or second day of the disease). Thereafter blood samples were taken once a week till the outcome.

The control material consisted of CSF and blood samples from 5 patients with transient ischemic attack (TIA) and 5 patients with muscle diseases.

The CSF and blood samples were centrifuged at 4000 rpm for 10 min. The supernatants were frozen at −70°C until used.

Table 1. Computer tomography and findings in the presented material

Case/age	Duration of the disease (days)	Outcome	CT on day	Ischemic lesion in CT	Ischemic lesion in autopsy	Contact with CSF pathways
MZ/80	70	death	14	+++	-	+++
NL/69	58	death	9	+++	+	+++
BI/67	32	survival	7	+	-	+
BH/81	44	death	5	+++	-	+++
SE/79	16	death	-	-	+++	+++
NM/67	11	death	-	-	+++	+++
CJ/57	5	death	2	+++	-	+++

+ – small ischemic lesion, + + – moderate size ischemic lesion,
+++ – large ischemic lesion.

Total protein concentration in CSF and serum was determined according to the method of Lowry et al. (1951). The samples were diluted with an equal volume of two times concentrated sample buffer consisting of 0.125 M Tris base, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.001% bromophenol blue (pH 6.8). The samples were heated in boiling water for 3 minutes and a volume corresponding to 100 μg of protein was applied to lanes on 1.5 mm thick SDS-polyacrylamide gels, using the discontinuous system of Laemmli (1970). The resolving gels were 10% acrylamide: bisacrylamide (30:0.8). The separated proteins were blotted on nitrocellulose 0.2 μm membranes, using a transfer buffer (0.25 M Tris-HCl, 1.92 M glycine, pH 8.3) at 0.8 mA per cm^2 per hour (Towbin et al. 1979). LKB Multiphor II Nova Blot electrophoretic transfer unit was used. Unreacted binding sites on dry nitrocellulose sheets were blocked with 3% bovine albumin (Sigma) in buffer containing 10 mM Tris-HCl, 0.15 NaCl and 0.05% (v/v) Tween 20 (pH 8.0), which was also used to wash the nitrocellulose sheets after albumin blocking and incubations with primary antibodies. Incubation steps during 3 hours were performed with primary antibodies (anti-S-100, dilution 1:80, Sigma; anti-GFAP, dilution 1:400, Dako; anti-$\gamma\gamma$enolase, dilution

1:200, Dako; anti-parvalbumin, dilution 1:1000, Sigma) and secondary antibodies (alkaline phosphatase conjugate goat anti-human IgG, dilution 1:1000, Sigma). The blots were finally exposed to alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl$_2$, nitroblue tetrazolium salt in 0.1 M Tris – HCl and dimethylformamide, pH 9.5) for 30 minutes, rinsed in distilled water and dried.

Results

CT examination was performed in all but two cases. In all cases examined areas of hypodensity localized within the area of middle cerebral artery supply were observed (Fig. 1). In two cases in which CT examination was not done, postmortem autopsy revealed focal tissue abnormalities within the same brain areas. In one case (SE) postnecrotic cavity was localized within inferior frontal gyrus, while in the second one (NM) early tissue necrosis involved a larger area supplied by middle cerebral artery. In only case (NL) in which both intravital CT-examination and postmortem autopsy were performed, slight discrepancy between changes found in these two procedures, was observed. This concerned the extend of tissue alterations. CT examination performed on the ninth day of the disease indicated rather larger area of hypodensity within the area of middle cerebral artery supply, while on autopsy postischemic cavity was confined to the inferior frontal lobe and adjacent white matter of the semioval centre. In all seven cases, the brain lesions as seen on the CT pictures and/or autopsy examination were contacting cerebrospinal fluid spaces, either the subarachnoid or ventricular.

NSE was not present in the control CSF and serum samples. In the patients with ischemic stroke it was present in both CSF and serum on the day of admission either as traces or in evidently increased amounts (Figs 2 and 3). The marker had a tendency to increase further in the serum during the first
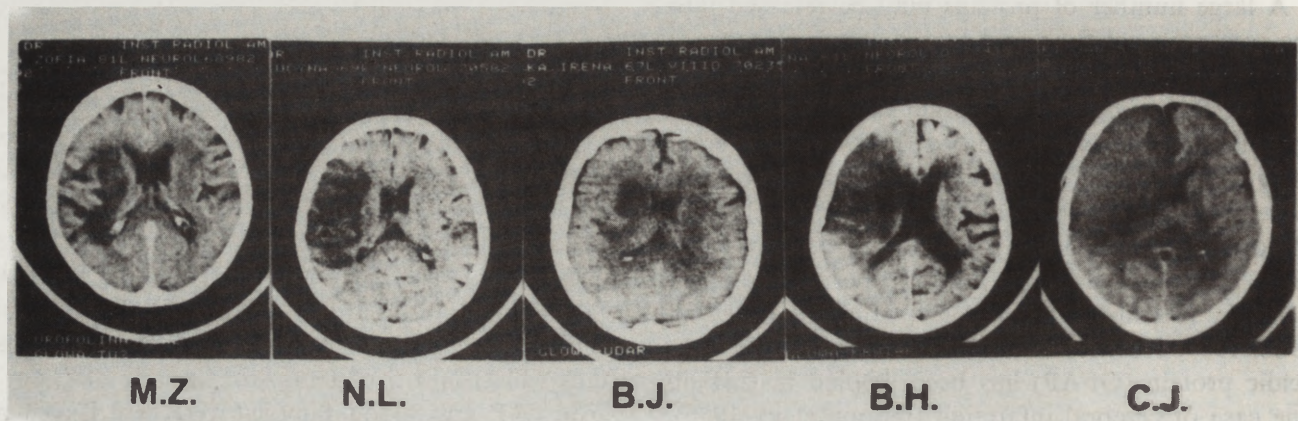

Fig. 1. Computer tomography scan of some of the investigated cases. In all cases the areas of hypodensity were localized within territory of middle cerebral artery supply.
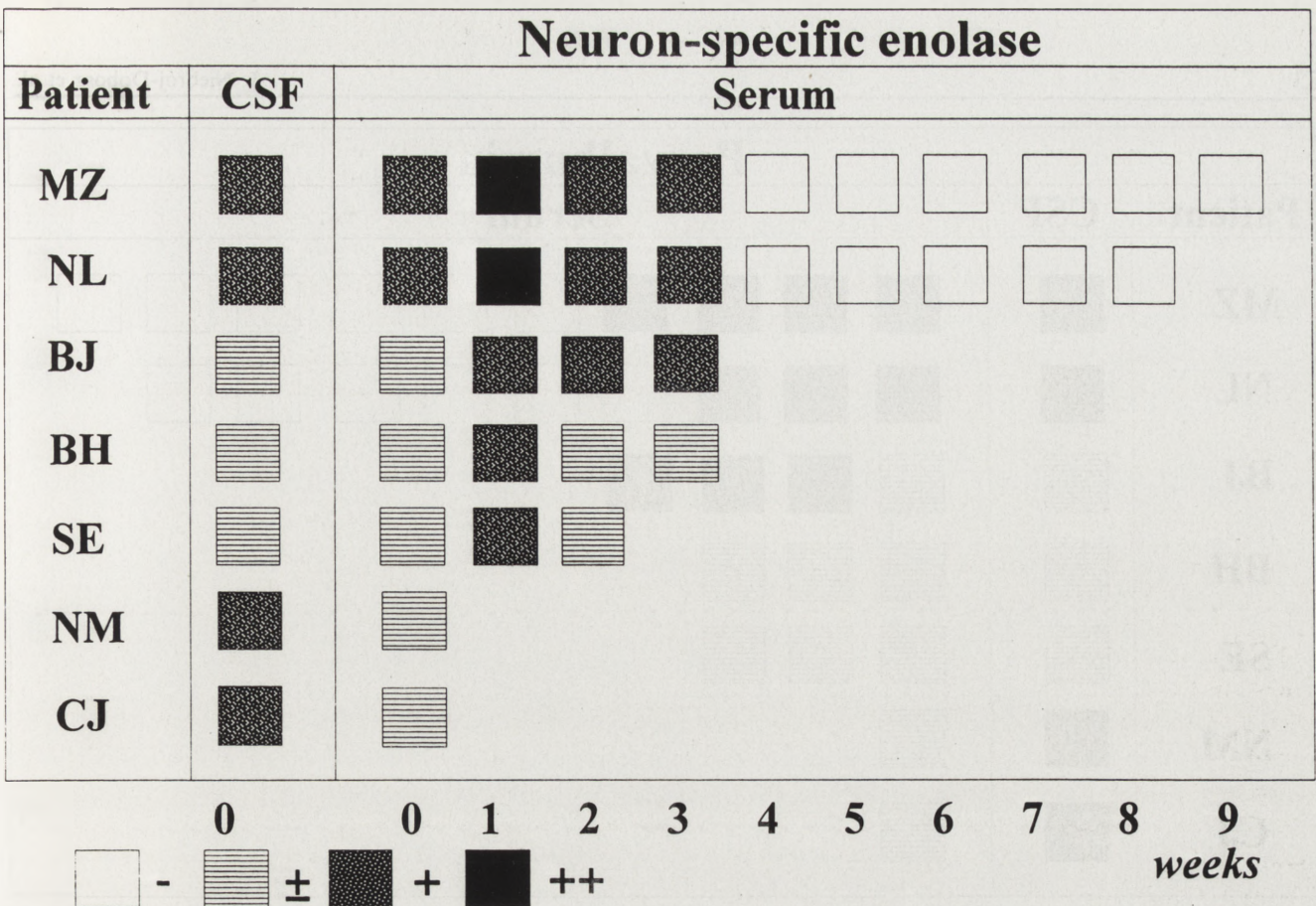
Neuron-specific enolase

Patient	CSF	Serum									
	0	0	1	2	3	4	5	6	7	8	9
MZ											
NL											
BJ											
BH											
SE											
NM											
CJ											

weeks

- □ - ▤ ± ▨ + ■ ++

Fig. 2. The appearance of neuron-specific enolase in CSF and serum in all cases examined.

NSE

CSF   SERUM

NL   0   0   1   2   3   4   5   6   7   8
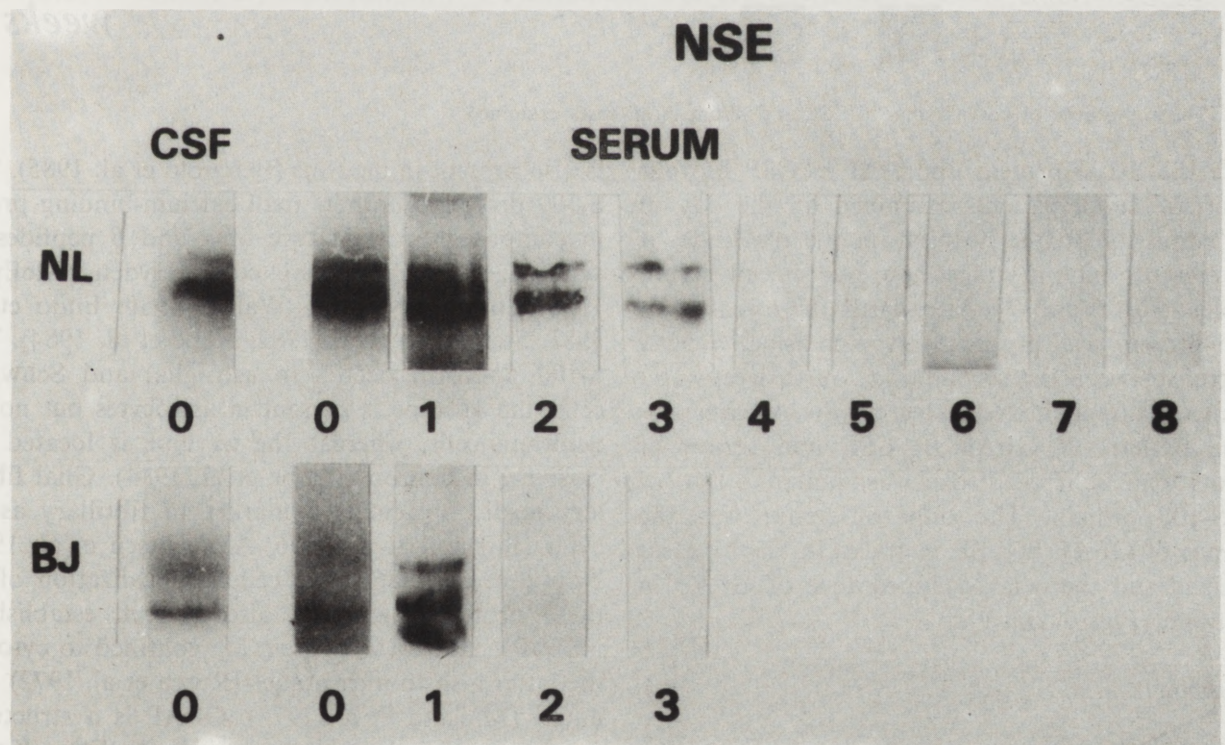
BJ   0   0   1   2   3

Fig. 3. Western-blotting analysis of neuron-specific enolase in CSF and serum in a mild (BI) and severe case (NL) in the course of ischemic stroke.

week of the disease, thereafter decreasing and disappearing after two to four weeks. The anti-NSE-antibodies possibly reacted not only with γγ-neuron-specific enolase (the lower intense band in Fig.3) but also to some extent with αα-enolase (the upper less intense band in Fig. 3).

A pattern similar to that of NSE was also seen for anti-parvalbumin antibodies (Figs 4 and 5). When compared to the NSE the staining intensity of parvalbumin was lower and its decrease in the serum appeared earlier.

No reaction with anti-S-100 protein antibodies was observed in the CSF and serum from the control material. In the course of the ischemic
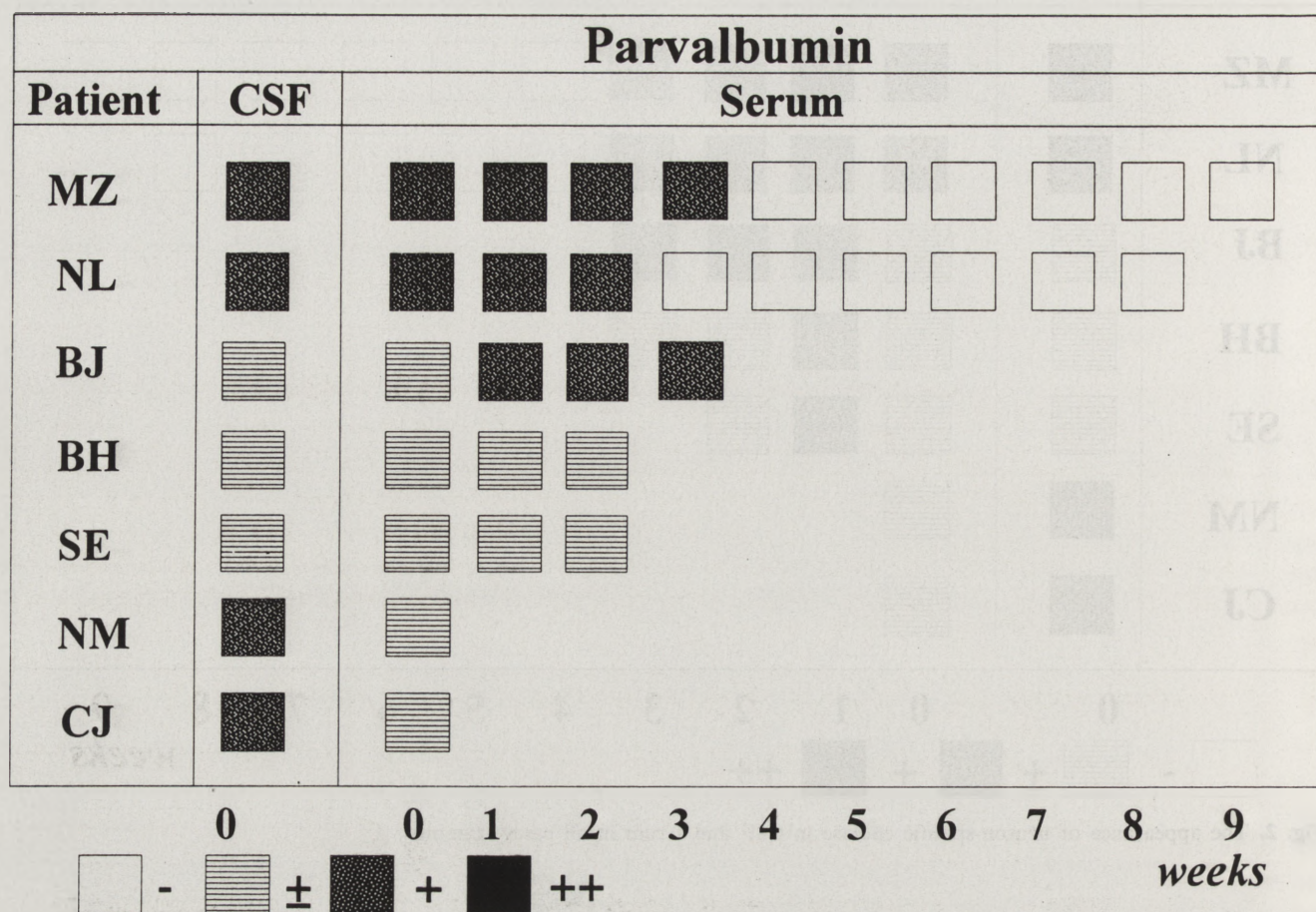
Parvalbumin

Patient	CSF	Serum									
	0	0	1	2	3	4	5	6	7	8	9
MZ	++	++	++	++	++	-	-	-	-	-	-
NL	++	++	++	++	-	-	-	-	-	-	
BJ	±	±	++	++	++						
BH	±	±	±	±							
SE	±	±	±	±							
NM	++	±									
CJ	++	±									

weeks

Legend: □ - □(±) ▒ + ■ ++

Fig. 4. The appearance of parvalbumin in CSF and serum in all cases examined.

stroke the S-100 protein appeared in CSF of four out of the seven patients examined on the day of their admission to the hospital, in the remainder it was present only in traces, so was in all serum samples (Figs 6 and 7). Afterwards the intensity of S-100 protein was progressively increasing; a peak occurred between the second and fourth weeks and the intensity remained elevated till the outcome.

The pattern of GFAP in CSF and serum of patients with ischemic stroke was similar to that of the S-100 protein. The only difference was the presence of GFAP in CSF in traces in all the cases examined and the delayed appearance of GFAP in the serum (Figs 8 and 9).

Discussion

Several substances are released into CSF and the blood stream in the course of brain injury. Among these substances are proteins which can be used as brain damage markers. They can also indicate whether the damage is of neuronal, glial or mixed origin.

$\gamma\gamma$-neuron-specific enolase is present predominantly in neurons (Schmechal et al. 1978; Marangos et al. 1978, 1979; Kato et al. 1982a, b), whereas the $\alpha\alpha$-form is confined to astroglial cells (Royds et al. 1982). Parvalbumin, a small calcium-binding protein is also present in neurons (Berchold et al. 1985). The S-100 protein, another small calcium-binding protein composed of a mixture of α and β peptides, is synthesized in astroglial cells (Hyden, McEwen 1966; Zomzely-Neurath, Walker 1980; Endo et al. 1981; Stefansson et al. 1982; Isobe et al. 1984). The S-100 $\beta\beta$ form occurs in astroglial and Schwann cells, the $\alpha\beta$ type is present in astrocytes but not in Schwann cells, whereas the $\alpha\alpha$ type is located exclusively in neurons (Isobe et al. 1984). Glial fibrillary acidic protein is a marker of fibrillary astrocytes (Bignami et al. 1976; Albrechtsen et al. 1985; Noppe et al. 1986). The cellular localization of all these proteins has been already well established. NSE, PV and S-100 protein are confined to cytosol, the latter also to membranes (Rusca et al. 1972) and nuclei (Michetti et al. 1974). GFAP is a structural component of the cell cytoskeleton (Eng, Kosek 1974; Bignami et al. 1976).

In normal conditions all these proteins are either absent or present in amounts undetectable by the methods used both in CSF and serum (Michetti et al. 1980, Albrechtsen et al. 1985; Aurell et al. 1989). In a variety of diseases of the central nervous system several groups have examined NSE and/or S-100 protein in CSF (Brown et al. 1980; Michetti et al. 1980; Parma et al. 1981; Kato et al. 1982b; Mokuno
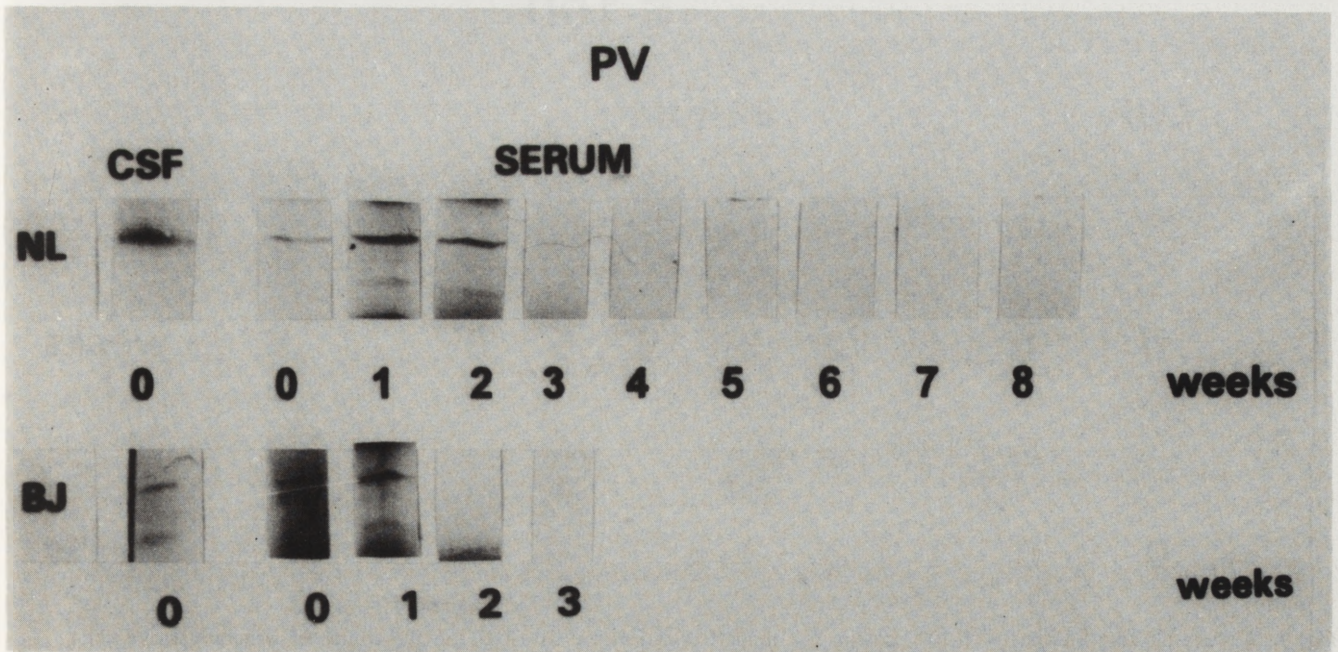
Fig. 5. Western-blotting analysis of parvalbumin in CSF and serum in a mild (BI) and severe case (NL) in the course of ischemic stroke.
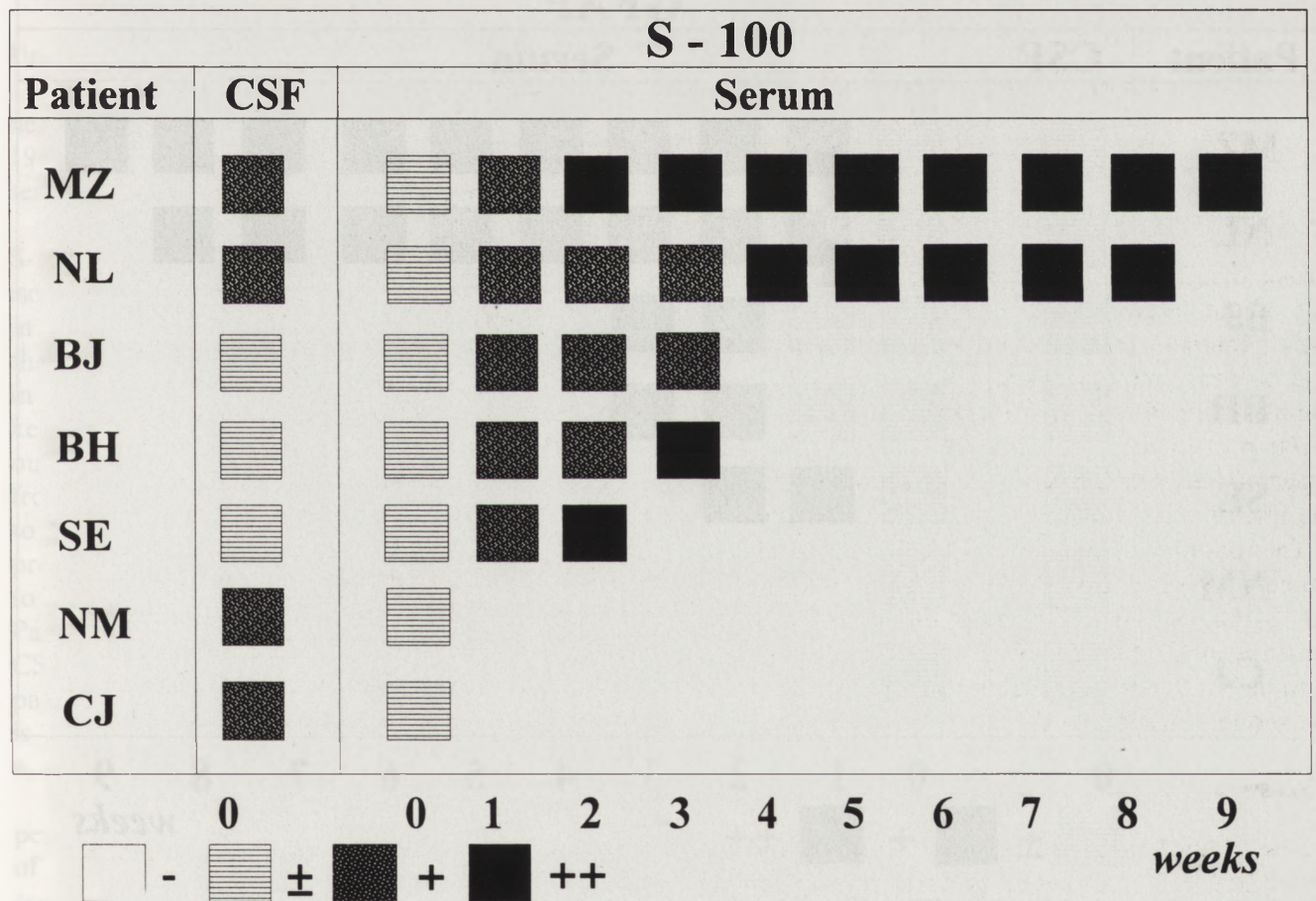

Fig. 6. The appearance of S-100 protein in CSF and serum in all cases examined.

et al. 1983). High NSE and S-100 protein levels in CSF were found in multiple sclerosis, inflammatory processes including different types of encephalitis and encephalomyelitis, brain tumors, dementia, Guillain-Barré syndrome, cervical spondylosis, amyotrophic lateral sclerosis, subarachnoid hemorrhage and cerebral infarction. The studies of both proteins showed a relationship between the degree of cell damage and their concentration in CSF (Royds et al. 1981, 1983; Sindic et al. 1982; Hay et al. 1984; Takayasu et al. 1985). After small infarcts and transient ischemic attacks only NSE, but not the
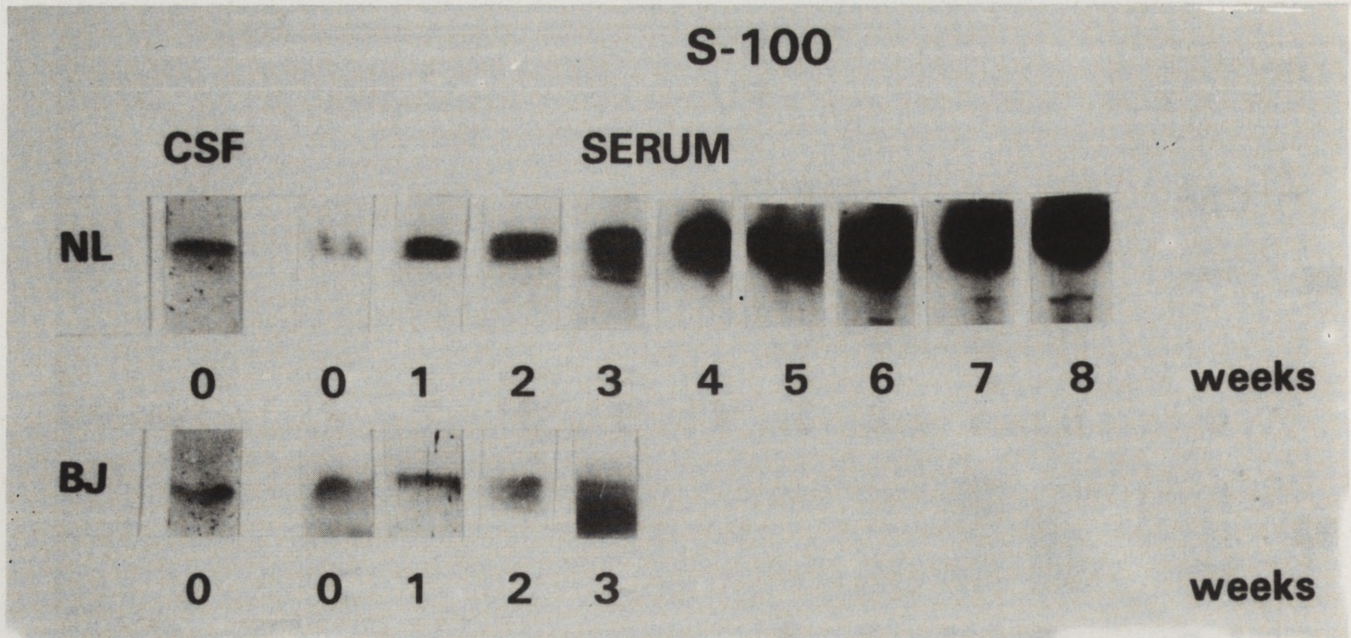
Fig. 7. Western-blotting analysis of S-100 protein in a mild (BI) and severe case (NL) in the course of ischemic stroke.
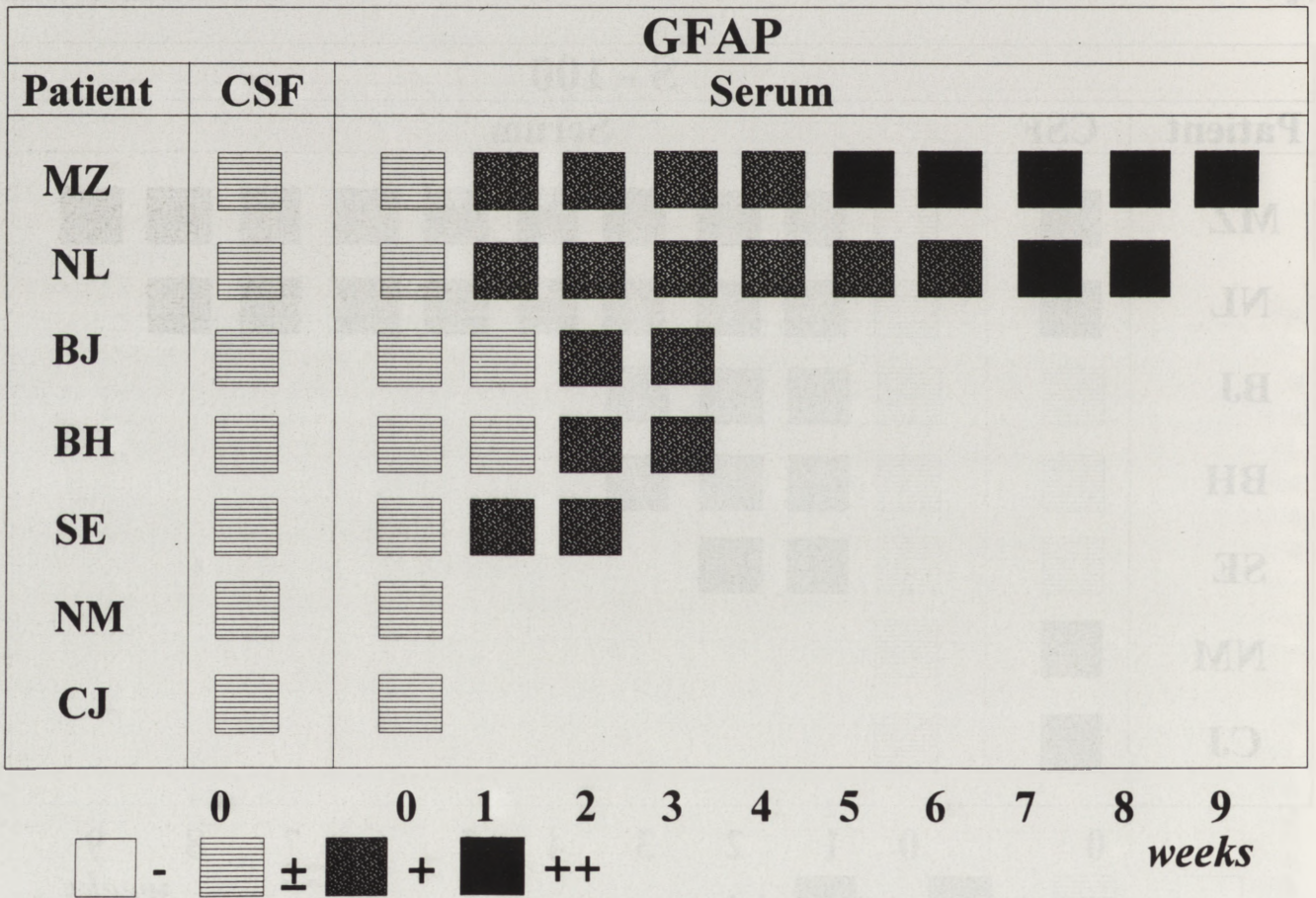

Fig. 8. The appearance of GFAP in CSF and serum in all cases examined.

S-100 protein, increases between 18 hrs to four days following the incident (Persson et al. 1987). Both NSE, and S-100 protein were also detected in the serum in ischemic strokes and head trauma. In the serum also their levels were related to severity of the disease during the first days after the incident (Dauberschmidt et al. 1983; Persson et al. 1987).

GFAP was shown to increase in CSF in multiple sclerosis, different types of encephalitis, epilepsy, Niemann-Pick disease, dementia, syringomyelia, brain tumors and cerebrovascular disease (Lowenthal et al. 1978; Noppe et al. 1986). It is supposed that appearance of GFAP in CSF is the most reliable marker of organic diseases of the central
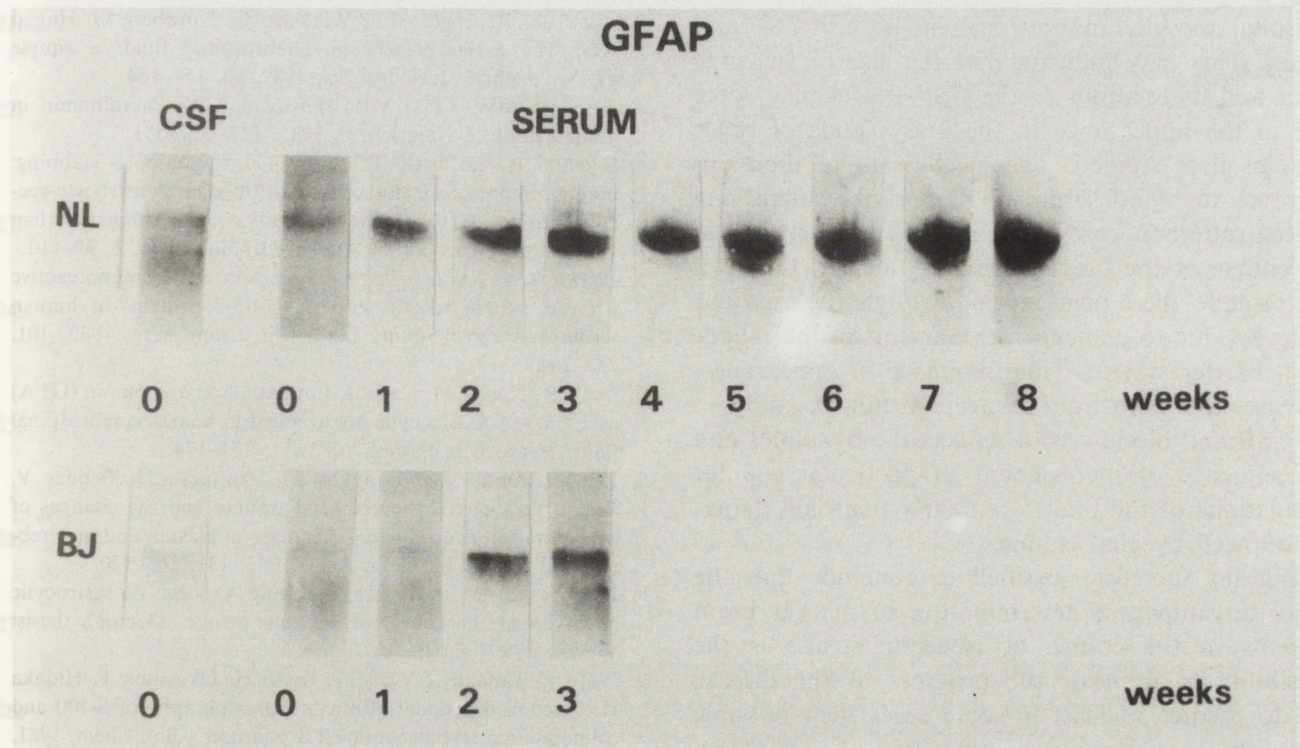
Fig. 9. Western-blotting analysis of GFAP in a mild (BI) and severe case (NL) in the course of ischemic stroke.

nervous system (Hayakawa et al. 1979; Crols et al. 1983). No data are published as yet on GFAP in the serum in CNS diseases.

Our results concerning appearance of NSE and S-100 in both CSF and blood serum in the course of ischemic stroke confirm the relevant data published in the literature. Both proteins appeared just after the onset of the disease in CSF and serum, although in small amounts. By examining both protein markers in serum at one week intervals, uptil the outcome we also confirmed that NSE disappears from body fluids earlier than does S-100. Contrary to the data presented before, in our material S-100 presence in serum was long-lasting, with a tendency to increase progressively in the course of the disease. Parvalbumin has not been so studied yet either in CSF or blood serum. Our data indicate that in patients with ischemic stroke, this neuronal protein is following the behaviour of NSE, although on a somewhat lower level.

We have also demonstrated that GFAP was appearing in traces both in CSF and serum at the time of onset of the disease and was progressively increasing in content, at least in the serum.

As NSE and PV are of neuronal origin their appearance in body fluids is a consequence of necrosis of the gray matter. S-100 protein and GFAP when present in CSF and serum indicate lesion of the white matter. Immunocytochemical studies of Rafałowska et al. (1991) and Dziewulska (1994) revealed that numerous cellular particles covering infarcted brain tissue and its neighbourhood

react with GFAP-antibodies already two days after an ischemic incident. These GFAP-positive particles were considered to represent products of astrocyte degeneration and breakdown. Normal astrocytes are revealing relatively low GFAP-immunoreactivity. In ischemic conditions it increases after few days and is followed by astrocytic degeneration (Rafałowska et al. 1991; Dziewulska 1994). Most probably at that time GFAP and S-100 protein escape to body fluids in larger amounts. The nearly identical intensity of GFAP and S-100 bands in the serum is possibly due to the fact that the necrotic lesions were located close to the subarachnoid space or the ventricular system.

The relatively small clinical material undoubtedly limits possibility to correlate of the disease and CT-pictures and/or autopsy findings on one side and the intensity of protein markers studied on Western-blots on the other. Only one patient with a small ischemic lesion survived while in other cases the clinical progress, CT and/or autopsy data were similar and the outcome was fatal. However, observations concerning patients with relatively longest disease course (MZ and NL), preceding fatal outcome are worth mentioning. These were the cases with very extensive focal brain lesions, contacting the CSF spaces, they were characterized by increased content of glial protein markers within blood serum, progressing till patients' death. On the other hand, the only surviver from our series, with definitely small ischemic focus and disputable contact with CSF-spaces showed relatively low levels of

neuronal and glial marker-proteins both in CSF and serum. This may indicate that the size of ischemic focus and its relations to the CSF spaces play some role in the appearance in the body fluids of brain proteins here studied. The mechanism of their appearance in blood serum (in remarkably increasing concentration in case of glial protein markers), in the course of the disease remains unclear. One can not exclude their penetration through the vascular pathway, due to damaged mechanisms of the blood-brain barrier system. Time sequence of appearance, increase and reduction of proteins under study in the CSF and blood serum reflects the dynamics and the sequence of involvement of particular cellular populations of the brain – an early neuronal damage followed by glial changes.

It seems therefore justified to conclude that the major advantage of determination of marker brain proteins in the course of ischemic stroke is the possibility to monitor the progress of the disease and to predict, at least in some cases their possible outcome.

Analiza immunochemiczna niektórych białek w płynie mózgowo-rdzeniowym i surowicy krwi chorych z udarem niedokrwiennym

Streszczenie

U chorych w wieku od 57 do 81 lat z niedokrwiennym udarem mózgu, przeprowadzono immunochemiczną identyfikację następujących białek: $\gamma\gamma$-enolazy neuronalnej, parwalbuminy, białka S-100 oraz kwaśnego białka włókienkowego gleju przy pomocy techniki „Western-blotting" w płynie mózgowo-rdzeniowym i w surowicy krwi. Płyn mózgowowo-rdzeniowy i pierwszą próbkę krwi pobierano w pierszym lub drugim dniu choroby. Następne próbki pobierano w odstępach tygodniowych aż do wypisu chorego lub jego zgonu. W płynie mózgowo-rdzeniowym i surowicy krwi badane białka w pierwszych dwóch dniach choroby występowały w niewielkiej ilości. Ilość enolazy i parwalbuminy w surowicy krwi wzrastała następnie w pierwszym tygodniu choroby. Oba białka zanikały między 2 i 4 tygodniem choroby. Ilość białka S-100 i GFAP wzrastała znacząco w okresie od 2 do 5 tygodnia i utrzymywała się na wysokim poziomie aż do zgonu chorego. Tomografia komputerowa mózgu i/lub badanie sekcyjne wykazały obecność rozległych ognisk martwicy niedokrwiennej w obszarze unaczynionym przez tętnicę mózgową środkową. W przedstawionym materiale nie stwierdzono wyraźnej korelacji między ilością białek w płynie mózgowo-rdzeniowym i surowicy krwi a rozległością i ciężkością uszkodzenia mózgu. Wydaje się jednak, że seryjne oznaczenie badanych białek w przebiegu udaru niedokrwiennego mózgu może być przydatne w monitorowaniu postępu procesu chorobowego, a być może, również w jego prognozowaniu, przynajmniej w poszczególnych przypadkach.

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