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The use of particle counting immunoassay for the diagnosis of neurological disease

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Abstract: Particle counting immunoassay (PACIA) is applied to the determination of proteins at trace levels in normal and pathological cerebrospinal fluid (CSF) samples. Performance characteristics for the assays are given and the results for immunoglobulins (Igs) expressed as Ig indices. Ig profiles characteristic of various neurological disorders have been studied, and anti-herpes antibodies detected using an agglutination inhibition method. Ferritin, C-reactive protein and the brain protein S-100 have also been studied.

Keywords: *Particle counting immunoassay; immunoglobulin G F(ab')2 fragments; proteins in cerebrospinal fluid; immunoglobulin assays; brain protein S-100; ferritin; C-reactive protein; agglutination inhibition assay; anti-herpes antibodies.*

Introduction

An important factor in the diagnosis of neurological disorders is the analysis of cerebrospinal fluid (CSF), especially the assay for minute concentrations of various proteins. CSF, a clear colourless liquid surrounding the spinal cord and the brain, has a total volume of about 150 ml. While the concentrations of low molecular weight materials, such as sodium, glucose, potassium, etc., are similar to those in serum, the concentrations of the proteins in CSF vary from 250 times lower than in serum for albumin to 11 000 times lower for IgM. The CSF is separated from the blood stream by the blood–brain barrier, a tightly knit network of endothelial and choroidal epithelial cells. The natural migration of substances across the blood–brain barrier is by diffusion which obviously favours the small molecules and virtually excludes the large molecules (IgM). As antibodies (IgG, IgM, IgA) are not normally produced in the brain, their concentration in CSF is very low, as are such other serum proteins as ferritin, C-reactive protein (CRP), α_2-macroglobulin, etc.

A useful assay for CSF must therefore use small volumes of sample and have a sensitivity some 1000–10,000 times greater than required for serum assays. Since particle counting immunoassay exhibits sensitivities in the high femtomolar range (10^{-15} M) and requires less than 100 μl of sample per assay, this technique has been applied to the assay of CSF proteins.

Elevated protein levels in CSF may arise from two principal causes, both of diagnostic significance. Proteins may diffuse at a faster rate than normal across the blood–brain

barrier from the blood, indicating an increased permeability of this network of cells most probably due to an inflammatory process such as meningitis, encephalitis, stroke, traumatic contusion, etc. Alternatively, the proteins may be produced locally, e.g. immunoglobulins synthesized by lymphocytes and plasma cells in response to an infection, or they may be products arising from damage to the nervous system. Such products will be myelin basic protein, S-100, glial fibrillary acidic protein, etc.

This paper describes a study of protein levels in CSF under several headings:

(i) Measurement of CRP and ferritin as indicators of inflammatory reactions within the central nervous system (CNS).

(ii) Assays of IgG, IgA, IgM and IgE to indicate immune reactions within the CNS, and the detection of anti-herpes antibodies in herpetic encephalitis and viral meningitis.

(iii) Assay of two brain specific proteins, the S-100 and glial fibrillary acidic protein, as indicators of acute damage to the nervous system.

Experimental

In all cases, $F(ab')_2$ fragments of IgG antibodies from an appropriate antiserum were covalently bound to 0.8 μm latex particles. When the latex was mixed with sample (serum or CSF) the antigen of interest agglutinated some of the latex after incubation at 37°C for 40 min. The mixture was then pumped through a particle counter electronically modified to recognize only the unagglutinated latex. The concentration of unagglutinated latex was inversely proportional to the concentration of antigen in the sample.

Each IgG fraction was separated from the antiserum by precipitation with half-saturated ammonium sulphate or rivanol, or by chromatography on Sepharose-Protein A. Controlled pepsin digestion of the IgG fraction produced $F(ab')_2$ fragments, which were separated on Ultro Gel AcA 4–4 and coupled to carboxylated latex. Trial and error determined the exact latex loading of $F(ab')_2$ and the optimum supporting solution for the required sensitivity. The use of $F(ab')_2$ fragments avoided interference from rheumatoid factor or the complement protein C1q. Details of these procedures and of other methods to avoid interference with the latex agglutination assay have been described elsewhere [1] and were employed as appropriate. A variant of this technique determining for herpes simplex antibody is described later.

Results were obtained on an early protype of the IMPACT® system (Acade SA, Brussels, Belgium) but no significant variation in technique is required to use the present apparatus which is considerably more automated. Performance characteristics of the assays are listed in Table 1.

Results and Discussion

Proteins of inflammatory reactions within the central nervous system (CNS): CRP and ferritin

At a detection limit of 1 μg/l, CRP was detectable only in 40% of the CSF samples, both in a control group of patients without neurological disorders, and in patients with multiple sclerosis. CRP levels were, however, elevated in all patients with pyogenic infections and in 90% of patients with viral infections of the CNS. In contrast to some results previously reported by other workers, our data showed an overlap of results between these two types of infection of the nervous system. Serum samples collected during the first days of the disease, had absolute levels of CRP much more elevated

Table 1
Performance characteristics for assays used

Protein	Serum[2–4]			CSF[5–9]		
	Sample size	Conc. range	Precision inter-assay R.S.D.	Sample size	Conc. range	Precision inter-assay R.S.D.
CRP	30 μl	0.5 mg l^{-1} –80 mg l^{-1}	6%	30 μl	1–100 μg l^{-1}	8–12%
Ferritin	30 μl	0–300 μg l^{-1}	5%	30 μl	0.5–50 μg l^{-1}	7.3%
IgM	50 μl	0.3–3 g l^{-1}*	6%	30 μl	11.5–400 μg l^{-1}	3.7–11.5%
IgA	50 μl	0.2–4 g l^{-1}*	6%	30 μl	4–130 μg l^{-1}	4.5–7.2%
IgE	30 μl	1 U ml^{-1}	10%	30 μl	0.2–40 IU ml^{-1}	8.5–12.8%
S-100				30 μl	1–50 μg l^{-1}	7%

*Nephelometric assays — Technicon Method No. SE4-0038FD6 (1976).

in pyogenic infections (50–480 mg/l) than in viral meningitis (all values except one below 50 mg/l and 11 values below the upper reference value of 10 mg/l). The assay of CRP in CSF is less discriminatory, presumably because of the uptake of the protein by bacteria in CNS, CRP being an opsonin.

The assay of CSF ferritin is clinically more relevant as a good marker of haemorrhage within the CNS. Its concentration in normal CSF is 3 μg/l with an upper normal limit of 7 μg/l. In vascular disease of the CNS, high levels of ferritin were observed in haematoma (up to 60 μg/l), subarachnoidal bleeding (up to 350 μgl) and hypertensive encephalopathy (up to 17 μg/l), but not in ischemic stroke or in transient ischemic attacks. Very high CSF ferritin levels (up to 2480 μg/l) were also observed in all cases of herpetic encephalitis but not in other viral infections of the CNS. The concentration in CSF was sometimes higher than in serum, clearly indicating a local biosynthesis of this protein.

Proteins of immune reactions within CNS: immunoglobulins G, A, M and E

Local production of immunoglobulins inside the CNS is always due to a pathological process. The local biosynthesis of IgG in multiple sclerosis and neurosyphilis has been known for 40 years but the biosynthesis of IgA and especially of IgM has only recently been studied. IgM is present in normal CSF at very low levels (mean concentration 130 μg/l, serum 1.1 g/l). The concentration of IgA (1.82 mg/l total) depends on its molecular form; the monomeric form obviously diffuses more rapidly than the dimer through the blood–brain barrier.

A high CSF immunoglobulin level must be identified as arising either from local production within the CNS or by accelerated diffusion through an impaired blood–brain barrier. Many workers have therefore used an 'index' for each protein which relates its normal concentration in serum to its normal concentration in CSF. Albumin is generally used as the reference protein since it is accepted that albumin is never produced in CSF and can only be present by diffusion through the blood–brain barrier. Such an index for immunoglobulin (Ig), may then be expressed as:

$$\text{Ig index} = \frac{\text{CSF Ig}}{\text{Serum Ig}} : \frac{\text{CSF albumin}}{\text{Serum albumin}}.$$

Table 2
Normal values for the Ig index

Immunoglobulin	Ig Index = $\frac{\text{CSF Ig}}{\text{Serum Ig}} : \frac{\text{CSF albumin}}{\text{Serum albumin}}$	
	Mean	Upper reference value
IgG	0.48	0.69
IgA	0.23	0.42
IgM	0.026	0.079
IgE	0.29	0.53

Normal values for the index have been determined (Table 2). If the barrier is damaged, the Ig and albumin will increase independently but the index will remain constant. If the index increases, immunoglobulins are being synthesized within the CNS.

The establishment of reference values requires the reliable assay of relatively large numbers of samples with high sensitivity. The automated PACIA system was obviously suited to this purpose since analysis rates of 60/hour with little or no sample pretreatment are a feature of the system. The present work, for the first time, establishes normal values for the IgM index and also for the monomeric and dimeric forms of IgA (separated by sucrose gradient ultra-centrifugation). In contrast, IgE was virtually absent from CSF.

With this information, immunological profiles characteristic of various neurological disorders may be defined. The means of the IgG, IgA and IgM indices calculated as log values were established for all patients with multiple sclerosis (MS), acute and chronic infections of the CNS, Guillain–Barré syndrome and lymphocytic meningo-radiculitis, and expressed as multiples of the means determined in non-neurological patients (Fig. 1). The IgM index is significantly increased in all patients with lymphocytic meningo-radiculitis and active neurosyphilis. Its determination is clinically relevant in these two

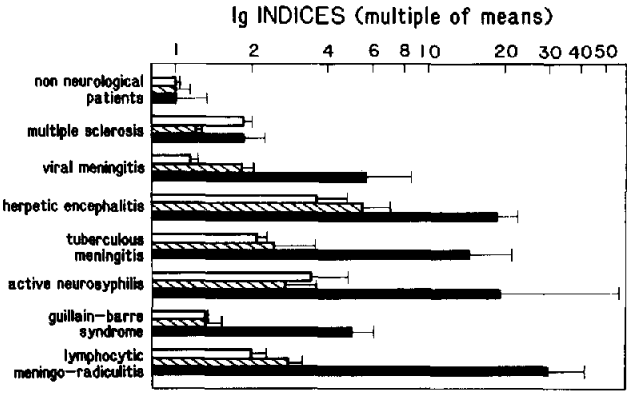

Figure 1
Means of IgG (white columns), IgA (hatched columns) and IgM (black columns) indices, calculated as log values, from patients with various neurological diseases, expressed as multiples of the means calculated in a group of 50 non-neurological patients. When several samples from the same patient were available, only the highest values of the Ig indices were considered. Bars indicate standard errors of the mean.

diseases because a high index signals an active process and its decrease parallels clinical recovery and successful treatment. Neurosyphilis, without local production of IgM, is a burnt out infection which does not require antibiotics. High IgM indices are also observed in herpetic encephalitis and tuberculous meningitis, but with herpetic encephalitis all the three Ig indices are elevated. In contrast, only the IgM index is frequently increased in viral meningitis whereas the IgG index remains nearly always in the normal range, in agreement with the concept that following infection IgM is the first antibody produced. The determination of the IgM index may thus be the first step towards a search for a specific antibody activity and the etiological agent. In Guillain–Barré syndrome, a disease of the peripheral nerves, the relatively elevated IgM was somewhat surprising and suggested a mild but frequent immune reaction within the CNS, with a consequent local production of IgM. Even more surprising, although only 26% of MS patients had an IgM index exceeding the upper reference value, the MS group as a whole had an IgM index comparable to the increase of the IgG index.

The increase of IgA index is usually associated with the increase of the IgG index, but with two exceptions: the IgA index is higher in viral meningitis and lower in MS. Enteroviruses are the most frequent etiological agents in viral meningitis and the local synthesis of IgA may arise from the recruitment of primed B cells from the peripheral blood. There is no immediate explanation for the relatively lower IgA index in MS.

The brain-specific protein in CSF as CNS damage marker

In the nervous system, nervous cell types (neurons, oligodendrocytes and astrocytes) have particular functions. These specific functions are, to a large extent, mediated by soluble or membrane-bound proteins which are unique to the nervous system, as shown by immunochemical methods. One could expect that these brain-specific proteins are released into the CSF and/or serum in CNS lesions and could be used as specific markers of brain damage. By assaying for these proteins, brain injury could be assessed and in some cases, the cell type of the brain structure affected by the pathological process could be identified.

In the present work, two CSF proteins specific to the astrocytes have been assayed: the S-100 protein and the glial fibrillary acidic protein (GFA). As the results were similar for the two proteins, only results for the S-100 protein will be summarized here.

S-100 is an acidic protein (Mr *ca* 21 000) found in the nervous system of vertebrates. More precisely, it is located in the cytoplasm of astrocytes and is called S-100 because of its solubility in 100% saturated ammonium sulphate at neutral pH. S-100 changes structurally in the presence of calcium ions. Its function, however, remains unknown. Using purified S-100, a specific antiserum has been produced in rabbits, and protein levels in CSF have been determined by particle counting immunoassay. The standard curve was typically sigmoid from 2.5 to 250 μg/l. For improved accuracy, samples containing more than 50 μg/l were serially diluted.

With a detection limit of 2.5 μ/l, CSF samples from 29 non-neurological patients and from patients with sciatica, cervicarthrosis myelopathy, lumbar stenosis, degenerative disorders of the nervous system and seizure were devoid of S-100. In contrast, high levels of S-100 and a high proportion of positive samples were found in four types of neurological disorders: tumoral compression of the spinal cord, ischaemic disorders, subarachnoidal bleeding and haematoma, and viral or suspected viral infections. All these disorders have in common acute damage of the nervous system. In most cases of multiple sclerosis, we failed to detect S-100. This was not surprising as recent

immunohistochemical studies failed to detect S-100 in oligodendrocytes and myelin sheaths which are selectively destroyed in multiple sclerosis.

The prognostic value of S-100 levels is clearly illustrated by a study of nine patients with herpetic encephalitis; their clinical outcome was closely related either to increasing levels or to the absence of CSF S-100. In three patients with transverse myelitis, poor prognosis was also associated with increased or persistently high levels of S-100. In an ongoing study of comatose patients after cardiac arrest or severe head trauma, it was found that a marked increase of S-100 levels was invariably associated with a fatal outcome. In contrast, progressively decreasing levels were observed in cases of partial or complete recovery. The results thus support the assumption that S-100 in CSF is a reliable index of the CNS injury and that its level has a prognostic value.

Detection of local production of anti-herpes antibodies

Herpes simplex virus type 1 is the most frequently recognized agent of sporadic fatal encephalitis in temperate climates and, more rarely, the causative agent of acute myelitis and aseptic meningitis. In infections of the CNS, detecting the local production of specific antibodies can be of great help in the identification of the causative agent, even retrospectively. In contrast, CSF antibodies derived from serum through an impaired blood–brain barrier are diagnostically irrelevant. However, discrimination between local or systemic production of specific antibodies is difficult, using complement fixation tests which express results in dilution titres. Again, the sensitivity and precision of the particle counting technique made it relatively simple to set up an assay for herpes antibodies in both serum and CSF.

The technique used for the detection of antibodies was an agglutination inhibition technique, quantitated by the addition of a known quantity of viral antigen. The rabbit IgG antibodies to the herpes simplex virus were purified, converted to the $F(ab')_2$ fragments and bound to the latex particles as previously described. Sufficient viral antigen was then added to agglutinate 70% of the latex [i.e. about one volume of latex to one volume of a commercially available solution of herpes antigen (Microbiological Associates) diluted 1:500]. Antibodies in the test samples will compete with the antibody fragments bound to the latex for the limited quantity of viral antigens. Viral antigen bound to free antibody obviously could not agglutinate the latex. While the concentration of IgG and IgM in serum is relatively constant, the concentration in CSF is highly variable and hence a more rigorous definition of antibody concentration than "X" ng/ml is necessary. The concentration of anti-herpes antibody was thus related to the concentration of IgG, using as a reference value a concentration of 10 μg/ml IgG.

In 20 pairs of serum/CSF samples from non-neurological patients, the mean difference of inhibition between CSF and serum after appropriate dilution was 2.7% with an upper reference limit of 9%. Differences between CSF and serum inhibition exceeding 9% were considered as suggestive of a local production of anti-herpes antibodies. This was, of course, observed in patients with herpetic encephalitis about 10 days after onset [10] but only in a few patients with other neurological disorders (three out of 37 patients with multiple sclerosis, one out of eight with sciatica and two out of 20 with aseptic meningitis).

In summary, the high precision and reliability of particle counting immunoassay allowed us to study CSF proteins and antibodies effectively. Work is continuing on the assay of other brain-specific proteins and on the quantitative detection of bacterial antigens in CSF.

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