Detection of IgG Fraction Bands in Cerebrospinal Fluid by Highly Sensitive and Specific Immunoperoxidase Staining Methods*

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SUMMARY Various highly sensitive and specific immunoperoxidase staining methods were introduced for the identification in detail of IgG bands in iso-electric focusing of cerebrospinal fluid (CSF). Avidinbiotin-peroxidase complex stain (ABCS) proved to be the most sensitive. It was about 2, 4, 32 and 4,800-6,400 times more sensitive than peroxidase anti-peroxidase stain (PAPS), direct immunoperoxidase stain (DIPS), silver stain (SS) and Coomassie blue stain (CBS) respectively. The mechanism of ABCS's high sensitivity and specificity is briefly described. The application and prospect of ABCS in cerebrospinal fluid research is briefly mentioned.

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The detection of IgG in cerebrospinal fluid (CSF) is an important diagnostic aid for some inflammatory neurological disorders.[1-9] It has been demonstrated in recent research that some IgG fraction bands in CSF of patients with multiple sclerosis and subacute sclerosing panencephalitis contain antivirus antibody, especially antimeasles virus antibody.[10] Therefore, such detection is not only helpful for making a diagnosis, but also for aetiological and epidemiological research.

So far, because the detecting method is not sensitive, it has usually been necessary to concentrate CSF 50 times[11] or to increase the amount (200 μg of protein per sample).[4] Silver stain (SS) is 150-200 times more sensitive than coomassie blue stain (CBS).

One of the most important specific methods for the detection, localisation and quantitation of antigens and antibodies is immuno-enzymatic technique using peroxidase reagents.[13-15] There are two commonly used methods: the indirect conjugate method and the peroxidase anti-peroxidase stain (PAPS) method.

Recently, the non-covalent but exceptionally strong interaction of avidin and biotin (dissociation constant $K_d=10^{-15}M$) has been used for a variety of purposes including immunohistochemistry,[16-27] ELISA[28] and hybridoma screening. Hsu *et al*[29] compared the sensitivities of immunohistochemical methods using different procedures, including bridge-avidin and avidin conjugate methods. One of the methods, avidin biotin-peroxidase complex stain (ABCS), was found to be more sensitive than the direct peroxidase and PAPS methods.

To detect the IgG fraction bands in CSF, the indirect peroxidase and PAPS methods were applied to iso-electrofocused polyacrylamide gel (PAG) by Mattson *et al.*[30] Although ABCS is more sensitive than DIPS and PAPS, so far it has not been used for the detection of IgG fraction bands.

We have compared various immunoperoxidase stains, trying to find a method which increases both sensitivity and specificity for the detection of IgG fraction bands in CSF.

MATERIALS AND METHODS

Repurification, identification and quantitation of rabbit anti-human IgG (heavy and light chains)

Commercially available IgG fraction of rabbit anti-human IgG (heavy and light chains) (Cappel Laboratories, Cochranville, PA,

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Abbreviations:

ABCS:	Avidin biotin-peroxidase complex stain
BSA:	Bovine serum albumin (globulin free)
CBS:	Coomassie blue stain
CSF:	Cerebrospinal fluid
DAB:	Diaminobenzidine
DIPS:	Direct immuno-peroxidase stain
IEF:	Isoelectric focusing
IgG:	Immunoglobulin G
PAG:	Polyacrylamide gel
PAPS:	Peroxidase anti-peroxidase stain
PBS:	Phosphate buffered solution
SS:	Silver stain
TNS:	Transblotted nitrocellulose sheet

U.S.A.) IgG bound to protein A-Sepharose CL-4B (*Staphylococcus aureus* protein A covalently bound to Sepharose CL-4B, Pharmacia, Sweden) was eluted with 3M potassium thiocyanate. The eluate was exhaustively dialysed against phosphate buffered solution (PBS), pH 8.0, and clarified by centrifugation (3,000 rpm for 20 minutes). Aliquots were kept in a -20°C freezer. The repurified rabbit anti-human IgG was identified by Ouchterlony test (against human whole serum and purified human IgG) and quantitated by spectrophotometric absorption (OD_{280}).

Preparation of patient's CSF absorbed by repurified rabbit anti-human IgG

CSF (100μl) from a patient with multiple sclerosis (IgG concentration was 10.8 mg%) was mixed with 200 μl of the repurified rabbit anti-human IgG (IgG concentration was 16.1 mg%) and incubated at 4°C for 24 hours. The incubated mixture was centrifuged for one minute in an Eppendorf centrifuge. The supernatant was concentrated to a final volume of 100 μl using a microdialysis system (Bethesda Research Laboratories, Inc., Rockville, MD, U.S.A.). The concentrated supernatant was used as antibody-absorbed CSF.

Isoelectric focusing (IEF) and Western blotting

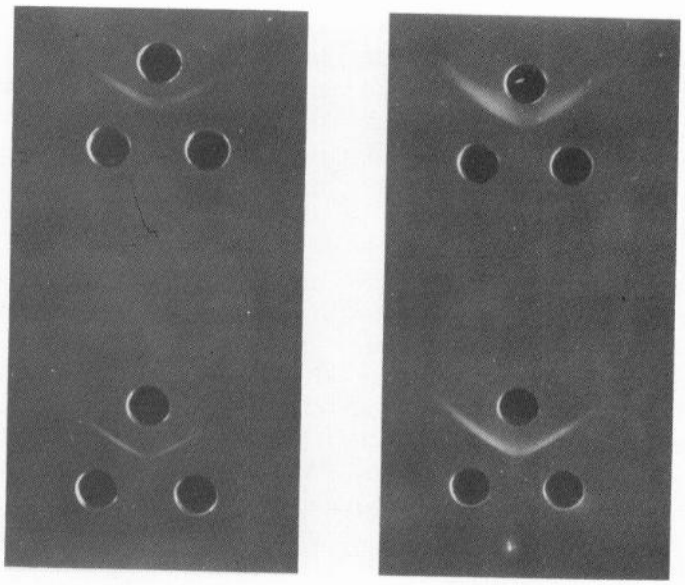
IEF was performed as previously described.[1] In brief, samples (20μl) were applied around a pH 5.7 portion of ampholine polyacrylamide gel (PAG) pH 3.5-9.5 (LKB, Stockholm, Sweden). Focusing was performed for three hours at 4°C at 20 watts. Immediately after IEF, proteins were transblotted from PAG to nitrocellulose sheets (Bio-Rad Laboratories) with a transblotting apparatus (Bio-Rad), in 25 mM tris buffer, pH 8.3, containing 92 mM glycine and 30% methyl alcohol (v/v). The process was run for three hours at 48 volts, 0.9 amperes.

Comparing three different methods of identification of iso-electrofocused IgG by direct immunoperoxidase stain (DIPS)

A CSF sample was isoelectrofocused in PAG in triplicate. One strip was fixed in 11.5% trichloroacetic acid/3.5% sulphosalicylic acid/30% methyl alcohol for one hour, rinsed overnight in tris-buffer (0.01 M Tris/0.85% sodium chloride, pH 7.8) and soaked in 3% gammaglobulin-free bovine serum albumin (BSA)/Tris buffer for 20 minutes. The proteins in the other two strips were transblotted to nitrocellulose sheet (TNS), one being treated just the same as PAG, the other being soaked directly in 3% BSA/Tris buffer for 20 minutes. The three samples were then incubated in a 1:200 dilution of rabbit anti-human IgG peroxidase (γ-chains, Dako, Copenhagen, Denmark; final rabbit IgG concentration was 6.5 μg/ml) for one hour at room temperature, and rinsed with 0.01 M Tris bugfer, pH 7.8, for 15 minutes (three changes, five minutes each). They were developed in a substrate containing 10 mg of 3,3'-diaminobenzidine 3,3', 4,4'-tetrahydrochloride, and 10 μl of 30% hydrogen peroxide in 200 ml of 0.01 M tris-buffer, pH 7.8, for five minutes or until the bands were visible.

Peroxidase anti-peroxidase stain (PAPS)

Transblotted nitrocellulose sheets (TNS) were incubated in succession in three solutions, each for one hour; the solutions consisted of a 1:20 dilution of the repurified rabbit anti-human IgG (final rabbit IgG concentration was 8.0 μg/ml) and a 1:1,000 dilution of swine immunoglobulins to rabbit immunoglobulins (Dako; final concentration of antibody protein was 15.7 μg/ml) in 1% normal swine serum, and a 1:200 solution complex of horseradish peroxidase and rabbit antihorseradish peroxidase (Dako, final concentration of rabbit anti-human IgG was 6.5 μg/ml); the two incubations were separated by three rinses of 0.01 M Tris buffer, pH 7.8, (five minutes each). After



A. 24 hours B. 72 hours

Fig. 1 Ouchterlony test of repurified rabbit anti-human IgG.

R. Repurified rabbit anti-human IgG — O R

S. Human (whole) serum — O S

I. Purified human IgG — O I

the final incubation, TNS was rinsed in 0.01 M Tris buffer, pH 7.8, three times and then developed as in DIPS.

Avidin biotin-peroxidase complex stain (ABCS)

TNS was incubated in succession in three solutions, each for one hour. They consisted of a 1:20 dilution of monoclonal mouse anti-human IgG serum (Bethesda Research Laboratories, Inc., Gaithersberg, MA, U.S.A.; final IgG concentration was 1 μg/ml), 1:200 dilution of biotinylated horse anti-mouse IgG serum (Vector Laboratories, Inc., Burlingame, CA, U.S.A.; final IgG concentration was 7.5 μg/ml) in 1% normal horse serum, and 1:200 dilution of ABC (Vector Laboratories, Inc.); the incubations were separated by three rinses of 0.01 M Tris buffer, pH 7.8, (five minutes each). After the final incubation, the TNS was rinsed three times in 0.01 M Tries buffer, pH 7.8, and then developed as in DIPS.

Negative control tests were performed by replacing the patient's CSF primary and secondary antibody with the patient's CSF absorbed by a repurified rabbit antihuman IgG, 1:2 normal SJL mouse and 1:2 normal horse serum respectively.

To detect IgG kappa and lambda light chain and gamma heavy chain fraction bands in CSF, the triplicated TNSs were incubated in succession in two solutions, each for one hour. They consisted of a 1:100 dilution of biotin conjugated goat anti-human kappa, lambda light chain or gamma heavy chain antibody (Tago, Inc., Burlingame; final IgG concentrations were 6.5, 7 and 6.2 μg/ml respectively) and a 1:200 dilution of ABC.

RESULTS AND DISCUSSION

Repurified rabbit anti-human IgG

The concentration of the repurified rabbit anti-human IgG was 16.1 mg/dl and its specificity was confirmed by Ouchterlony test (Fig. 1).

Three different DIPSs

TNS unpretreated with acid (Fig. 2C) was twice as sensitive as the acid-pretreated one (Fig. 2B). This might be due to the fact that acid pretreatment partly destroyed the antigenicity of IgG in CSF. Although both were pretreated with acid, the TNS (Fig. 2B) was twice as sensitive as PAG (Fig. 2A). This

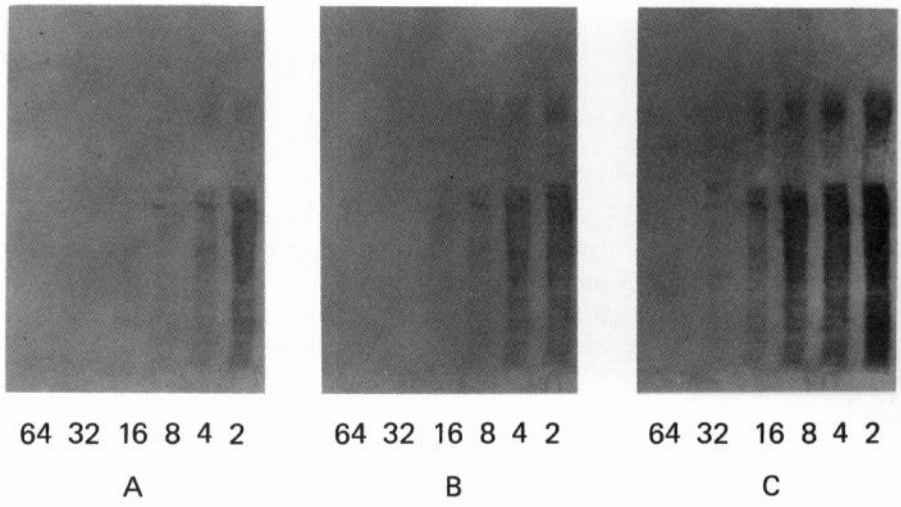


Fig. 2 Comparison of three different direct immunoperoxidase staining methods.
A. Proteins were isoelectrofocused on PAG, which was pretreated with acid and followed by DIPS.
B. Proteins were isoelectrofocused on PAG, then transblotted from PAG to a nitrocellulose sheet. The TNS was pretreated with acid and followed by DIPS.
C. Proteins were isoelectrofocused on PAG, then transblotted from the PAG to nitrocellulose sheet. The TNS was directly stained with DIPS without undergoing pretreatment with acid.
"C" is two and four times more sensitive than "B" and "A" respectively.

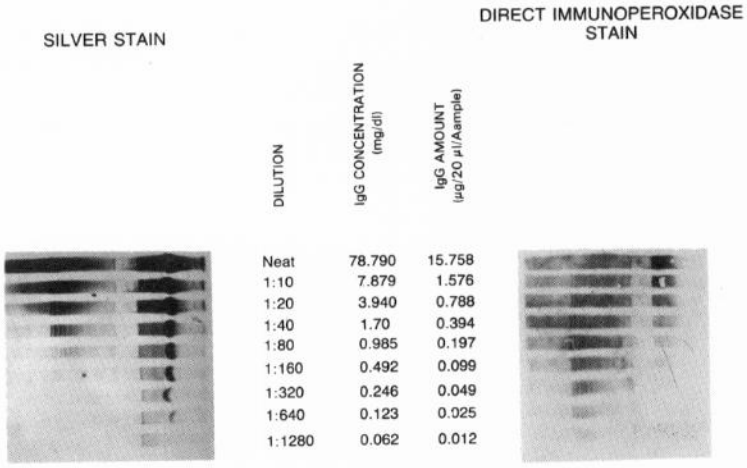


Fig. 3 Comparison of silver stain with direct immunoperoxidase stain. The neat CSF from a patient with neurosyphilis contained 78.79 mg/dl of IgG which was serially diluted with PBS. IgG was separated by isoelectric focusing on PAG in duplicate. Of the duplicates, one was stained by silver stain, another was transblotted to nitrocellulose sheet and followed by direct immunoperoxidase stain. The IgG fraction bands could be detected with SS at an IgG concentration of 0.985 mg/dl (197 ng/20 μl) and with DIPS at an IgG concentration of 0.123 mg/dl (25 ng/20 μl).

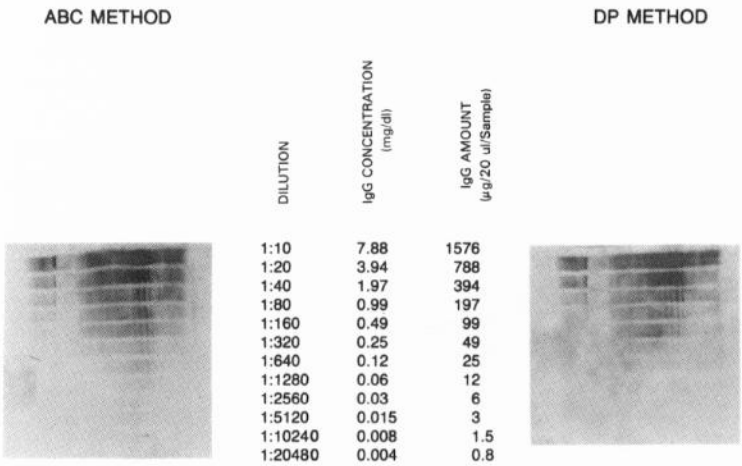


Fig. 4 Comparison of ABCS and DIPS methods. The same CSF as in Figure 3 was serially diluted with PBS. IgG was separated by IEF, transblotted to nitrocellulose sheets, stained with ABCS and DIPS. The IgG fraction bands could be detected with the DIPS method at an IgG concentration of 0.25 mg/dl (50 ng/20 μl); with the ABCS method, at an IgG concentration of 0.03 mg/dl (6 ng/20 μl).

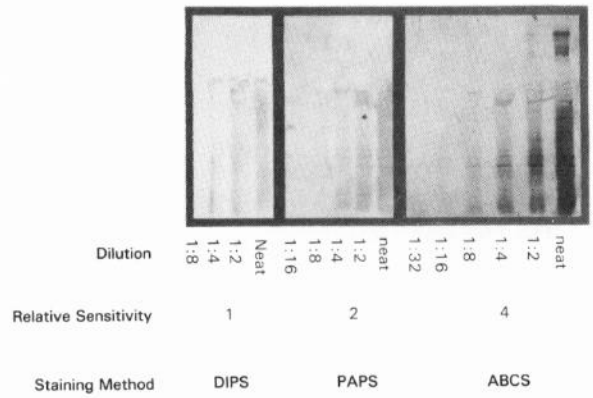


Fig. 5 Comparison of sensitivities of three different immunoperoxidase staining methods, DIPS, PAPS and ABCS. The CSF from a patient with multiple sclerosis was serially diluted with PBS. IgG was separated by IEF, transblotted to nitrocellulose sheets, stained with DIPS, PAPS and ABCS. The relative sensitivity of DIPS, PAPS and ABCS in the detection of IgG fraction bands was 1, 2 and 4 respectively.

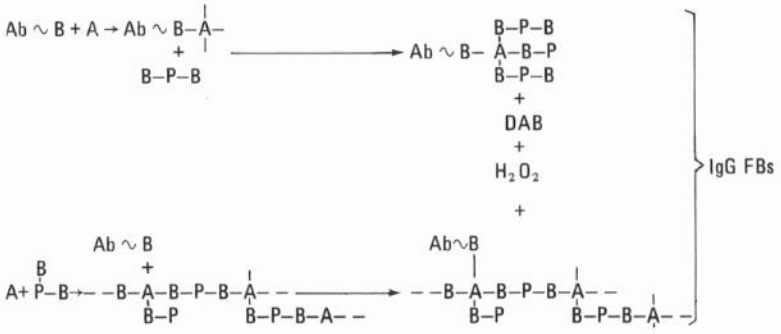


Fig. 6 Mechanism of ABCS method

A:	Avidin	DAB:	Diaminobenzidine
Ab:	Antibody	IgG FBs:	IgG fraction bands
B:	Biotin	P:	Peroxidase

might be because the IgG was aggregated on the sheet surface, i.e., relatively concentrated, and that the brown bands were easily visualised on the white TNS. The DIPS mentioned later on is the DIPS on the TNS unpretreated with acid.

Sensitivity of various immunoperoxidase staining methods

DIPS (25 ng detected) is eight times more sensitive than SS (197 ng, Fig. 3). ABCS (6 ng detected) is four times more sensitive than DIPS (25 ng, Fig. 4), and twice as sensitive as PAPS (Fig. 5).

One IgG molecule binds to one peroxidase molecule in DIPS; two IgG molecules bind to three peroxidase molecules in PAPS. With the ordinary avidin-biotin staining method, IgG binds to biotin first, then the biotinylated IgG is successively incubated with avidin and biotin-peroxidase complex; one IgG molecule can bind to more peroxidase molecules, because one avidin has four binding sites for biotin and one peroxidase molecule has multiple binding sites for biotin. With ABCS, avidin-biotin peroxidase complex is preformed by avidin and biotin-peroxidase complex in the proportion of 1 to 4, there are numerous peroxidase molecules in one ABC; the biotinylated IgG is incubated with ABC, then one IgG molecule binds to many more molecules of peroxidase. That is the mechanism which explains why ABCS is much more sensitive than any other known immunoperoxidase staining method (Fig. 6).

Specificity of ABCS

The results obtained with our negative controls proves that ABCS is highly specific for the stain of human IgG.

Lowering the background stain

Pretreatment of the transblotted nitrocellulose sheet with gamma-globulin-free bovine serum albumin reduced the background remarka-

	Patient's CSF	Mouse anti-human IgG monoclonal antibody	Horse anti-mouse IgG antibody conjugated biotin	Result
A	+	+	+	+
B	+	Normal SJL mouse serum	+	—
C	+	+	Normal horse serum	—
D	absorbed by repurified rabbit anti-human IgG	+	+	—

A. Normal complete ABCS
B. Normal mouse serum substitutes monoclonal mouse anti-human IgG
C. Normal horse serum substitutes biotinylated horse anti-mouse IgG
D. Repurified rabbit anti-human IgG absorbed patient's CSF substitutes patient's CSF

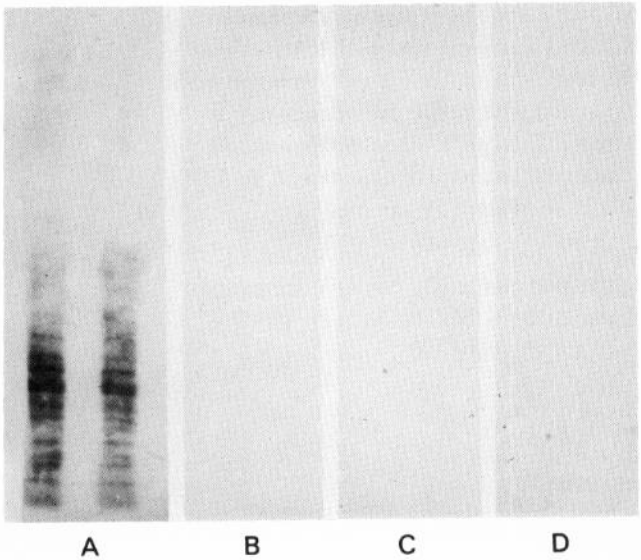


Fig. 7 Specificity of ABCS.

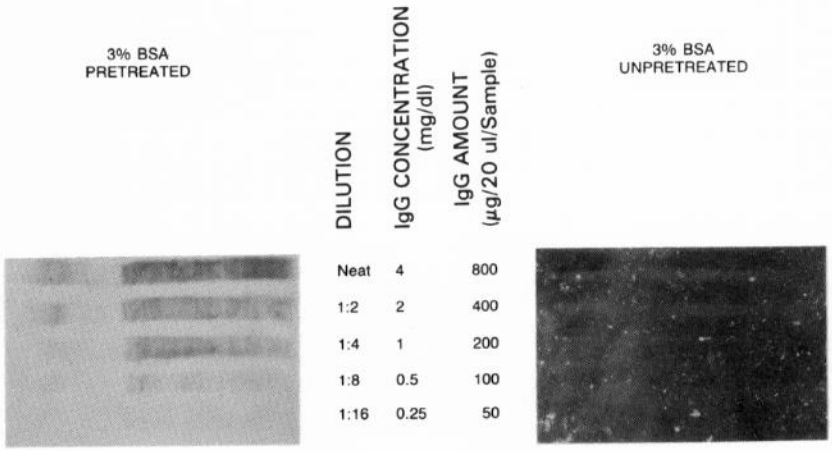


Fig. 8 Effect of pretreatment with 3% BSA (globulin free)

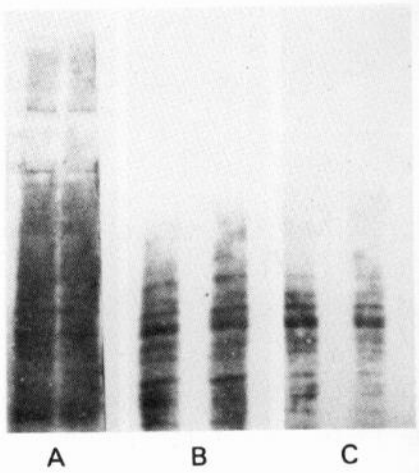


Fig. 9 Comparison of the background stain of ABCS using antibodies from different sources as first antibody.
A. Commercially available rabbit anti-human IgG as first antibody.
B. Repurified rabbit anti-human IgG as first antibody.
C. Mouse anti-human IgG monoclonal antibody as first antibody.

bly (compare Fig. 8A with Fig. 8B). The monoclonal mouse anti-human IgG antibody was chosen to be the primary antibody because the background was the least pronounced, and the number of IgG fraction bands was similar; the monoclonal mouse anti-human IgG antibody was compared with commercially available rabbit anti-human IgG and repurified rabbit anti-human IgG. In addition, the use of a highly diluted second antibody and ABC is also a very important measure for lowering the non-specific background stain and increasing sensitivity.

The application and prospect

When ABCS is used, IgG kappa and lambda light chain fraction bands can be detected, even if the CSF sample contains as little as 35-70 ng of IgG (Fig. 10), i.e., IgG kappa and lambda light chain bands can be detected without concentration of CSF.

It has been recently recognised that some neurological diseases are related to chronic viral infection. These highly specific and sensitive immunoperoxidase staining

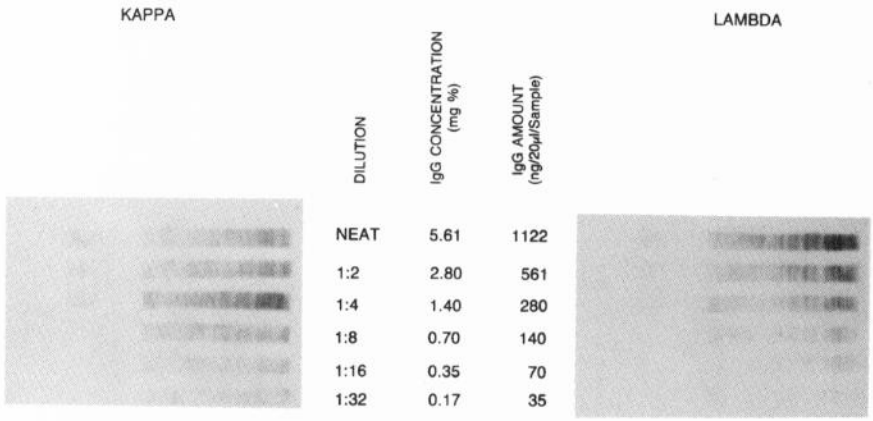


Fig. 10 IgG kappa and lambda light chain fraction bands detected by the ABCS method in CSF from a patient with multiple sclerosis.

Analysis of CSF from a patient with multiple sclerosis. The neat specimen contained 5.61 mg/dl of IgG which was serially diluted with PBS. The proteins were separated by IEF, followed by transblotting and ABCS. TNS was incubated with a 1:100 goat anti-human kappa or lambda antibody conjugated with biotin for one hour, rinsed three times in 0.01 M Tris buffer, pH 7.8, incubated with 1:200 preformed ABC for one hour, rinsed three times in the aforementioned Tris buffer, then developed. The IgG kappa and lambda light chain fraction bands can be clearly detected at an IgG concentration of 0.17 mg/dl, i.e., 35 ng of IgG/20 μl of the CSF sample.

methods can be used for identifying the antigens and antibodies of relevant viruses in CSF.

Recommended methods for the identification of IgG in isoelectrofocused CSF

1. The CSF IgG concentration should be approximately 0.5 mg/dl. Isoelectric focus the CSF sample (20 μl of each sample) in PAG. Transblot the isoelectrofocused proteins from the PAG to a nitrocellulose sheet. Soak TNS in 3% gammaglobulin-free bovine serum albumin for 30 minutes.

2. Incubate with primary antibody (IgG concentration about 1-8 μg/ml) for one hour.

3. Wash three times (five minutes each time) with 0.01 M Tris buffer, pH 7.8. Incubate with biotinylated secondary antibody (IgG concentration about 5-8 μg/ml) for one hour.

4. Wash three times (five minutes each time) with 0.01 M Tris buffer, pH 7.8. Incubate with preformed ABC (1:200) for one hour.

5. Wash three times (five minutes each time) with 0.01 M Tris buffer, pH 7.8. Develop in substrate containing diaminobenzidine and hydrogen peroxide for five minutes. Rinse in 0.01 M Tris buffer, pH 7.8; then air dry.

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