Purification and characterization of a protein antigen (p94) from human brain and its identification in other species

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A protein antigen was chromatographically purified from human brain by its immunoaffinity to 44E3 monoclonal IgG and its chemical nature was investigated. The yield of antigen was estimated at 71%, and a 3160-fold purification was achieved relative to the homogenate. The antigen preparation from brain showed a very high degree of purity when analysed by SDS/polyacrylamide-gel electrophoresis and was composed of a single polypeptide of M_r 94000. Amino-sugar and neutral-sugar analyses indicated that the protein was not glycosylated. The amino acid composition of the purified protein from brain was compared with that of the analogous protein purified from an acute-lymphoblastic-leukaemic cell line, HOON. The compositions were very similar, suggesting that the two proteins were closely related. Both purified proteins were equivalent in their ability to inhibit the reactivity of monoclonal antibodies 44E3 and 44H4 with leukaemic cells. These two antibodies appear to recognize spatially related, if not identical, epitopes on the same molecule. The antibodies were shown to cross-react with a polypeptide of M_r 94000 in homogenates of human, bovine and guinea-pig brain white matter. Indirect immunoperoxidase staining of human grey- and white-matter acetone-fixed tissue sections incubated with either antibody indicated that the antigen was present on neuronal and glial cells; the staining was seen as clusters in the cytoplasm, starting at the plasma membrane, but leaving the nucleus unstained. The concentration of the protein in human brain was shown to be similar throughout postnatal development and aging.

INTRODUCTION

In a previous investigation (Quackenbush *et al.*, 1985), we identified three distinct proteins of human brain by using monoclonal antibodies (MAbs) prepared against the non-T, non-B acute-lymphocytic-leukaemia cell line HOON (Quackenbush & Letarte, 1985). A glycoprotein of M_r 80000, normally restricted to white matter, was identified with MAb 44D10 (Quackenbush *et al.*, 1985). This glycoprotein has been shown to bear several antigenic epitopes and to correspond to a previously identified brain protein (Dalchau *et al.*, 1980; McKenzie *et al.*, 1982; Letarte *et al.*, 1985; Letarte, 1986). The 44D10 antigen was increased in concentration in white matter and was present in low levels in grey matter of brains of individuals over the age of 55 years (Cruz *et al.*, 1985). It was also detected in grey matter and at elevated levels in white matter of brains from victims of multiple sclerosis (Cruz *et al.*, 1986).

A glycoprotein complex of M_r > 120000 (composed of two major subunits of M_r 80000 and 41000), present in both grey and white matter, was isolated chromatographically from white matter by affinity chromatography on MAb 44D7-IgG–Sephrose (Quackenbush *et al.*, 1985).

A protein of M_r 94000 was reactive with two MAbs (44H4 and 44E3) by Western-blot analysis of a white-

matter homogenate. It migrated with the homologous protein of HOON cells (Quackenbush *et al.*, 1985). This antigen was also detected in homogenates of grey matter.

All three of these proteins were absent from the multilamellar compact myelin of white matter, but were associated with a membrane fraction of higher density. All three antigens were found in several other tissues (Letarte *et al.*, 1985, 1986; Quackenbush & Letarte, 1985; Quackenbush *et al.*, 1986) and cannot be considered as brain-lymphocyte shared antigens.

In the present study we report the purification and characterization from human brain of the polypeptide antigen reactive with MAbs 44E3 and 44H4 and designated 'p94'. We also demonstrate the presence of this protein in brains of other species.

MATERIALS AND METHODS

Preparation of MAbs

MAbs 44E3 and 44H4 were derived by immunization of Balb/c mice with HOON leukaemic cells. Both antibodies are of IgG₁ subclass and react with the same protein on leukaemic cells (Quackenbush & Letarte, 1985). MAb 44E3 was purified and the IgG coupled to CNBr-activated Sepharose 4B as described previously (Quackenbush & Letarte, 1985).

Abbreviations used: RAM-Fc, F(ab')₂ pepsin fragment of rabbit IgG produced against the purified papain Fc fragment of mouse IgG and purified by affinity to mouse IgG; MAb, monoclonal antibody; phosphate-buffered saline, 0.01 M-phosphate buffer, pH 7.4, containing 0.15 M-NaCl, 1 mM-CaCl₂, 0.5 mM-MgCl₂ and 1 mM-KCl.

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Purification of antigen reactive with MAb 44E3

White matter (30 g) dissected from adult human brain was homogenized in 10% (w/v) sucrose. Fraction P3, a 0.88 M-sucrose pellet containing membranes depleted of multilamellar compact myelin (Quackenbush *et al.*, 1985), was solubilized in a final concentration of 1.5% Triton X-100 in 0.05 M-Tris/HCl, pH 7.8, containing 0.04% NaN₃, 0.001 M-phenylmethanesulphonyl fluoride and 0.01 M-iodoacetamide for 2 h at 4 °C. The soluble fraction was recovered by centrifugation at 100 000 g for 60 min. It was applied sequentially to a column of non-immune mouse IgG-Sepharose (5 ml at 2 mg of IgG/ml), to remove proteins binding non-specifically, and to a column of 44E3-IgG-Sepharose (5 ml; 2 mg of IgG/ml). The columns were washed with 0.05 M-Tris/HCl, pH 7.8, containing 0.15 M-NaCl, 0.04% NaN₃ and 0.6% cholate. The antigen specifically bound to the 44E3-IgG-Sepharose column was eluted with 0.05 M-diethylamine, pH 11.2, containing 0.6% cholate and neutralized immediately with 0.5 M-phosphate buffer, pH 7.6. The antigen was concentrated 5–6-fold by Amicon filtration, and 10% (w/v) SDS was added to give a final concentration of 1% SDS. The preparation was heated for 10 min at 37 °C, dialysed successively against 0.1% SDS for 16 h, 0.05% SDS for 3 h and 0.02% SDS for 3 h. Samples were freeze-dried and stored at –20 °C.

The 44E3 antigen from HOON cells was purified as previously described (Quackenbush & Letarte, 1985) and subjected to dialysis as described above. Protein was estimated by the method of Peterson (1979).

Cellular radioimmunoassay

The presence of antigen reactive with MAb 44H4 (or 44E3) in different brain fractions was assayed by inhibition of the cellular radioimmunoassay described previously (Quackenbush & Letarte, 1985; Quackenbush *et al.*, 1985). Briefly, serial dilutions of the brain fractions in phosphate-buffered saline containing 0.5% bovine serum albumin (100 µl) were incubated for 16 h at 4 °C with 100 µl of MAb 44H4 culture supernatant (1:80). After centrifugation for 6 min at 10 000 g, 25 µl aliquots were transferred to microtitre plates and incubated for 2 h at 4 °C with 5 × 10⁵ glutaraldehyde-fixed HOON cells (25 µl). MAb 44H4 bound to the target cells was detected by incubating for 2 h at 4 °C with ¹²⁵I-RAM-Fc (25 ng/well, 100 000 c.p.m.). Cells were washed four times, the wells cut out and radioactivity determined. The concentration of antigen was determined from the inhibition curves obtained for each sample and expressed as units per mg of protein. All assays were done in triplicate and the mean plotted; the S.D. was less than 10%. One unit of activity was defined as the amount of antigen giving 50% inhibition of binding to target cells. Yield was estimated by comparison of the units of antigen recovered in a given fraction with that of the total units in the original brain homogenate.

For the antibody competition assay, 400 µg of 44E3-IgG was labelled with ¹²⁵I by using a chloramine-T/protein molar ratio of 10:1 and a 50 000-fold excess of dimethyl sulphoxide. Glutaraldehyde-fixed HOON cells (5 × 10⁵ per well) were incubated for 16 h at 4 °C with the different antibodies. Hybridoma culture supernatants were concentrated by ultrafiltration (Amicon) and titrated to standardize the reactivity of each pool with HOON cells. ¹²⁵I-labelled 44E3-IgG (40 ng) was added to

each well, and the incubation continued for 2 h at 4 °C. The cells were then washed four times in phosphate-buffered saline containing 0.1% bovine serum albumin, and the contents of each well were transferred to tubes and counted for radioactivity.

Amino acid composition

Samples containing 100 µg of protein were freeze-dried in acid-washed glass ampoules and hydrolysed in 1.0 ml of 5.7 M-HCl for 24 h at 110 °C under vacuum (Cockle *et al.*, 1978). The hydrolysates were dried three times on a flash evaporator, washed with water each time and resuspended in 200 µl of 0.01 M-HCl. Hydrolysates containing 10–20 µg of protein were used to determine the amino acid compositions on a Dionex D-500 amino acid analyser.

Carbohydrate analysis

The presence of galactosamine and glucosamine was determined on a Dionex D-500 amino acid analyser after the hydrolysis of 200 µg of protein with 4.0 M-HCl at 105 °C for 4 h under vacuum. Galactosamine and glucosamine standards can be separated from amino acids and quantified. The carbohydrates fucose, galactose, glucosamine and sialic acid were analysed in a Varian Aerograph series 2100 g.l.c. analyser by the method of Zanetta *et al.* (1972). Purified mucin was used as a positive control and was analysed for carbohydrate content in the same experiment. Both procedures detected 1–2 nmol of each carbohydrate.

Indirect immunoperoxidase staining

Portions of adult brain were frozen in isopentane and sectioned on a cryostat (International Equipment Co.). Indirect immunoperoxidase staining was carried out as described previously (Quackenbush & Letarte, 1985). Acetone-fixed sections were washed in Tris-buffered saline, pH 7.6, and incubated with MAb 44H4 (or 44E3) culture supernatant for 40 min at 23 °C. Slides were washed and incubated for 30 min with 100 µl of a 40-fold dilution of peroxidase-conjugated RAM-IgG. Slides were then treated for 4 min with 0.5% diaminobenzidine in 0.05 M-Tris/HCl, pH 7.6, containing 0.006% H₂O₂, washed with Tris-buffered saline and counterstained with Mayer's haematoxylin. MAb 44H9, which does not react with human brain, was used as a negative control. Positive immunoperoxidase reaction gives a brown coloration clearly distinguished from the blue coloration given by the haematoxylin staining.

Western-blot analysis

Samples containing 80 µg of protein from brain white-matter homogenate were separated by electrophoresis on 10%-(w/v)-polyacrylamide gels (Laemmli, 1970). The proteins were transferred to nitrocellulose sheets and the p94 protein identified by Western blot (immunoblotting) with MAb 44H4 (or 44E3) essentially as described by Towbin *et al.* (1979). The blots were allowed to react for 16 h at 4 °C with 100-fold dilution of culture supernatant containing MAb 44H4. The blots were washed and then treated with ¹²⁵I-RAM-Fc (1 × 10⁷ c.p.m.; 3 µg). Autoradiography was performed with XRO-1 film and du Pont Cross Hi-Plus intensifying screen.

Table 1. Purification by adsorption to monoclonal 44E3-IgG-Sepharose of a protein from white matter of human brain

White-matter homogenate was fractionated by sucrose-density-gradient centrifugation, and the 0.88 M-sucrose fraction, P₃, was solubilized and fractionated on a 44E3-IgG-Sepharose column. All fractions were assayed for 44H4 antigenic activity by inhibition of the cellular radioimmunoassay.

Fraction	Protein (mg)	Antigenic activity			
		Total units	Yield (%)	Units/mg of protein	Relative specific activity
Homogenate	2225.0	6900	100	3.1	1
P ₃ (0.88 M-sucrose)	458.0	5400	78	11.8	4
Triton X-100 extract	145.0	5100	74	35.2	11
44E3-IgG-Sepharose bound and eluted fraction	0.5	4900	71	9808.0	3160

RESULTS

Purification of p94 protein from human white matter

Fraction P₃, a membrane fraction obtained from human white matter and depleted of multilamellar compact myelin, was found to be enriched 44-fold relative to homogenate in the antigen reactive with MAb 44H4 (Table 1). All of the antigen in the P₃ fraction was recovered in the Triton X-100 extract, resulting in an 11-fold purification relative to homogenate. The soluble extract was then applied to a non-immune IgG-Sepharose column, followed by the 44E3-IgG-Sepharose column. The enrichment in antigen in the bound and eluted fraction relative to the Triton X-100 extract was of the order of 280-fold. The final purification achieved

was 3160-fold, with a yield of 71% of the total antigen present in the white-matter homogenate (Table 1).

Fig. 1 shows the purified 44E3 antigen analysed by SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue. This purified antigen migrated as a single polypeptide with an apparent M_r of 94000, designated 'p94'.

Reactivity of MAb 44H4 and 44E3 with purified p94 protein

We have previously mentioned that MAb 44H4 was reactive with the 44E3 antigen purified from the HOON

Fig. 1. Analysis of the purified protein reactive with MAb 44E3 from brain by SDS/polyacrylamide-gel electrophoresis

Approx. 10 µg of 44E3 antigen, purified from brain as described in Table 1, was run on a 10%-polyacrylamide gel and stained with Coomassie Blue. M_r markers were run in parallel and are indicated on the right.

Fig. 2. Competitive inhibition of the binding of ¹²⁵I-44E3 IgG to HOON cells

Glutaraldehyde-fixed HOON cells (5×10^5 cells/25 µl) were incubated overnight at 4 °C with serial dilutions of MAb 44C10 (□), 44E3 (●) and 44H4 (△). The pools of culture supernatants were standardized to equivalent titres before serial dilution. ¹²⁵I-44E3 IgG was added to each well (approx. 40 ng/25 µl) without prior washing of the cells. After 2 h, the cells were washed four times in phosphate-buffered saline containing 0.1% bovine serum albumin, transferred and counted for radioactivity. The 100%-binding value was 47000 c.p.m. and the percentage bound was calculated by dividing the radioactivity bound with competing antibody by that bound to HOON with ¹²⁵I-44E3 IgG alone.

Fig. 3. Inhibition of the binding of MAb 44H4 and 44E3 to the leukaemic cells by 44E3 antigen purified from human brain and leukaemic cells

The 44E3 antigen was purified by affinity to 44E3-IgG-Sepharose from either human brain (●) or HOON leukaemic cells (△). Portions of the neutralized eluates were tested for their ability to block the binding of MAb 44H4 (a) or 44E3 (b) to HOON leukaemic target cells in the cellular radioimmunoassay. An antigenic preparation purified by affinity to 44D10 IgG-Sepharose from the same brain extract (□) was used as a negative control. The concentration of purified antigen used in the inhibition assay is expressed in μg of protein/ml present during absorption.

leukaemic cell line (Quackenbush & Letarte, 1985). In Fig. 2, we demonstrate that MAb 44H4 and 44E3, used at equivalent titres, can block the reactivity of purified and radiolabelled 44E3-IgG to leukaemic cells. A control MAb, 44C10, reactive with the CALLA antigen present on HOON cells (Quackenbush & Letarte, 1985), was unable to block the reactivity of 44E3-IgG to the same cells. These results imply that both MAb 44H4 and MAb 44E3 are reacting with spatially related, if not identical, epitopes on the protein.

To demonstrate that MAb 44H4 and 44E3 were indeed reacting with the same molecule, purified antigens from brain and leukaemic cells were tested for their ability to

Table 2. Amino acid compositions of the brain and HOON cell antigens reactive with MAb 44E3

Results are means for seven to eight independent amino acid analyses. Carbohydrate analyses were performed for two antigen preparations. Abbreviation: und, undetectable levels.

Amino acid	Composition (residues/ 100 residues)	
	Brain	HOON
Asx	11.0 ± 0.8	12.2 ± 1.2
Thr	3.6 ± 0.7	4.8 ± 0.1
Ser	3.6 ± 0.6	6.0 ± 0.8
Glx	12.7 ± 1.4	13.1 ± 0.7
Pro	6.8 ± 0.9	7.7 ± 0.3
Gly	10.7 ± 1.2	9.8 ± 1.0
Ala	8.9 ± 0.9	7.9 ± 0.1
Cys	und	und
Val	6.6 ± 0.6	5.5 ± 0.3
Met	und	und
Ile	5.1 ± 0.4	4.7 ± 0.3
Leu	9.3 ± 0.5	8.1 ± 0.4
Tyr	3.3 ± 0.1	3.1 ± 0.4
Phe	4.8 ± 0.6	3.9 ± 0.3
Lys	7.4 ± 0.4	7.6 ± 0.9
His	2.1 ± 0.3	1.9 ± 0.7
Arg	5.0 ± 0.6	5.2 ± 0.1
Carbohydrate	und	und

inhibit the reactivity of these antibodies with target cells. Fig. 3 shows that at protein concentrations of $0.8 \mu\text{g}/\text{ml}$ (a) or $1.0 \mu\text{g}/\text{ml}$ (b), the purified 44E3 antigens of either brain or HOON cells could block 50% binding of corresponding dilutions of 44H4 and 44E3 MAb to HOON target cells. These results indicate that the 44E3 antigenic preparations were of a similar degree of purification (confirmed by SDS/polyacrylamide-gel electrophoresis). Furthermore, p94 proteins of human brain or leukaemic cells bear both 44E3 and 44H4 epitopes.

Amino acid composition and carbohydrate analysis of purified p94 protein from brain and HOON cells

The amino acid compositions of the p94 proteins purified from brain and HOON cells are shown in Table 2. An interesting observation was the absence of detectable methionine and cysteine residues in all preparations of both proteins. The two amino acid compositions were similar (since serine is poorly resolved on the chromatograms, this apparent difference was not emphasized). When the amino acid compositions were compared for protein relatedness ($S\Delta Q$) by the formula:

$$S\Delta Q = \sum_j (X_{a,j} - X_{b,j})^2$$

where X is the content of a given amino acid of type j for two proteins, a and b (HOON and brain respectively), an $S\Delta Q$ value of 14 units was observed (Marchalonis & Weltman, 1971). According to this equation, a value of < 100 indicates that the two proteins are related. Any differences which may exist between these polypeptides will only be revealed by amino-acid-sequence analysis. Both proteins contain 40% hydrophilic residues and are less hydrophobic than some membrane proteins (Steck &

Fig. 4. Reactivity of MAb 44H4 with white-matter homogenates prepared from human, guinea-pig and bovine brain

White-matter homogenates were prepared from human (●), guinea-pig (○) and bovine (△) brain and adjusted to a final concentration of 10 mg/ml containing 1.5% Triton X-100 in 0.05 M-Tris/HCl, pH 7.8. Portions were tested for their ability to inhibit the binding of 44H4 MAb to HOON leukaemic target cells in the cellular radioimmunoassay.

Fig. 5. Identification of the protein reactive with 44H4 (p94) in brain homogenates at different species

Samples containing equal amounts of protein (80 μg) from white-matter homogenates of human (lane 1), bovine (lane 2) and guinea-pig brain (lane 3) were solubilized in 1.5% SDS containing 2.5% β-mercaptoethanol and separated on a SDS/10%-(w/v)-polyacrylamide gel. The proteins were transferred to nitrocellulose sheets and incubated with MAb 44H4 and ¹²⁵I-RAM-Fc. The reactive antigens were revealed by autoradiography after 60 h exposure. *M_r* markers (not shown) were run in parallel.

Fox, 1972). However, hydrophobicity in amino acid composition is not an overall property of membrane proteins, as revealed for band 3, the major glycoprotein of red cells, and Thy-1, the major glycoprotein of thymocytes (Marchesi *et al.*, 1972; Barclay *et al.*, 1976).

Fig. 6. MAb 44H4 reacts with cells in grey and white matter of human brain

Frozen and acetone-fixed cryostat sections of human brain were stained with MAb 44E3 by using an indirect immunoperoxidase technique. MAb 44E3 reacted with neuronal cells (N) and glial cells (G) of grey matter, as shown in (a), and with glial cells (G) of white matter, as shown in (b). A cluster-like pattern of staining can be seen in the cytoplasm and/or near the plasma membrane.

Carbohydrate analysis of the purified p94 preparations from brain and HOON cells by amino acid analyses for glucosamine and galactosamine and by g.l.c. for fucose, galactose and sialic acid failed to detect any of these sugars. Therefore we concluded that the antigens were not glycoproteins.

Reactivity with white-matter homogenates from different species

In order to establish if these antigens were present in other species, white-matter homogenates from guinea-pig, human and bovine brain were compared for reactivity with MAb 44H4 by cellular radioimmunoassay. Fig. 4 demonstrates that the brain homogenates of all three species were able to block the reactivity of MAb 44H4 with leukaemic cells. However, the concentration of antigen was three times higher in white-matter homogenates from guinea-pig than from human and bovine white matter, as calculated from the protein concentrations required to obtain 50% inhibition of binding (Fig. 4). Similar results were observed with 44E3 MAb.

To demonstrate that the antigens found in white-matter homogenates of the different species were associated with similar proteins, the homogenates were fractionated by SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose sheets and examined by Western-blot analysis. Fig. 5 indicates that MAb 44H4 reacts with a similar protein of *M_r* 94000.

Fig. 7. Concentration of p94 protein in human white matter during postnatal development and aging

White-matter homogenates were prepared from brains of humans whose age varied between 8 months and 84 years and tested in the same experiment for reactivity with MAb 44H4 as described in the Materials and methods section. Eight dilutions were tested for each homogenate, and an inhibition curve was drawn to calculate the 50% -inhibition value. The results are expressed as units/mg of protein, where one unit is the concentration of protein required to inhibit 50% of the binding of the MAb to HOON target cells in the cellular radioimmunoassay.

Localization of 44E3 antigen in human brain and in other tissues

Indirect immunoperoxidase staining of acetone-fixed tissue sections of brain grey and white matter incubated with MAb 44E3 revealed that all cellular elements were stained. In grey matter, neuronal cells and glial cells reactive with 44E3 can be observed (Fig. 6a), whereas in

white matter, glial cells can be seen (Fig. 6b). The staining seems to be in clusters extending from the plasma membrane to the nucleus, but absent from the nucleus. Observations of the slides at higher magnification suggested that the plasma membrane (most likely on its internal surface) and some intracellular membranes are expressing the antigen. Control sections incubated with MAb 44H9 or 44H6, both of IgG₁ isotype, derived from the same fusion and negative with brain tissue, gave no detectable staining.

Table 3 summarizes the distribution of 44E3 antigen in other human tissues. All cellular elements in tissues tested to date are reactive, whereas extracellular elements such as stroma and interstitium are negative. The type of staining observed is described as cytoplasmic, as it expands from the plasma membrane to the nuclear membrane, leaving the nucleus unstained.

Concentration of p94 in human white matter during development and aging

White-matter homogenates were prepared from 24 brains of individuals of ages varying from 8 months to 84 years, and the concentration of antigen reacting with MAb 44H4 was determined in a single experiment by inhibition of the cellular radioimmunoassay. The results shown in Fig. 7 demonstrate that the concentration of p94 was the same at all ages during postnatal development and with aging.

DISCUSSION

In the present study we report the purification of a protein from human brain reactive with both MAb 44H4 and 44E3. The antigen was identified as a single

Table 3. Identification of 44E3 antigen in several human tissues

Fresh frozen acetone-fixed tissue sections were stained with 44E3 or 44H4 monoclonal antibodies as described in the Materials and methods section.

Tissue	Staining observed	Reference
Tonsils	Germinal centres and mantle zones of lymphoid follicles and lymphocytes of interfollicular areas are stained; stroma is negative	Quackenbush & Letarte (1985)
Thymus	Cortical thymocytes show stronger staining than medullary thymocytes	Quackenbush & Letarte (1985)
Lymph nodes	Lymphocytes in follicles and interfollicular areas are reactive; extracellular regions unstained	E. J. Quackenbush & M. Letarte (unpublished work)
Kidney	All cellular glomerular components and all portions of the renal tubules were reactive; interstitium was negative, except for scattered foci of cells	Quackenbush <i>et al.</i> (1986)
Lung	Bronchial epithelium and alveolar macrophages reactive; septal staining of pneumocytes	E. J. Quackenbush, A. Gougos, R. Baupal & M. Letarte (unpublished work)
Breast	Duct epithelium and stromal cells positive; extracellular elements of stroma negative	E. J. Quackenbush, A. Gougos, R. Baupal & M. Letarte (unpublished work)
Liver	Diffuse cytoplasmic staining of hepatocytes	E. J. Quackenbush, A. Gougos, R. Baupal & M. Letarte (unpublished work)

polypeptide of M_r 94000 and was similar in antigenicity and amino acid composition to the protein purified from HOON leukaemic cells. Both MABs are reactive in Western-blot analysis, suggesting that they recognize epitopes on the unfolded polypeptide.

During the isolation of this protein from human kidney, two polypeptide bands, of M_r 94000 and 90000, were detected that reacted with either MAB 44H4 or 44E3 (Quackenbush *et al.*, 1986). More recent studies have indicated that the band at M_r 90000 can also be observed in preparations of brain and leukaemic cells and is likely derived from p94. The existence of the 90000- M_r polypeptide might be related to a functional state of this molecule and/or represent a degradation product.

Previous studies have indicated that the concentration of p94 was higher in grey than in white matter (Quackenbush *et al.*, 1985). Indirect immunoperoxidase staining of frozen and acetone-fixed sections of brain indicated that neuronal cells of grey matter appear more heavily stained than do other cellular elements of both white and grey matter, including glial and endothelial cells. Nuclei were unstained, and the brown coloration given by immunoperoxidase staining was found in clusters at or near the plasma membrane and extending throughout the cytoplasm. Within white matter, we established previously that the antigenic activity was associated with membrane fractions composed of loosely packed myelin and of many smaller membrane fragments (Quackenbush *et al.*, 1985). The examination at higher magnification of tissue sections of brain suggested that plasma membrane and intracellular membranes of cytoplasmic organelles were reactive with the antibodies.

One of the interesting features of the p94 protein is that its antigenic determinants, 44E3 and 44H4, are not usually detectable on intact cells when analysed by flow cytometry, but are detectable by cellular radioimmunoassay on cells fixed with 0.1% glutaraldehyde (Quackenbush & Letarte, 1985). These results suggested that the antigens are present on the internal side of the plasma membrane, on membranes of cytoplasmic organelles or the determinants are sequestered in the plasma membrane.

The results on the species distribution suggested that the proteins detected by 44E3 and 44H4 MAB might be highly conserved. The observations that the antigens are present on all cellular elements of tissues analysed to date suggested that this protein is an important functional component of membranes. Immuno-electron-micro-

scopic studies should reveal the type of intracellular organelles stained with MAB 44E3.

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