IMMUNOELECTRON MICROSCOPIC CHARACTERIZATION OF GLIAL INTERMEDIATE FILAMENTS IN HUMAN GLIOMAS

by

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Declaration

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature: ........................................ Date: 17/02/1893.
To Lola, Margeaux, Rudolf and Armin.

Out of chaos will emerge confusion, then clarity

(Anonymus)
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Wisselende hoeveelhede gliale fibrillère suur proteïen (GFAP) word in die sitoplasma van baie normale en neoplastiese selle van astrogliale oorsprong aangetref. Alhoewel vimentien in bykans alle weefselfipes aangetref word, is hierdie proteïen saam met GFAP immunoelektronmikroskopies as die monomere van gliale intermedière filamente aangetoon. Die ontwikkeling van hierdie strukture is onomkeerbaar en toon 'n stadige metaboliese omset.

Alhoewel hulle tans as astrogliale merkers aangewend word, bestaan die moontlikheid dat hierdie proteïene die funksionele status sowel as die graad van ontwikkeling en differensiasie van die selle waarin hulle voorkom, kan weerspieël. Sommige skrywers is van mening dat hierdie eienskappe diagnosties aangewend kan word ter gradering van maligniteit en anaplasie, terwyl andere wisselende GFAP konsentrasies met verschillende astrogliale selftipes en entiteite assosieer.

Verskeie weefselvoorbereidingsmetodes, waaronder die gebruik van epoksie- en akrielhars, weglating van osmiumtetroksied en variasie van konsentrasie sowel as inkubasietye van primêre fikseermiddels, is ondersoek om die mees aanvaarbare balans tussen antigeen beskikbaarheid en ultrastrukturele morfologie te verkry. Die indirekte immunogoudmetode is op dun snitte ("on grid") aangewend om
monsters vir GFAP (Dako A561) en vimentien (Dako M725) te merk. Vir semi-kuantifisering van relatiewe antigeen konsentrasies, is 'n metode ontwerp waarmee die goudpartikeldigtheid, die persentasie heterogeniteit van die partikelverspreiding sowel as die area van weefsel wat ondersoek is, bereken kon word. Resultate kon sodoende as 'n drie-syfer eenheid uitgedruk word.

Gestandaardiseerde immuno-elektronmikroskopie is op 11 normale en neoplastiese weefselmonsters van menslike oorsprong uitgevoer. Die weefsel is voorberei deur konvensionele fiksering (indompeling) met glutaraldehied en osmiumtetroksied, gevolg deur aangepaste inbedding in "LR White" akrielhars. Die resultate is met konvensionele histopatologiese, immunohistochemiese en kliniese data van die ooreenstemmende monsters vergelyk om die waarde daarvan te bepaal.

Resultate het getoon dat die aanwesigheid van epoksiehars in dun snitte antigeen beskikbaarheid sodanig inhibeer dat baie swak tot negatiewe immunoreaktiviteit verkry is. Beter resultate is met LR White akrielhars verkry, maar ten koste van swakker ultrastrukturele morfologie en verlaagde stabiliteit van dun snitte tydens elektronmikroskopiese blootstelling. Dit het daartoe geleid dat die effek van glutaraldehied fiksering (vernietiging van die vimentien antigeen en sommige GFAP geassosieerde epitope sowel as verhoogde digtheid van die weefsel-fikseermiddel matriks),
minder opvallend was. Alhoewel osmiumtetroxied 'n vereiste is vir aanvaarbare ultrastrukturele morfologie, het dit immunoreaktiviteit met 20% verlaag en grootliks bygedra tot te vroeë verharding van akrielhars tydens impregnering van weefsel.

Ondanks die voordele van elektronmikroskopie en semi-kwantifisering van resultate, is die immuno-sensitiwitie van hierdie tegniek egter swakker as die van ligmikroskopiese immunohistochemie.

Immuno-elektronmikroskopie het die verband tussen GFAP en gliale intermedière filamente in bykans al die gevalle wat ondersoek is, bevestig. Die resultate het ooreengestem met GFAP positieit wat op ligmikroskopiese vlak met immunohistochemie aangetoon is. In die afwesigheid van intermedière filamente, kon geen GFAP of vimentien positieit in oligodendrogliale komponente van gemengde tumore aangetoon word nie.

In astrositome is GFAP positieit gekenmerk deur tussen 17 en 125 partikels / μm², maar was heelwat laer in die glioblastoom (PD = 8) en sommige van die gemengde gliome (PD = 6). Rosenthal vesels het perifere sowel as sentrale GFAP positieit getoon, en dus 'n bykomende bydrae tot die hipotetiese degeneratiewe, astrogliale aard van hierdie strukture gelewer. Die meningioom was GFAP negatief, maar het lae digtheid vimentien positieit opgelewer.
Die voorkoms van beide GFAP en vimentien in die astroblastoom en degeneratiewe breinweefsel (Alexander se siekte), dui daarop dat beide proteïne betrokke is by die ontwikkeling van gliale strukture. Alhoewel areas van waarskynlik aktiewe intermedière filament sintese gevind is, was die antigeen beskikbaarheid vir vimentien egter te laag om enige sinvolle gevolgtrekkings te maak met betrekking tot vimentien lokalisering en die verhouding tussen GFAP en vimentien in individuele intermedière filamente en / of astrogliale vesels.

Verskille in partikel digthede (PD) waardeur GFAP in die verskillende entiteite aangetoon is, is toegeskryf aan veranderlike tegniese- en weefselvoorbereidingsfaktore eerder as aan werklike verskille in GFAP voorkoms in individuele intermedière filamente. Hieruit word afgelei dat die GFAP konsentrasie / gliale intermedière filament-oppervlakte in ten volle ontwikkelde intermedière filamente heel waarskynlik konstant is en dus nie as diagnostiese maatstaf gebruik kan word om tussen verskillende astrogliale selle of entiteite te onderskei nie. Alhoewel die aantal intermedière filamente per sel heel waarskynlik verskil, kon hierdie aspek tot dusver nie oortuigend aangetoon word nie.

Teseame met aanvullende, konvensionele immunohistochemie, is hierdie tegniek waardevol vir die elektronmikroskopiese lokalisering van hoë konsentrasies, aldehydweerstandige,
sitoplasmiese antigenen in geskikte, deeglik georiënteerde weefselmonster.
SUMMARY

Glial fibrillary acidic protein (GFAP) is found in varying amounts in the cytoplasm of most normal and neoplastic cells of astroglial origin. Though not glial specific, immunoelectron microscopy has shown that vimentin and GFAP are coexpressed as monomers of glial intermediate filaments. These structures display irreversible assembly and a slow metabolic turnover.

Although currently applied as astroglial markers, these intermediate filament proteins may reflect the functional and developmental differentiation status of the cells in which they are expressed. Some authors have tried to apply these aspects as diagnostic parameters for grades of malignancy and anaplasia whilst other workers have indicated variable concentrations of GFAP in different astroglial cell types and entities.

Different processing protocols, including the use of epoxy and acrylic resins, omission of osmium tetroxide and variations in concentration and incubation time of primary fixatives, were evaluated to find a compromise between antigen availability and acceptable ultrastructure. Thin sections were labelled on grid for GFAP (Dako A561) and vimentin (Dako M725) by means of the indirect immunogold method. For semi-quantification of relative antigen concentrations, a novel method was devised to calculate the labelling density, percentage heterogeneity of the particle
distribution and the surface area investigated. This allowed expression of labelling results as a three figure unit.

Standardized post-embedding immunoelectron microscopy was performed on 11 normal and neoplastic human tissue specimens. The tissue was exposed to conventional immersion fixation in glutaraldehyde and osmium tetroxide prior to modified embedding in LR White resin. The validity of these results was verified by correlation with conventional histopathological, immunohistochemical and clinical data obtained for each specimen.

The presence of epoxy resin in thin sections was shown to reduce antigen availability to such an extent that very low to negative labelling was encountered. Acrylic LR White resin allowed more acceptable immunodetection, but at the cost of inferior ultrastructure and greater instability of thin sections in the electron beam. This masked the effects of glutaraldehyde fixation on the density of the tissue-fixative matrix which included destruction of the vimentin and some GFAP associated epitopes. Although osmium tetroxide was required for acceptable ultrastructure, it reduced the labelling sensitivity by 20% and was responsible for premature curing of acrylic resin during impregnation of tissue.

Despite superior resolution gained by electron microscopy and the advantage of semi-quantification of labelling
results, the labelling sensitivity of this technique is lesser than that of light microscopical immunohistochemistry.

Immunoelectron microscopy confirmed the association between GFAP and glial intermediate filaments in almost all the glial tumours studied, correlating well with GFAP expression in matching specimens demonstrated at light microscopical level. In the absence of intermediate filaments, no positivity for GFAP or vimentin was found in oligodendrogial components of mixed tumours.

GFAP positivity in astrocytomas was demonstrated by between 17 and 126 particles / μm², whilst lower figures were obtained for the glioblastoma (PD = 8) and some of the mixed gliomas (Pd = 6). Rosenthal fibres showed both peripheral and central positive labelling for GFAP, thus providing more evidence for their hypothetical degenerative, astroglial nature. The meningioma studied, was GFAP negative, but produced low density positivity for vimentin.

Coexpression of GFAP and vimentin was demonstrated in an astroblastoma and degenerative infant brain tissue, thus supporting the presence of both these proteins during development of glial structures.

Although sites of likely glial intermediate filament synthesis were found, the antigen availability for vimentin
was too low to allow a reliable assessment of specific vimentin localization and determination of the GFAP : vimentin ratio in individual intermediate filaments and/or astroglial fibres.

Variations in particle densities (PD) which demonstrated GFAP in the various astroglial entities studied, were considered to be a result of variable technical and tissue processing factors rather than truly significant differences in expression of GFAP in individual intermediate filaments. This lead to the conclusion that the GFAP concentration/glial intermediate filament area is likely to be constant for mature glial intermediate filaments and therefore cannot be used to distinguish between different astroglial cells or entities. Whether each cell has a different number of glial intermediate filaments, has not been established satisfactorily.

Following complementary conventional immunohistochemistry and careful orientation of biopsy material, the procedure can be applied to suitable specimens for the electron microscopical localization of high concentrations of aldehyde resistant, cytoplasmic antigens.
INTRODUCTION

Intermediate filaments are an integral part of the cytoskeleton and are found in most eukaryotic cells. The distribution of biochemically and immunologically distinct types of intermediate filaments has been associated with different cell types in normal and neoplastic tissue. This has found increasing diagnostic application in histopathology and other related fields, (Ramaekers et al, 1986; Bolen & McNutt, 1987; Corwin et al, 1989).

Individual intermediate filaments, with a diameter of about 10 nm and only visible at electron microscopical level, occur in bundles in the cytoplasmic processes of astroglial cells. An indirect association between astroglial fibres (seen at light microscopical level), and glial intermediate filament monomers (glial fibrillary acidic protein (GFAP) and vimentin), has been established by conventional transmission electron microscopy in combination with immunofluorescent, immunohistochemical and/or Western blot techniques. These glial intermediate filament proteins have been associated with the functional and developmental characteristics of the cells in which they are expressed (Dahl & Bignami, 1988; Trojanowski, 1986).

Direct immunoelectron microscopical demonstration of molecular configurations associated with cytoplasmic structures such as intermediate filaments, has been difficult. This is influenced by the effects of
conventional tissue processing on immunodetection sensitivity (Van Noorden, 1986), as encountered with post embedding immunolabelling of epoxy embedded specimens.

Where conventional procedures are altered in favour of improved immunodetection, the quality of ultrastructure is significantly reduced. Frequently, and most prominent at electron microscopical level, suboptimal and controversial immunolabelling, such as irregular staining, total absence of staining or unexpected "false" positivity, is found. Immunoelectron microscopy therefore requires a compromising approach towards the preservation of both morphology and antigenicity. Interpretation of labelling results is influenced by the limited knowledge of the combined effect of tissue processing factors on immunolabelling as well as the striking absence of an acceptable means for standardized measuring, expression and comparison of immunodetection sensitivity.

Although GFAP has been demonstrated in individual glial intermediate filaments by post embedding immunoelectron microscopy of perfused, acrylic resin embedded rat brain specimens, (Gräber & Kreutzberg, 1985; Goto et al, 1987;), it is still uncertain whether this approach is applicable to immersion fixed human brain biopsies.

In this study, the effects of individual processing factors on immunodetection as well as other technical aspects were addressed to establish a standardized, cost effective
technique for routine diagnostic application. This would not only allow semi-quantitative investigation of intracellular expression and coexpression of intermediate filaments, but also the characterization of various antibodies as immunohistochemical markers to distinguish between certain histopathological entities.

This was done by comparatively studying the semi-quantitative post embedding immunoelectron microscopical localization of GFAP and vimentin in normal, degenerative and neoplastic specimens. Various combinations of conventional histological procedures were applied to these normal rat brain and human glioma biopsies.

A better understanding of the major factors influencing the immunodetection threshold for post embedded tissue, may lead to better definition of standardized criteria for good "normal" ultrastructure and high density specific labelling. The current knowledge, mainly obtained from in vitro studies on animal tissue, could then be verified and applied to human material, thus becoming part of established histopathology.
Chapter 1
LITERATURE REVIEW

1A PRINCIPLES OF IMMUNOLABELLING

1A.1 Antibody - antigen interactions

One of the features of the mammalian immune system is the genetically controlled recognition of antigens (molecular substances foreign to the individual) by globular proteins, known as immunoglobulins or antibodies.

Reversible, mainly covalent, high affinity interactions with association constants in the range of $10^5$ to $10^9$ (Sternberger, 1979), exist at molecular level between the variable and hypervariable regions of the antibody molecule and the epitopes (antigenic determinants) of the antigen. In the presence of interleukins and cells such as T lymphocytes, this interaction can stimulate proliferation of specialized, antibody associated lymphocytes (B cells). Such a clone of plasma cells will secrete immunoglobulins of homogeneous specificity, idiotype and allotype. Eventually, antibody binding to the preselected epitopes, will trigger mechanisms which lead to antigen destruction.

This phenomenon can be exploited by immunization of suitable animals with any molecular substance specific for particular cells, organs or subcellular structures to obtain useful reagents (markers) for the characterization of biochemical, serological and histological specimens.
1A.2 Production of antisera

In order to raise useful antibodies, a particular antigen has to be isolated and subjected to biochemical purification and characterization prior to immunization of suitable animals. Subsequently, different regions or three dimensional configurations (epitopes) on the periphery of the antigen molecule are recognized by different B cells, thus yielding a "cocktail" of antibodies of polyclonal origin which can be retrieved from the immunized animal's circulation.

Attempts to obtain antibodies with strong, specific binding potential (affinity), often yield variable results which are influenced by the complicated, often unpredictable regulation of the immunological response of the immunized animal. The yield and specificity of antibodies raised to a particular antigen, depend upon the kind of antigen, schedules of immunization and the host species involved. Here, aspects such as the size of the epitope, expression of epitopes on other molecules (cross-specificity), their availability for recognition (antigenic potential), as well as the efficiency of isolation techniques for the retrieval of high and low affinity antibodies from serum, are of major importance (Sternberger, 1979).

In a recent review, De Mey & Moeremans (1986) discuss the advantages and disadvantages of various biochemical and immunological techniques that are currently applied for the
raising and testing of polyclonal antibodies for immunocytochemistry. This includes the selection and immunization of suitable animals, purification and characterization of antigens and antibodies as well as specificity screening procedures required to produce reliable markers. Despite recent developments to improve the production and characterization of polyclonal antibodies, the possibility of false positivity or false negative reactions will remain and has to be considered during interpretation of results. Other disadvantages include the mixture of specificities found in one particular polyclonal antiserum and the inability to reproduce such a reagent on a large scale due to variations in immune response to the same immunogen by individual animals of the same species.

The development of monoclonal antibodies, which was awarded with the Nobel Prize for Medicine in 1984, has provided advanced specificity for immunolabelling techniques. This principle relies on the hybridization of activated plasma cells with suitable myeloma cells, where successful hybrids will produce immunoglobulins specific for the epitope recognized by the original plasma cell and will continue to proliferate in tissue culture or in implantation sites in laboratory animals in similar fashion to the original tumour. Ritter (1986) describes the principles and procedures currently used for the long term production, screening and selection of high affinity
monoclonal antibodies that can be used for the demonstration and localization of single epitopes in a wide range of specimens.

1A.3 Immunolabelling techniques

Since 1941 appropriate antibodies have been applied to establish the presence of the corresponding antigen in a particular specimen. This concept of antibody histochemistry includes the blocking of non-specific labelling and the visualization of the binding site. Immunolabelling is applied to histological specimens to demonstrate cells (immunocytochemistry), tissue (immunohistochemistry) and/or subcellular structures (immuno-electron microscopy). Over the last decades a large variety of immunolabelling methods and assays have been developed and to find wide application in various disciplines of biology and pathology (reviews: Mason & Gatter, 1987; Howanitz, 1988; Pettigrew, 1989). Recent advances include automation for staining large quantities of slides and the application of hard and software for stain quantification.

Non-specific reactions

Non-specific adherence of antibodies to binding sites not associated with the antigen, may occur due to low affinity covalent, hydrophobic and/or ionic interaction between immunoglobulins and tissue components. By preincubation of tissue sections with bovine serum albumin (BSA), normal
serum or non-fat milk, substances (mainly proteins) present in these reagents, will occupy sites of such interaction without interfering with further labelling procedures. Antisera are diluted to an optimal point where exclusive binding of remaining high affinity antibodies is favoured. These precautions minimize competition from low affinity and non-specific antibodies present in a particular antiserum.

Visualization

For the purpose of visualization, appropriate reagents or labels such as radioactive, fluorescent, enzyme or heavy metal complexes, are coupled or conjugated to the antibody by means of suitable coupling reagents.

Tritiated or iodinated labelling reagents find application in radioimmunoassays and autoradiography. Radioactive data analysis of radioimmunocytochemical tissue specimens is done by ultrasensitive photographic techniques and/or computer controlled densitometry. Light and dark field microscopy is used for morphological examination.

Immunofluorescence techniques refer to procedures where fluorescent substances such as fluorescein (FITC) and rhodamine (RITC) isothiocyanates are coupled to immunolabelling reagents to visualize binding sites during examination with an ultraviolet light microscope or by means of immunophotoelectron microscopy.
Enzymes and chromogens

Major contributions by Paul Nakane (Farr & Nakane, 1981) have lead to the widespread application of horseradish peroxidase and other visible enzyme complexes such as alkaline phosphatase, beta-D-galactosidase, glucose oxidase and cytochrome C to visualize antibody binding sites at light and electron microscopical levels. Immunoperoxidase staining refers to the application of horseradish peroxidase, conjugated to primary antibodies or suitable coupling reagents, followed by the enzyme substrate (hydrogen peroxide) and an appropriate chromogen for visualization of antibody binding sites. Interfering signals from endogenous peroxidase normally found in tissue, are eliminated by incubation with hydrogen peroxide and methanol prior to immunolabelling procedures.

The enzyme/substrate reaction creates a primary complex in which the haemeprosthetic group of the enzyme is oxidized. In the presence of a third substance, an electron donor used as chromogen, a secondary complex is formed, which in the third step, dissociates to release the oxidized electron donor and the enzyme in a reduced state (Sternberger, 1979). Useful chromogens include amines such as 3-amino-9-ethylcarbazole (AEC), diaminobenzidine (DAB) and 4-chloro-1-naphthol (CN), which acquire a specific colour when oxidized.

Initiated by Graham and Karnovsky (1966), DAB has become
the most widely used chromogen despite its assumed carcinogenic properties. This reliable reagent forms a polymer when oxidized and is characterized by a distinct brown colour and insolubility of the reaction product. Its ability to chelate with osmium tetroxide can be used to intensify the reaction product and also allows the use of this electron dense label for immunoelectron microscopy (Sternberger, 1979). The intensity of DAB staining can be enhanced by treatment with imidazole, chlorides of heavy metals such as gold and cobalt or by the use of interference filters for photography.

**Colloidal gold**
Since the initiation of colloidal gold as immunoelectron microscopical label, interest in earlier metal tracers such as ferritin and uranium, has faded. Visible as spherical, electron dense particles in electron microscopical preparations, colloidal gold particles with a diameter of 5 - 40 nm, form very stable complexes with proteins and can easily be distinguished from structures such as ribosomes and glycogen. The exploitation of these characteristics has lead to the widespread use and commercial availability of such particles conjugated to appropriate coupling reagents (Warhol, 1989; Willingham, 1989). Application of these probes at light microscopical level, with or without silver enhancement, forms the basis of an ultrasensitive marker system.
**Signal amplification**

Visualization of reaction sites by means of direct or indirect coupling of suitable reagents can be used for further classification of the immunolabelling techniques mentioned above. In the direct approach, the label is conjugated to the primary antibody, thus using only one reagent with both immunological and visual properties. A low signal to noise ratio is the major disadvantage of this approach. The indirect approach leads to the use of two-step methods exploiting the high affinity, covalent bonds between secondary immunoglobulins against the primary antibody species. Due to its ability to bind to various immunoglobulins, protein A (Goding, 1978) is also used as a secondary labelling reagent. The two-step methods are well suited for the application of gold and silver labels conjugated to either secondary antibodies or protein A (Bendayan, 1984; Roth, 1986).

A major breakthrough to increase the sensitivity and specificity of immunolabelling techniques by the inclusion of a third step involving an enzyme-anti-enzyme complex, was introduced with the development of the PAP (peroxidase-antiperoxidase) method (Sternberger, 1979). This principle was also applied to some other enzyme systems such as GAG (glucose oxidase-antiglucose oxidase complexes, and APAAP (alkaline phosphatase-anti-alkaline phosphatase complexes).

The remarkably high affinity between avidin and biotin was
exploited by Hsu to develop avidin-biotin-enzyme complexes which find application in the ABC method (Hsu et al, 1981). More recently, the ABC methods have been improved and expanded to include streptavidin-biotin-enzyme complexes and labels other than enzymes as mentioned previously.

Major advantages of the indirect approach include amplification of the signal and reduction of the number of different labelling reagents required for visualization of antibody panels.

Selection of suitable techniques depends on the type of specimen, antigen to be demonstrated as well as the apparatus and the antisera available and has to be determined by the individual user. By appropriate selection of techniques, multiple labelling of antigens in the same section of a particular specimen can be achieved. The use of different labels in combination with primary antibodies of different species by simultaneous or sequential labelling, are just a few examples that have been documented.

1A.4 Applications of immunolabelling in histopathology

Presently a great variety of antibodies are being applied on a histopathological routine and research basis to localize and identify a growing list of cellular and tissue antigens. In an overview, Damjanov (1987) points out the potential value of cell, tissue and organ specific markers
In cell lineage studies, the demonstration of particular cells and tissues and the determination of cell phenotype, class and state of differentiation. Such information is useful for the determination of early neoplasia, histochemical grading of malignancy, distinction between benign and malignant tumours as well as the histogenetic and phenotypic classification of tumours. Guidelines to useful markers in this respect are given by Mason & Gatter (1987) and Corwin et al, (1989).

The extent and value of application in diagnostic immunohistopathology, including cell and tissue typing and histochemical differential diagnosis of tumours, was evaluated by Pettigrew (1989), showing that the H&E diagnosis was confirmed in 53% of routine cases submitted to immunolabelling. Further, a definitive diagnosis could be made from a list of differential diagnoses (14,5%), while contributary information was obtained in 18% of the cases. Two of these 200 cases evaluated, provided an unsuspected diagnosis, while the remaining 13,5% of cases made no contribution due to suboptimal fixation and/or preservation of the specimen.

Recently, the role of intermediate filament markers for typing of neuroid, epithelial, glial, muscular, mesenchymal and neuroid tissues has received special attention and is discussed in more detail in section 1D.1.

Various authors such as Gullotta et al (1985), Trojanowski
(1986) and Perentes & Rubinstein (1987), point out that although immunohistochemistry is a powerful tool with a wide spectrum of application, the results obtained from each specimen have to be interpreted with utmost care as many pitfalls exist in the preparation of samples and reagents as well as in the histopathological application of such results. This calls for careful correlation with all other morphologic methods available. Such methods, on which existing histological and histopathological principles have been based, are prone to similar pitfalls (Scott, 1972). This often leads to even greater confusion. However, future availability of superior antibodies along with more recent concepts such as cluster designation of antisera, are positive steps towards the development of useful probes for application in histopathology.

1B ASPECTS OF TISSUE PREPARATION FOR MICROSCOPY

1B.1 Tissue sampling and conventional preparation of specimens for light and electron microscopy

The principle of passing light or electrons through cells or small bits of tissue to obtain representative images, forms the basis of various microscopical techniques for the visualization of cellular and subcellular detail. Although valuable information can be obtained by studying the cell in its natural state or by in vitro examination of tissue cultures, the micro-anatomic structural aspects of cells, tissues and organs are commonly studied in sections of dead
cells by light microscopy and transmission electron microscopy.

When collecting single cells or lumps of tissue during macroscopical examination, the emphasis is on representative sampling for microscopical analysis. However, the sample size, referring to both number of specimens as well as the physical dimensions required for adequate preservation of the tissue, is often neglected. The objectives and restrictions which dictate the sampling procedure, as summarized by Jakstys (1988), show that human clinical specimens from biopsies and autopsies do not lend themselves to adequate sampling, thus requiring utmost care and reservation when making comparisons of data obtained from such samples. As ideal, carefully controlled laboratory sampling conditions are generally not applicable, this is a common restriction which affects most data obtained in this field.

The physical dimensions of the specimen are determined by the selected procedures for preservation of tissue according to set criteria and the addition of structural support to enable the cutting of sections thin enough to allow penetration by light or electrons for subsequent image production. Tissue has to be removed from a living organism with minimal trauma to the specimen, while the rapid onset of autolysis at cellular level has to be prevented by physical and/or chemical preservation of the
tissue.

Although the criteria for ideal tissue preservation are not met by any known procedure, a compromise between artifactual effects, practical applicability and acceptable morphology has lead to the conventional application of formalin fixation; dehydration through graded ethanols, clearing with xylol and impregnation and embedding of tissue in paraffin wax for light microscopical analysis. Microtomy allows the preparation of uniform, representative sections of tissue, approximately 1 cell layer (4 - 6 μm) thick, which are attached to glass slides. Alternatively, freezing of small tissue samples allows cryostat sectioning for quick analysis.

As cellular components have similar optical densities, various dyes or histological stains are used for visualization and emphasis of nuclear, cytoplasmic and intercellular substances. A combination of haematoxylin and eosin (H&E) reveals a general picture and is commonly used as a routine stain. Additional stains such as silver or haematoxyllins, may be applied to obtain detail of specific features that might be present in a particular specimen.

Removal of wax and rehydration of the tissue precedes staining of tissue. Stained sections are dehydrated and mounted with a suitable mounting medium and coverslip to obtain a permanent preparation which is examined at different magnifications with a light microscope.
For conventional embedding of specimens for transmission electron microscopy, a hard medium is required to support the specimen to allow precision ultramicrotomy. Here plastic embedding is appropriate, with the epoxy resins offering the best resolution, good sectioning qualities and stability when exposed to the electron beam. To allow impregnation of the tissue with the hydrophobic resin, complete dehydration through graded ethanols, followed by absolute ethanol and or acetone, is required. Prior to dehydration and embedding of specimens, primary fixation of very small tissue samples in glutaraldehyde, followed by secondary fixation in osmium tetroxide, will allow conventionally accepted micro-anatomical preservation of tissue components.

Semithin sections (1 μm on glass slides for light microscopical orientation of the specimen) and thin sections (50 - 300 nm on copper or nickel grids for electron microscopy), are cut with an ultramicrotome from the resin blocks. Contrast of subcellular structures is enhanced by staining with heavy metal solutions such as uranylacetate and lead citrate prior to and/or after sectioning.

1B.2 Different approaches towards preservation of both morphology and antigenicity

Optimum preservation procedures have been developed from a blend of experience, rationalization and empiricism in an
attempt to immobilize and preserve in death the structure and content of living cells. The degree to which a fixed specimen reflects its former living state, is the single ultimate measure of the quality of the preservation (Farr & Nakane, 1981; Bowers & Maser, 1988). With no single protocol best suited for all specimens, the search for optimal preservation of morphology in combination with practical applicability, has dominated the evolution of histological techniques and methodology over more than 100 years. This approach has lead to gross morphological preservation of tissue rather than keeping specific, molecular configurations intact.

The more recent introduction of immunolabelling techniques, (IA.3), has provided a new means of more specific histological analysis of tissue components at molecular level, whereby the limitations of basic conventional preparative procedures have become apparent (Miller, 1972). Although the multitude of membrane, cytoplasmic and nuclear antigens in tissue specimens can be immunolabelled at almost any stage of tissue processing, (generally categorized as non-embedding, pre-embedding and post-embedding labelling), each processing step influences antigenicity as well as morphology. Autolysis will cause eventual destruction of epitopes and poor morphology where proper preservation and processing of tissue specimens is delayed or absent. This emphasizes the need for a selective, theoretical and/or empirical compromising
approach towards optimal fixation, processing and preservation of antigenicity. The availability of tissue specimens and the nature and characteristics of antigens to be demonstrated (Van Noorden, 1986), are the dominant factors to be considered.

Whereas the criteria for optimal processing of tissue samples are well established, only relative indications of the antigen availability in a particular specimen can be obtained by arbitrary judgement of the degree of positive reaction that can be visualized. This reflects the number of antigen binding sites that remain available to react with the selected immunolabelling reagents after tissue processing has been completed. Such antigen availability depends largely on the initial concentration of antigen in a particular specimen, the resistance to destruction, alteration or masking of its epitopes by processing factors and the sensitivity of the immunolabelling technique and optical system used for detection. Though not as prominent at light microscopical level, this is best seen at ultrastructural level.

Fixation has been pointed out by various authors as the most crucial event in the preservation of antigenicity and morphology in tissue samples (Miller, 1972; Hopwood, 1985; Pettigrew, 1889). Fixatives should cause minimal denaturation, rearrangement or diffusion of the antigen, while chemical reactions between molecular configurations
of a particular epitope and the fixative, resulting in masking, alteration or destruction of the antigen, are undesirable. Along with adequate preservation of morphology and antigenicity, the antigen to be localized, must be accessible to reagents used in the immunolabelling procedure (Farr & Nakane, 1981). Truly resistant epitopes can therefore be defined as molecular configurations associated with substances preserved by proper fixation without being involved in the actual chemical bond formation, thus making such antigens suitable for detection by post-embedding immunolabelling techniques. This group will include antigens altered by fixation, but retaining their specificity. However, intermediate conditions occur frequently and are responsible for the variable and often controversial labelling results found in routinely processed tissue specimens. This is due to delayed or incomplete fixation, which may lead to some loss of fixation-resistant antigens and/or partial masking or destruction of vulnerable epitopes, while antigens not bound by the fixative, could be lost during further processing. Inferior preservation of molecular structures in such intermediate conditions is not always apparent and may not be reflected as poor morphology. Once tissue has been removed from a living organism, the rapid onset of autolysis at cellular level has to be prevented. This can be achieved by physical preservation, such as snap freezing, which causes coagulation of protein and prevents
the loss of lipids, or chemical preservation in one of a large variety of fixatives.

Theoretically, immunolabelling for the demonstration of membrane antigens in mildly fixed cell suspensions, which can be obtained in the fresh state at demand, would be the near to ideal situation and can be followed by further fixation and embedding in fixatives and resins normally preferred for electron microscopy. However, handling and availability of fresh tissue, as well as the need for sophisticated equipment as for ultramicrotomy, may severely limit the application of these techniques.

Despite variations in tissue density and texture, ultramicrotomy allows the preparation of thin sections from frozen material, which can be immunolabelled on grid following cryoprotection, freeze drying and/or mild fixation. Further fixation and/or staining as well as improvement of the stability of the sections in the electron beam by critical point drying, the use of formvar coated grids and/or carbon coating of sections, will complete the protocol for such non-embedding techniques.

Pre-embedding techniques rely on the preparation of small fractions of tissue for immunolabelling before further processing and embedding in suitable media from which thin sections can be obtained in a conventional way. Though antigenicity is generally well preserved by both these approaches (Hölund et al, 1981), the ultrastructure is
relatively poor due to freeze artifact, poor fixation and sectioning. The use of suitable detergents for the penetration and partial destruction of cell membranes to expose cytoplasmic epitopes prior to labelling, leads to further loss of ultrastructure.

Good morphological preservation is the main priority in post-embedding immunolabelling techniques, which are applied to thick sections on glass slides and thin sections on grid after fixation and embedding has been completed. However, this preservation of morphology is obtained at the cost of limited antigen availability (Hemming et al, 1983), but has emerged as the most practical way to obtain permanent preparations in laboratories handling fixed tissues. Damage to tissue and the onset of autolysis can be limited by efficient selection and removal of specimens before rapid immersion into a suitable fixative. Alternatively, though for obvious reasons impractical for use on human tissue, fixation by perfusion will allow access of fixative to tissue in the living state, thus minimizing autolysis.

Any attempt to preserve an antigen along with acceptable morphology in a combined protocol, will therefore be subject to the factors mentioned above. Either inferior ultrastructure or suboptimal immunolabelling results as well as variable reproducibility and limited application are inevitable. As exact mechanisms for the molecular
interaction between tissue components and fixatives as well as other processing reagents have not been established, the currently useful protocols serve as starting points for further development. They may be based on a trial and error approach as well as theoretical and empirical motivations. Although new, more suitable processing techniques might be found for optimal antigen preservation and optimal morphology, they could alter the histologic picture to which all histologists and histopathologists have become accustomed.

1B.3 Some aspects of fixation and individual fixatives that influence antigen availability and morphology in post embedding immunoelectron microscopy

Optimal fixation is a function of time and specimen thickness, depending on the penetration rate of the fixative (Straus, 1979), the type of chemical reaction induced by the fixative and the nature of the specimen.

As the semi-permeability of cell membranes is altered by contact with the fixative, the actual osmolarities and pH at cellular level differ from the specifications of the original fixative solution as prepared in the laboratory. Apparently osmotic effects are minimal, as swelling is caused and controlled by electrolytes present in the fixative as they bind electrostatically to charged macromolecules (Bowers & Maser, 1988). Therefore, fixative concentration, osmolality and pH are useful, but not
critical, fixative properties. They may serve as starting points for a reasoned modification of the formulation to obtain the total effective osmolality following proper fixation, thus allowing reproduction of satisfactory combinations, (Bowers & Maser, 1988). Although poorly defined and variable for different tissues, this is of major importance. Depending on the tissue, some osmotic pressure at neutral pH 6-8 has to be created to allow penetration of the fixative into tissue, while significant swelling and poor preservation, associated with hypotonicity, and gross shrinkage due to extreme hypertonicity, should be avoided. Spongy and highly vascular tissue will allow faster penetration of the fixative, while dense tissue will require a longer period of fixation.

Although specimen fixation would be optimal at natural temperature, lower temperatures are preferred to minimize autolysis. However, low temperatures decrease the penetration rate of fixatives and slow down the chemical reactions involved in fixation. The opposite effect can be achieved at higher temperatures. Where the thickness of the specimen exceeds the maximum penetration depth of the fixative, the interior of such a bulky specimen is less well fixed than the exterior, thus resulting in a fixation quality gradient from surface to centre. Thus, conventionally excised and immersion fixed specimens display fixation quality which is not consistent throughout.
the block and generally suffer from shrinkage of tissue as well as loss of some tissue components during fixation (Bowers & Maser, 1988).

In the absence of an ideal fixative, the formation of cross-linking bonds between neighbouring proteins (Artvinli, 1975; Peters & Richards, 1977) by aldehyde fixation has become the accepted way of primary chemical tissue preservation.

Formalin

Formalin, (4% formaldehyde or 10 % formalin), commonly buffered with sodium phosphate to prevent formation of formic acid, has become the most widely used fixative due to fast penetration of tissue and acceptable morphology at light microscopical level. Formalin is involved in numerous and complex reactions with proteins, while lipids are neither destroyed nor preserved. Generally, carbohydrates are not fixed, but partially trapped in the protein network, where as other cell constituents are lightly precipitated.

Though many antigens do not survive formalin fixation, formol resistant epitopes have been reported (Puchtler & Meloan, 1985; Van Noorden, 1986). It is generally accepted that a certain degree of reduction of antigenicity occurs for a third group of antigens, which can be exploited under well controlled conditions (Straus, 1979; Hølund et al, 1988).

Major disadvantages of the fixative include the relative slow formation of cross-linking bonds (Bowers & Maser, 1988) and the disruption of cell membranes, which makes this an inferior fixative for electron microscopy and the preservation of most cell membrane antigens. Despite the availability of numerous formalin derived fixatives known for improved immunolabelling, storage, rapid preservation of mucosubstances and brain tissue, the basic limitations of formalin fixation have remained.

**Glutaraldehyde**

Since the introduction of glutaraldehyde, this reagent has received much attention and has emerged as the best micro-anatomical fixative known. It is characterized by slow penetration of about 1mm per hour and synchronous, rapid formation of cross-linking bonds which mainly involve the lysine residues of various proteins. Glutaraldehyde does not preserve lipid antigens, while soluble antigens are often washed out. Although cell membranes remain intact due to slow penetration, poor morphological preservation and/or loss of antigenicity may occur in specimens thicker than 1.5 mm due to autolysis in areas not reached by the fixative. While the osmolarity of glutaraldehyde fixatives seems to be important (Bone & Ryan, 1972), Bowers & Maser (1988) reported little morphologic degradation in specimens that have been exposed to prolonged (months and years)
storage in aldehyde fixatives. Apparently cells are still osmotically reactive after glutaraldehyde fixation (Wen & Wisniewski, 1987).

Epitopes masked by their involvement in the formation of cross-linking bonds during aldehyde fixation, may be partially recovered through etching by means of proteolytic enzymes such as trypsin, pepsin and pronase. As for other etching reagents such as hydrogen peroxide (Straus, 1979) and sodium (meta) periodate (Bendayan & Zollinger, 1983), the mechanisms for these reactions are not known and generally have a destructive effect on morphology and certain antigens. However, in the absence of fresh tissue and under well controlled conditions, such treatment can be beneficial for the exposure and demonstration of a limited number of epitopes.

**Osmium tetroxide**

Introduced as the first electron microscopical fixative by Palade in 1952, osmium tetroxide has set a standard of lipid preservation, staining and contrast enhancement (Friedenberg & Seligman, 1973) which is included in the criteria for good ultrastructural preservation of tissue. Though not suitable as a primary fixative, these are the main reasons for its conventional application as a secondary fixative, following primary aldehyde fixation. The complex chemical reactions of osmium tetroxide with tissue components are poorly characterized, and involve
mainly lipid preservation, but also initial cross-linking of proteins, which are solubilized and degraded when exposed to the fixative for longer than 30 minutes (Bowers & Maser, 1988). Penetration is at an approximate rate of 1 mm per hour during which the osmolarity of the fixative is not very important (Bone & Ryan, 1972). According to Wen & Wisniewski (1987), osmium tetroxide rapidly eliminates osmotic responses of cells, stops volume changes and leads to improved preservation.

Despite its toxicity, partial reduction of antigenicity (Hemming et al., 1983) as well as destruction of enzyme activity, microtubules and proteins such as actin, osmium tetroxide cannot be replaced by any other known fixative. To allow improved immunolabelling, but at the expense of resulting poor ultrastructure, some investigators prefer to omit osmium tetroxide from their protocols. Alternatively, osmium may be bleached out by using oxidizing reagents, thus allowing immunodetection of some antigens in sections cut from osmicated blocks. Where osmium vapours are applied to sections after immunolabelling, only remaining osmiophilic substances will be stained, as most lipids are extracted during dehydration. This results in dark, inferior ultrastructure (Erickson et al., 1987).

Precipitating fixatives

Precipitating fixatives such as acetone, ethanol and methacarn (Puchtler et al., 1970) are recognized for
improved preservation of antigens and are commonly applied after freezing of tissue samples. All non-aqueous fixatives and dehydrating fluids cause excessive shrinkage of tissue, extraction of lipids and loss of other soluble tissue components. These major disadvantages, which are more prominent with fixatives than for dehydrating solvents, influence morphology and have special effects on morphometry.

Fixative combinations

As all tissue components cannot be preserved by one fixative, combinations of more than one fixative have been used with partial efficiency. Simultaneous application of formalin and glutaraldehyde to achieve fast penetration and good morphology, proved to be unsuccessful due to chemical modification of tissue components by formalin preceding glutaraldehyde fixation (Bowers & Maser, 1988). Similarly, combinations of aldehyde fixatives and osmium tetroxide suffer from interactions between the fixatives, which lead to inferior preservation of tissue (Hopwood, 1970). Other combinations such as periodate/lysine/paraformaldehyde for the preservation of glycoproteins and acetone/periodate-lysine-paraformaldehyde for improved morphology of cryostat sections, have been applied. These procedures require careful attention to detail at every stage of preparation and fixation, thus having limited application.
Additives

The use of small quantities of additives such as picric acid (Newman et al., 1983), will improve preservation of membrane systems in some cells without further destroying antigenicity and enzyme activity in the fixed tissue. Erickson et al. (1987) have promoted the en bloc application of uranyl acetate to improve tissue preservation, contrast (staining) and labelling possibilities when using LR White resin. This needs careful observation as precipitation may occur due to overstaining (Bell, 1988). According to Bowers & Maser (1988), the use of such additives is usually not required for adequate fixation and probably should be avoided to prevent needless complications.

Buffers

The contribution made by various buffers used in combination with the different fixatives, has been put in perspective by Coetzee & Van der Merwe (1984). They found that good contrast obtained by cacodylate buffers was due to excessive extraction of tissue components, while the use of phosphate buffers ensured a minimal loss of such components. As good contrast no longer depends on the buffer used, phosphate buffers are applied conventionally despite the formation of precipitates with cations such as calcium and magnesium, which are responsible for granular precipitates in some tissues.
1B.4 Embedding media:
Their influence on antigenicity and morphology in post embedding immunoelectron microscopy

The mechanical and chemical nature of a particular embedding medium (resin) will determine its sectioning properties, stability in the electron beam and resolution, but also the degree of access of stains and labelling reagents to tissue components after sectioning (Acetarin et al, 1987). All known resins have significant flaws and there is little or no understanding of the chemical interaction between embedding substances and tissue components (Mollenhauer, 1988). This explains the often empirical selection of resins to satisfy particular needs for various applications (Causton, 1984). Hard, tough resins section best, but with current microtomy techniques some deformations, internal dislocations or crevices will always be present, thus presenting severe limitations in retrieving high resolution information.

The extreme heat required for polymerization of unsaturated polyesters is harmful to antigenic sites and other tissue components while methacrylates do not bind to tissue, thus being prone to cleavage rather than sectioning (Acetarin et al, 1987).

Epoxy resin

Epoxy resins react with tissue, require 100% dehydration of
specimens and are well suited for sectioning. However, they are surprisingly unstable, mutagenic, (some may be carcinogenic) and are prone to artifacts caused by improper dehydration. This leads to differential swelling or shrinkage of organelles due to improper resin impregnation. Although immunolabelling is inhibited (Acetarin et al, 1987), probably due to the hydrophobic nature of the resin which minimizes interaction of aqueous immunolabelling reagents with antigenic determinants the superior tissue preservation of epoxy resins is a strong motivation for their use where possible (Bendayan, 1984). When immunolabelling is done on epoxy thin sections, etching is required to expose antigenic determinants on the surface of the 70 nm sections. This approach has been successfully applied for the demonstration of mainly hormonal antigens in secretory granules of a large variety of neuroendocrine cells. Successful immunolabelling with or without etching on thick sections after removal of the epoxy resin, indicates that hydrophobic embedding media are efficient barriers against immunolabelling reagents rather than destructors of antigenicity. However, as staining intensity is increased proportionally by incubation time in etching reagents, the consequent loss in membrane definition and image quality has yet to be overcome.

Great potential exists for the simultaneous morphological and immunocytochemical investigation of a single specimen at both light and electron microscopical levels by using
thin and semithin epoxy sections from the same block. Here major restrictions in the form of the interaction between osmium tetroxide and diaminobenzidine and the efficiency of removal of resin from the thick sections, have been encountered (Mar & Wight, 1988).

Non-epoxy resins

Although certain antigens are best localized in epoxy resins and glycol methacrylate, significant increased post-embedding labelling potential for non-epoxy resins has emerged. Indications are that the sensitivity of epoxy resin embedded material for immunolabelling is very low to zero when compared to Lowicryl K4M or LR White resins (Mollenhauer, 1988).

Acrylic LR White resin

Acrylic resins allow immunolabelling of cytoskeletal proteins (Valentino et al, 1985) which cannot be demonstrated satisfactorily with epoxy resins. Although inferior in quality to the epoxy resins, acrylic resins with hydrophilic properties such as LR White and Lowicryl K4M (Causton, 1984; Newman, 1987) have proved to be useful alternatives as they do not need etching, allow partial dehydration as well as low temperature embedding. They are considered to be less toxic than epoxy resins.

Very little data, if any, is known on the removal of LR White resin from thick sections, whereas reactivity in
thick sections after prolonged incubation with immunolabelling reagents as claimed by the manufacturers, remains to be a continuing problem (Slater, 1989).

Investigators applying LR White resin, usually exclude osmium tetroxide from their protocol (Newman & Jasani, 1984b), but where this is not the case, prolonged impregnation is done at low temperatures (Craig & Miller, 1984; Gräber & Kreutzberg, 1985). Poor morphology due to poor penetration of resin at low temperatures, or destruction of antigens by high temperatures caused by heat or chemical curing of resin, are some of the physical aspects that can affect the quality of ultrastructure and immunolabelling.

Incorrect interpretation of mainly morphological data may occur due to a number of factors, summarized below, which are associated with the preparation of specimens for electron microscopy as well as viewing and recording such images.

**Ultrathin sections**

Ultrathin sectioning, an integral part of biological specimen preparation for TEM, requires some considerable practice and dedication to recognize, minimized or eliminate the various artifacts which are associated with each knife, block, specimen, microtome, and set of environmental conditions (Klomparens, 1988). Investigators
should also be aware of deposits caused by rough section surfaces as well as other staining artifacts (Bell, 1988). Charging of specimens due to poor contact between section and grid, grid and holder and/or holder and stage can cause drift or breaking of the specimen in slightly damaged areas of the section. When using the transmission electron microscope, factors such as resolution, astigmatism, contamination, etching, contrast, coherence, a 10% fluctuation in accuracy of image magnification on digital readout as well as drift in accelerating voltage, have to be considered (Chapman, 1988). The accuracy of the final image produced, will depend on the lenses of the microscope, camera, film as well as photographic enlargement and manipulations such as spotting, burning and dodging that are used to eliminate or reduce the impact of artifacts in the final print (Wergin & Pooley, 1988).

1B.5 Evaluation of immunoelectron microscopical results

When evaluating immunoelectron microscopical results, the major objective is to establish the validity of the morphological localization of a particular immunoprobe. After consideration of method, tissue and reagent controls, the specificity of the particular immunolabelling protocol can be determined, thus allowing arbitrary judgement of a positive or negative result.

For morphological characterization of the labelling site, acceptable ultrastructure is required as proposed by Bowers
& Maser (1988). These authors state that, "over the last 20 years, there has been general agreement among students of fine structure over the extent to which a biological specimen has been well prepared for electron microscopy". Thin sections prepared with a diamond knife from tissue fixed in phosphate buffered glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy resin, will under normal conditions provide such an acceptable image. This consensus will allow evaluation of comparable specimens with relation to living and fixed specimens, specimens fixed by different means and compatible morphological analysis of fixed specimens with respect to biochemical and physiological information obtained from living specimens.

As labelling conditions have to be compromised with the requirements for acceptable morphology, neither antigen availability nor morphology can be considered to be optimal, which requires additional methodology to establish these criteria. Therefore, by application of protocols favouring immunolabelling with subsequent inferior ultrastructure, correlation with relevant, additional data obtained from conventional electron microscopy, light microscopy and immunohistochemistry, is necessary to confirm the origin and histological nature of specimens to support the immunogold labelling result. Positive localization of an antigen to a well characterized site, can thus be established by means of acceptable ultrastructure and specific, high density immunogold
labelling.

Limited information on antigen concentration and labelling efficiency can be obtained by additional, often cumbersome procedures to attempt quantification of labelling deposits. This has limited comparative value and application due to the diversity found in specimens and methodology. As immunogold labelling techniques offer the advantage of quantitative measurement of the visualized reaction product, gold particles can be counted and correlated with the structures with which they have been associated, thus expressing the relative antigen concentration for comparison with other areas in a particular specimen (Slot et al, 1989). This requires some form of manual or automated morphometrical analysis of structures. Here the quality of the ultrastructure plays a crucial role, making such procedures prone to various errors.

Signs of suboptimal morphology cannot be ignored and are found in most immunolabelled specimens. They include swelling and vesiculation of rough endoplasmic reticulum, disruption of smooth endoplasmic reticulum and the mitochondrial matrix, the appearance of irregular mitochondrial profiles with myelin figures, loss of mitochondrial granules and the dense matrix, crystalline inclusions in microbodies, as well as coarse clumping and margination of nuclear chromatin.

By calculating the standard mean deviation for the number
of arbitrary particle counts made, the significance of the observations made can be verified, but does not include critical analysis of factors influencing the preparation of micrographs that are used for such observations. This includes limited antigen detection (Acetarin et al, 1987; Slot et al, 1989), loss of specimen volume, drift and specimen damage (Chapman, 1988), resolution, photographic manipulation and final print magnification (Wergin & Pooley, 1988). All these aspects directly influence the particle density. Although particle densities and related statistical calculations were part of the results given by some investigators, many recent reports lack such figures. This indicates the absence of an useful, standardized method for comparison of labelling densities obtained by different protocols on comparable specimens.

1C INTERMEDIATE FILAMENTS

1C.1 The filamentous composition of the cytoskeleton

The cytoskeleton of eukaryotic cells divides the cytoplasm of a cell into water rich fluid and polymerized, protein rich phases.

Slender 4 - 10 nm microtrabeculae are suspended in cytoplasmic ground substance and form a three dimensional, cytoskeletal lattice. These microtrabeculae incorporate microfilaments (7nm), intermediate filaments (10 nm) and microtubules (24nm), appear to be dynamic and are involved
in sol/gel transformations. In this respect, they serve as frameworks for the maintenance of cell anisometry (Weber, Osborn, 1980; Anderton, 1982).

Both microfilaments (actin, 4 - 6 nm, m.w. 43 kD) and the relatively larger proteins myosin and tubulin, associated with 25 nm microtubules, are essential for cell motility and determination of cell shape. Subtle antigenic differences of microfilaments can be exploited to demonstrate 4 isotypes of actin specific for cardiac, smooth and skeletal muscle, while the remaining two isotypes are found in non-muscle cells (Corwin et al, 1989).

1C.2 Classification of intermediate filaments

The intermediate filaments have been divided into 4 subgroups, being keratins (Type I and II), non-epithelial desmin, GFAP and vimentin (Type III) and neurofilaments (Type IV). Based on their biochemical and immunological differences, 5 major groups of intermediate filaments have been defined, including cytokeratins (epithelial cells, m.w. 40-68 kD), desmin (smooth, cardiac and skeletal muscle, m.w. 55 kD), glial fibrillary acidic protein (GFAP, astroglia, m.w. 52 kD), neurofilaments (neurons, m.w. 68, 150 and 200 kD) and vimentin (wide range of mesenchymal cells, m.w. 57 kD).

At least 19 different cytokeratin polypeptides with
different isoelectric points and molecular weights of 40 - 70 kd have been associated with tonofilaments. On frozen tissue, high molecular weight cytokeratins can be demonstrated in complex stratified epithelium, while the low molecular weight cytokeratins usually occur in simple epithelia. Both high and low molecular weight cytokeratins are found in ductal epithelia of breast, bronchogenic mucosa, skin appendages, pancreatic ducts, transitional mucosa, ovarian serosa and mesothelium. The potential subclassification of carcinomas based on such a distribution has not been established.

GFAP, previously known as α-albumin, was first isolated by Eng from fibrous astrocyte-rich multiple sclerosis plaques (Eng, 1985). Various molecular weights have been determined for a single GFAP peptide, including specific figures of 49 kd, 51 kd and 52 kd) or average figures of 48 - 52 kd and 50 - 52 kd. Other workers have found 6 polypeptides of 39, 40, 42, 44, 20 50 kD, a group of 7 peptides (40-54 kD) as well as 11 polypeptide bands of 37 - 49 kd which included both particulate and soluble forms. GFAP shows a high degree of interspecies conservation (Wikstrand & Bigner, 1980; Dewhurst et al, 1987) and has a stability in situ well in excess of normal post mortem times.

Neurofilaments consist of a triplet of distinct, but related proteins with molecular weights 68 - 70 kd, 150 kd and 200 - 210 kd which occur in most neurons (Corwin et al,
1989). Primitive neuroblasts express vimentin, then the 68 kd unit, whilst the other 2 subunits only appear with maturation. Although expression of all 3 proteins in neoplastic cells is often incomplete, they may occur in some neuroendocrine tumours and apparently are the only intermediate filaments found in oat-cell carcinomas and the paired helical filaments associated with Alzheimer's disease (Wen & Wisniewski, 1987). According to Smith et al (1984), neurofilament proteins are more active metabolically and less resistant to degradation than GFAP at all stages of maturation.

Vimentin, a single protein of 57 - 58 kd, has been demonstrated in many tissues and is useful as an indicator of mesenchymal differentiation. It is further characterized by typical randomly coiled fibres and involvement in coexpression and shifts during embryonic development (Leader et al, 1987; Corwin et al, 1989).

1C.3 Molecular structure of intermediate filaments

Known from protein and DNA sequencing work, a prototype structure consisting of a central α-helical rod region of about 310 residues in length with terminal head and tail domains of contrasting hypervariability in both sequence and length, has been associated with the intermediate group of filaments. Here the central rod domain is responsible for the common features of all intermediate filaments, which include electron microscopical characteristics such
as the apparently similar morphologies and a 7 - 12 nm diameter for all types of intermediate filaments, both in vivo and after in vitro assembly. A 21 nm repeat was observed for metal shadowed, in vitro reconstituted intermediate filaments. Differences found in function, distribution and in stability of different intermediate filament proteins are therefore likely to reside in the hypervariable end domains (Altmannsberger & Osborn, 1987).

After trypsin treatment of intact filaments, Geisler et al (1986) isolated an octameric complex of the terminal domains of desmin, which was shown to be a portion of the carboxyl-terminal half of the α-helical rod domain. This indicates that rapid digestion of the terminal domains into small peptides during trypsin treatment, does not interfere with the basic structure of the intermediate filament.

Partial and complete amino acid sequences determined for desmin and vimentin, show that the central α-helical rod domain contains a 7 residu (a - g) repeat pattern in its linear sequence, which is conserved in sequence principle and in length, rather than in actual sequence. The hydrophobic character of residues a and d argue for coiled coil formation of helical domains, implicating the formation of tetrameric rods from antiparallel, double stranded coiled coils with a length of 50 nm. Although the exact arrangements of the tetramer units are still under discussion, it is believed that each 10 nm filament
contains about 8 tetramers or 32 polypeptide chains per diameter.

Other useful data has emerged from DNA sequence studies, showing that the number and position of introns is strongly conserved for types I, II, and III, while the neurofilaments of type IV have a totally different intron arrangement. Despite the similarity in amino acid sequences of desmin and vimentin, these two proteins are products from different genes. Non-epithelial types III and IV can form homopolymers, while contrastingly the epithelial group is involved in obligatory heteropolymers, requiring one type I and one type II polypeptide for filament formation.

The close relationship between these intermediate filaments can be illustrated by comparison of their central rod sequence identities. A 63% similarity is found between the rod sequences of desmin, vimentin and GFAP in the non-epithelial subgroup (Type III), while comparison of sequences from Type III and either Type I or Type II yield a 30% similarity. The 3 Type IV neurofilament proteins share the same structural motif for their rod sequence, which shows a similarity of 53% when compared to vimentin.

1C.4 Functional intermediate filament expression

By extraction of cells with detergents or solutions of high or low ionic strength, intermediate filaments remain while cytosol and other protein filaments are lost, showing
that cytoskeletal filaments are very stable and least soluble of the cytoskeletal units.

In contrast with a fairly rapid turnover previously indicated in vitro, De Armond et al (1986) have shown a relatively slow turnover of glial intermediate filaments in vivo. Their assembly is thought to be irreversible, as no dynamic equilibrium between soluble and polymerized forms has been documented. This is supported by the absence of a pool of unpolymerized intermediate filament protein in cells (Bolen & McNutt, 1987).

Treatment with colchicine caused intermediate filaments to reorganize into perinuclear caps and whirls of perinuclear fibres (Weber & Osborn, 1980). The metabolic activity of all CNS intermediate filaments was shown to decrease with age along with an increased susceptibility to degradation (Smith et al, 1984).

Due to incomplete developmental studies and limited molecular information on their behaviour in vivo, the function of intermediate filaments is still uncertain. Involvement in cell shape, internal organization and movement of eukaryotic cells (Bolen & McNutt, 1987) or restriction of individual cell mobility (Altmannsberger & Osborn, 1987) indicates that intermediate filaments are involved in the mechanical integration of cytoplasmic space, contributing to the skeletal framework of the cytomatrix.
De Armond et al (1986) suggest that the stability provided by bundles of glial intermediate filaments in astroglial cells creates a framework for proliferation and extension of astrocytic processes. Wikstrand & Bigner (1980) believe that GFAP, being the major component of the astroglial fibres in fibrous astrocytes, provides structural support for myelinated CNS axons.

1C.5 Mechanisms of intermediate filament expression

Cellular control of these phenomena by a yet unknown mechanism, has been suggested and is supported by data indicating that a single site specific phosphorylation of vimentin is important in the modulation of intermediate filament structures and organization. Goldman & Chiu (1984) showed that GFAP and vimentin expression could be modulated by cyclic AMP dependant mechanisms. Dramatic disassembly of vimentin could also be induced through phosphorylation by a cyclic AMP dependant kinase for which vimentin was shown to be a substrate in vitro (Geiger B, 1987).

According to Backhovens et al (1987), the addition of 1 mM dibutyryl cyclic AMP to a glioma cell culture to induce such GFAP formation, did not require cell to cell contact nor cell proliferation. This induction occurred at a low vimentin concentration, thus indicating a shift from vimentin to GFAP synthesis. Pixley & De Vellis (1984) support a hypothetical correlation between vimentin positivity and cell contact with large volumes of
extracellular fluid, thus indicating communication between fluid and adjacent tissue via intracellular transport involving cells containing vimentin.

Although found in most cells, intermediate filaments are more abundant in nervous tissue where they appear in bundles forming neurofibrils and glial fibres at light microscopical level. Immunocytochemical characterization of different intermediate filaments is best done on frozen tissue using specific antibodies. Alternatively, ethanol and/or methacarn fixed tissue (not suitable for prolonged storage) or aldehyde fixed paraffin embedded tissue combined with proteolytic digestion, may be utilized, but shows decreased sensitivity and at its best will not render optimal results (Bolen & McNutt, 1987; Corwin et al, 1989).

Careful characterization of antibodies is required to eliminate possible cross-reactivity due to shared epitopes. However, the specificity of currently used antibodies, although having been tested on a large number of cells and tumours, has not been definitely determined (Altmannsberger & Osborn, 1987).

1C.6 Coexpression of intermediate filaments

Various reports indicate coexpression of two or more intermediate filaments in many different tissues. Domagala et al (1988) have classified three different types of coexpression of intermediate filaments in tissues. True
coexpression involves more than one intermediate filament in the same cell, whilst pseudo-coexpression refers to different intermediate filaments in different cells of the same tumour. The third type involves false coexpression where only one type of intermediate filaments is present in tumour cells, while benign cells in the specimen contain a different type of intermediate filament. This is commonly seen in fine needle aspirates.

Double labelling immunoelectron and light microscopy techniques have been applied to determine whether the different polypeptides occur in the same 10 nm filament during coexpression of intermediate filaments. Initial results indicate that vimentin and cytokeratins occur in different intermediate filaments, while coexpression of vimentin and GFAP in gliomas as well as vimentin and desmin in rhabdomyosarcomas seem to occur in the same intermediate filament (Altmannsberger & Osborn, 1987).

The functional status of a cell may be reflected by true coexpression of intermediate filaments by yet unknown mechanisms, as indicated by various workers (Pixley & De Vellis, 1984; Pixley et al 1984b; Dahl & Bignami, 1986a; Herpers et al, 1985; Backhovens et al, 1987; Miettinen, 1987). However, this data also supports the possibility that a combination of shared epitopes, cross-reacting antisera, sensitivity limitations of immunolabelling techniques and masking or destruction of certain epitopes
in routinely processed tissue, may present a distorted picture of the true intermediate filament antigenic character.

Despite coexpression, interspecies differences, cross-reactivity and other technical problems, the close relationship between the 5 major groups of intermediate filaments, as emphasized by the available biochemical, immunological and morphological data, seems be functionally and developmentally significant and may be of diagnostic aid in some of the so called histopathological problem cases (Damjanov, 1987). However, the full potential of intermediate filaments as immunohistochemical markers, is not yet available to the histopathologist who has to rely on routinely processed tissue.

1D. NEUROGLIA

1D.1 Astrocytes: characterization, function and reactions to disease or injury

Astrocytes, along with oligodendrocytes and ependymal cells, differentiate from primitive ectodermal cells (spongioblasts) of the subependymal layer of the developing nervous system.

Spongioblasts

In tissue sections, spongioblasts are smaller than astrocytes or oligodendrocytes and have extremely scanty
cytoplasm and spherical, dark staining nuclei which contain much chromatin.

Ependymal cells
These cells line the lumen of the neural tube, proliferative and have a supporting function before they form an epithelial lining (ependyma) for ventricles of the brain and central canal of the spinal cord.

Astroblasts
Immature astroblasts have the ability to divide and may be recognized by their sparse content of intermediate filaments. Their nuclei are of low density with clumps of chromatin usually near the nuclear envelope.

Astrocytes
These star-shaped cells, known since 1889, are found in brain and spinal cord where their processes and other glial elements occupy interneuronal spaces to form the neuropil. They are the most numerous glial cells in grey matter and it is thought that they mediate some metabolic exchange between neurons and blood.

Generally, astrocytes are characterized by large, pale staining, ovoid or spherical nuclei which have no obvious nucleoli and a watery cytoplasm of low density which extends into the processes. At electron microscopical level the nuclei contain fine, sparse chromatin granules lying
adjacent to the nuclear envelope. Sparse, occasionally elongated mitochondria, few laminated membranes of rough endoplasmic reticulum, dense bodies (lysosomes) and bundles of intermediate filaments, stretching from one process through perinuclear cytoplasm to another, are present in the cytoplasm. Many elongated, usually thicker processes, are involved with the formation of conical foot-plates, (pedicles, sucker-feet or perivascular feet). Some of these extend to the surfaces of neurons, blood vessels and basement membrane at the surface of the CNS, (basal lamina under the pia mater).

Morphologically, two types of astrocytes can be distinguished. The large, star-shaped protoplasmic astrocytes commonly associated with grey matter, have cytoplasmic filaments and microtubules, interconnect by gap junctions and seem to have beaded margins which fit tightly into neighbouring structures. Although similar to protoplasmic astrocytes, fibrous astrocytes contain more filaments and glycogen and have fine, firm fibres in their cell bodies and processes. They are found in white matter and in a few areas of grey matter. The processes have a smooth outline, are longer, and show less frequent branching occurring at more acute angles. Gap and desmosomal type junctions exist between individual cells.

Astrocytes react to all noxae which damage neurons, and although less vulnerable than nerve cells and fibres, they
are more easily damaged than connective tissue. Stimuli such as necrotic brain tissue, degenerative changes, demyelination, neuroglial hyperplasia, edema and minor degrees of ischaemia may cause a variety of astroglial changes. These range from necrosis and disintegration of the cells to proliferation and new formation of fibrous processes, (glial scar formation, diffuse fibrous gliosis, sclerosis). Transitional stages may include regressive changes such as swelling of astrocyte cell bodies, breaking off of processes, increase in lipid granules, pyknotic, irregular nuclei and eventual disintegration. Progressive changes are marked by enlarged cell bodies, no loss of processes, larger, darker staining nuclei and the formation of giant swollen astrocytes. Such gemistocytic astrocytes have intact intermediate filaments in their processes, but none in the perinuclear, swollen cell bodies. Although all the astrocytes in such an area show similar changes, the degree of swelling is very variable. Elongated or piloid astrocytes are formed by the attachment of their processes to collagenous tissue associated with brain wounds.

Oligodendroglial cells

Oligodendroglia, commonly seen in white matter, have heterochromatic ovoid or spherical nuclei which are darker than those of neurons. Their plump cell bodies have a perinuclear rim of scanty, fairly dense cytoplasm which contains moderate amounts of rough endoplasmic reticulum,
mitochondria, microtubules, some free ribosomes and a small Golgi region. These cells have fine, tree-like processes which are shorter, fewer and non-branching in comparison to astrocytic processes.

Three types of oligodendrocytes occur in the developing brain, being large, light cells, small dark ones and cells of intermediate size and density. Only the dark cells are seen in adult CNS tissue, occurring in perivascular areas as well as directly adjacent to nerve cell bodies in gray matter, (perineuronal or satellite cells). In white matter they are found in rows between myelinated fibres (interfascicular), where each cell is involved in the formation of multiple myelin sheaths around several nerve fibres. In the PNS, cells known as Schwann cells, have a similar function.

Glial cell behaviour

During their development, glial cells proliferate and migrate to specific areas where they differentiate cytologically into mature cells. Astroglial and oligodendroglial cells in different stages of development have been demonstrated in various in vitro studies. Although Kennedy & Fok-seang (1986) showed that the proportions of developing glial cells were dependant on the tissue culture medium, this developmental potential, which is marked by the formation of radial glia, progressive myelination and gliofibrillogenesis, appears to continue.
throughout life. It represents a stereotypic response of the CNS to injury as a result of many different etiologies (De Armond et al., 1986). Proliferation and increased intracytoplasmic differentiation of astrocytes as well as the induction of a wide spectrum of neoplasms including astrocytomias, has been demonstrated by in vitro stimulation with glial growth factor or chemicals such as dibutyryl cyclic AMP, dexamethasone or benzo(a)pyrene. GFAP has also been synthesized in vitro by using messenger RNA from a human glioma cell line. As glioma cells are capable of adapting their cytoskeleton to their microenvironment, contact with dense collagenous tissue appeared to be an important factor to induce increased production of GFAP by adjacent glial cells. Other findings include decreased GFAP production by glioma cells along with increased expression of fibronectin, a relatively slow turnover of glial intermediate filaments in vivo in contrast to a rapid turnover previously indicated in vitro (De Armond et al., 1986) and changes in GFAP expression of some cell lines maintained by long-term tissue culture (Dewhurst et al., 1987).

1D.2 Classification of neuroglial tumours

Almost 50% of intracranial tumours, commonly referred to as gliomas, are derived from cells of central neurogenic origin. These intrinsic elements of the central nervous system consist of neuronal cells, neuroglial elements,
TABLE 1.01
CLASSIFICATION OF TUMOURS OF THE CENTRAL NERVOUS SYSTEM
(Zülch, 1979)

<table>
<thead>
<tr>
<th>I. TUMOURS OF NEUROEPITHELIAL TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Astrocytic:</td>
</tr>
<tr>
<td>Astroblastoma; Astrocytoma, fibrillary, protoplasmic,</td>
</tr>
<tr>
<td>gemistocytic, pilocytic, subependymal giant cell,</td>
</tr>
<tr>
<td>anaplastic (malignant).</td>
</tr>
<tr>
<td>B. Oligodendroglial:</td>
</tr>
<tr>
<td>Oligodendroglioma, benign, anaplastic (malignant);</td>
</tr>
<tr>
<td>Mixed oligo-astrocytoma.</td>
</tr>
<tr>
<td>C. Ependymal and choroid plexus:</td>
</tr>
<tr>
<td>Ependymoma, myxopapillary, papillary, malignant,</td>
</tr>
<tr>
<td>subependymoma; Choroid plexus papilloma, benign,</td>
</tr>
<tr>
<td>malignant.</td>
</tr>
<tr>
<td>D. Pineal:</td>
</tr>
<tr>
<td>E. Neuronal:</td>
</tr>
<tr>
<td>F. Poorly differentiated and embryonal:</td>
</tr>
<tr>
<td>Glioblastoma, sarcomatous, giant cell; Medullo-</td>
</tr>
<tr>
<td>epithelioma; Medulloblastoma, medullomyoblastoma,</td>
</tr>
<tr>
<td>desmoplastic; Primitive polar spongioblastoma;</td>
</tr>
<tr>
<td>Gliomatosis cerebri.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II TUMOURS OF NERVE SHEATH CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>III TUMOURS OF MENINGEAL AND RELATED TISSUES</td>
</tr>
<tr>
<td>IV PRIMARY MALIGNANT LYMPHOMAS</td>
</tr>
<tr>
<td>V TUMOURS OF BLOOD VESSEL ORIGIN</td>
</tr>
<tr>
<td>VI GERM CELL TUMOURS</td>
</tr>
<tr>
<td>VII OTHER MALFORMATIVE TUMOURS AND TUMOUR-LIKE LESIONS</td>
</tr>
<tr>
<td>VIII VASCULAR MALFORMATIONS</td>
</tr>
<tr>
<td>IX TUMOURS OF THE ANTERIOR PITUITARY</td>
</tr>
<tr>
<td>X LOCAL EXTENSIONS FROM REGIONAL TUMOURS</td>
</tr>
<tr>
<td>XI METASTATIC TUMOURS</td>
</tr>
<tr>
<td>XII UNCLASSIFIED TUMOURS</td>
</tr>
</tbody>
</table>

63
(astrocytes, oligodendrocytes and ependymal cells), and their primitive bipotential precursors.

Although the etiology of these gliogenous tumours is not clear, it is thought that they are the result of malignant proliferation of well differentiated adult elements, due to either a mutation in the genetic apparatus of the cell or the transformation of its genome by the introduction of extraneous oncogenic nucleic acid material.

The eventual classification of a particular tumour is based on clinical and radiological data, age and sex of the patient, topography of the lesion and the cytological and histological appearance of tissue samples. This includes general biologic characteristics such as malignancy, growth patterns, secondary and tertiary structures and tumour cell behaviour in tissue culture.

In an attempt to explain the morphologic complexity and diversity of these neoplasms, their embryonic derivation is used to align tumour types with various stages of cell ontogenesis in the CNS. Additionally, phenomena such as dedifferentiation and anaplasia may be responsible for rapid increases in tumour growth, thus influencing the morphologic character of tumour cell populations.

This has lead to modification of the historical concepts through the introduction of a system of grading from 1 to 4 in an ascending order of malignancy, as applied to
astrocytomas, oligodendrogliomas and ependymomas by Kernohan and Sayre (Rubinstein, 1972). According to the histological classification and grading of central nervous system tumours recommended by the World Health Organization (Zülch, 1979), most gliogenous tumours are found in group I as a result of their neuroepithelial origin (Table 1.01).

Mixed tumour cell populations in the glioma group are frequently encountered, probably derived from truly primitive embryonal neoplasms such as the medulloblastoma, which is capable of differentiating along more than one cell line (Roessmann et al, 1983). These mixed tumours, together with tumours such as glioblastomas, medulloepitheliomas, neuroblastomas and spongioblastomas, often present difficulties towards diagnosis, prognosis and classification within these concepts. Detection of astroglial elements in mixed neoplasms by means of immunolabelling for markers such as GFAP, may help to solve at least some of the diagnostic problems encountered (Rubinstein, 1982). However, the presence of trapped or reactive cells may lead to misinterpretations, thus requiring critical evaluation of results. These concepts also emphasize the utmost importance of representative sampling when collecting biopsy material (Rubinstein, 1972).
1D.3 Distribution of GFAP and vimentin in normal and neoplastic glial tissue

Immunohistochemical studies by a large number of workers have established a typical astroglial distribution for GFAP. Their findings are summarized in Table 1.02 and show that cytoplasmic GFAP positivity is present in varying amounts in most normal and neoplastic cells of astrocytic origin. Coexpression of vimentin and GFAP has been shown in most astroglial tissues.

Of the various controversies that have emerged from this data, many can be traced back to fundamental concepts of histology such as pluripotential differentiation of precursor cells, multiple cell populations involved with neoplasms, antigen expression associated with the functional state of a cell as well as the degree of sensitivity and the variability of the immunolabelling methodology applied.

Commonly, different workers report controversial results on GFAP positivity in ependymomas, oligodendrogliomas and poorly differentiated tumours such as medulloblastomas and spongioblastomas. According to Duffy et al (1979), some GFAP positive cells are present in ependymomas and are thought to be mainly perivascular cells, cuboidal cells forming tubules or cavities as well as scattered neoplastic and reactive astrocytic cells. The absence of such cells in an ependymal tumour, as reported by
TABLE 1.02
DISTRIBUTION OF GFAP AND VIMENTIN
IN NORMAL AND NEOPLASTIC GLIAL TISSUE

<table>
<thead>
<tr>
<th>Normal cells</th>
<th>GFAP</th>
<th>VIMENTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>astrocytes, (fibrous and protoplasmic)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>reactive astrocytes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pineal astrocytes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>stellate cells of anterior pituitary, pituicytes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ganglionic cell layer, retina</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>ependymal cells</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>subependymal cells</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>choroid plexus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>oligodendrocytes, microglia, neurons</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>meningeal cells, perivascular mesenchymal cells</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neoplasms</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>malign human astrocytomas, low and high grade</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>astroblastoma, peri-vascular cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>glioblastomas</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>gliosarcomas: glioblastomatous areas</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>fibrosarcomatous areas</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ependymomas, low and high grade</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>poorly differentiated tumours</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>meningealomas</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>neurinomas</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>adenomas of hypophysis</td>
<td>-</td>
<td>?</td>
</tr>
</tbody>
</table>

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Tascos et al (1982), therefore indicates developmental and/or functional variation of the tumour cell population (Royds et al, 1986), thus questioning the validity of the general concept of ependymal cells being GFAP positive.

Similarly, variable GFAP positivity in oligodendrogliomas has been reported, while normal mature oligodendrocytes are considered to be GFAP negative (Rasmussen et al, 1980; Kumpulainen et al, 1983; Gullotta et al, 1985; Yung et al, 1985; Royds et al, 1986). Assuming that the presence of mixed tumours or trapped astrocytes had been excluded, Herpers & Budka (1984) have described GFAP positive oligodendrocytes in oligodendrogliomas occurring in areas of classical honeycomb texture within a vascular stroma. Although the clinical significance has not been determined, the existence of such a transitional cell type and/or tumour is indicated by the observation of gradual morphological transition from such cells to gemistocytic astrocytes in some tumours. These workers have proposed the use of the term gliofibrillary oligodendrocyte (gfoc) for these immunoreactive cells, suggesting the return to fetal behaviour by some neoplastic oligodendrocytes, thus resembling the GFAP expression by myelin forming glia during normal development (Choi & Kim, 1984).

Poorly differentiated tumours such as medulloblastomas, spongioblastomas and neuroblastomas, are characterized by the presence of many small cells of which only a few show
positivity for GFAP, thus indicating their pleuripotential stage of development (Rasmussen et al, 1980; Tascos et al, 1982; Carlei et al, 1984; Yung et al, 1985).

GFAP uptake by stromal cells (Royds et al, 1986) and GFAP positivity found in both giant cells and histiocyctic infiltrate of giant cell astrocytomas (Tsao et al, 1983; Gullotta et al, 1985), are aspects which may lead to misinterpretation of labelling results. Similarly, some reports indicate that necrosis has no obvious effect on GFAP concentrations (Rasmussen et al, 1980) while others indicate binding of immunoglobulin labelling reagents to swollen-bodied astrocytes in the absence of sufficient blocking procedures (Paasivuo & Saksela, 1983).

The considerable amounts of GFAP in reactive glia found in craniopharyngioma and haemangioblastoma (Rasmussen et al, 1980), as well as the indication that at least some of the heterogeneous group of stromal cells in haemangioblastomas are astrocytes (Royds et al, 1986), indicates that the presence of trapped and/or reactive astroglia due to their response to injury and disease, cannot be ignored. Here, the GFAP concentration (Herpers et al, 1984) and the difference in the distribution of GFAP (in cellular processes) and vimentin (juxta-nuclear) in reactive gliosis as observed by Herpers et al (1986), may contribute to solve the issue. Based on immunohistochemical observations (Duffy et al, 1979) and the determination of GFAP
concentrations by quantitative electrophoresis in normal, reactive and neoplastic astrocytes, (Rasmussen et al, 1980), a descending order of GFAP positivity ranging from reactive fibrillary astrocytes through astrocytoma, glioblastoma, normal fibrillary astrocytes to protoplasmic astrocytes, was established. Although Rasmussen et al (1980) could not support the theory due to possible differences of grading in areas of tumour specimens, this lead to the application of concepts relating GFAP concentrations to degrees of malignancy and differentiation (Herpers et al, 1984; Royds et al, 1986). More recently, characterization of mature and immature glia on the basis of differences concentration and coexpression of vimentin and GFAP (Schachner et al, 1984; Yung et al, 1985; Dahl & Bignami, 1986; Perentes & Rubinstein, 1987), have followed. However, no clear-cut differences between grade of malignancy and immunostaining pattern were seen by Luevano et al (1986), while a more recent report by Herpers et al (1986) showed no change in vimentin or GFAP expression with increasing anaplasia. A transition from vimentin to GFAP expression has been indicated by some authors (Pixley & De Vellis, 1984; Backhovens et al, 1987), while others propose two different, related, but yet unknown mechanisms that regulate the expression of these intermediate filaments (Goldman & Chiu, 1984). Therefore, these theories remain to be proved by future investigations.

Although the distinct biochemical and immunological
character of GFAP intermediate filaments and its value as potential astrocytic marker has been established and partially confirmed by application of various methods, controversial results due to the use of various polyclonal and monoclonal antibodies of commercial and non-commercial origin, differences in sensitivity of detection systems and application of more than one processing protocol on tissue samples from different sources, have emerged. These include low sensitivity and specificity of vimentin antisera in aldehyde fixed, paraffin-embedded tissues (Herpers et al., 1986; Leader et al., 1987), classification of different astrocytes based on the use of some monoclonal antibodies (Miller & Raff, 1984; Kennedy & Fok-seang, 1986) and variable sensitivity of some GFAP antisera (Eng, 1985).

Variable sensitivity and specificity of different detection and processing procedures is probably best illustrated by the considerable controversy that exists about the presence of GFAP in Rosenthal fibres. These structures are abnormal inclusions that occur within astrocytes and are characteristically associated with Alexander's disease (Borrett & Becker, 1985). While Gullotta et al. (1985) reported strong, partial or weak positivity in both the centre and peripheral areas of these structures, Papasozomenos (1983) and Towfighi et al. (1983) found no staining at all. Johnson & Bettica (1986) found little or no reactivity at light microscopical level, but were successful in obtaining convincing immunogold labelling at
electron microscopical level. A third group of workers believe that the central, amorphous core is GFAP negative, while the filamentous periphery is strongly positive (Tascos et al, 1982; Luevano et al, 1986). By raising various antibodies to a 22 kd Rosenthal fibre protein, Iwaki et al (1989) have shown variable Western blot reactivity for GFAP which could be eliminated by affinity purification of the particular reagents. Further studies from these workers indicate that this protein is αB-crystallin, a major lenticular protein which is also found in muscle and other tissues of most vertebrate species. Whether Rosenthal fibres represent breakdown products of GFAP or whether they consist of plasma proteins engulfed by astrocytes under pathologic conditions, are issues which remain to be resolved.

These findings correlate well with similar reports on neurofilament positive accumulations of fibrillary structures in neurons, excluding Hirano bodies (Gambetti et al, 1983). In Lewy bodies, peripheral positivity is seen at light microscopical level, while both core and periphery show positivity at electron microscopical level (Pappolla, 1986).

Reports of GFAP reactivity outside the CNS, include GFAP positive cells seen in peripheral nerve sheath tumours such as schwannomas and neurofibromas, pleomorphic adenomas of the salivary gland, in certain myoepithelial cells as well
as spindle-shaped cells in the perivascular region of hyalinized thick blood vessels. These findings may be due to a combination of factors indicating coexpression, cross-reactivity as well as general, poor characterization of antisera by some of the investigators and manufacturers involved.

The distribution of vimentin includes almost all tissues, thus limiting its diagnostic potential. However, it may be useful in some cases when combined with GFAP and other markers (Leader et al, 1987).

Proper interpretation of results obtained by carefully selected antisera, may therefore serve as diagnostic criteria for the characterization of most astroglial neoplastic cells in benign, malignant and mixed tumours, thus distinguishing them from other primary brain tumours as well as mesenchymal and metastatic neoplasms (Duffy et al, 1979). Particular cell types can be identified independently of morphological criteria, showing close correlation with classical methods such as Weigert's stain for astroglia and Cajal's silver nitrate methods for neurofibrils, (Dahl & Bignami, 1985). However, Gullotta et al (1985) emphasize that GFAP is not astrocyte specific, but a glial specific protein. These workers also state that its concentration is not a reliable parameter for prediction of degrees of malignancy and that the pathogenetic problems of undifferentiated tumours cannot be
solved by its expression. Single cell positivity should be treated with utmost care, while small samples are generally not suitable for diagnostic purposes. Therefore, a cautious approach to the histopathological significance of glial intermediate filaments in brain tumours and neurodegenerative disorders is expressed by Trojanowski (1986), indicating that various hypotheses still need further investigation.

1E ULTRASTRUCTURAL IDENTITY OF GLIAL INTERMEDIATE FILAMENTS

1E.1 Post-embedding immunoelectron microscopical characterization of glial intermediate filaments

The typical fibrillar staining pattern for GFAP at light microscopical level (Eng, 1985) was associated immunoelectron microscopically with intermediate filaments and diffuse cytoplasmic positivity in astrocytic processes. This was done on perfused tissue by means of an indirect pre-embedding immunoperoxidase procedure using DAB as an electron dense label (Schachner et al, 1977; Maunoury et al, 1979; D'Amelio et al, 1985). Although these results were an improvement on earlier reports lacking proper illustrations, the typically diffuse DAB staining pattern and relatively poor quality of the ultrastructure remained unsatisfactory.

A major contribution was made by Gräber & Kreutzberg (1985)
when they succeeded in demonstrating GFAP immunoreactivity on astrocytic intermediate filaments of rat cerebellum. This was achieved by a post-embedding technique using LR White resin following intracardial perfusion with formalin, post-fixation in glutaraldehyde as well as osmium tetroxide. Impregnation with resin was done in the cold and thin sections were mounted on Formvar grids. Monoclonal antibodies were applied and detected by using an indirect immunogold technique on grid. No etching was required and reasonably good morphology as well as significant labelling was obtained. As previous attempts using epon or araldite embedded tissues had failed, these results proved that GFAP could be demonstrated immunoelectron microscopically on post-embedded tissue by using an acrylic resin. Lipid rich cell components such as membranes and myelin sheaths, were less well preserved. The clear definition obtained with gold labels did not show the non-specific staining of microtubules and outer membranes of mitochondria as was seen with diffuse DAB labelling in pre-embedding procedures.

This principle was adapted by Goto et al (1987) to obtain double labelling for myelin basic protein and GFAP on the same thin section. They confirmed the results obtained by Gräber & Kreutzberg (1985) and demonstrated that the thin sections were not totally penetrated by labelling reagents, thus allowing different labelling protocols on each side of a section or alternatively double density labelling by
immersion of the grid into labelling solutions. Vimentin was also demonstrated while combinations of pre- and post-embedding procedures were successfully attempted. It appeared that incompletely dehydrated specimens required more effective fixation or other stabilizing methods while best labelling results were obtained in the absence of osmium tetroxide.

Using Lowicryl K4M embedded cultured fetal astrocytes, Renau-Piqueras et al (1989) obtained immunogold labelling results comparable to those found by Goto et al (1987). The limitations of post-embedding immunodetection of GFAP in epoxy embedded material were also recognized and documented by Johnson & Bettica (1989), who managed to demonstrate GFAP in Poly/Bed 812 embedded specimens of an Alexander's disease brain biopsy (Bettica & Johnson, 1990). These workers used a modified mixture of the resin which was partially removed from the grid before immunolabelling. Their procedures included extensive pretreatments with sodium (m) periodate, hydrogen peroxide and protease, modified washing solutions, prolonged antisera incubations and the application of 5 nm gold probes. No reactivity for vimentin was found.

After almost a decade, post-embedding immunodetection of limited sensitivity for GFAP in glial intermediate filaments has been achieved by modified, often cumbersome procedures. The presence of aldehyde fixation, but to a
greater extent osmium tetroxide and resin, seem to be the major obstacles for tissue penetration by labelling reagents. This implies that a standardized protocol is not yet available for routine application to immersion fixed, human tissues.
Chapter 2

MATERIALS AND METHODS

The various methods and techniques applied to prepare, label and evaluate rat and human brain tissue specimens are summarized in Table 2.01.

<table>
<thead>
<tr>
<th>TABLE 2.01</th>
<th>PREPARATION, LABELLING AND EVALUATION OF SPECIMENS FOR SEMI-QUANTITATIVE DETERMINATION OF RELATIVE GFAP AND VIMENTIN CONCENTRATIONS IN GLIAL INTERMEDIATE FILAMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TISSUE</strong></td>
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<tr>
<td>Section 2A</td>
<td>Sources and selection of</td>
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<tr>
<td></td>
<td>NORMAL RAT BRAIN</td>
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<td></td>
<td>HUMAN BRAIN BIOPSIES</td>
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<td><strong>TISSUE PREPARATION</strong></td>
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<td>for</td>
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<td>ELECTRON MICROSCOPY</td>
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<td>2C</td>
<td>LIGHT MICROSCOPY</td>
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<tr>
<td><strong>IMUNOLABELLING</strong></td>
<td></td>
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<tr>
<td>Section 2D</td>
<td>IMMUNOELECTRON MICROSCOPY</td>
</tr>
<tr>
<td>2E</td>
<td>IMMUNOHISTOCHEMISTRY</td>
</tr>
<tr>
<td><strong>DATA EVALUATION</strong></td>
<td></td>
</tr>
<tr>
<td>Section 2F</td>
<td>SEMI-QUANTITATIVE ASSESSMENT OF LABELLED GLIAL INTERMEDIATE FILAMENTS</td>
</tr>
</tbody>
</table>
Each of these steps influence antigen availability in their own right, thereby contributing to the overall effect of processing on the sensitivity of immunodetection. As single factors can only be evaluated in combination with other processing factors, various processing protocols, each differing from the others in one or two aspects, were evaluated to determine the effect on both quality of the ultrastructure and immunoreactivity obtained.

The routine processing protocol for electron microscopy was used as a standardized basis for comparison. Optimal conditions were determined for the basic immunolabelling techniques prior to application. Based on the results and findings obtained from these experimental phases, a modified, standardized protocol was established and applied to randomly selected histopathological human specimens.

2A TISSUE

2A.1 Sources and preparation of rat tissue

For the immunoelectron microscopical localization of glial intermediate filaments, astrocytes present in the habenular nuclei lateralis and medialis (Pellegrino et al., 1979) of normal rat brain were chosen as the target cell type (figure 2.01).
Habenular nucleus [case R] GFAP [x 480]

Immunohistochemistry (ABC method) demonstrates GFAP positive astrocytes and their processes (arrow-heads), while neurons (arrows) and oligodendrocytes (squares) show no reactivity. This area was small enough for microscopical orientation after initial processing and could be identified macroscopically in the fresh state.

Several normal male Wistar rats (case R) were obtained from the department of Anatomical Pathology, Tygerberg Hospital, as well as the departments of Biochemistry and Pharmacology, US Medical School. The rats were killed by decapitation before their brains were removed from the skull with minimum delay. To allow cutting without trauma to the normally soft brain tissue, the rat brains were
chilled in cold fixative for 5 minutes. The habenular nuclei were then exposed by means of a coronal cut through the ventral surface of the brain between the optic chiasm and the infundibulum (Pellegrino et al., 1979; Fig 2.02).

NORMAL RAT BRAIN

FIGURE 2.02

[Ventral view of normal rat brain showing the coronal cut line (---) between the infundibulum (arrow) and the optic chiasm (arrow-head).]
Habenular nuclei as exposed by the coronal cut through the brain as shown in figure 2.02.

Haematoxylin stain (left) shows the general morphology of the dentate gyrus (square), habenular nuclei medialis (arrow-head) and lateralis (L).

Myelinated fibres of the stria medullaris thalami (arrow) are illustrated by the Cresyl violet / Luxol blue stain (right).

One or two 2 mm thin slices were cut off the exposed areas, after which the habenular nuclei were removed from the surrounding brain tissue. The tissue was then diced into 1-1.5 mm x 1-1.5 mm cubes and left in the appropriate fixatives.
2A.2 Criteria for the selection of human tissue

Case reports indicating normal, reactive and/or neoplastic glial tissue were drawn from the files of the Neuropathology unit at Tygerberg Hospital. Selection of biopsy material depended on the availability of fresh tissue, routinely processed epoxy embedded specimens or glutaraldehyde fixed tissue which had not been embedded during the initial routine investigation. Final selection of tissue was determined by the presence of intermediate filaments on immunogold labelled, thin sections of each specimen.

2A.3 Sources, preparation and description of selected human brain tissue biopsies

Fresh human glioma biopsies were obtained after neurosurgery from theatres of the Tygerberg Hospital in Parow (cases I, J and K), as well as the City Park Medical Centre (case L) and Volkshospital (case M) in Cape Town. The tissue from case L was snap frozen before being placed into fixative. Half of each biopsy was removed for paraffin embedding. The remaining tissue was cut into cubes of about 1 mm x 1 mm and replaced into the fixative in which the tissue had been collected.

A further 11 human brain biopsies (cases B - H and N - Q) were selected from the Neuropathology files of this institution. For these cases, the case reports indicated
the presence of glial intermediate filaments along with the availability of fixed, unprocessed tissue. These remaining fractions of tissue were divided to expose the largest possible area of each biopsy. Where sufficient tissue was available, one half was processed and paraffin embedded for light microscopy. This served as a mirror image for the tissue used for the immunoelectron microscopical investigation. Remaining tissue was cut into small cubes (1 mm x 1 mm) and stored in fresh fixative awaiting further processing.

Cases A and S were chosen on the basis of a normal or neoplastic astroglial component being indicated by the histopathological report. Intermediate filaments were abundant on thin sections cut from routinely fixed, epoxy resin embedded tissue. For these cases no tissue was available for embedding in acrylic resin.

In addition to the normal brain histology of the rat habenular nucleus, the heterogeneous, randomly selected group of human brain biopsies represented different histopathological conditions. These include various neoplastic and degenerative disorders such as astrocytomas, malignant gliomas, a meningioma and a rare case of Alexander's disease. A brief summary of histopathological and some clinical data for each individual case, is presented in Table 2.02.
<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Anatomic site</th>
<th>Age</th>
<th>Sex</th>
<th>Race</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
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<td>(L) parieto-occipital</td>
<td>25</td>
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<tr>
<td>B</td>
<td>ASTROBLASTOMA</td>
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<tr>
<td>C</td>
<td>ASTROCYTOMA</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>Glioblastoma</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>G</td>
<td>GLIOMA</td>
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<td>GLIOMA</td>
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<tr>
<td>J</td>
<td>GLIOMA</td>
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<td>K</td>
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<tr>
<td>L</td>
<td>GLIOMA</td>
<td>site unknown</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>M</td>
<td>GLIOMA</td>
<td>site unknown</td>
<td>*</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>N</td>
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<tr>
<td>O</td>
<td>MIXED GLIOMA</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>DISEASE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>SSPE Gliosis</td>
<td>cerebrum</td>
<td>19</td>
<td>Female</td>
<td>White</td>
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</tbody>
</table>
2B TISSUE PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

2B.1 Summary of processing protocols

A basic protocol for preparation of tissue for transmission electron microscopy (TEM) consists of tissue processing (primary and secondary fixation, followed by dehydration and impregnation of the tissue with resin) and embedding of the specimen in resin. This allows cutting of thin sections suitable for viewing with the electron microscope.

The combinations of processing protocols that were used to prepare specimens for TEM, are summarized in Table 2.03. The chronological order of protocols starts with the routine technique for epoxy embedded material as used in this institution. Various primary and secondary fixation procedures were applied as modifications, first with epoxy resin and then with acrylic resin. Complete, as well as partial dehydration was applied to acrylic resin procedures.

Finally, the modified technique was standardized by using the basic fixation protocols, 96% dehydration and an improved impregnation protocol for the acrylic resin.
### TABLE 2.03
#### SUMMARY OF PROCESSING PROTOCOLS APPLIED

<table>
<thead>
<tr>
<th>FIXATION and DEHYDRATION</th>
<th>RESIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,5% glutaraldehyde &gt;24h, Osmium, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &gt;24h, None, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>1% glutaraldehyde &lt;24h, Osmium, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>1% glutaraldehyde &lt;24h, None, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>1% glutaraldehyde &gt;24h, None, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>1% glut/picric acid &lt;24h, Osmium, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>1% glut/picric acid &lt;24h, None, 100%</td>
<td>Epoxy 18h</td>
</tr>
<tr>
<td>1% glut/picric acid &lt;24h, None, 100%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>1% glut/picric acid &lt;24h, None, 70%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>1% glut/picric acid &lt;24h, Osmium, 70%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>1% glut/picric acid &lt;24h, Osmium, 100%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &lt;24h, None, 100%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &lt;24h, None, 70%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &lt;24h, Osmium, 70%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &gt;24h, None, 70%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &gt;24h, Osmium, 70%</td>
<td>Acrylic 18h</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &gt;24h, Osmium, 96%</td>
<td>Acrylic 48h 4°C</td>
</tr>
<tr>
<td>2,5% glutaraldehyde &gt;24h, Osmium, 96%</td>
<td>Acrylic 48h 0°C</td>
</tr>
</tbody>
</table>

### KEY to TABLE 2.03

**PRIMARY FIXATIVES:** Buffered 2,5% glutaraldehyde or 1% glutaraldehyde with or without picric acid.

**SECONDARY FIXATION:** 1,5% buffered osmium tetroxide.

**DEHYDRATION:** 100%, 96% or 70% with graded ethanol.

**RESIN:** Spurr’s epoxy resin (18 hour impregnation) or London White acrylic resin, medium grade (18 hours at room temperature, 48 hours at 4°C or 48 hours at 0°C.)
2B.2 **Primary fixation of tissue**

A routine protocol for primary fixation i.e. 2.5% glutaraldehyde (Taab Laboratories, Reading, England) in 0.1 M phosphate buffered saline pH 7.4 for at least 4 hours at room temperature, was selected as the standard procedure. For tissue collected in the fresh state, the period of fixation was recorded accurately (rat tissue [R] and cases I - M). For biopsies received in fixative, an approximate figure was calculated to represent the fixation time, including the storage period in glutaraldehyde at 4 - 8 °C (cases A - H, N - Q and S). Mild primary fixation was characterized by application of lower concentrations of glutaraldehyde (1%) and/or 1% buffered glutaraldehyde, picric acid and PBS pH 7.4 (BGPA) at 4 °C according to the method of Newman et al (1983). Alternatively, two rats were perfused with normal saline, followed by glutaraldehyde or BGPA. This procedure was not pursued due to the occurrence of extensive swelling of subcellular structures (poor ultrastructure) and being impractical for use with human tissue.

2B.3 **Secondary fixation of specimens**

Two buffer washes (PBS 0.1 M pH 7.4) preceded secondary fixation in osmium tetroxide (O - 5500, Sigma Chemical Co., St Louis, USA). A 1 - 1.5% solution of osmium tetroxide in veronal-acetate-HCl (Palade's) buffer for 1 hour at room temperature was applied to some of the specimens. Where
secondary fixation was not done, the tissue remained in buffer awaiting further processing. Whilst all the available tissue from cases A and S was fixed in osmium tetroxide, none of the tissue from case K was exposed to secondary fixation. For the remaining cases, half the primarily fixed tissue was exposed to secondary fixation in osmium tetroxide. After a further two buffer washes in PBS (or 3 changes of distilled water), all tissue was ready for one of two procedures followed for dehydration and impregnation with resin.

2B.4 Processing of specimens for embedding in epoxy resin

Standardized methods for dehydration and embedding of tissue in Spurr's epoxy resin (Taab Laboratories, Reading, England) were used. Incubation in 50% ethanol for 10 minutes and 2% uranyl acetate in 70% ethanol (optional), was followed by complete dehydration of tissue with graded ethanols. Two changes of 5 minutes each with 70 - 75% and 80 - 96% ethanol, then 2% uranyl nitrate in 96% ethanol for 15 minutes, preceded incubation in 2 changes of 100% ethanol (dried with sodium sulphate) of between 10 and 30 minutes each. This was followed by a third change of absolute alcohol (alternatively acetone) for 30 minutes before impregnation of the tissue with resin. This was done at room temperature by a 1:1 mixture of 100% ethanol and resin for 1 - 1.5 hours, (alternatively with a 1:1 mixture of acetone and resin for 30 minutes. Further impregnation
was accomplished by two changes of pure resin for 1 - 1.5 hours with the last 30 - 60 minutes of each change at 60 °C. Alternatively, addition of an equal volume of pure resin to the acetone / resin mixture for overnight incubation, was followed by a further change in fresh resin for 3 hours under vacuum at room temperature. Finally, tissue was placed in gelatin capsules (Size 0, Lilly SA) or polyethylene (Taab) capsules, filling the capsule to the rim with resin.

Spurr's epoxy resin was prepared according to the manufacturer's specifications and heat cured at 60 °C for 18 hours. All washes and incubations with reagents and resin were carried out in processing bottles at room temperature, the reagents being removed from the bottles by vacuum suction after completion of incubation times. A Taab rotator was available for rotation of processing bottles whilst using the alternative method as described above.

2B.5 Processing of specimens for embedding in acrylic resin

Partial or complete dehydration of tissue was achieved with graded ethanols before incubation with resin. All washes and incubations with reagents and resin were carried out in processing bottles at room temperature, the reagents being removed from the bottles after completion of incubation times with vacuum suction. All tissue embedded in acrylic resin, was exposed to graded ethanols (2 changes of 5 minutes each in 30% and 50% ethanol) and a saturated
solution of uranyl acetate in 70% ethanol for 30 minutes (optional). Further partial dehydration was limited to 70% ethanol (2 changes of 1 hour each, or 96% ethanol (2 changes of 5 minutes each). Alternatively, tissue was completely dehydrated through 2 changes of 5 minutes each in 70% and 80% ethanol, 2 changes of 10 minutes each in 90% and 96% ethanol and 2 changes of 30 minutes each in absolute (100%) ethanol.

Following dehydration, the tissue was transferred to LR White medium grade resin (London resin Co, Basingstoke, Hampshire) for an initial incubation of one hour at room temperature. Although care was taken to remove all resin from the surface of the tissue blocks before adding fresh resin, spontaneous curing of osmicated specimens occurred during the second incubation of 16 hours (overnight). The Taab rotator that was used for rotation of processing bottles, did not prevent spontaneous curing of the resin in these specimens. A final 1 hour incubation in fresh resin preceded embedding of these specimens.

Eventually prolonged, low temperature impregnation of tissue was attempted, first at 4 - 8 °C and finally at 0 °C. The initial incubation was extended to 3 hours, while during the second incubation of at least 45 hours, the resin was changed twice, first after 3 hours and again within 20 hours during the following day.

Tissue was embedded with resin in gelatin capsules (Size 0,
Lilly SA) and heat cured in the oven at 55 - 60 °C for 24 hours. Initial precautions to exclude oxygen from the acrylic resin by vacuum embedding or embedding under liquid nitrogen, proved to be unnecessary, provided that the gelatin capsules were filled to the rim and sealed off by their appropriate caps.

2B.6 Selection and sectioning of specimens for TEM

Thick sections were used to select the most suitable, representative blocks for each specimen. The presence of intermediate filaments on thin sections was used as the ultimate criterium for this selection.

Blocks were trimmed by hand using a steel blade, or with a mechanical trimmer (TM 60, Reichert, Austria) to expose the cutting surface. Thin (70 nm / gold) sections were cut on a LKB ultratome III using glass knives made on a LKB Type 7801B knifemaker. These sections were heat stretched (LR White resin) or exposed to chloroform fumes (Spurr's resin) before being picked up on 200 mesh copper grids (routine electron microscopy) or 300 mesh nickel or 400 mesh gold grids for immunoelectron microscopy. No coating of sections or additional support was given. Grids were kept dry in a place free of dust. Some grids with LR White resin sections were dried in a dessicator under vacuum to limit contamination of the electron microscope column by excessive water retained in the resin.
Due to the hydrophilic nature of the resin, stains and coverslip mounting media applied to thick sections often produced shrinkage and metachromasia. Smaller, additional thick sections, cut after thin sectioning, often improved the quality of the thick section, allowing better light microscopical correlation with the area selected on the thin sections.

2B.7 Staining and TEM of thin sections

Uranyl acetate and lead citrate stains were applied to all grids used for electron microscopy. Saturated (2%) uranyl acetate or uranyl nitrate in 50% ethanol and Reynold's lead citrate were used. Grids were floated on 50 μl drops of reagent placed on dental wax sheets covered by a petri dish. A minimum of 5 minutes was allowed for each stain, followed by two dip washes in distilled water and an additional dip wash in 50% ethanol immediately after uranyl acetate staining. Possible precipitation of reagents was prevented by microfiltration or low speed centrifugation of reagents before use.

Specimen grids were viewed with a Hitachi H600-2 or Philips 420 electron microscope, using AGFA or Kodak film for photography at original magnifications ranging from x 3000 to x 90 000. Negatives were enlarged (x 3) to obtain prints on various grades of paper. Different control voltages and condenser lens values of the particular electron microscopes, as well as various grades of paper and
exposure times for photography were applied to enhance contrast lost due to etching or the omission of osmium tetroxide fixation.

2C PREPARATION OF TISSUE FOR LIGHT MICROSCOPY

2C.1 Processing of specimens for paraffin embedding

Fresh tissue was immersed in 10 % buffered formalin for at least 1 - 24 hours, then completely dehydrated through graded ethanols (9 hours). This was followed by 2 changes of 1 hour each in xylol and impregnation in paraffin wax for 3 hours at 58 °C. The specimens were then orientated and embedded in paraffin wax. Some tissue from each specimen fixed for electron microscopy (section 2B.), was dehydrated, impregnated and embedded in paraffin wax according to the same protocol. Routinely prepared formalin fixed, paraffin embedded tissue, processed as above, was available for cases drawn from the files of this institution.

C2.2 Sectioning of paraffin embedded specimens

Sections of 4 - 6 μm were cut with a microtome, floated on a warm water bath and picked up on glass slides. Gelatin or poly (L) lysine (Sigma P 1399, mol.wt> 150 000) coated slides were used for sections intended for immunohistochemistry. Before labelling, sections were incubated at 58 °C for at least 3 hours, deparaffinized in 2 changes of xylol and rehydrated in descending grades of
ethanols to water.

2C.3 Sectioning of resin embedded tissue

Thick (1 μm) epoxy and acrylic resin sections were cut on a LKB ultratome III, using glass knives made on a LKB Type 7801B knifemaker. The sections were transferred to drops of water on glass slides, dried on a warmplate or air dried before staining (section 2F). Epoxy resin was removed from the sections by incubation for 30 - 60 seconds in a solvent containing 5 g sodium, 100 ml methanol and 50 ml benzene (sodium methoxide). After washing in two changes of methanol, then distilled water, slides were ready for immunolabelling and / or other stains. Acrylic resin could not be removed from the sections.

2C.4 Stains used for light microscopy

<table>
<thead>
<tr>
<th>Stain</th>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cresyl violet</td>
<td>Acrylic, Paraffin</td>
<td>Cox G, 1977</td>
</tr>
<tr>
<td>Haematoxylin (Mayer's)</td>
<td>Acrylic, Paraffin</td>
<td>Stevens A, 1977</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Acrylic, Paraffin,</td>
<td>Stevens A, 1977</td>
</tr>
<tr>
<td>Luxol blue</td>
<td>Acrylic, Paraffin</td>
<td>Cox G, 1977</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Acrylic, Epoxy</td>
<td>Robinson G, 1977</td>
</tr>
<tr>
<td>PTAH</td>
<td>Paraffin</td>
<td>Cox G, 1977</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Acrylic, Epoxy,</td>
<td>Robinson G, 1977</td>
</tr>
</tbody>
</table>
Staining procedures other than immunolabelling that were applied to some deparaffinized sections as well as epoxy and acrylic resin sections, are summarized in Table 2.04.

2C.5 Viewing and photography of LM specimens

Slides were viewed with a Zeiss Standard microscope and photographed with a MC83 photomicrographic camera using PAN F (Ilford) film. An original magnification of x 250 was selected for all figures presented in chapter 3.

2D IMMUNOGOLD LABELLING

The labelling protocol for thin sections on grid included etching (optional) and blocking of non-specific labelling prior to incubation with primary antiserum. Bound antibodies were visualized with the appropriate "auroprobes".

The initial concentrations and dilutions for the various reagents were obtained from previously standardized protocols, manufacturer specifications or as proposed by other workers. These were tested and modified where necessary.

2D.1 Apparatus, washing and precautions

Labelling procedures were performed on batches of ten to fifteen grids in microtitre plates at room temperature. Following an initial rinse with 20 ml distilled water, grids were ready for incubation. Using alternative wells of
the microtitre plate, grids were either floated (first rinse and etching steps), or immersed (all other incubations) in 20 μl droplets of the appropriate reagents.

A 0.5 M TRIS stock buffer containing 0.5 M NaCl, was prepared and brought to pH 7.2 with HCl. Washing and blotting of grids was done with TRIS wash buffer at pH 7.2 (TRIS stock diluted 1:10 with distilled water) during transfer between reagent incubations to prevent sections from drying out. Grids were handled with a fine forceps and washed with a gentle jet of appropriate washing solution produced from a 20 ml non-grease syringe. Filter paper or fibre free blotting paper was initially used to blot grids after washing steps, but was eventually replaced by paper towel. The superior absorption qualities of the paper towel allowed more efficient removal of fluid from the forceps and grids. The increased flow of reagents away from the grid minimized contamination with dust particles, fibres or remaining reagents. Microtitre plate lids were used during incubations, used pipette points were discarded immediately and the forceps was cleaned before contact with different grids and reagents to prevent possible contamination.

2D.2 Etching

Three different approaches to etching were investigated.

Firstly, etching was done with a saturated solution of sodium (meta) periodate in distilled water as proposed by
Bendayan & Zollinger (1983). This reagent was applied to both, but not all epoxy and acrylic embedded specimens. Different times of incubation ranging from 10 to 180 minutes, showed that a 30 minute incubation was preferable.

Secondly, a 5 minute incubation with 5% NaOH was used, either alone or in combination with sodium (meta) periodate. This approach was not pursued as NaOH caused dislocation of sections from the grids, thus disrupting further labelling steps.

Finally, etching was omitted as it did not improve immunogold labelling of acrylic embedded specimens. This is in accordance with the findings of other workers (Causton 1984; Newman & Jasani, 1984a).

2D.3 Blocking of non specific labelling

A solution of 1% BSA (bovine serum albumin, fraction V, BDH Chemicals Ltd, Poole, UK) in TRIS stock buffer or distilled water, was applied for 30 minutes to block non-specific binding of labelling reagents to the sections. Grids were blotted but not washed prior to incubation with primary antiserum.

2D.4 Primary antiserum

Polyclonal rabbit anti bovine GFAP (DAKO A561), was used throughout the study on all specimens labelled for GFAP. Various dilutions ranging from 1:50 to 1:10000 were
tested, showing that a dilution of 1:1000 was optimal. Incubations were done at room temperature for 3 hours or overnight at 4 °C.

Little or no immunoreactivity was obtained with undiluted monoclonal mouse anti GFAP (Amersham RPN 1111). No other suitable monoclonal antibodies were available.

Monoclonal mouse anti vimentin (DAKO M725) was applied at a dilution of 1:100 for 3 hours at room temperature on specimens that were not etched.

All the antisera used were diluted with TRIS stock buffer containing 0.5% BSA. The selection of specimens provided inbuilt positive (glial intermediate filaments) and negative (all other subcellular organelles) control tissue for each labelled section. Absorption controls were not done for the antisera used as these reagents were already tested and characterized by the suppliers. Additional reagent negative controls were performed for each labelling batch. These consisted of substitution of primary antiserum with diluting buffer, normal serum or an antiserum from another species.

2D.5 Immunogold probes

Following a 20 ml wash with TRIS wash buffer and a 5 ml wash with TRIS wash buffer containing 0.1% BSA at pH 7.2, grids were incubated for 5 minutes with TRIS stock buffer brought to pH 8.2 using NaOH. This solution contained 1.0%
BSA.

Vimentin antibodies were visualized by goat anti mouse immunoglobulins conjugated to 10 nm colloidal gold particles (GAM 10). This probe was also used in combination with the monoclonal anti GFAP reagent.

Similarly, 15 nm colloidal gold particles conjugated to goat anti rabbit immunoglobulins (GAR 15), were used to visualize bound rabbit anti GFAP antiserum. Both "Auroprobes", obtained from Janssen Life Sciences Products (Belgium), were diluted 1:15 with TRIS stock buffer brought to pH 8.2 with NaOH, containing 1.0% BSA. The grids were immersed in this reagent for 1 hour at room temperature. At dilutions of 1:10 or lower, increased non-specific deposition of gold particles occurred.

In addition, an alternative immunogold probe, prepared according to the method of Frens (Roth, 1982), was used on some specimens. This reagent, Protein A gold 20 nm (PAG20), was obtained by conjugation of Protein A (Staphylococcus aureus, Cowan strain from Sigma Chemical Co., St. Louis, USA) to 20 nm colloidal gold particles prepared from a solution of gold chloride (Merck 1582, Darmstadt, West Germany) and aqueous sodium citrate.

The labelling process was completed by a 5ml TRIS 1% BSA wash and a 20 ml wash with TRIS wash buffer, both at pH 7.2.
IMMUNOHISTOCHEMISTRY

Immunohistochemistry was done on paraffin embedded material as well as "thick sections" of resin embedded tissue. All steps were performed at room temperature. Phosphate buffered saline (PBS) 0,5 M at pH 7,4, was used for the preparation of various reagents as indicated.

2E.1 Etching

No etching was done with proteolytic enzymes such as trypsin or pronase.

2E.2 Blocking of endogenous peroxidase

Endogenous peroxidase activity was blocked with 2% peroxide in methanol for 20 minutes.

2E.3 Blocking of non specific binding

A solution of 5% non fat milk in 0,5 M PBS, pH 7,4 was prepared to block non-specific binding of reagents to the tissue sections. This solution, at a concentration of 0,5%, was used for dilution of method reagents.

2E.4 Primary antisera

Incubation with primary antisera was done at room temperature for 30 minutes. Polyclonal rabbit anti bovine GFAP (DAKO A561, dilution 1 : 1000) and monoclonal mouse anti vimentin (DAKO M725) at a dilution of 1:100 were used. No satisfactory labelling was achieved with a
monoclonal mouse anti GFAP (Amersham RPN 1111, dilution 1:1).

Additional immunohistochemistry was performed with antibodies to S100 (DAKO Z311, dilution 1:500) and NSE (DAKO A589, dilution 1:500).

Negative reagent controls were performed for each block by substituting primary antisera with the diluting solution (0.5% non fat milk in 0.5 M PBS, pH 7.4). Positive control sections (positivity previously recorded for each antiserum), were included in each batch to ensure optimal performance of the reagents utilized.

2E.5 ABC method

Horse anti mouse biotinylated immunoglobulins (BA 2000) and goat anti rabbit biotinylated immunoglobulins (BA 1000) were used to link the primary antisera to the Avidin-Biotin-Peroxidase complex (PK 4000), applied according to the specifications of the manufacturer, (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine (DAB) was the chromogen used for the visualization of ABC complexes. This was followed by counterstaining in Mayer's Haematoxylin for 5 minutes. Slides were washed, dehydrated and mounted with DPX.
2F DATA EVALUATION

2F.1 Protocol and precautions for evaluation of immunogold labelling

Labelled specimens were viewed electron microscopically searching for intermediate filaments as well as areas showing localization of gold particles. Micrographs were taken in order to document intermediate filaments, positively labelled structures, the particular area for correlation with light microscopy and the overall quality of the ultrastructure.

A simple labelling evaluation system was devised whereby transparent graph paper was placed on contact and/or enlarged prints for counting gold particles in 1 cm² blocks. Characteristic bundles of glial intermediate filaments were chosen for evaluation of immunogold labelling, as single intermediate filaments could not be counted effectively with this method. All other subcellular structures such as the nucleus, myelin sheaths, neurofilaments, mitochondria, lysosomes and neuropil were considered as built in negative control areas and were counted as being one structure. High density labelling in areas other than intermediate filaments was analysed in order to identify a structure or organelles reacting with the labelling reagents. The largest possible area for a particular structure was counted, leaving out only blocks partially covering it or containing other structures.
Repeated counts of the same area were prevented by selecting one micrograph only for a particular structure and by keeping the graph paper in the same position during the counting process. A minimum final magnification of x 8200 was required to prevent particles from being obscured by the bars of the graph paper. A magnifying lens was used for visualization of particles when counting at lower magnifications.

A minimum area (A) of 1 μm² was evaluated for each specimen. Hereby artifacts that occur through conversion of figures obtained from small areas at high magnification with a heterogeneous labelling distribution, were minimized. For each micrograph the number of particles per block (x) and the number of blocks counted (n) were recorded for further calculations.

2F.2 Mean labelling density

The mean labelling density (y) expressing the average number of particles per block was calculated by dividing the total number of particles counted for a particular structure (Σx) by the number of observations or 1 cm² blocks counted (n).

\[ y = \frac{\Sigma x}{n} \quad \text{(average number of particles per block)} \]

2F.3 Labelling Density (PD)

The mean labelling density (y) has to be converted to a
unit of average number of particles per \( \mu m^2 \) (PD) to allow comparison of results from different specimens. This was done by multiplication of the average number of particles per 1 cm\(^2\) block by the square of the print magnification before division by 1 x 10 (cm\(^2\) converted to \( \mu m^2 \)).

\[
PD \text{ (mean particles / } \mu m^2) = \frac{y \cdot (\text{print magnification})^2}{100 \, 000 \, 000}
\]

2F.4 Area counted (A)

Each observation made, represented an arbitrary viewing field (area) equal to 1 cm\(^2\). The actual area evaluated was found by converting the number of cm\(^2\) blocks counted for a particular structure (n) to \( \mu m^2 \), (multiplication by 100 000 000, division by the square of the print magnification). This figure represents the true area evaluated.

\[
A \text{ (} \mu m^2) = \frac{n \cdot 100 \, 000 \, 000}{(\text{print magnification})^2}
\]

2F.5 Deviation and variance of the observations

The deviations (differences from the mean) for each observation (d), were found by subtracting the mean value calculated for the particular structure (y) from the particle count per block (x).

\[
d = x - y
\]

The sum of the differences can be calculated by \( \Sigma d \) or
\[\Sigma (x-y), \text{ but equals 0 due to the positive } \Sigma^+ (x-y) \text{ and negative } \Sigma^- (x-y) \text{ deviations around the mean. Therefore,} \]

\[\Sigma (x-y) = \Sigma^+ (x-y) + \Sigma^- (x-y) = 0\]

Normally, in order to calculate the variance and standard deviation of the observations made, the differences (d) have to be squared to get rid of the negative values. \(\Sigma (x-y)\) will then give a positive value, which gives the variance of the observation when divided by \(n - 1\). The standard deviation (SD) is obtained by taking the square root of the variance.

As these figures relate to the observations made, they give low \(<1\) values for a number of similar observations (homogeneous labelling), but fail to define the actual particle distribution of a positive or negative labelling result. Therefore, calculation of the variance and standard deviation for the observations made, were omitted.

2F.6 Heterogeneity of the particle distribution (\% H)

In order to evaluate the particle distribution, including the variance/deviation of observations, a formula was devised whereby the negative differences \(\Sigma^- (x-y)\) were subtracted from the positive deviations \(\Sigma^+ (x-y)\) obtained from particle counts for \(n\) observations. This figure represents the number of particles falling either side of
(outside) the mean \((z)\) for \(n\) observations. This allows expression of heterogeneity of the particle distribution for \(n\) observations as reflected by the ratio between the total number of particles not equal to the mean \((z)\) and the total amount of particles counted \((\Sigma x)\).

Ratio of Heterogeneity \((H)\) of the particle distribution

\[
\frac{z}{\Sigma x}
\]

By using two ultimate examples, absolute values for this ratio can be established.

**Example 1.** If \(n\) observations were made, where \(x\) would have the same value for all the observations, the mean \(y\) would be equal to \(x\) so that \(z = 0\).

Therefore

\[
\frac{z}{\Sigma x} = 0
\]

\(\Sigma x\) = complete homogeneity or zero heterogeneity.

**Example 2.** It was observed that with immunogold labelling at least one (non-specific) particle would be present on the specimen as a result of very low resin affinity for the labelling reagents (section 3B.2, Fig 3.22). Therefore it can be concluded that \(\Sigma x\) does not equal 0, even if the particle is not present in a particular viewing field.

\(\Sigma x > 0\) for any negative labelling result.
In a case where any large number, say 100,000 observations were made, revealing only 1 particle, $\Sigma x$ would be 1, $y = 0.00001$ and $z = 2 (1.99998)$. For such a heterogeneous particle distribution (H), the ratio represents ultimate heterogeneity or zero homogeneity.

$$H = \frac{z}{\Sigma x} = 2$$

Examples 1 and 2 show that H has now values between 0 and 2. With a known maximum value of 2, the ratio can be divided by 2 and expressed as a percentage:

$$\%H = \frac{100 \times z}{2 \Sigma x}$$

Provided that the number of observations is >1, figures obtained in this way provide a way to measure the heterogeneity of the total particle distribution for a particular labelled structure, expressing the result as a value between 0 and 100. The percentage figure allows arbitrary comparison of the heterogeneity or homogeneity of the particle distribution as obtained for different labelled specimens and structures.

2F.7 Immunohistochemistry: evaluation of staining

Granular, cytoplasmic, brown immunoperoxidase labelling was recorded as positive [+], irrespective of the number of positive cells or the intensity of the stain. Other results were shown as [-] (all cells negative) or [±] (dubious
positivity). Further descriptions of the staining pattern with respect to intensity of stain, number, type and distribution of positive cells, were added.

2F.8 Data correlation and result presentation

After evaluation of the individual components of this investigation, the findings were compiled to present a total picture for each specimen.

Using the PD, %H and A figures obtained from the individual counted micrographs, immunogold labelling figures for a particular specimen were composed by calculating the averages for labelling density and percentage heterogeneity respectively. The total area of the specimen investigated, was found by adding up the areas counted for each individual micrograph. These figures were linked with the quality of the ultrastructure as well as the immunohistochemical, histological and clinical data that was available for each case.

Final determination of the significance of immunogold labelling of intermediate filaments and effect of processing and labelling factors on such results was therefore based on an overall picture seen in all the specimens studied.
Chapter 3

RESULTS

<table>
<thead>
<tr>
<th>TABLE 3.01</th>
<th>EFFECT OF PROCESSING FACTORS ON IMMUNOLABELLING, SECTION STABILITY AND ULTRASTRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section 3A.1</td>
<td>STANDARD FIXATION, OSMIUM, EPOXY RESIN, STANDARD ETCHING</td>
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<tr>
<td></td>
<td>poor labelling sensitivity [± 4 particles / μm²]</td>
</tr>
<tr>
<td></td>
<td>normal ultrastructure, very stable sections</td>
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<tr>
<td>Section 3A.2 &amp; 3</td>
<td>MILD FIXATION, OSMIUM, EPOXY RESIN, PROLONGED ETCHING</td>
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<tr>
<td></td>
<td>poor labelling sensitivity [± 1 particles / μm²]</td>
</tr>
<tr>
<td></td>
<td>inferior ultrastructure, stable sections</td>
</tr>
<tr>
<td>Section 3A.4</td>
<td>MILD FIXATION, NO OSMIUM, EPOXY RESIN, STANDARD ETCHING</td>
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<td></td>
<td>poor labelling sensitivity [± 47 particles / μm²]</td>
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<tr>
<td></td>
<td>pale, inferior ultrastructure, stable sections</td>
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<tr>
<td>Section 3A.5</td>
<td>MILD FIXATION, NO OSMIUM, ACRYLIC RESIN normal, NO ETCH</td>
</tr>
<tr>
<td></td>
<td>increased labelling sensitivity [± 281 particles / μm²]</td>
</tr>
<tr>
<td></td>
<td>pale, inferior ultrastructure, stable sections</td>
</tr>
<tr>
<td>Section 3A.6</td>
<td>STANDARD FIXATION, OSMIUM, ACRYLIC RESIN normal, (ETCH)</td>
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<td>intense labelling [± 275 (1072) particles / μm²]</td>
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<tr>
<td></td>
<td>inferior ultrastructure, sections very unstable</td>
</tr>
<tr>
<td>Section 3A.7</td>
<td>STANDARD FIXATION, OSMIUM, ACRYLIC RESIN Cold, NO ETCH</td>
</tr>
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<td>Selected method for standardized protocol [section 3B]</td>
</tr>
<tr>
<td></td>
<td>labelling low sensitivity [± 50 particles / μm²]</td>
</tr>
<tr>
<td></td>
<td>relatively normal ultrastructure, stable sections</td>
</tr>
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</table>

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The results obtained from the various processing protocols studied, are presented in section 3A. These methods are summarized in Table 3.01, showing the factors that influenced the development of the standardized protocol (section 3A.7). This method was used to prepare the different histopathological specimens for which the labelling results are given in section 3B.

3A FACTORS INFLUENCING IMMUNOLABELLING

3A.1 Immunoelectron microscopy of glutaraldehyde fixed, osmicated, epoxy embedded specimens

Human biopsy tissue from case A (astroblastoma), case K (malignant glioma) and case S (SSPE, gliosis) was fixed in 2.5% glutaraldehyde. Secondary fixation in osmium tetroxide preceded complete dehydration before the specimens were embedded in epoxy resin as for routine electron microscopy. Thin sections were treated for 30 minutes with sodium meta periodate before overnight incubation with GFAP 1:1000.

Strongly positive immunohistochemical results were in sharp contrast with the apparently negative immunogold labelling counts found irrespectively of entity or number of intermediate filaments present (Table 3.01). The ultrastructure obtained from these cases was characterized by a slight washed out appearance in comparison to material not exposed to immunogold labelling, but was regarded to be normal.
TABLE 3.02
GFAP IMMUNOLABELLING OF VARIOUS SPECIMENS PROCESSED FOR ROUTINE ELECTRON MICROSCOPY:

<table>
<thead>
<tr>
<th>INTERMEDIATE FILAMENTS</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECIMENS</td>
<td>Density Distribution Area</td>
<td>Area</td>
</tr>
<tr>
<td>ASTROBLASTOMA case A</td>
<td>1</td>
<td>81%</td>
</tr>
<tr>
<td>GLIOMA case K</td>
<td>4</td>
<td>23%</td>
</tr>
<tr>
<td>SSPE (gliosis) case S</td>
<td>0</td>
<td>84%</td>
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<table>
<thead>
<tr>
<th>OTHER STRUCTURES</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
<th>IHC</th>
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</thead>
<tbody>
<tr>
<td>SPECIMENS</td>
<td>Density Distribution Area</td>
<td>Area</td>
</tr>
<tr>
<td>ASTROBLASTOMA case A</td>
<td>4</td>
<td>82%</td>
</tr>
<tr>
<td>GLIOMA case K</td>
<td>1</td>
<td>57%</td>
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<tr>
<td>SSPE (gliosis) case S</td>
<td>0</td>
<td>97%</td>
</tr>
</tbody>
</table>

KEY TO TABLE 3.02

**Immunoelectron microscopy**

**Density** 15 nm gold particles / μm²

**Distribution** % heterogeneity of particle distribution, low figures indicate homogenous labelling.

**Area** Total specimen area (μm²) counted.

IHC : Light microscopical immunohistochemistry results given as [+] positive, [-] negative or [±] dubious.
Formalin fixed, paraffin embedded tissue showing a fairly monomorphic tumour composed of cells with round-oval nuclei, even chromatin and well defined eosinophilic cytoplasm. Perivascular rosettes (arrow-heads) were a striking feature, their cells showing elongated processes pointing towards the vessel (square) in a palisaded manner.
FIGURE 3.02

Astroblastoma (case A), GFAP

[ x 250 ]

The perivascular rosettes (arrow-heads) were strongly positive for GFAP (ABC method), while the vascular structures (squares) were devoid of any staining. Striking absence of staining was seen towards the centre of the section. Focal, weak vimentin positivity was found in some rosettes (not illustrated).

FIGURE 3.03

Astroblastoma (C05), GFAP, Methylene blue

[ x 250 ]

Thick sections representing the epoxy resin embedded specimen used for electron microscopy, showed similar histology to that found in the paraffin embedded tissue (fig. 3.01). Immunohistochemical localization of astroglial cells was partially successful, showing positive staining for GFAP in some perivascular rosettes (arrow-head), but often weak or dubious in others. Sporadic, non-specific staining was seen mainly in osmiophilic areas.
Conventional ultrastructure was obtained with 2.5% glutaraldehyde fixed, osmicated, epoxy embedded material, confirming the presence of widespread fibrillar material within cells. Prominent intermediate filaments (arrowhead), were seen. Numerous cells, such as the necrotic cell illustrated (square), contained lipid-like material.
FIGURE 3.05

Astroblastoma (case A), GFAP 1 : 1000
[ x 11400 ]
Inset: [ x 18500 ]

The same specimen shown in fig 3.04, was labelled for GFAP (optimal dilution 1 : 1000) following etching with sodium (meta) periodate. A few 15nm gold particles were found on intermediate filaments (arrow). No affinity for gold particles of osmiophilic structures was seen, whilst the particle counts for large areas of other structures were similar to those found for intermediate filaments. Artifactual deposits and occasional clumping of gold particles were considered to be insignificant.

A slightly pale electron microscopical appearance, due to etching, was noted in the labelled specimen. In comparison to the previous figure, the ultrastructure was inferior, but still good enough to demonstrate sub-cellular structures such as polysomes (circle) and part of a nucleus (square).
Different antibody concentrations ranging from GFAP 1 : 50 to 1: 10000 were applied to the same specimen as in fig. 3.05. At very low antibody concentration (GFAP 1 : 8000), the particle densities were 0 with heterogeneity of the particle distribution being > 92%. This was the case for both glial intermediate filaments (square) and other structures such as the nucleus (arrow). The presence of scattered, 15nm gold particles (circles), were applied to define calculation of absolute values for the ratio of the heterogeneity of the particle distribution (section 2H.7).
Astroblastoma, (case A) GFAP 1 : 50
[ x 31500 (print magnification) ]

Increased primary antibody concentration showed higher particle counts on all structures. For equal areas (18 µm²) counted, 15 nm gold particles (circles) were found on glial intermediate filaments (square; 32 particles / µm²) and negative control structures such as the nucleus (arrow; 17 particles / µm²). The distribution of particles over intermediate filaments was less heterogeneous (49%) than for other structures (64%). Although these figures indicate some specificity for GFAP in glial intermediate filaments, the high false positive background labelling is sub-optimal and demonstrates the low specificity and poor sensitivity for GFAP in the specimen.

The quality of the ultrastructure was not altered by application of different dilutions of antibody.
FIGURE 3.08

Astroblastoma (case A), 3 hours etching, GFAP 1:1000

Improvement of previous results (table 3.02) was attempted by prolonged etching (60 minutes to 3 hours) of sections prior to labelling. No increase in specific immunogold labelling for GFAP was found. These results as well as the negative influence on ultrastructure and an increase in non-specific labelling with 15 nm gold particles (circle), are shown in Table 3.3. This effect was progressively proportional to incubation with sodium (meta) periodate, to such an extent that intermediate filaments could not be identified after 3 hours of etching.
**TABLE 3.03**

**EFFECT OF PROLONGED SODIUM (met) PERIODATE ETCHING ON GFAP IMMUNOLABELLING OF ASTROBLASTOMA (case A) PROCESSED FOR ROUTINE ELECTRON MICROSCOPY**

**INTERMEDIATE FILAMENTS**

<table>
<thead>
<tr>
<th>ETCHING minutes</th>
<th>ULTRASTRUCTURE</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
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<tbody>
<tr>
<td></td>
<td>Density</td>
<td>Distribution</td>
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<tr>
<td>60</td>
<td>inferior</td>
<td>1</td>
</tr>
<tr>
<td>75</td>
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<td>1</td>
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<tr>
<td>90</td>
<td>inferior</td>
<td>1</td>
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<tr>
<td>105</td>
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<td>1</td>
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<tr>
<td>120</td>
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<tr>
<td>180</td>
<td>poor</td>
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**OTHER STRUCTURES**

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<th>ULTRASTRUCTURE</th>
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<tr>
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<td>inferior</td>
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<td>90</td>
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</tr>
<tr>
<td>120</td>
<td>poor</td>
<td>2</td>
</tr>
<tr>
<td>180</td>
<td>poor</td>
<td>6</td>
</tr>
</tbody>
</table>

**KEY TO TABLE 3.03**

**Immunoelectron microscopy**

**Density**

15 nm gold particles / μm²

**Distribution**

% heterogeneity of particle distribution, low figures indicate homogenous labelling.

**Area**

Total specimen area (μm²) counted.
This formalin fixed, paraffin embedded specimen showed a cellular, malignant glioma. Areas of extensive necrosis and tumour vascularization were present (not illustrated).

GFAP immunohistochemistry of the same area as in figure 3.09 produced distinct, strongly positive staining in pleomorphic cells (arrows). Similar staining in all tumour areas confirmed the astroglial character of the neoplasm.
FIGURE 3.11

Malignant glioma (case K), GFAP  
[x 250]

Thick sections of the epoxy embedded blocks showed a partially necrotic specimen. Scattered GFAP positive cells (circles), indicated an astroglial pattern in some areas.

FIGURE 3.12

Malignant glioma (case K), GFAP  
[x 250]

Further examination of the partially necrotic specimen shown in fig. 3.11, revealed dubious GFAP positivity as well as areas of non-specific staining (squares). These findings were associated with incomplete resin removal and/or the presence of osmium tetroxide in the section.
Immunoelectron microscopy for GFAP produced very low counts of 15 nm particles (circle), localized to intermediate filaments in cell processes. Some specificity for GFAP was indicated by a slightly higher (± 3 particles / μm²) particle density and a 34% more homogenous particle distribution for intermediate filaments in comparison to other structures. Whether necrosis present in the specimen had an effect on fixation and immunolabelling, could not be determined.
GLIOSIS / SUBACUTE SCLEROSING PANENCEPHALITIS (SSPE)
- cerebrum    [ CASE S ]

FIGURE 3.14
SSPE, gliosis (case S), H&E
[ x 250 ]

Formalin fixed, paraffin embedded tissue showed the picture of inclusion body encephalitis with perivascular lymphocytic infiltration (arrow-head), neuronal and oligodendroglial intranuclear inclusions (square) as well as gliosis.

FIGURE 3.15
SSPE, gliosis (case S), GFAP
[ x 250 ]

Gliosis seen in the paraffin embedded specimen, was characterized by reactive astrocytes that were strongly positive for GFAP (arrows).
FIGURE 3.16

SSPE, gliosis (case S), GFAP

[ x 250 ]

Reactive astrocytes (arrows) were also seen in thick sections of the corresponding epoxy embedded specimen. Here, focal, often dubiously positive staining for GFAP, indicated lesser labelling sensitivity than for the paraffin embedded specimen.

FIGURE 3.17

SSPE, gliosis (case S), GFAP

[ x 13500 ]

Despite the prominent ultrastructural abundance of filamentous glial processes, glial intermediate filaments (arrow) failed to show any significant labelling. A few scattered, non-specific 15nm gold particles (circle) were seen on neurofilaments (squares) in association with myelin figures (arrow-heads).
3A.2 Effect of low concentration primary fixative on immunogold labelling of osmicated, epoxy embedded specimens

MALIGNANT GLIOMA - site unknown [ CASE M ]

FIGURE 3.18
Malignant glioma (case M), H&E
[ x 250 ]

Histological examination of the 1% glutaraldehyde fixed, paraffin embedded tissue, showed a poorly differentiated, malignant tumour.

FIGURE 3.19
Malignant glioma (case M), PTAH
[ x 250 ]

Although the specimen shown in figure 3.18, failed to produce GFAP immunoreactivity, glial processes (arrow-head) with gemistocytic changes and possible Rosenthal fibre formation were indicated by PTAH staining.
Malignant glioma (case M), GFAP

[ x 6900 ]

Thin sections of the 1% glutaraldehyde fixed, osmicated epoxy embedded specimen, were exposed to immunogold labelling for GFAP. Bundles of lightly packed intermediate filaments (squares) were positive, (gold particles not visible at this low magnification). Signs of improper resin impregnation (large arrow) and lesser contrast were prominent. The lower glutaraldehyde concentration in comparison to the previous cases, caused instability of the section due to improper penetration of fixative into the tissue. The micrograph was overexposed to compensate for lesser specimen contrast.

The area indicated by the dark arrow, is shown at higher magnification in figure 3.21.

Inset: Malignant glioma (case M), GFAP

[ x 250 ]

Immunohistochemistry on the corresponding thick resin section showed extensive labelling for GFAP in a fibrillar, astroglial pattern (arrow). Some areas displayed a non-specific staining reaction (encircled square) between DAB and osmium tetroxide. This result was in sharp contrast to the negative result obtained for the paraffin embedded specimen, (figures 3.18 & 3.19).
FIGURE 3.21

Malignant glioma (case M), GFAP

Inset: [x 30000]

Higher magnification of the area shown in Fig 3.20 (arrow), revealed intermediate filaments (squares) decorated with 15nm gold particles. Although a relatively low particle density (14 particles / µm^2) was found, the figure was tenfold higher than for the previous cases. This illustrates slightly better antigen availability as result of milder fixation.
3A.3 Effect of etching on immunogold labelling of BGPA fixed, osmicated, epoxy embedded normal rat brain

NORMAL RAT BRAIN - Habenular nucleus [CASE R]

FIGURE 3.22
Rat habenular nucleus, (case R) GFAP
[ x 250 ]
GFAP positive astrocytes and astrocytic processes (squares) as well as ependymal cells (arrows) were demonstrated immunohistochemically on formalin fixed, paraffin embedded tissue from the habenular nucleus of normal rat brain.

FIGURE 3.23
Rat habenular nucleus, (case R) GFAP
[ x 250 ]
More of the same tissue was fixed in buffered glutaraldehyde / picric acid (BGPA). Secondary fixation in osmium tetroxide preceded embedding in epoxy resin. Thick resin sections produced dubious GFAP positivity due to excessive background staining (arrow-heads).

Thin sections were etched with 5% NaOH (5 minutes) or with a similar protocol which included incubation with sodium (meta) periodate (75 minutes). These conditions were so harsh that sections were completely removed from the grid. Milder etching (sodium periodate, 30 minutes, omission of NaOH) was performed, followed by incubation with GFAP (1 : 1000, 3 hours or overnight). Intermediate filaments,
less abundant in these specimens, showed no significant GFAP positivity. Reagent negative controls (replacement of the primary antibody with buffer), produced negative labelling results for all structures. Immunogold labelling without etching was equally unsuccessful (Table 3.04).

<table>
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<tr>
<th>INTERMEDIATE FILAMENTS</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Density Distribution Area</td>
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<tr>
<td>No etching</td>
<td>1</td>
</tr>
<tr>
<td>Sodium periodate (30 min)</td>
<td>1</td>
</tr>
<tr>
<td>NaOH (5 min)</td>
<td>*</td>
</tr>
<tr>
<td>Sodium periodate (75 min) and NaOH (5 min)</td>
<td>*</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>OTHER STRUCTURES</th>
</tr>
</thead>
<tbody>
<tr>
<td>No etching</td>
</tr>
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</tr>
<tr>
<td>NaOH (5 min)</td>
</tr>
<tr>
<td>Sodium periodate (75 min) and NaOH (5 min)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>KEY TO TABLE 3.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoelectron microscopy</td>
</tr>
<tr>
<td>Density</td>
</tr>
<tr>
<td>Distribution</td>
</tr>
<tr>
<td>Area</td>
</tr>
</tbody>
</table>
3A.4 Effect of omission of osmium tetroxide on immunogold labelling of mildly fixed, epoxy embedded gliomas and normal rat brain

Tissue from cases K and M (gliomas, fixation > 24 hours) and case R (rat brain, fixation < 24 hours), were fixed in 1% glutaraldehyde. Specimens from case L (glioma) and case R (rat) were fixed in BGPA for < 24 hours. These protocols were all characterized by the omission of secondary fixation in osmium tetroxide. Immunogold labelling was done by etching for 30 minutes with sodium (meta) periodate and overnight incubation with GFAP (1:1000).

GFAP immunohistochemistry on corresponding paraffin embedded specimens showed negative, focally positive, weakly positive or dubious staining. This is a typical phenomenon associated with incomplete preservation of tissue components. Labelled thick resin sections cut from the block used for electron microscopy, showed definite GFAP positive staining, thus confirming the astroglial pattern seen on formalin fixed, paraffin embedded tissue. There was no sign of the false positive background staining previously seen in the presence of osmium tetroxide.

Thin sections cut from specimens exposed to shorter, milder fixation, showed signs of incomplete impregnation with resin, thus causing instability in the electron beam. Under these conditions, suitable viewing fields were limited.
The immunoelectron microscopical results for this group, (Table 3.05), show low density specific labelling of glial intermediate filaments in both human and rat tissue. In comparison to previous results, (tables 3.02-4), substantially increased labelling was observed due to omission of osmium tetroxide. Additional labelling sensitivity was gained by shorter incubation with the primary fixatives.

NORMAL RAT BRAIN - Habenular nucleus [CASE R]

FIGURE 3.24

Rat habenular nucleus [ case R ] GFAP
[ x 108000 ]

The pale appearance of this specimen illustrates the lack of contrast and relatively low labelling intensity found in this group. Here, mild BGPA fixation for less than 24 hours, allowed improved labelling at the cost of acceptable ultrastructure. In the absence of lipid fixation / staining with osmium tetroxide and worsened by the effect of etching, myelin figures (arrow-head) were difficult to identify. A small number of 15 nm gold particles decorated the intermediate filaments (square). Reagent negative controls (substitution of primary antiserum with buffer) were negative for all structures, produced no labelling on any structures.
### TABLE 3.05
GFAP IMMUNOLABELLING OF EPOXY EMBEDDED SPECIMENS: COMBINED EFFECT OF MILD FIXATION AND OMISSION OF OSMIUM TETROXIDE

#### INTERMEDIATE FILAMENTS

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<thead>
<tr>
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<th>IMMUNOELECTRON MICROSCOPY</th>
<th>IHC</th>
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</thead>
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<tr>
<td></td>
<td>Density</td>
<td>Distribution</td>
</tr>
<tr>
<td>Specimen</td>
<td>Fixation</td>
<td></td>
</tr>
</tbody>
</table>

- **GLIOMA case K**
  - > 24h: 2, 36%, 14, +

- **GLIOMA case M**
  - > 24h: 4, 25%, 20, +

- **GLIOMA case L**
  - BGPA < 24h: 12, 56%, 6, +

- **RAT case R**
  - BGPA < 24h: 35, 52%, 2, +
  - < 24h: 47, 24%, 2, +

#### OTHER STRUCTURES

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Density</td>
<td>Distribution</td>
</tr>
<tr>
<td>Specimen</td>
<td>Fixation</td>
<td></td>
</tr>
</tbody>
</table>

- **GLIOMA case K**
  - > 24h: 0, 78%, 47, -

- **GLIOMA case M**
  - > 24h: 0, 63%, 29, -

- **GLIOMA case L**
  - BGPA < 24h: 1, 95%, 49, -

- **RAT case R**
  - BGPA < 24h: 0, 92%, 109, -
  - < 24h: 0, 94%, 82, -

#### KEY TO TABLE 3.05

**Immunoelectron microscopy**

- **Density**: 15 nm gold particles / µm²
- **Distribution**: % heterogeneity of particle distribution, low figures indicate homogenous labelling.
- **Area**: Total specimen area (µm²) counted.

**IHC**: Light microscopical immunohistochemistry results given as [+] positive, [-] negative or [±] dubious.
GLIOMA – site unknown [ CASE L ]

FIGURE 3.25

Glioma (case L), H&E
[ x 250 ]

Paraffin embedded tissue, fixed in BGPA for < 24 hours, showed the signs of poor fixation/preservation of the specimen that prevented definitive diagnosis. However, the highly cellular, malignant tumour had features suggesting a glial origin.

FIGURE 3.26

Glioma (case L), GFAP
[ x 250 ]

The same specimen as in figure 3.25, produced a focally GFAP positive area (arrow-head) which provided more evidence towards the glial origin of the tumour. False negative staining due to the mild, incomplete preservation of the specimen, was suspected.
Immunoelectron microscopy did not convincingly confirm the astroglial character indicated by GFAP positive astrocytic processes (arrow-heads) seen on immunolabelled thick resin sections (inset). Intermediate filaments were decorated with 15nm gold particles (squares) with a density of 12 particles / \mu m^2 and a heterogeneous distribution of 56%. Prominent signs of improper resin impregnation (large arrow) were seen.
3A.5 Immunolabelling of specimens embedded in acrylic resin with the omission of osmium tetroxide

Processing of tissue from case R (rat), case I (malignant astrocytic glioma) and cases J and L (gliomas), included fixation in BGPA (< 24 hours) and dehydration (100%).

A second protocol, including fixation in 2.5% glutaraldehyde (> 24 hours) and 70% dehydration, was applied to tissue from cases K and M (gliomas). All these specimens were embedded in acrylic resin. Following overnight incubation with antiserum, GFAP was demonstrated by means of 15 or 20 nm colloidal gold particles.

As acrylic thick sections were not suitable for immunohistochemistry, light microscopical immunolabelling results were obtained from paraffin embedded tissue representing these cases. As shown in tables 3.06-7, these results correlated well with immunoelectron microscopical findings. Exceptions were case L (figure 3.26) and case M (figures 3.18-9), where poor tissue preservation resulted in dubious or false negative immunohistochemical results. For case L (glioma), only a few intermediate filaments were found in the specimen selected for electron microscopy.

Despite a slight increase in non-specific labelling, glial intermediate filaments were demonstrated by a fairly homogeneous, high density particle distribution (Table 3.06). These figures were substantially higher than those
obtained for similar tissue in previous experiments.

No definite pattern could be identified when comparing specimens exposed to the different mild fixation procedures. However, all the specimens showed prominent signs of incomplete impregnation of tissue with resin. This caused occasional instability of thin sections, which limited the areas available for viewing.

The absence of fixation and staining with osmium tetroxide, was characterized by the pale appearance and lack of contrast of the sections. Due to the hydrophilic character of the acrylic resin, thin sections were not as stable as epoxy sections in the electron beam.

For the Protein A method, reagent negative controls where primary antiserum was replaced with either buffer (2 particles / \( \mu m^2 \)) or normal serum (11 particles / \( \mu m^2 \)) were done on case 24. These results demonstrated the reaction between Protein A and immunoglobulins present in the tissue and in the normal serum that was applied to the sections. This lead to increased non-specific labelling of all structures.

137
### TABLE 3.06

**GFAP IMMUNOLABELLING OF ACRYLIC EMBEDDED SPECIMENS**

**PROCESSED WITH MILD FIXATION AND**

**OMISSION OF OSMIUM TETROXIDE**

<table>
<thead>
<tr>
<th>SPECIMENS</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Density Distribution Area</td>
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</tr>
<tr>
<td><strong>INTERMEDIATE FILAMENTS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixation: BGPA &lt; 24 h</td>
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<td></td>
</tr>
<tr>
<td>Dehydration 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASTROCYTIC GLIOMA case I</td>
<td>260 13% 14 +</td>
<td></td>
</tr>
<tr>
<td>GLIOMA case L</td>
<td>5 6% 2 ±</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde &gt; 24 h</td>
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<td></td>
</tr>
<tr>
<td>Dehydration 70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLIOMA case K</td>
<td>281 13% 14 +</td>
<td></td>
</tr>
<tr>
<td>GLIOMA case M</td>
<td>96 9% 4 −</td>
<td></td>
</tr>
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<td><strong>OTHER STRUCTURES</strong></td>
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<td></td>
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<tr>
<td>Fixation: BGPA &lt; 24 h</td>
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<td></td>
</tr>
<tr>
<td>Dehydration 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASTROCYTIC GLIOMA case I</td>
<td>2 95% 7 −</td>
<td></td>
</tr>
<tr>
<td>GLIOMA case L</td>
<td>0 73% 30 −</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde &gt; 24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydration 70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLIOMA case K</td>
<td>7 81% 53 −</td>
<td></td>
</tr>
<tr>
<td>GLIOMA case M</td>
<td>2 82% 4 −</td>
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</tr>
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</table>
## TABLE 3.07

**GFAP PROTEIN A GOLD IMMUNOLABELLING OF**
**BGPA FIXED, ACRYLIC EMBEDDED SPECIMENS**
**WITH OMISSION OF OSMIUM TETROXIDE**

### INTERMEDIATE FILAMENTS

<table>
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<td></td>
<td>Density</td>
<td>Distribution</td>
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<tr>
<td>RAT BRAIN</td>
<td>144</td>
<td>25%</td>
</tr>
<tr>
<td>ASTROCYTIC GLIOMA case I</td>
<td>262</td>
<td>26%</td>
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<td>GLIOMA</td>
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### OTHER STRUCTURES

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<td>Density</td>
<td>Distribution</td>
</tr>
<tr>
<td>RAT BRAIN</td>
<td>4</td>
<td>90%</td>
</tr>
<tr>
<td>ASTROCYTIC GLIOMA case I</td>
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<tr>
<td>GLIOMA</td>
<td>2</td>
<td>79%</td>
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### KEY TO TABLES 3.06 & 3.07

- **Immunoelectron microscopy**
  - **Density**: 15 nm (table 3.06) or 20 nm (table 3.07) gold particles / μm²
  - **Distribution**: % heterogeneity of particle distribution, low figures indicate homogenous labelling.
  - **Area**: Total specimen area (μm²) counted.
  - **IHC**: Light microscopical immunohistochemistry results given as [+] positive, [-] negative or [±] dubious.
The specimen was fixed in glutaraldehyde, partially dehydrated (70%) and embedded in LR White resin. Osmium tetroxide was omitted. The micrograph was over-exposed to compensate for the low contrast of the specimen. Despite the poor ultrastructure, part of a nucleus (arrow-head) and fibrillar structures, decorated with 15 nm gold particles (circle), were identified. These structures were thought to be artifactually distorted intermediate filaments (arrows). Whereas the corresponding paraffin embedded specimen failed to produce GFAP positivity (figures 3.18-9), thick, non-osmicated epoxy sections (figure 3.20) indicated the astroglial character of the tumour.
GLIOMA - temporal lobe [CASE J]

FIGURE 3.29

Glioma (case J), GFAP

[ x 250 ]

GFAP positive astroglial processes (arrow-heads) were demonstrated on this BGPA fixed, paraffin embedded specimen. Despite drifting of thin sections in the electron beam, a positive count of 200 particles / $\mu m^2$ was obtained for glial intermediate filaments (not illustrated), which confirmed the light microscopical result. The reagent negative control (primary antibody replaced by buffer) showed slight non-specific labelling (1 particle / $\mu m^2$) on all structures.
The formalin fixed, paraffin embedded specimen showed the features of a malignant astrocytic glioma (inset top right). Immunohistochemistry done on the same specimen, (inset lower right), demonstrated GFAP positive astroglial cells (arrow-heads). Immunoelectron microscopy on the BGPA fixed, acrylic embedded specimen, showed a homogenous distribution of 20 nm gold particles at a density of 262 particles/μm² on glial intermediate filaments (arrow). The specimen was also labelled with the GAR 15 auroprobe, producing 260 particles/μm². Replacement of primary antiserum with buffer provided reagent negative controls for both the Protein A method (2 particles/μm²) and the GAR 15 protocol (0 particles/μm²), showing that the slightly higher particle density obtained with the Protein A technique, was due to increased non-specific labelling.
FIGURE 3.31

Malignant glioma (case K), GFAP
[ x 31500 ]

Bundles of glial intermediate filaments (arrows), heavily labelled with 15nm gold particles (square), illustrated the high labelling intensity and poor ultrastructure which is characteristic of glutaraldehyde fixation, omission of osmium tetroxide, partial dehydration (70%) and embedding in acrylic resin. Massive restriction of antigen availability by epoxy resin, was illustrated by comparing this specimen to corresponding epoxy embedded tissue of this glioma (Tables 3.02-3). The labelling intensity improved from 2-4 particles / μm² (epoxy embedded specimens) to 281 particles / μm² (acrylic embedded specimens).
Immunolabelling of osmicated, acrylic embedded gliomas and normal rat brain

Tissue from two gliomas (cases M and K) were fixed in glutaraldehyde (＞24) and osmium tetroxide before partial dehydration (70%) and embedding in acrylic resin. Except for primary fixation in BGPA for <24 hours, rat habenular nucleus (case R) was processed in the same way.

Immunogold labelling for GFAP (1:1000 overnight) with or without etching, was performed.

A spontaneous exothermic reaction between osmicated tissue and acrylic resin occurred during impregnation of tissue with resin, causing premature, thermal curing of the resin. The resulting incomplete resin impregnation lead to instability of sections in the electron beam. Selection of suitable viewing fields was difficult, as tearing, stretching and/or shrinkage of thin sections was commonly encountered. This phenomenon is reflected by the relative small areas counted for each specimen (Table 3.08).

Glial intermediate filaments with acceptable ultrastructure were demonstrated by high intensity labelling for GFAP. Etching was seemingly correlated with increased specific and non-specific particle counts for similar specimens.

With no means available to remove acrylic resin from thick sections, immunohistochemistry could not be performed on these specimens. However, with the exception of case M...
(figures 3.18-9), the GFAP positivity found in corresponding paraffin embedded specimens, correlated well with the immunoelectron microscopical findings.

**TABLE 3.08**

GFAP IMMUNOLABELLING OF ACRYLIC EMBEDDED GLIOMAS: EFFECT OF MILD FIXATION AND OSMIUM TETROXIDE

<table>
<thead>
<tr>
<th>INTERMEDIATE FILAMENTS</th>
<th>IMMUNOELECTRON MICROSCOPY</th>
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</tr>
</thead>
<tbody>
<tr>
<td>SPECIMENS</td>
<td>Density</td>
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<tr>
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<td>1092</td>
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<td>14%</td>
</tr>
<tr>
<td>case M etched specimen</td>
<td>753</td>
<td>5%</td>
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<table>
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<th>Area</th>
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<td>54%</td>
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<td>case K etched specimen</td>
<td>7</td>
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<td>11</td>
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<td>case M no etching</td>
<td>2</td>
<td>50%</td>
<td>6</td>
</tr>
<tr>
<td>case M etched specimen</td>
<td>10</td>
<td>41%</td>
<td>3</td>
</tr>
</tbody>
</table>

**KEY TO TABLE 3.08**

- **Immunoelectron microscopy**
  - **Density**: 15 nm gold particles / \( \mu \text{m}^2 \)
  - **Distribution**: % heterogeneity of particle distribution, low figures indicate homogenous labelling.
  - **Area**: Total specimen area (\( \mu \text{m}^2 \)) counted.

- **IHC**: Light microscopical immunohistochemistry results given as [+J positive, [-J negative or [± J dubious.
This glutaraldehyde fixed, osmicated specimen was partially dehydrated (70%) prior to LR White embedding at room temperature. Due to the premature curing of the resin, complete impregnation of tissue was not achieved. Instability of thin sections cut from these blocks, was illustrated by the torn section (inset), showing the effect of stretching and/or shrinkage (square) on the quality of the ultrastructure. The partial absence of resin within the section allowed greater antigen availability, as illustrated by the high intensity labelling (1092 particles / μm²) of intermediate filaments with 15 nm gold particles (arrows). The interaction between sodium (meta) periodate and osmium tetroxide is also favoured by the partial absence of resin. Omission of etching from the labelling protocol resulted in a fourfold decrease in labelling sensitivity (275 particles / μm²) as observed in comparable labelled sections (not illustrated).
Normal rat brain, fixed in BGPA and osmium tetroxide, prior to partial dehydration and embedding in LR White resin, illustrated the typical ultrastructure that was obtained with this procedure. With support from the grid bars as well as the absence of incubation with sodium (meta) periodate, the effects of stretching/tearing and etching on the stability of the section were minimized. The large arrow shows the distorted area where the section was torn. The remaining, more stable area (circle), illustrated the size and quality of viewing field studied (inset).

Higher magnification of the encircled area revealed 15nm gold particles (density 112 particles/μm²) on glial intermediate filaments (arrows). Myelin figures (arrowheads) and neurofilaments (squares) were prominent.
3A.7 Prolonged impregnation with acrylic resin at low temperatures following secondary fixation in osmium tetroxide

Following primary fixation in 2.5% glutaraldehyde for > 24 hours, secondary fixation in osmium tetroxide and ethanolic dehydration (96%), tissue was impregnated with resin at 4°C for at least 45 hours. The low temperature counteracted the exothermic reaction between osmium and acrylic resin and allowed slow, but more effective impregnation of the tissue. The results obtained with this protocol, were considered to represent the minimum ultrastructural requirements for post embedding immunoelectron microscopy.

Despite air tight cold storage of LR White resin over a period of 18 months, prolonged exposure of resin to air in the half filled storage container increased the cross-linking density of the resin. In this state even lower temperatures were required (0°C) to prevent premature curing of the resin. Here the lower temperature and viscosity of the resin lead to slower impregnation. Isolated signs of incomplete impregnation were seen on some thin sections. Simultaneously, the increased cross-linking density of the resin caused greater steric hinderance and did not optimally favour reaction with immunogold labelling reagents. The last number of specimens embedded in LR White resin, increasingly suffered from this phenomenon.
Prior to viewing, the stability of thin sections obtained from these blocks, was greatly improved by exposing the sections at lowest magnification to a gradual increase in intensity of the electron beam.

MENINGIOMA - cranial fossa / falx [ CASE P ]

FIGURE 3.34

Meningioma (case P) TEM
[ x 9000 ]

After impregnation with resin at 0 °C for 45 hours, this acrylic embedded specimen produced stable thin sections showing improved, acceptable ultrastructure as expected for tissue fixed in 2.5% glutaraldehyde and osmium tetroxide. Nuclei (arrow-heads), intermediate filaments (squares) and desmosomes (arrows), were clearly distinguished.
3B. GFAP AND VIMENTIN EXPRESSION IN DIFFERENT HISTOPATHOLOGICAL ENTITIES

3B.1 Semi-quantitative demonstration of GFAP and vimentin in astrocytomas and an astroblastoma

Tissue from an astroblastoma (case B) and 3 astrocytomas (cases C - E) was processed according to the standardized protocol which included fixation in glutaraldehyde and osmium tetroxide, followed by 96% dehydration and prolonged impregnation in acrylic resin at low temperature.

Sections were labelled for vimentin (1: 100) and GFAP (1: 1000) for 3 hours at room temperature.

Immunoelectron microscopical investigation of the intermediate filaments in astrocytomas revealed poorly to negative reactivity for vimentin. The astroblastoma showed slightly higher levels of vimentin (5 particles / \mu m²). Both the astroblastoma and the astrocytomas were GFAP positive. Normal, acceptable ultrastructure was found in these cases.

Immunohistochemistry performed on formalin fixed, paraffin embedded tissue, showed GFAP positivity in all the specimens, but negative, dubious or weakly positive staining for vimentin. These findings correlated well with the immunoelectron microscopical results (Table 3.09).
TABLE 3.09

GFAP AND VIMENTIN EXPRESSION IN ROUTINELY PROCESSED, ACRYLIC EMBEDDED ASTROCYTOMAS AND AN ASTROBLASTOMA

INTERMEDIATE FILAMENTS

<table>
<thead>
<tr>
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<th>IMMUNOELECTRON MICROSCOPY</th>
<th>IHC</th>
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<td>GFAP</td>
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<td>VIMENTIN</td>
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<td>Density Distrib Area</td>
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<td>30% 10</td>
<td>5 78% 32 χ</td>
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<td>23% 3</td>
<td>2 93% 5 ± χ</td>
</tr>
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<td>36% 66</td>
<td>0 100% 28 -</td>
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<td>E</td>
<td>+ 17</td>
<td>29% 22</td>
<td>1 99% 11 -</td>
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OTHER STRUCTURES

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<td>1 91% 95</td>
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<tr>
<td>E</td>
<td>-</td>
<td>0 97% 75</td>
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</table>

KEY TO TABLE 3.09

**Immunoelectron microscopy**

- **Density**: 10 nm (vimentin) or 15 nm (GFAP) gold particles/μm²
- **Distribution**: % heterogeneity of particle distribution, low figures indicate homogenous labelling.
- **Area**: Total specimen area (μm²) counted.

**IHC**: Light microscopical immunohistochemistry results given as [+] positive, [-] negative or [±] dubious.
A perivascular rosette, indicated by arrows, was one of many found in this glutaraldehyde fixed, paraffin embedded specimen. These structures were characterized by a prominent papillary growth pattern and pronounced vascularity. Scattered mitoses were present. The unipolar tumour cells, with uniform nuclei, even chromatin content and small, easily visible nucleoli, were thickly clustered around the vessels. The perikaryon was often removed from the vessel (square) with a thick processes extending vertically to the vessel wall (arrow-head). GFAP and vimentin immunohistochemistry done on this specimen, produced dubious positivity (not illustrated).
Contrastingly, immunohistochemistry on similar, formalin fixed, paraffin embedded tissue revealed perivascular rosettes with strongly GFAP positive cells (arrow). The positivity was seen mainly in the processes, but also in perikarya of the cells.

Immunohistochemical labelling for vimentin of the formalin fixed, paraffin embedded specimen showed a similar, but much weaker staining pattern as was seen for GFAP. Here, labelling was focal, weakly positive in some processes (arrow-heads) of the cells found in the perivascular rosettes.
FIGURE 3.38

Astroblastoma, (case B) GFAP

[ x 9000 ]

Immunoelectron microscopy of the glutaraldehyde fixed, osmicated specimen produced thick processes with 15 nm gold particles (not easily visible at this magnification) on intermediate filaments (arrows). A characteristic nucleus with a prominent nucleolus (arrow-head) was also demonstrated.
FIGURE 3.39

Astroblastoma, (case B) GFAP

[ x 60000 ]

Higher magnification clearly demonstrated 15 nm gold particles (44 particles / μm² with a 30% heterogeneity in distribution) on glial intermediate filaments (arrow).

Polysomes (square) and rough endoplasmic reticulum were observed in close association with intermediate filaments. The specimen appeared to be well preserved with as membranes were relatively well defined and adequate contrast was obtained.
FIGURE 3.40

Astroblastoma, (case B) vimentin

[ x 36000 ]

Sparsely as well as densely arranged glial intermediate filaments were decorated with 10 nm gold particles (arrow), indicating vimentin positivity (5 particles / \( \mu m^2 \)). The particle distribution was almost 50% more heterogeneous than the labelling figures obtained for GFAP. By replacing the primary antibody with buffer, a reagent negative control was obtained that yielded 1 particle / \( \mu m^2 \) on intermediate filaments. The abundance of these structures and their association with polysomes (circle) and granular endoplasmic reticulum (dark arrow-heads), indicated active intermediate filament synthesis (S). These conditions of very low labelling sensitivity restricted proper demonstration of the postulated vimentin / GFAP shifts during active filament synthesis.
ASTROCYTOMA - spinal cord [ CASE C ]

FIGURE 3.41

Astrocytoma, (case C) H&E
[ x 250 ]

Formalin fixed, paraffin embedded tissue showed masses of well differentiated fibrillary astrocytes, characterized by cellularity and glial process formation (arrows) including Rosenthal fibres (arrow-heads).

FIGURE 3.42

Astrocytoma, (case C) GFAP
[ x 250 ]

Immunohistochemistry done on the specimen shown in figure 3.35, produced GFAP positivity localized to the cell bodies and processes of the fibrillary astrocytes (arrows) as well as Rosenthal fibres (arrow-heads). A few cell processes showed focal, weakly positive staining for vimentin (not illustrated).
Despite prolonged primary fixation, immunoelectron microscopy revealed satisfactory ultrastructure with a relatively high density of 15nm gold particles (arrows). These were localized to well defined astroglial bundles of GFAP positive intermediate filaments. Neurofilaments (squares) and myelin figures (arrow-heads) did not attract any significant labelling. Immunogold labelling for vimentin was negative (not illustrated).

The inset (lower left), has a large arrow overlying the area studied. The encircled myelin figure and adjacent bundle of filaments within the large arrow, are shown at higher magnification (dark arrows) as well as in figure 3.44.
FIGURE 3.44

Astrocytoma, (case C) GFAP

[ x 60000 ]

Inset: [ x 30000 ]

The structures marked by dark arrows in figure 3.43, are shown here at higher magnifications. The inset (top left) shows the myelin figure (arrow-head) and GFAP negative neurofilaments (square). GfAP positive structures included a Rosenthal fibre (dark arrow-head) and the bundle of glial intermediate filaments (arrow), both decorated with 15nm gold particles.
Additionally, overnight incubation with antiserum at 4 °C was performed on this specimen. The 15 nm gold particles (arrows) were localized to bundles of glial intermediate filaments. These particle counts (111 particles / μm² with a 17% heterogeneity in distribution over an area of 13 μm² counted, [0 particles / μm² on other structures]), were similar to those obtained by a 3 hour GFAP incubation at room temperature (126 particles / μm² with a 23% heterogeneity in distribution over an area of 3 μm² counted, [1 particle / μm² on other structures]) as shown in the previous figures. This illustrated slightly better specificity, but lower sensitivity for the overnight procedure.
FIGURE 3.46

Astrocytoma, (case C) GFAP

[ x 66000 ] (Philips)

Improved ultrastructure was obtained by photographing the same specimen whilst using a more advanced electron microscope. Bundles of glial intermediate filaments, decorated with 15nm gold particles (arrows), were clearly visible. The relatively low labelling sensitivity was illustrated by fairly large areas of intermediate filaments which were devoid of any particles.
ASTROCYTOMA - cerebellum [CASE D]

FIGURE 3.47

Astrocytoma, (case D) H&E

[ x 250 ]

Formalin fixed, paraffin embedded tissue from this cerebellar astrocytoma was characterized by astroglial processes (arrow-heads) and prominent Rosenthal fibres (arrows). Pilocytic areas and focal microcystic degeneration were other features observed (not illustrated).

FIGURE 3.48

Astrocytoma, (case D) GFAP

[ x 250 ]

Immunohistochemistry produced strong positivity for GFAP localized to Rosenthal fibres (arrows) and astroglial processes (arrow-heads). While staining for vimentin was negative, these astrocytic processes showed S100 positivity and diffuse dubiously positive staining for NSE (results not illustrated).
FIGURE 3.49

Astrocytoma, (case D) GFAP

[ 30000 ]

This specimen produced 15 nm particles localized to well defined bundles of glial intermediate filaments (arrowhead) as well as an area of Rosenthal fibre formation (arrow). This indicated specific, low density GFAP positive immunogold labelling (28 particles / μm² with a 36% heterogeneity in distribution). Here individual intermediate filaments were still recognizable within the dense core of the fibre. This clearly showed that the intermediate filaments were actively involved in the formation of Rosenthal fibres.

Labelling for vimentin gave a negative result (not illustrated). The specimen displayed normal ultrastructure.
FIGURE 3.50

Astrocytoma, (case D) GFAP

[ 90000 ]

Rosenthal fibres (arrows) showed peripheral as well as central, low density labelling for GFAP. 15 nm particles (arrow-head) of similar density and distribution were present on surrounding glial intermediate filaments. The satisfactory morphological detail seen at high magnification, correlated well with proper resin impregnation. This made a fairly large, stable area (66 \( \mu \text{m}^2 \)) available for counting. Relatively large areas of intermediate filaments and Rosenthal fibres were not labelled, which accounted for the low labelling density, (28 particles / \( \mu \text{m}^2 \)).
ASTROCYTOMA - cerebellum [ CASE E ]

FIGURE 3.51

Astrocytoma, (case E) H&E

[ x 250 ]

This glutaraldehyde fixed, paraffin embedded specimen showed the light microscopical appearance of the specimen area that was used for immunoelectron microscopy. Formalin fixed, paraffin embedded tissue was characterized by vascular proliferation, large necrotic areas and infiltration of the leptomeninges (not illustrated).

FIGURE 3.52

Astrocytoma, (case E) GFAP

[ x 250 ]

The glutaraldehyde fixed, paraffin embedded specimen revealed strongly GFAP positive cells (arrows). Contrastingly, immunohistochemistry on formalin fixed, paraffin embedded tissue produced focal, often dubious positivity for GFAP. Labelling for vimentin was negative while some S100 positive nuclei and a few NSE positive cells were observed (results not illustrated).
FIGURE 3.53

Astrocytoma, (case E) GFAP

[ x 15000 ]

Rather small, but numerous bundles of glial intermediate filaments (arrows), were closely associated with polysomes (circle), which indicated areas of active intermediate filament synthesis (s). This area (square) is shown at higher magnification in figures 3.54-5.
These areas indicated filament synthesis (s) associated with polysomes (circles) and loosely arranged intermediate filaments. Only a few, scattered 15 nm gold particles were found on these filaments opposed to more intense GFAP positive labelling of more prominent bundles of intermediate filaments (arrow). The quality of the ultrastructure was reasonable although not all membranes were clearly defined. Fixation artifact was indicated by the fuzzy nuclear envelope of the partially exposed nucleus (arrow-head, top left). Signs of incomplete impregnation with resin (dark squares) were also present.
FIGURE 3.55

Astrocytoma, (case E) GFAP

[ x 60000 ]

Higher magnification of the area shown in the previous two figures (S), illustrated the relationship between polysomes (circle) and a bundle of intermediate filaments (arrow). The intermediate filaments were decorated with 15 nm gold particles of low labelling density. The reagent negative control (primary antiserum substituted by buffer) was negative while no significant immunogold labelling for vimentin was seen (results not illustrated). Signs of section instability were present (dark squares).
3B.2 Semi-quantitative demonstration of GFAP and vimentin in gliomas

Specimens from case F (glioma/glioblastoma), cases G and H (gliomas) and cases N and O (mixed gliomas, mainly oligodendroglial), were prepared according to the standardized processing and labelling protocols.

As presented in Table 3.10, the intermediate filaments of all gliomas showed a very low to negative reactivity for vimentin. This pattern, similar to the one found in the astrocytomas, was confirmed by negative, dubious or weakly positive results obtained for paraffin embedded tissue. Provided that an astroglial component was present, all the gliomas in this group displayed positive labelling for GFAP at light as well as electron microscopical levels. These specimens produced low sensitivity labelling and were characterized by relatively normal ultrastructure.
# Table 3.10

GFAP and Vimentin Expression in Routinely Processed, Acrylic Embedded Gliomas

## Intermediate Filaments

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<td>Density Distrib Area</td>
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<tr>
<td>G</td>
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<td>H</td>
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<tr>
<td>N</td>
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<td>O</td>
<td>± *</td>
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## Other Structures

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<td>Density Distrib Area</td>
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<td>0</td>
</tr>
<tr>
<td>G</td>
<td>- 1</td>
<td>98% 36</td>
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</tr>
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<td>H</td>
<td>- 1</td>
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<td>0</td>
</tr>
<tr>
<td>N</td>
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<td>0</td>
</tr>
<tr>
<td>O</td>
<td>- 1</td>
<td>95% 26</td>
<td>0</td>
</tr>
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</table>

**Key to Table 3.10**

**Immunoelectron microscopy**

- **Density**: 10 nm (vimentin) or 15 nm (GFAP) gold particles / μm²
- **Distribution**: % heterogeneity of particle distribution, low figures indicate homogenous labelling.
- **Area**: Total specimen area (μm²) counted.
- **IHC**: Light microscopical immunohistochemistry results given as [+] positive, [-] negative or [±] dubious.
GLIOMA / GLIOBLASTOMA - (L) frontal lobe [ CASE F ]

FIGURE 3.56

Malignant glioma / Glioblastoma, (case F) H&E
[ x 250 ]

Formalin fixed, paraffin embedded tissue presented a pleomorphic glial tumour (shown here). Areas of necrosis were also present (not illustrated).

FIGURE 3.57

Malignant glioma / Glioblastoma, (case F) S100
[ x 250 ]

Immunohistochemistry done on this specimen produced S100 positivity in astroglial (arrow-heads) as well as some oligodendroglial cells. A positive reaction for NSE was found in a variety of cell types, including astrocytes (not illustrated).
FIGURE 3.58

**Malignant glioma / Glioblastoma, (case F) GFAP**

[ x 250 ]

Immunohistochemically, the astroglial component was best demonstrated by a strong GFAP positive reaction (arrow-heads). The oligodendroglial cells were GFAP negative.

---

FIGURE 3.59

**Malignant glioma / Glioblastoma, (case F) vimentin**

[ x 250 ]

Weak vimentin positivity was found in some of the astroglial cells (arrow-heads), while all other cells were negative.
Contrastingly, glutaraldehyde fixed, paraffin embedded tissue representing the small specimen that was available for immunoelectron microscopy (inset, middle right), showed no reactivity for either GFAP or vimentin.

Ultrastructurally, the specimen was characterized by densely packed cells and processes. Gemistocytes, stuffed with glial intermediate filaments, were prominent (arrowheads). The 15 nm gold particles on these structures are more visible at higher magnification.
Well defined bundles of densely packed as well as more loosely arranged intermediate filaments were sparsely decorated with 15 nm gold particles (arrow-heads). The labelling density for GFAP (8 particles / \( \mu m^2 \)) was rather low, while no significant labelling for vimentin was seen. By using the rabbit anti GFAP antiserum in combination with the goat anti mouse 10 nm gold probe, a reagent negative control was performed which produced 3 particles / \( \mu m^2 \) on intermediate filaments and 1 particle / \( \mu m^2 \) on other structures. As this figure was slightly higher than normal non-specific labelling levels, it indicated some cross reactivity between rabbit and mouse immunoglobulins.

The intermediate filaments were in close association with numerous polysomes (square) and mitochondria (arrow), indicating active growth of the tumour. Individual necrotic cells and macrophages, features commonly seen in glioblastomas, were also noted (not illustrated).
GLIOMA - frontal lobe and meninges [CASE G]

FIGURE 3.62

Malignant glioma, (case G) HE

[ x 250 ]

Glutaraldehyde fixed, paraffin embedded showed a cellular malignant glioma in which there were florid vascular proliferative structures (arrow-heads), haemorrhage and elaboration of more or less connective tissue. These vasoformative cells did not appear cytologically malignant. Formalin fixed, paraffin embedded tissue displayed similar histology (not illustrated).

FIGURE 3.63

Malignant glioma, (case G) GFAP

x 250

Both glutaraldehyde (shown here) and formalin fixed, paraffin embedded specimens produced strongly GFAP positive cells (arrow-heads). Focal areas were S100 positive, but no reaction was seen with antisera to NSE and vimentin (not illustrated).
FIGURE 3.64

Malignant glioma, (case G) GFAP

[ x 12000 ]

Immunoelectron microscopy revealed GFAP positive, glial intermediate filaments (small arrows, gold particles not clearly visible at this magnification) in association with mitochondria (encircled arrow-heads) and collagen (square). A phagocyte (dark arrow-heads) which engulfed a necrotic cell (large arrow), is shown at higher magnification in figure 3.59. These features are often associated with glioblastomas.
Figure 3.65

Malignant glioma, (case G) GFAP
[ x 24000 ]

Higher magnification showed part of the phagocyte (arrowheads) seen in the previous figure, surrounding the necrotic cell (large arrow). This large, almost oval structure contained irregular filamentous material, which attracted some 15 nm gold particles (circle). Although the possible presence of glial remnants with an artifactual appearance within the necrotic cell could not be excluded, the labelling was interpreted as being non-specific.
FIGURE 3.66

Malignant glioma, (case G) GFAP

[ x 45000 ]

This specimen displayed low density GFAP positivity (6 particles / μm²), with 15 nm gold particles (arrow-heads) localized to loosely arranged glial intermediate filaments. Immunogold labelling for vimentin was negative (not illustrated).
FIGURE 3.67

Malignant glioma, (case H) H&E

[ x 250 ]

Formalin fixed, paraffin embedded sections of this large, cystic mass revealed a pleomorphic malignant glioma with focal gemistocytic (shown above) and oligodendroglial areas. Prominent background vacuolation (almost microcystic) and a gradual transition from gliotic brain to tumour, was seen in all the cyst wall tissue examined (not illustrated).

FIGURE 3.68

Malignant glioma, (case H) GFAP

[ x 250 ]

Immunohistochemistry done on this specimen showed GFAP positive gemistocytes (arrow-heads) and gliosis, whilst other markers gave dubiously positive (S100, NSE) or negative (vimentin) results.
FIGURE 3.69

Malignant glioma, (case H) GFAP
[x 30000 ]
Inset: H&E [ x 250 ]

The glutaraldehyde fixed, paraffin embedded section representing the area studied immunoelectron microscopically (inset, top left), also displayed pleomorphism, with a greater oligodendroglial content (smaller nuclei, (arrow-head) and a few gemistocytes (dark arrow-head). Immunohistochemistry done on this specimen produced no significant labelling for GFAP or vimentin.

Contrastingly, numerous small bundles of glial intermediate filaments (arrows), were demonstrated at ultrastructural level by GFAP positive 15 nm immunogold labelling. No vimentin positivity was observed whilst replacement of the vimentin monoclonal antibody with buffer yielded a negative labelling result (not illustrated). Signs of incomplete impregnation with resin were prominent (squares). A few non-specific particles were present on the nucleus (arrow-heads, top right), which showed clearly defined membranes.
MIXED GLIOMA  [ CASE N ]

Predominantly oligodendroglial, - cortex, corpus callosum

FIGURE 3.70

Mixed glioma, (case N) H&E

[ x 250 ]

The formalin fixed, paraffin embedded specimen showed histological features of a mixed glioma with diffuse infiltration of the cortex by oligodendroglial cells and prominent neoplastic astrocytes elsewhere. The particular area shown here, was characterized by clusters of cells with pleomorphic, often large nuclei, prominent nucleoli and eosinophilic cytoplasm.

FIGURE 3.71

Mixed glioma, (case N) S100

[ x 250 ]

Immunohistochemically, the cells shown in figure 3.70. were S100 positive, including astrocytic as well as oligodendrocytic components.
FIGURE 3.72

**Mixed glioma, (case N) GFAP**

[ x 250 ]

A different area of the same specimen was characterized by a more cellular, less pleomorphic appearance. Clusters of astroglial cells were GFAP positive (arrow-heads) while oligodendroglial cells (squares) were not stained. Negative immunolabelling was observed for vimentin, while neurons in the cortical areas, infiltrated by tumour cells, stained positively for NSE (not illustrated).

FIGURE 3.73

**Mixed glioma (case N) H&E**

[ x 250 ]

Glutaraldehyde fixed, paraffin embedded sections of the tissue corresponding with the specimen selected for immunoelectron microscopy, had a similar appearance to the area shown in the previous figure. Here astroglial fibres (arrow-head) were more prominent.
FIGURE 3.74

Mixed glioma, (case N) GFAP

[ x 10500 ]

Inset: [ x 250 ]

(Inset, bottom left): Immunohistochemically, GFAP positive cells (arrows) and astrocytic processes were demonstrated on the glutaraldehyde fixed, paraffin embedded specimen, thus confirming the astroglial character of the specimen.

At electron microscopical level rather small bundles of glial intermediate filaments (arrow-heads) along with numerous hyaline granules (squares) were scattered throughout the cytoplasm. The area adjacent to the capillary (large arrow, middle right) and the Rosenthal fibres (arrow, top right), are shown at higher magnification in figures 3.75-6.
FIGURE 3.75

Mixed glioma, (case N) GFAP
[ x 30000 ]

Higher magnification of the area indicated by a large dark arrow in fig 3.74, revealed small bundles of densely packed glial intermediate filaments (dark arrow-heads) adjacent to the erythrocyte (e) within the capillary (top right). The 15nm gold particles (arrow-head) indicate GFAP positivity. The intermediate filaments were dispersed amongst hyaline granules (squares) and areas of possible Rosenthal fibre formation (dark arrows). Polysomes (circle) occurred within the endothelial cell cytoplasm. Immunogold labelling for vimentin failed to produce any significant positivity (not illustrated).
FIGURE 3.76

Mixed glioma, (case N) GFAP

[ x·60000 ]

At higher magnification, the area marked by the smaller dark arrow in figure 3.74 demonstrated glial intermediate filaments decorated with 15nm gold particles (arrow-heads). This represented a labelling density of 51 particles / μm². A hyaline granule (square) and signs of degeneration (Rosenthal fibre formation, arrows) were present. Although distinct individual intermediate filaments were not clearly distinguished within the Rosenthal fibres, their GFAP positivity strongly suggested glial involvement in these structures.
MIXED GLIOMA [ CASE 0 ]

Predominantly oligodendroglial, temporo-parietal

The formalin fixed paraffin embedded specimen showed an oligodendroglioma with characteristic vascularity and perivascular pseudorosetting. Prominent mitosis, calcification, necrosis and foci of vacuolated cells were present. The presence of an astroglial component was indicated by GFAP and S100 positivity found in isolated areas of the specimen.

However, glutaraldehyde fixed, paraffin embedded tissue representing the block used for electron microscopy, produced negative labelling for GFAP and vimentin. Thorough electron microscopical investigation of glutaraldehyde fixed, acrylic embedded tissue revealed numerous oligodendrocytes, thus confirming the oligodendroglial character of the tumour. Necrotic areas were found, but no intermediate filaments were seen in any of the 13 blocks that were examined. This case illustrates the utmost importance of proper tissue sampling for electron microscopy when studying glial neoplasms.
3B.3 Semi-quantitative demonstration of GFAP and vimentin in meningioma and Alexander's disease

Tissue from case P (meningioma) and case Q (Alexander's disease) was processed and labelled according to the standardized protocols. Ultrastructure with good contrast and membrane definition was obtained for both cases.

As illustrated in Table 3.11, the intermediate filaments found in the meningioma were GFAP negative, but showed low density positivity for vimentin. In the case of Alexander's disease, positive immunogold labelling for GFAP and vimentin was localized to glial intermediate filaments present in relatively normal brain tissue as well as areas characterized by degenerative changes. Rosenthal fibres, representing degenerative filamentous structures, showed evenly distributed peripheral and central GFAP positivity, but were vimentin negative. These results correlated well with immunohistochemical findings at light microscopical level.
TABLE 3.11

GFAP AND VIMENTIN EXPRESSION IN ROUTINELY PROCESSED, ACRYLIC EMBEDDED MENINGIOMA (case P) AND ALEXANDER’S DISEASE (case Q).

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OTHER STRUCTURES

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<td>Q</td>
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KEY TO TABLE 3.11

Immunoelectron microscopy

Density 10 nm (vimentin) or 15 nm (GFAP) gold particles / \( \mu \text{m}^2 \)

Distribution % heterogeneity of particle distribution, low figures indicate homogenous labelling.

Area Total specimen area (\( \mu \text{m}^2 \)) counted.

IHC: Light microscopical immunohistochemistry results given as [+] positive, [-] negative or [±] dubious.
MENINGIOMA - cranial fossa / falx  [ CASE P ]

FIGURE 3.77

Formalin fixed, paraffin embedded tissue displayed a meningeal tumour with the histological features of a meningioma. Intense cellularity and distinct, interlocking cytoplasmic cell processes (arrow-heads) were prominent. Vimentin immunohistochemistry done on this specimen revealed diffuse, often focal positivity, while staining for GFAP was negative (not illustrated).
The electron microscopical investigation showed the distinct, interlocking, sinuous cytoplasmic cell processes which contained loosely packed intermediate filaments, decorated with a few isolated 15 nm gold particles (arrow). Numerous desmosomes (arrow-heads) and other cytoplasmic structures were present.
FIGURE 3.79

Meningioma, (case P) vimentin

[ x 60000 ]

Heterogeneously distributed (78%) 10 nm gold particles with a density of 9 particles / μm², were localized to loosely arranged glial intermediate filaments (squares) in close association with polysomes (circle). Despite the low labelling sensitivity, this result indicated significant positivity for vimentin. The reagent negative control (primary antiserum substituted for by buffer), produced a negative result (0 particles / μm²) on all structures.
Alexander's disease, (case Q) H&E

[250]

Formalin fixed, paraffin embedded tissue of a rare example of Alexander's disease, showed diffuse demyelination of white matter with paucity of oligodendrocytes and relative little myelinoclastic activity. In addition, homogeneous eosinophilic bodies (Rosenthal fibres) were present, either as granular deposits within perikarya of astrocytes, or as elongated tapering rods in astrocytic processes (arrows).
FIGURE 3.81
Alexander's disease, (case Q) GFAP
[ x 250 ]

Immunohistochemistry done on the formalin fixed, paraffin embedded specimen, demonstrated GFAP positive Rosenthal fibres (arrows) and astrocytic processes (arrow-heads) in association with vascular structures (square). Other markers done on this specimen (not illustrated), included S100 (positive in some oligodendrocytes, diffusely in astrocytes and neuropil) and NSE (diffusely positive staining of neuropil and cells).

FIGURE 3.82
Alexander's disease, (case Q) vimentin
[ x 250 ]

A few vimentin positive astrocytes (arrow-heads) were found in the glutaraldehyde fixed, paraffin embedded specimen that represented the tissue selected for immunoelectron microscopy. Rosenthal fibres showed no immunoreactivity for vimentin.
Examination of the immunoelectron microscopical specimen showed Rosenthal fibres (arrows) and glial intermediate filaments (arrow-heads) decorated with 15 nm gold particles. Both core and periphery of Rosenthal fibres were labelled, the labelling density being similar to that found for adjacent intermediate filaments. Due to the electron density of these structures, the gold particles on these degenerative structures were not clearly visible. With the aid of under-exposed photographs, the fibres marked by dark arrows, are shown at higher magnification in figures 3. 84-5. Instability of the thin sections was encountered mainly in osmiophilic areas associated with the Rosenthal fibres.

To establish a reagent negative control, the rabbit anti GFAP antiserum was replaced by the mouse anti vimentin monoclonal antibody whilst using the goat anti rabbit 15 nm gold probe. The negative labelling result (0 particles / \(\mu\text{m}^2\)) supported the specificity of the labelling protocol.
FIGURE 3.84

Alexander's disease, (case Q) GFAP

[ x 60000 ]

Higher magnification of the Rosenthal fibre marked by a dark arrow in the previous figure, illustrated the distribution of 15 nm particles associated with this structure. The electron dense core of the Rosenthal fibre attracted more particles (large arrow) than the filamentous periphery (smaller dark arrows) or the adjacent bundle of glial intermediate filaments (arrow-head). Here the close relationship between these structures is evident.

As the filamentous components could be lying deeper within the section than the larger, electron dense core of the fibre, this could explain the different labelling sensitivities for these structures.
FIGURE 3.85

Alexander's disease, (case Q) GFAP

[ x 75000 ]

This Rosenthal fibre (arrow) and the adjacent glial intermediate filaments (arrow-head) were decorated with 15 nm gold particles. Here the labelling density on the electron dense core of the fibre is greater than for the closely associated, surrounding filamentous periphery. Other structures such as neurofilaments within myelin figures were not labelled (not illustrated). Whether the filamentous periphery of the fibre was an extension of adjacent bundles of glial intermediate filaments, could not be established with certainty.
FIGURE 3.86

Alexander's disease, (case Q) vimentin
[x 75000]

Immunogold labelling for vimentin produced scattered 10 nm gold particles (5 particles/μm²) over loosely arranged intermediate filaments (arrow-head). In contrast with the GFAP positivity demonstrated in the previous figures, here the electron dense core of the Rosenthal fibre (arrow) was negative.
Chapter 4
DISCUSSION

4A THE INFLUENCE OF METHODOLOGY ON LABELLING RESULTS

4A.1 Semi-quantitative post embedding immunoelectron microscopy

The abundance of astroglial fibres in the central nervous system was exploited to study various morphological and immunochemical aspects of glial intermediate filaments by application of improved immunodetection technology. In this respect, the need for post-embedding immunoelectron microscopy has been emphasized, but remained to be one of the difficulties which have not been solved satisfactorily.

The requirements for comparative evaluation of immunoelectron microscopic methodology as expressed by Slot et al (1989), were met by the novel, semi-statistical method that was devised to measure, express and compare immunogold labelling obtained from different specimens. Figures expressing particle densities are semi-quantitative indications of labelled antigen associated with intermediate filaments present in a particular specimen. This is complemented by expression of the percentage heterogeneity of the particle distribution.

High percentage heterogeneity figures (%H > 50%) indicate negative labelling with a random distribution of particles, while low figures indicate homogeneous distribution of
particles associated with positive labelling. By definition, the examined area as defined by the borders of a bundle of intermediate filaments, includes the clear spaces between individual filaments. Particle densities for loosely arranged filaments will therefore be slightly lower than for areas characterized by densely packed filaments.

Similarly, visible glial intermediate filaments lying beneath the surface of the section will not be labelled as intensely as those lying at the surface (Goto et al, 1987). The density and stability of sections in the electron beam (Chapman, 1988) as well as mathematical aspects concerning photography, magnification (Wergin & Pooley, 1988) and formula calculations, may also contribute to minor variability of figures. Although these relative figures do not solve the question of true antigen concentration, they may be applied to express positivity and/or efficiency of immunogold labelling when comparing different specimens.

As specimen processing and immunodetection technology has a major influence on labelling results, these aspects must be considered before any conclusions on expression and coexpression of GFAP and vimentin in glial intermediate filaments can be drawn.

4A.2 Effect of matrix density on antigen availability and morphology

Complex matrix formation is the combined effect of the
various cross-linking reactions associated with fixation (primary matrix) and impregnation of fixed tissue with embedding media (secondary matrix). Formation of such a high density matrix ensures high quality ultrastructure and is additional to the normal density of tissue in the fresh state.

Contrastingly, the high density matrix creates a degree of steric hindrance which limits access of immunolabelling reagents to antibody binding sites. This is the major restriction on immunocytochemical recognition of surviving tissue antigen after chemical alteration or destruction of epitopes during tissue processing.

These aspects imply that embedding media as well as primary and secondary fixation procedures have to be applied selectively to manipulate the density of the tissue-fixative-resin matrix to obtain a compromise between antigen availability and morphology. As both the molecular interactions involved in tissue processing and the initial (true) antigen concentration in a tissue section cannot be quantified, this leads to the conclusion that antigen availability is a relative, variable value for epitopes present in tissue specimens.

The combined, complex effect of tissue processing factors on antigen availability is illustrated by case C (Table 3.09, Figures 3.41 - 6), which produced significant positivity for GFAP after incubation of the specimen in
glutaraldehyde for 2 years. Here the abundance of glial intermediate filaments, possible loss of cytoplasmic components and the fibrous nature of the tissue may have lead to matrix formation of lesser density. This confirms the findings of Bowers & Maser (1988) that prolonged fixation is not proportional to negative labelling. It also implicates that the nature of the tissue-fixative-resin matrix for a given specimen will determine the phenotypic characterization of an epitope as recognized by a particular antibody.

4A.3 The effect of embedding media on matrix density

In comparison to acrylic embedded specimens (Sections 3A.5-7), thin sections cut from the corresponding Spurr's epoxy resin embedded blocks repeatedly produced very low to negative labelling results for GFAP (Section 3A.1-4). This was best illustrated by case A, a known positive specimen which produced constantly negative results, as well as cases K and M, where, despite mild fixation, particle densities were at least 20 times greater for acrylic embedded specimens.

Although epoxy resin is preferred for optimal ultrastructure where possible (Bendayan, 1984), the density and hydrophobic nature of the secondary matrix formed by epoxy resins prevents penetration of any aqueous solutions into the section (Acetarin et al, 1987; Mollenhauer, 1986). At the cost of "pale sections" and increased non-specific
labelling (sections 3A.2-3), this may be partially overcome by etching of the section surface (Straus, 1978; Bendayan & Zollinger, 1983). This limits labelling to portions of relatively large structures such as zymogen granules, which, due to their size, will be exposed at the surface of the section.

For central nervous system tissue (Valentino et al., 1985), in particular intermediate filaments (Gräber & Kreutzberg, 1985; Bettica & Johnson, 1990), this is not applicable as these structures, due to their small diameter, are only randomly exposed at the section surface. These aspects illustrate and confirm the need for a resin with greater immunolabelling potential.

Due to its hydrophilic character and lesser cross-linking density, acrylic LR White resin (Newman et al., 1983; Causton, 1984; Newman, 1987; Mollenhauer, 1988) was selected as embedding medium for this study. However, incomplete dehydration and the incompatibility between osmium tetroxide and LR White resin prevented proper impregnation of tissue with resin, thus contributing to malformation of the secondary matrix which is required for stability of sections and good ultrastructure (Table 3.07). Improved stability of the sections was obtained by prolonged, low temperature impregnation of osmicated tissue (Craig & Miller, 1984, Gräber & Kreutzberg, 1985). Despite these precautions, premature curing of the resin still
occurred in isolated areas. Enhanced cross-linking density was also acquired by the resin due to repeated exposure to air as well as the limited shelf life of the product.

In specimens where the exothermic reaction between osmium tetroxide and LR White resin was not counteracted, focal areas of intense labelling (Table 3.08), characterized by signs of poor resin impregnation, were seen. Although the instability of the sections made identification of subcellular structures almost impossible, labelled areas produced labelling densities > 200 particles / \(\mu m^2\). This phenomenon indicated the presence of large concentrations of antigen, thus representing optimal labelling conditions for areas where the secondary matrix was of low density. At light microscopical level, similar positive labelling was observed in deresined thick epoxy sections.

These results clearly indicate that for acrylic embedded specimens, the embedding medium effectively restricted access of labelling reagents to the antigenic binding sites.

More recently, Johnson & Bettica (1989) and Bettica & Johnson (1980) attempted to reduce the density of the tissue-resin matrix by formula modification of a new epoxy resin. The procedure included partial removal of the resin from thin sections and oxidation of osmium tetroxide prior to application of 5 nm gold probes to penetrate the
remaining matrix. Their labelling results for GFAP in a case of Alexander's disease were comparable or less sensitive than those obtained for the glutaraldehyde fixed, osmicated, acrylic embedded specimen (case Q; table 3.11) shown in figures 3.80-6).

4A.4 Acrylic LR White resin for light microscopy

At light microscopical level, the density and hydrophilic character of LR White medium grade resin is not fully compatible with known aqueous and non-aqueous reagents used for staining and mounting of thick resin sections.

Although thick sections are easily cut and were used for initial orientation of embedded tissue after staining with toluidine blue, their retention of water soon resulted in metachromasia, shrinkage and folding. As aqueous solutions do not completely penetrate the resin, such stains and mounting media gave suboptimal results characterized by inferior resolution. The absence of a suitable solvent for removal of the resin from tissue sections, emphasized these incompatibilities (Slater, 1988).

4A.5 Effect of primary fixation on antigen availability and morphology

Despite masking and destruction of some antigenic binding sites by formation of cross-linking bonds, (Miller, 1972; Bolen & McNutt, 1987; Corwin et al, 1989), glutaraldehyde
was chosen as primary fixative to ensure efficient preservation and conventional ultrastructure of labelled tissue specimens (Artvinli 1975; Peters & Richards, 1977; Hopwood 1985).

In the absence of a suitable monoclonal antibody, reduced antigen availability was partially overcome by application of a widely used, commercially available polyclonal antibody (Dako A561) which demonstrated the aldehyde resistant epitopes associated with GFAP.

As most vimentin associated epitopes are destructed by aldehyde fixation, currently available vimentin antisera display low sensitivity and specificity when applied to aldehyde fixed, paraffin-embedded tissues (Herpers et al., 1986; Leader et al., 1987). Under these circumstances it is no surprise that application of the monoclonal antibody to vimentin (Dako M725) produced an average of only 2 particles/µm² with 92% heterogeneity in distribution for the glial intermediate filaments that were found in 11 specimens (total area of 19 µm²). These results are supported by the manufacturer's recent suggestion for application of monoclonal antibody M725 to microwave processed specimens and current development of new antibodies that recognize aldehyde resistant epitopes. For labelling of post-embedded specimens, this implies that the detection sensitivity for aldehyde fixed vimentin associated epitopes by this reagent (or any other known
antibody to vimentin) is too low and therefore not suitable for investigation of vimentin-GFAP shifts or reliable demonstration of coexpression with GFAP in the same intermediate filament.

Controlled mild primary fixation procedures (Sections 3A.2-5) showed that low concentrations of fixative and shorter incubation times as promoted by Bone & Ryan (1972), result in incomplete formation of cross-linking bonds, thus leaving relatively more epitopes unaffected. In these specimens subjected to such suboptimal or altered processing protocols, high density positivity in small areas, characterized by poor morphology and instability of sections, was found for GFAP and vimentin. This reflects false negative results for well preserved, stable areas containing initial low concentrations of antigen.

Although reports indicate that GFAP is fairly stable under post mortem conditions and that necrosis had no obvious effect on GFAP concentrations (Rasmussen et al, 1980), inconsistent labelling was found in specimens that were exposed to delayed fixation and in routine specimens where tissue blocks exceeded the maximum thickness (1.5 mm) for fixative penetration (Straus, 1979). Under these conditions autolytic changes at molecular level result in incomplete primary matrix formation and destruction of epitopes which lead to false negative and variable immunolabelling results. This is in agreement with the findings of
Pettigrew (1989), who showed that up to 13.5% of specimens studied were not suitable for immunohistochemistry due to suboptimal fixation.

Proteolytic digestion of formalin fixed, paraffin embedded specimens with hydrolytic enzymes such as trypsin or pronase, will produce increased antigen availability for GFAP (Dako A561), but not for vimentin (M725) (results not shown). Although the exact mechanisms for both aldehyde fixation and proteolytic digestion are not known, the hydrolytic reactions are aimed at breakdown of lysine associated cross linking bonds. Such random destruction of proteins (often including specific epitopes) can be interpreted as non-specific, "reversed" fixation which will allow labelling of masked epitopes. Vimentin associated epitopes destroyed by fixation cannot be restored by this procedure.

At electron microscopical level, this approach is not applicable due to the larger number of cross linking bonds associated with glutaraldehyde and/or restriction of the enzymatic reaction by the presence of osmium tetroxide and embedding medium (Johnson & Bettica, 1988).

Though some GFAP associated epitopes were unmasked, others were destroyed by pronase digestion, indicating that destruction of epitopes located in the hypervariable terminal domains of the intermediate filaments (Geisler et al, 1988) can reduce the specificity of antibodies applied
to distinguish between different intermediate filaments. This could indicate minimized chances for recognition of type specific epitopes on the remaining central rod domain, which shows high percentages of similarity in amino acid sequences as determined for the different types of intermediate filaments (Altmannsberger & Osborn, 1987).

Although it favours increased antigen availability in the absence of cross linking bonds, application of a precipitating fixative such as methacarn (Puchtler et al, 1970) was not considered as poor ultrastructure due to shrinkage and loss of some cytoplasmic components is not suitable for routine electron microscopy.

Despite the various shortcomings mentioned, the labelling results from this study showed that immersion fixation can replace perfusion fixation (Gräber & Kreutzberg, 1987), thus providing access to routine human brain biopsy specimens for post embedding immunoelectron microscopy.

4A.6 Influence of secondary fixation on morphology and antigen availability

Secondary fixation with osmium tetroxide was included in the processing protocol to obtain acceptable, conventional ultrastructure (Bone & Ryan, 1972; Friedenberg & Seligman, 1973; Bowers & Maser, 1988). Despite the exothermic interaction between osmium tetroxide and LR White resin, application of this fixative/stain in combination with
primary fixation in glutaraldehyde produced a primary matrix which was characterized by relatively normal ultrastructure with good contrast and sharp definition of membranes.

Omission of osmium tetroxide produced ultrastructure of inferior quality, but still acceptable with most detail visible. Poor contrast, due to the absence of lipid staining, resulted in "pale" sections. These specimens confirmed the findings of Hemming et al (1983) and Wen & Wisniewski (1987), showing increased immunolabelling reactivity (± 20%) for non-osmicated specimens.

Although Newman et al (1983) promoted the use of picric acid to obtain better contrast in non-osmicated, LR White embedded specimens, this study shows that this additive in combination with glutaraldehyde (BGPA) did not meet the expectations of improved immunolabelling or being an efficient substitute for osmium tetroxide.

In unstable sections where the secondary matrix is of lesser density (Table 3.08), efficient oxidation of osmium tetroxide with sodium (m) periodate dramatically increased antigen availability. For epoxy embedded specimens, similar oxidation served a dual purpose by primarily etching of the resin as well as limited oxidation of osmium tetroxide. Here the high density of the secondary matrix prevents proper penetration of the reagent, thus limiting the efficiency of the reaction to the surface of the section.
Prolonged incubation with sodium (meta) periodate will cause non-specific destruction of all tissue components in the section, with resulting poor ultrastructure and increased non-specific labelling (Table 3.03).

In thick sections submitted to immunohistochemistry after removal of the epoxy resin, oxidation of osmium tetroxide with sodium (meta) periodate improved labelling intensity, but suffered from non-specific staining in osmiophilic areas. This was caused by the reaction between diaminobenzidine and osmium tetroxide as described by Sternberger (1979). Here, unwanted enhancement of traces of osmium tetroxide remaining in the section after oxidation (Mar & Wight, 1988) was obtained with DAB during visualization of peroxidase complexes as part of the immunolabelling procedure.

4A.7 Sensitivity of immunodetection systems

Immunodetection sensitivity defines the ability to detect the few accessible epitopes within the high density tissue-resin matrix at the resolution available for viewing specimens by application of high affinity primary antibody in combination with low noise signal amplification and visualization.

Although international quality assurance has shown that many laboratories have not achieved an acceptable standard of immunolabelling (Reynolds, 1989), visualization
techniques such as the ABC method and indirect immunogold methods are well established and may be regarded as a constant factor in immunodetection protocols.

In comparison to 28 \( \mu m^2 \) of other structures examined, (particle density 0 with a 99% heterogeneous distribution), the average vimentin labelling figure of 2 particles / \( \mu m^2 \) at a percentage heterogeneity of 92% for glial intermediate filaments found in 11 specimens (total area examined 19 \( \mu m^2 \)), clearly demonstrates very low labelling sensitivity. This result reflects very low immunolabelling sensitivity due to the destruction of vimentin epitopes by aldehyde fixation, thus producing a false negative staining pattern for vimentin.

Whereas no immunoreactivity was obtained for thick acrylic sections at light microscopical level, GFAP labelled glial intermediate filaments in acrylic thin sections were detected by superior resolution of the electron microscope. Although these specimens produced an average of 50 gold particles / \( \mu m^2 \), the complementing heterogeneity of the particle distribution (20%) indicated that the labelling sensitivity was suboptimal. This is supported by strong immunohistochemical GFAP positivity obtained for corresponding deparaffinized specimens where limited resolution of the light microscope has been partially overcome by greater antigen availability and superior signal amplification offered by the ABC method.
4A.8 Specificity of immunodetection systems

Although equal in sensitivity, auroprobes show greater specificity and are preferred to Protein A gold systems for immunoelectron microscopy (Bendayan, 1984; Roth, 1986). This was demonstrated by the Protein A gold probe (Section 3A.5) which, due to the nature of the reagent, reacted with non-specific immunoglobulins present in tissue sections, their source being either endogenous immunoglobulins and/or proteins originating from the normal non-immune serum that was used to substitute for primary antibody. This finding explained the slightly higher labelling figures shown in Table 3.07, thus implicating reduced specificity due to specific, but unwanted labelling of non-specific immunoglobulins.

The constant negative labelling produced by negative tissue and reagent controls (< 1 particle / \mu m^2 with a 95 - 100% heterogeneity of the particle distribution), indicated high specificity for the immunodetection methodology that was applied to obtain GFAP and vimentin labelling results. Non-specific reactions such as those mentioned by Paasivuo & Saksela (1983), were therefore virtually absent. Here the low immunodetection sensitivity favoured the signal to noise ratio, thus adding credit to the specificity of the polyclonal anti GFAP antibody. These aspects were confirmed by figure 3.45 which illustrated that overnight incubation of specimens with primary antiserum at 4°C produced
slightly improved specificity at the cost of reduced immunodetection sensitivity.

However, such acquired specificity due to low immunodetection sensitivity can lead to poor phenotypic characterization of antigens where low concentrations of antigen including non-specific and/or shared epitopes may not be detected. Similarly, optimal high immunodetection sensitivity may reveal labelling results which could be interpreted as phenotypic expression/coexpression of the antigen in particular tissues and/or structures. This is often seen at light microscopical level where many workers claim novel findings such as GFAP in myoepithelial cells and various reports of coexpression of intermediate filaments in different tissues.

4A.9 Specificity of antisera

Against a background of different known GFAP associated epitopes (Miller & Raff, 1984; Eng, 1985, Kennedy & Fokseang, 1986) and a 63% similarity in the central rod sequence of GFAP, vimentin and desmin (Altmannsberger & Osborn, 1987), it is likely that antibodies raised to these intermediate filaments proteins will have variable specificity and sensitivity. This aspect is additional to specificity and sensitivity manipulation due to antigen availability and immunodetection efficiency.

Many workers include data on the origin, chemical
characteristics, molecular weights and preparation of the antigen used to raise a particular antibody. However, cluster designation of antigens and absorption controls, commonly performed to state the specificity of a particular antibody, cannot secure the possibility that antibodies have been raised against non-specific epitopes present in the antigen preparation. These guidelines associate antibodies with particular antigens, but have little effect on the actual specificity of particular antibodies. As the epitopes seen by the antibody are not defined, one has to rely on their labelling pattern within standardized immunodetection systems to determine their specificity.

The specificity and sensitivity of such standardized labelling patterns can easily be disturbed by a combination of technical factors. These include the disadvantages associated with polyclonal antisera (De Mey & Moeremans, 1986), variable antibody dilutions and titres, the presence of anti-desmin autoantibodies in polyclonal antisera, low affinity of monoclonal antibodies and alteration of staining patterns by proteolytic digestion. Therefore, well characterized, high affinity antibodies, preferentially monoclonal to ensure specificity and a constant supply, should be recognized as unique reagents rather than being associated with antigens in a broad sense.

As most users of these probes are guided by the literature, an internationally standardized format, such as attempted
by NEQAS (Reynolds, 1989), should be used for documentation of specific antibodies, associated labelling patterns and methodology.

4B EXPRESSION OF GFAP AND VIMENTIN IN ASTROGLIAL ENTITIES

4B.1 Relative vimentin concentration in glial intermediate filaments of different astroglial entities

Although at least some vimentin positivity was expected for all the specimens presented in section 3B (Tables 3.09-11), immunodetection with monoclonal anti vimentin (Dako M725) produced a disappointing average particle density (PD) of 2 particles / \( \mu m^2 \) with the heterogeneity in distribution (%H) being 92%. These figures were similar to those obtained for epoxy embedded specimens labelled for GFAP (Table 3.02), which clearly demonstrated false negative results due to very low immunodetection sensitivity.

In relation to these false negative results, low intensity vimentin positivity was found in meningioma (PD = 9, %H = 79%), astroblastoma (PD = 5, %H = 78%) and infant brain tissue associated with Alexander’s disease (PD = 5, %H = 70%). Due to low immunodetection sensitivity, these figures indicate high vimentin concentrations in the glial intermediate filaments of these specimens.

The coexpression of vimentin and GFAP demonstrated in infant frontal lobe brain tissue and astroblastoma, was associated
with embryonal conditions which are represented by these entities.

If intermediate filament formation is irreversible as proposed by De Armond et al (1986) and Bolen & McNutt (1987), then a transition from vimentin to GFAP expression as indicated by Pixley & De Vellis (1984), Backhovens et al (1987) and Dewhurst et al (1987), is likely to be regulated by two different, related, but yet unknown mechanisms (Goldman & Chiu, 1984). Areas indicating active protein synthesis were seen in astroblastoma (case B) and astrocytoma (case E) where polysomes were found in close association with loosely arranged intermediate filaments. Although the low labelling sensitivity did not allow characterization of the intermediate filaments involved, this could be the actual site where the regulatory mechanisms proposed by Goldman & Chiu, (1984) take place. Despite their absence in other astrocytomas or glioblastoma, these features were considered to be random observations which could also occur in other astroglial areas.

Due to limited labelling sensitivity, double labelling attempted on cases B and Q, failed to demonstrate significant coexpression of GFAP and vimentin on the same section (results not shown). As other workers have encountered similar problems for detecting vimentin in aldehyde fixed tissues, these results indicate that
investigation of vimentin-GFAP shifts and demonstration of coexpression with GFAP in the same intermediate filament (Altmannsberger & Osborn, 1987; Domagala et al, 1988), are aspects which cannot be accessed with post embedding techniques applying currently available antibodies to vimentin.

As final consensus on the function of glial intermediate filaments has not been reached (Geiger, 1987), the functional state indicated by the vimentin : GFAP ratio (Pixley & De Vellis, 1984; Pixley et al 1984b; Dahl & Bignami, 1986; Herpers et al, 1986; Backhovens et al, 1987; Miettinen, 1987) remains to be defined by future investigations.

4B.2 Relative GFAP concentration in glial intermediate filaments of different astroglial entities

Analysis of all the specimens presented in section 3B (Tables 3.09-11), produced an average particle density (PD) of 41 particles / \mu m^2 (%H = 39%) for GFAP associated epitopes as detected by the antibody (Dako A561) used.

Except for the meningioma (GFAP negative), relatively uniform GFAP positive labelling was found in 3 astrocytomas (PD = 57, %H = 29%, A = 91 \mu m^2), 2 gliomas (PD = 47, %H = 28%, A = 17 \mu m^2), Alexander's disease (PD = 46, %H = 50%, A = 16 \mu m^2) and astroblastoma (PD = 44, %H = 30%, A = 10 \mu m^2).
Low labelling figures were obtained for the glioblastoma (PD = 8, %H = 49%, A = 37 \text{ \mu m}^2) and case G, a glioma with some features of a glioblastoma (PD = 6, %H = 75%, A = 32 \text{ \mu m}^2).

As the parameters for this semi-quantitative determination are not absolute, variability due to density of filament arrangement, processing factors and mathematical calculations may account for substantial differences in particle densities for the various specimens labelled.

The average 38% heterogeneity in particle distribution found in this group of specimens, indicates relatively low antigen availability for GFAP in comparison to specimens examined under more favourable labelling conditions (tables 3.06-8).

Whether the low figures for cases F and G were due to suboptimal sampling and preservation of the specimens (Pettigrew, 1989), or whether they indicate the coexpression of different intermediate filament monomers in immature glia in glioblastomas (Schachner et al, 1984; Yung et al, 1985; Dahl & Bignami, 1986; Perentes & Rubinstein, 1987), could not be determined with certainty.

As a vimentin negative glioblastoma and two astrocytomas with low labelling figures for GFAP were found, the significance of slightly lower GFAP concentrations as well as indications of increased vimentin expression in the
astroblastoma and infant brain (Alexander's disease), remains to be uncertain.

Although glial intermediate filaments were more abundant in astrocytomas and astroblastoma, with fewer, smaller bundles in normal rat brain and glioblastoma, the actual GFAP concentrations determined for the various entities did not correlate with the descending order of GFAP positivity ranging from reactive fibrillary astrocytes through astrocytoma, glioblastoma, normal fibrillary astrocytes to protoplasmic astrocytes, as established by Duffy et al (1979) and Rasmussen et al (1980).

These workers used immunohistochemistry for counting positive cells in a particular tumour (Duffy et al, 1979) or quantitative electrophoresis to determine GFAP concentrations in homogenized tumour or tissue samples (Rasmussen et al, 1980), which has lead to the application of concepts relating GFAP concentrations to degrees of anaplasia (Herpers et al, 1986), malignancy or differentiation (Herpers et al, 1984; Royds et al, 1986). Their findings relate to the number of astroglial cells present within a particular tumour or perhaps the total number of intermediate filaments in an astroglial cell, but not to the GFAP concentration for a given number of glial intermediate filaments as seen in a small part of a cell.

As glial tumours seldomly are of uniform consistency,
quantification of GFAP is prone to errors as sampling will effect histopathological diagnosis and grading as well as the efficient determination and correlation of the total GFAP concentration. These aspects were recognized by Rasmussen et al (1980) and were confirmed by Luevano et al (1986), who could not find any clear-cut differences between grade of malignancy and immunostaining pattern.

These findings indicate that the GFAP concentration for a given number of mature glial intermediate filaments is constant, irrespective of the astroglial entity in which they occur. Particle densities as recorded with immunoelectron microscopy, therefore reflect suboptimal labelling conditions due to the influence of tissue processing factors (Section 3A).

4B.3 Rosenthal fibres

At light microscopical level, Rosenthal fibres were constantly negative for vimentin, whilst peripheral as well as central positivity for GFAP was observed.

This confirms the findings of Gullotta et al (1985), who reported strong, partial or weak positivity in both the centre and peripheral areas of these structures, but contradicts the earlier reports of Papasozomenos (1983) and Towfighi et al (1983), who found no staining at all. A third group of workers believe that the central, amorphous core is GFAP negative, while the filamentous periphery is
strongly positive (Tascos et al, 1982; Luevano et al, 1986). Western blot reactivity for the 22 kd Rosenthal fibre protein αB-crystallin, demonstrated by Iwaki et al (1989), has not been confirmed by immunoelectron microscopy.

Figure 3.84 shows the close association between the filamentous periphery and the amorphous, electron dense core of the Rosenthal fibre and clearly illustrates GFAP positivity in both parts of the structure. This finding is supported by Johnson & Bettica (1986), who found little or no reactivity at light microscopical level, but were successful in obtaining convincing immunogold labelling at electron microscopical level.

These findings correlate well with similar reports on neurofilament positive accumulations in Lewy bodies, where Pappolla (1986) has shown a positive periphery (light microscopy) as well as both core and peripheral positivity at immunoelectron microscopical level.

Although these findings strongly indicate an astroglial nature for these structures associated with degenerative changes in nervous tissue, the issue whether Rosenthal fibres represent breakdown products of GFAP or whether they consist of plasma proteins engulfed by astrocytes under pathologic conditions, remains to be resolved. Variable staining seen by other workers is likely to reflect the degree of degeneration represented by individual structures
as well as the specificity and sensitivity of their immunodetection methodology applied.

4B.4 GFAP expression in oligodendroglia and ependymal cells

As both astrocytic and oligodendroglial components could not always be demonstrated in the available, small biopsies studied, conflicting labelling results were obtained for mixed tumours (cases N and O). These aspects were expressed by Rubinstein (1982), showing that GFAP in mixed tumours helps diagnosis, but depends on proper sampling, which is often not adequate (Jakstys, 1988).

In predominantly oligodendroglial mixed gliomas, gemistocytic (case H) and neoplastic (case N) astrocytes were prominent, thus explaining the variable GFAP positivity reportedly found in oligodendrogliomas. Negative labelling for GFAP and vimentin was found in the oligodendroglial portion of the mixed glioma (case O). No glial intermediate filaments were found in this specimen. As GFAP positivity in all the cases studied was always associated with the presence of glial intermediate filaments, this finding was as expected. This is in accord with the findings of Rasmussen et al (1980), Kumpulainen et al (1983), Gullotta et al (1985), Yung et al (1985) and Royds et al (1986), that normal mature oligodendrocytes are GFAP negative.

Contrastingly, Herpers & Budka (1984) described GFAP positive oligodendrocytes in oligodendrogliomas occurring
in areas of classical honeycomb texture within a vascular stroma. Here, demonstration of glial intermediate filaments in these GFAP positive cells could solve the issue whether they are true oligodendrocytes, astrocytes or gliofibrillary oligodendrocytes as suggested by Choi & Kim (1984). The possibility of polyclonal differentiation of primary (spongioblastic) embryonal tumours along more than one line (Roessmann et al, 1983), has not been ruled out.

To determine whether true GFAP positivity occurs in ependymal cells, ependymomas (Duffy et al, 1979) and stromal cells (Royds et al, 1986), the presence of GFAP positive glial intermediate filaments at electron microscopical level is required. The nature of both giant cells and histiocytic infiltrate of giant cell astrocytomas (Tsao et al, 1983; Gullotta et al 1985) should be assessed similarly.

Ultrastructural examination of these cells should prove whether they are trapped, reactive or neoplastic astroglial cells. Here, the difference in the distribution of GFAP (in cellular processes) and vimentin (juxta-nuclear) in reactive gliosis as observed by Herpers et al (1986), may contribute to solve the issue.

4B.5 Semi-quantitative post embedding immunoelectron microscopy as diagnostic aid in histopathology

Post embedding immunoelectron microscopy is superior to
other labelling techniques as it offers precise anatomic localization of labelled antigens with ultrastructure comparable to routine TEM.

Despite superior resolution gained by electron microscopy and the advantage of semi-quantification of labelling results, the labelling sensitivity of light microscopical immunohistochemical immunodetection methodology cannot be equaled by post embedding immunoelectron microscopy. Here, GFAP labelling densities of at least 500 particles / μm² for glial intermediate filaments are required to obtain acceptable immunodetection sensitivity.

As the specificity of immunohistochemical markers remains to be a relative parameter, the cautious approach to diagnostic immunochemistry, as expressed by workers such as Scott (1972), Gullotta et al (1985), Trojanowski (1986), Damjanov (1987) and Perentes & Rubinstein (1987), has to be emphasized.

Despite the many shortcomings, the association between GFAP as astroglial marker and glial intermediate filaments has been confirmed in routine human brain biopsies.

However, stable ultrathin sections, stringent morphometry and greater antigen availability for vimentin are required to determine whether differences in the arrangement of glial intermediate filaments are artifactual (shrinkage and/or stretching of the specimen) or whether they
represent true ultrastructural differences between vimentin (loose arrangement) and GFAP (more dense packing) expression. A more practical approach to overcome the low detection thresholds of post embedding immunoelectron microscopy, remains to be found.

The post-embedding immunoelectron microscopical method described, complemented by immunohistochemical and other histological techniques, can therefore be recommended for the demonstration of relatively high concentrations of aldehyde resistant, cytoplasmic antigens in biopsies of uniform cellular content.
REFERENCES


Hopwood D. The reactions between formaldehyde, glutaraldehyde and osmium tetroxide, and their fixation effects on bovine serum albumin and on tissue blocks. Histochemie 1970; 24: 50-64.


