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CELL PROLIFERATION, CELL DEATH, AND DIFFERENTIATION IN GLIOMAS

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ABSTRACT

FACULTY OF MEDICINE

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by David William Ellison

Gliomas including the commonest primary brain tumour, the glioblastoma, are incurable; any therapeutic advances will depend on an increased understanding of their biology. Gliomas are currently classified on the basis of histology, but analysis of regulators and markers of cell proliferation and programmed cell death, and of the events surrounding tumour progression may provide additional information of prognostic value, allow a more rational use of conventional therapies, and will be invaluable for identifying new therapies in the future.

This thesis consists of several studies testing the hypothesis that the biological behaviour of gliomas correlates with the expression of specific neuroectodermal proteins, cell-cycle markers, and genetic regulators of cell proliferation and programmed cell death.

The expression of neuronal proteins has been observed in about one third of high-grade astrocytic tumours from patients who have survived 2 years beyond operation, at which time less than 10% of patients with these tumours are alive. The hypothesis that 2 groups of cerebral astrocytic tumours either showing or not showing a neuronal immunophenotype can be distinguished on the basis of clinical profile, prognosis, and incidence of p53 mutations was tested.

Control of the cell cycle may be lost when multiple genetic abnormalities occur in a cell. The growth fraction of tumours can now be assessed by immunohistochemistry with an antibody, Ki-67, to a cell cycle specific antigen. The tumour suppressor gene p53 is involved in DNA repair and the regulation of apoptosis. Abnormalities of this gene are frequently found in astrocytic tumours and are associated with accumulation of the p53 protein. Apoptosis in cells containing irreparable DNA damage is facilitated by p53, but inhibited by upregulation of the *bcl* -2 gene. The hypotheses that immunolabelling with Ki-67 and p53 antibodies is of prognostic significance in a range of cerebral astrocytic tumours and that apoptosis in these tumours is related to growth fraction, and to the expression of the p53 and *bcl* -2 proteins were tested.

Glioma cells exhibit phagocytic activity; in order to differentiate between the manufacture or endocytosis of neuronal proteins in gliomas with a neuronal immunophenotype, a non-isotopic *in situ* hybridisation method using oligonucleotide probes was developed to assess the expression of synaptophysin mRNA. A series of 170 cerebral astrocytic tumours and oligodendrogliomas was examined by immunohistochemistry with antibodies to neuron-specific proteins, p53, and *bcl* -2, and the Ki-67 antibody.

A neuronal immunophenotype was demonstrated in 11% of high-grade astrocytic tumours, but not in low-grade astrocytomas, and in about one third of oligodendrogliomas. The expression of synaptophysin mRNA was detected in 23% - 33% of tumours that contained synaptophysin protein. Mutations of the p53 gene were present in about one third of astrocytic tumours with a neuronal immunophenotype. A neuronal immunophenotype did not distinguish a subgroup of astrocytic tumours with distinct clinicopathological attributes.

Conventional histological assessment of astrocytic tumours gave the best guide to survival, but growth fraction was also a strong prognostic indicator. Accumulation of the p53 protein was unrelated to prognosis. The index of apoptotic cells increased across the range of cerebral astrocytic tumours, and was significantly correlated with growth fraction. No relationship was found between apoptosis and accumulation of the

p53 or *bcl* -2 proteins.

Accumulation of genetic abnormalities is a feature of astrocytic tumour progression; undefined genetic abnormalities lead to the aberrant production of neuronal proteins at the anaplastic end of the range of these tumours, but are not associated with other cellular abnormalities of prognostic significance.

Oligodendrogliomas appear to be heterogeneous, and those that manufacture synaptophysin should be reclassified as cerebral neurocytomas.

Ki-67 is a useful adjunct to conventional histological assessment of astrocytic tumours. The demonstration of p53 protein accumulation has no prognostic value; gene abnormalities on chromosomes 9p, 19q, and 10 may be more important for astrocytic tumour progression and outcome. While apoptosis is a feature of astrocytic tumours that correlates with growth fraction, its regulation is complex and unrelated to accumulation of the p53 and *bcl* -2 proteins in these tumours.

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Preface

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Abbreviations

AI	apoptotic index
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BUdRLI	bromodeoxyuridine labelling index
BSA	bovine serum albumin
CNS	central nervous system
CTAB	cetyl-trimethyl ammonium bromide
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EORTC	European Organisation for Research and Treatment of Cancer
GFAP	glial fibrillary acidic protein
HPLC	high performance liquid chromatography
ISEL	*in situ* end labelling
ISH	*in situ* hybridisation
LI	labelling index (Ki-67 / p53)
LOH	loss of heterozygosity
MAP-5	microtubule associated protein - 5
NBT	nitro-blue tetrazolium
NFP	neurofilament protein
NTP	ribonucleotide
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PNET	primitive neuroectodermal tumour
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	standard saline citrate buffer
SSCP	single strand conformational polymorphism
ssDNA	sheared salmon sperm DNA
TBS	Tris buffered saline
TdT	terminal deoxyribonucleotide transferase
TLI	thymidine labelling index
UTP	uridine triphosphate
WHO	World Health Organisation

Introduction

1.1 Untreatable brain tumours pose a scientific challenge

The primary nervous system tumour most commonly diagnosed in neurosurgical centres is the glioblastoma (Weller, 1990). It is the most malignant of a range of cerebral astrocytic tumours, having a poor prognosis and no cure. The infiltrative nature of the glioblastoma implies that resection without profound neurological deficit is impossible. Survival at 2 years is less than 10%, and many trials of adjuvant therapy have shown that various regimens of radiotherapy and chemotherapy at best prolong median survival by only a few months. This situation has remained unchanged for many years, and can only be challenged by an increased understanding of the biology of these tumours.

At present, the management of patients with nervous system tumours depends upon an accurate pathological diagnosis. Histological characteristics are used to classify primary brain tumours; the macroscopic and microscopic features of a tumour are assessed, and a diagnosis is made from a recognised classification (Ellison, 1993). An increased understanding of tumour biology may aid patient management by enhancing this process in 2 principal ways:

(i) facilitating the distinction between tumours that closely resemble each other in conventional histological preparations

(ii) identifying histologically novel cellular abnormalities that have implications for prognosis or therapeutic intervention

1.1.1 Classifications of central nervous system tumours

Primary tumours of the central nervous system (CNS) represent approximately 1.5% and 3.5% of all cancers in adults and children respectively (Russell and Rubinstein, 1989; Weller, 1990; Ellison, 1993). All ages are affected, and nervous system tumours are the second most frequent category of malignant tumour in children, following haemopoietic malignancies. Most primary nervous system tumours occur in adults, and the glioblastoma and the meningioma are the commonest.

The classification of tumours allows their histopathology to be related to therapeutic success or failure. In the World Health Organisation (WHO) classification, they are grouped into neuroepithelial tumours, meningeal tumours, mesenchymal tumours excluding meningeal tumours, melanocytic tumours, haemopoietic tumours, and germ cell tumours (Kleihues et al., 1993). A major classification of CNS tumours, by Bailey and Cushing in 1926, used an ontogenetic scheme, matching tumour cells to cells from developmental periods of the CNS (Bailey and Cushing, 1926). Though the authors did not claim an embryogenetic origin, the role of cytological anaplasia was underplayed. In contrast, the scheme of Kernohan (1949) emphasised measures of cytological anaplasia for the classification of gliomas into just five categories; with the result that rare entities were excluded (Kernohan et al., 1949). The advantages of both systems were combined in the WHO classification of nervous system tumours (1979 and 1993). Table 111 lists the broad categories of neuroepithelial tumours in the WHO classification.

1.1.2 Astrocytic tumours

Astrocytic tumours included in the WHO classification (1993) are: astrocytoma (fibrillary, protoplasmic, and gemistocytic subtypes), anaplastic astrocytoma, glioblastoma, and pilocytic astrocytoma. This abbreviated list encompasses tumours that present in children and young adults, such as the pilocytic astrocytoma, and those that present in older adults such as the glioblastoma; it omits several rare variants, the clinical significance of which is unclear. Some varieties of astrocytoma are found at

Table 111
WHO classification (1993) of
neuroepithelial tumours

astrocytic tumours

oligodendroglial tumours

ependymal tumours

mixed gliomas

choroid plexus tumours

neuronal and neuronal - glial tumours

pineal tumours

primitive neuroectodermal tumours

particular sites in the brain, exemplified by juvenile pilocytic astrocytomas in the cerebellum and hypothalamus.

The commonest astrocytic tumour is the glioblastoma which contributes about half of all astrocytic tumours, if all age groups are surveyed. The glioblastoma has a prevalence in the Wessex Region of 7.3 / 100,000 with a peak incidence in the sixth decade, and represents the most malignant in a range of cerebral astrocytic tumours that includes the fibrillary astrocytoma and anaplastic astrocytoma (Weller, 1990). The fibrillary astrocytoma is a well differentiated tumour that presents most frequently in the third and fourth decades (Burger and Scheithauer, 1995). Epileptic seizures tend to be an early symptom, followed by focal neurological deficits and raised intracranial pressure. It is a diffusely infiltrating tumour which may occur in every lobe of the cerebrum at a frequency proportional to the size of each lobe (Burger et al., 1991). Fibrillary astrocytomas are composed of astrocytic cells with elongated bodies and fine processes. Microcysts are frequently present in these tumours. Pleomorphic nuclei may be evident in fibrillary astrocytomas, but nuclear hyperchromasia in many cells and an increased nuclear : cytoplasmic ratio are regarded as anaplastic features. Focal anaplasia and mitotic activity eventually develop in about 70% of fibrillary astrocytomas; the diagnosis becomes anaplastic astrocytoma and carries a much poorer prognosis (Burger et al., 1985). Capillary endothelial proliferation may be present in some anaplastic astrocytomas, but this is a more characteristic feature of the glioblastoma which also contains areas of necrosis (Weller, 1990; VandenBerg, 1992).

As well as representing the end-point of tumour progression for diffuse cerebral astrocytomas, the glioblastoma also presents without clinical evidence of having passed through this sequence. There is no difference in the histological appearance or behaviour of glioblastomas presenting in either way (VandenBerg, 1992; von Deimling et al., 1993). The glioblastoma has a large repertoire of histological appearances; giant and spindle-cell forms are recognised as separate diagnoses in the WHO classification. An extensive histological examination of a glioblastoma either postmortem or by multiple biopsies may reveal a striking range of appearances. Some portions of the tumour may be composed of small, undifferentiated cells, while others resemble the

cells of an astrocytoma, oligodendroglioma or ependymoma (Russell and Rubinstein, 1989).

A diversity of histological appearances is also found at the benign end of the spectrum of glial tumours (Burger and Scheithauer, 1995). Divergent neuroepithelial differentiation is a feature of the mixed glioma and a mixed neuronal - glial tumour termed the ganglioglioma. Any combination of astrocytoma, oligodendroglioma, or ependymoma may theoretically constitute a mixed glioma, but the oligoastrocytoma is the only tumour seen with any regularity. A diagnosis of mixed glioma is made when a distinct population of glial cells makes up more than 25% of the tumour, though this criterion is entirely arbitrary (Burger et al., 1991).

The ganglioglioma is rare, and is composed of ganglion cells and astrocytic cells (Diepholder et al., 1991). Both mixed gliomas and gangliogliomas may occasionally progress to anaplastic tumours, the astrocytic component of the ganglioglioma undergoing anaplastic change (Russell and Rubinstein, 1989).

1.1.3 Oligodendrogliomas

The oligodendroglioma presents throughout a wide age-range, but its peak incidence is in the sixth decade (Mørk et al., 1985; Shaw et al., 1992). Headache and epilepsy are the commonest presenting features. A more circumscribed appearance than an astrocytoma and deposits of calcification are characteristic features of this tumour on computerised tomography (CT) scans of the brain (Margain et al., 1991; Nijjar et al., 1993). Like other neuroepithelial tumours, the oligodendroglioma was named on the basis of its cytology; the small, round cells that comprise the tumour look like oligodendrocytes (Russell and Rubinstein, 1989). The cells of an oligodendroglioma, however, do not elaborate myelin, and their ultrastructural characteristics only show broad similarities with those of oligodendrocytes. Histochemical markers of mature oligodendrocytes, such as myelin basic protein, myelin associated glycoprotein, galactocerebrosidase, and 2,3 - cyclic nucleotide 3 - phosphohydrolase, are not found in oligodendrogliomas (Cameron and Rakic, 1991; Schwechheimer et al., 1992).

Oligodendrogliomas may show anaplastic histological characteristics, including cellular pleomorphism, mitotic activity, capillary endothelial proliferation, and necrosis (Mørk et al., 1986; Kros et al., 1988; Cascino, 1991). The prognosis of oligodendrogliomas as a group is better than that of astrocytomas (Devaux et al., 1993).

1.1.4 Neuronal tumours

The WHO classification of neuroepithelial tumours includes the following neuronal tumours: gangliocytoma, ganglioglioma, anaplastic ganglioglioma, central neurocytoma, and neuroblastoma (cerebral and cerebellar). The hallmark of the first 3 tumours is the ganglion cell which has the cytological and ultrastructural features of a neuron: large cell body, large nucleus with prominent nucleolus, neurofilaments, and synaptic vesicles (Russell and Rubinstein, 1989; VandenBerg, 1992; Kleihues et al., 1993). Ganglion cells in neuronal tumours are haphazardly arranged, are often misshapen, and sometimes contain 2 nuclei (Burger and Scheithauer, 1995). A ganglioglioma consisting of ganglion cells and astrocytic cells must be distinguished from an astrocytoma that invades grey matter and entraps native neurons (Diepholder et al., 1991). Attention to the architectural and cytological features of the tumour usually permits this distinction. The gangliocytoma and ganglioglioma grow very slowly, though the astrocytic component of the latter may rarely undergo anaplastic transformation.

The central neurocytoma is an indolent, well circumscribed tumour of the third ventricle; its cells are monomorphic, round, and reminiscent of small neurons, though they also resemble oligodendrocytes and the tumour had initially been regarded as an intraventricular glioma. In the last 5 years, several studies examining this tumours ultrastructural and immunohistochemical characteristics have confirmed its neuronal nature (von Deimling et al., 1990; Figarella-Branger et al., 1992; Vaquero et al., 1992).

The categories of primitive neuroectodermal tumours (PNET's) and of neuronal tumours overlap, and include the cerebral neuroblastoma and cerebellar

medulloblastoma (VandenBerg et al., 1987; Maleci et al., 1992; Trojanowski et al., 1992; VandenBerg, 1992; Burger and Scheithauer, 1995). A posterior fossa medulloblastoma is the commonest PNET, and most present in the first decade. PNET's are regarded as embryonal neuroepithelial tumours, but the medulloblastoma, and particularly the cerebral neuroblastoma, may show neuronal differentiation. The cells of PNET's are small, and round or polyhedral with a high nuclear : cytoplasmic ratio. Mitotic activity and apoptosis are prominent. Ganglion cells and rosettes are characteristic features of the neuroblastoma, but may be sparse; the small cells of these tumours do exhibit neuronal ultrastructural features which align them to neuronal precursors. PNET's have a high proliferation rate (Tsanaclis et al., 1991), and 5 year survival is only about 30%, despite a response to radiotherapy and chemotherapy in some cases.

1.2 The application of developments in tumour biology to the assessment of neuroepithelial tumours

Tumour diagnosis and assessment of biological activity may be enhanced by using immunohistochemical and molecular biological techniques to distinguish subgroups of tumours on the basis of distinctive protein production or molecular genetic changes. Such refinement in classification, beyond a simple histological assessment, may reveal tumours that respond better to contemporary treatments, and the discovery of pivotal oncogenic mechanisms may yield new therapies, such as gene manipulation (Hynes and Groner, 1995; Lowy and Willumsen, 1995).

1.2.1 The analysis of differentiation in neoplastic cells

All somatic cells in an eukaryotic organism, with the exception of lymphocytes which can rearrange their antigen receptor genes, possess the same genome (the genotype). The restricted expression of genes in a cell determines its form and function, or differentiation (the phenotype). Patterns of gene expression therefore underlie the histological characteristics of neoplastic cells within a tissue (the morphophenotype). Assessment of these characteristics forms the basis of conventional tumour diagnosis. This process may now be supplemented by an immunohistochemical analysis of protein production (the immunophenotype), or by molecular biological analysis of the genotype or phenotype (Hall, 1992).

During the development of an organism, the spatial and temporal expression of genes is carefully regulated as cells differentiate along predetermined lines. Once this process is completed in the adult, restricted gene expression is reflected by cellular differentiation. In tumour cells, abnormal cellular differentiation is not random; deregulation of gene expression often reflects the neoplastic cells lineage, in other words, dedifferentiation frequently occurs along the cells developmental pathway (Trojanowski et al., 1992). In addition, while tumours are regarded as clonal, phenotypic variation within a tumour is well recognised (as it is in clonally derived normal cells); but this variation is usually restricted to developmental phenotypes. Until

the oncogenic process is ultimately dissected and detailed, this phenotypic variation can still complement histological assessment if it correlates with a change in the tumours behaviour.

1.2.2 The immunophenotype in the diagnosis of neuroepithelial tumours

The histological assessment of tumours benefited significantly from the development of immunohistochemistry because morphophenotype could be related to another indicator of differentiation, the immunophenotype (Matthews, 1987; Magdelenat, 1992; Ellison, 1993).

Antibodies to the intermediate filament of astrocytes, glial fibrillary acidic protein (GFAP), and to the intermediate filament of neurons, neurofilament protein (NFP), were first used as aids to the diagnosis of neuroepithelial tumours alongside antibodies to less cell-specific nervous system proteins such as S-100 (Rubinstein, 1986). It soon became apparent, however, that dedifferentiation in neuroepithelial tumours produced an immunophenotype in tumour cells that differed from the immunophenotype in normal cells. GFAP production is not confined to astrocytic tumours (McLendon and Bigner, 1994); many glial tumours and other neuroepithelial tumours, such as the choroid plexus papilloma, are GFAP-positive (Ang et al., 1990; Scott and Knightly, 1995). Mature oligodendrocytes contain very few intermediate filaments and do not label with GFAP antibodies, but some oligodendrogliomas are GFAP-positive (Cruz-Sanchez et al., 1991a; McLendon and Bigner, 1994).

Antibodies to synaptophysin, a 38 kD transmembrane protein of synaptic vesicles, and antibodies to neurofilament proteins, components of the neuronal cytoskeleton, initially appeared to be more specific than antibodies to GFAP; they labelled only neurons in CNS tissue and ganglion cells in neuronal tumours (Gould et al., 1987; Miller et al., 1990). But exceptions began to emerge. Tumours containing cells with no resemblance to neurons labelled with antibodies to neuronal proteins; not only did some PNET's label with neuronal antibodies as well as with antibodies to GFAP (Kleinert, 1991), a reflection of dedifferentiation to an early phase of neuroepithelial cell development

(Trojanowski et al., 1992), but the idiosyncratic intraventricular glioma was reclassified as a central neurocytoma when labelling of its cells with neuronal antibodies was demonstrated (von Deimling et al., 1990).

Phenotypic variation in neoplastic cells is demonstrated not only by upregulation of protein expression, but by downregulation of the proteins found in their normal counterparts. Loss of GFAP production is not uncommon in anaplastic astrocytomas and glioblastomas (Kajiwara et al., 1992).

Few studies have looked for a relationship between immunophenotype and the behaviour or response to treatment of neuroepithelial tumours. The labelling of neuroblastomas with an antibody to S-100, for example, has been proposed as a favourable prognostic feature (Misugi et al., 1985), but this has been disputed by others (Carter et al., 1990). The expression of GFAP in oligodendrogliomas does not appear to be of prognostic significance (Kros et al., 1990).

A key observation for the present studies was made when anaplastic astrocytomas and glioblastomas from patients who had survived more than 2 years beyond operation were examined for the presence of neuronal proteins (Weller and Garfield, 1989). A neuronal immunophenotype was evident in 9 / 26 (35%) of these tumours. The expression of neuronal proteins in these tumours had not previously been demonstrated, and the study raised the possibility that a neuronal immunophenotype might be associated with prolonged survival. Such a concept is embodied in one of the hypotheses to be tested in this thesis.

1.2.3 Oncogenesis

Advances in tumour biology over the last decade have revealed many aspects of oncogenesis (Bishop, 1987; Wynford-Thomas, 1991). Oncogenesis has a genetic basis; abnormalities in the genome that predispose to neoplastic transformation may be hereditary, as in Li-Fraumeni syndrome (Srivastava et al., 1990), or may be caused by external agents, such as oncogenic viruses (Shields, 1993). The mutagenicity of some

chemicals correlates with their carcinogenicity, and susceptibility to cancer correlates with an inability to repair DNA. The discovery of cellular proto-oncogenes has now been supplemented by the discovery of tumour suppressor genes (Levine, 1990).

Almost all proto-oncogenes code for components of signalling pathways that regulate cell division. Their gene products may be divided into nuclear oncoproteins, growth factors, growth factor receptors, and transducers: GTP binding proteins and non-receptor tyrosine kinases (Wynford-Thomas, 1991; Watkins and Rouleau, 1992). Abnormal overexpression, a change of function following mutation, and loss of normal regulatory mechanisms may all cause a proto-oncogene to malfunction and to provide a stimulus for neoplasia.

The retinoblastoma and p53 genes are examples of tumour suppressor genes which negatively regulate cell division or abnormal cellular growth (Levine, 1990; Seizinger, 1991; Chang et al., 1993). If, through a variety of mechanisms such as mutation or deletion, neither allele of a tumour suppressor gene produces a functional protein, then its regulatory action will be abolished. Some DNA tumour viruses, such as SV40, produce proteins that bind and inactivate the p53 and retinoblastoma gene products (Bartek et al., 1992; Henson et al., 1994).

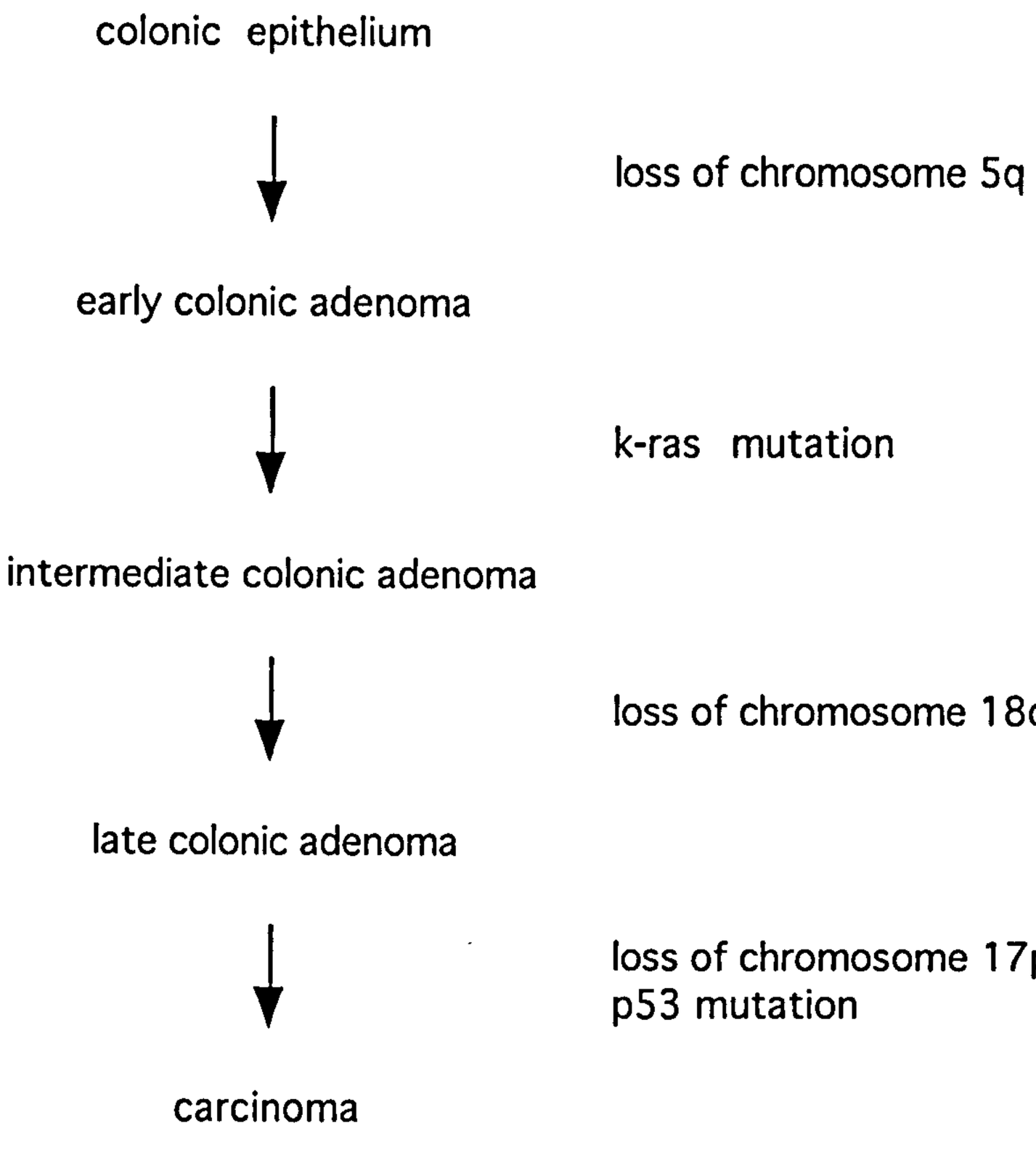
Oncogenesis is now regarded as a multi-step process characterised by the progressive acquisition of genetic defects. The development of colorectal carcinoma illustrates this process (Table 123); each step is associated with the appearance of particular genetic abnormalities, some of which must account for the progressively abnormal appearance and behaviour of neoplastic cells as polyp evolves into cancer (Fearon and Jones, 1992).

1.2.4 Astrocytic tumours and abnormalities of p53

Antibodies to proteins expressed in specific phases of the cell-cycle and to the products of oncogenes and tumour suppressor genes have advanced the study of tumour biology, enabling the proliferation rate of tumours and the activity of genes implicated

Table 123

Acquisition of genetic abnormalities during progression of colonic tumours



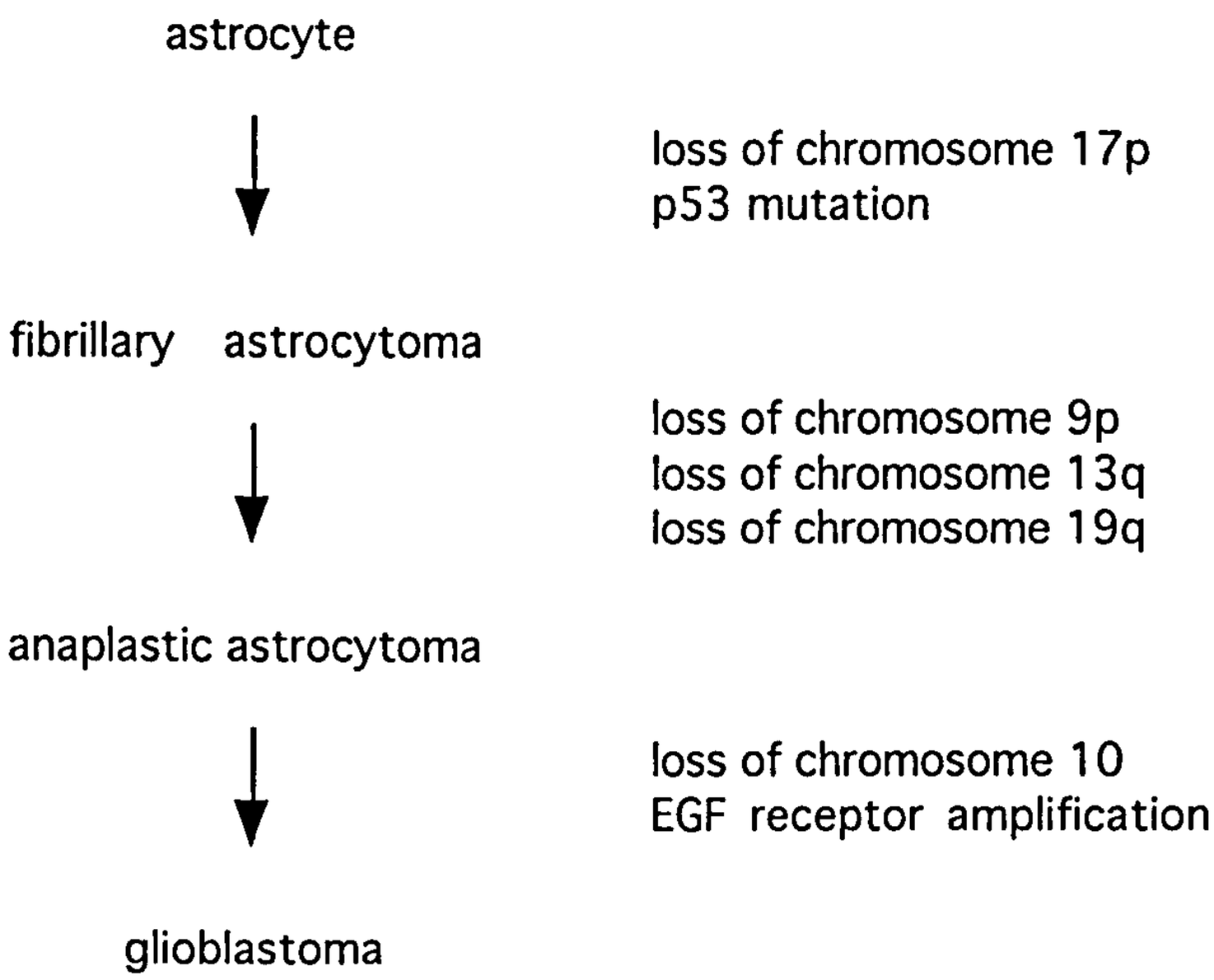
in the genesis of nervous system tumours to be assessed (Hall, 1991; Magdelenat, 1992; Yu et al., 1992; Hall and Lane, 1994; Harrington et al., 1994; Rose et al., 1994).

Advances in DNA technology have revealed that extensive molecular genetic abnormalities may be present in many types of tumour (Bishop, 1987; Wynford-Thomas, 1991; Fearon and Jones, 1992). In the range of cerebral astrocytic tumours (von Deimling et al., 1993; Louis, 1994), the progression of astrocytoma to anaplastic astrocytoma and on to glioblastoma is associated with the acquisition of specific genetic abnormalities (Table 124).

About one third of fibrillary astrocytomas, anaplastic astrocytomas, and glioblastomas harbour a mutation of the p53 gene (Louis, 1994), suggesting that this is an early event in the development of these tumours (Table 124). The p53 tumour suppressor gene encodes a nuclear phosphoprotein of 393 amino acids which functions as a transcription factor (Fields and Jang, 1990; Lane, 1994). It is involved in the initiation of DNA repair, and in order to optimise this, p53 halts the cell cycle in G_1 before DNA replication occurs in S phase (Marx, 1993; Prives, 1993). Cells beyond repair may be stimulated by p53 to undergo apoptosis (Clarke et al., 1993). Mutations of the p53 gene are currently the most frequent genetic abnormalities found in human tumours, and produce inactive proteins that fail to bind DNA (Kern et al., 1992; Vile, 1993). This permits the replication of damaged DNA which may then act to promote the mutational activation of proto-oncogenes, with subsequent deregulation of the cell-cycle. Further mechanisms that prevent the sequence-specific binding of wild-type p53 to DNA include the formation of inactive hetero-oligomers between some mutant and wild-type proteins (dominant-negative action), and the overexpression of genes, such as *mdm* -2, the products of which bind the p53 protein (Vogelstein and Kinzler, 1992; Wu et al., 1993).

In normal cells, p53 protein is present in minute quantities that are undetectable by immunohistochemistry. Mutations of the p53 gene and stabilisation of the wild-type protein by a variety of mechanisms increase the half-life of the protein and produce

Table 124
Acquisition of genetic abnormalities during cerebral astrocytic tumour progression



immunohistochemically detectable levels (Hall and Lane, 1994). Nuclear accumulation of the p53 protein has now been demonstrated in many tumours, and various patterns of immunolabelling are seen. A few positively labelled cells may be scattered through histological sections of tumour, or nearly all cells may be labelled (Lane, 1992; Vogelstein and Kinzler, 1992; Vile, 1993).

Studies on prognostic indicators in malignant melanomas and carcinomas of the breast, lung, and prostate gland have shown that immunohistochemistry using antibodies to the p53 protein is of value in predicting the behaviour of these tumours (Quinlan et al., 1992; Barnes et al., 1993; McGregor et al., 1993; Thomas et al., 1993a). A considerably worse prognosis is found for patients with breast carcinomas that express the p53 protein in the majority of tumour cells (Barnes et al., 1993).

About one third of fibrillary astrocytomas contain scattered p53-labelled cells, while nearly two thirds of anaplastic astrocytomas and glioblastomas contain p53-positive cells (Ellison et al., 1992; Louis, 1994). A positive correlation between accumulation of p53 and indices of cell proliferation has been proposed for astrocytic tumours (Barbareschi et al., 1992; Haapasalo et al., 1993), but the prognostic significance of patterns of p53 accumulation is unknown. The correlation between p53 accumulation and survival will be assessed in this thesis.

1.2.5 Tumour growth - cell proliferation versus cell death

The growth of a tumour depends upon the balance between cell proliferation and cell death. Though the contribution of cell death to tumour growth may be very significant, more emphasis has been placed, during histopathological assessment, on the measurement of cell proliferation than of cell death. Measures of cell proliferation include mitotic count, and thymidine (TLI) and bromodeoxyuridine (BUdRLI) labelling indices which identify cells in S phase of the cell cycle (Quinn and Wright, 1990; Hall, 1991; Magdelenat, 1992; Yu et al., 1992). The mitotic count is not a standardised method; it is frequently measured as counts per high-power field. Variability in the definition of a high-power field, variability between microscopes, and

variability in tumour cell density will all affect this measure. It is additionally affected by any delay in fixation (Cross et al., 1990), and, overall, cannot be regarded as an independent parameter of cell proliferation. TLI is a proven prognostic indicator in breast carcinoma and plasma cell dyscrasias, but requires fresh tissue and the use of autoradiographs; it can also be time consuming. The bromodeoxyuridine method has several advantages over thymidine labelling; exposure to radioactive compounds is avoided and the potential doubling time of the tumour can be calculated. It is, however, only applicable to prospective studies because bromodeoxyuridine which is a pyrimidine analogue must be incorporated into the tumour cells *in vivo* or *in vitro* .

In recent years, the value of antibodies to proteins expressed in proliferating cells, such as Ki-67, has been investigated in astrocytic tumours (Morimura et al., 1989; Raghavan et al., 1990; Louis et al., 1991; Ellison and Weller, 1992). The Ki-67 antibody recognises a non-histone, nuclear protein that is present as a doublet of 345 kD and 395 kD and expressed throughout all phases of the cell cycle except G_0 and the early part of G_1 (Gerdes et al., 1991). Several antibodies have now been raised against this antigen and used successfully to measure the growth fraction of tumours (Brown and Gatter, 1990), and the Ki-67 labelling index (LI) correlates well with the behaviour of many different malignant tumours (Sawhney and Hall, 1992). Immunolabelling with Ki-67 initially required frozen tissue sections, but the development of new antibodies and the advent of microwave-based antigen retrieval techniques have produced good results with formalin fixed, paraffin embedded tissues (Cattoretti et al., 1992).

There is a strong relationship between histological type of astrocytic tumour and Ki-67 LI's (Louis et al., 1991; Ellison and Weller, 1992), and a few studies have related Ki-67 LI's to survival (Zuber et al., 1988; Raghavan et al., 1990; Jaros et al., 1992). It is difficult to draw conclusions from them, however, because a relationship between Ki-67 LI and survival was either found (Jaros et al., 1992), or not found (Zuber et al., 1988; Raghavan et al., 1990). The analysis is devalued in one of these studies by a small number of patients, and in another by a small number of low grade astrocytomas.

The contribution of cell death to the retardation of a tumours growth is significant, and is one explanation for the lack of correlation between cell proliferation and growth rate (Quinn and Wright, 1990). Many factors may influence tumour cell death, including host immune responses, integrity of vascular supply, and iatrogenic intervention with chemotherapy or radiotherapy; but the underlying mechanism of cell death is one of two processes: necrosis or apoptosis (Arends et al., 1994). These processes differ in many respects. In contrast to necrosis which is a pathological phenomenon, apoptosis is a physiological process of particular significance during morphogenesis, at which time the programmed death of many cells is required (Cohen, 1993; Wyllie, 1993). It occurs in single cells while surrounding cells survive. In histological sections the characteristic features of apoptosis are nuclear condensation, crescents of collapsed chromatin along the nuclear envelope, and fragmentation of the nucleus into spheres. The histological features are accompanied by biochemical changes, including disruption of the link regions between nucleosomal cores, which, on gels, produces a characteristic ladder of DNA fragments (Cohen, 1993; Stewart, 1994). Apoptosis requires both energy and the expression of apoptosis-related genes; but, perhaps the most important difference between necrosis and apoptosis is that apoptosis can be regulated (Kerr et al., 1994). Two genes that can regulate apoptosis are *bcl* -2 and p53 (Williams, 1991; Stewart, 1994).

The B-cell leukaemia / lymphoma -2 (*bcl* -2) gene was first identified in studies on the pathogenesis of human, follicular, non-Hodgkin's lymphoma (Korsmeyer, 1992). It codes for a 26kD, intracellular membrane-associated protein which confers a survival advantage on B-lymphocytes by inhibiting apoptosis (Tsujimoto and Croce, 1986; Carson and Ribeiro, 1993; Akao et al., 1994). The t(14;18)(q32;21) translocation that occurs in certain follicular lymphomas juxtaposes the *bcl* -2 gene and the immunoglobulin heavy chain gene locus to produce a fusion gene under the control of the heavy chain gene promoter (Aisenberg et al., 1988). This results in increased levels of *bcl* -2, prolonged survival of lymphocytes, and follicular lymphoproliferation. The potential of *bcl* -2 to generate lymphoid tumours has been demonstrated in transgenic mice (McDonnell and Korsmeyer, 1991); and it can combine with rearrangements of other oncogenes such as *myc* to produce high-grade lymphomas (Vaux et al., 1988).

bcl -2 is currently the only oncogene that acts by controlling cell death rather than cell proliferation (Korsmeyer, 1992).

The regulation of apoptosis is critical for morphogenesis and cellular differentiation in the developing nervous system, where *bcl* -2 is expressed in neurons and some neuroglia (Hockenbery et al., 1990; LeBrun et al., 1993; Merry et al., 1994). Despite the possible implications for nervous system neoplasms, the expression of *bcl* -2 has only been studied in neuroblastomas, in which its significance is uncertain (Castle et al., 1993; Ramani and Lu, 1994), and in the cell lines of some neural tumours, in which moderate levels of *bcl* -2 expression were detected in glioblastoma and neuroblastoma cell lines.

To supplement its role in DNA repair, the p53 gene is required for the induction of apoptosis in cells where DNA has been irreparably damaged (Lane, 1992). Expression of exogenous wild-type p53 induces apoptosis in a variety of tumour cell lines that lack functional p53 (Ramqvist et al., 1993; Wang et al., 1993; Yanai and Obinata, 1994). In some tumours with *myc* overexpression, apoptosis may occur in response to conflicting cellular signals from *myc* which stimulates proliferation, and from p53 which arrests growth (Lane, 1994). The relationship between apoptosis and p53 status in astrocytic tumours has not been established, despite its theoretical importance for response to radiotherapy. The relationship between apoptosis and expression of the p53 and *bcl* -2 proteins will be studied in this thesis.

1.3 Hypothesis based studies

As abnormalities of the genome accumulate in neoplastic cells (Cavenee and White, 1995), the phenotype and clinical behaviour of a tumour evolve (tumour progression). Morphophenotype or immunophenotype may mark critical phases in this evolution, and act as indirect signals of undefined molecular genetic defects directly linked to the oncogenic process. If a genotypic or phenotypic marker that coincides with a significant change in the behaviour of a tumour can be demonstrated in a histological preparation, then it becomes a valuable prognostic indicator. If the indicator has implications for therapy, it becomes still more valuable. A combination of immunohistochemical and molecular biological markers for critical cellular events, such as control of the cell cycle or cell death, could eventually prove to be as important for tumour classification as conventional histological assessment.

The hypothesis that the biological behaviour of gliomas correlates with the expression of specific neuroectodermal proteins, cell-cycle markers, and genetic regulators of cell proliferation and programmed cell death was tested in several studies of biopsied cerebral astrocytic tumours and oligodendrogliomas from patients with well documented clinical details.

1.3.1 Neuronal differentiation in cerebral astrocytic tumours and oligodendrogliomas

Based on the observation that a neuronal immunophenotype was observed in 35% of high grade astrocytic tumours from patients who survived 2 years beyond operation (Weller and Garfield, 1989), a study of neuronal markers would be undertaken in a range of astrocytic tumours. Groups of patients distinguished by having astrocytic tumours with and without a neuronal immunophenotype would be compared on several clinical and pathological parameters, including survival and tumour growth fraction.

Because astrocytes have phagocytic activity (Noske et al., 1982; Shuangshoti et al., 1989), and astrocytomas are infiltrative tumours, neoplastic glia might incorporate

neuronal proteins that are released from disrupted neuropil or fibre tracts by the destructive effects of the tumour. A method of *in situ* hybridisation (ISH) using non-isotopic oligonucleotide probes for synaptophysin mRNA would be developed to assess whether synaptophysin protein is manufactured or incorporated in neoplastic glia.

Astrocytic tumours with a neuronal immunophenotype would also be examined for the presence of p53 mutations. About one third of standard cerebral astrocytic tumours contain p53 mutations, and a similar finding in astrocytic tumours with a neuronal immunophenotype would ally them with their standard counterparts.

Based on the observation that the central neurocytoma, which closely resembles an oligodendroglioma, and several other rare neuroepithelial tumours have an immunophenotype characterised by both glial and neuronal markers (von Deimling et al., 1990; VandenBerg, 1992), a study would be conducted on the expression of neuron-specific proteins in oligodendrogliomas. Immunohistochemistry for synaptophysin protein would again be undertaken in conjunction with ISH for synaptophysin mRNA. The clinical parameters of 2 groups of patients, with and without standard oligodendrogliomas, would be compared.

1.3.2 Ki-67 and p53 as prognostic indicators in cerebral astrocytic tumours

Immunohistochemistry with a Ki-67 antibody provides a measure of the growth fraction in a tumour. Its value as a prognostic indicator would be compared with conventional histological assessment in a range of astrocytic tumours.

Accumulation of the p53 protein is detectable by immunohistochemistry in two thirds of astrocytic tumours. It can result from a p53 mutation, the concurrent stabilisation and inactivation of wild-type p53 protein that follows the aberrant accumulation of a protein to which it binds, or the physiological increase in wild-type p53 associated with DNA damage (Hall et al., 1993). The first two processes entail p53 dysfunction which may predispose a cell to the acquisition of DNA errors; in contrast, the third is

an attempt by a cell to rectify damage to the genome. The inability or ability to effect DNA repairs might be reflected in accelerated or retarded tumour progression respectively. Because wild-type p53 suppresses transformation by a variety of oncogenes (Finlay et al., 1989), and is a potent inducer of cell-cycle arrest (Lin et al., 1992), the relationship between patterns of p53 accumulation, growth fraction, and survival would be studied in these tumours.

1.3.3 Apoptosis in astrocytic tumours

Apoptosis is an important determinant of tumour growth (Kerr et al., 1994), and also has critical functions in the developing nervous system (Cohen, 1993; Soriano et al., 1993). Regulators of apoptosis include the p53 and *bcl* -2 genes. In this study, apoptosis would be measured in a range of astrocytic tumours, and related to accumulation of the p53 and *bcl* -2 proteins. A relationship between apoptosis and growth fraction would also be sought.

Methods

2.1 Selection and histological classification of tumours

2.1.1 Selection of cases

For 15 years, between 1976 and 1991, patients with cerebral gliomas from the Wessex Neurological Centre in Southampton were entered into treatment trials supervised by the European Organisation for Research and Treatment of Cancer (E.O.R.T.C.). Cases in the present studies were sought from:

(i) E.O.R.T.C. trials 26751, 26781, 26812, 26851 (appendix 2.1.1)

(ii) records in the Neuropathology department of Southampton General Hospital

Clinical details of patients from the Wessex Neurological Centre that had been entered into E.O.R.T.C. treatment trials were generally available from E.O.R.T.C. headquarters in Brussels.

Clinical data were incomplete for a few E.O.R.T.C. cases, and Wessex Neurological Centre medical notes for these patients were retrieved. Clinical data on those patients not included in E.O.R.T.C. trials, but selected initially on the basis of the pathology report, were also sought in the medical notes.

Data were entered into a computer (*Apple Macintosh*) database on which statistical analyses could subsequently be undertaken (*Statview 4.0.2, Abacus Concepts*).

2.1.2 Histological classification of tumours

All patients had cerebral (supratentorial) tumours, and had undergone craniotomies to establish the histopathological diagnosis. A limited resection of the tumour had been performed in nearly all cases. Many patients had subsequently received radiotherapy, with or without adjuvant chemotherapy (appendix 2.1.1). The histology of each cerebral tumour was reviewed for the present study, and classified according to World Health Organisation (W.H.O.) criteria (Kleihues et al., 1993) into: astrocytoma (fibrillary subtype only), anaplastic astrocytoma, glioblastoma, oligodendroglioma, or anaplastic oligodendroglioma. Paraffin embedded tissue blocks for further study were selected at this stage (see 2.2.2).

2.1.3 Division of cases among studies

A series of 145 biopsies of astrocytic tumours from 139 patients and 25 biopsies of oligodendrogliomas were used for the study of neuronal protein expression. The astrocytic tumours were subdivided histologically as detailed in Table 213-1. Of the 6 tumours that had been biopsied on 2 separate occasions, 4 had been classified as fibrillary astrocytomas at initial biopsy and had progressed to anaplastic astrocytomas by the second biopsy, while 2 had been classified as anaplastic astrocytomas at first biopsy and had progressed to glioblastomas by the second biopsy.

The 6 paired biopsies were included in the study of apoptosis, but were omitted from the study of the relationship between growth fraction, p53 accumulation and survival in astrocytic tumours because only one time point for surgery / diagnosis could be included in the survival analysis.

Uncertain follow-up data applied to 2 / 145 patients, so these cases were excluded from the survival analysis.

The 14 tumours that had shown expression of neuronal proteins were also excluded from this analysis on the basis that they should be regarded as non-standard.

Table 213-1
Cases in each study

	neuronal phenotype study	Ki-67, p53 and survival study	apoptosis, p53, and bcl-2 study
Biopsies	170	123	81
Patients	164	123	75
Fibrillary astrocytoma	24	24	16
Anaplastic astrocytoma	40	31	19
Glioblastoma	81	68	46
Oligodendroglioma	14	-	-
Anaplastic oligodendroglioma	11	-	-

The demographic details of the patients with astrocytic tumours included in each of the separate studies were almost identical (Table 213-2), and representative of those previously reported for cerebral astrocytic tumours (Burger et al., 1985; Burger and Green, 1987; Kallio et al., 1991; Kim et al., 1991; VandenBerg, 1992; Devaux et al., 1993).

Table 213-2
Details of patients entered into studies

		neuronal phenotype study	Ki-67, p53 and survival study	apoptosis, p53, and bcl-2 study
age (years)*	Fibrillary astrocytoma	35.1 ± 2.8	35.3 ± 1.9	38.9 ± 2.1
	Anaplastic astrocytoma	45.0 ± 2.3	40.7 ± 2.1	45.2 ± 2.9
	Glioblastoma	50.7 ± 1.1	50.6 ± 1.1	50.4 ± 1.4
survival (days)†	Fibrillary astrocytoma	1026 (469)	1026 (469)	1280 (1902)
	Anaplastic astrocytoma	362 (384)	377 (367)	445 (439)
	Glioblastoma	318 (245)	321 (247)	323 (267)
gender	% male	67	63	68
tumour site	% frontal	42	44	45
	% temporal	25	25	23
	% parieto-occipital	33	31	32

* mean ± s.e.m.
† median (interquartile range)

2.2 Histochemical methods

2.2.1 Fixation and processing of tissue

Archival biopsy material had been fixed in 10% neutral phosphate-buffered formalin (pH 7.0) for 24 hours, before being processed in alcohol with chloroform as the clearing agent, and embedded in paraffin blocks. These tissue blocks were used for all immunohistochemistry, *in situ* hybridisation (ISH), and for the p53 gene analysis.

2.2.2 Standard histology

Sections stained in a routine manner with haematoxylin and eosin were used to classify all tumours and to select the best block of tissue for further study.

The chosen blocks contained tumour with histological features that were representative of tumour throughout available blocks. The infiltrative edge of the tumour and large areas of necrosis were avoided where possible. A preparation for reticulin was used to assess capillary endothelial proliferation in some cases.

Haematoxylin was used as a counterstain for immunohistochemistry and for some ISH preparations.

2.3 Immunohistochemistry

2.3.1 Choice of antibodies

The panel of antibodies to neuronal proteins consisted of 2 antibodies to synaptophysin, an antibody to chromogranin-A, and 2 antibodies to neurofilament proteins. Antibodies to the γγ-subunit of enolase, ß-tubulin, and microtubule associated protein 5 (MAP-5) were considered for the panel on the basis of claimed neuronal specificity (Reiderer et al., 1986; Marangos and Schmechel, 1987; Cameron and Rakic, 1991; Hessler et al., 1992; Maraziotis et al., 1992; Murray et al., 1993), but a preliminary study of these antibodies on a series of neuroepithelial tumours disclosed labelling of almost all tumours (Table 231-1). Such widespread labelling indicated that these antibodies would not identify a subgroup of astrocytic or oligodendroglial tumours, and they were excluded from the panel. An antibody to GFAP was used alongside the panel of neuronal antibodies (Table 231-2).

A polyclonal Ki-67 antibody was used as a measure of tumour growth fraction, having equal and superior performances on paraffin sections when compared with a monoclonal MIB-1 antibody and a monoclonal Ki-67 antibody respectively (Rose et al., 1994).

An antibody to the N-terminal portion of the p53 protein that works well on paraffin sections, DO-7, was preferred (Vojtesek et al., 1992).

2.3.2 Immunohistochemical method

Near adjacent sections (5 μm) were cut from formalin fixed, paraffin embedded tissue, floated onto slides, and dried at 37°C overnight. Slides destined for the microwave pretreatment step had been coated previously (appendix 2.3.2) with 3-aminopropyltriethoxysilane (Maddox and Jenkins, 1987). The sections were then dewaxed, rehydrated to 70% methanol, and treated for 10 minutes in freshly prepared 1% H_2O_2 in methanol to block endogenous peroxidase activity.

Table 231-1
Immunolabelling of a series of neuroectodermal tumours with antibodies to "neuron-specific" proteins

	total	MAP-5	NSE	ß-tubulin
fibrillary astrocytoma	n = 9	7	7	2 (focal)
anaplastic astrocytoma	n = 6	6	6	4
glioblastoma multiforme	n = 8	8	8	8
pilocytic astrocytoma	n = 3	3	3	3
ganglioglioma	n = 4	4	4	4
medulloblastoma	n = 6	6	6	4
neuroblastoma	n = 4	4	4	4
neurocytoma	n = 3	3	3	0
oligodendroglioma	n = 9	5	3	6
ependymoma	n = 7	7	7	7
schwannoma	n = 3	3	3	3
neurofibroma	n = 3	3	3	3
paraganglioma	n = 3	3	3	3

Figures in columns represent the numbers of positively labelled tumours

Table 231-2
Characteristics of a panel of antibodies to neuronal proteins

antibody	antigen	molecular weight	function
synaptophysin	synaptophysin	38 kD	presynaptic vesicle protein
S2	synaptophysin	38 kD	presynaptic vesicle protein
chromogranin-A	chromogranin-A	49 kD	neuromodulator
NFP	neurofilament protein peptide subunits	70 kD & 200 kD	cytoskeletal element
N52	neurofilament protein peptide subunit	200 kD	cytoskeletal element

Depending on the antibody (Table 232), 1 of 3 pretreatment protocols was now followed:

(i) **standard**:

blocking step in 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) for 30 minutes (appendix 2.3.2)

(ii) **trypsin**:

wash in water at 37°C for 5 minutes

enzymatic digestion in a solution of 0.1% trypsin in 0.1% $CaCl_2$ at 37°C for 10 - 30 minutes (depending on length of fixation, and batch of trypsin)

rinse in cold running water

2 washes in TBS (pH 7.6) for 5 minutes each

(iii) **microwave**:

place slides in plastic Coplin jars containing 0.01 M citrate buffer (pH 6.0)

heat in microwave oven (Panasonic 6400) for either 2 or 4 (Ki-67 only) cycles of 5 minutes (medium power setting)

replace buffer lost through boiling between cycles

cool for 15 minutes

Monoclonal or polyclonal antibodies in TBS were applied separately to serial sections, at the stated concentrations (Table 232). Biotinylated anti-mouse antibody (Amersham)

Table 232
Antibodies for immunohistochemical studies

Antibody	Manufacturer	Type	Clone	Dilution	Method
synaptophysin	DAKO	m	SY38	1:200	mw
synaptophysin	*	p	-	1:400	mw
NFP	DAKO	m	2F11	1:400	mw
chromogranin-A	DAKO	m	DAKA3	1:400	mw
N52	Sigma	m	N52	1:2000	mw
NSE	§	m	NSE-P1 / 2	1:5	st
MAP - 5	Sigma	m	AA6	1:2000	st
ß - tubulin	Eurodiagnostics	m	DM1B	1:50	st
GFAP	#	p	-	1:400	t
Ki-67	DAKO	p	-	1:50	mw
p53	DAKO	m	DO-7	1:100	mw
bcl-2	†	m	100	neat	mw

Type: m = monoclonal; p = polyclonal
Method: mw = microwave; t = trypsin; st = standard

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§ Dr. G Murray, Aberdeen University
# Neuropathology, Southampton General Hospital
† Dr. K. Gatter, Oxford University

diluted 1:200 in TBS was used in the second stage with monoclonal antibodies. Biotinylated anti-rabbit antibody (DAKO) diluted 1:400 in TBS was used in the second stage with polyclonal antibodies. Streptavidin-biotin horseradish peroxidase complex (DAKO) prepared 30 minutes before use and diluted 1:200 in TBS, was used in the third stage. Each stage was performed at room temperature, lasted 30 minutes, and was separated by 3 x 5 minutes washes in TBS. Antigen-antibody complexes were revealed with a standard diaminobenzidine technique: diaminobenzidine (5 mg) was dissolved in 10 ml of Tris buffer (pH 7.6), and 0.1 ml of freshly prepared 1% H_2O_2 was added just before use.

Control sections were processed without the application of primary antibody, and all sections were counterstained with haematoxylin.

2.4 Immunoblotting

The specificity of a novel polyclonal antibody to synaptophysin (courtesy of Prof. R. Thompson, University of Southampton) was assessed on immunoblots of normal cerebral grey matter and synaptophysin-positive glioblastomas.

2.4.1 Material

Frozen tissue was available from 2 synaptophysin-positive glioblastomas in the study of neuronal immunophenotype. Frozen sections were first cut from the available biopsy material and stained with haematoxylin and eosin to confirm that tumour without infiltrated grey matter was to be used for immunoblotting.

Normal cerebral grey matter was used as a positive control for the presence of synaptophysin, and a synaptophysin-negative glioblastoma was used as a negative control. The suitability of the control material was also first examined in frozen sections.

2.4.2 Method

Approximately 150 mg of tumour tissue was placed in a mixture of 600 µl of suspension buffer and 300 µl of 3 x sodium dodecyl sulphate SDS gel-loading buffer (appendix 2.4.2). The tissue was disrupted by sonication and the suspension (total volume ~ 1 ml) was centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed and stored at -20°C. Aliquots (1 µl, 5 µl, 10 µl) of the supernatant were run on a 12.5% polyacrylamide minigel (appendix 2.4.2) which was chosen for its capacity to resolve proteins in the 35 - 45 kD range. The gel was stained with Coomassie blue dye (appendix 2.4.2) and used to determine the amount of each sample that contained an optimal quantity of protein for immunoblotting.

Once the optimal volume of supernatant had been established, gels (12.5% polyacrylamide) carrying molecular weight markers, negative and positive controls,

and the test samples were run at 50 - 70V for approximately 5 hours. Proteins from the gels were transferred to nitrocellulose paper in a second electrophoresis apparatus. In this, the nitrocellulose was placed next to the gel and sandwiched between layers of 3MM™ and Scotchbrite™ paper while care was taken to exclude bubbles. The transblot chamber was filled with blotting buffer (appendix 2.4.2), and electrophoresis performed (initial current ~ 110 mA) overnight at room temperature.

The nitrocellulose paper was removed and placed in Ponceau S solution for 5 - 10 minutes before several washing steps in ultrapure water. The pink molecular weight protein bands were marked with pin-holes. Nitrocellulose paper strips were placed in a blocking solution of phosphate buffered saline (PBS) + 2% Marvel™ (appendix 2.4.2) for 1 hour before incubation with first antibody (monoclonal antibody 1:200, polyclonal antibody 1:20) in blocking solution + 0.5% Tween for 2 hours. As a control for the non-specific binding of second antibody to other proteins, non-immune serum instead of primary antibody was applied to a strip of nitrocellulose that contained proteins from normal cerebral cortex. Washing steps preceded incubation with peroxidase-conjugated second antibody in blocking solution + 0.5% Tween for 2 hours at room temperature. Further washing steps preceded the application of substrate: a solution of diaminobenzidine in Tris buffer (appendix 2.4.2) to which 2 µl of H_2O_2 (30% v:v) had been added just prior to use. The reaction was stopped when a brown reaction product had developed.

2.5 *In situ* hybridisation (ISH) for synaptophysin mRNA in 13 astrocytic tumours and 9 oligodendrogliomas labelled with synaptophysin antibodies

2.5.1 Probe manufacture

High quality chemicals (Molecular Biology or Analar grade) were used for ISH, and nearly all were purchased from BDH or Sigma.

Oligodeoxyribonucleotide probes consisting of 30 (deoxy)adenosine bases (poly-d(A)) or 30 (deoxy)thymidine bases (poly-d(T)) were manufactured by the department of Virology, University of Southampton, on an Applied Biosystems DNA synthesiser. The synthesis of each probe involved the assembly of a string of bases by phosphoramidite reactions. The probes were de-protected, eluted from their columns, and purified either on OPC cartridges, or by high performance liquid chromatography (HPLC). The HPLC was undertaken on a Hamilton PRP-1 300 mm x 7 mm, reverse-phase, C-8 polymer column. Separation was achieved with an acetonitrile gradient (15% - 100%) in a triethylamine ammonium buffer, and the detection step employed a spectrophotometer set at 260 nm.

A cocktail (appendix 2.5.1) of oligonucleotide probes (Table 251-1) to the 16S (large) subunit of mitochondrial ribosomal RNA (Hilton et al., 1994) was kindly donated by Dr. H. Pringle of the University of Leicester.

Antisense and sense probes to 9 different regions (appendix 2.5.1) of synaptophysin mRNA (Table 251-2) were manufactured by British Biotechnology Ltd., again using an Applied Biosystems DNA synthesiser. These probes were supplied with a single moiety of digoxigenin-11-dUTP at the 5' end, having been purified by HPLC. The concentration of probes was checked by spectrophotometry. Probes were initially stored at -160°C.

Table 251-1
Anti-sense probes to mitochondrial rRNA

bases	1921 - 1950
mito rRNA	5′ acctaagaacagctaaaagagcacacccgt 3′
	3′ tggattcttgtcgattttctcgtgtgggca 5′
probe 1	5′ acgggtgtgctcttttagctgttcttaggt 3′

bases	2001 - 2030
mito rRNA	5′ cgagcctggtgatagctggttgtccaagat 3′
	3′ gctcggaccactatcgaccaacaggttcta 5′
probe 2	5′ atcttggacaaccagctatcaccaggctcg 3′

bases	2051 - 2080
mito rRNA	5′ aatttgcccacagaaccctctaaatcccct 3′
	3′ ttaaacgggtgtcttgggagatttagggga 5′
probe 3	5′ aggggatttagagggttctgtgggcaaatt 3′

bases	2101 - 2130
mito rRNA	5′ caaagaggaacagctctttggacactagga 3′
	3′ gtttctccttgtcgagaaacctgtgatcct 5′
probe 4	5′ tcctagtgtccaaagagctgttcctctttg 3′

bases	2161 - 2190
mito rRNA	5′ acaccccatagtaggcctaaaagcgccacc 3′
	3′ tgtggggtatcatccggattttcgcggtgg 5′
probe 5	5′ ggtggcgcttttaggcctactatggggtgt 3′

These probes were manufactured and kindly donated by
Dr. H. Pringle, University of Leicester.
The probes were supplied as an unlabelled cocktail.

Table 251-2
Anti-sense probes to synaptophysin mRNA

bases	809 - 848
syn mRNA	5′ gtggctacggccctcagggcgactatgggcagcaaggcta 3′
	3′ caccgatgccgggagtcccgctgatacccgtcgttccgat 5′
probe A2	5′ tagccttgctgcccatagtcgccctgagggccgtagccac 3′
bases	52 - 81
syn mRNA	5′ gtcttcgccatcttcgcctttgccacatgc 3′
	3′ cagaagcggtagaagcggaaacggtgtacg 5′
probe A3	5′ gcatgtggcaaaggcgaagatggcgaagac 3′
bases	248 - 277
syn mRNA	5′ actcctcgtcagccgaattctttgtcaccg 3′
	3′ tgaggagcagtcggcttaagaaacagtggc 5′
probe A4	5′ cggtgacaaagaattcggctgacgaggagt 3′
bases	321 - 351
syn mRNA	5′ ctacatcttcctgcagaacaagtaccgagag 3′
	3′ gatgtagaaggacgtcttgttcatggctctc 5′
probe A5	5′ ctctcggtacttgttctgcaggaagatgtag 3′
bases	512 - 542
syn mRNA	5′ acacatgcaaggagctgagagaccctgtgac 3′
	3′ tgtgtacgttcctcgactctctgggacactg 5′
probe A6	5′ gtcacagggtctctcagctccttgcatgtgt 3′
bases	821 - 849
syn mRNA	5′ ctcagggcgactatgggcagcaaggctac 3′
	3′ gagtcccgctgatacccgtcgttccgatg 5′
probe A7	5′ gtagccttgctgcccatagtcgccctgag 3′
bases	1296 - 1325
syn mRNA	5′ ccttggttcctccagcaatgatgcctgaac 3′
	3′ ggaaccaaggaggtcgttactacggacttg 5′
probe A9	5′ gttcaggcatcattgctggaggaaccaagg 3′
bases	1578 - 1607
syn mRNA	5′ ctttaagcgaggcagaatggtgggctccag 3′
	3′ gaaattcgctccgtcttaccacccgaggtc 5′
probe A10	5′ ctggagcccaccattctgcctcgcttaaag 3′
bases	1940 - 1968
syn mRNA	5′ gtggcctgagaagagtcgagtgatatggc 3′
	3′ caccggactcttctcagctcactataccg 5′
probe A11	5′ gccatatcactcgactcttctcaggccac 3′

Probe A2 is identical to a probe used successfully for in situ hybridisation in brain by Prof. C. Pearson, University of Sheffield. A2 was used on its own in some preliminary experiments, but was not used in the synaptophysin probe cocktails, the shorter A7 probe being used instead.

2.5.2 Probe labelling

Terminal transferase (Boehringer Mannheim) was used for the template independent addition of deoxyribonucleoside triphosphates to the 3'-OH end of the DNA strand of all probes (Deng and Wu, 1981; Pringle et al., 1989). Digoxigenin-11-dUTP (Boehringer Mannheim) was attached to the oligonucleotides both with and without unlabelled dATP (if dATP is included in the reaction, 2 - 4 bases of digoxigenin-11-dUTP are incorporated in a tail of approximately 50 bases, otherwise the 3'-OH end of the probe is labelled with 1 or occasionally 2 bases of digoxigenin-11-dUTP).

The tailing reactions (appendix 2.5.2) contained:

sterile deionised water
Boehringer tailing reaction buffer*
5 mM $MnCl_2$
50 µM digoxigenin-11-dUTP
(500 µM dATP)†
terminal transferase (50 u. for each 1 µg DNA)
1 µg (100 pmol) oligonucleotide probe

* Tris-HCl, 25 mM; potassium cacodylate, 200 mM; BSA, 0.25 mg / ml

† optional (see above & appendix 2.5.2)

Incubation of the reaction mixture at 37°C proceeded over 2 hours. The reaction was stopped by adding 1 µl of glycerol solution (20 µg glycerol in 200 µl of 0.2 M EDTA pH 8.0). Labelled DNA was either extracted from the reaction mixture by ethanol precipitation at -20°C overnight or separated on commercial columns.

The concentration of labelled probes was checked by spectrophotometry. Labelled probes were stored at -20°C.

The efficiency of the tailing reaction was determined by test blots (Figure 252). Serial dilutions (1 µg / ml, 100 ng / ml, 50 ng / ml, 10 ng / ml, 1 ng / ml, and 0.1 ng / ml) of a small amount (40 ng) of labelled probe in buffer (50 mM Tris-HCl, pH 7.5; 0.3 M NaOH; 1.5 mg / ml ssDNA) were boiled for 10 minutes, and 1µl of each dilution was spotted on to nitrocellulose paper, together with 1 µl of buffer alone to act as a control. The nitrocellulose paper was baked at 80°C for 2 hours before being placed in a preheated blocking solution (TBS with 3% BSA and 0.1% Triton-X 100) at 50°C for 20 minutes. The blot was again baked at 80°C for 20 minutes before being immersed in ultrapure water and put back into blocking solution for 10 minutes. The blot was placed in a solution of anti-digoxigenin antibody (1:1200 in blocking solution) for 1 hour. After a rinse in water, the blot was transferred to Tris buffer (pH 9.5) for 5 minutes before being placed in substrate for 1 hour (see 2.5.7). The developed test blots were used to select optimum conditions for labelling (appendix 2.5.2), and the best labelled probes to be used on tissue sections.

2.5.3 Fixation and processing of tissue

Optimally fixed and processed CNS tissue which contained neurons was essential both for assessment of components of the ISH method and to act as control tissue for the ISH method when biopsy material was examined. Fresh cerebral lobectomy specimens were fixed in either 10% neutral buffered formalin or paraformaldehyde for either 24 or 48 hours. Incisions approximately 20 mm long and 5 mm deep were made into superficial areas of normal cerebral cortex to ensure rapid fixation. Small blocks, approximately 10 x 5 x 2 mm, were subsequently taken from these regions. Previous studies had reported good results for both fixatives (Singer et al., 1986; Larsson and Hougaard, 1990; Wilkinson, 1992), and no appreciable difference was found between them in this study. However, there was a more uniform signal from all probes in sections from tissue that had been fixed for 48 hours, and this was subsequently used as control tissue for the synaptophysin probes. Archival, normal postmortem spinal

Figure 252

Test blot for the efficiency of probe labelling

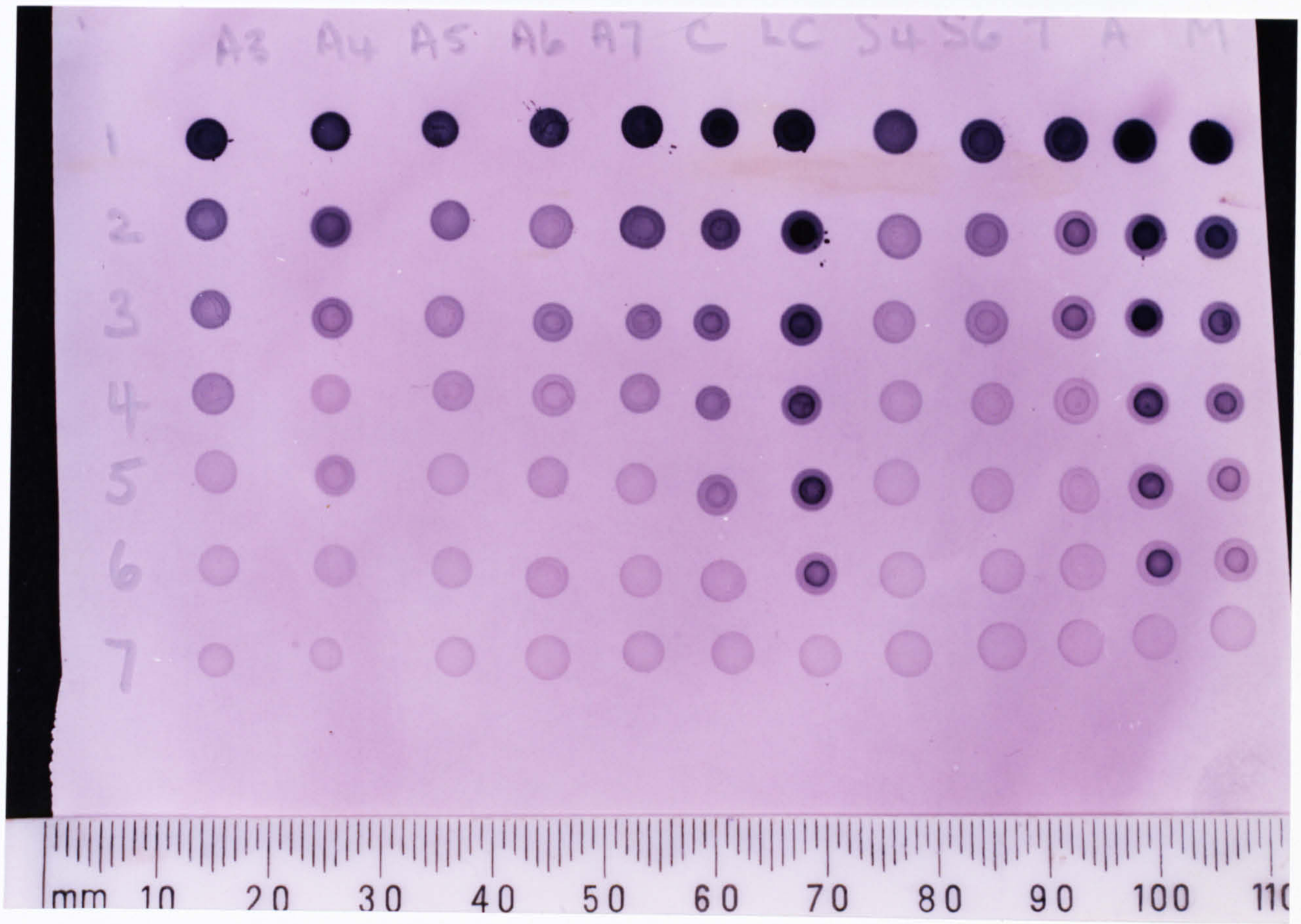
y axis

Serial dilutions (1 μg / ml - 0.1 ng / ml; **1 - 6**) of probe, plus buffer only; **7**

x axis

Different probes, showing different degrees of labelling

NBT / BCIP chromogen


A3 A4 A5 A6 A7 C LC S4 S6 T A M
1
2
3
4
5
6
7
mm 10 20 30 40 50 60 70 80 90 100 110

cord fixed for 4 weeks in 10% neutral buffered formalin was sometimes used as an alternative to the blocks of cerebral cortex.

2.5.4 Pretreatment of sections for ISH

Sections (5 μm) cut from paraffin embedded tissue were floated onto slides coated with 3-aminopropyltriethoxysilane (appendix 2.3.2) using water treated by reverse osmosis. The coating of slides was mandatory because of the large number of steps in the method. After being dried overnight at 37°C, the sections were dewaxed in xylene, and taken through graded alcohols before being rinsed once in diethylpyrocarbonate (DEPC)-treated water. All solutions and glassware used before the hybridisation step were RNAase-free (appendix 2.5.4). For the pretreatment of sections of spinal cord, slides were first immersed in 0.2N HCl for 20 minutes (appendix 2.5.4). All slides were placed in 2 x standard saline citrate (SSC) at 70°C for 10 minutes (appendix 2.5.4). After one rinse in DEPC-treated water, the slides were washed twice in 50 mM Tris buffer (pH 7.65) for 2 minutes at room temperature. Proteinase K (0.02 - 15 μg / ml in 50 mM Tris buffer, pH 7.65) was then applied to the sections at predetermined concentrations (appendix 2.5.4), the slides being incubated for 60 minutes at 37°C. After a double rinse in DEPC-treated water, the slides were washed once in cold 1 x PBS (appendix 2.4.2) for 2 minutes. The sections were next placed in pre-cooled paraformaldehyde (0.4% in 1 x PBS) for 20 minutes at 4°C. The slides were rinsed twice in DEPC-treated water before prehybridisation solution was applied.

2.5.5 Hybridisation

For each ISH run, a solution consisting of the following reagents (appendix 2.5.5) was freshly prepared at 4°C in 1.5 ml Eppendorf tubes:

sterile deionised water	to make a total volume of 1 ml	
5M NaCl	120 µl	(600 mM)
10 x PE (appendix 2.5.5)	100 µl	(1 x PE)
dextran sulphate	200 µl	(10% dextran sulphate)
formamide	200 / 300 µl *	
ssDNA (appendix 2.5.5)	50 µl	(0.5 mg / ml)

*20% formamide	poly-d(T) / poly-d(A) and synaptophysin probes
*30% formamide	mitochondrial probes

Solubilisation was achieved when the reagents were added in the above order. The solution was thoroughly mixed by vortexing before and after addition of DNA. For each fresh batch of solutions, ≈ 0.5 ml of stock ssDNA (sheared salmon sperm DNA) was thawed to 4°C, then boiled for 10 minutes before immediately being put back onto ice in preparation for its addition to the other reagents. A total of 4 x 1 ml was sufficient for both prehybridisation and hybridisation steps in each ISH run.

Half the solution was set aside for the prehybridisation step, while probes were added to the remainder at the following optimal final concentrations:

poly-d(T) / poly-d(A)	1 µg / ml
mitochondrial	0.1 µg / ml
synaptophysin	2 µg / ml

Several combinations (cocktails) of synaptophysin antisense and sense probes were assessed on cerebral cortex before being used on runs with tumour sections (appendix 2.5.5).

The prehybridisation step involved saturating the sections with ≈ 50 µl of the above solution. After 1 hour at 37°C, the solution was run off the slides and replaced by a similar quantity of hybridisation solution with added probes. The hybridisation solution on each slide was covered with a coated coverslip (appendix 2.5.5) and incubated at 37°C overnight.

2.5.6 Stringency washes

The coverslips were washed off with a solution of prewarmed (37°C) 2 x SSC, and the slides were placed in Coplin jars. Prewarmed solutions of 2 x SSC plus 30% formamide were used for 3 washes at 37°C lasting 5 minutes each (appendix 2.5.6).

2.5.7 Detection

After the stringency washes, the slides were rinsed in water, and placed in a blocking solution (appendix 2.5.7) for 10 minutes. Alkaline phosphatase conjugated anti-digoxigenin antibody (appendix 2.5.7) at a dilution of 1:600 in the blocking solution was applied to the slides which were then placed on a rocker tray for 1 hour. Slides were rinsed (2 x 5 minutes) in TBS + 0.1% BSA, and then in water, before being placed in buffer, 0.1M Tris chloride (pH 9.5) + 50 mM $MgCl_2$ + 100 mM NaCl, for 5 minutes. This buffer must be freshly made, and the pH should be precisely adjusted (appendix 2.5.7).

The substrate solution which uses a 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate, nitro-blue tetrazolium salt (NBT) chromogen combination was prepared as follows:

Tris buffer pH 9.5	2 ml
levamisole 1M	2 µl
NBT (75 mg / ml in 70% DMF)	8.8 µl
BCIP (50 mg / ml in 70% DMF)	6.6 µl

Approximately 100 µl of substrate was applied to each section, and the slides were placed in trays in a light-proof environment for 1 - 20 hours. The development of the reaction was monitored during this time; when the reaction was complete, the slides were rinsed in water, and coverslips were applied.

One of the disadvantages of the alkaline phosphatase system is the necessity for aqueous mounting media. However, if a drop of Crystalmount™ is placed on the section and baked for 15 minutes at 80°C, the slides can be mounted in DPX.

2.5.8 Controls

Each ISH run included (appendix 2.5.8):

(i) sections of optimally processed normal cerebral cortex or spinal cord (control tissue) in which neurons and glia would act respectively as positive and negative tissue controls for the synaptophysin probes

(ii) sections of control and tumour tissue to which no probe had been applied

(iii) sections of control and tumour tissue to which either poly-d(T) or mitochondrial probes had been applied

(iv) sections of control and tumour tissue to which a cocktail of sense probes (synaptophysin or poly-d(A)) had been applied

2.6 Analysis of the p53 gene in 8 astrocytic tumours with neuronal immunophenotype

2.6.1 Extraction of DNA from tumour tissue

Sections, 18 x 10 µm thick and 2 x 5 µm thick (first and last), were cut from formalin fixed, paraffin embedded tissue. Fresh water treated by reverse osmosis was used to float the sections onto uncoated slides. The first and last sections were stained with haematoxylin and eosin, and examined for suitable areas of tumour to use for p53 gene analysis. Normal cerebral cortex and the infiltrating edge of the tumour where present were avoided as before (see 2.2.2). The chosen areas were identified on the unstained sections, and scraped into sterile 1.5 ml Eppendorf tubes with a scalpel blade. The scrapings were suspended in 1 ml xylol, and the tubes put on a mechanical shaker for 30 minutes, before a centrifugation step at 14,000 rpm for 10 minutes. The supernatant was discarded, and the tissue resuspended in 1 ml absolute ethanol. This step was repeated before the ethanol was drawn off the tissue pellets using vacuum extraction and until the pellets were slightly damp. The pellets were resuspended in a solution containing: 200 mM Tris-HCl (pH 8.3), 10 mM NaCl, 1 mM EDTA, to which 1% SDS, and then proteinase K (500 µg / ml) at 4°C were added (final volume ~ 0.5 ml). After gentle mixing, the tubes were sealed with Parafilm™, and placed in a waterbath at 50°C overnight. Fresh proteinase K was added the next day, and the incubation proceeded for a further 24 hours. An isovolume of phenol : chloroform was added to the solutions which were well mixed and taken through a centrifugation step, 14,000 rpm for 10 minutes. Supernatants were placed in new Eppendorf tubes and the phenol : chloroform extraction was repeated. A chloroform extraction step preceded DNA precipitation which used 0.1 x *supernatant volume* of 3 M sodium acetate (pH 6), and 2.5 x *supernatant volume* of absolute ethanol. DNA was precipitated at -20°C, and a DNA pellet produced by prolonged centrifugation, 14,000 rpm for 45 minutes. The pellet was washed three times with 0.5 - 1 ml of 70% ethanol at 4°C, before resuspension in sterile water (15 µl). DNA was stored at 4°C.

2.6.2 Polymerase chain reaction (PCR) to amplify exons 5 - 8 of the p53 gene

A batch of reaction mixtures was prepared, each distinguished by containing primers for just one of the four p53 exons (Table 262). DNA (1 or 2 µl) from each tumour to be tested, from reference samples, and from controls which contained either normal p53 or p53 with a mutation in the exon to be amplified, was added separately to a solution (made up originally to supply all reaction mixtures) of the following composition:

1 µl of dNTP's

1 µl of 1 mM $MgCl_2$ in PCR buffer (appendix 2.6.2)

0.5 µl of each primer

5 µl of sterile water

0.07 µl of Gibco Taq polymerase

(total volume 9 / 10 µl with DNA added).

Solutions were kept on ice while the reaction mixtures were prepared, and Taq polymerase was added just before addition of the DNA. A drop of machine oil was placed on top of each solution, and a 5 second centrifugation step ensured that all bubbles were removed before the PCR.

An Omnigene automated PCR cycler was used for the reactions. After a denaturing step at 94°C which lasted 3 minutes, each PCR ran through 30 cycles (Figure 262-1) with the following characteristics:

94°C	30 seconds		
62°C	60 seconds	exon 5 and exon 6:	60°C
72°C	60 seconds	exon 5:	40 seconds

A 10 minute step at 72°C ended the reaction. The presence of PCR reaction products was confirmed by running the reference samples on a 2% agarose mini-gel. Ethidium

Table 262
Details of PCR primers for exons 5 - 8 of the p53 gene

	primer A	primer B	product length
exon 5	GTGCCCTGACTTTCAACTCTG	GGGCAACCAGCCCTGTCG	260 bp
exon 6	CAGGGCTGGTTGCCCAGGGTCCCCA	ACTGACAACCACCCTTAACCCCTCC	221 bp
exon 7	CTCCTAGGTTGGCTCTGACTGT	GAGGCTGGGGCACAGCAGGCCAGTG	167 bp
exon 8	TAGGACCTGATTTCCTTACTGCCTC	AACTGCACCCTTGGTCTCCTCCACC	236 bp

	p53 gene (bases)	primer A matches (5'-3')	primer B complementary (3'-5')
exon 5	13055 - 13238	13014 - 13034	13279 - 13262
exon 6	13320 - 13432	13265 - 13289	13485 - 13461
exon 7	14000 - 14109	13993 - 14014	14159 - 14135
exon 8	14452 - 14588	14400 - 14424	14635 - 14611

Figure 262-1
PCR: hybridisation and extension of primers

primer →

caggg ctggttgccc agggtcccca

3′ ctgctgtccc gaccaacggg tcccaggggt ccggagacta aggagtgact aacgagaatc CA GAC CGG GGA GGA GTC GTA GAA

5′ gacgacaggg ctggttgccc agggtcccca ggcctctgat tcctcactga ttgctcttag GT CTG GCC CCT CCT CAG CAT CTT

13260

3′ TAG GCT CAC CTT CCT TTA AAC GCA CAC CTC ATA AAC CTA CTG TCT TTG TGA AAA GCT GTA TCA CAC CAC CAC

5′ ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG TAT TTG GAT GAC AGA AAC ACT TTT CGA CAT AGT GTG GTG GTG

3′ AAA ATA CTC GGC GGA CTC cagacca aacgttgacc ccagagaccc tcctccccaa ttcccaccaa cagtcaccgg gaggcccact

5′ CCC TAT GAG CCG CCT GAG gtctggt ttgcaactgg ggtctctggg aggaggggtt aagggtggtt gtcagtggcc ctccgggtga

cctccccaa ttcccaccaa cagtca 13499

← **primer**

PCR primers flank the coding region as illustrated for exon 6 of the p53 gene
The template dependent addition of bases proceeds in the direction of the arrows

bromide had been added to the electrophoresis buffer (0.6 x TBE), and was used to demonstrate the DNA under ultraviolet light (Figure 262-2).

2.6.3 Preparation of single-strand conformational polymorphism (SSCP) gels

For each p53 exon, gel composition and electrophoretic conditions varied as set out in Table 263. Two glass plates were coated on one side (next to gel) with 5% dimethyldichlorosilane, separated by 0.35 mm spacers, and taped in preparation for pouring the acrylamide gels. Gel polymerisation was triggered by adding 180 µl of 20% ammonium persulphate and 20 µl of TEMED to 70 ml of stock gel solution. A comb was inserted into the gel solution to form sample wells. Once a gel had set, it was loaded into a Poker Face ™ 2 (Hoefer) apparatus, and samples in gel loading buffer were placed in the wells. A size standard (Boehringer V), and positive and negative control samples as defined above (see appendix 2.6.2) were run on the gel together with the samples under investigation.

2.6.4 Gel development

After electrophoresis, the Poker Face apparatus was disassembled, and the gel was left on one glass plate. This was placed in a home-made development apparatus that trapped the plate and gel in the bottom of a sealed wooden well. The gel is developed by a sequence of solutions in the same way as a black and white photograph:

10 % ethanol	5 minutes
1% KNO_3	3 minutes
2 x dH_2O	5 minutes each
7.2 mM Ag_2NO_3	20 minutes
2 x dH_2O	5 minutes each
≈ 4 x 0.4M Na_2CO_3	replace solution when it turns light brown
10% acetic acid	5 minutes
2 x dH_2O	5 minutes each

Table 263
Conditions for gel electrophoresis

	gel characteristics			electrophoresis	
	% acrylamide	bisacryl. : acryl.	5% glycerol	power	duration
exon 5	8	1 : 80	-	2 W	14 hours
exon 6	12	1 : 29	+	20 W	14 hours
exon 7	8	1 : 80	-	2 W	14 hours
exon 8	10	1 : 29	+	5 W	14 hours

Half the width of any shifted band detected on developed gels and thought to be indicative of a mutation within the exon was removed from the gel using a sterile scalpel blade. The other half of the band remained as a permanent record of the shift. Once developed, gels were dried by transferring them first to large sheets of absorbent (blotting) paper, and then placing them on a vacuum assisted heating plate for 1 hour.

2.6.5 Gene sequencing

Having been cut from the gels, the shifted bands were placed in Eppendorf tubes to which 50 µl of DNA elution buffer (appendix 2.6.5) was added. The tubes were placed on a Thermomixer (~ 50°C) for at least 8 hours. DNA was precipitated at -20°C overnight after the addition of 125 µl of absolute ethanol. After a centrifugation step, 14,000 rpm for 30 minutes, the supernatant was discarded, and the pellet was washed twice with 70% ethanol as before. Once dried, the pellet was resuspended in 20 µl of sterile water.

Reamplification of the DNA by PCR followed, using the same conditions and primers as detailed above (see 2.6.2), but for 35 cycles. The presence of amplification product was confirmed as before, and the DNA was purified on a 1% low-melting point agarose gel. The DNA was recovered using agarase digestion, and precipitated at -20°C overnight. After washing steps performed as before, the DNA pellet was resuspended in 30 µl of sterile water. One tenth of this was run on an agarose gel where the localisation and intensity of the DNA band were compared with those of a standard. The concentration of the solution was thus calculated approximately, so that ≈15 ng of DNA template could be used in the sequencing reactions.

The sequencing PCR reaction was run on a Perkin-Elmer TC 9600 cycler. In a final volume of 20 µl, ~ 15 ng DNA was added to 9.5 µl of Terminator mix, 10 pmol of primer, and water. Incorporation of the fluorescent bases proceeded over 25 cycles, and the annealing temperature was identical to that for the PCR reactions above:

96°C	15 seconds
50°C - 60°C	15 seconds
60°C	4 minutes

After PCR, cetyl-trimethyl ammonium bromide (CTAB), a cationic detergent, was used in conjunction with ethanol precipitation to remove excess dye terminators from the sequencing reaction prior to loading the sequencing gel. Each reaction mixture was removed to a new 0.5 ml Eppendorf tube containing 2.5 µl of 5% CTAB. After a centrifugation step, 14,000 rpm for 4 minutes, the supernatant was removed, and the pellet was resuspended in 50 µl of 1.2 M NaCl. Absolute ethanol (125 µl) was added, and the centrifugation step was repeated after vortexing. Washing steps using 70% ethanol followed as before. After drying, the samples were resuspended in 4 µl of loading buffer (formamide / 50 mM EDTA, pH 8, in a ratio of 5 : 1), and loaded on the sequencer gel.

DyeDeoxy Terminator sequencing was performed on an Applied Biosystems International 373 A, computer (*Apple Macintosh*) linked, automated sequencer.

2.7 Statistical analysis and measurement

2.7.1 Indices of immunolabelling

An immunohistochemical labelling index (LI) was calculated for the DO-7 (p53) and Ki-67 antibodies. At least 2 high-power (x40 objective) fields of each section were assessed with the aid of a counting grid. The denominator was at least 1,000 tumour cells. A tumour with a p53 LI < 1% was regarded as negative. Areas of each section that were free from necrosis or capillary endothelial proliferation were chosen for the calculation of LI's. If present in the section, the infiltrative edge of the tumour where neoplastic cells surround normal neurons and glia was also avoided. Where an uneven distribution of immunohistochemical labelling was evident, fields from the area of maximal labelling were chosen for counting; otherwise, fields were randomly chosen while avoiding areas of the tumour specified above.

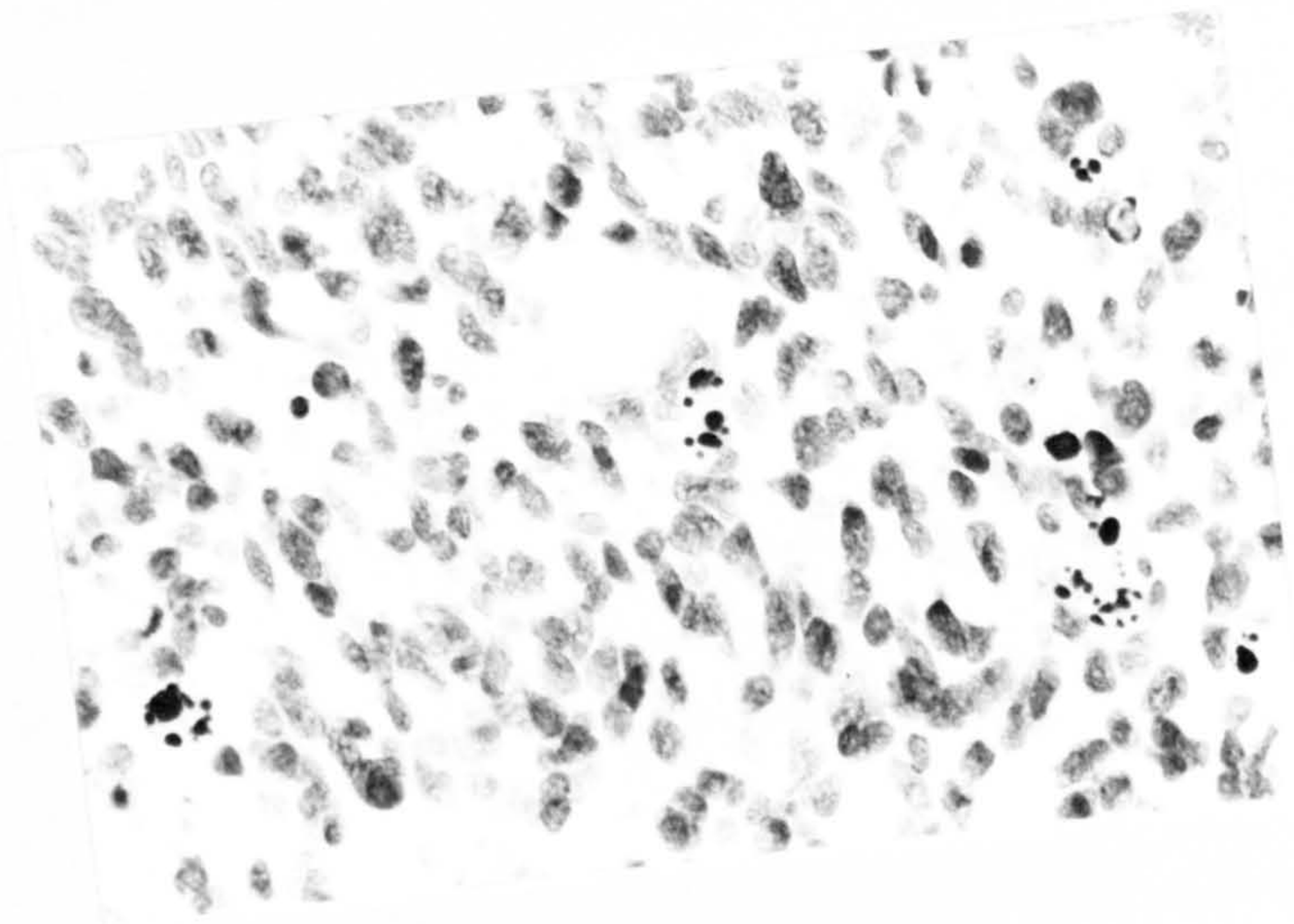
2.7.2 Indices of apoptosis

Apoptotic cells were identified in haematoxylin stained sections by their characteristic nuclear features, such as crescents of collapsed chromatin along the nuclear envelope, and fragmentation of the nucleus into spheres (Figure 272). An index of these cells (AI) was calculated with the aid of a counting grid, and with reference to the total number of cells in at least 6 high-power (x40 objective) fields of each section, representing over 10,000 cells in 15% of tumours, and over 5,000 cells in the remainder. More than 6 fields were chosen if the total number of cells assessed was below 5,000. As for LI's (see 2.7.1), areas of each section that were free from necrosis or capillary endothelial proliferation were chosen for the calculation of AI's, and the infiltrative edge of the tumour where neoplastic cells surround normal neurons and glia was also avoided.

Figure 272

Cells undergoing apoptosis are evident in this glioblastoma; nuclear fragmentation and crescents of collapsed chromatin are both seen.

Haematoxylin; x 200.



2.7.3 Statistical methods

For the analysis of clinicopathological data, continuous variables, with one exception, were not normally distributed, and non-parametric statistical terms and tests were therefore used. The median and interquartile range were used as descriptive statistics. The Mann-Whitney test for 2 (unpaired) groups and the Kruskal-Wallis test for more than 2 groups were used to evaluate differences between groups of data. One variable, age, was normally distributed, and the mean and its standard error were used as descriptive statistics. Differences among nominal groups were tested in contingency tables (χ^2 test).

Direct comparisons between my data and those of most previous studies are slightly hampered by the inappropriate use of parametric statistical tests in previous studies.

Survival times were calculated as time from surgery to death, or time to last follow-up appointment in the case of survivors. Kaplan-Meier curves were produced to compare length of survival between groups. Prognostic factors were entered into a Cox proportional hazards model; each term being tested on its own and in the presence of others. Ki-67 LI and p53 LI were considered both as continuous variables, and categorised into 3 levels. Only the categorial version was considered in the adjusted Cox model. Likelihood ratio tests were undertaken for each variable, and relative hazards with corresponding 95% confidence intervals were produced.

Results

3.1 Immunohistochemistry

3.1.1 Immunolabelling of neuronal elements in normal cerebral cortex with the panel of neuronal antibodies

The panel of neuronal antibodies used to study astrocytic tumours and oligodendrogliomas immunolabelled neuronal elements in normal cerebral cortex. Both synaptophysin antibodies labelled the neuropil of the cortex (Figure 311). Almost undetectable labelling of neuronal cytoplasm by these antibodies was enhanced by the addition of the microwave pretreatment step. The NFP antibody labelled both neuronal cell bodies and large axons; but, unlike the synaptophysin antibodies, did not reveal the dense meshwork of small neuritic processes in the neuropil. N52 labelled neuronal cell bodies, but displayed fewer axons than the NFP antibody. Chromogranin-A only labelled some of the larger cortical neurons.

None of these antibodies labelled meninges, glia or blood vessels.

3.1.2 Immunolabelling of astrocytic tumours with a panel of neuronal antibodies

All gliomas are infiltrative tumours, and despite the considered selection of paraffin blocks for this study (see 2.2.2), some care was required to differentiate the labelling of neoplastic cells from the labelling of native entrapped neuronal elements; the differentiation was nearly always clear on cytological grounds (Figure 312-1).

None of the 24 fibrillary astrocytomas was labelled with any of the neuronal antibodies. Both synaptophysin antibodies labelled 13 / 121 (11%) of the high-grade astrocytic tumours (Table 312-1), 3 of which were anaplastic astrocytomas. Only 1 synaptophysin-negative tumour was labelled with other neuronal antibodies (Table 312-1). The antibody to 70 kD & 200 kD neurofilaments labelled just over half of the

Figure 311

Uniform labelling of the neuropil with synaptophysin antibodies is a characteristic of the cerebral grey matter. Some neuronal cell bodies also label faintly. Glia are not labelled.

Synaptophysin antibody; haematoxylin counterstain; x 100.

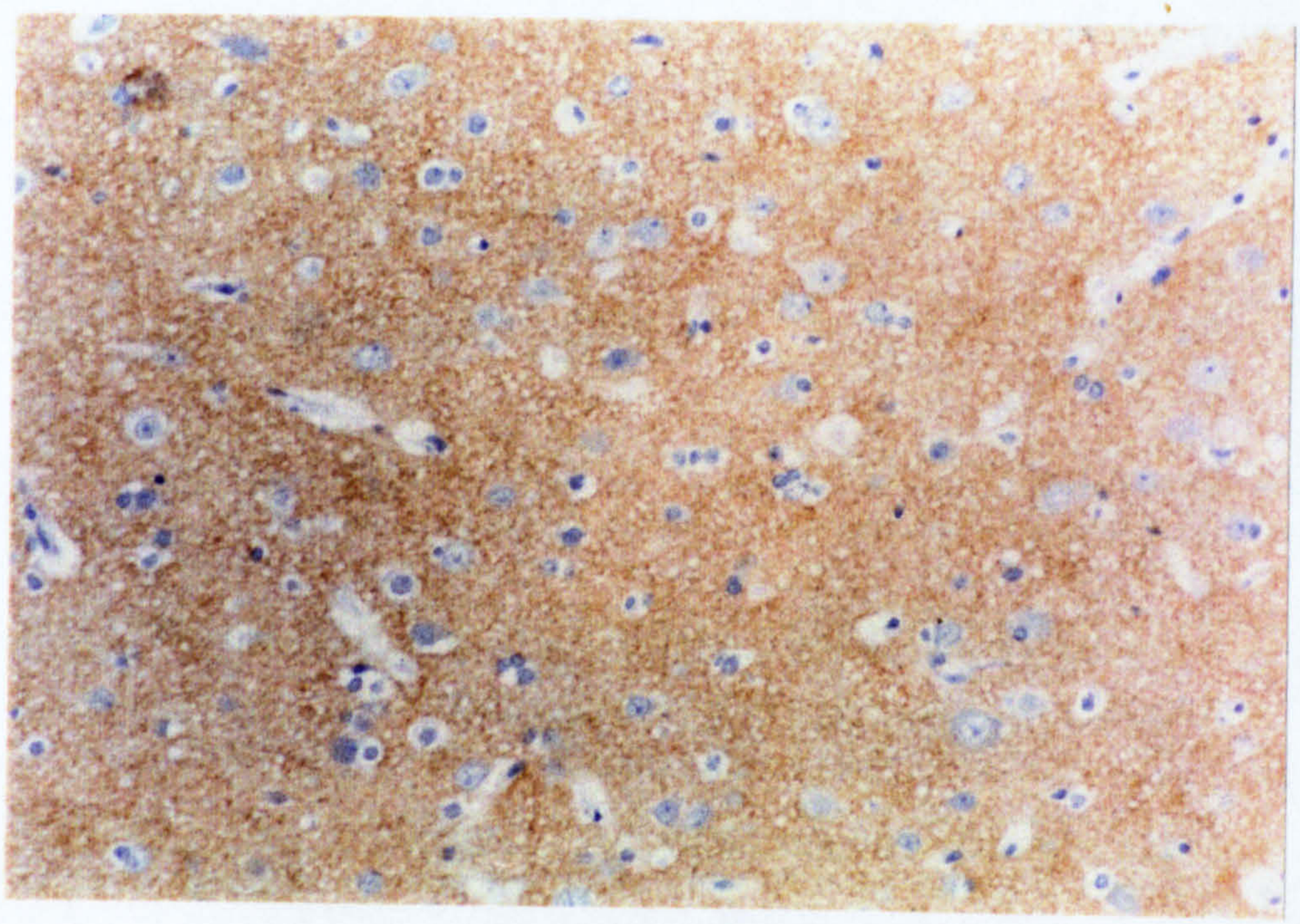


Figure 312-1a

A glioblastoma infiltrates the cerebral grey matter. Tumour cells are not labelled, but enmeshed neuritic processes and neuronal cell bodies are clearly identified.

Synaptophysin antibody; haematoxylin counterstain; x 100.

Figure 312-1b

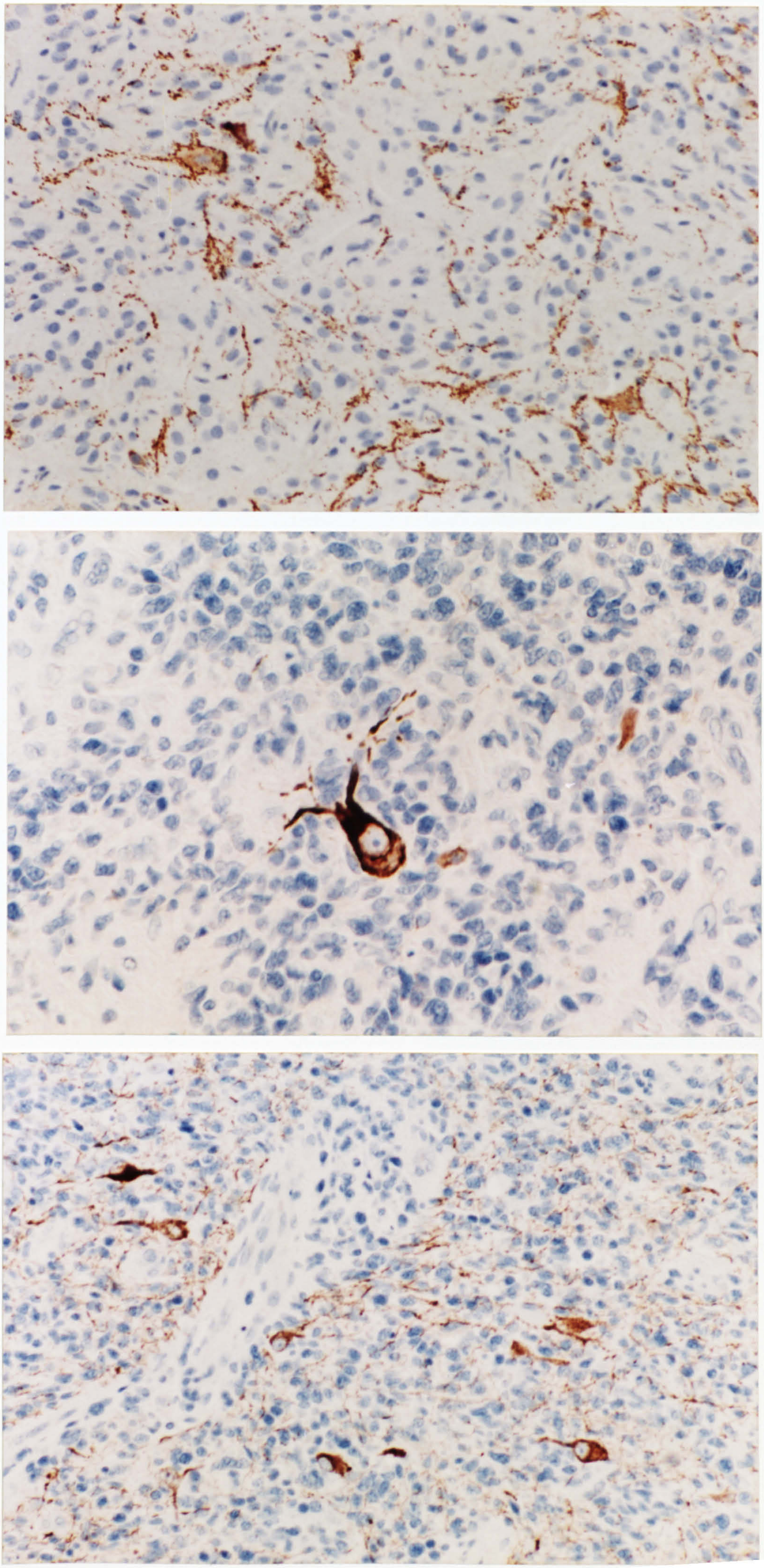
Unlabelled tumour cells surround a solitary pyramidal, cortical neuron which has been labelled with a neurofilament antibody.

Neurofilament antibody (200 kD); haematoxylin counterstain; x 200.

Figure 312-1c

Neuritic processes and cortical neurons are dispersed in a sea of unlabelled cells that form part of an infiltrating glioblastoma.

Neurofilament antibody (70 kD & 200 kD); haematoxylin counterstain; x 100.



synaptophysin-positive tumours; the N52 and chromogranin-A antibodies labelled fewer tumours (Table 312-1). The remaining 107 tumours did not label with any of the antibodies to neuronal proteins, but the majority of cells in 89% of these tumours labelled with the GFAP antibody (Table 312-2).

No high-grade astrocytic tumour was universally labelled with any neuronal antibody; instead, labelling was generally confined to small foci of cells or a few, scattered cells (Figure 312-2). Synaptophysin-positive tumour cells were frequently round to oval with circular nuclei; but, in some instances, showed typical astrocytic features such as eccentric nuclei and an elongated, slightly irregular shape. Small foci of oligodendroglial differentiation in a few glioblastomas were occasionally synaptophysin-positive. Other neuronal antibodies usually labelled cells with an astrocytic morphology. The large, GFAP-positive, neoplastic cells that are found in some glioblastomas and that frequently have a ganglionic appearance, with a large nucleus and prominent nucleolus, did not label preferentially with neuronal antibodies; except in one glioblastoma, where a small number of large cells was labelled with the chromogranin-A antibody (Figure 312-2f).

Some anaplastic astrocytomas and glioblastomas contained large areas where GFAP immunolabelling was lost. This phenomenon was significantly ($P < 0.02$) associated with a neuronal immunophenotype (Table 312-2). Astrocytic tumours with a neuronal immunophenotype did not differ on various clinical and pathological variables from standard (high-grade) astrocytic tumours (Table 312-3).

3.1.3 Immunolabelling of oligodendrogliomas with a panel of neuronal antibodies

Just over one third (9 / 25) of the oligodendrogliomas in this study was synaptophysin-positive (Table 313-1). However, none of the other neuronal antibodies labelled these tumours. The synaptophysin labelling took a distinctive form in many oligodendrogliomas, being confined to a small perinuclear focus (Figure 313c).

Oligodendrogliomas have a propensity to spread along the subarachnoid space above

Table 312-1
Profile of neuronal immunophenotype in 14 high-grade astrocytic tumours

W.H.O. diagnosis	synaptophysin	NFP	N52	chromogranin-A	GFAP
anaplastic astrocytoma	++	+	+	+	+
glioblastoma	+	+	+	+	+
anaplastic astrocytoma	+	+	-	+	++
glioblastoma	+	+	-	+	++
glioblastoma	+	+	-	+	+
glioblastoma	+	+	+	-	++
glioblastoma	+	+	-	-	++
glioblastoma	+	+	-	-	+
glioblastoma	++	-	-	-	++
anaplastic astrocytoma	+	-	-	-	++
glioblastoma	+	-	-	-	++
glioblastoma	+	-	-	-	++
glioblastoma	+	-	-	-	+
glioblastoma	-	++	++	-	++

- no labelling
+ labelled cells in a minority
++ labelled cells in a majority

Table 312-2
Percentage of astrocytic tumours with or without neuronal immunophenotype versus 2 categories of GFAP immunolabelling

		GFAP +	GFAP ++
neuronal IHC +	n = 14	36 %	64 %
neuronal IHC -	n = 107	11 %	89 %

$\chi^2 = 5.53$; P = 0.019

GFAP + labelled cells in a minority
GFAP ++ labelled cells in a majority

Figure 312-2a

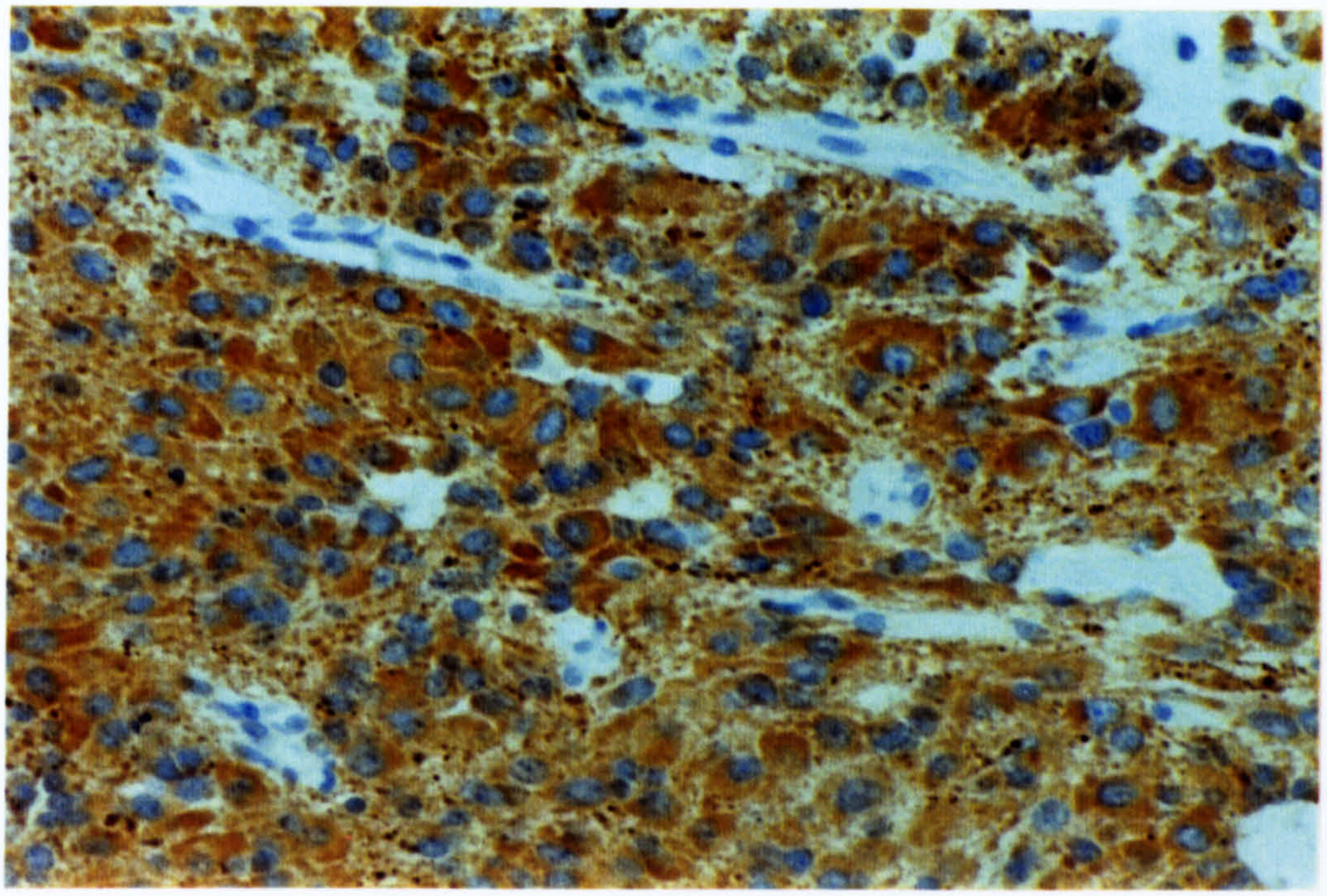
A group of neoplastic astrocytes in this glioblastoma labels with an antibody to synaptophysin.
Some displaced neuritic processes also label strongly and have a beaded appearance.

Synaptophysin antibody; haematoxylin counterstain; x 200.

Figure 312-2b

Round and oval cells in this glioblastoma are immunolabelled by a synaptophysin antibody. Small dots of dense perinuclear labelling are reminiscent of the synaptophysin immunolabelling in some oligodendrogliomas (Figure 313), though small areas of oligodendroglial differentiation in glioblastomas were not invariably labelled with synaptophysin antibodies.

Synaptophysin antibody; haematoxylin counterstain; x 200.



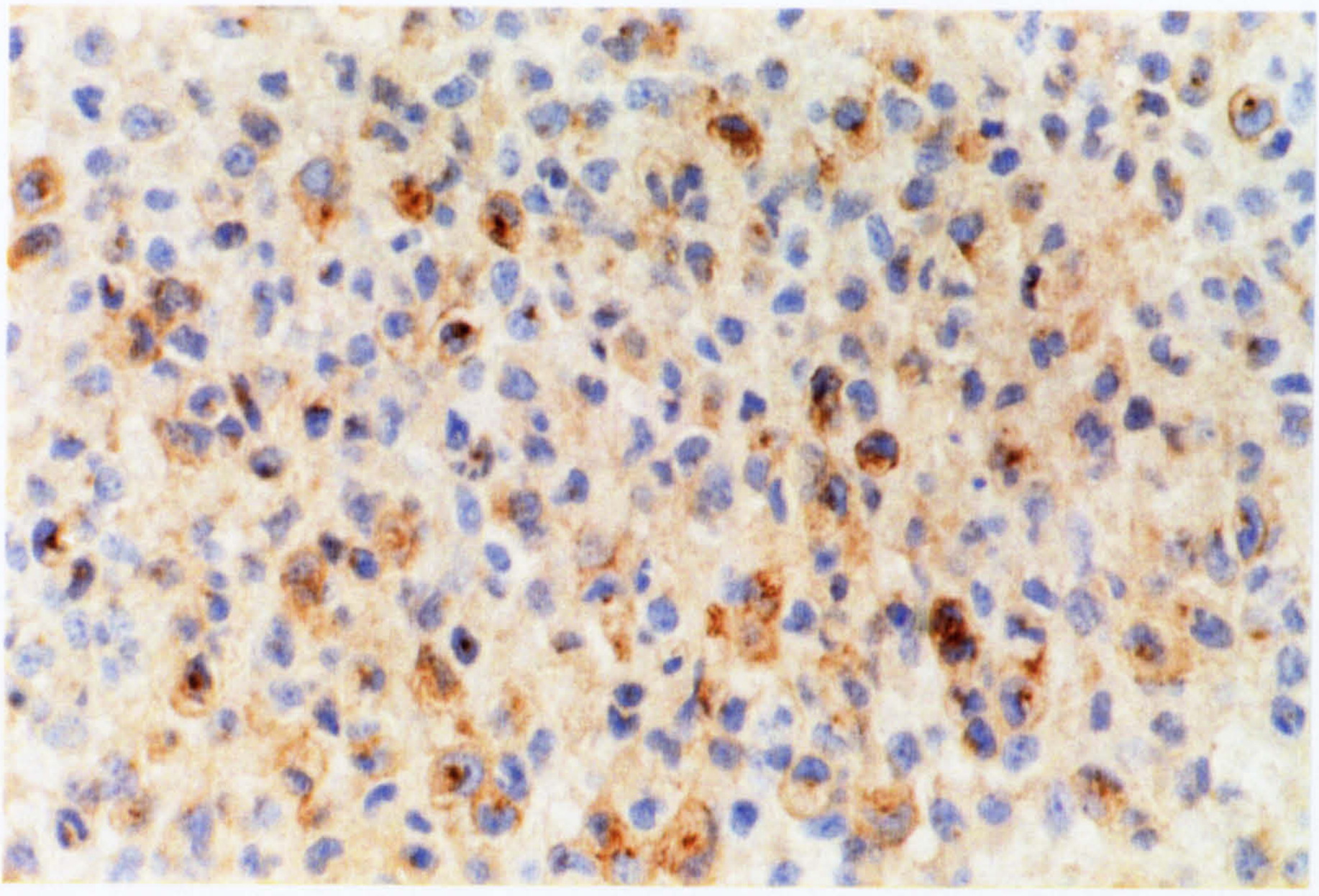


Figure 312-2c

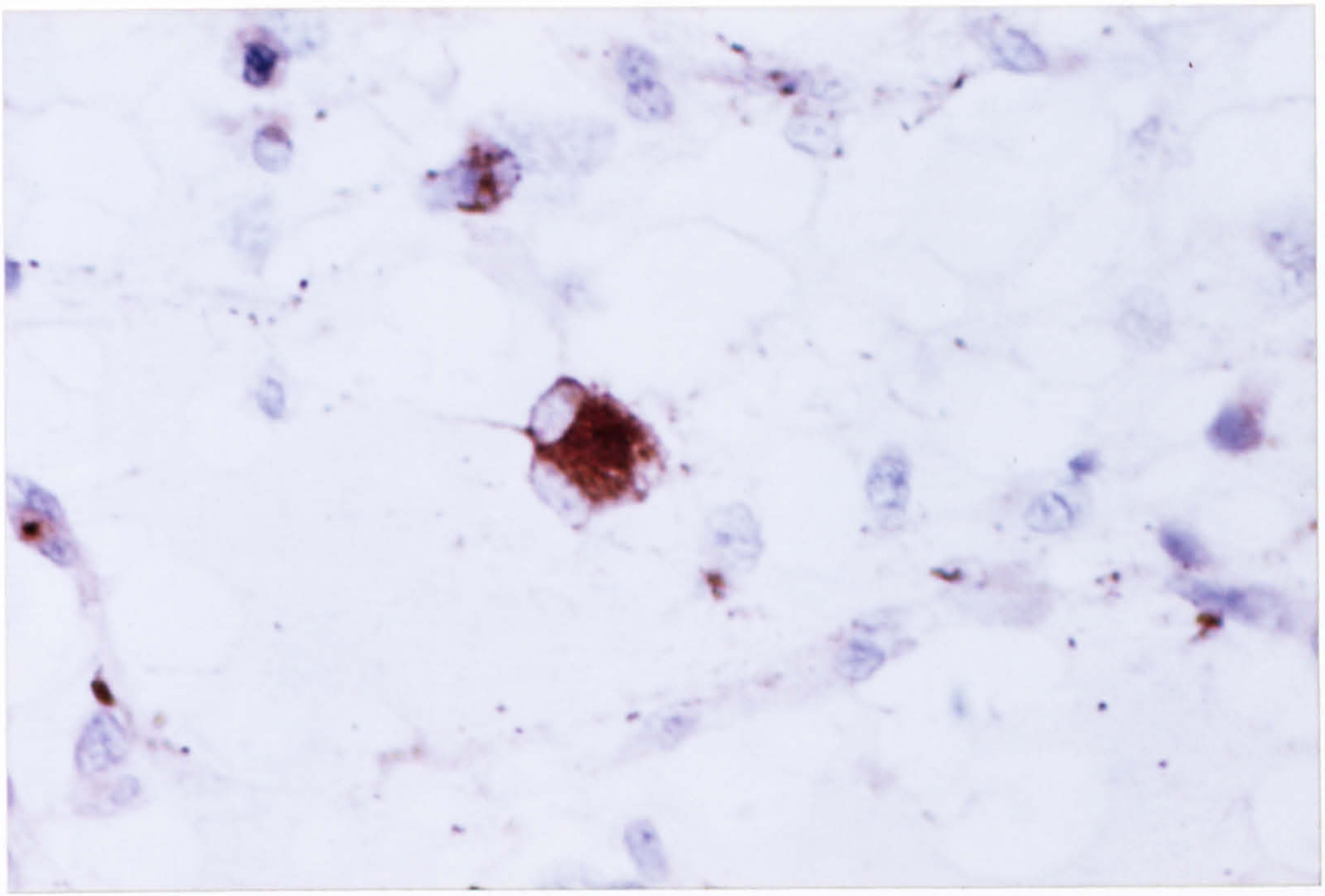
Some cells in this glioblastoma labelled with a neurofilament protein antibody. The astrocytic morphology of the cells is apparent, though the cellularity in this region is clearly low.

Neurofilament protein antibody (70kD & 200 kD); haematoxylin counterstain; x 400.

Figure 312-2d

Scattered cells in this glioblastoma are labelled with a neurofilament protein antibody.

Neurofilament protein antibody (70kD & 200 kD); haematoxylin counterstain; x 200.



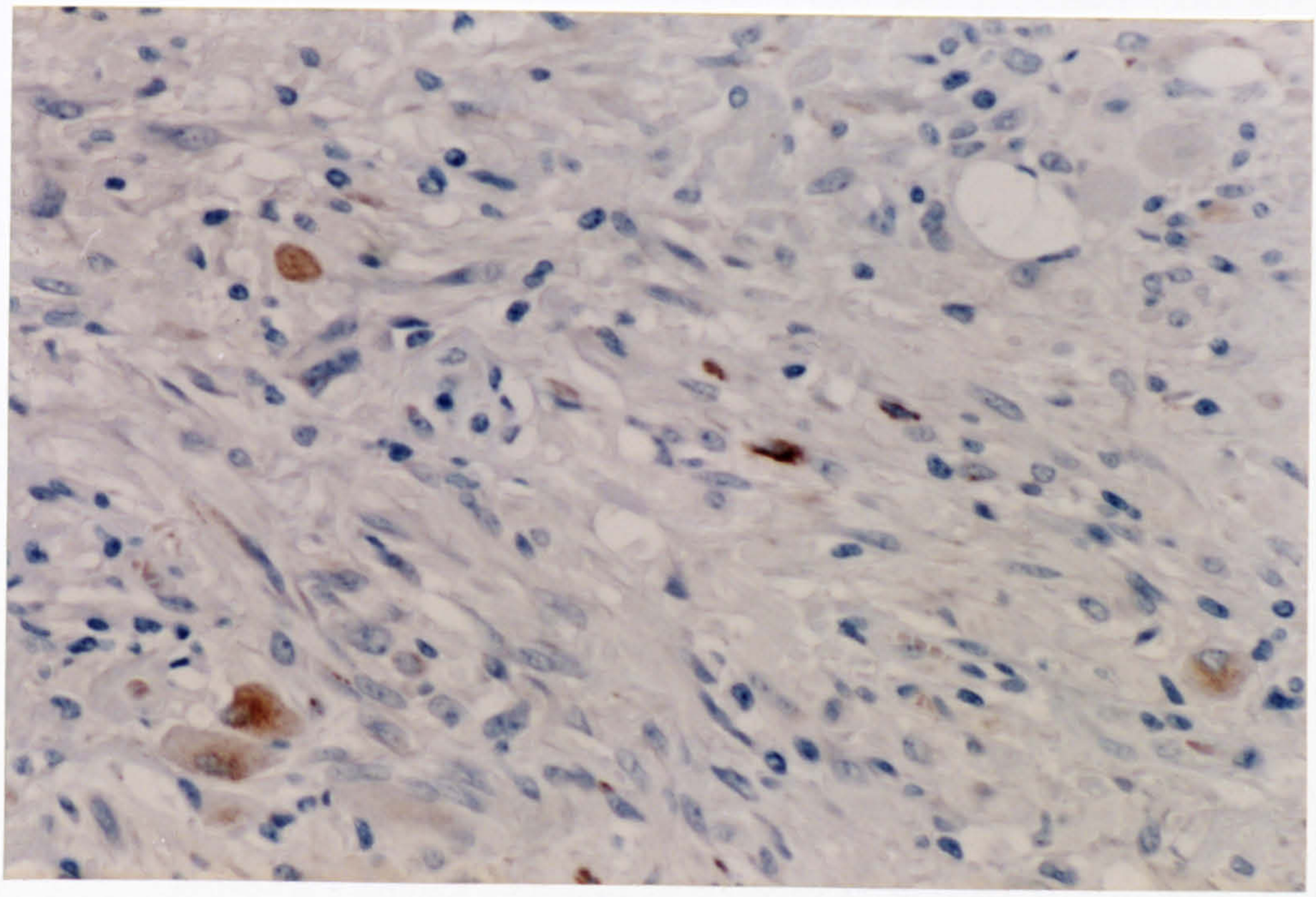


Figure 312-2e

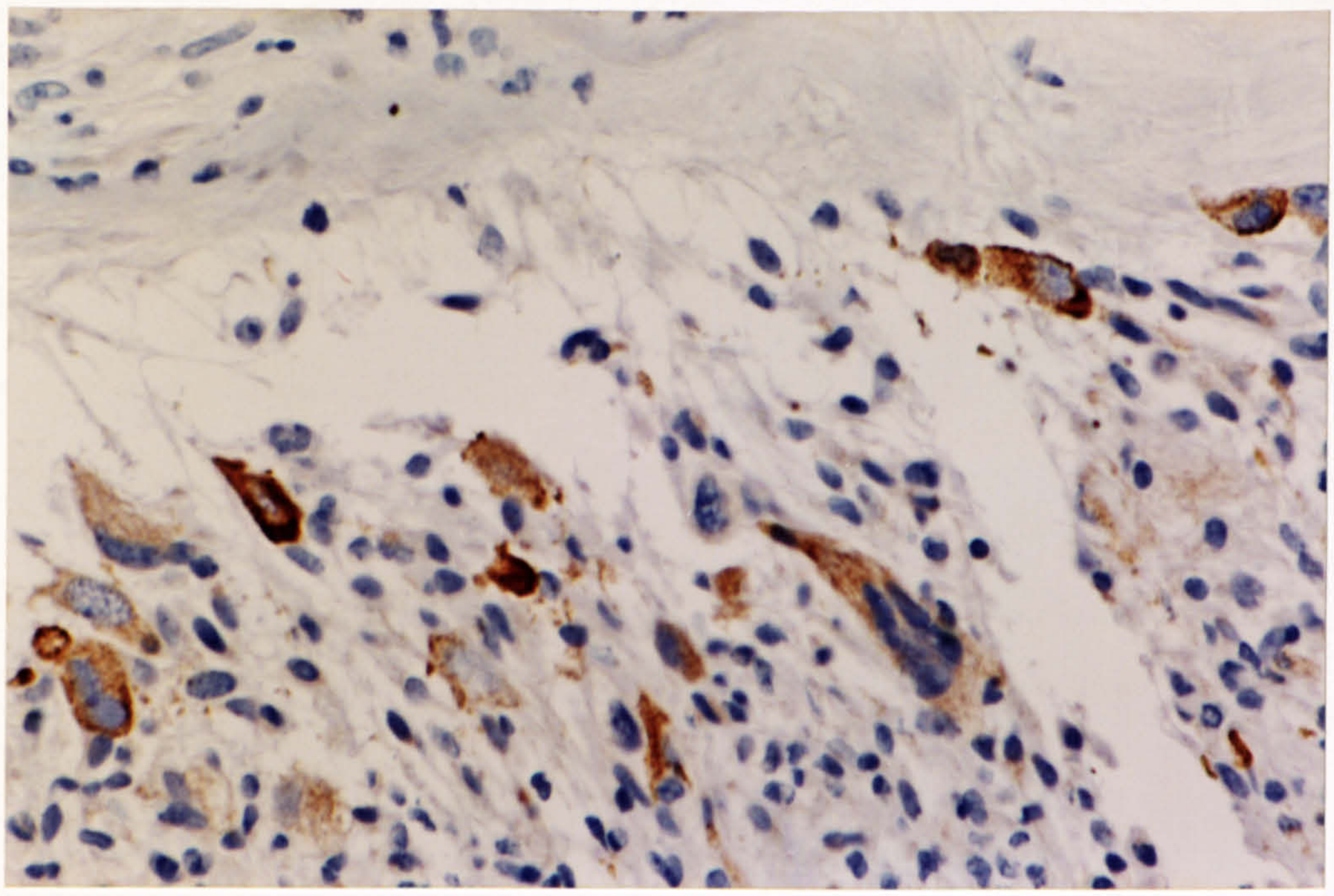
The processes of many spindle shaped cells in this glioblastoma have labelled with an antibody to neurofilament protein.

Neurofilament antibody (200 kD); haematoxylin counterstain; x 100.

Figure 312-2f

Large cells in this glioblastoma label with an antibody to chromogranin-A.

Chromogranin-A antibody; haematoxylin counterstain; x 200.



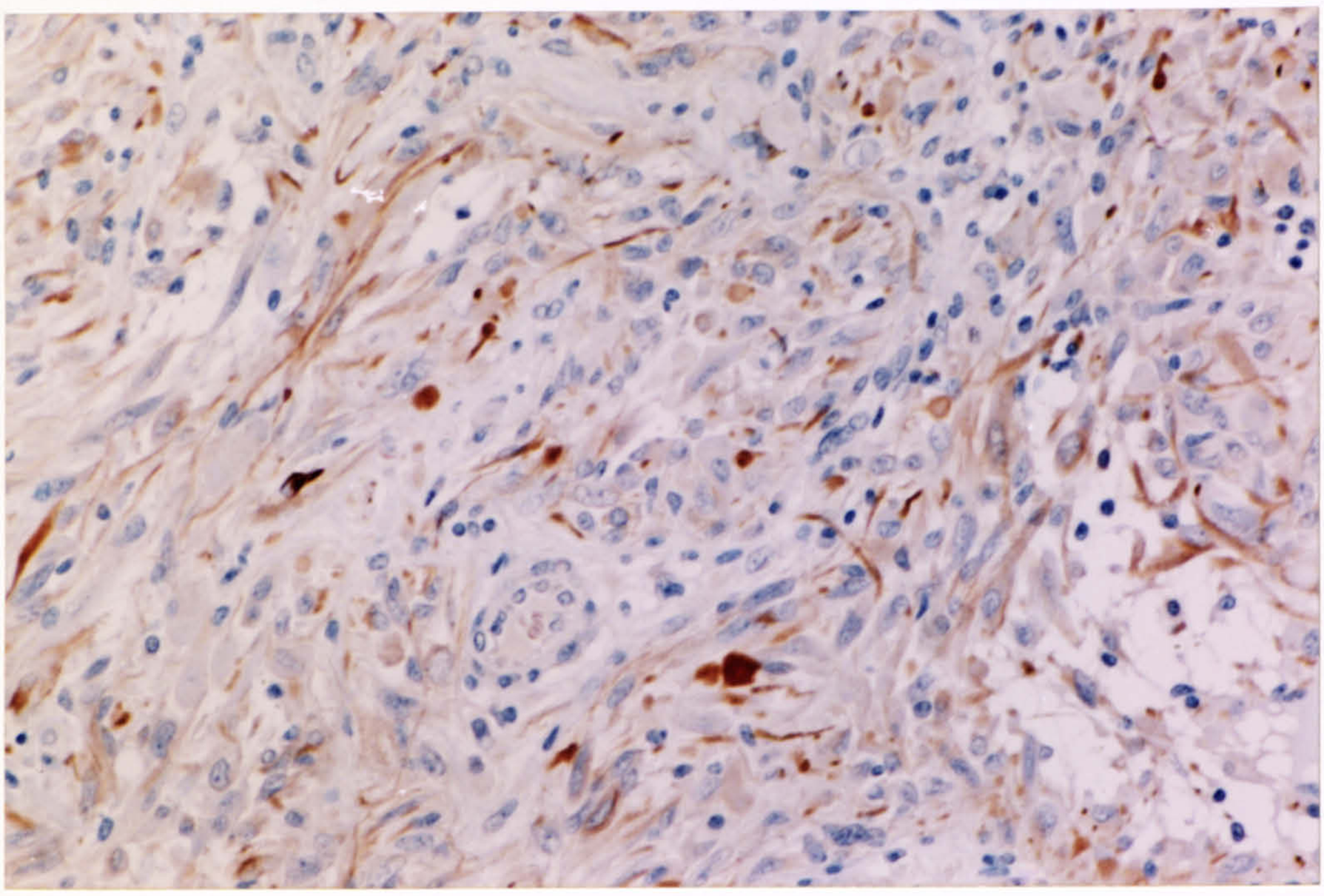


Table 312-3

Clinicopathological characteristics of high-grade astrocytic tumours with a neuronal or standard immunophenotype

	neuronal (n = 14)	standard (n = 99)	P value
age (years)*	49 ± 2.5	48 ± 1.1	0.97
gender (M : F)†	8 : 6	63 : 36	0.26
Ki-67 LI§	5.0 (4.8)	4.8 (7.6)	0.44
survival (days)§	252 (283)	326 (303)	0.40
frontal#	9 (64)	41	
temporal#	1 (7)	25	0.09
parieto-occipital#	4 (29)	33	
p53 negative#	3 (21)	35	
p53 LI < 40%#	5 (36)	40	0.37
p53 LI > 60%#	6 (43)	24	

The group of standard astrocytic tumours excludes 8 cases (see section 2.1.3)

* mean ± s.e.m.
† male : female
§ median (interquartile range)
# number (percentage)

P values refer to parametric and non-parametric comparisons between neuronal and standard groups

Table 313-1
Profile of immunolabelling with GFAP and neuronal antibodies in 25 oligodendrogliomas

GFAP	synaptophysin	NFP	N52	chromogranin-A	total
++	-	-	-	-	4
++	++	-	-	-	3
+	-	-	-	-	12
+	++	-	-	-	6

- no labelling
+ labelled cells in a minority
++ labelled cells in a majority

Figure 313a

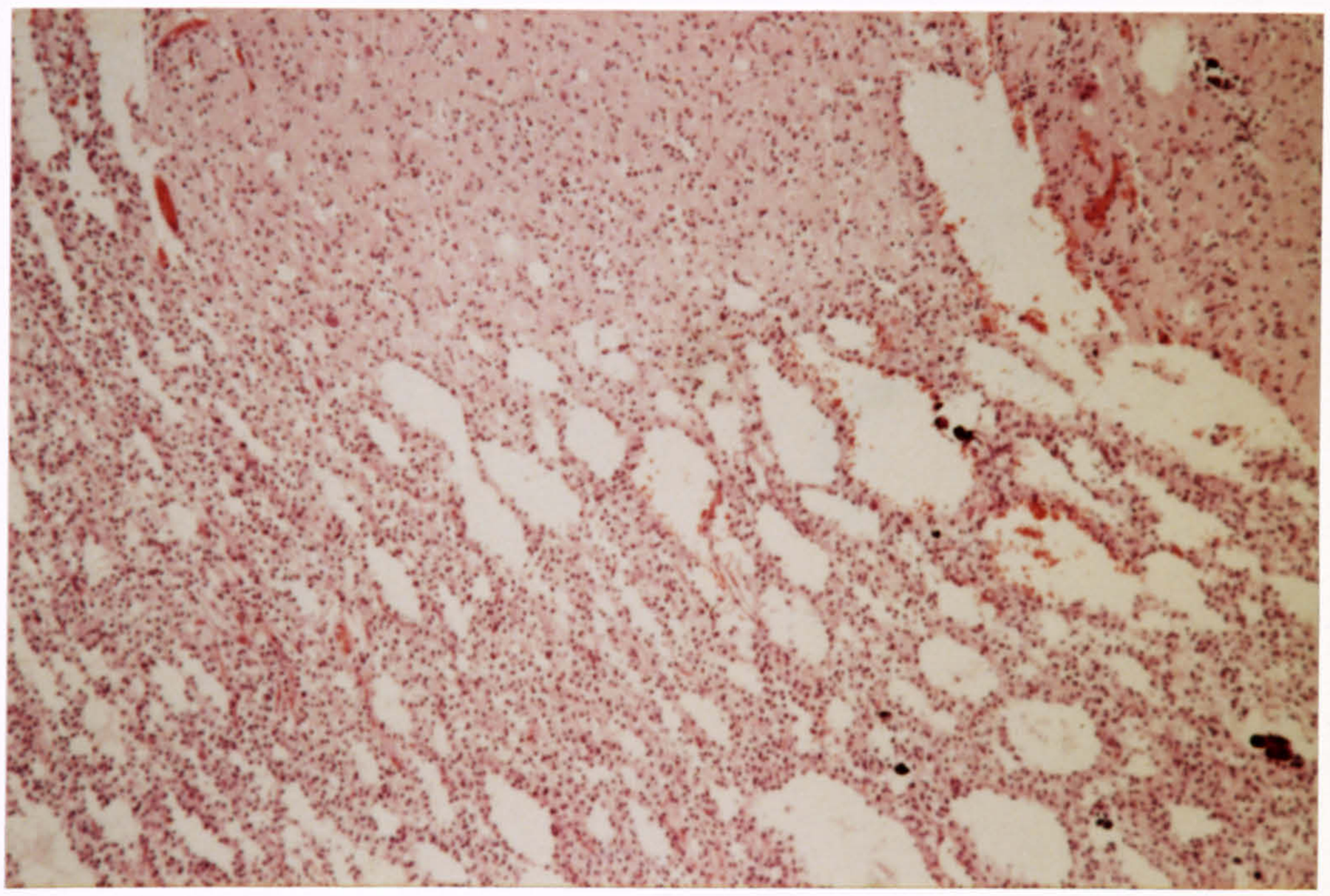
An oligodendroglioma composed of round cells diffusely infiltrates the cerebral cortex. Microcysts and dystrophic calcification are evident.

Haematoxylin and eosin; x 50.

Figure 313b

Cells in this anaplastic oligodendroglioma show the artefactual “fried egg” appearance that can be a distinctive feature of oligodendrogliomas.
Foci of anaplasia were present in this tumour; mitotic figures, capillary endothelial proliferation and foci of necrosis were found.

Haematoxylin and eosin; x 200.



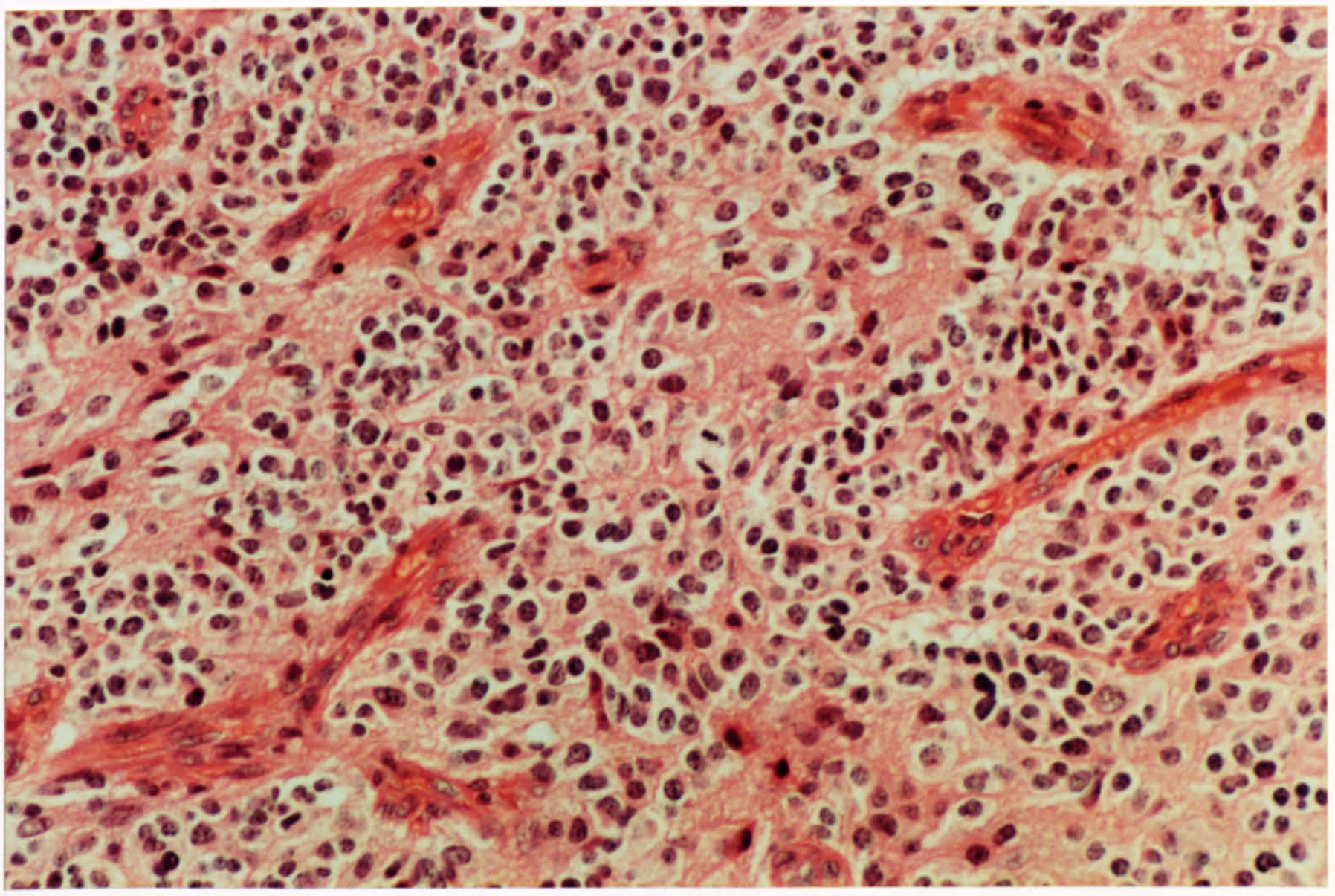


Figure 313c

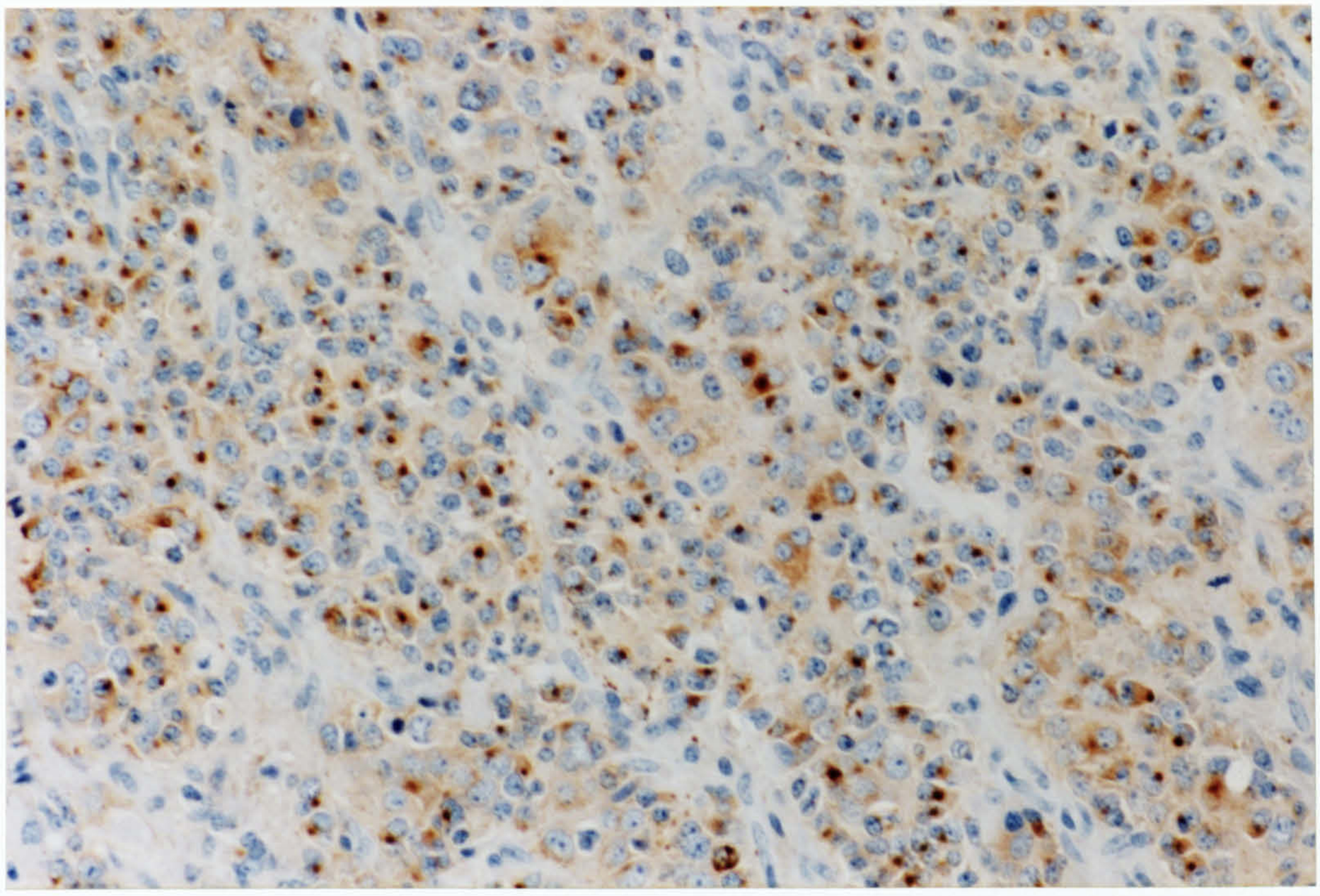
A distinctive, punctate, perinuclear labelling with the synaptophysin antibodies was observed in several oligodendrogliomas.

Synaptophysin antibody; haematoxylin counterstain; x 100.

Figure 313d

Many synaptophysin-positive oligodendrogliomas also labelled with the GFAP antibody.

GFAP antibody; haematoxylin counterstain; x 200.



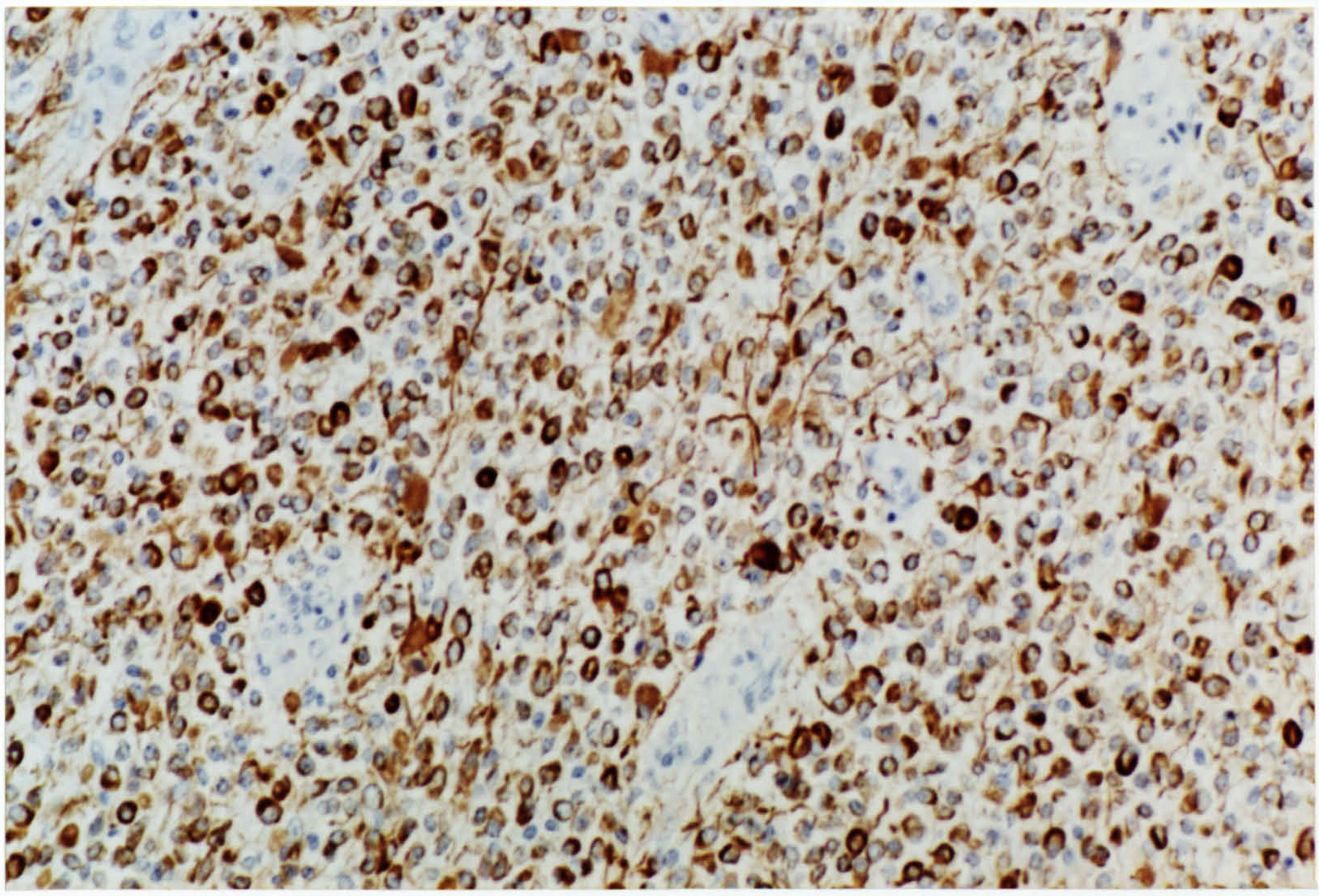
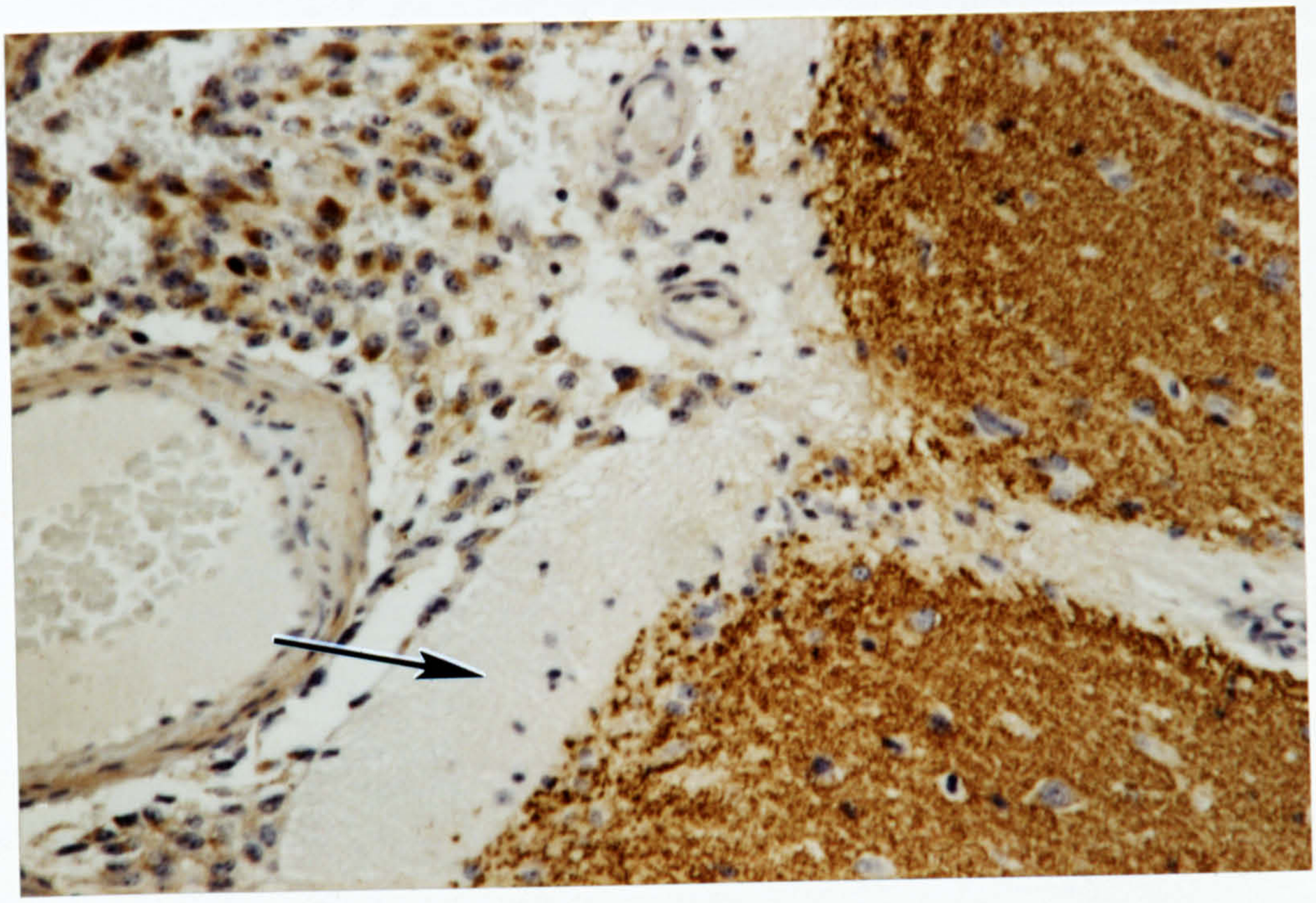


Figure 313e

Synaptophysin labelling of cortical grey matter and an oligodendroglioma that has infiltrated the subarachnoid space. The tumour in the subarachnoid space is separated from the labelled neuropil by the unlabelled pial surface of the cortex (arrow).

Synaptophysin antibody; haematoxylin counterstain; x 200.



the pial surface of the cortex. When this phenomenon was observed in synaptophysin-positive cases, tumour cells in the subarachnoid space and in the underlying cortex labelled in an identical manner (Figure 313e).

Equal proportions (36%) of oligodendrogliomas (5 / 14) and anaplastic oligodendrogliomas (4 / 11) were synaptophysin-positive.

Oligodendrogliomas with a neuronal immunophenotype did not differ on various clinical and pathological variables from standard oligodendrogliomas (Table 3132). Almost half of the patients with oligodendrogliomas were alive at the time of data entry, and an analysis of survival was not practical with this small number of cases.

A greater proportion of anaplastic oligodendrogliomas than oligodendrogliomas was GFAP-positive (Table 313-3), but a reciprocal relationship between GFAP and synaptophysin labelling was not seen in these tumours, in contrast to the astrocytic tumours (Table 313-4).

3.1.4 Immunolabelling of astrocytic tumours and oligodendrogliomas with the Ki-67 antibody

Distinct labelling of nuclei in tumour cells was observed with the Ki-67 antibody in all astrocytic tumours (Figure 314). While only small numbers of Ki-67 positive cells were found in entire sections from some fibrillary astrocytomas, one glioblastoma had a Ki-67 LI of 41.5%. The percentage of tumours with Ki-67 LI's above and below 2% is given in Table 314-1 for the 3 types of astrocytic tumour. Nearly all (96%) of the fibrillary astrocytomas had Ki-67 LI's below 2%, whereas 83% of glioblastomas had Ki-67 LI's above 2%. Median Ki-67 LI was significantly different ($P < 0.0001$) between the 3 types of astrocytic tumour (Table 314-2).

A significant difference ($P = 0.002$) was evident between the median Ki-67 LI's for oligodendrogliomas (1.4) and anaplastic oligodendrogliomas (6.8).

Table 313-2

Clinicopathological characteristics of oligodendrogliomas with a neuronal or standard immunophenotype

	neuronal (n = 9)	standard (n = 16)	P value
age (years)*	44 ± 3.3	48 ± 2.6	0.25
gender (M : F)†	4 : 5	9 : 7	0.55
Ki-67 LI§	2.9 (9.6)	3.7 (5.8)	0.86
survival (days)	1686	292	
frontal#	9 (100)	10 (62)	
temporal#	0 (0)	3 (19)	0.09
parieto-occipital#	0 (0)	3 (19)	

* mean ± s.e.m.
† male : female
§ median (interquartile range)
# number (percentage)

P values refer to parametric and non-parametric comparisons between neuronal and standard groups

Table 313-3
Percentage of oligodendrogliomas or anaplastic oligodendrogliomas versus 2 categories of GFAP immunolabelling

		GFAP +	GFAP ++
oligodendrogliomas	n = 14	43 %	57 %
anaplastic oligodendrogliomas	n = 11	9 %	91 %

$\chi^2 = 3.48$; P = 0.062

GFAP + labelled cells in a minority
GFAP ++ labelled cells in a majority

Table 313-4
Percentage of oligodendrogliomas with or without synaptophysin labelling versus 2 categories of GFAP immunolabelling

		GFAP +	GFAP ++
synaptophysin +	n = 9	33 %	67 %
synaptophysin -	n = 16	25 %	75 %

$\chi^2 = 0.20$; P = 0.66

GFAP + labelled cells in a minority
GFAP ++ labelled cells in a majority

Figure 314a

A single Ki-67 labelled nucleus is evident in this fibrillary astrocytoma.

Ki-67 antibody; haematoxylin counterstain; x 200.

Figure 314b

A very high Ki-67 labelling index is apparent in this glioblastoma, where the nuclei of many giant cells are positive.

Ki-67 antibody; haematoxylin counterstain; x 100.

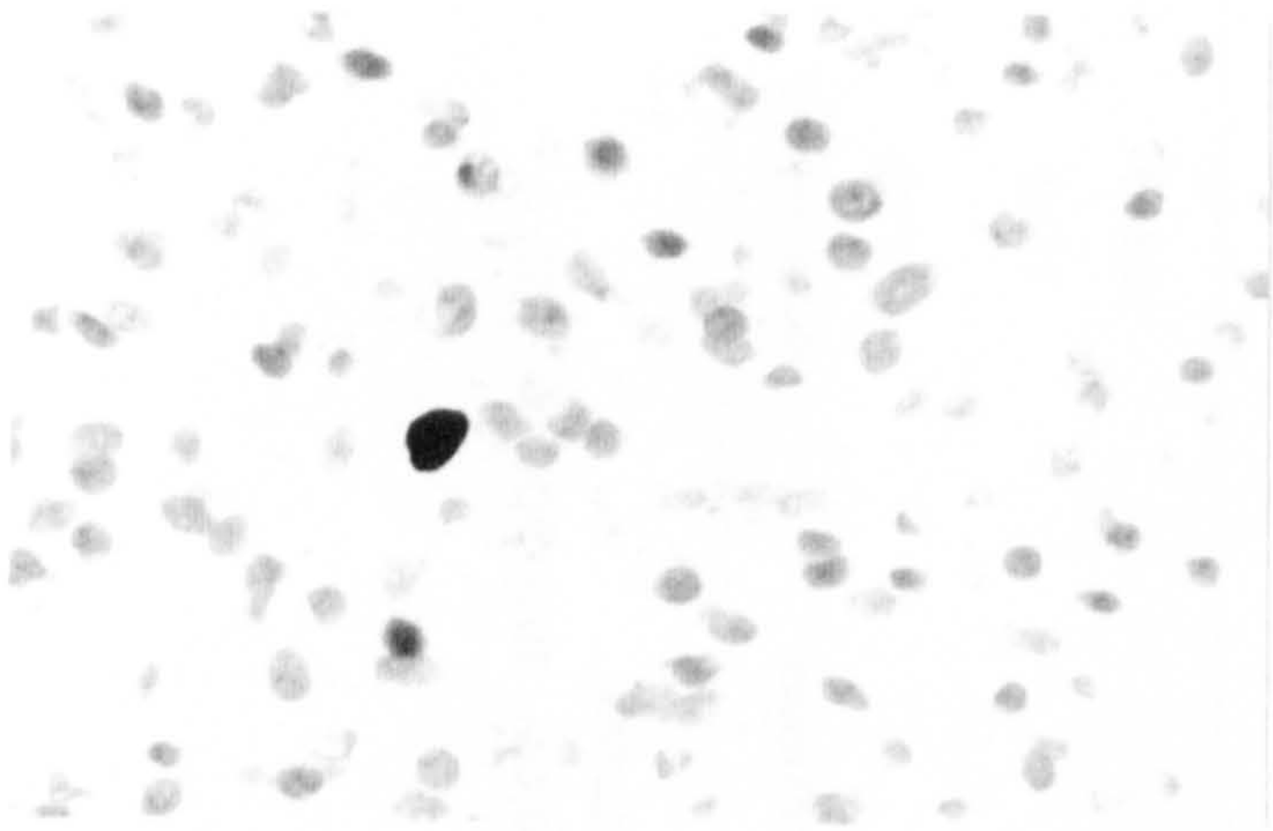


Table 314-1

Percentage of tumours with a Ki-67 LI below and above 2%
in 3 types of astrocytic tumour

	astrocytoma n = 24	anaplastic astrocytoma n = 31	glioblastoma n = 68
Ki-67 LI < 2 %	96 %	45 %	17 %
Ki-67 LI > 2 %	4 %	55 %	83 %

Table 314-2
Ki-67 LI by histology of astrocytic tumour

		total	median (IQR)	P value
all cases		123	3.39 (6.88)	
astrocytoma		24	0.38 (0.92)	
anaplastic	astrocytoma	31	2.48 (3.87)	0.0000*
	glioblastoma	68	5.71 (6.63)	

Ki-67 LI was not normally distributed. Median and interquartile range were used in a nonparametric analysis (* Kruskal-Wallis) of the above groups.

3.1.5 Immunolabelling of astrocytic tumours with an antibody to p53

Labelling of nuclei with the DO-7 antibody to p53 was found in tumours from all 3 histological types of astrocytic tumours (Figure 315-1). The intensity of the immunolabelling and the distribution of positively labelled cells within a section of tumour could be variable; however, the majority of p53-positive tumours contained an even distribution of labelled cells from which a labelling index could readily be calculated. Intensely labelled cells were found in tumours with LI's < 10% as well as in tumours with LI's > 90%. The range of p53 LI's was large: 0% - 98%, and the frequency distribution of LI's was U-shaped (Figure 315-2). Thus, many p53-positive tumours were located at each end of the p53 LI range, but none had a value between 40% and 60%. Therefore, all p53-positive tumours could be divided into 2 categories: p53 LI < 40% and p53 LI > 60%. The percentage of tumours in 3 categories of p53 immunolabelling is displayed in Table 315-1 for the 3 types of astrocytic tumour. A smaller proportion of fibrillary astrocytomas (8%) than anaplastic astrocytomas (32%) and glioblastomas (21%) was found in the p53 LI > 60% group.

The median Ki-67 LI's of 3 categories of p53 immunolabelling were compared (Table 3152), and the group of astrocytic tumours with a p53 LI > 60% was found to have a significantly ($P < 0.04$) greater Ki-67 LI. A combination of the relationship between Ki-67 LI and type of astrocytic tumour, and the different proportions of each type of astrocytic tumour in the p53 categories might confound this analysis. However, when glioblastomas were analysed alone, the median Ki-67 LI of the p53 LI > 60% group, 9.28, was also significantly ($P = 0.03$) greater than the median Ki-67 LI's for the p53 LI < 40% group, 6.05, and the p53-negative group, 4.4.

3.1.6 Immunolabelling of astrocytic tumours with an antibody to *bcl* -2

A rim of dark brown, cytoplasmic staining in perivascular lymphocytes served as a positive control for *bcl* -2 immunolabelling in sections from all tumours (Figure 316). The assessment of *bcl* -2 labelling in neoplastic astrocytes was slightly complicated by clear labelling of reactive astrocytes in adjacent cerebral tissue, and within some

Figure 315-1a

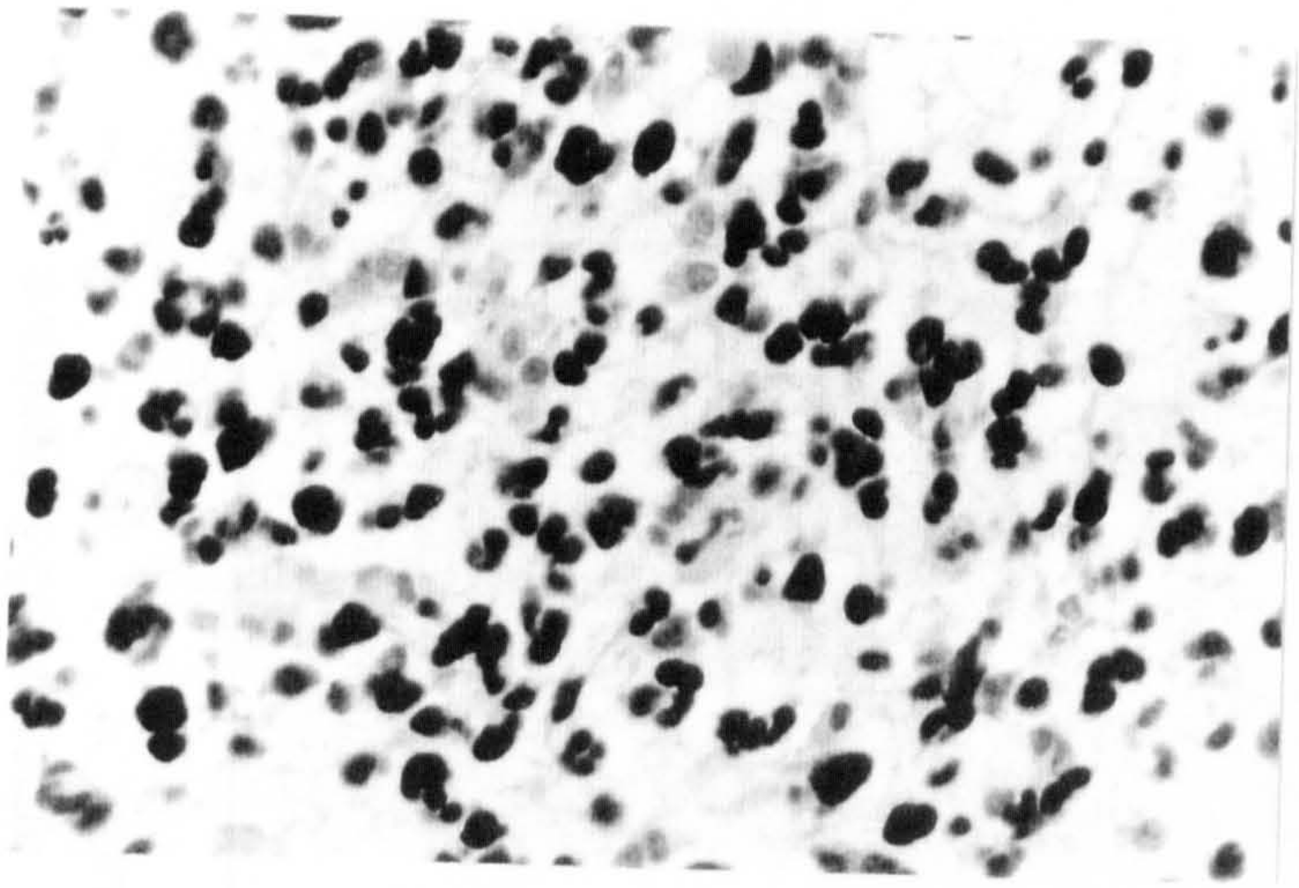
A high proportion of cells in this glioblastoma is labelled with the DO-7 antibody to p53 protein.

DO-7 antibody; haematoxylin counterstain; x 200.

Figure 315-1b

The p53 LI in this glioblastoma approaches 100%. Giant cells and small cells are both labelled.

DO-7 antibody; haematoxylin counterstain; x 100.



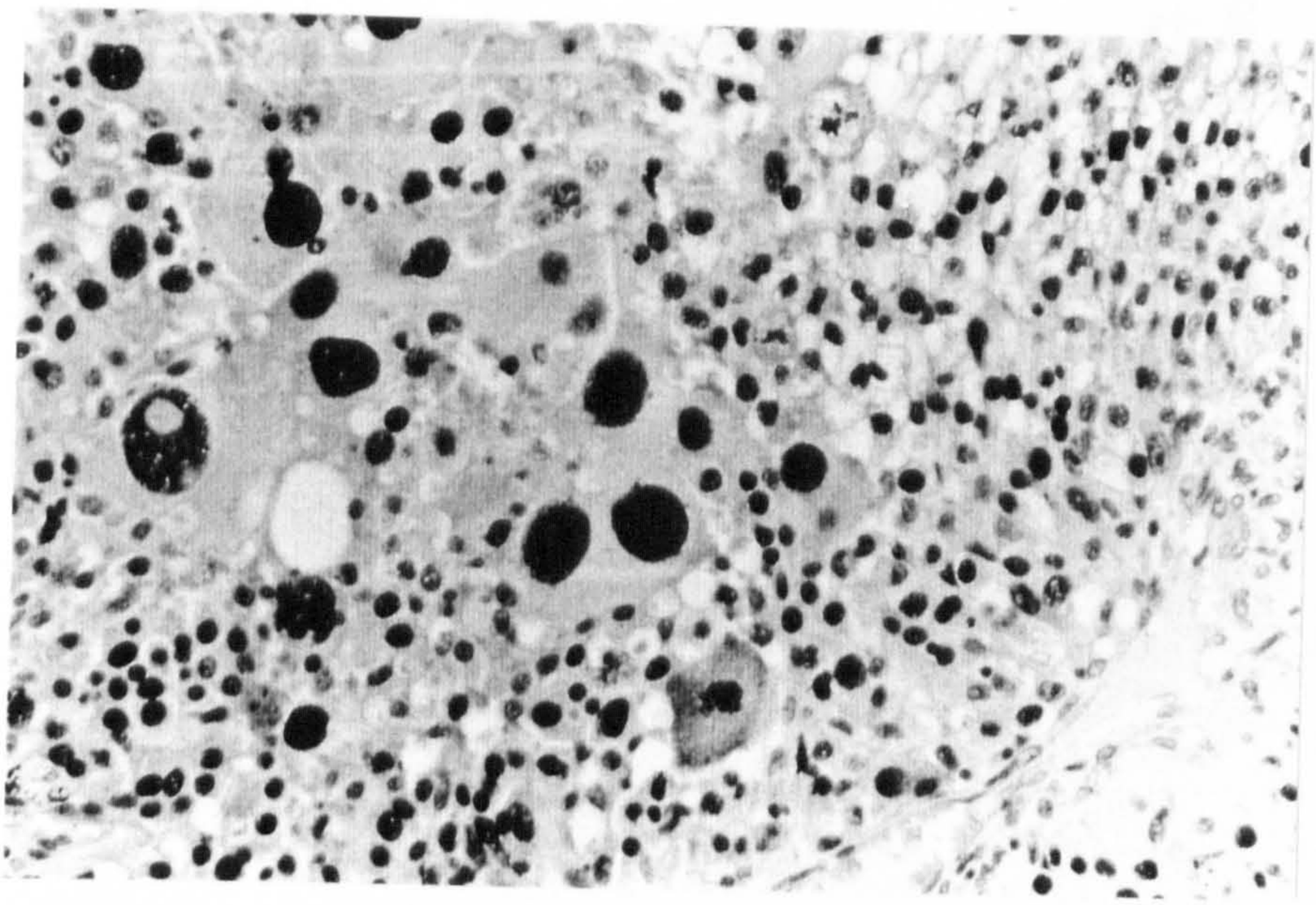


Figure 315-2
Frequency distribution of p53 LI's among p53-positive astrocytic tumours

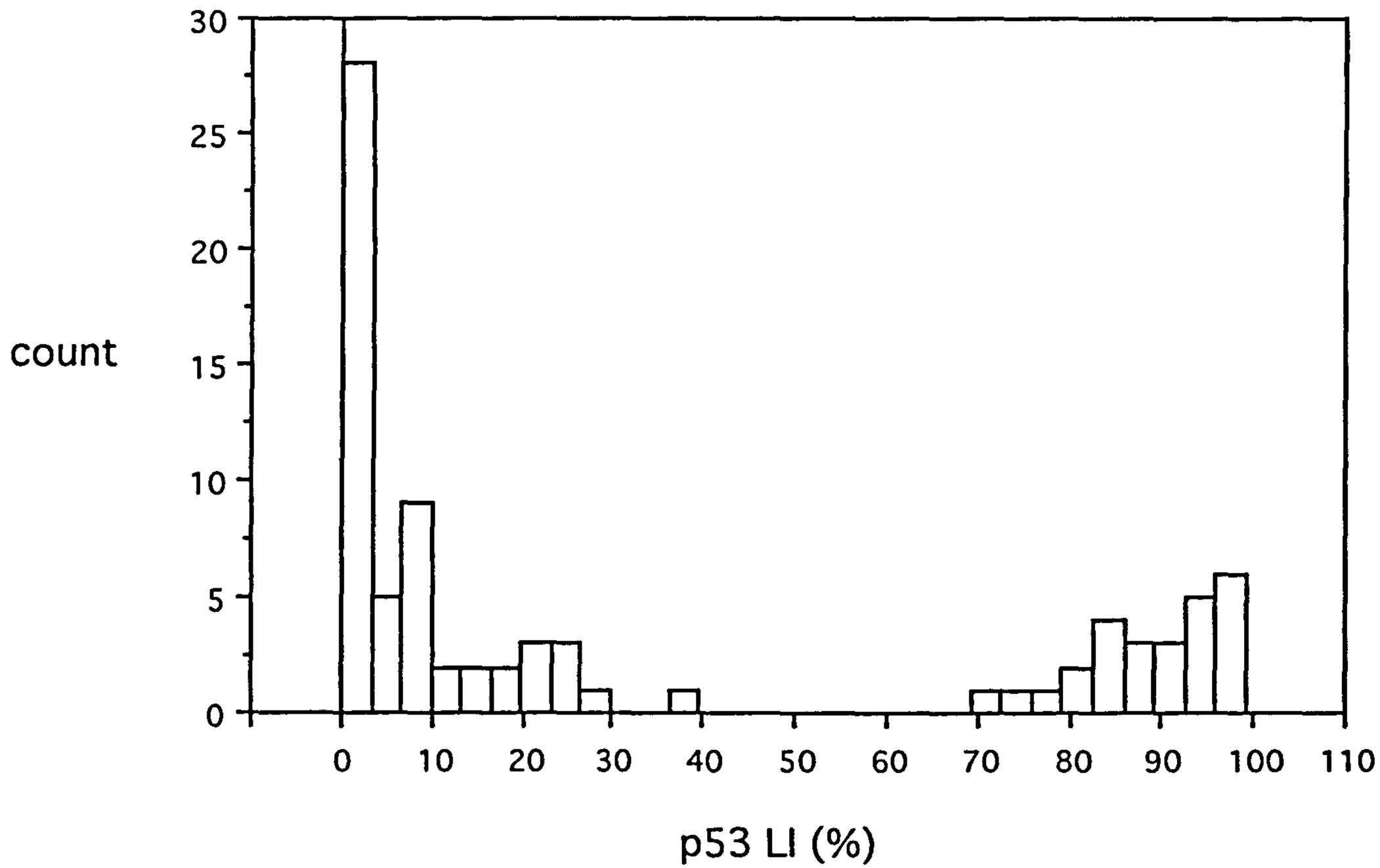


Table 315-1
Percentage of tumours in 3 categories of p53 immunolabelling in 3 types of astrocytic tumour

	astrocytoma n = 24	anaplastic astrocytoma n = 31	glioblastoma n = 68
p53 negative	34 %	26 %	39 %
p53 LI < 40%	58 %	42 %	40 %
p53 LI > 60%	8 %	32 %	21 %

Table 315-2
Ki-67 LI by category of p53 immunolabelling

	total	median (IQR)	P value
all cases	123	3.39 (6.88)	
p53 negative	43	3.30 (4.46)	
p53 LI < 40%	54	3.05 (5.04)	0.0377*
p53 LI > 60%	26	7.05 (11.6)	

Ki-67 LI was not normally distributed. Median and interquartile range were used in a nonparametric analysis (* Kruskal-Wallis) of the above groups.

tumours. Reactive astrocytes in many non-neoplastic conditions, including abscesses, arteriovenous malformations, demyelination, and encephalitis, show a moderate degree of labelling with the *bcl* -2 antibody (appendix 3.1.6) that is less than the level of staining in lymphocytes. Reactive astrocytes can be identified by their characteristic radial arrangement of many fine processes, and could nearly always be distinguished from neoplastic astrocytes that were clearly not labelled with the *bcl* -2 antibody (Figure 316c).

Some tumours were ubiquitously labelled by *bcl* -2 (Figure 316b); but, in others, focal *bcl* -2 labelling was evident. Several of the *bcl* -2 positive tumours contained large groups of "gemistocytic" cells which labelled strongly around their cytoplasmic borders (Figure 316c). The proportions of each type of astrocytic tumour that either did not label with the *bcl* -2 antibody or labelled in a focal or ubiquitous manner are displayed in Table 316-1. Convincing focal *bcl* -2 labelling was evident in 2 fibrillary astrocytomas, 3 anaplastic astrocytomas, and 5 glioblastomas. In 3 of the glioblastomas, the focal labelling took a distinctive form, being confined to multinucleated, giant cells (Figure 316c).

A striking feature of some glioblastomas and a few anaplastic astrocytomas was *bcl* -2 labelling of the glomeruloid capillary endothelial proliferation that characterises these tumours (Figure 316c). The vessels in adjacent normal brain were not labelled, but the endothelium of some vessels in the cortex invaded diffusely by tumour showed up-regulation of*bcl* -2.

Of the 6 tumours biopsied on 2 occasions, 4 were classified as fibrillary astrocytomas on the first biopsy and as anaplastic astrocytomas on the second biopsy; no labelling with the *bcl* -2 antibody was evident in 3 of these 4 tumours. The one labelled tumour contained a few foci of *bcl* -2 positive cells as a fibrillary astrocytoma and ubiquitous *bcl* -2 labelling when it recurred as an anaplastic astrocytoma. The remaining 2 tumours were classified as anaplastic astrocytomas at first biopsy, and as glioblastomas at second biopsy. One of these tumours contained *bcl* -2 positive giant cells.

Figure 316a

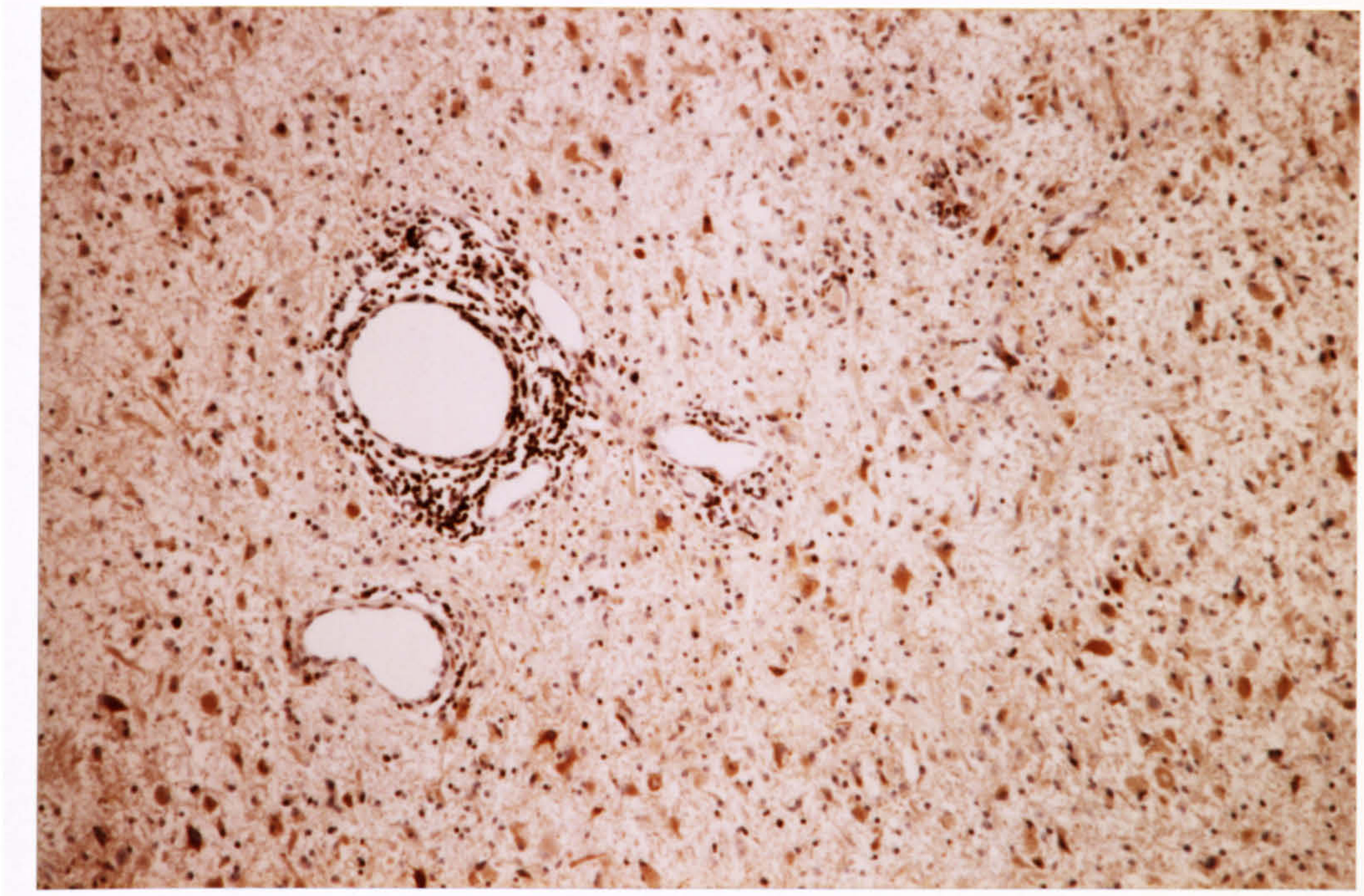
Perivascular lymphocytes are strongly labelled and neoplastic astrocytes are moderately labelled with the *bcl*-2 antibody in this fibrillary astrocytoma.

bcl-2 antibody; haematoxylin counterstain; x 50.

Figure 316b

Nearly all tumour cells in this glioblastoma are labelled with the *bcl*- 2 antibody.

bcl-2 antibody; haematoxylin counterstain; x 100.



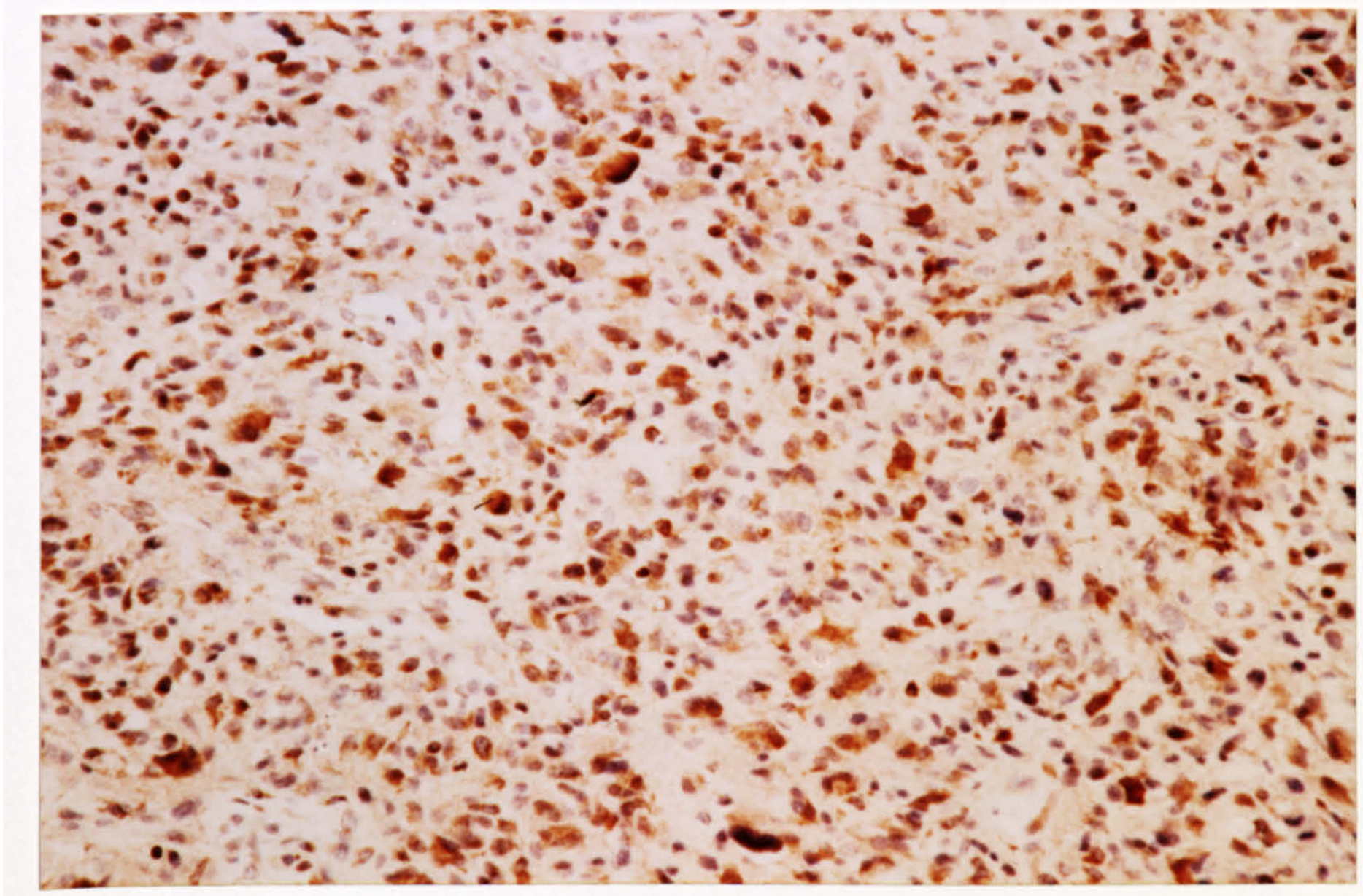


Figure 316c

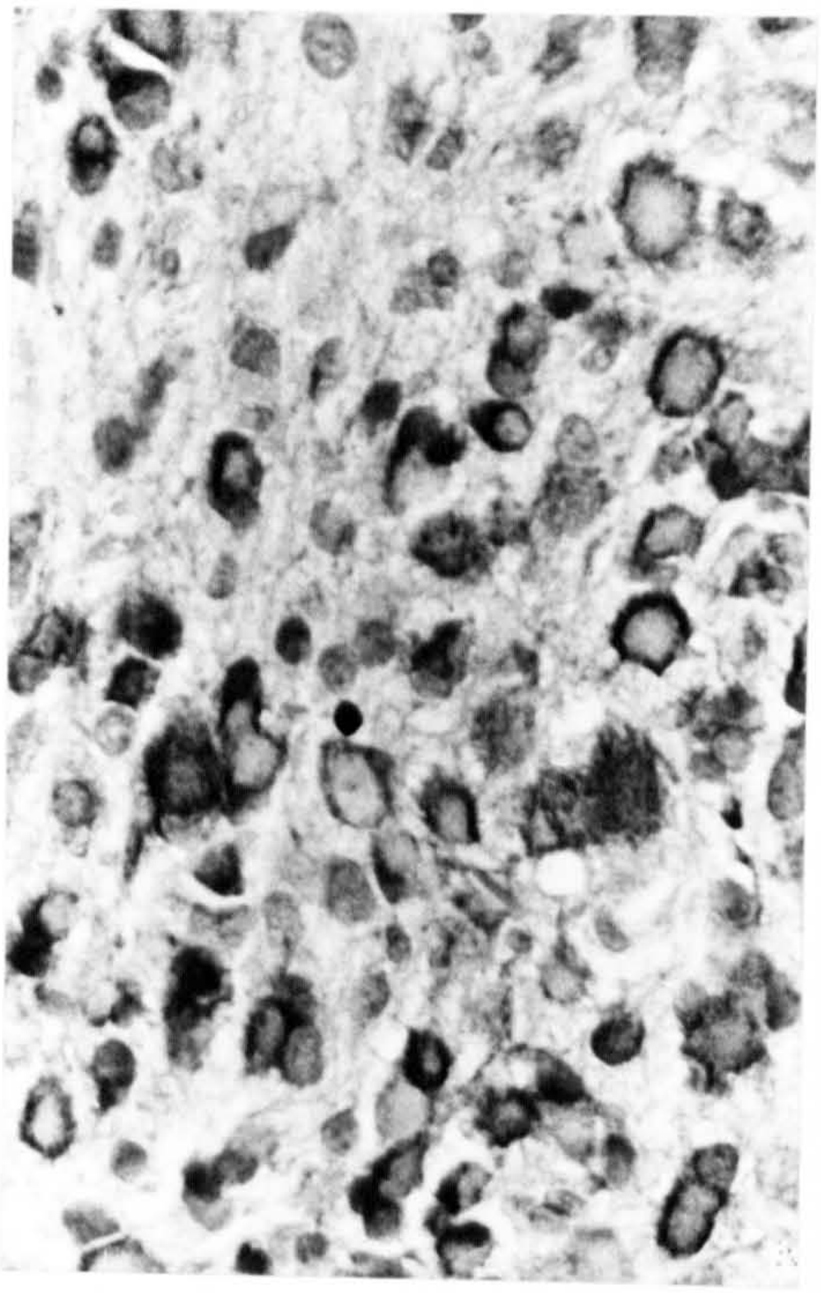
top left: several *bcl-2* positive tumours contained gemistocytes which showed strong labelling around the periphery of their cytoplasm; x 400.

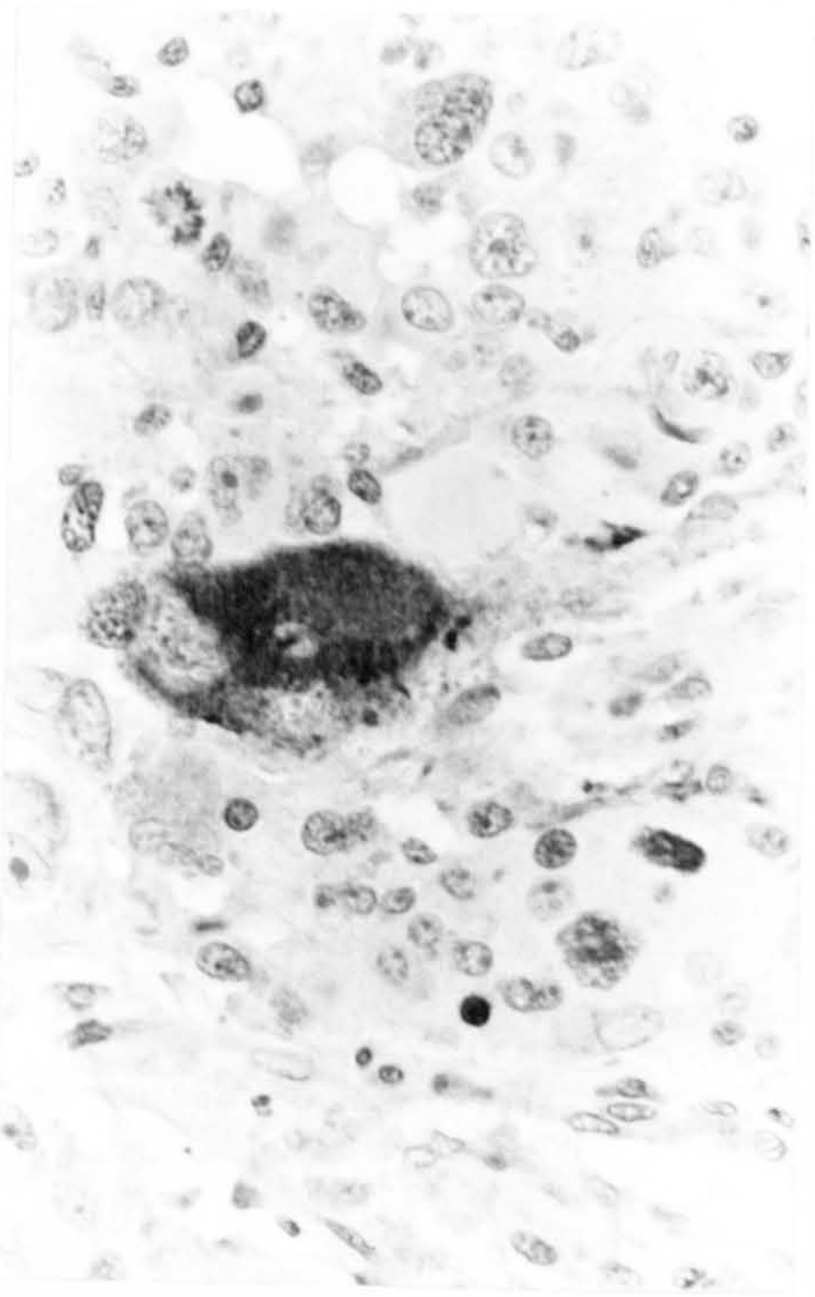
top right: *bcl-2* labelling in 3 glioblastomas was confined to multinucleated, giant cells; x 400.

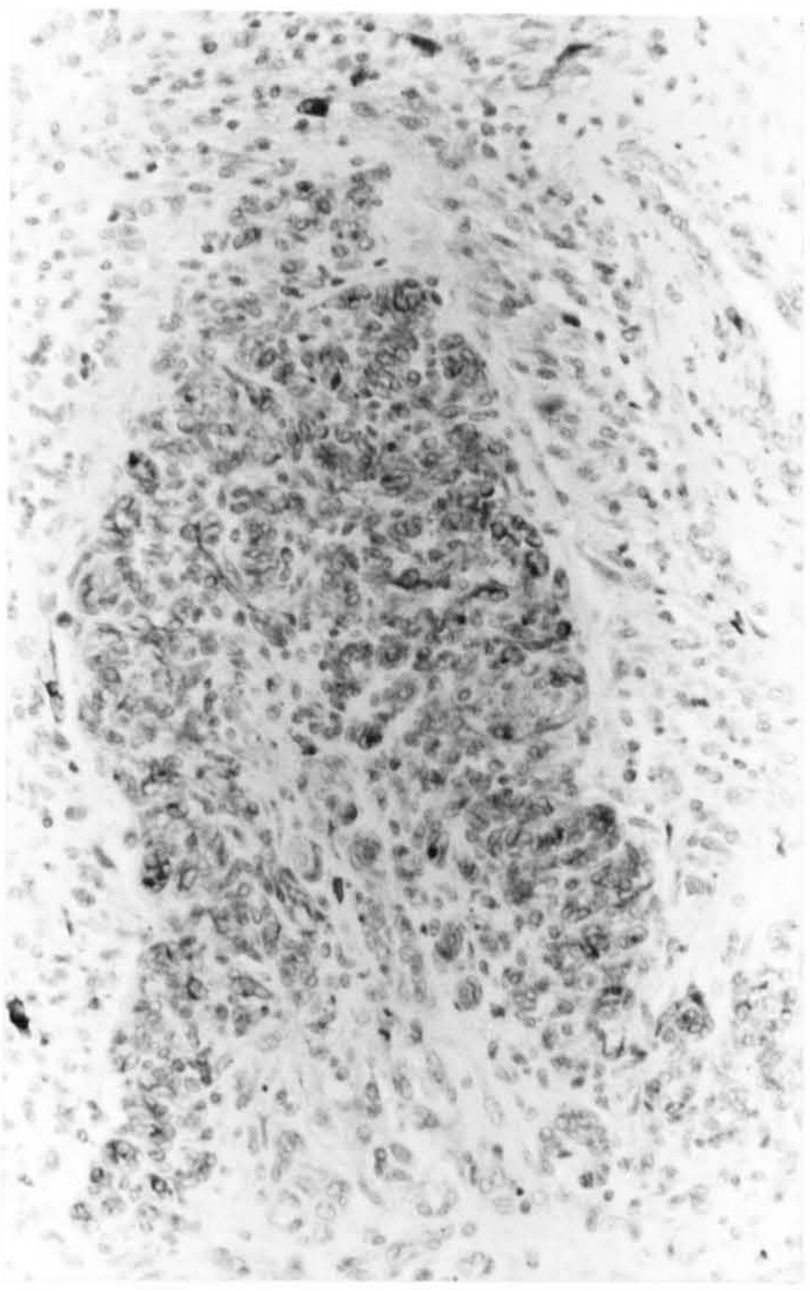
bottom left: in some astrocytic tumours, capillary endothelial proliferation was strongly *bcl-2* positive; x 100.

bottom right: lymphocytes (arrowhead) and reactive astrocytes (arrow) were labelled with the *bcl-2* antibody, and were sometimes scattered through otherwise *bcl-2* negative tumours; x 100.

bcl-2 antibody; haematoxylin counterstain.







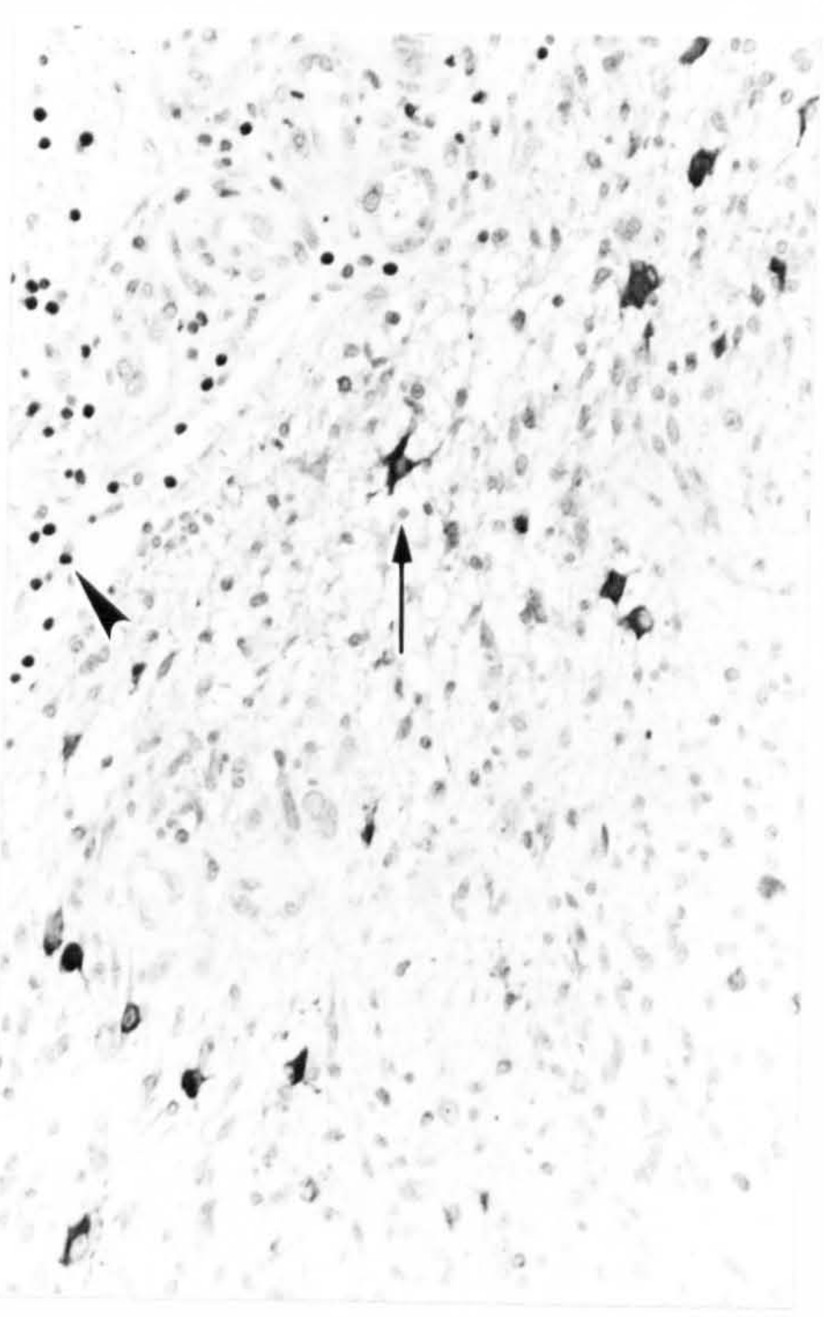


Table 316-1

bcl-2 labelling patterns by histology of astrocytic tumour

	astrocytoma n = 16	anaplastic astrocytoma n = 19	glioblastoma n = 46
bcl-2 negative	9 (56)	11 (58)	33 (72)
bcl-2 focal	2 (13)	3 (16)	5 (11)
bcl-2 ubiquitous	5 (31)	5 (26)	8 (17)

Figures are: count (percentage)

Median Ki-67 LI's for the *bcl* -2 positive (3.1) and bcl -2 negative (3.6) groups were not significantly different. Approximately equal proportions of p53-positive tumours were *bcl* -2 negative and *bcl* -2 positive; in contrast, a smaller proportion of p53-negative tumours was *bcl* -2 positive (Table 316-2).

Table 316-2
Distribution of astrocytic tumours
by *bcl*-2 and p53 status

	bcl-2 negative	bcl-2 positive
p53 negative	27	7
p53 LI < 40%	17	14
p53 LI > 60%	9	7

Figures are counts

3.2 *In situ* hybridisation (ISH)

3.2.1 ISH of normal cerebral cortex and spinal cord

Optimal fixation of tissue and pretreament of sections before hybridisation allowed a reproducible and even distribution of ISH signal in normal cerebral cortex (Figure 321). Poly-d(T) and mitochondrial probes produced a cytoplasmic signal from polyadenylated mRNA and mitochondrial rRNA in neurons, glia, and blood vessels across the grey and white matter. The signal was especially prominent in large, pyramidal neurons. Faint labelling of nuclear mRNA was occasionally seen with the poly-d(T) probe; but this was always much less than the labelling of cytoplasmic mRNA. No signal was detected in sections to which either poly-d(A) probes or no probe had been applied.

In sections of normal cerebral cortex to which a cocktail of synaptophysin probes had been applied, the best signal was also found in large, pyramidal neurons, but its intensity never matched that of the poly-d(T) and mitochondrial probes (Figure 321). Glia were not labelled with the synaptophysin probes, and this distinction produced a border between grey and white matter. No labelling of any cell was observed in sections to which a cocktail of synaptophysin sense probes had been applied.

Similar results were observed in sections of postmortem spinal cord. The mitochondrial probes strongly labelled the large, ventral horn motor neurons. Synaptophysin mRNA was clearly demonstrated in these cells and their proximal neuritic processes, and was frequently found in smaller neurons. The signal with the cocktail of synaptophysin probes was again weaker than that with the mitochondrial probes. In both cerebral cortex and spinal cord, the distribution of synaptophysin mRNA was in contrast to the distribution of synaptophysin protein which was evident mainly in the neuropil (Figure 321).

Figure 321a

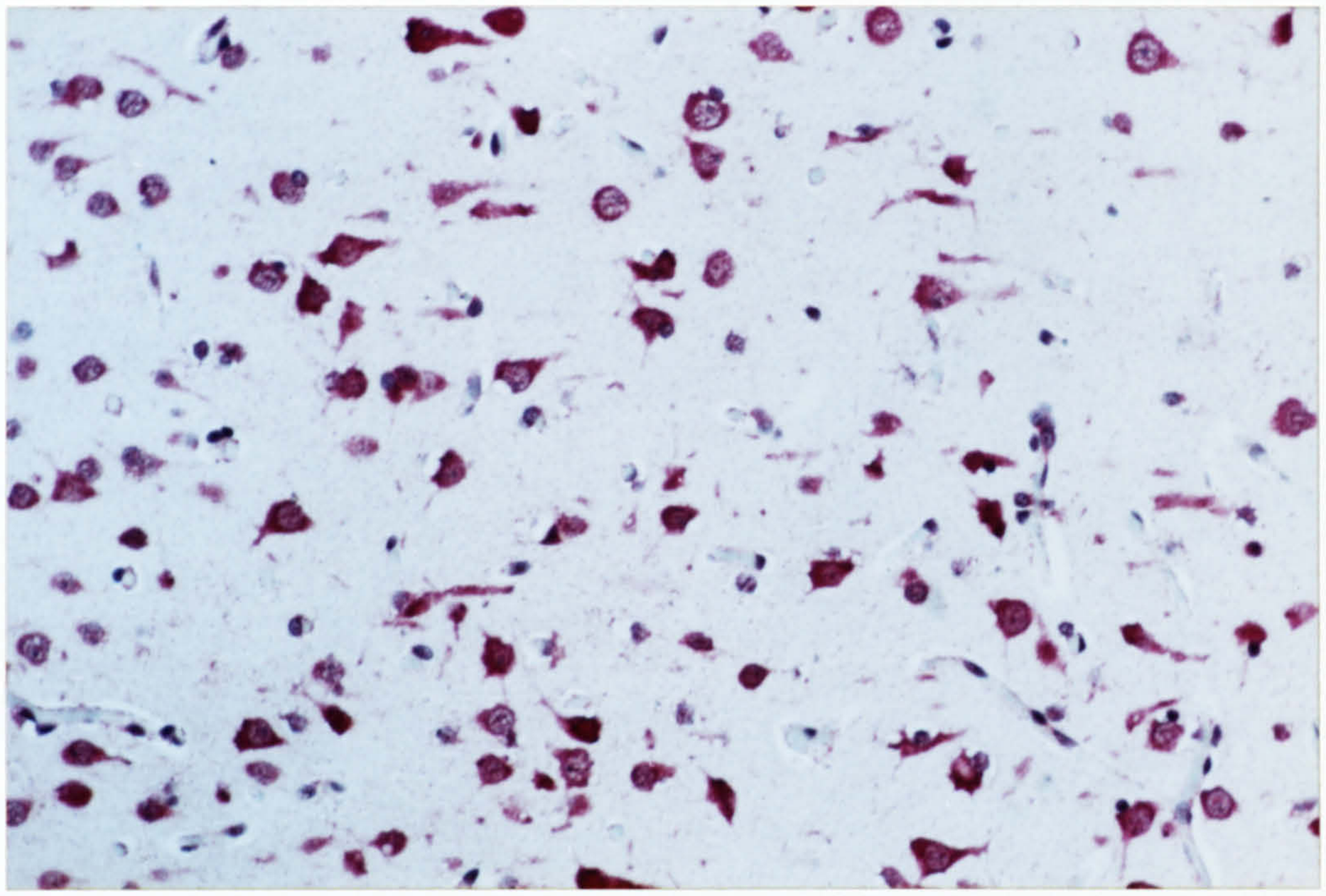
In situ hybridisation for polyadenylated mRNA has revealed strong signals from neurons and glia in the cerebral grey matter. A faint signal from the nuclei of some pyramidal neurons is evident, but much weaker than the signal from the cytoplasm of these cells.

poly-d(T) probe; fast red chromogen & haematoxylin counterstain; x 100.

Figure 321b

A sense probe control for the preparation in Figure 321a shows no significant signal.

poly-d(A) probe; fast red chromogen & haematoxylin counterstain; x 100.



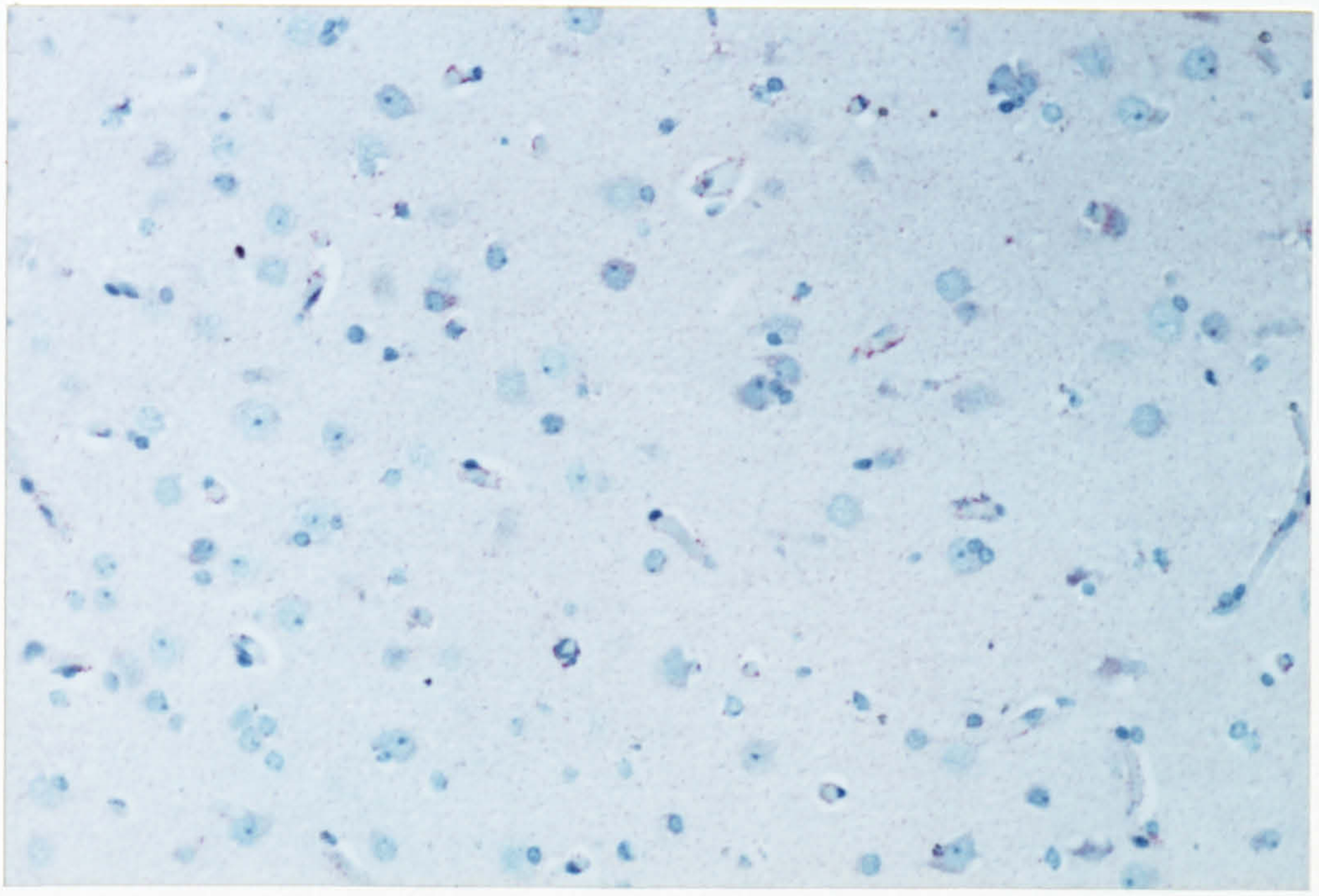


Figure 321c

A cocktail of oligonucleotide probes to mitochondrial tRNA has labelled neurons (arrowhead) and glia (arrow) in this section of the grey / white matter interface of the cerebral cortex.

Mitochondrial probes; NBT / BCIP chromogen; x 100.

Figure 321d

A cocktail of 5 oligonucleotide antisense probes to synaptophysin mRNA has labelled neurons (arrowheads), but not glia (arrows), in this section of cerebral cortex.

Synaptophysin antisense probes; NBT / BCIP chromogen; x 200.

Figure 321e

Once the correct conditions for hybridisation and the post-hybridisation washes had been established, cocktails of 5 oligonucleotide sense probes to synaptophysin mRNA never produced a signal in sections of cerebral cortex.

Synaptophysin sense probes; NBT / BCIP chromogen; x 100.

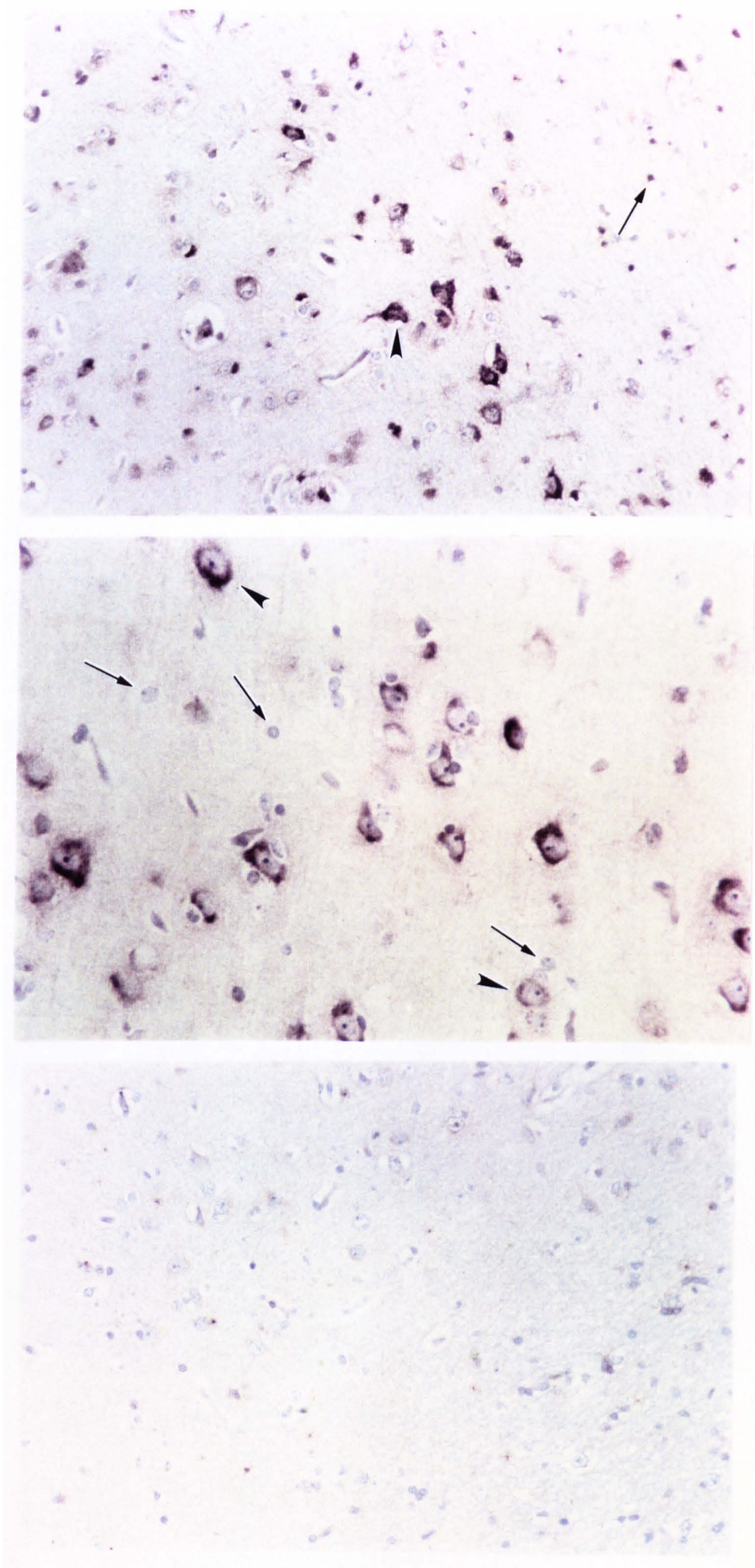


Figure 321f

In situ hybridisation for mitochondrial tRNA has revealed a strong signal in the ventral horn motor neurons of the spinal cord.

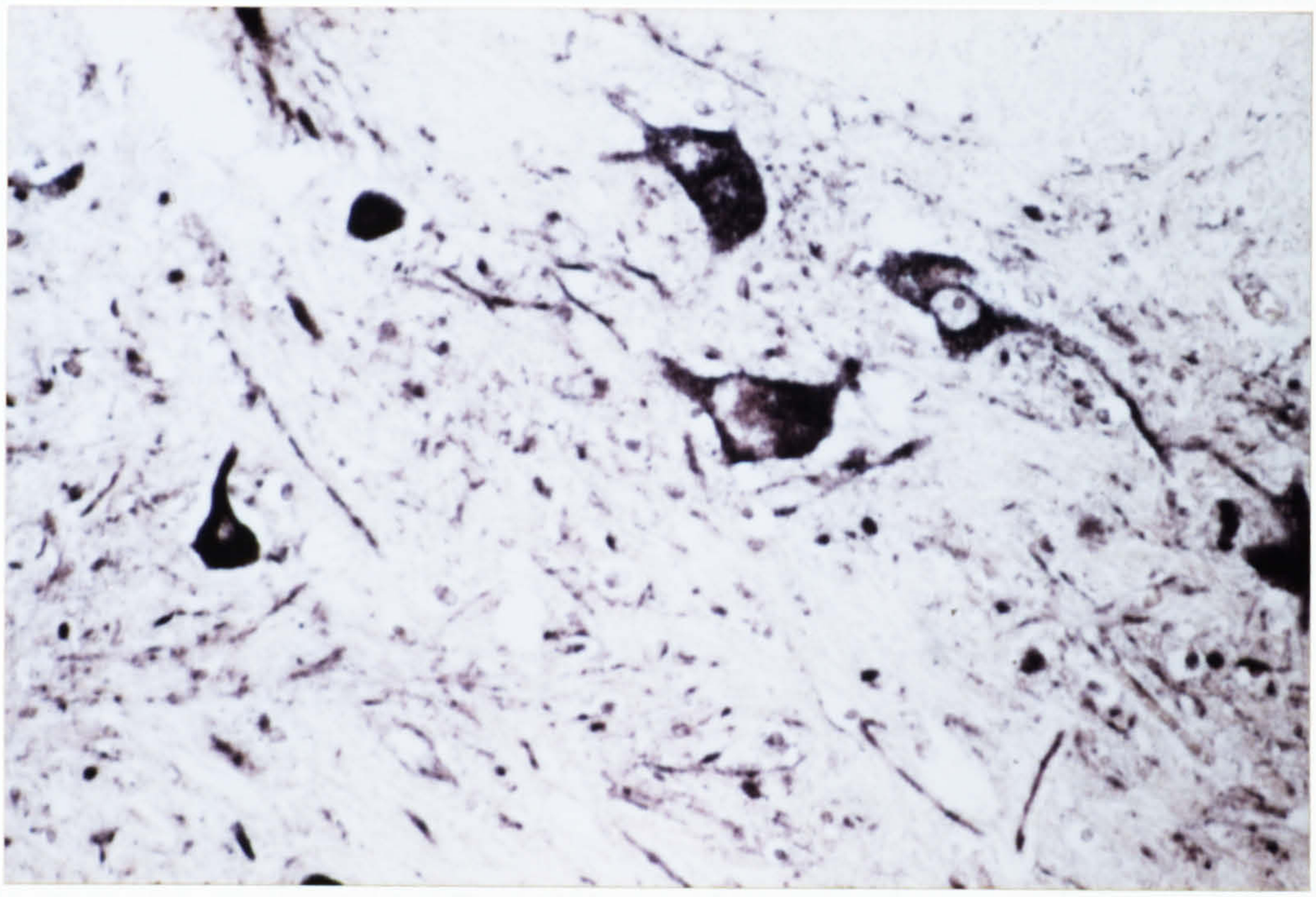
Mitochondrial probes; NBT / BCIP chromogen; x 200.

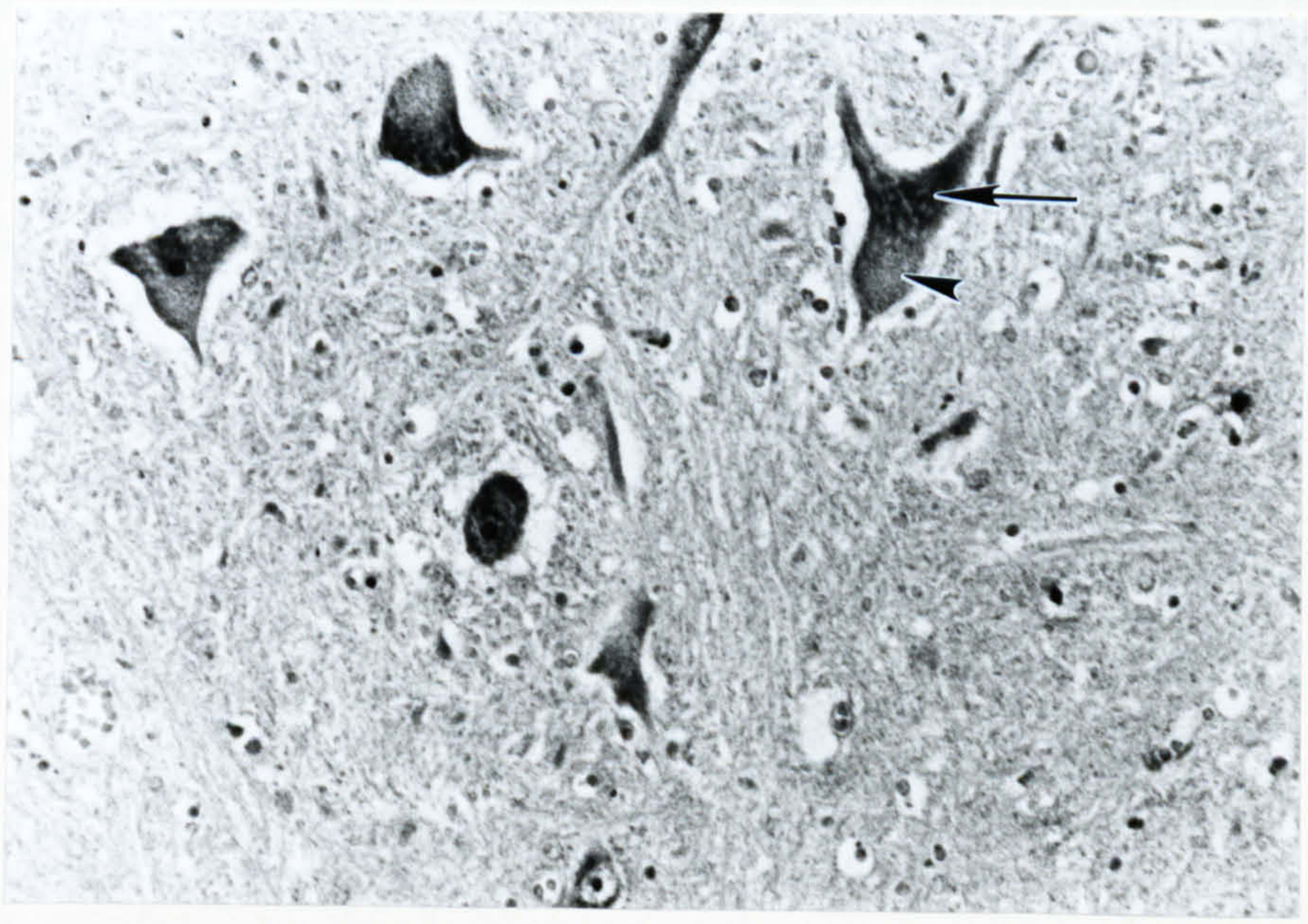
Figure 321g

In situ hybridisation for synaptophysin has shown a signal from the ventral horn neurons that is weaker than that with the cocktail of mitochondrial probes; the dark grey signal from the cytoplasm (arrow) is not apparent where lipofuscin has accumulated in the cell (arrowhead).

A cocktail of complementary sense probes gave no signal.

Synaptophysin antisense probes; NBT / BCIP chromogen; x 200.





3.2.2 Tumour biopsies

Of 145 astrocytic tumours, 14 high-grade examples had shown a neuronal immunophenotype, and all but one of these 14 had labelled with synaptophysin antibodies (Table 322). Mitochondrial rRNA was unquestionably detected in 6 / 13 synaptophysin-positive astrocytic tumours. Specific signal with the synaptophysin probes (*antisense probe cocktail - positive, sense probe cocktail - negative*) was reliably detected in a small proportion of cells in only 3 of these 6 tumours. Of 25 oligodendrogliomas, 9 had immunolabelled with both synaptophysin antibodies (Table 322). Mitochondrial rRNA was detected in 5 / 9 of these oligodendrogliomas, and synaptophysin mRNA was detected in some cells in 3 / 5.

Few sections from tumours showed an even distribution of ISH signal with the poly-d(T) or mitochondrial probes (Figure 322). Instead, the only signal was frequently found towards the edge of a section. In these cases, attempts to optimise the pretreatment protocol for each biopsy by using different concentrations of proteinase K still did not produce specific signal throughout the sections. Indeed, increasing the amount of enzymatic digestion at the pretreatment stage served only to produce a non-specific (sense probe) signal. In some of these suboptimal biopsies, mitochondrial rRNA was detected in vascular endothelial cells (Figure 322g), and in tumour cells surrounding blood vessels, but nowhere else. Some tumour sections contained areas where no signal was seen with any of the probes, other areas where non-specific signal was seen, and further areas where specific signal was seen. At times, the synaptophysin antisense signal was so weak that it was barely discernible above background staining (Figure 322d).

Table 322

Flow diagram relating expression of synaptophysin at different levels in high-grade astrocytic tumours and oligodendrogliomas

121 anaplastic astrocytomas and glioblastomas

↓

13 tumours with synaptophysin-positive cells

↓

6 tumours with rRNA detectable in tumour cells

↓

3 tumours with synaptophysin mRNA detectable in tumour cells

25 oligodendrogliomas

↓

9 tumours with synaptophysin-positive cells

↓

5 tumours with rRNA detectable in tumour cells

↓

3 tumours with synaptophysin mRNA detectable in tumour cells

Figure 322a

In situ hybridisation in sections of a synaptophysin-positive glioblastoma.

top: mitochondrial rRNA.

A strong signal is seen in many of the larger tumour cells.

Mitochondrial probes; NBT / BCIP chromogen; x 200.

middle: synaptophysin mRNA.

The antisense probes have labelled the cytoplasm of some of the larger tumour cells (arrow). A faint signal is seen in some cells (arrowhead).

Synaptophysin antisense probes; NBT / BCIP chromogen; x 200.

bottom: sense probes for the above preparation.

No signal is evident.

Synaptophysin sense probes; NBT / BCIP chromogen; x 200.

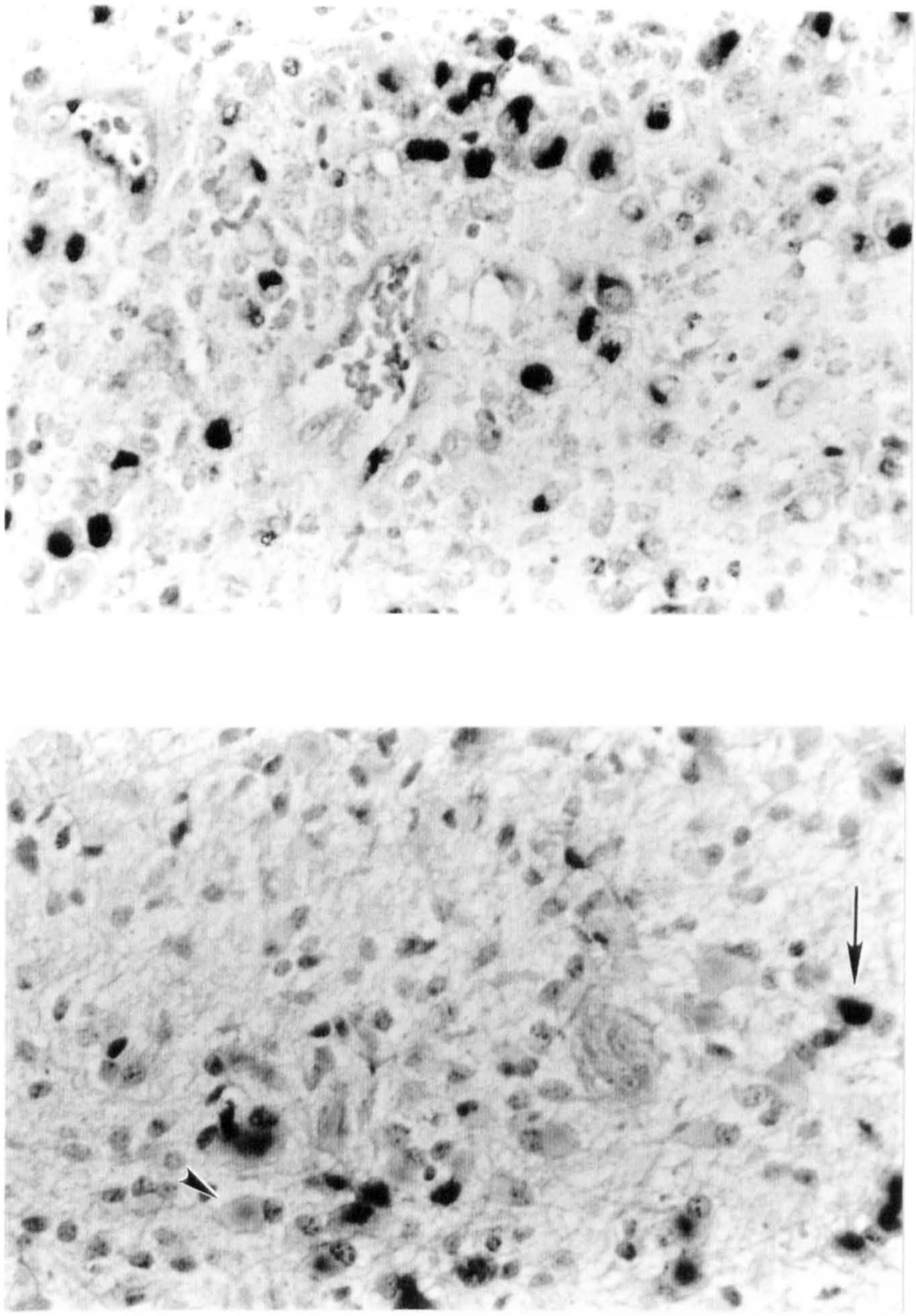


Figure 322b

In situ hybridisation has revealed abundant mitochondrial rRNA in many cells in this glioblastoma.

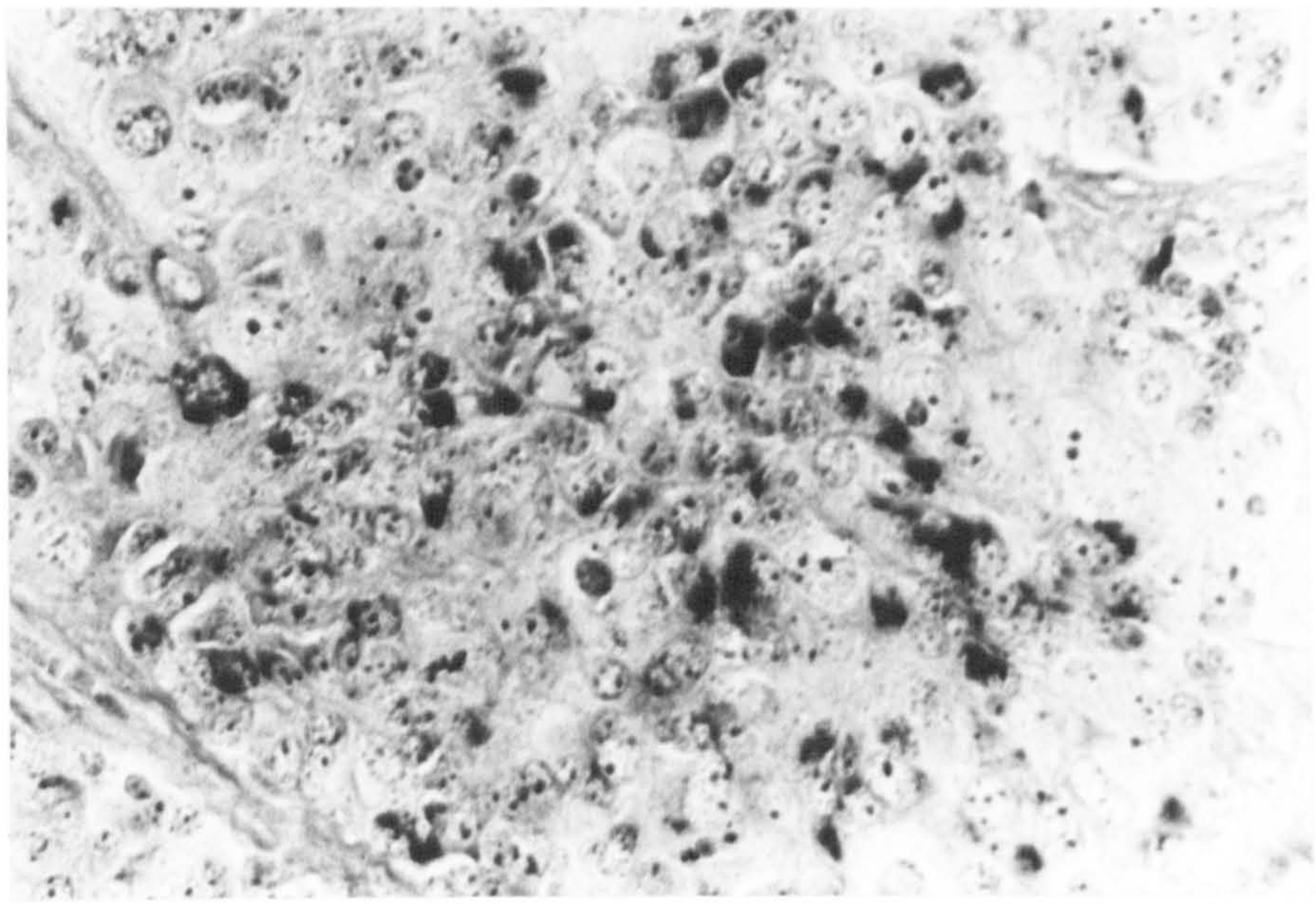
Mitochondrial probes; NBT / BCIP chromogen; x 200.

Figure 322c

In a near adjacent section to the one above, the cocktail of synaptophysin probes has labelled several tumour cells with an intensity that does not match that of the mitochondrial probe cocktail; but clearly indicates that the synaptophysin present in this tumour is manufactured in these cells.

Sense probes produced no signal.

Synaptophysin antisense probes; NBT / BCIP chromogen; x 400.



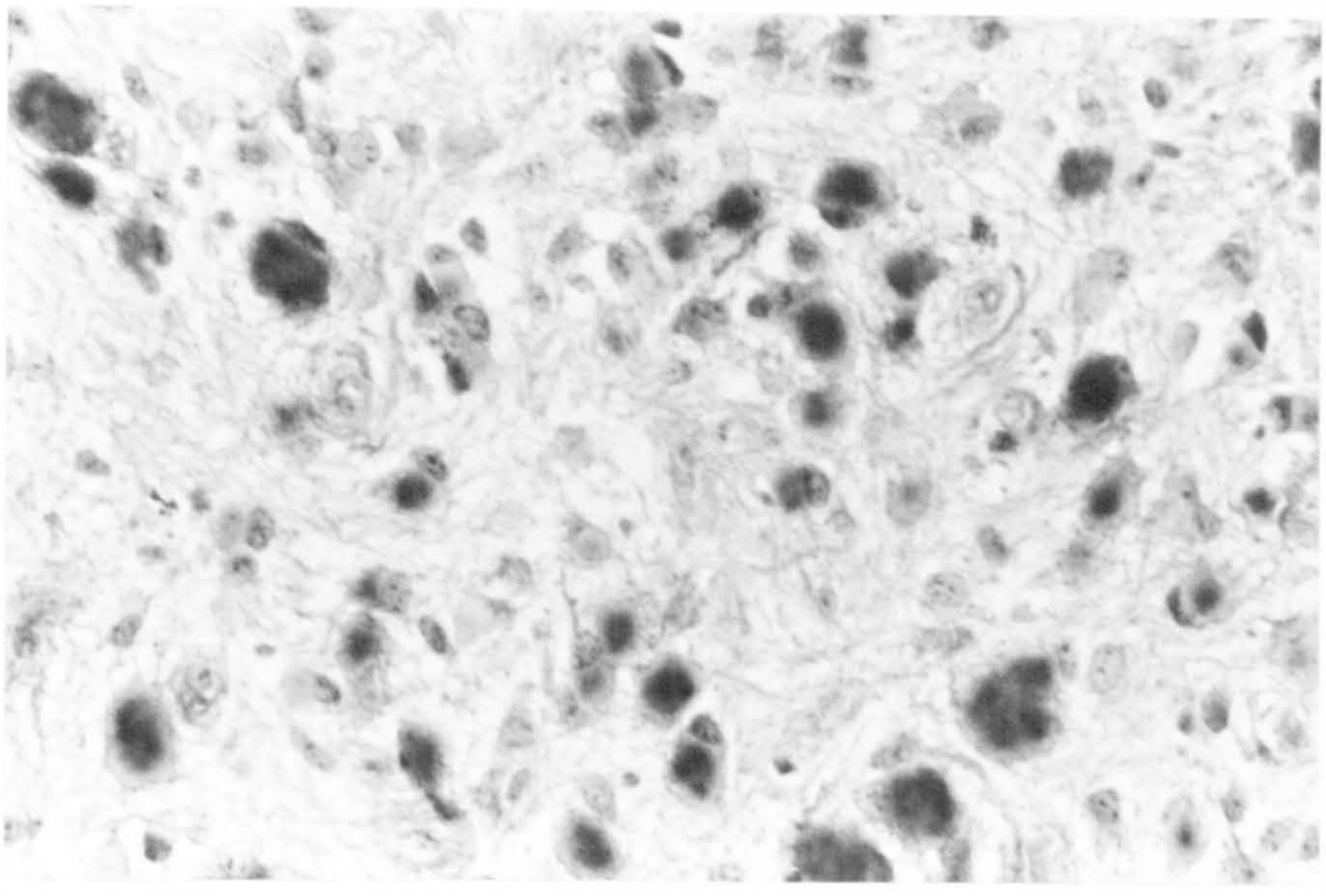


Figure 322d

In situ hybridisation in these sections of a synaptophysin-positive glioblastoma illustrates the equivocal nature of the signal from the synaptophysin antisense probes that was a feature of a few biopsies.

top: mitochondrial rRNA.

A strong signal is seen in many tumour cells.

Mitochondrial probes; NBT / BCIP chromogen; x 200.

middle: synaptophysin mRNA.

A faint signal is found in a few cells (arrowhead), but is only just distinguishable from background staining.

Synaptophysin antisense probes; NBT / BCIP chromogen; x 200.

bottom: sense probes for the above preparation.

No signal is evident.

Synaptophysin sense probes; NBT / BCIP chromogen; x 200.

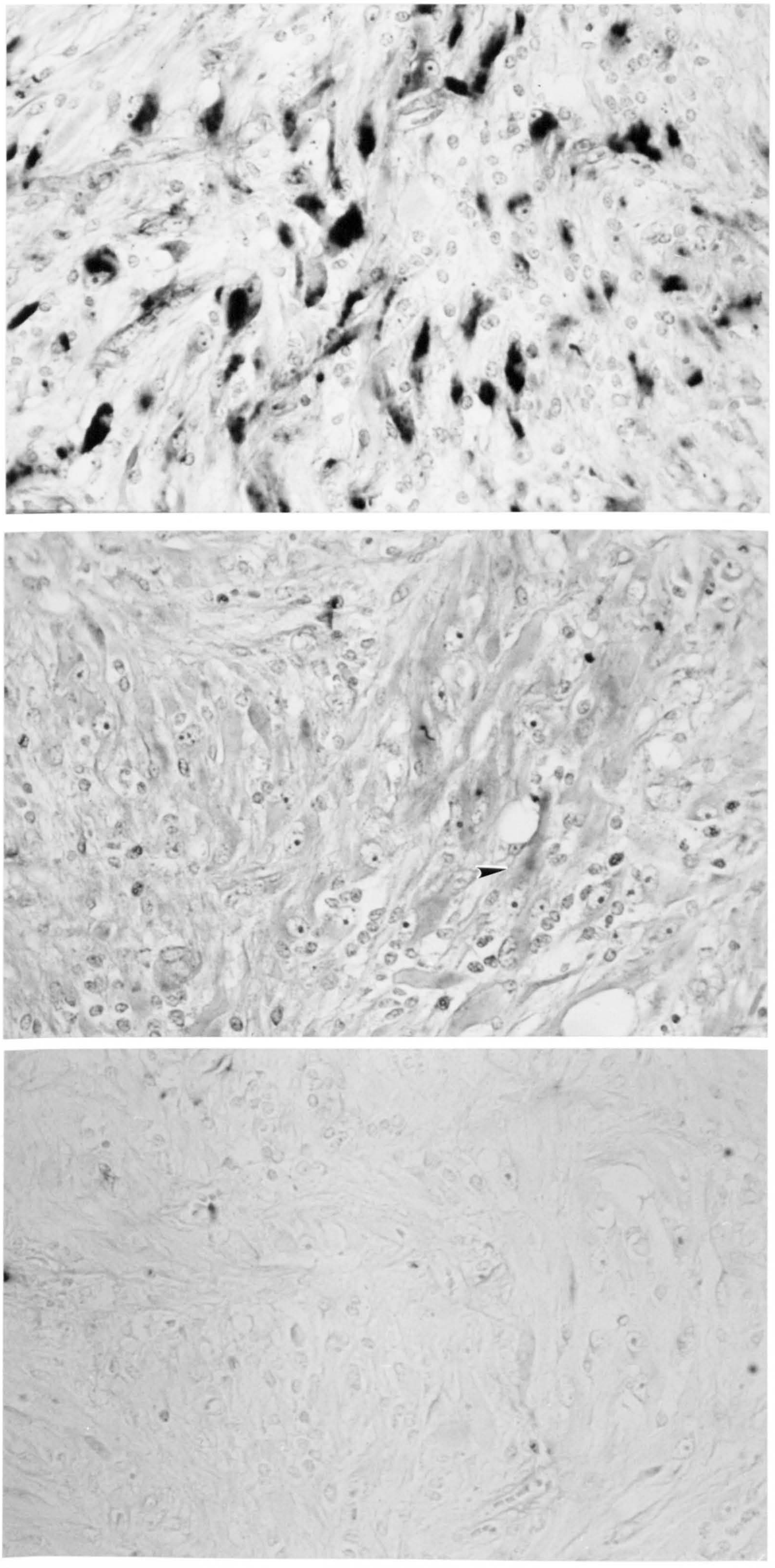


Figure 322e

In situ hybridisation has shown a patchy signal for mitochondrial rRNA in the cells of this oligodendroglioma.

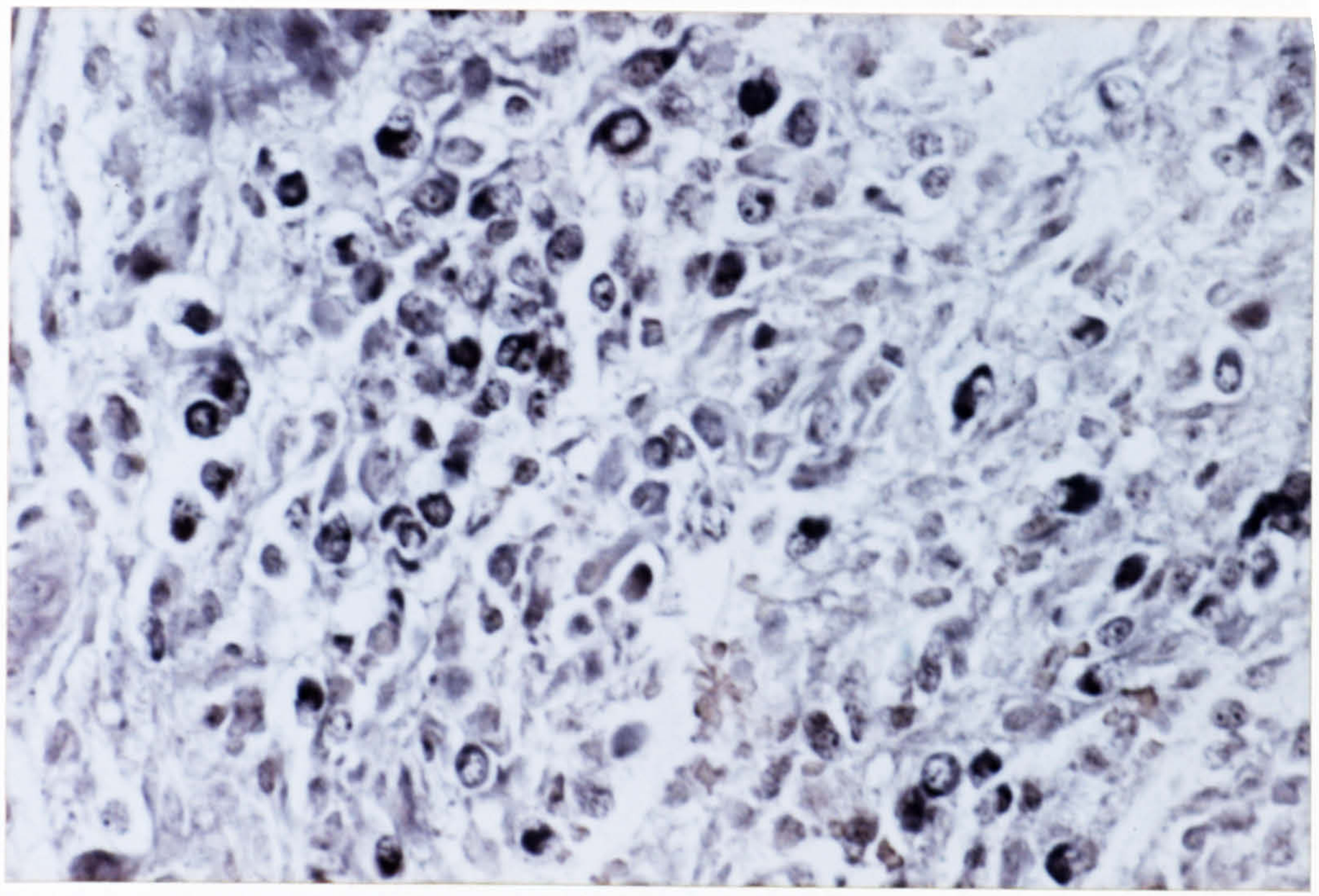
Mitochondrial probes; NBT / BCIP chromogen; x 200.

Figure 322f

In a near adjacent section to the one above, the cocktail of synaptophysin probes has labelled cells in one area of the tumour, but has not labelled cells in an adjacent area. The capricious, focal nature of the specific signal was typical of results with these cases.

Sense probes produced no signal.

Synaptophysin antisense probes; NBT / BCIP chromogen; x 200.



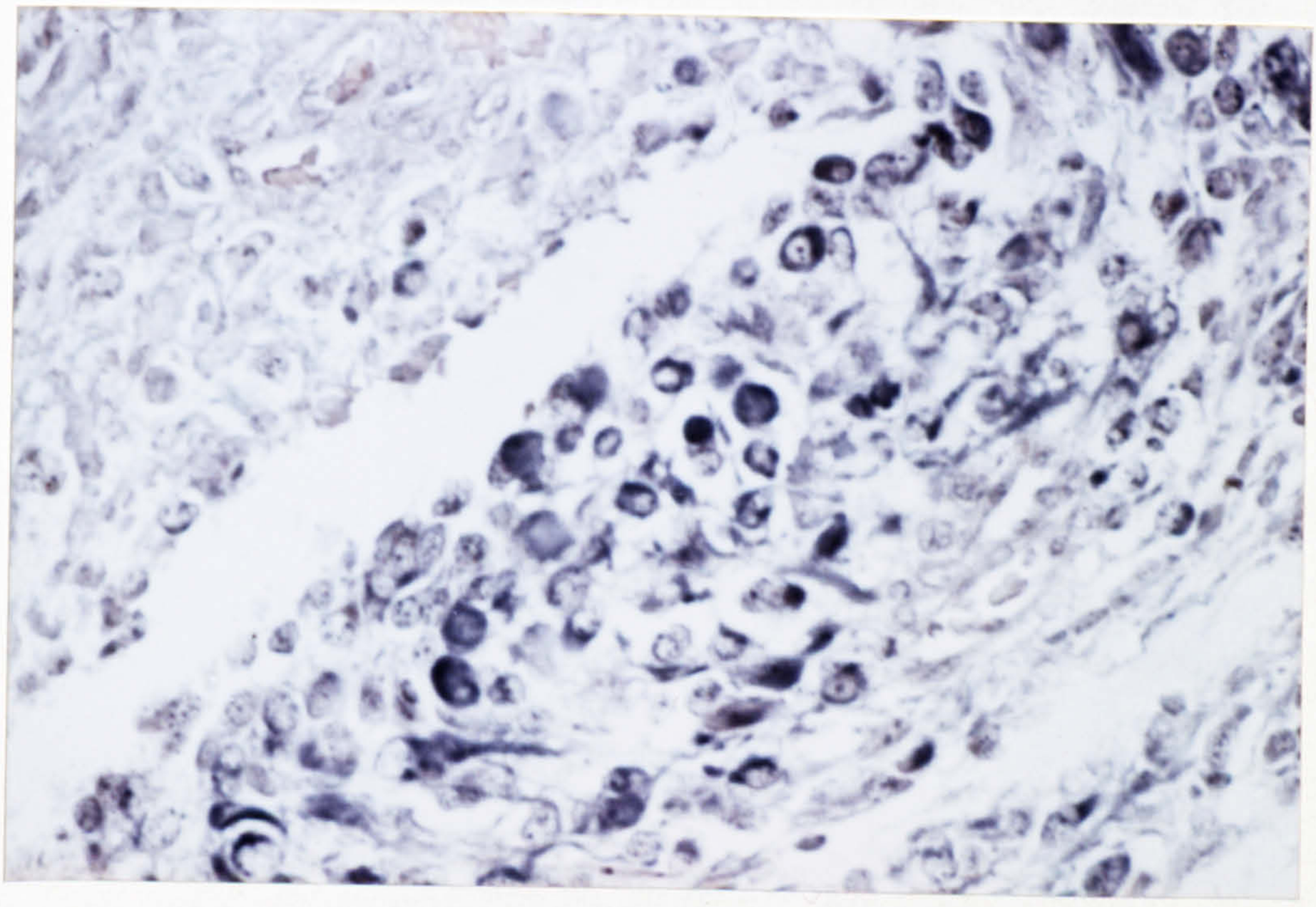
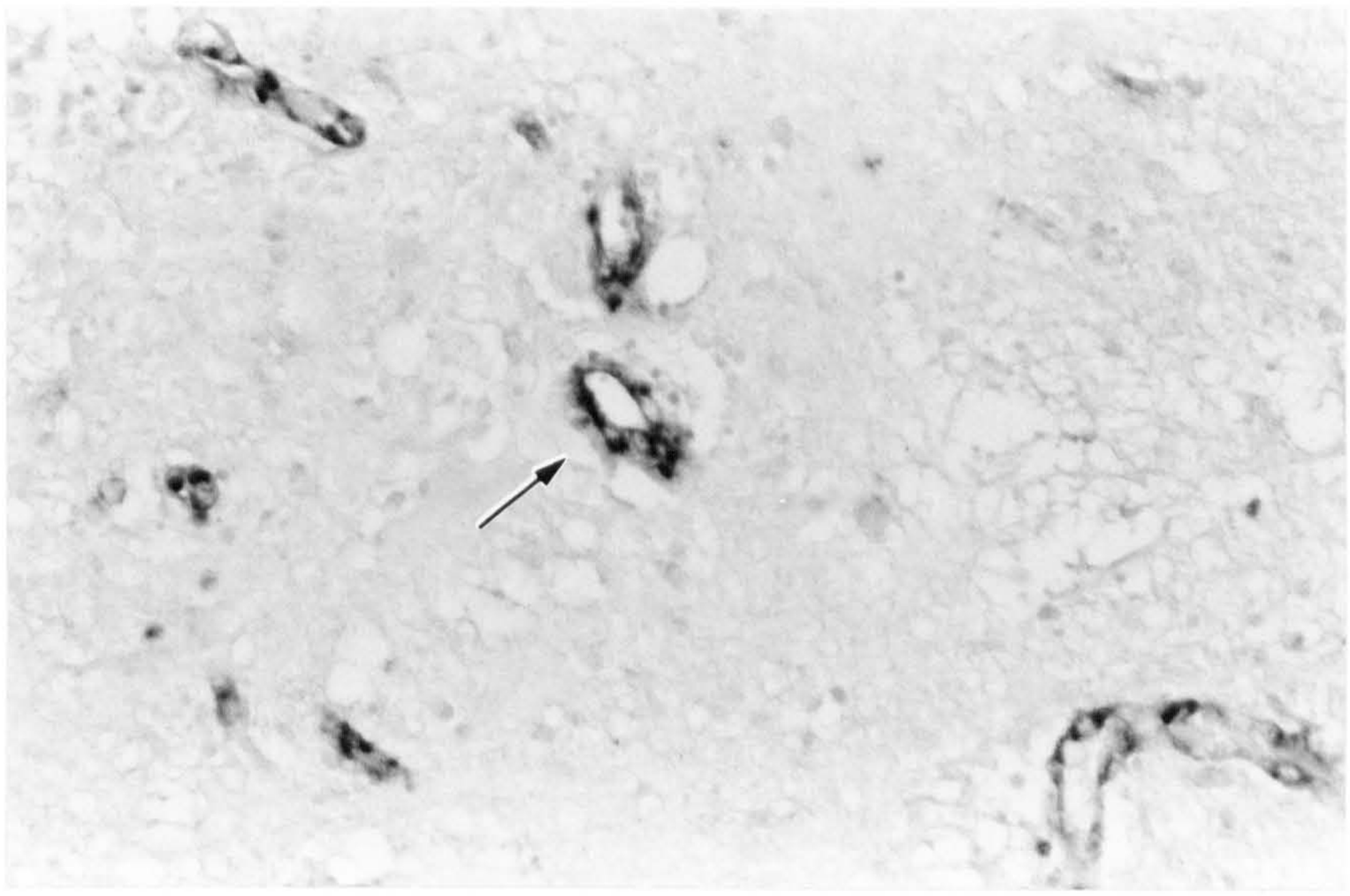


Figure 322g

Many of the tumour biopsies assessed with in situ hybridisation contained little RNA. Only endothelial cells (arrow) in this glioblastoma have labelled with the cocktail of probes to mitochondrial rRNA. No tumour cells showed a signal.

Mitochondrial probes; NBT / BCIP chromogen; x 100.



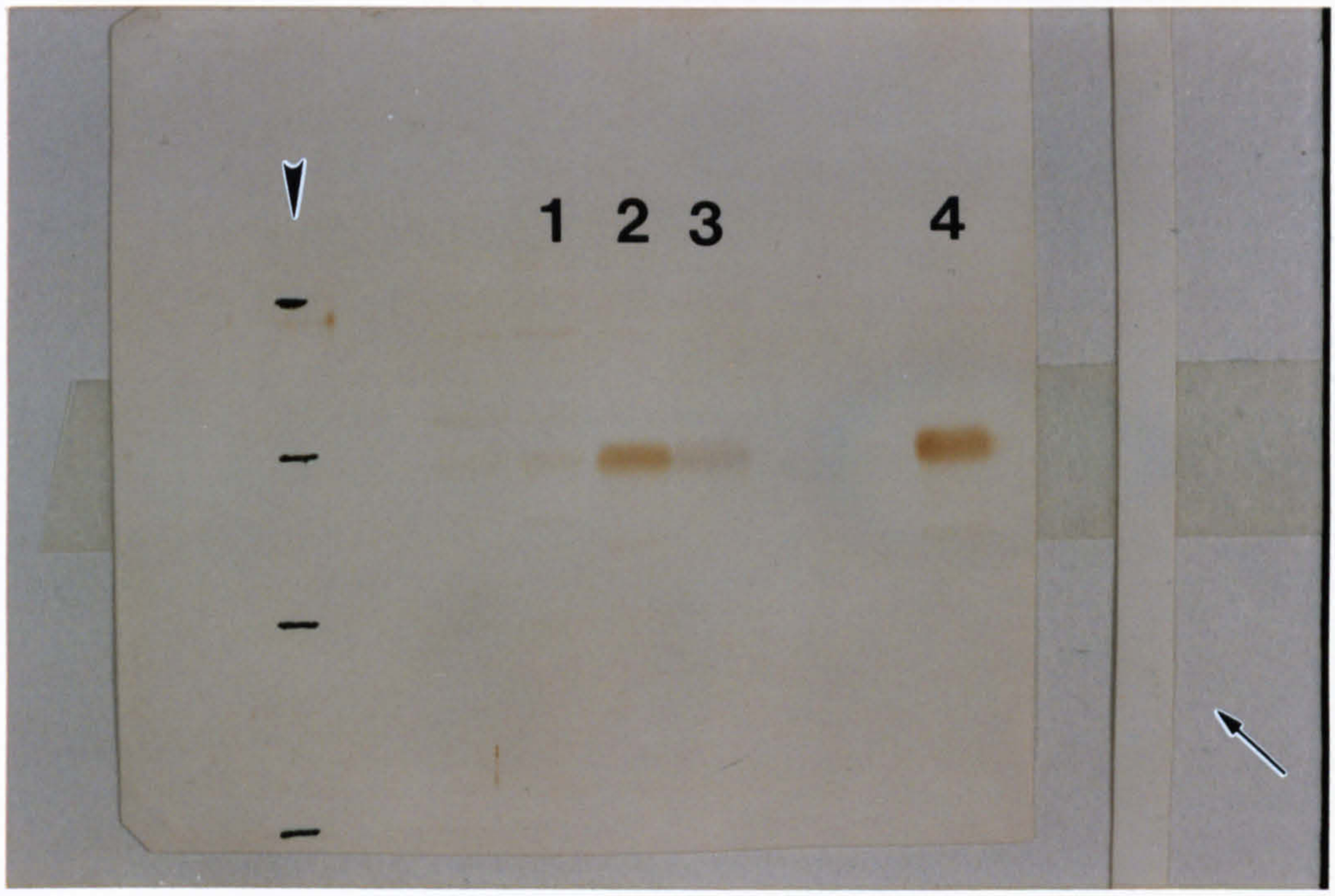
3.3 Immunoblotting

The glioblastomas that had labelled immunohistochemically with synaptophysin antibodies gave a moderately strong, single band and a faint, single band on the immunoblot (Figure 33). A strong single band was seen for cerebral grey matter (positive control). These bands migrated at approximately 41 - 42 kD. The synaptophysin-negative glioblastoma (negative control) and the nitrocellulose strip to which only non-immune serum had been applied showed no bands.

Figure 33

Immunoblot with molecular weight markers (arrowhead), plus a nitrocellulose strip containing proteins from normal cerebral grey matter to which non-immune serum has been applied (arrow).

Lane 1	synaptophysin-negative glioblastoma
Lane 2	synaptophysin-positive glioblastoma
Lane 3	synaptophysin-positive glioblastoma
Lane 4	normal cerebral grey matter


1 2 3
4

3.4 Apoptosis

3.4.1 Indices of apoptosis and Ki-67 immunolabelling

Many fibrillary astrocytomas contained no apoptotic cells. However, apoptotic cells were regularly, if infrequently, found in anaplastic astrocytomas and glioblastomas (Figure 272), which showed median AI's of 0.072% and 0.12% respectively (Table 341). The range of AI's across all tumours was 0% - 0.68%. Median Ki-67 LI's for the 3 histological types of astrocytic tumour were significantly ($P < 0.0001$) different (Table 341). In a regression analysis of all tumours, AI was correlated with Ki-67 index ($\rho = 0.55$; $P < 0.0001$).

3.4.2 Indices of apoptosis and labelling with *bcl* -2 and p53 antibodies

The relationship between patterns of *bcl* -2 labelling and AI is displayed in Table 342, where tumours showing focal and ubiquitous *bcl* -2 labelling have been combined into one *bcl* -2 positive group. No significant difference in median AI's was evident between *bcl* -2 positive and *bcl* -2 negative groups for all astrocytic tumours together and for astrocytomas and glioblastomas alone. A comparison of p53 labelling patterns and AI's in all astrocytic tumours (Table 342) showed some association ($P = 0.06$) between a high AI and a p53 LI > 60%, and between a low AI and a p53 LI < 40%. However, this association may reflect the uneven distribution of tumour types across the p53 labelling categories because it was not a significant finding for glioblastomas alone ($P = 0.27$). No correlation was observed between AI and p53 LI ($\rho = 0.25$; $P = 0.22$).

Table 341
Apoptotic indices and Ki-67 labelling indices by type of astrocytic tumour

	astrocytoma n = 16	anaplastic astrocytoma n = 19	glioblastoma n = 46
apoptotic index	0 (0)	0.072 (0.146)	0.120 (0.207)
Ki-67 LI	0.40 (1.06)	2.3 (3.6)	7.2 (6.7)

Figures are: median (interquartile range)
A significant difference ($p < 0.0001$) is found between histological types of astrocytic tumour for both AI and Ki-67 LI (Kruskal-Wallis)

Table 342

Apoptotic indices by *bcl* -2 and p53 labelling patterns for all astrocytic tumours, astrocytomas, and glioblastomas

	all astrocytic tumours n = 81	astrocytomas n = 35	glioblastomas n = 46
bcl-2 negative	0.110 (0.236)	0.100 (0.096)	0.167 (0.239)
bcl-2 positive	0.069 (0.172)	0.053 (0.147)	0.116 (0.177)
p53 negative	0.116 (0.184)	0.100 (0.131)	0.121 (0.190)
p53 LI < 40%	0.060 (0.117)	0.040 (0.076)	0.113 (0.250)
p53 LI > 60%	0.218 (0.268)	0.053 (0.258)	0.241 (0.296)

Figures are: median (interquartile range)

Comparison between bcl-2 groups:

all astrocytic tumours, P = 0.48 (Mann-Whitney)
astrocytomas, P = 0.29 (Mann-Whitney)
glioblastomas, P = 0.28 (Mann-Whitney)

Comparison between p53 groups:

all astrocytic tumours, P = 0.06 (Kruskal-Wallis)
astrocytomas, P = 0.32 (Kruskal-Wallis)
glioblastomas, P = 0.27 (Kruskal-Wallis)

3.5 p53 gene analysis

Of the astrocytic tumours with neuronal immunophenotype that were assessed for p53 gene mutation, 4 / 8 showed shifts on SSCP gels (Figure 35), and 3 / 8 showed point mutations (Table 35). A common polymorphism in exon 6 was identified in 3 tumours (Figure 35).

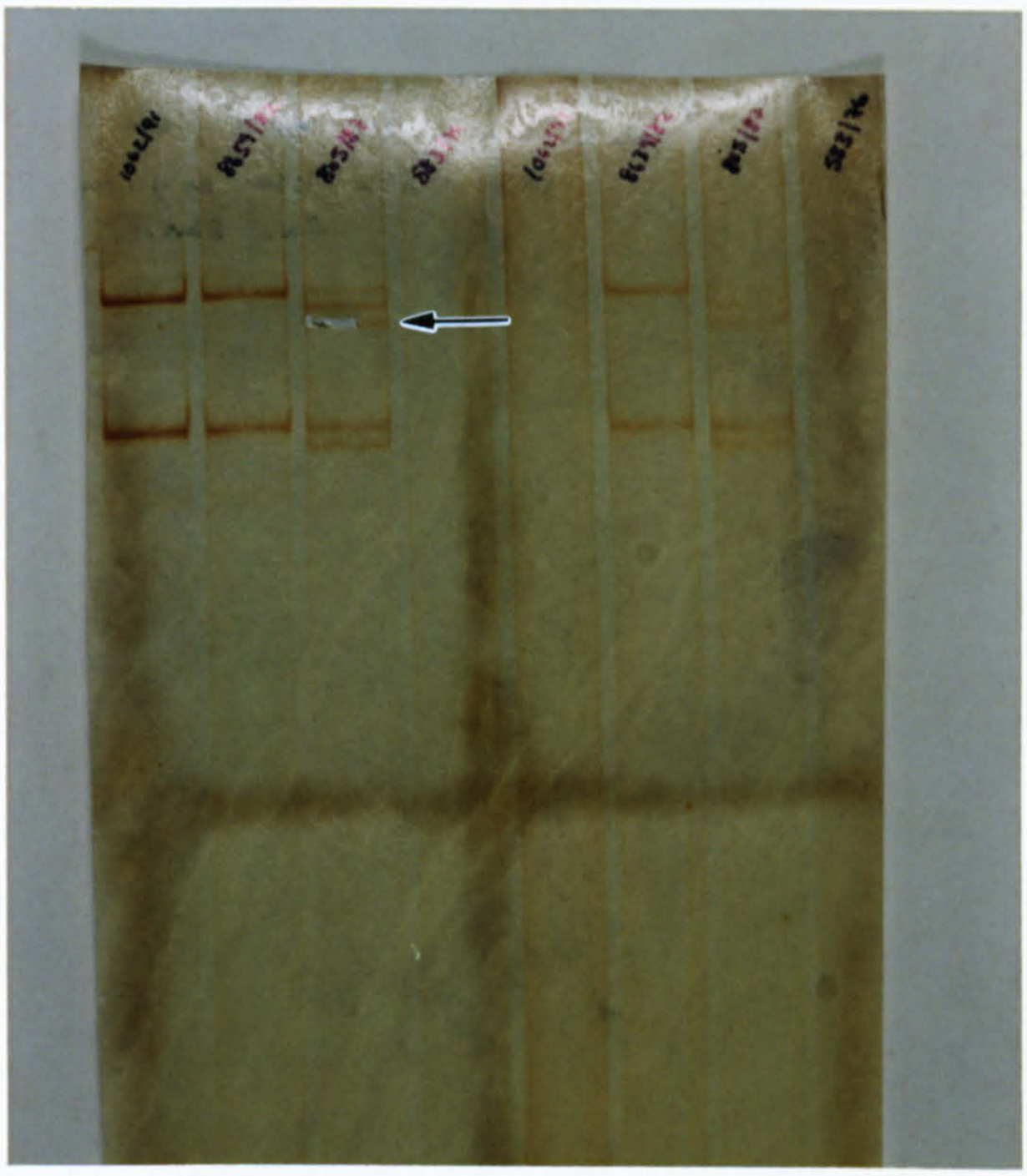
Figure 35a

SSCP gel for exon 5 of the p53 gene.

A shift for tumour 805.87 is evident (lanes 3 & 7), and part of the band has been removed (arrow) for gene analysis.

Figure 262-2

The presence of PCR reaction products was confirmed on mini-gels.
Ethidium bromide under ultraviolet light was used to demonstrate the bands.



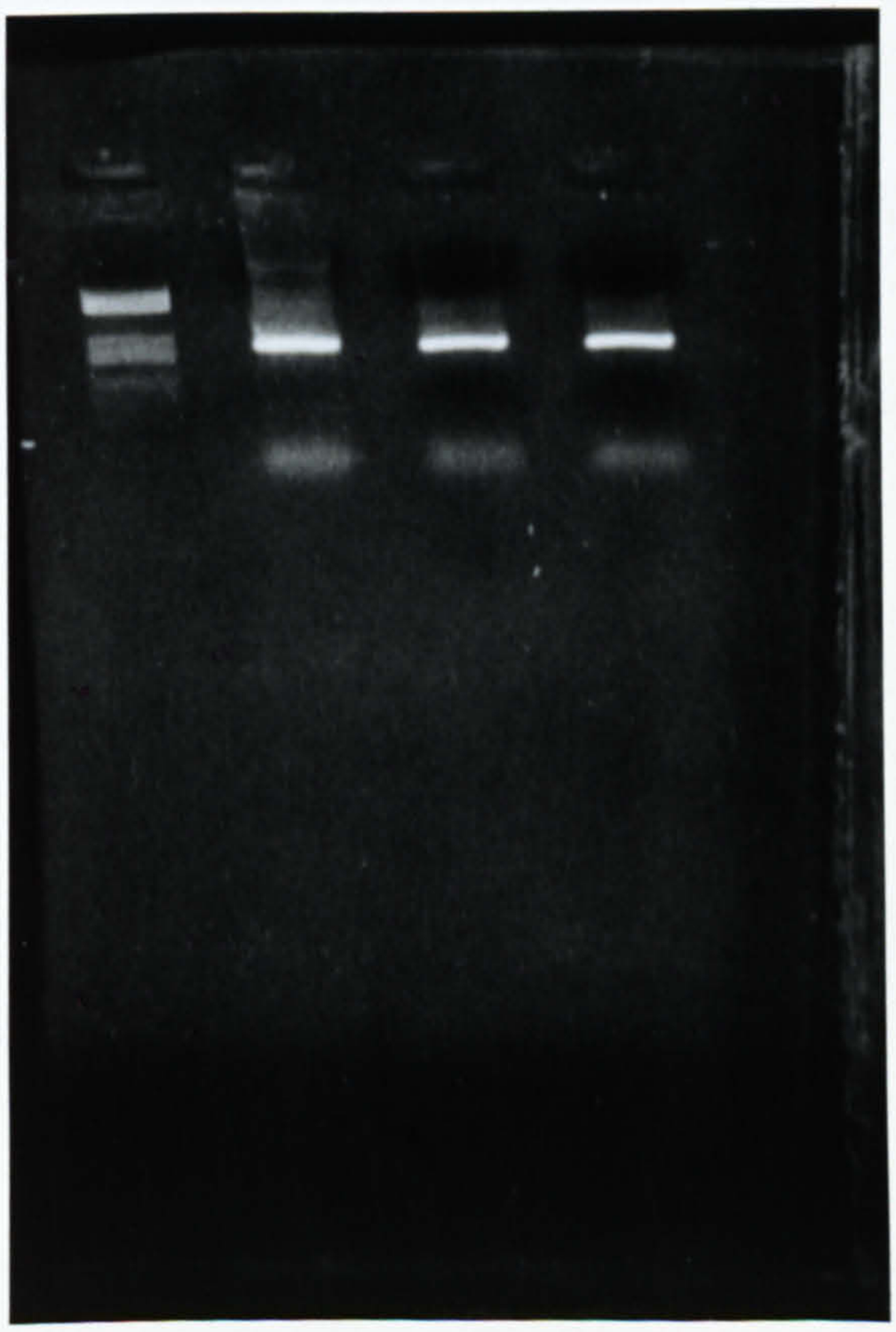


Figure 35b

SSCP gel for exon 8 of the p53 gene.

Lane 1 size markers
Lane 4 control tumour with known mutation (arrow)

Shifts (arrowheads) were evident for tumours

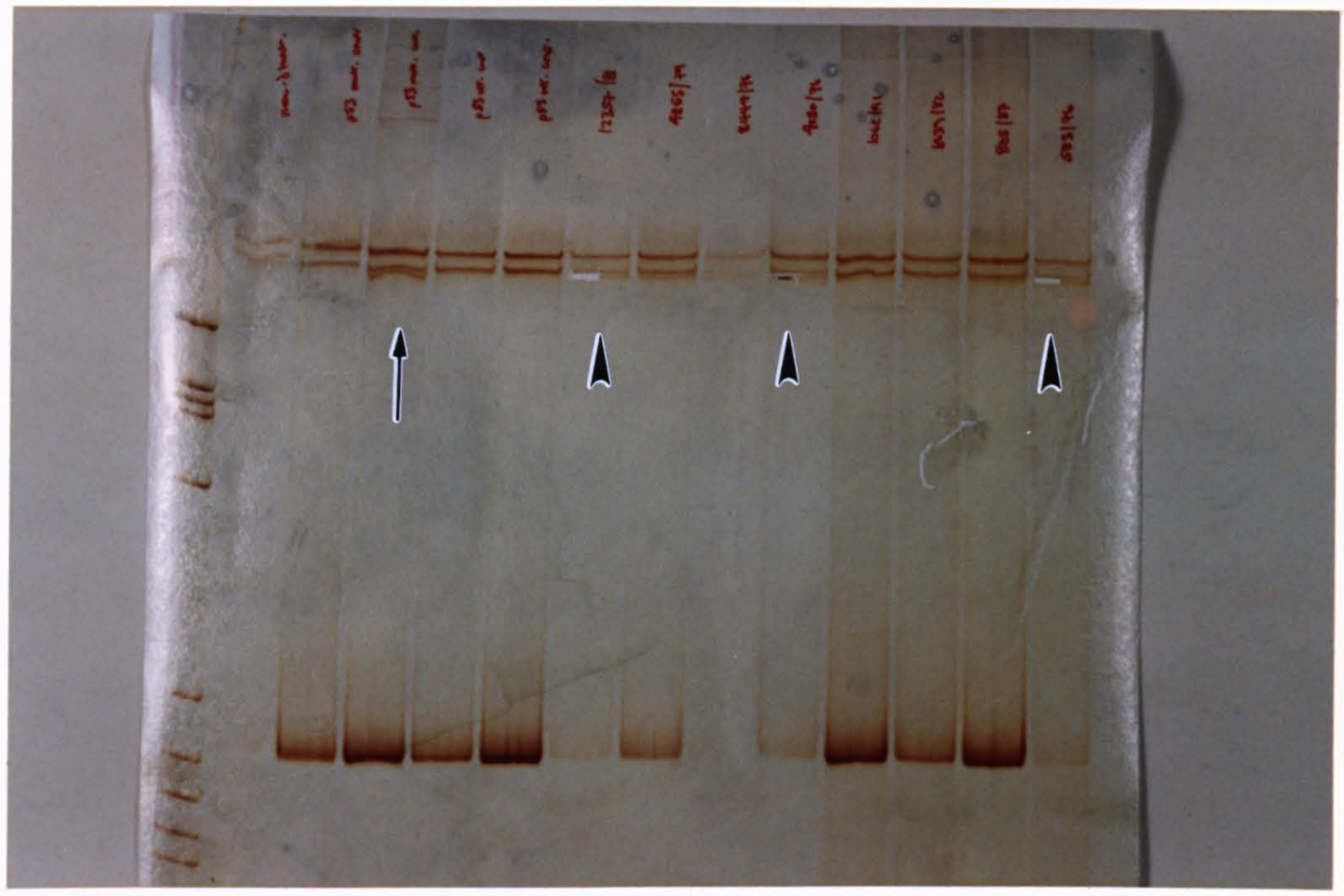
12357.81	(lane 7)
4030.76	(lane 10)
583.76	(lane 14)

The shifted band in lane 10 was faint, and not indicative of a mutation once the genes had been sequenced.

Figure 35c

SSCP gel for exon 6 of the p53 gene.

The different patterns of banding seen among samples (arrow and arrowhead) is due to a common polymorphism.



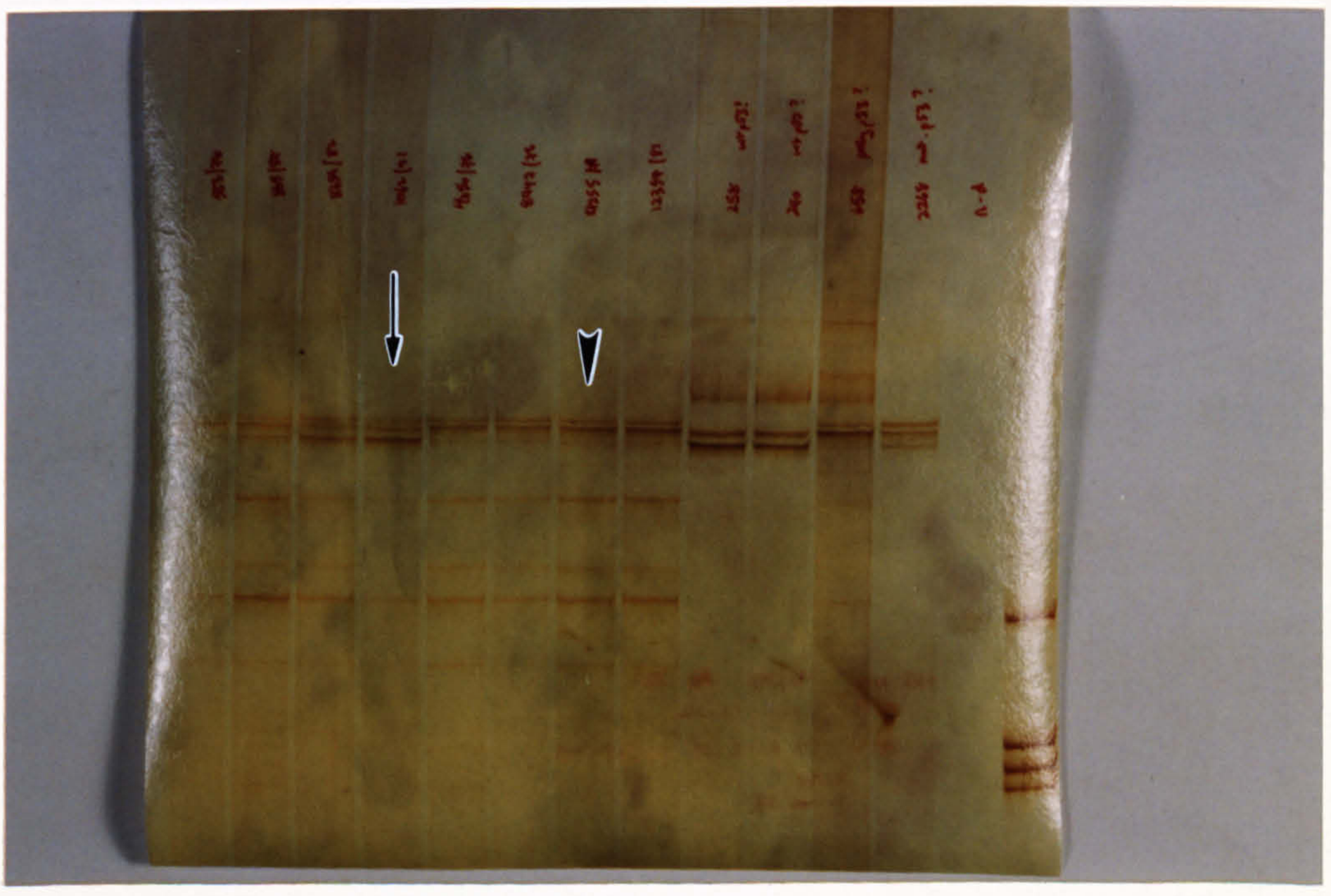


Table 35

p53 gene analysis of 8 astrocytic tumours with neuronal immunophenotype

exon 5	exon 6	exon 7	exon 8	number	mutation
normal	normal	normal	normal	3	NA
normal	poly.	normal	normal	1	NA
normal	normal	normal	**shift**	2	codon 273; Arg - Cys
shift	poly.	normal	normal	1	codon 168; His - Arg
normal	poly.	**shift**	normal	1	wild type gene

poly. = polymorphism
NA = not analysed

3.6 Survival studies

3.6.1 Clinical variables

Of the 123 patients in the study of survival and immunohistochemistry with Ki-67 and p53 antibodies, 100 had died at the time of data entry. Median survival times for the 3 types of astrocytic tumour were: fibrillary astrocytoma, 1026 days; anaplastic astrocytoma, 377 days; and glioblastoma, 321 days. Table 361 shows the unadjusted and adjusted relative hazards to survival for several clinical and pathological variables. There was no significant relationship ($P = 0.87$) between treatment regimen and survival (not shown). Neither gender nor tumour site was a prognostic indicator; however, age was a significant and independent hazard to survival.

3.6.2 Conventional histological classification and survival

The most important prognostic indicator was type of astrocytic tumour. Anaplastic transformation of an astrocytoma or a diagnosis of glioblastoma multiforme significantly ($P < 0.0001$) shortened survival, and this effect was independent of other variables. The substantial differences in survival associated with the 3 types of astrocytic tumour and with a Ki-67 LI above and below 2% are seen in Kaplan-Meier survival curves (Figures 362-2a & 362-2b).

3.6.3 Ki-67 immunolabelling and survival

Ki-67 LI as a continuous variable carried a significant ($P = 0.0043$) unadjusted hazard to survival, the 2 nominal categories with Ki-67 LI > 2% showing relative hazards above 3. This effect was reduced and insignificant when adjusted for other variables, because of the close relationship between Ki-67 LI and histological type of astrocytic tumour.

Table 361
Unadjusted and adjusted relative hazards to survival in a spectrum of astrocytic tumours

n = 123		UNADJUSTED			ADJUSTED		
VARIABLE	CATEGORY	RELATIVE HAZARD (95% CI*)		P	RELATIVE HAZARD (95% CI*)		P
Age	R.H. / U.I. †	1.06	(1.04, 1.08)	0.0000	1.03	(1.01, 1.06)	0.0100
Gender	male	1.00			1.00		
	female	0.94	(0.62, 1.42)	0.7719	1.13	(0.73, 1.76)	0.5595
Tumour Site	frontal	1.00			1.00		
	temporal	1.42	(0.87, 2.32)	0.3257	1.25	(0.74, 2.12)	0.3972
	occipito-parietal	1.28	(0.80, 2.06)		0.85	(0.51, 1.42)	
Histology	glioblastoma	1.00			1.00		
	anaplastic astrocytoma	0.40	(0.24, 0.67)	0.0000	0.59	(0.31, 1.15)	0.0037
	fibrillary astrocytoma	0.14	(0.07, 0.27)		0.22	(0.09, 0.56)	
Ki-67 LI	R.H. / U.I. †	1.04	(1.01, 1.06)	0.0043		-	
Ki-67 group	Ki-67 LI < 2%	1.00			1.00		
	Ki-67 LI 2 - 5%	3.13	(1.81, 5.44)	0.0000	1.43	(0.76, 2.66)	0.4038
	Ki-67 LI > 5%	3.21	(1.95, 5.28)		1.04	(0.55, 1.95)	
p53 LI	R.H. / U.I. †	1.00	(0.99, 1.01)	0.8576		-	
p53 group	p53-negative	1.00			1.00		
	p53 LI < 40%	0.86	(0.55, 1.34)	0.6502	0.90	(0.55, 1.47)	0.8722
	p53 LI > 60%	1.07	(0.62, 1.85)		1.02	(0.58, 1.82)	

Hazards to survival are relative either to one group with a value of unity, or to a unit increase in a continuous variable†. *confidence intervals

Figure 362-2a
Survival curves for a spectrum of astrocytic tumours divided by histologcial type

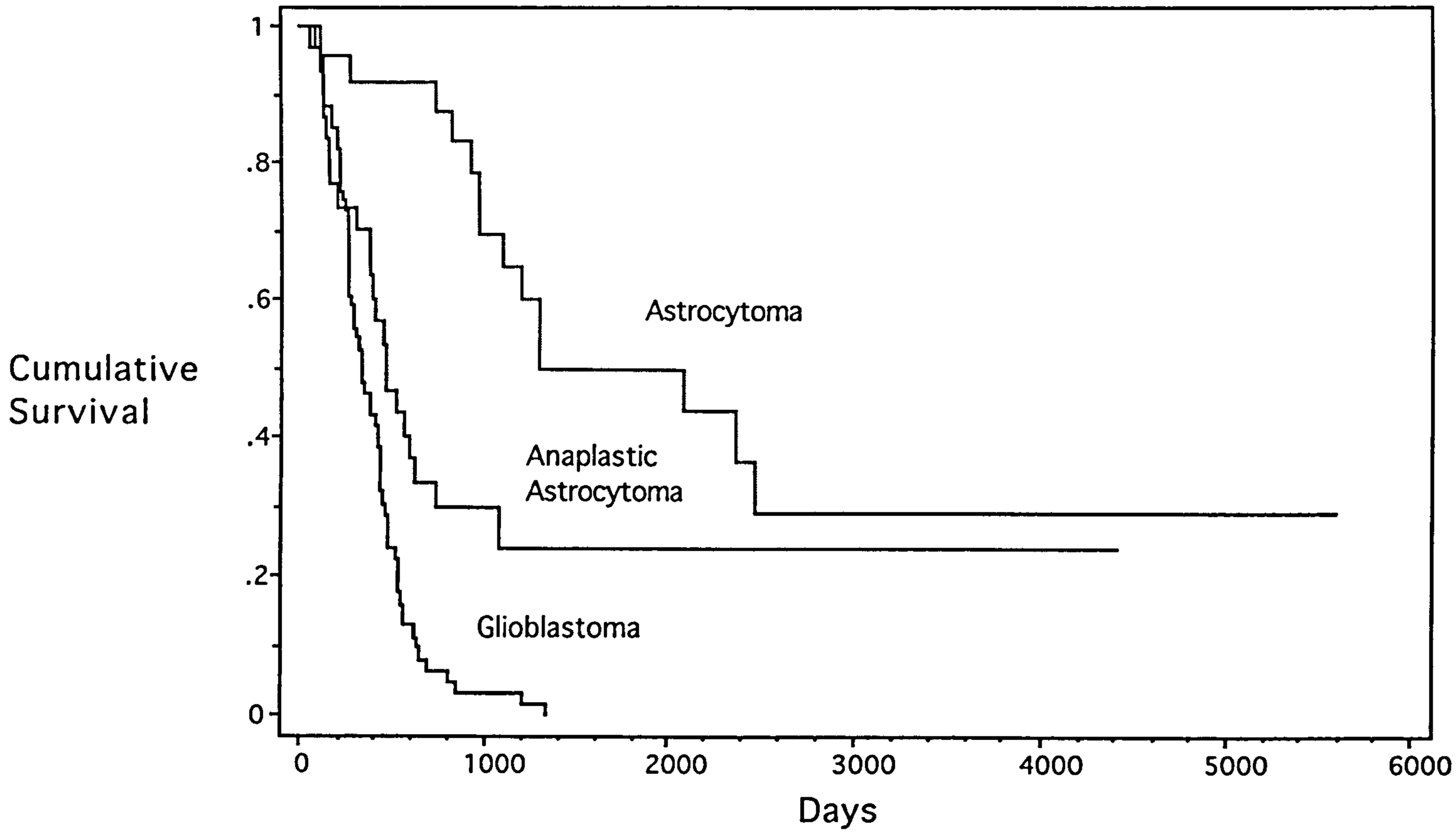
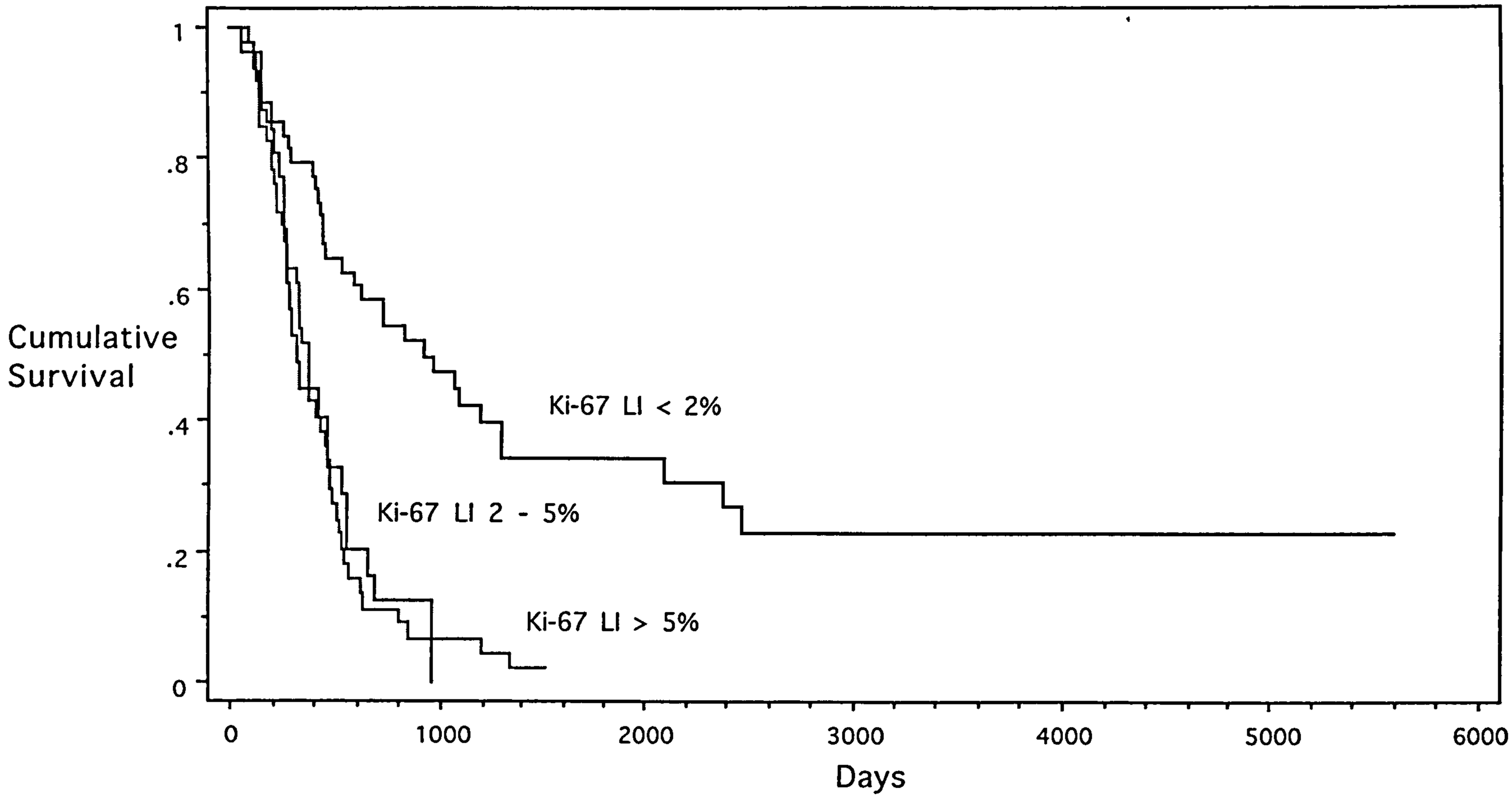


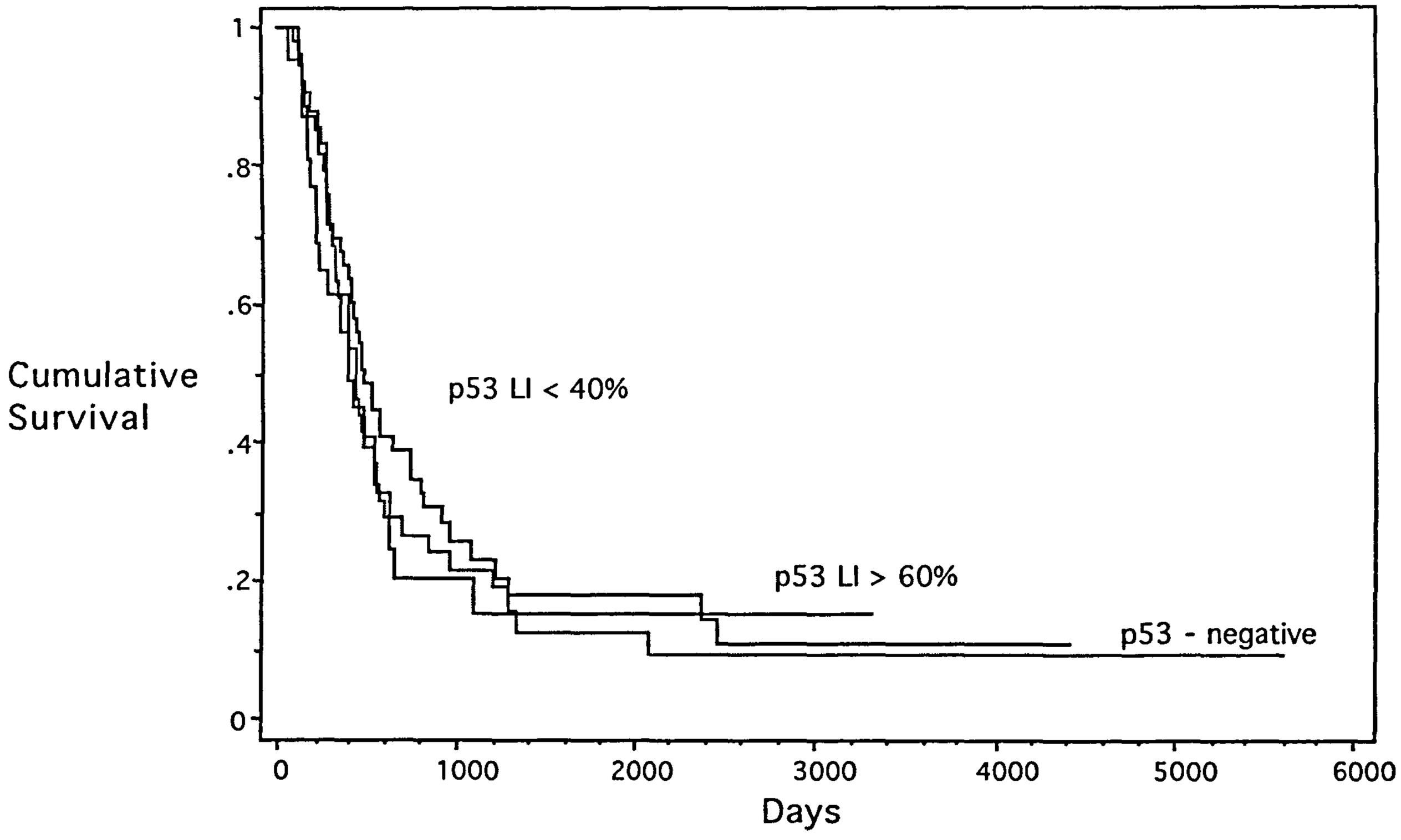
Figure 362-2b
Survival curves for a spectrum of astrocytic tumours divided by Ki-67 LI group



3.6.4 p53 immunolabelling

As a continuous variable or divided into 3 nominal categories, p53 LI showed no significant effect on survival (Table 361). Survival curves for patients with tumours divided according to category of p53 labelling are very close (Figure 364). This pattern was maintained when the 3 categories of p53 labelling were compared for each type of astrocytic tumour (survival curves not shown).

Figure 364
Survival curves for a spectrum of astrocytic tumours divided by category of p53 LI group



Discussion

4.1 Restatement of hypothesis and major findings

This thesis consists of several studies that test the hypothesis that the biological behaviour of gliomas correlates with the expression of specific neuroectodermal proteins, cell-cycle markers, and genetic regulators of cell proliferation and programmed cell death.

The ultimate aims of this research are to identify neuroepithelial tumours that respond well to established therapies and to define opportunities for new therapies, and it is likely that new systems of neuroepithelial tumour classification will follow the development of histological techniques to identify the specific cellular defects that directly affect or indirectly reflect the growth and behaviour of these tumours.

4.1.1 The expression of neuronal proteins in cerebral astrocytic tumours and oligodendrogliomas

Previous studies had suggested that a subgroup of cerebral astrocytic tumours exhibits neuronal differentiation and that such tumours might be characterised by a better prognosis than tumours without neuronal differentiation.

The present study has shown that:

(i) a small population (11%) of high grade, cerebral astrocytic tumours does express neuronal proteins

(ii) neuronal differentiation is not associated with an improved prognosis

(iii) the groups of tumours with and without neuronal differentiation show a similar rate of p53 mutation

The demonstration of combined glial and neuronal differentiation in some, rare neuroepithelial tumours and the similarity between the central neurocytoma of the third ventricle and the cerebral oligodendroglioma prompted the examination of a series of oligodendrogliomas for neuronal differentiation.

The present study has shown that:

(i) about one third of oligodendrogliomas expresses synaptophysin

(ii) this subgroup shows similar clinical attributes to those of standard tumours

4.1.2 Prognostic indicators in cerebral astrocytic tumours

Putative prognostic indicators, including Ki-67 LI, a measure of growth fraction, and patterns of p53 protein accumulation were examined in a range of cerebral astrocytic tumours, and the results have shown that:

(i) age and histological type are the principal, independent determinants of survival

(ii) Ki-67 LI correlates well with survival, and shares a close relationship with histological type

(iii) despite a significant association between a high p53 LI and a high Ki-67 LI in cerebral astrocytic tumours, patterns of accumulation of the p53 protein are unrelated to outcome, either for each type of astrocytic tumour or for all astrocytic tumours together

4.1.3 Apoptosis in cerebral astrocytic tumours

Apoptotic cell death was studied in cerebral astrocytic tumours and related to the expression of two regulators of apoptosis, p53 and *bcl* -2. The results have shown that:

(i) apoptotic index (AI) increases in parallel with increasing anaplasia across the range of these tumours

(ii) there is a strong correlation between AI and Ki-67 LI

(iii) there is no clear relationship between AI and the expression of the *bcl* -2 and p53 proteins

4.2 Expression of neuron-specific proteins in cerebral astrocytic tumours and oligodendrogliomas

Immunohistochemistry has been used over the last decade to supplement electron microscopy as an aid to histological interpretation. Both techniques, however, have been used mainly to study undifferentiated neoplastic cells for the expression of cell-specific proteins or the presence of cell-specific ultrastructural features which might elucidate the nature of the cell from which the tumour is derived.

Immunohistochemistry can offer more information if the expression of targeted proteins reflects the oncogenetic process either directly or indirectly; the products of abnormal genes may be detected or the deregulation of cellular processes caused by multiple gene abnormalities may be examined. If the appearance of a distinctive immunophenotype in a tumour is associated with a particular change in its behaviour or response to treatment, then immunohistochemistry is more valuable to patient management than if it merely allows a dedifferentiated tumour to be classified.

4.2.1 Immunohistochemistry and the classification of neuroepithelial tumours

For many years, the classification of CNS tumours and the management of patients with CNS tumours have depended upon the interpretation of histological features in tissue sections treated with a limited number of histochemical stains. After histological analysis, a tumour can be assigned a diagnosis from one of several, established systems of classification (Ellison, 1993). In the classification of Bailey and Cushing, the morphology of neoplastic cells was related to that of normal cells in the developing or mature CNS, and in the classification of Kernohan, the tumour was assessed for atypical cytological features (Russell and Rubinstein, 1989). When a recent international consensus on the classification of CNS tumours was reached (Kleihues et al., 1993), the diagnostic process still depended upon conventional histological assessment and categorisation of tumours by cell type. There was no recourse to modern histological methods that allow the division of tumours by immunohistochemical markers of tumour growth or the oncogenetic process itself,

because the usefulness of these markers as guides to prognosis or therapy has yet to be fully established.

Early immunohistochemical studies confirmed preconceptions about well differentiated and poorly differentiated tumours. Neoplastic astrocytic cells usually manufacture the intermediate filament of astrocytes, GFAP, and neoplastic ganglion cells manufacture the intermediate filament of neurons, NFP (Rössmann et al., 1983). Undifferentiated, rapidly proliferating tumours, such as PNET's, that were thought to be derived from stem cells were shown to express a broad range of neuroepithelial proteins; focal labelling with antibodies to GFAP, synaptophysin, and NFP was assumed to reflect the pluripotential nature of the neoplastic cells (Cruz-Sanchez et al., 1991b; Kleinert, 1991; Maraziotis et al., 1992). These findings were considered to uphold the principle that dedifferentiation, of which immunophenotype is one measure, occurs along developmental lines (Rössmann et al., 1983; Rubinstein, 1986; Russell and Rubinstein, 1989; Fung and Trojanowski, 1995).

The presence of GFAP in various neuroepithelial tumours, such as oligodendrogliomas and ependymomas, as well as astrocytomas was not inconsistent with this principle because of the transient appearance of GFAP in the progenitors of glial cells (Cameron and Rakic, 1991). GFAP was thus the marker for gliomas (VandenBerg, 1992; McLendon and Bigner, 1994), and NFP was the marker for neuronal tumours. Other neuronal markers, such as synaptophysin, were considered equally specific (Miller et al., 1990). The ganglioglioma was the only tumour at the border between these groups, and its components labelled in the expected manner: ganglion cells were NFP-positive, and astrocytic cells were GFAP-positive (Diepholder et al., 1991).

As new antibodies targeted to neuronal proteins were developed and then tested on neuroepithelial tumours, gliomas were used primarily as controls in which no labelling could be expected (Gould et al., 1987; Kleinert, 1991). Only small numbers of astrocytic tumours or oligodendrogliomas were generally used for this purpose, and no glioma was initially reported to label positively with the range of neuronal antibodies used in the present study. However, when immunohistochemistry with extensive panels of antibodies was used to elucidate the nature of some rare neuroepithelial tumours, examples of tumours, apart from PNET's, were discovered that simultaneously manufacture GFAP and neuronal proteins (Hirose et al., 1992; VandenBerg, 1992; Ellison et al., 1993). The central neurocytoma and desmoplastic infantile ganglioglioma are notable among these, both for behaving benignly, in contrast to PNET's, and for containing synaptophysin-positive tumour cells that do not have the morphology of ganglion cells (von Deimling et al., 1990; Louis et al., 1992). Such tumours raise difficulties for conventional classifications. Should these tumours be classed as gliomas (as they were before immunohistochemistry), or neuronal tumours, or put into an intermediate category with the ganglioglioma?

Molecular genetic abnormalities underlie the uncontrolled proliferation of neoplastic cells; but, though these bring an abnormal clone of cells to clinical attention by producing a tumour, disruption of other cellular functions, not vital to a neoplastic cells survival, may result from separate, concurrent faults in the genome. As tumour progression occurs, these abnormalities accumulate and are responsible for alterations in the cytoskeleton that produce cytological atypia, and for the invasive behaviour of tumour cells. The expression of neuron-specific proteins in astrocytic tumours and oligodendrogliomas may be of prognostic or, possibly, therapeutic value if it is linked with the dysfunction of other genes that affect growth or behaviour.

4.2.2 Subgroups of cerebral astrocytic tumours and oligodendrogliomas express neuron-specific proteins

The demonstration of neuronal proteins in some gliomas with the histological features of astrocytic tumours and oligodendrogliomas, and of a neuronal immunophenotype in 35% of high grade astrocytic tumours from patients who had survived 2 years beyond diagnosis, prompted the following hypotheses:

(i) cerebral astrocytic tumours that express neuronal proteins have a better prognosis than standard tumours

(ii) some tumours with a histological diagnosis of oligodendroglioma express neuronal proteins

All the gliomas in the present study showed histological features typical of the WHO categories to which they were assigned, yet labelling of a proportion of cells in cerebral astrocytic tumours and oligodendrogliomas with antibodies to neuronal proteins has clearly been demonstrated. A smaller proportion of astrocytic tumours than oligodendrogliomas expressed neuronal proteins, but the astrocytic tumours labelled with a wider range of neuronal antibodies.

In support of the present findings, an immunohistochemical study of 24 gliomas with antibodies to various intermediate filaments demonstrated labelling of tumour cells in 1 anaplastic astrocytoma and 1 glioblastoma with an antibody to NFP (Hirato et al., 1994). In a separate study of childhood astrocytomas, 6 / 14 tumours labelled with an antibody to NFP (Bodey et al., 1990). No attempt was made to establish the prognostic significance of these findings, but small numbers of tumours in both studies would have impaired such analysis.

Expression of neuronal proteins in astrocytic tumours is a phenomenon that coincides with tumour progression; none of the fibrillary astrocytomas labelled with any neuronal antibody. The principle that the extent of aberrant protein expression parallells cytological anaplasia is therefore observed in these tumours. The cells of fibrillary

astrocytomas have the appearance of astrocytes, express GFAP, and show mild to moderate pleomorphism. As anaplastic transformation occurs, the cells appear less like astrocytes and may stop manufacturing GFAP. Aberrant expression of neuronal proteins occurs in a small proportion of tumours once this step has been reached.

Though dedifferentiation to a point where tumour cells adopt the immunophenotype of primitive neuroepithelial cells is considered to characterise oncogenesis in PNET's (Fung and Trojanowski, 1995), this may not necessarily represent the end-point of dedifferentiation in all neuroepithelial tumours; protein expression may just become more disorganised in astrocytic tumour cells as more genetic abnormalities are acquired.

The basis of neuronal protein expression in astrocytic tumours is undetermined. Some growth factors, such as epidermal growth factor, platelet-derived growth factor (PDGF), and insulin-like growth factor-1, promote the expression of a neuronal immunophenotype and a neuronal morphology in cultured neuroepithelial cells (Pahlman et al., 1991; Heasley and Johnson, 1992; Reynolds and Weiss, 1992). Overexpression of these growth factors is a feature of some neuroepithelial tumours (Libermann et al., 1985; Mapstone et al., 1991; Hurtt et al., 1992; von Deimling et al., 1992a; Sandberg-Nordqvist et al., 1993), and may account for the concurrent up-regulation of neuronal proteins and down-regulation of GFAP in some high grade astrocytic tumours.

The present study shows that no benefit to patient management is derived from subdividing astrocytic tumours according to the presence or absence of neuronal proteins; the clinical characteristics of the subgroups, including survival, were almost identical. Though neuronal protein expression was confined to high-grade tumours in this study of cerebral astrocytic tumours, this phenomenon cannot be used with confidence to grade gliomas from which only small biopsies have been taken; the existence of several slowly growing neuroepithelial tumours that exhibit dual expression of GFAP and neuronal proteins precludes this.

Synaptophysin positivity was found in equal proportions of standard and anaplastic oligodendrogliomas. That a neuronal immunophenotype in these tumours is not associated with the development of anaplasia was also supported by similar Ki-67 LI's for the subgroups with and without neuronal immunophenotype. Though expression of synaptophysin may possibly be switched on in oligodendrogliomas in response to the overexpression of growth factors that promote neuronal differentiation, an alternative possibility is that the cerebral tumours composed of small round cells and classified as oligodendrogliomas are actually heterogeneous. The synaptophysin-positive tumours should probably be considered cerebral neurocytomas; a proposal supported by the close morphological and immunohistochemical similarities between the tumours in the present study and the intraventricular neurocytoma.

The oligoastrocytoma consists of cells with astrocytic and oligodendroglial morphology. By (arbitrary) definition (Burger and Scheithauer, 1995), at least 25% of the tumour must show a separate glial morphology to the rest of the tumour. None of the tumours selected for the present study fulfilled the criteria for a diagnosis of oligoastrocytoma or anaplastic oligoastrocytoma; however, small foci of oligodendroglial differentiation were noted in some glioblastomas, and small foci of astrocytic differentiation were noted in some oligodendrogliomas. The presence of some synaptophysin-positive foci of oligodendroglial differentiation in glioblastomas proves only that an overlap of morphophenotypes in gliomas may sometimes be matched by appropriate immunophenotypes. It does not necessarily imply a continuum that encompasses all astrocytic tumours and oligodendrogliomas and along which exists an intermediate variant, the oligoastrocytoma. Though such a relationship could be proposed, a wider range of neuronal protein expression in astrocytic tumours sets them apart from oligodendrogliomas, and might suggest the existence of several divergent paths of dedifferentiation in these tumours.

Further evidence that astrocytic tumours are distinct from oligodendrogliomas or oligoastrocytomas comes from molecular genetic studies; the abnormalities found in oligoastrocytomas align them with oligodendrogliomas rather than cerebral astrocytic tumours. Oligoastrocytomas and oligodendrogliomas frequently show partial loss of

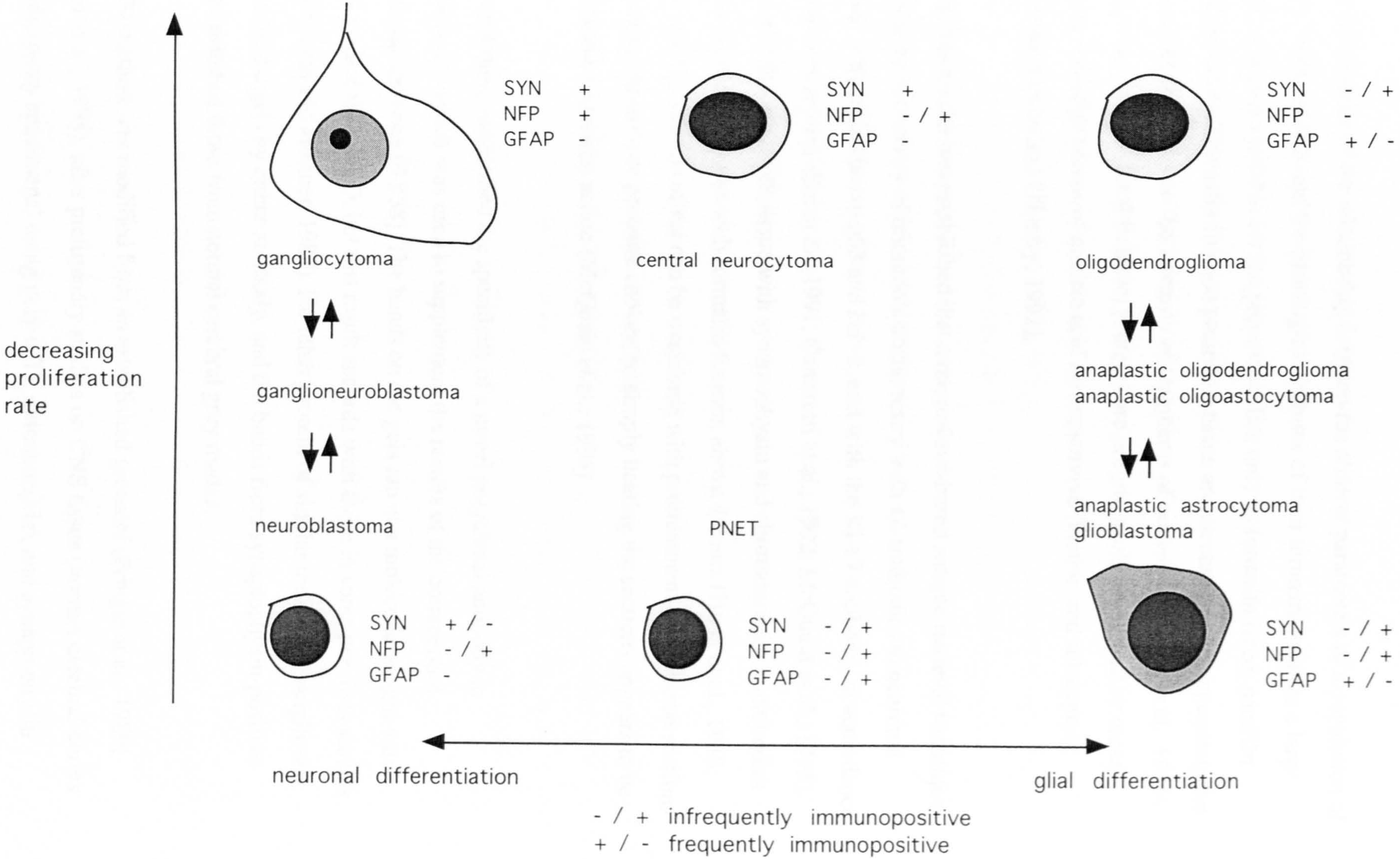
chromosomes 1p and 19q (von Deimling et al., 1992b; Bello et al., 1994), but very rarely show loss of chromosome 17p or p53 mutations (Kyritsis and Saya, 1993; Ohgaki et al., 1993). The high grade astrocytic tumours with a neuronal immunophenotype in the present study showed the same frequency of p53 mutations as other astrocytic tumours. There is thus no justification from these results for drawing together oligodendrogliomas and astrocytic tumours with a neuronal immunophenotype into a single category (of mixed neuronal - glial tumour), in the manner of oligoastrocytomas.

From the results of the present and previous studies, the following list of neuroepithelial tumours that express neuronal proteins can be compiled:

ganglion cell tumours
primitive neuroectodermal tumours, including the neuroblastoma
anaplastic astrocytomas and glioblastomas
oligodendrogliomas
neurocytomas
rare tumours: dysplastic infantile ganglioglioma
dysembryoplastic neuroepithelial tumour
subependymal giant cell astrocytoma of tuberous sclerosis

The overlap of the immunophenotypes and histological features of these tumours is outlined in Table 422.

Table 422
Tumours manufacturing neuronal proteins



- / + infrequently immunopositive
+ / - frequently immunopositive

4.2.3 Methodological aspects - immunohistochemistry and *in situ* hybridisation

Adequate analysis of the relationship between the clinical parameters of a population of patients with tumours and the histological features of their tumours requires a large number of cases, available for the present studies only as formalin fixed, paraffin embedded tissue. Formalin fixation preserves tissue architecture, cell components, the reactivity of proteins, and the integrity of fragments of nucleic acid (Fox et al., 1985). It is not, however, an ideal fixative; proteins are progressively enmeshed by cross-linkages, and large pieces of nucleic acid do not survive fixation and subsequent processing (Doyle and O'Leary, 1992).

Preliminary studies had established that a microwave-based antigen retrieval technique improved the sensitivity of immunohistochemistry with all antibodies to neuronal proteins, with antibodies to p53 and *bcl* -2, and with the Ki-67 antibody, in accordance with previous reports (Shi et al., 1991; Cattoretti et al., 1992; McQuaid et al., 1995). Immunolabelling of CNS tissue with synaptophysin and chromogranin-A antibodies progressively deteriorates with formalin fixation above 4 hours (Hoog et al., 1988; Munoz, 1990); but, this effect can be overcome with pretreatment of the tissue sections in a microwave oven or pressure cooker, so simply heating the sections appears to be the common, beneficial action (McQuaid et al., 1995).

Immunoblotting established the specificity of a novel polyclonal antibody to synaptophysin which was used to supplement the results of the commercial monoclonal antibody (SY38). The bands on the gels ran at a molecular weight value slightly greater than 38kD, but this result accords with those in some previous studies (Wiedenmann and Huttner, 1989). No other protein of similar molecular weight was labelled on the gels by either antibody, and the bands from synaptophysin-positive tumours matched those from normal cerebral grey matter.

The ISH method was modified from an established protocol (Pringle et al., 1989; Kendall et al., 1993), after preliminary studies on CNS tissue (normal cerebral cortex from lobectomy specimens) using poly-d(T), mitochondrial, and synaptophysin

probes. The elements of any ISH method are:

selection of probe and label

preparation of the tissue section

hybridisation and post-hybridisation stringency washes

visualisation of the probe

Oligonucleotide (< 100 bases) probes were preferred to larger probes, such as riboprobes, because they penetrate tissue sections well; though the degree of this advantage depends on the nature of the tissue, fixative, and pretreatment regimens (Wilkinson, 1992). Oligonucleotide probes are significantly less sensitive than riboprobes because more labels can be incorporated into the latter. Increased sensitivity can be achieved with oligonucleotide probes by using a cocktail of probes complementary to different regions of the nucleic acid of interest; this produced a significant improvement in signal : noise ratio in the present study. The short length of oligonucleotide probes necessitates a computer search to identify possible mismatches of probe to spurious sequences of mRNA.

Non-isotopic labels were preferred to isotopic labels because they are less expensive and avoid the need for special laboratory facilities that are subject to particular safety regulations. They also give better cellular resolution, and avoid the background activity that can make the detection of specific signal from isotopic probes difficult where copy number is low (Emson and Gait, 1992). There is some loss of sensitivity when using non-isotopic rather than isotopic labels, but this difference can be reduced if digoxigenin is used instead of biotin (Morris et al., 1990; Komminoth, 1992). End-labelling is desirable with oligonucleotide probes because the haptens interfere very little with hybridisation.

Pretreatment of sections is required for unmasking nucleic acid sequences within fixed tissues. This was the most critical step of the method because the optimal amount of protein digestion was different for each block of tissue, and needed to be determined quite precisely, in advance, to avoid non-specific binding of probes. A very narrow

range of protein digestion produced optimal results for each block of CNS tissue, and relatively small concentrations of proteinase K were required; a ten-fold difference was sometimes found between the concentrations required in this study and the concentrations required for other tissues (Larsson and Hougaard, 1990; Pringle et al., 1990; Fleming et al., 1992; Kendall et al., 1993). All biopsies in the study had been fixed in formalin for 24 - 48 hours before processing according to standard laboratory practice, yet the optimum concentrations of proteinase K might vary by five-fold between blocks of tumour tissue. Some studies have identified considerable variation in the preservation of RNA species in human CNS samples (Ross et al., 1992); though control sections of cerebral cortex worked consistently well with the same protocol and little variation in proteinase K concentration. Therefore, the use of reagents of variable quality in the 15 years over which the biopsies were taken is also a possible explanation for this phenomenon (Koshiba et al., 1993).

Optimal conditions for hybridisation and post-hybridisation stringency washes were established in preliminary studies on cerebral cortex. The objective of these steps is to achieve a high level of specific binding of probe to target sequences during hybridisation that will not be disrupted too much by washes that remove probe attached non-specifically to sections. The precise determinants of hybridisation in tissue sections are not understood, and many studies that recommend certain conditions have an empirical basis (Cox et al., 1984; Wilkinson, 1992). The kinetics of hydrogen bond formation between nucleic acids in a hybridisation solution (denoted by the melting temperature, T_m) are influenced by a number of factors, including:

length of probe	-	longer probes form more stable hybrids
GC content	-	the greater the GC content, the higher the T_m
composition of the hybridisation and washing solutions		

For DNA-RNA hybrids, the following formula, which only applies to nucleic acids in solution, predicts the T_m:

$$T_m = 79.8 + 18.5 \lg (\text{molarity of monovalent cations}) + 0.58 (\%\text{GC content of probe})$$
$$+ 0.0012 (\%\text{GC content of probe})^2 - 820 / (\text{probe length in bases}) - 0.5 (\%\ \text{formamide})$$

The molarity of monovalent cations, %GC content of probes, and length of probes were all constant. The optimal concentration of formamide for use with poly-d(T), poly-d(A) and mitochondrial probes had been established in previous studies (Pringle et al., 1989; Hilton et al., 1994). The optimal concentration of formamide for use with cocktails of synaptophysin probes was determined empirically on sections of cerebral cortex; but complied with the principle that the hybridisation temperature should be 10 - 20^{o}C below the T_m as calculated by the above formula (Pringle et al., 1990).

Experiments on the composition, temperature, and duration of the post-hybridisation washes revealed that formamide was an absolute requirement, in contrast to the experience of other groups (Thomas et al., 1993b).

Experiments with different detection methods established the combination of anti-digoxigenin alkaline phosphatase conjugated antibody and BCIP / NBT as most sensitive for detection of the labelled probes. Confirming results in other studies (Morris et al., 1990; Breitschopf et al., 1992; Fleming et al., 1992; Komminoth, 1992), this combinations performance was superior to methods that employed streptavidin-biotin / fast-red (Talbot et al., 1989), and streptavidin-biotin / aminoethylcarbazole (Herrington et al., 1992). The present method also circumvents the problem of cross-reactivity with endogenous streptavidin binding sites (Morris et al., 1990).

No mRNA was detectable in a large proportion of the cases, despite prolonged attempts to optimise conditions for its visualisation. The stability of mRNA postmortem is relatively good (Walker and McNicol, 1992), and polyadenylated mRNA and synaptophysin mRNA were readily demonstrated by the present method in postmortem human spinal cord. The presence of mRNA in some blocks only at the tissues edge and in a perivascular distribution suggests that rapid fixation is necessary to stabilise mRNA in a biopsy specimen; these are sites that will come quickly into contact with the formalin. Fixation of biopsy specimens is quite often delayed for a variety of reasons, including the time required for a perioperative, frozen section diagnosis. This and the time taken for the fixative to penetrate solid tumour tissue may account for the mRNA-negative cases. If lysosomes in the tissue are damaged and

RNAases are consequently active in the tissue, then significant destruction of mRNA could take place before adequate penetration of the fixative (Ross et al., 1992).

Synaptophysin mRNA was demonstrated in only about half the cases in which synaptophysin protein and mitochondrial or polyadenylated mRNA had been found. Either the sensitivity of the ISH method was insufficient to detect small quantities of synaptophysin mRNA in the negative cases (especially if a proportion of this mRNA had degraded prior to fixation), or the synaptophysin protein had been exogenously acquired. Given that synaptophysin mRNA was demonstrated in some synaptophysin-positive tumours, the former explanation appears more reasonable.

4.3 Prognostic indicators in cerebral astrocytic tumours

The results of the present study of prognostic indicators in cerebral astrocytic tumours match those of previous reports which show that age and histological diagnosis are the most important independent indicators of survival (Byar et al., 1983; Medical Research Council Brain Tumour Working Party, 1990). Preoperative (Karnovsky) performance score is also an independent prognostic indicator for cerebral astrocytic tumours, but was not evaluated in the present study. Other variables, such as duration of symptoms, tumour size, extent of surgical resection, and radiotherapy protocol have all been advanced as prognostic indicators in some, but not every study, and there independence is equivocal (Medical Research Council Brain Tumour Working Party, 1990; Kaplan, 1993). The prognostic value of immunohistochemistry with a polyclonal Ki-67 antibody and an antibody to the protein product of the tumour suppressor gene, p53, was tested in the present study alongside clinical parameters.

4.3.1 Ki-67 as a prognostic indicator

An increasing median Ki-67 LI was found across the range of astrocytic tumours from fibrillary astrocytoma to glioblastoma. Other studies have shown an increasing mean Ki-67 LI across the same spectrum of cerebral astrocytomas (Zuber et al., 1988; Louis et al., 1991; Jaros et al., 1992; Onda et al., 1994). In the present study, a clear difference in survival was demonstrated between patients with astrocytic tumours showing a Ki-67 LI of less than 2%, and patients with astrocytic tumours showing a Ki-67 LI of more than 2%, the significance of which was lost after adjusting for other variables. That this reflects the close association between histological features accompanying low and high Ki-67 LI's and histological features used for classifying astrocytic tumours is supported by the distribution of fibrillary astrocytomas and glioblastomas across the Ki-67 LI categories. Even though the use of Ki-67 LI as a truly objective measure of astrocytic tumour type is precluded by the overlap of Ki-67 LI's across the histological spectrum, the association between Ki-67 LI, tumour type, and prognosis makes Ki-67 immunohistochemistry a valuable adjunct to the histological diagnosis of astrocytic tumours. Biopsies of cerebral astrocytic tumours

obtained by stereotactically guided needles have become commonplace, and immunohistochemistry with Ki-67 may aid the histological interpretation of this limited material.

Ki-67 has been used to assess the growth fraction of many types of tumour, and some studies have also examined its value as a prognostic indicator (Hall et al., 1988; Brown and Gatter, 1990). While there appears to be a correlation between Ki-67 labelling and survival in low-grade lymphomas (Hall et al., 1988), no such correlation exists for several types of carcinoma (Cole et al., 1992; Kubota et al., 1992). Only two studies have examined the relationship between Ki-67 LI and survival in astrocytic tumours (Jaros et al., 1992; Torp et al., 1992). Both showed a significant difference between the survival of groups divided by Ki-67 LI's above and below 5% and 1.5% respectively. Only small numbers of patients were included in these studies.

Immunohistochemistry with Ki-67 antibodies on paraffin sections has been facilitated by microwave-based antigen retrieval techniques, and is now the method of choice for assessment of a tumours growth fraction (Rose et al., 1994). It has clear benefits over the mitotic count which is not a standardised method, and is more straightforward than assessment of the proportion of cells in S-phase by thymidine or bromodeoxyuridine labelling (Quinn and Wright, 1990). A popular alternative to Ki-67 antibodies has been the PC10 antibody to proliferating cell nuclear antigen (PCNA) which is the polymerase ∂ accessory protein (Bravo et al., 1987). Levels of this protein rise in S-phase. Though its popularity was founded on its ability to label cells in paraffin sections at a time when Ki-67 was restricted to frozen sections, its capacity to overestimate the growth fraction was recognised as a significant limitation (Scott et al., 1991). PCNA expression is regulated at the mRNA level, and may be stabilised by overexpression of growth factors, such as PDGF. PDGF is frequently overexpressed in glioblastomas (Pantazis et al., 1985), and may account for the lack of correlation between Ki-67 and PC10 labelling indices in these tumours (Ellison and Weller, 1992).

4.3.2 p53 as a prognostic indicator

Following the publication of studies that have reported a correlation between p53 immunohistochemistry and survival in patients with malignant melanomas and some carcinomas (Quinlan et al., 1992; Barnes et al., 1993; Bosari et al., 1993; McGregor et al., 1993; Thomas et al., 1993a), the value of p53 immunohistochemistry in predicting outcome in a range of cerebral astrocytic tumours was examined. In agreement with several previous reports (Jaros et al., 1992; Haapasalo et al., 1993; Karamitopoulou et al., 1993; Louis et al., 1993; Danks et al., 1995), this study of 123 cases has shown that nuclear accumulation of the p53 protein occurs in about two thirds of anaplastic astrocytomas and glioblastomas, and that high p53 LI's are not a common feature of p53-positive fibrillary astrocytomas, but tend instead to be found in anaplastic astrocytomas and glioblastomas. Despite the latter association, no relationship was found in the present study between patterns of p53 immunolabelling and survival; either for all astrocytic tumours together, or for each type of astrocytoma alone.

In a study by Jaros and others (Jaros et al., 1992), a small difference was detected between the survival curves for patients with p53-positive (undefined) and p53-negative astrocytic tumours; patients with p53-positive tumours had a shorter median survival. Similar results have been reported in a study of glioblastomas in which p53 LI's above and below 5% distinguished positive and negative tumours respectively (Soini et al., 1994). When the present data are analysed using this distinction, no difference in survival between positive and negative tumours is revealed.

The present results agree with those in a study by Chozick and others who examined the range of cerebral astrocytic tumours and showed no independent association between p53 protein accumulation and survival (Chozick et al., 1994). A recent study of anaplastic astrocytomas alone also showed no association between patterns of p53 accumulation and survival (Danks et al., 1995).

- Accumulation of p53 protein in tumour cells can be multifactorial, and is not only a consequence of p53 mutation (Vogelstein and Kinzler, 1992; Anker et al., 1993; Louis

et al., 1993; Lang et al., 1994). In cells with damaged DNA, levels of wild-type p53 increase to immunohistochemically detectable levels as part of the reparative processes in which p53 participates by halting the cell cycle in G_1 (Lane, 1992). Increased levels may also occur when wild-type p53 is bound to overexpressed quantities of cellular proteins, such as MDM-2. In normal cells, there is a fine balance between the levels of these two proteins which can regulate each other, but excess MDM-2 produced by amplification of the *mdm* -2 gene inhibits the function of wild-type p53 (Momand et al., 1992; Wu et al., 1993).

Accumulation of p53 protein in the absence of p53 gene mutations is a feature of a variety of tumours, and only about 50% of the astrocytomas that exhibit p53 accumulation contain a detectable p53 gene mutation to explain it (Louis et al., 1993; Newcomb et al., 1993; Lang et al., 1994). Overexpression of the *mdm* -2 gene is, however, rare in astrocytomas, and cannot account for this discrepancy (Newcomb et al., 1993; Reifenberger et al., 1993; Rubio et al., 1993). The abnormal regulation of other, as yet undefined, proteins to which p53 binds may be responsible for its increased stability. An increase in wild-type p53 in response to the DNA damage that accumulates progressively in the range of astrocytomas is an alternative explanation (von Deimling et al., 1993).

Inactivation of p53 may follow p53 gene mutation or the binding of p53 protein to other proteins (Vogelstein and Kinzler, 1992). Studies on p53-deficient mice that develop normally but are prone to develop neoplasia have demonstrated that, while p53 regulates the cycle of stressed cells, its presence is not crucial for embryogenesis (Donehower et al., 1992). However, inactivation of the p53 protein might be responsible for the replication of DNA errors that promote the mutational activation of oncogenes and a subsequent increase in cell proliferation. Two studies have looked at the relationship between p53 labelling and growth fraction in astrocytomas. Haapasalo and others have reported that, across all grades of astrocytoma, p53-positive tumours have a higher growth fraction than p53-negative tumours, as assessed by immunolabelling with an antibody against PCNA (Haapasalo et al., 1993). Jaros and others found that p53-positive astrocytomas of all grades had a higher Ki-67 LI than

p53-negative astrocytomas (Jaros et al., 1992). Some association between the pattern of p53 expression and growth fraction as assessed by immunohistochemistry with a Ki-67 antibody was observed in the present study. Tumours in the p53 LI > 60% category showed a higher median Ki-67 LI than the other 2 categories. However, this phenomenon was *not* translated into shorter survival.

The status of other tumour suppressor genes may be more critical to the progression of astrocytic tumours and to the survival (and treatment) of patients. Loss of heterozygosity (LOH) on chromosome 13q is found in 30 - 50% of anaplastic astrocytomas and glioblastomas, but in very few fibrillary astrocytomas (Fults et al., 1990). The retinoblastoma gene is located at 13q14 and about 20% of astrocytic tumours with LOH at 13q also harbour a retinoblastoma gene mutation (Henson et al., 1994). Homozygous or heterozygous losses of chromosome 9p are present in 30 - 50% of astrocytomas (James et al., 1991). The *MTS1* (*CDKN2*) gene has recently been mapped to 9p, and encodes p16, a cell cycle regulatory protein that binds to CDK4 and inhibits CDK4 / cyclin D phosphorylation of retinoblastoma protein (Serrano et al., 1993). While the majority of glioma cell lines show homozygous deletions involving *MTS1* , mutations of this gene are rare in astrocytic tumours themselves (Ueki et al., 1994). Recent evidence suggests that allelic inactivation of *MTS1* by methylation of an upstream CpG island, which is in turn caused by deregulation of an imprinter gene, can produce dysfunction without the necessity of a mutation in the *MTS1* gene (Little and Wainwright, 1995). This mechanism may operate to disrupt the activity of p16 in gliomas, and may be involved in the development of anaplasia in astrocytomas (Little and Wainwright, 1995). LOH of chromosome 19q occurs in about one third of anaplastic astrocytomas, but in less than 10% of fibrillary astrocytomas; detailed mapping studies suggest that a tumour suppressor gene may reside at 19q13.3 (Yong et al., 1995). Whether the presence of any of these molecular genetic abnormalities correlates with prognosis is yet to be determined.

4.3.3 Methodological aspects - immunohistochemistry

A reassuring aspect of the present immunohistochemical analysis with Ki-67 and DO-7 antibodies is that the results match those of previous studies undertaken by myself on frozen sections of cerebral astrocytic tumours (Ellison et al., 1992; Ellison and Weller, 1992). Though the addition of a microwave pretreatment step had greatly enhanced labelling with both antibodies in paraffin sections, the question of whether the target antigens were completely revealed to give results equivalent to those with frozen sections remained unanswered.

Median Ki-67 LI's were slightly lower for anaplastic astrocytomas and glioblastomas in paraffin sections than frozen sections when the present and previous studies were compared, but the range of Ki-67 LI's for each type of tumour was similar (Ellison and Weller, 1992). A similar proportion of each type of astrocytic tumour was p53-positive in the present study and a previous study using 5 antibodies to p53 on frozen sections (Ellison et al., 1992). This observation is backed up by a study that compared p53 staining in frozen and paraffin sections of gliomas and colorectal carcinomas (Bruner et al., 1993). No labelling of paraffin sections was seen in the absence of labelling in frozen sections, and the converse was true for no colorectal tumours and only a small proportion of gliomas.

4.4 Apoptosis in cerebral astrocytic tumours

In recent years, as much emphasis has been placed on apoptosis as a determinant of cell growth as on cell proliferation (Williams, 1991; Carson and Ribeiro, 1993; Cohen, 1993; Wyllie, 1993). A combined effect on these two processes appears to be a property of several oncogenes, such as *myc* (Harrington et al., 1994), implying that a tumours growth depends on a fine balance of regulatory forces that favours cell proliferation over cell death. An increased understanding of this regulatory balance may provide an insight into how more specific anti-tumour therapies may be developed.

4.4.1 Apoptosis and tumour progression in cerebral astrocytic tumours

A significant difference in the median number of apoptotic cells exists between the 3 categories of astrocytic tumour. Apoptotic bodies were rarely seen in fibrillary astrocytomas which have a low growth fraction as assessed by the Ki-67 antibody. Higher AI's in anaplastic astrocytomas and glioblastomas were associated with progressively higher median Ki-67 LI's, and a significant correlation between these 2 measures was confirmed.

In some normal, differentiated tissues and in the developing organism, cell proliferation which may be sustained by growth factors is inversely correlated with apoptosis which is the natural response of some tissues to withdrawal of these factors (Kerr et al., 1994). However, previous studies have shown that growth fraction and apoptosis are positively correlated in several types of malignant tumour (Kerr and Lamb, 1984; Leoncini et al., 1993; Harrington et al., 1994), and that AI increases with dedifferentiation of tumours (Kerr and Lamb, 1984). A correlation between these measures, whether positive or negative, is not invariably found; for example, no relationship was shown between AI and indices of proliferation in medulloblastomas (Schiffer et al., 1994).

A cellular mechanism that obligatorily links cell proliferation and apoptosis in some tissues could provide a powerful, inbuilt system for suppressing neoplastic transformation; but, its control must become deregulated in tumours. When this occurs, cooperation among dysfunctioning oncogenes may promote tumour formation and progression. For example, overexpression of c-*myc* produces both increased proliferation and increased sensitivity to apoptosis (Evan et al., 1992); but, it becomes a strong stimulus to neoplastic growth in the setting of overexpression of *bcl* -2 or PDGF, both of which can inhibit apoptosis (Harrington et al., 1994).

4.4.2 Apoptosis and upregulation of *bcl* -2 and p53

The hypothesis that AI is related to expression of the *bcl* -2 protein in astrocytic tumours was not substantiated. Apoptosis is inhibited by the upregulation of *bcl* -2 in several cell systems (Korsmeyer, 1992; McDonnell et al., 1993), and although AI's were lower in *bcl* -2 positive than in *bcl* -2 negative astrocytic tumours, this difference was not significant (P = 0.48). Just over one third of astrocytic tumours in the series labelled with the *bcl* -2 antibody. Examples of *bcl* -2 positive tumours were present in each histological category of astrocytic tumour, and the ratio of positive to negative tumours was greater for astrocytomas than glioblastomas. Similar observations have recently been reported in a study of nervous system tumours that additionally demonstrated *bcl* -2 labelling of reactive astrocytes, and vascular endothelial proliferation (Nakasu et al., 1994).

It is now evident that *bcl* -2 is upregulated in many tumours apart from lymphomas, and that the t(14;18) translocation is not necessary for this upregulation (Pezzella et al., 1990). Some carcinomas of the lung (Pezzella et al., 1993), breast (Silvestrini et al., 1994), and prostate (McDonnell et al., 1992), and many neuroblastomas are labelled with *bcl* -2 antibodies (Castle et al., 1993; Ramani and Lu, 1994); but it is still unclear how the expression of *bcl* -2 is related to the behaviour of many tumours. In breast carcinomas, strong *bcl* -2 immunolabelling correlates positively with oestrogen receptor expression, and inversely with indicators of poor prognosis, such as necrosis, and p53 and epidermal growth factor receptor immunolabelling (Leek et al., 1994;

Lipponen et al., 1995). However, accumulation of *bcl* -2 is not an independent prognostic indicator when analysed alongside p53 immunolabelling (Silvestrini et al., 1994), and, as in the present study, shows only a trend towards an effect on apoptosis (Lipponen et al., 1995). The expression of *bcl* -2 in high grade B-cell lymphomas does not correlate with survival, but accumulation of the p53 protein is associated with a poor prognosis. Patients with tumours that are labelled with both *bcl* -2 and p53 antibodies have a significantly poorer prognosis than patients with tumours that are p53-positive (Piris et al., 1994). There is some association between *bcl* -2 status and survival in non-small cell carcinoma of the lung (Pezzella et al., 1993), but whether this relates to any effect of *bcl* -2 on apoptosis is undetermined.

The role of *bcl* -2 in the control of cell maturation and terminal differentiation is related to its inhibition of apoptosis (Garcia et al., 1992; Lu et al., 1993; Merry et al., 1994). Upregulation of *bcl* -2 was observed in reactive astrocytes, in multinucleated giant cells within otherwise *bcl* -2 negative glioblastomas, and in the capillary endothelial proliferation that characterises glioblastomas and some anaplastic astrocytomas. A trend for ubiquitous *bcl* -2 labelling in astrocytic tumours that contain cells with abundant cytoplasm and eccentric nuclei ("gemistocytes") was also noticed. Whether these phenomena represent the capacity of certain types of cell to protect themselves from promoters of cell death by upregulation of *bcl* -2 is yet to be determined.

No clear relationship was found between AI and patterns of p53 accumulation in cerebral astrocytic tumours. In agreement with previous studies, p53-positive cells were found in approximately two thirds of astrocytic tumours (Ellison et al., 1992; Louis, 1994). When all astrocytic tumours were grouped together, there was an association ($P = 0.06$) between the p53 LI > 60% category and a high AI. Such a result was not found for glioblastomas alone; thus, the association found when astrocytic tumours are analysed together may primarily reflect the different proportions of tumours from each histological group in the 3 categories of p53 immunolabelling. Evidence that p53 might regulate apoptosis comes from finding that the reintroduction of wild-type p53 into p53-negative tumour cells triggers apoptosis (Yonish-Rouach et al., 1991). However, p53 cannot be a critical element of all pathways leading to

apoptosis, because p53-negative transgenic mice develop normally (Lane et al., 1994), and the cytocidal effects of glucocorticoids are independent of p53 status (Lowe et al., 1993). Also, apoptosis occurs in tumour cells with dysfunctioning p53; so, while p53 may play a role in the death of cells with DNA damage, other factors are certainly important and may mask any effect that p53 might have on apoptosis in astrocytic tumour cells.

About half of the p53-positive tumours in the present series were *bcl* -2 positive, whereas only one fifth of the p53-negative tumours was *bcl* -2 positive. This finding accords with the proposal that up-regulation of *bcl* -2 is an advantage in those tumours where increased levels of wild-type p53 protein reflect the promotion of apoptosis in response to DNA damage, and not required in tumours with disabled (mutant) p53. This proposal was advanced in a recent study of astrocytic tumours that demonstrated up-regulation of *bcl* -2 in 16 / 28 tumours (Alderson et al., 1995). A p53 mutation was found in 8 / 28 tumours, but only one of these was *bcl* -2 positive.

The complexity of the situation militates against establishing a unifying hypothesis from the results. Apoptosis is regulated by factors other than p53 and *bcl* -2, such as *ras* , *bax* , and c-*myc* (Harrington et al., 1994; Stewart, 1994), and a reciprocal relationship exists between the regulatory functions of p53 and *bcl* -2 (Miyashita and Reed, 1993; Chiou et al., 1994), and between the actions of *bcl* -2 and *bax* (Miyashita et al., 1994). We have not found the reciprocity between *bcl* -2 and p53 protein expression that appears to exist in carcinomas of the thyroid gland (Pilotti et al., 1994). Well differentiated thyroid carcinomas and normal thyroid epithelium express the *bcl* -2 protein; however, it is not detected in undifferentiated tumours in which p53 frequently accumulates.

4.4.3 Methodological aspects - the identification of apoptotic cells

Apoptotic cells were identified in this study by their characteristic histological features, such as crescents of collapsed chromatin along the nuclear envelope, and fragmentation of the nucleus into spheres (Cohen, 1993). Cells destined to die by apoptosis exhibit

biochemical and ultrastructural changes before they show nuclear condensation or fragmentation (Carson and Ribeiro, 1993; Cohen, 1993). The cells that were counted in the present study are therefore in the later stages of apoptosis, and represent an unknown proportion of the total number of cells destined for apoptosis (Stewart, 1994). *In situ* end-labelling to detect internucleosomal DNA fragmentation identifies cells at a slightly earlier stage of apoptosis, but may not demonstrate many more cells than are found using conventional histological criteria (Ansari et al., 1993). Furthermore, evidence suggests that *in situ* end-labelling is neither entirely specific because necrotic cells are detected (Ansari et al., 1993; Migheli et al., 1994), nor very sensitive (Stewart, 1994). An assessment of the extent to which apoptosis might retard tumour growth must relate AI's to the duration of apoptosis. Estimates for the duration of apoptosis vary and are tissue specific. None has been calculated for astrocytic tumours; however, in some situations, the duration of apoptosis is short enough for AI's below 1% to reflect a profound effect of cell death on tumour growth (Kerr and Lamb, 1984; Arends et al., 1994).

4.5 Future directions

4.5.1 Neuronal expression in gliomas

The existence of a subgroup of cerebral astrocytic tumours expressing neuronal proteins has been confirmed in this study; but the designation of a new class of neuroepithelial tumour is not warranted because distinctive clinical features were not demonstrated for this subgroup. A neuronal immunophenotype in cerebral astrocytic tumours therefore shares with other diverse histological characteristics, such as lipidisation and desmoplasia (gliosarcoma), or the rare expression of cytokeratins, the property of having no prognostic significance in astrocytic tumours.

The significance of synaptophysin immunoreactivity in about one third of oligodendrogliomas requires assessment in a further study. A large series of patients with oligodendrogliomas would be required to test the hypothesis that a neuronal immunophenotype in these tumours is associated with distinctive biological activity. In addition, the presence of GFAP and synaptophysin immunoreactivity in tumours, the cells of which show none of the histological characteristics of oligodendrocytes, prompts reclassification of these tumours, perhaps as cerebral neurocytomas.

A study of oligoastrocytomas is also warranted. At present, these tumours are aligned with oligodendrogliomas on the basis of shared molecular genetic abnormalities. As the proportion of astrocytic differentiation increases and the proportion of oligodendroglial differentiation decreases in these tumours, is there a switch in the range of neuronal protein expression, if present, to parallel the switch in molecular genetic abnormalities?

4.5.2 Prognostic indicators

Attempts to find immunohistochemical prognostic indicators in cerebral astrocytic tumours using antibodies to Ki-67 and p53 have shown that there is a close relationship between Ki-67 LI and histological subtype, the latter remaining the

principal determinant of prognosis in these tumours. The close association between Ki-67 LI and histological subtype of astrocytic tumour suggests that immunohistochemistry with the Ki-67 antibody would aid the diagnostic process in small cerebral biopsies such as those obtained using stereotactic surgery. As the number of tumours examined by this technique increases, Ki-67 LI could be compared with conventional histological assessment as a prognostic indicator.

The tumour suppressor gene p53 has achieved a remarkable prominence in cancer research over recent years because it shows abnormalities in many types of tumour. Mutation of the p53 gene, frequently in association with loss of heterozygosity at the p53 gene locus, occurs early in the evolution of cerebral astrocytic tumours. Several causes of p53 protein accumulation in these tumours are recognised, and may explain the lack of prognostic significance found in this study for different patterns of p53 accumulation.

Progression of fibrillary astrocytomas to anaplastic astrocytomas is associated with loss of portions of chromosomes 9p and 19q, and with abnormalities of the retinoblastoma gene on 13q, and the presence of one or more of these genetic abnormalities may prove to be of prognostic significance. Identifying the genes on chromosomes 9p and 19q responsible for this tumour progression will be particularly important in the next few years.

4.5.3 Apoptosis

Since the start of the present study of apoptosis in cerebral astrocytic tumours, improved *in situ* end labelling (ISEL) methods for the assessment of cells committed to apoptosis have been described, and could be used in conjunction with a study of newly found regulators of apoptosis that show homology with *bcl* -2, such as *bax* , and *bcl* -x. The results of the present study indicate that regulation of apoptosis in cerebral astrocytic tumours is complex. Any progress towards promoting apoptosis in anaplastic astrocytomas and glioblastomas as a therapeutic measure will require identification of the important regulators of apoptosis in these tumours, and how any

dysfunction of this regulation might enhance tumour growth.

Normal p53 production and function are required for the surveillance and correction of DNA damage in cells. While no significant relationship was found between patterns of p53 accumulation and AI in cerebral astrocytic tumours, further study of p53 status and apoptosis is warranted in patients receiving radiotherapy. Response to radiotherapy is dependent upon normal p53 status which will induce apoptosis in cells that have sustained irreparable, radiotherapy induced DNA damage, and the relationship between p53 gene status, p53 protein accumulation, apoptosis, and survival could be studied in these patients.

Appendix

2.1.1 E.O.R.T.C. trials

E.O.R.T.C. trials of adjuvant therapy for malignant gliomas:

26751

Evaluation of CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea), VM-26 (4-dimethyl-epipodophyllotoxine-D-therylidine-glucoside), and procarbazine in supratentorial brain gliomas (EORTC Brain Tumour Group, 1981).

26781

Misonidazole in radiotherapy of supratentorial malignant brain gliomas in adult patients: a randomised double-blind study (EORTC Brain Tumour Group, 1983).

26812

Adjuvant chemotherapy followed by radiotherapy versus radiotherapy alone in brain gliomas (EORTC Brain Tumour Group, 1987).

26851

Effect of cisplatinum given during radiation therapy in patients with supratentorial malignant brain gliomas (EORTC Brain Tumour Group, 1991).

All of these trials had shown no benefit for the treatment regimens.

2.3.2 Immunohistochemical method

Coating of slides with 3-aminopropyltriethoxysilane

(i) Immerse slides in 1% Decon 90 for 30 minutes.

(ii) Wash in running tap water for 30 minutes.

(iii) Wash in ultra-pure water: 2 x 5 minutes.

(iv) Wash in 95% ethanol: 2 x 5 minutes.

(v) Air dry.

(vi) Coat slides in freshly prepared 2% 3-aminopropyltriethoxysilane in dry acetone for 5 seconds.

(vii) Rinse quickly twice in dry acetone.

(viii) Wash twice in DEPC-treated water.

(ix) Air dry at 42°C overnight.

(x) Store boxed at room temperature.

Tris buffered saline (TBS)

(i) Make up 0.05 M Tris and adjust to pH 7.6 with hydrochloric acid.

(ii) Dissolve NaCl and $MgCl_2$ hexahydrate to give concentrations of 0.15 M and 2 mM.

(iii) Add 0.1% bovine serum albumin.

2.4.2 Immunoblotting

Suspension buffer

Mix reagents to the following concentrations:

100 mM NaCl

10 mM Tris-HCl buffer (pH 7.6)

1 mM EDTA (pH 8.0)

100 µg / ml phenylmethylsulfonylfluoride (PMSF)

Gel loading buffer (3 x SDS)

Mix reagents to the following concentrations:

100 mM Tris-HCl buffer (pH 6.8)

200 mM dithiothreitol

6% SDS (electrophoresis grade)

0.2% bromophenol blue

20% glycerol

SDS polyacrylamide gel (12.5%)

Mix the following reagents, adding the 10% ammonium persulphate and TEMED (polymerising agents) just before pouring the gel:

40% acrylamide	9.375 ml
1% bis-acrylamide	3.1 ml
3 M Tris (pH 8.7)	3.75 ml
20% SDS	0.15 ml
deionised water	13.275 ml
10% ammonium persulphate	200 µl
TEMED	20 µl

Coomassie protein gel stain

Make up the following to 1 litre with deionised water:

methanol	500 ml
glacial acetic acid	50 ml
Coomassie brilliant blue	250 mg

Blotting buffer

Make up the following to 3 litres with deionised water:

Tris base	7.26 g
glycine	33.78 g
methanol	600 ml

Stock 10 x phosphate buffered saline (10 x PBS)

(i) Make up a solution containing:

1.3 M NaCl

70 mM NaH_2PO_4

30 mM Na_2HPO_4 (dihydrate).

(ii) Store at room temperature.

Diaminobenzidine solution

Add 5 mg diaminobenzidine to 10 ml of 50 mM Tris-HCl (pH 7.6)

2.5.1 Probe manufacture

A cocktail of probes combines more than one oligonucleotide antisense or sense probe.

Antisense probes are complementary to the mRNA of interest. Sense probes are complementary to the antisense probes and therefore have the same sequence as the target sequence of mRNA (except that T replaces U). Sense probes control for the non-specific binding of labelled oligonucleotides to nucleic acids; but this does assume that the sense probes are not, by chance, complementary to specific sequences in unrelated strands of mRNA.

Antisense and sense probes to 9 different regions of synaptophysin mRNA were constructed after a computer search of a human gene database had identified sequences with:

a GC content of 50% - 65%
no potential for the formation of hair-pin loops
a maximum of 3 identical bases in sequence
no adenine or thymine based nucleoside at the end of the sequence
no known correspondence to other human mRNA

The 9 probes are complementary to the underlined sequences in the following sequence of synaptophysin cDNA from the EMBL data library.

The number of the first base of each line is provided.

Synaptophysin cDNA

```
1       cagttccggg  tggtcaagga  gcccctcggc  tttgtgaagg  tgctgcaatg  ggtcttcgcc
61      atcttcgcct  ttgccacatg  cggcagctac  agtggggagc  tccagctgag  cgtggattgt
121     gccaacaaga  ccgagagtga  cctcagcatc  gaggtcgagt  tcgagtaccc  cttcaggctg
181     caccaagtgt  actttgatgc  acccacctgc  cgaggggcca  ccaccaaggt  cttcttagtt
241     ggggactact  cctcgtcagc  cgaattcttt  gtcaccgtgg  ccgtgtttgc  cttcctctac
301     tccatggggg  ctctggccac  ctacatcttc  ctgcagaaca  agtaccgaga  gaataacaaa
361     gggcccatgc  tggactttct  ggccacggct  gtgttcgcct  tcatgtggct  agttagctca
421     tcggcatggg  ccaaggggct  gtcagatgtg  aagatggcca  cagacccaga  gaacattatc
481     aaggagatgc  ctgtctgccg  ccagacaggg  aacacatgca  aggagctgag  agaccctgtg
541     acctcgggac  tcaacacctc  ggtggtgttc  ggcttcctga  acctggtgct  ctgggtcggc
601     aacctgtggt  tcgtgtttaa  ggagacaggc  tgggccgccc  cgttcctgcg  cgcgcctccc
661     ggcgcccccg  agaaacaacc  ggcacccggg  gacgcctacg  gcgatgcagg  ctacgggcag
721     gggcccggcg  ggtacgggcc  ccaggattcc  tacgggcctc  agggcggcta  ccagcctgac
781     tatggtcaac  cagccggcag  cggtggcagt  ggctacgggc  ctcagggcga  ctatgggcag
841     caaggctacg  gcccgcaggg  tgcacccacc  tccttctcca  atcagatgta  gtctggtcag
901     tgaagcccag  gaggacctgg  gggggcaag   agcctcagga  gaaggcctgc  ccccttccc
961     acccctatac  cctaggtctc  cacccctcaa  gccaggagac  cctgtctttg  ctgtttatat
1021    atatatatat  tatatataaa  tatctattta  tctgtctgag  ccctgccctc  actccactcc
1081    cctcatccac  taggtgccca  gtcttgagtg  ggcccctct   cttaccccgt  ccctttccct
1141    gcatcccttg  gcccctctct  gtttaccctc  cctgtccct   gaggttaagg  ggatctaaaa
1201    ggaggacagg  gagggaacag  acctcggctg  tgtggggagg  gtgggcgtga  cttcagactc
1261    tctcctctct  ctccctccac  tcctcccaac  tctggccttg  gttcctccag  caatgatgcc
1321    tgaacaaagg  ccgttaggaa  atccaactcc  agggttaaag  aaaggccaga  gattgggggg
1381    gcttgggggg  gcttggggta  gagaggacag  tttaggaccc  aaggtggtgt  tggagaggag
1441    gtgtggagtg  gaggggtcag  caggggggtt  gggttccaga  cagagtggat  ctggagtctg
1501    aaggagagga  gtgcgctaga  gcattctggg  gtggggcttg  gaagggcgct  gagggcaggg
1561    ttctagaagg  ggcgaggctt  taagcgaggc  agaatggtgg  gctccagagt  aggtgggtct
1621    tggattggta  ccagagccta  tggaaagggt  gtggcttgga  acatttggga  gactgagctt
1681    gattctaaag  gggacagatc  ttgagcaagg  caagaagtgg  gattcaggaa  tgggccaagc
1741    cagggttcca  gacagggtgg  ggcttagaat  ggggcttcca  tggtggtttc  agaaagggca
1801    gcccctcccc  atggtgcagt  gaagaaaatg  ttttacaatg  gctgggtttg  ggcagtggag
1861    aggggacttg  gataggagct  tccagatggg  ttttgttagg  ggtgggggag  aatggctctg
1921    gctacgactt  gggacggaag  tggcctgaga  agagtcgagt  gatatggctt  gtagggtgag
1981    gcgtgggatc  cagagagaag  caccccacca  cacacaccct  tccccactcc  cgtgatgaaa
2041    cagctaggtt  aataggagga  cagaaccaac  gggtctgtgg  gactggccca  cccctcttcc
2101    cccttccccc  gcgcctcccc  ccctccacac  ctccacccgt  cctggggtgg  ttggaggcct
2161    ggctctggag  cccctat
```

2.5.2 Labelling of probes

The effect of altering some variables in the tailing reaction on the incorporation of digoxigenin into probes was assessed by test blots. The following were compared:

50 μM digoxigenin-dUTP	25 μM digoxigenin-dUTP
50 u. of TdT	25 u. of TdT
$MnCl_2$	$CoCl_2$

50 μM digoxigenin-dUTP and 50 u. of TdT gave better labelling of probes.

$CoCl_2$ was equivalent to $MnCl_2$.

A 3-OH tail combining digoxigenin-dUTP and dATP gave no advantage over a short 3-OH tail composed only of digoxigenin-dUTP when synaptophysin probes labelled in each manner were compared on sections of cerebral cortex. Most of the labelled probes used in the study of tumours had only the short tail.

2.5.4 Pretreament of sections for ISH

RNAase-free water for ISH solutions:

(i) Dissolve 0.1% diethylpyrocarbonate (DEPC) in ultra-pure water.

(ii) Let the solution stand overnight.

(iii) Autoclave.

(iv) Use DEPC water for solutions in all steps of the protocols up to and including hybridisation.

Tris base is destroyed by DEPC, but DEPC is inactivated by a period in the autoclave. Tris buffers must therefore be made from pretreated water. DEPC may be added to other buffers which may then be processed as above.

Treated glassware:

(i) Rinse glassware in 95% ethanol.

(ii) Soak in 3% hydrogen peroxide for 10 minutes.

(iii) Rinse in DEPC water.

(iv) Dry in a dust free environment.

Glassware can be baked overnight at 180°C as an alternative to the above.

Stock 20 x standard saline citrate (20 x SSC)

(i) Make up a solution of 3 M sodium chloride and 0.3 M tri-sodium citrate.

(ii) Adjust to pH 7.0 with 1 M sodium hydroxide.

Paraformaldehyde (0.4%) in PBS

(i) Dissolve paraformaldehyde to a concentration of 4% in 10 x PBS by heating to 60°C and agitating.

(ii) Add sodium hydroxide to the cloudy solution until it just begins to clear.

(iii) Allow the solution to cool, and then filter it.

(iv) Dilute 1:10 with DEPC water.

Pretreatment steps for ISH

The following steps that unmask and fix mRNA in preparation for hybridisation were assessed to determine optimal conditions for the pretreatment of CNS tissue:

- to solubilise basic proteins:

  0.2 N HCl for 20 minutes

- to unravel the secondary structure of mRNA:

  2 x SSC at 70°C for 10 minutes

- to remove cross-linking of proteins:

  proteolytic digestion at 37°C for 60 minutes

- post-fixation:

  0.4% paraformaldehyde in PBS at 4°C for 20 minutes

Acid digestion before proteolytic digestion adversely affected sections of normal cerebral cortex and tumour biopsies, producing an uneven signal from poly-d(T) and mitochondrial probes. However, the signal in sections of postmortem spinal cord which had been fixed in formalin for 4 - 6 weeks was not decreased when this step was included.

Proteolytic digestion was the most critical step in the method. The following enzymes were tested, and gave similar results: proteinase K, protease XXIV, and protease VIII. Lower concentrations of enzyme than previously reported (Pringle et al., 1989; Larsson and Hougaard, 1990; Fleming et al., 1992; Wilkinson, 1992) for formalin-fixed biopsy material were required to produce an optimal signal in cerebral cortex and tumour biopsies. Too much enzymatic digestion produced increased non-specific binding, and ultimately disrupted the sections. Each tissue block had to be tested with a range of enzyme concentrations to find the best pretreatment conditions; and optimal requirements sometimes appeared to vary not only between tumour biopsies ostensibly processed in the same manner, but across the block. A hot wash in SSC and post-fixation in paraformaldehyde were essential pretreatment steps for CNS tissue.

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2.5.5 Hybridisation

Stock 10 x modified PE

(i) Make up 0.5 M Tris-HCl buffer (pH 7.5) and 50 mM disodium EDTA with 1% sodium pyrophosphate, 2% polyvinylpyrrolidone (MW 40,000) and 2% Ficoll (MW 400,000).

(ii) Dissolve by heating to 65°C.

(iii) Maintain solution at 65°C for 15 minutes.

(iv) Store at room temperature.

Sheared salmon sperm DNA (ssDNA)

(i) Add 100mg of ssDNA to 10ml of ultra-pure water.

(ii) Dissolve by rotating on a roller mixer overnight at 4°C.

(iii) Sonicate in an ultrasound bath using 30 cycles of 30 seconds each. Plunge the solution into an ice-bath between cycles.

(iv) Aliquot into 100µl quantities, and store at -20°C.

The composition of the prehybridisation (minus probes) and hybridisation (plus probes) solutions was modified with some improvements from that described in previous reports (Pringle et al., 1989; Pringle et al., 1990).

Dextran sulphate has been used by several groups as an accelerator to improve the rate and efficiency of hybridisation (Breitschopf et al., 1992; Fleming et al., 1992; Komminoth, 1992), and gave cleaner results than polyethyleneglycol in sections of cerebral cortex.

The addition of sheared DNA from salmon sperm gave reduced non-specific binding, and several concentrations were tested to find an optimal concentration for CNS tissue of 0.5 mg / ml.

A critical element of the hybridisation solution was the concentration of formamide. As this was increased, the signal : noise ratio first increased until all non-specific binding with a given set of stringency washes was eliminated, and then decreased as specific signal was reduced. Optimal concentrations of formamide for the poly-d(T) and poly-d(A) probes (20%), and the cocktail of mitochondrial probes (30%) were predetermined, but the formamide concentration for the cocktail of synaptophysin probes (20%) was established by examining signal : noise ratios in cerebral cortex. An optimal signal : noise ratio was also used to determine the concentration of all probes in the hybridisation mixture, once the composition of this had been established.

Synaptophysin antisense and sense probes that had been well labelled as assessed by test blots were used in combinations (cocktails). Combinations of 5 probes gave reliable, strong, specific signals in cerebral cortex and spinal cord. Cocktails with more than 5 probes did not appreciably enhance the signal. Successful cocktails that were subsequently used for ISH runs on tumour sections were:

A3, A5, A6, A7, and A9

A4, A6, A7, A9, and A10

Coating of coverslips in dimethyldichlorosilane:

(i) Immerse in 1% Decon 90 for 30 minutes.

(ii) Wash in running tap water for 30 minutes.

(iii) Wash in ultra-pure water: 2 x 5 minutes.

(iv) Wash in 95% ethanol: 2 x 5 minutes.

(v) Air dry.

(vi) Dip coverslips in 2% dimethyldichlorosilane in 1,1,1-trichloroethane, and allow to dry.

(vii) Rinse twice in DEPC-treated water, and dry overnight at room temperature.

(viii) Store boxed at room temperature.

2.5.6 Stringency washes

The stringency of a washing solution may be increased by reducing the salt concentration, raising its temperature, or by adding formamide. Multiple washes may be performed over varying lengths of time and at different temperatures. Several protocols have been described for this crucial stage (Cox et al., 1984; Pringle et al., 1989; Fleming et al., 1992; Komminoth, 1992; Wilkinson, 1992). The following variables were assessed in various combinations:

SSC	0.5 x, 1 x, 2 x†
temperature	37°C†, 43°C, 52°C
formamide	0% - 30%†
duration of wash	5 mins.†, 10 mins., 60 mins.

Triple washes were used for each run, and all wash solutions had been heated to temperature before use.

Altering the above variables affected specific and non-specific signal in a predictable manner, and the labelled (†) options were chosen for optimal stringency. With sections of cerebral cortex, it proved impossible to eliminate non-specific signal entirely without the use of formamide. With 30% formamide in the washes, low salt concentrations, high temperatures, and prolonged washes were not required to remove non-specific binding.

2.5.7 Detection of labelled probe

An alternative detection method that has been reported to enhance the signal from digoxigenin labelled probes was assessed (Herrington et al., 1992).

Sections were first incubated for 30 minutes in a solution of monoclonal antidigoxin antibody (also active against digoxigenin) at a concentration of 1:3000 in TBT (50 mM Tris-HCl; 100 mM NaCl, pH 7.2; with 3% bovine serum albumin and 0.05% Triton-X 100). After 2 washes in TBS for 10 minutes each, a biotinylated anti-mouse antibody to F(ab')2 fragments (1:200 in TBT) was applied to the sections for 30 minutes. Further washes preceded the application of avidin-peroxidase (1:75 in TBT with 5% w:v Marvel™) for 30 minutes. The substrate was aminoethylcarbazole.

This modification produced no discernible advantage.

TBS blocking solution

(i) On the day before use, add 2.9% bovine serum albumin and 0.1% Triton X-100 to TBS.

(ii) Filter.

Tris-HCl buffer

(i) Make up a fresh solution with preprepared reagents.

(ii) Mix the reagents as follows:

1 M Tris-HCl buffer at pH 9.5	10ml
1 M magnesium chloride hexahydrate	5ml
5 M sodium chloride	2ml
ultra-pure water	83ml

2.5.8. Controls for *in situ* hybridisation (ISH)

Suitable controls for ISH ensure that the signal visualised in tissue sections truly indicates the presence of target nucleic acid:

(i) No probe

This excludes the nonspecific visualisation by digoxigenin antibody of molecules other than labelled probe. It also acts as a control for the presence of native alkaline phosphatase. A section of every tissue block to which no probe had been applied was included in each ISH run.

(ii) Tissue known to contain target message

Entrapped neurons were occasionally seen at the edges of some tumour sections, and these acted as an internal control for the synaptophysin antisense probes. Control cerebral cortex or spinal cord was included in all ISH runs to act as an external control for the synaptophysin probes.

(iii) Tissue without target message

The endothelial cells of blood vessels in the sections of tumours, and glia in cerebral cortex and spinal cord acted as controls without target message.

(iv) RNAase pretreatment

In preliminary studies, RNAase (Sigma type 1A) pretreatment of sections of cerebral cortex and a small selection of tumour biopsies abolished signals from all the probe cocktails.

(v) Probes identical to target sequence (sense probes)

Poly-d(A) and synaptophysin sense probes were included whenever poly-d(T) and synaptophysin antisense probes were used respectively.

(vi) Poly-d(T) or mitochondrial probes to assess abundance of mRNA / rRNA

These were included in every ISH run.

(vii) Competitive inhibition with unlabelled probe

Preliminary studies established that a 5x concentration of unlabelled probe abolished the specific signal.

2.6.2 Polymerase chain reaction (PCR)

PCR buffer:

10 mM Tris-HCl

50 mM KCl

0.02% gelatin

2.6.5 Gene sequencing

DNA elution buffer:

500 mM ammonium acetate

10 mM magnesium acetate

1 mM EDTA, 0.1% SDS

3.1.6 Non-neoplastic conditions and *bcl* -2 labelling

In a preliminary study, labelling of the following non-neoplastic conditions with the antibody to *bcl* -2 was assessed:

infarction
demyelination
arteriovenous malformation
congophilic angiopathy
encephalitis
bacterial abscess

Enlarged, reactive astrocytes with abundant cytoplasm, a radial array of processes, and eccentric nuclei showed a moderate degree of *bcl* -2 labelling in all of these conditions.

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