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# Immunocytochemistry of neuronal and glial markers in retinoblastoma

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**Summary.** An immunocytochemical study of 30 retinoblastomas was carried out using antibodies to neuronal and glial markers. The tumours were found to react with antibodies to neuron-specific enolase (NSE), a marker for neuronal elements, and S-100 and glial fibrillary acidic protein (GFAP), both of which are proteins present in glia. Two distinct cell populations were found within the tumour: the first, composed of anaplastic tumour cells at various stages of differentiation, showed both NSE and S-100 immunoreactivity; the second cell type, which immunostained for S-100 and GFAP, resembled mature glial cells. The results of this study indicate that the retinoblastoma may arise from a pluripotential primitive cell partially retaining neuronal and glial characteristics.

**Key words:** Immunocytochemistry – GFAP – NSE – Retinoblastoma – S-100

Retinoblastoma is an ocular malignant tumour which generally develops in infancy (Sang et al. 1982; Gallie et al. 1983). Histologically the tumour presents many embryonic features, with constituent cells showing different degrees of anaplasia and differentiation (Tso 1980; Sang et al. 1982). Despite numerous morphological and biochemical investigations (see Tso 1980 for review) the histogenesis of this tumour is still controversial.

Virchow (1864) proposed a glial cell origin of the tumour, a view which was later shared by other authors (Parkhill et al. 1941; Dunphy 1964). A different school of thought proposed an origin from neuronal cells, particularly on the basis of the ultrastructural similarities between tumour and normal retinal cells (Tso et al. 1969, 1970 and 1980; Albert et al. 1970; Popoff et al. 1971; Reid et al. 1974). A similar conclusion was reached fol-

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lowing immunocytochemical studies using antibodies to either glial fibrillary acidic protein (GFAP) (Lane et al. 1983) or regulatory peptides (Sano et al. 1981; Tarkkanen et al. 1983), which have also been shown to be present in glial elements and amacrine cells of normal retina, respectively (Dixon et al. 1981; Stell et al. 1980; Lane et al. 1983). However, a definite answer to the problem has not yet been reached.

In recent years immunocytochemistry has been successfully applied in the study of neuroendocrine and glial tumours. Neuron-specific enolase (NSE), a reliable marker for all elements of neuroendocrine origin (Wharton et al. 1981; Marangos et al. 1982; Bishop et al. 1982; Sheppard et al. 1983) has been found to be present in different types of neuroendocrine tumours (Tapia et al. 1981; Sheppard et al. 1983; Gu et al. 1983). Antibodies to S-100 and glial fibrillary acidic protein (GFAP), two proteins present in glia, have successfully been used to characterise glial cells in normal and pathological tissues (Perez et al. 1970; O'Dowd et al. 1979; Bignami et al. 1980; Dixon et al. 1981; Ferri et al. 1982; Tascos et al. 1982; Terenghi et al. 1983; Cocchia et al. 1983; Lane et al. 1983; Holland et al. 1983).

In an attempt to clarify the histogenesis of retinoblastomas, we have studied several cases of retinoblastoma by immunocytochemistry, using antibodies to both neuronal and glial markers.

## Materials and methods

Thirty cases of retinoblastoma were investigated. Surgically enucleated eyes containing the tumour were fixed in formalin and embedded in wax. 5 μm sections were cut and collected on poly-L-lysine coated slides (Huang et al. 1983). Following dewaxing, the sections were immunostained according to the peroxidase/antiperoxidase (PAP) method (Sternberger 1979) using antibodies to NSE (Marangos et al. 1979) (dil. 1/400 to 1/800), S-100 (Cocchia et al. 1981) (dil. 1/1600) and GFAP (Dahl et al. 1976) (dil. 1/500). In all cases, immunostaining with the above antibodies was also carried out on sections which underwent trypsin digestion (Huang et al. 1976) for 30 min prior to immunocytochemistry.

Controls for the immunostaining were carried out using antisera pre-absorbed with specific antigens (NSE: 0.5 nmol/ml; S-100: 1 nmol/ml diluted antiserum). The antiserum to GFAP had been previously characterised and found to be specific for glial cells (Dahl et al. 1976). A portion of normal retina was also present with some of the tumours; in these cases the retina was used as a positive internal control for the immunostaining of neuronal and glial elements. As a furhter control of immunostaining specificity, immunocytochemistry was also carried out on formalin-fixed samples of ganglioneuroblastomas, paragangliomas and astrocytomas, using the same antibodies and methods as for the retinoblastomas.

## Results

Strong positive immunostaining for NSE, S-100 and GFAP was seen in numerous cells in all the retinoblastomas studied. The characteristics of the tumours and the results of the immunostaining are summarised in Table 1. The tumours were classified histologically according to Rorke (1983) as undifferentiated (type 1) or differentiated (type 5). Positive immunostaining for neuronal and glial markers was also obtained in elements of normal retina (Fig. 1, 2 and 3). Further confirmation of the specificity of the immunostaining was given by the negative results obtained with preabsorbed

**Table 1.** Characteristics and positive immunostaining of the retinoblastomas investigated

| Case No. | Histology[a] | Necrosis[b] | NSE+ve[c] | | S-100+ve[c] | | GFAP+ve[c] | |
|---|---|---|---|---|---|---|---|---|
| | | | ret. | tum. | ret. | tum. | ret. | tum. |
| 1 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 2 | Undiff. | − | + | + | + | + | + | + |
| 3 | Diff. | ++ | n.p. | + | n.p. | + | n.p. | + |
| 4 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 5 | Undiff. | ++ | + | + | + | + | + | + |
| 6 | Undiff. | − | n.p. | + | n.p. | − | n.p. | − |
| 7 | Diff. | + | + | + | + | + | + | + |
| 8 | Undiff. | ++ | n.p. | + | n.p. | − | n.p. | − |
| 9 | Diff. | + | + | + | + | + | + | + |
| 10 | Diff. | + | + | + | + | + | + | + |
| 11 | Undiff. | + | n.p. | + | n.p. | + | n.p. | + |
| 12 | Undiff. | ++ | + | + | + | + | + | + |
| 13 | Undiff. | ++ | n.p. | + | n.p. | + | n.p. | + |
| 14 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 15 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 16 | Undiff. | + | + | + | + | + | + | + |
| 17 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 18 | Diff. | ++ | n.p. | + | n.p. | + | n.p. | + |
| 19 | Undiff. | ++ | n.p. | + | n.p. | − | n.p. | − |
| 20 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 21 | Diff. | + | + | − | + | − | + | − |
| 22 | Undiff. | + | n.p. | + | n.p. | + | n.p. | + |
| 23 | Diff. | + | + | + | + | + | + | + |
| 24 | Undiff. | + | + | + | + | + | + | + |
| 25 | Undiff. | + | n.p. | + | n.p. | + | n.p. | + |
| 26 | Undiff | − | n.p. | + | n.p. | + | n.p. | + |
| 27 | Undiff. | + | n.p. | + | n.p. | − | n.p. | − |
| 28 | Diff. | + | n.p. | + | n.p. | + | n.p. | + |
| 29 | Diff. | + | + | + | + | + | + | + |
| 30 | Diff. | + | + | + | + | + | + | + |

[a] Differentiated or undifferentiated tumour
[b] + = necrotic tumour; ++ = very necrotic
[c] Positive immunostaining present in normal retina (ret.) and/or tumour (tum.); np = not present

antisera. Ganglioneuroblastomas and paragangliomas showed positive immunostaining for NSE. Positive immunostaining for glial markers, but not for NSE, was observed in astrocytomas.

When the same immunostaining was compared in sections with or without trypsin digestion, a noticeable improvement was seen in the trypsin-treated sections. In particular, GFAP-positive cells appeared to be more numerous and more strongly immunostained. For NSE and S-100, the intensity of immunostaining was only slightly improved. Within the tumour, the positive immunostaining for NSE, S-100 and GFAP was seen in morphologically distinct subpopulations of cells.

NSE-positive cells were seen in all but one (case no 21) of the retinoblas-

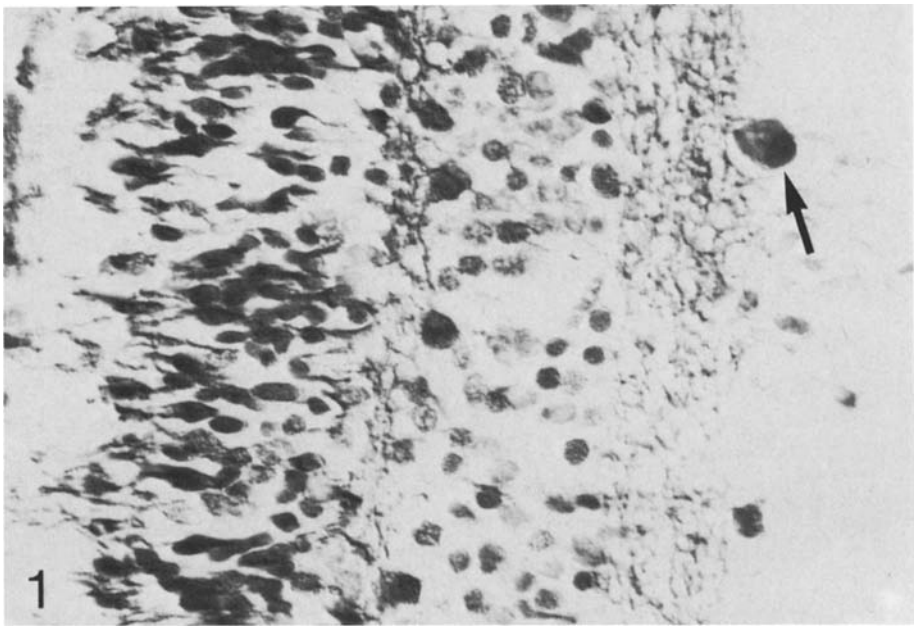


**Fig. 1.** NSE-immunoreactivity in normal retina: positive immunostaining can be seen in the cell bodies of photoreceptors, several cells of the inner nuclear layer and in a large ganglion cell (*arrow*). Several fibres in both inner and outer plexiform layers are also immunostained for NSE. (Nomarski optics, × 760)

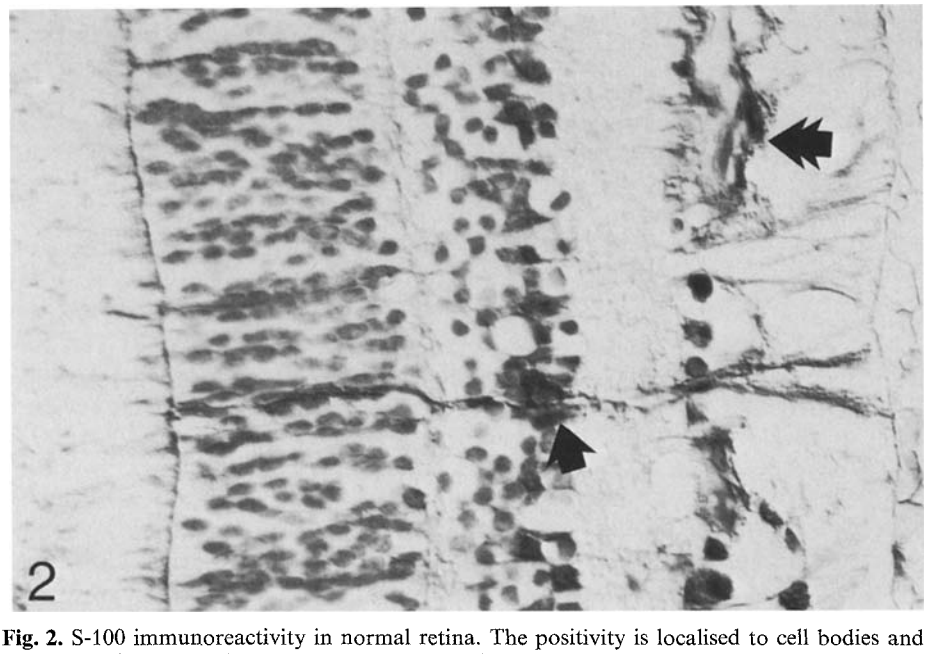


**Fig. 2.** S-100 immunoreactivity in normal retina. The positivity is localised to cell bodies and processes of both Müller cells (*single arrow*) and perivascular glia (*double arrow*). (Nomarski optics, × 760)


**Fig. 3.** GFAP-immunoreactivity in normal retina. Positive immunostaining is seen in numerous glial cells in the inner nuclear (*single arrow*) and ganglion cell layers (*double arrow*). The processes of the Müller cells extend through the full thickness of the retina. (Nomarski optics, × 760)

tomas. The cells were generally small, each with a round nucleus surrounded by scant cytoplasm. Some of these cells also showed short cytoplasmic processes, which gave the cell a club-shaped appearance (Fig. 4). The NSE-positive cells were very numerous, and could be seen either in groups of different sizes or scattered throughout the tumour. Positive staining for NSE was also seen in rosette formations whenever this cell arrangement was present in the tumour (Fig. 5). In these cases, NSE positive staining could often be observed in undifferentiated tumour cells and in rosette cells in the same microscopic field. In those areas of the retina infiltrated by the tumour, but where the retina still partially retained its original morphological organisation, NSE-positive staining was seen in the non-neoplastic retinal neuronal cells. These cells showed long axo-dendritic processes projecting into the more undifferentiated areas of the tumour, where NSE-containing tumour cells were also observed. Although the neuronal processes could extend deeply into the tumour, in only one case (No. 17) did they form close connections with blood vessels accompanying the tumour growth.

S-100 positive cells were observed in 25 out of 30 retinoblastomas. Most of the tumours found to be unreactive to S-100 antibodies appeared histologically to be of the undifferentiated type, generally with extensive necrotic areas. Numerous S-100-containing cells showed morphological characteris-

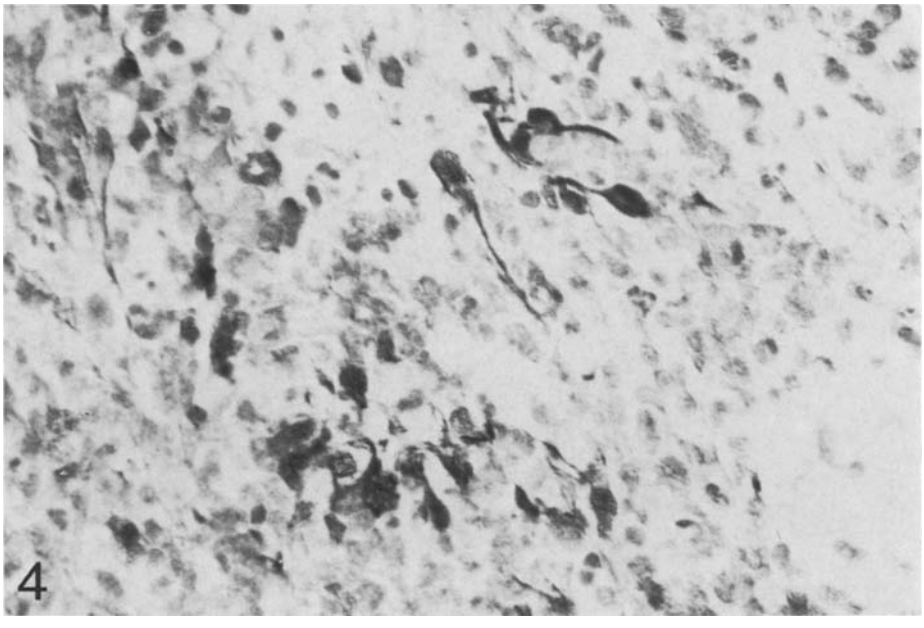


**Fig. 4.** NSE-immunoreactivity in retinoblastoma. The positive tumour cells are small in size with scant cytoplasm; some cells show a short cytoplasmic process. (Nomarski optics, × 720)


**Fig. 5.** NSE-immunoreactivity in retinoblastoma. Positive staining of different intensity can be seen in cells in rosette formations (*arrows*). (Nomarski optics, × 840)

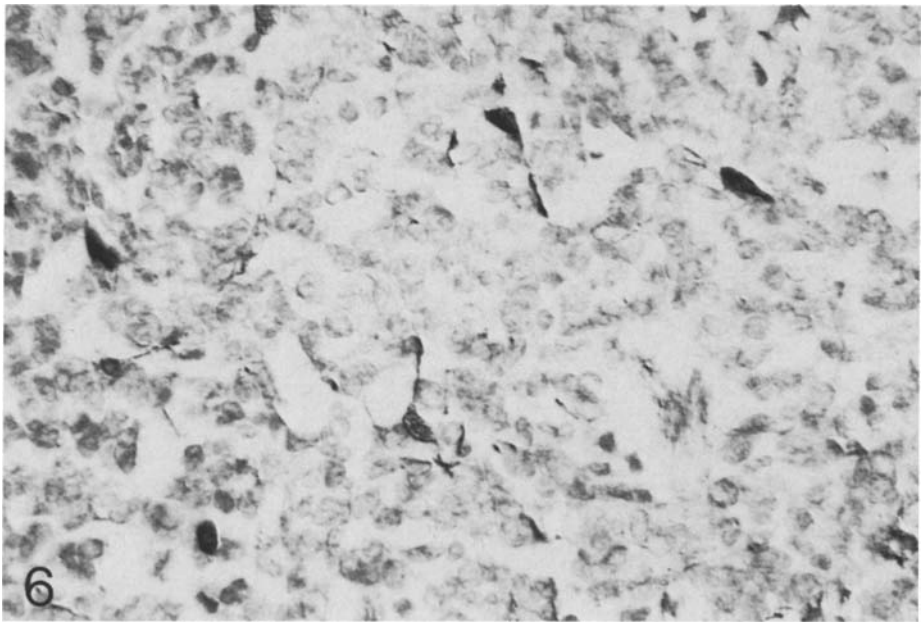


**Fig. 6.** S-100-immunoreactivity in retinoblastoma. The immunoreactive tumour cells appear morphologically similar to NSE-immunoreactive tumour cells: they are small in size and with a short cytoplasmic process. (Nomarski optics, × 720)

tics similar to those of the NSE-containing tumour cells, with a round nucleus, little cytoplasm and, occasionally, a short thick process (Fig. 6). Immunostaining for S-100 was not found in rosettes.

A separate population of cells immunostained for S-100 could also be seen interspersed between the previously described type of cells. They were spindle shaped, medium to large in size, with a round or oval nucleus, a variable amount of cytoplasm, and with long, thin cytoplasmic processes. The processes extended for a long distance between the tumour cells and, more often, surrounded the blood vessels embedded in the tumour mass (Fig. 7). S-100-containing glial cells of the normal retina projected extensive processes to the adjacent tumour areas.

Immunostaining for GFAP showed similarities to that for S-100, although the intensity of the immunostaining was greater, GFAP-containing cells were seen in 25 retinoblastomas, corresponding to those tumours also positive for S-100.

Only a single population of cells was positive for GFAP. The cells were large, with an oval or pleomorphic nucleus and also had very long cytoplasmic processes penetrating between unstained tumour cells and surrounding blood vessels (Fig. 8). Large groups of GFAP-containing cells and fibres were present throughout the tumour, particularly in proximity to the disrupted retina, where strong positivity for GFAP was seen in Muller and astroglial cells, projecting long processes into the adjacent tumour. No im-

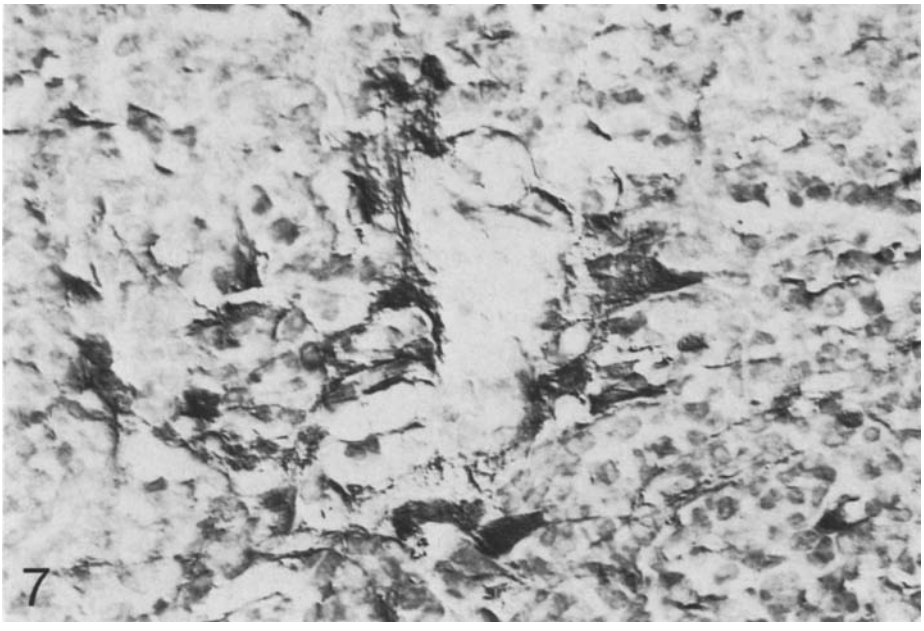


**Fig. 7.** S-100-immunoreactivity in retinoblastoma. The immunoreactive perivascular glial-type cells show large irregular cell bodies and numerous cytoplasmic processes, which surround the blood vessels. (Nomarski optics, × 720)

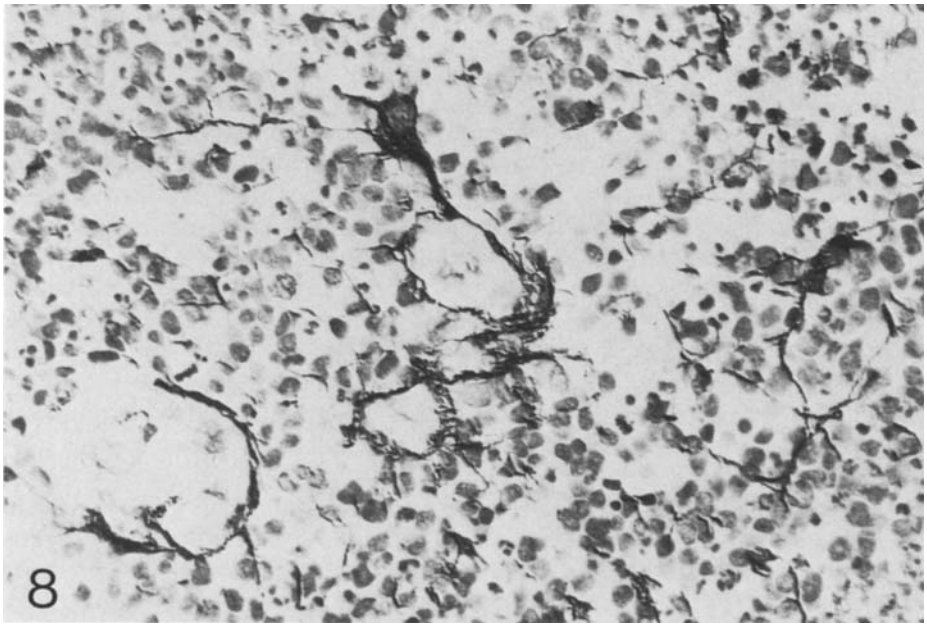


**Fig. 8.** GFAP-immunoreactivity in retinoblastoma. The very large immunoreactive glial-type cell shows long cytoplasmic processes which surround a nearby blood vessel. (Nomarski optics, × 610)

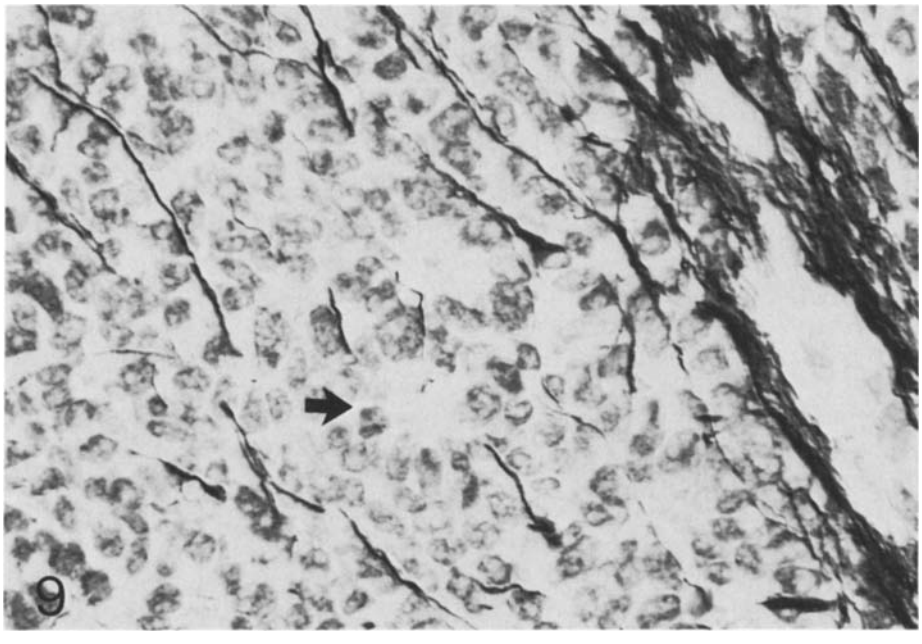

**Fig. 9.** GFAP-immunoreactivity in retinoblastoma. Several immunostained processes extend around a blood vessel and within the tumour. None of the undifferentiated tumour cells or the cells in the rosette formation (*arrow*) show any positive immunoreactivity for GFAP. (Nomarski optics, × 720)

munoreactivity for GFAP was seen in either the small undifferentiated cells or in rosettes (Fig. 9).

## Discussion

Our studies show positive immunostaining for both NSE and S-100 in tumour cells of retinoblastomas. While NSE is present in tumour cells at different stages of differentiation, antibodies to S-100 appeared to stain two distinct cell populations: one composed of undifferentiated or poorly differentiated tumour cells and the other formed by astrocyte-like cells, interspersed between the tumour cells and around the blood vessels. The immunoreactivity for NSE and S-100 in a variety of tumour cell types would be a clear indication of the capacity of the retinoblastoma cells to retain, at least partially, neuronal and/or glial characteristics. Some authors reported S-100 immunoreactivity in a wide range of tumour types, indicating that S-100 might not be a fully specific marker for tumours of glial origin (Nakamura et al. 1983; Stefansson et al. 1982). However, the immunostaining for S-100 noted in retinal glial cells, but not in neurons, seems to indicate the specificity of our antiserum towards glial elements. The pluripotentiality of retinoblastoma cells was first suggested by Ginsberg in 1899 (according to Duke-Elder 1967), and it has recently been reiterated by electron micro-

scopical studies (Ohnishi 1977; Taylor et al. 1979). The hypothesis of the tumour origin from a pluripotential undifferentiated cell is also consistent with the results of in vitro studies (Kyritsis et al. 1984), which showed primitive retinoblastoma cells to immunoreact with both neuronal and glial markers. The primitive tumour cell could possibly be the result of a de-differentiation process from mature retinal elements of either type to rapidly proliferating retinoblasts. Alternatively, it might arise from dormant embryonic stem cells in the normal retina. It is still a matter of conjecture as to whether a de-differentiation process causes the tumour development and, if so, which are the triggering circumstances. However, it is interestin to note that a viral influence has been suggested to play an important part in the onset of the tumour (Zimmerman 1970; Albert et al. 1972; Reid et al. 1974).

It was noticed in this study that in anaplastic types of retinoblastomas there was a noticeable decrease in both the number and intensity of staining of tumour cells for either NSE or S-100. A negative correlation between anaplasia and immunostaining has also been observed in other tumours of the central nervous system (Bignami et al. 1980; Tascos et al. 1982). On the other hand, NSE- but not S-100-immunoreactivity was present in cells in rosette formations, which are believed to represent a more differentiated cell type with close links to mature photoreceptor cells (Tso et al. 1969, 1970; Popoff et al. 1971).

S-100 immunoreactivity was also observed in astroglial elements within the tumour. GFAP-immunoreactivity was also present in glial cells, but not in undifferentiated tumour cells, a finding consistent with the results of Lane and Klintworth (1983). If GFAP was to be a more labile component than S-100, that could account for their different reactivities in tumour cells. It has been reported that undifferentiated samples of both normal retina and tumour of the central nervous system showed poor immunostaining for GFAP (Bignami et al. 1980; Dixon et al. 1981; Tascos et al. 1982). Conversely, S-100 and NSE have been found to be present in cells of developing tissues from early stages (Wharton et al. 1981; Sheppard et al. 1983). The staining of GFAP-positive cells is also influenced by the concentration of aldehyde-containing fixative (Dixon et al. 1981). Our investigation consistently showed a considerable improvement of GFAP immunostaining of glial elements after trypsin digestion of tissues. The immunostaining for S-100 and GFAP in similar types of mature glial elements in normal retina and retinoblastoma confirms the usefulness of these glial markers.

The number of positively stained tumour cells observed varied from case to case, often reflecting the undifferentiated and/or necrotic state of various tumours. In addition, some loss of tissue antigenicity might be due to the delay between surgery and tissue fixation.

In the tumours, it was always apparent that NSE-immunoreactive tumour cells were more numerous than S-100 immunoreactive ones. This pattern of immunoreactivity seems to reflect the situation observed in normal retina where neuronal cells, as identified by NSE antisera, have a numerical preponderance over glial cells. The persistence of this difference in immuno-

staining in the tumour suggests that biochemical and morphological differentiation of the tumour cells do not coincide, the two processes showing a different time course.

Several studies, mainly based on traditional histological or electron microscopical methods, have attempted to establish the cell of origin of retinoblastomas. By using immunocytochemical methods at the light microscopical level we have now been able to show pluri-reactivity of the tumour cells. This could be an indication that retinoblastoma might originate from a multipotential primitive cell which retains neuronal and glial characteristics and is potentially capable of differentiating in either way.

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