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Immunohistochemical demonstration of neuronal and astrocytic markers and oncofoetal antigens in retinoblastomas

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Summary. General opinion is that retinoblastomas, though not everyone agrees with that view. Some authors suggest that retinoblastomas are derived from a primitive retinal cell able to differentiate into both neuronal and glial cell lines.

The aim of the present work was to study immunohistochemically the expression of neuronal and astrocytic markers in retinoblastomas and at the same time the presence of the oncofoetal antigens carcinoembryonic antigen (CEA) and alpha Foeto Protein (AFP), since patients with retinoblastomas often show high oncofoetal antigen in serum levels. For this purpose we employed the streptavidin-biotin immunoperoxidase technique in 13 cases of retinoblastoma to evaluate the presence and distribution of neuron-specific enolase (NSE), neurofilament protein (NF), glial fibrillary acidic protein (GFAP), S-100 protein, CEA and AFP. All 13 tumours studied stained for NSE. Seven of them showed GFAP- and S-100 positive perivascular glial cells as well as cells distributed randomly in the tumour that were interpreted as non tumour cells. All 13 retinoblastomas lacked detectable NF, CEA, and AFP. These results support the idea that retinoblastomas are neuronal tumours, although retinal glial cells may become incorporated in the tumour and proliferate in response to the tumour.

Key words: Retinoblastoma, Immunohistochemistry, Neuronal and astrocytic markers, Oncofoetal antigens

Introduction

Retinoblastoma, a tumour of neuroectodermal origin, is the most frequent intraocular neoplasm in childhood (Sang and Albert, 1982), making up 1-1.5% of malignant infantile tumours (Albert, 1982). Genetic transmission is well established in some groups of patients on the basis of the multicentricity of the retinial tumours, high incidence of other tumours and the presence, in a large number of cases, of deletion in the long arm of chromosome 13 (Knudson et al., 1975; Sparkes and Sparkes, 1979).

Histologically, the majority of retinoblastomas are undifferentiated, appearing as clusters and infiltrates of small round cells with large hyperchromatic nuclei and scant cytoplasm. Differentiation of these tumours produces three characteristic cell arrangements: Homer-Wright rosettes, Flexner-Wintersteiner rosettes and fleurettes. All three are analogous to normal photoreceptor cell structures (Tsó et al., 1970; Popoff and Ellsworth, 1971; Choux et al., 1972; Dickson et al., 1976; Díaz-Flores et al., 1979).

More than a century ago, Virchow suggested that retinoblastomas originated in the supporting glial cells of the retina and designated the tumour «glioma of the retina». This theory was originally based on the assumption that neurons lose the capacity for mitosis after birth and hence should not give rise to neoplasms. Using glial cell and neuron markers, different authors have found different patterns in retinoblastomas, but the capacity of these tumours for glial cell differentiation is now, at best, controversial (Lane and Klintworth, 1983; Terenghi et al., 1984; Molnar et al., 1984; Craft et al., 1985; Messmer et al., 1985; Russell and Rubinstein, 1989). Mature astrocytes can easily be found in retinoblastomas as isolated cells or in small groups, being more numerous in the more highly differentiated parts of the tumours (Messmer et al., 1985), but most retinoblastoma cells clearly lack GFAP. Since many tumour cells lose the capacity to synthesize specific cell markers, it is nevertheless possible that retinoblastoma cells lacking GFAP during their descent from primitive retinal glial cells have lost the ability to synthesize this antigen (Lane and Klintworth, 1903; Molnar et al., 1984).

In the work described here we used the streptavidin-biotin immunoperoxidase technique to evaluate the

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presence and distribution of neuron specific enolase (NSE), neurofilament protein (NF), glial fibrillary acidic protein (GFAP) and S-100 protein in retinoblastomas from 13 eyes. We also investigated the presence of carcinoembryonic antigen (CEA) and alpha-foetoprotein (AFP) in the tumours cells because of reports of raised oncofoetal antigen levels in the plasma of patients with retinoblastoma and their relatives (Sang and Albert, 1982).

Materials and methods

The globes of 13 surgically enucleated eyes with retinoblastoma and metastasized intracraneal tissue from one of these patients were immersion-fixed in 10% formalin for 24 h, dehydrated with a graded ethanol series and embedded in paraffin. Sections 5 µm thick were cut, mounted on glass slides coated with chromealum-gelatin and dried overnight at 37° C. After dewaxing and rehydration, the sections were surrounded by Sigmacote (Sigma, St. Louis, USA) and rinsed with 0.01 M phosphate-buffered saline, pH 7.4 (PBS). The streptavidin-biotin bridge technique was used for immunohistochemistry. The sections were successively incubated, following blocking steps: 1) rabbit antiserum to NSE (Dako) at a dilution of 1:1,500, mouse antiserum to 70 Kd and 200 Kd polypeptide neurofilament (Biogenex) at a dilution of 1:200, mouse antiserum to S-100 (Biogenex) at a dilution of 1:500, mouse antiserum to GFAP (Biogenex) at a dilution of 1:200, rabbit antiserum to AFP (Biogenex) at a dilution of 1:400, rabbit antiserum to CEA (Dako) at a dilution of 1:1,000 (in each case for 2 h at 4° C); 2) biotinylated antibody to rabbit or mouse (Biomakor, Rehovot, Israel) at a dilution of 1:30 (for 30 minutes at room temperature); 3) streptavidin-peroxidase (Biomakor, Rehovot, Israel) at a dilution of 1:30 (for 30 minutes at room temperature); and 4) 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA) at a dilution of 0.06% with 0.003% H_2O_2 (for 10 minutes at room temperature).

Between steps, the sections were rinsed with PBS (2 x 5 minutes). After step 4, they were rinsed with distilled water, dehydrated, cleared and mounted.

All cases studied were examined in parallel with positive and negative controls. Preabsorption controls were performed by preincubation of each antiserum with the homologous antigen.

Fresh tissue from one case was fixed in cacodylate-

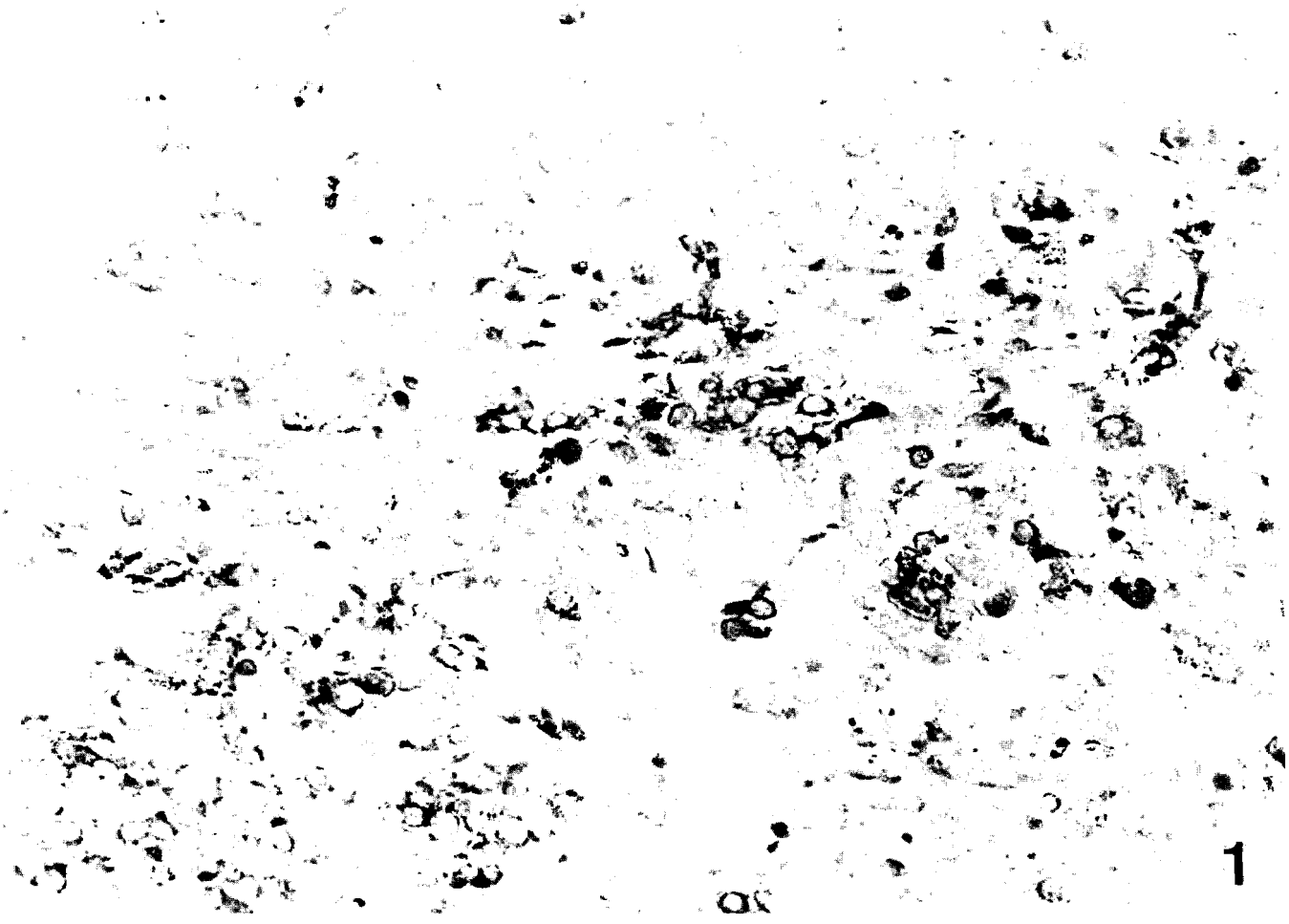

Fig. 1. Diffuse cytoplasmic staining for NSE in most tumour cells. × 400

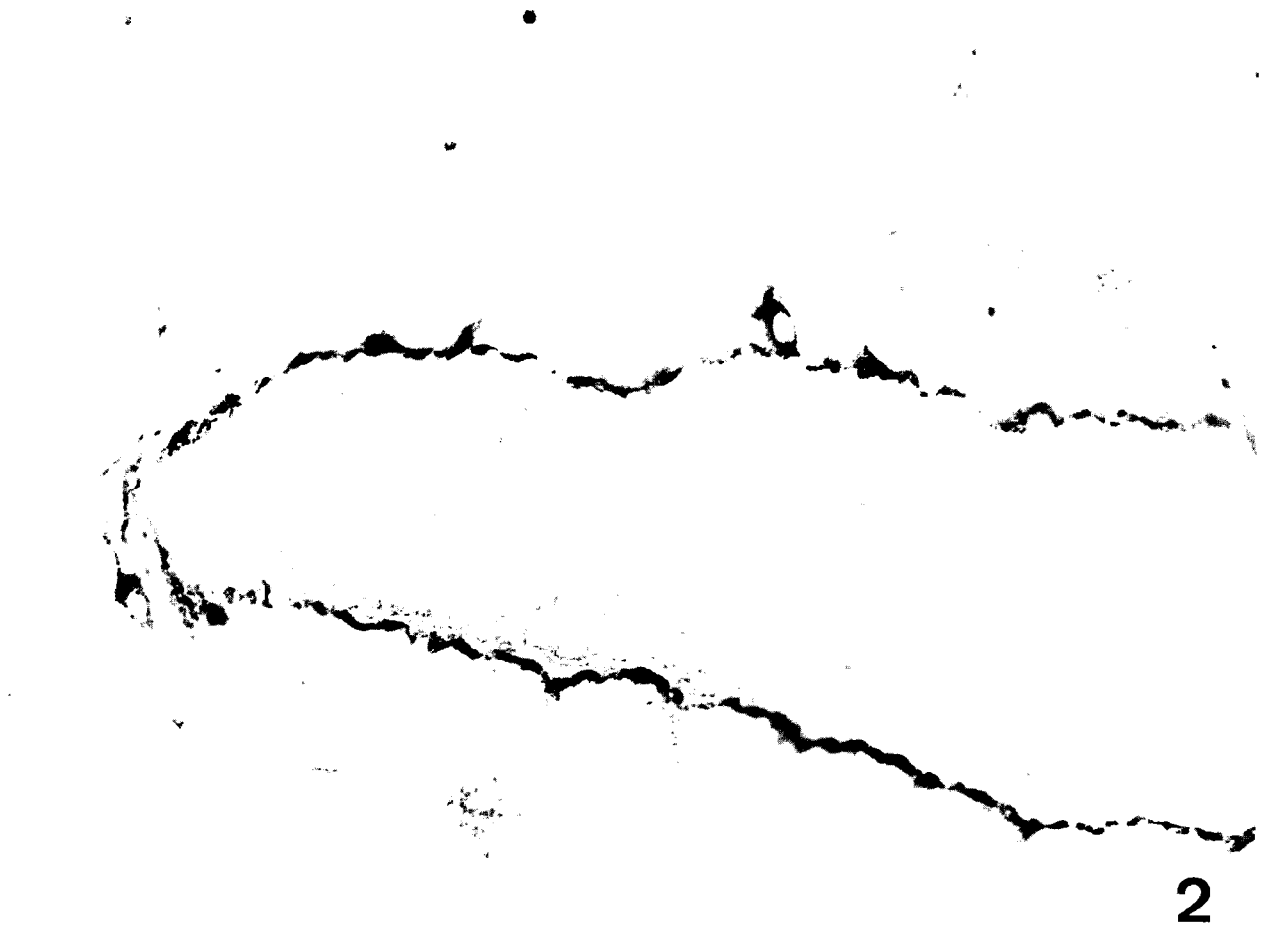

Fig. 2. Groups of strongly GFAP-positive cells arranged around blood vessels. × 400

buffered 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and embedded in spurr for electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate for ultrastructural study.

Results

The 13 retinoblastomas studied were positive for NSE, most tumour cells showing diffuse cytoplasmic staining, though intensity varied from one field to another (Fig. 1). In two tumours with appreciable photoreceptor differentiation faint staining in areas containing Flexner-Wintersteiner rosettes was observed. None of the tumours stained with NF.

Seven of the retinoblastomas studied contained groups of strongly GFAP-positive cells. The predominant pattern (Fig. 2) was a perivascular distribution of glial cells with rounded vesicular nuclei and abundant cytoplasm with occasional extensive GFAP-containing cytoplasmic processes. The adjacent undifferentiated tumour cells were small, with round hyperchromatic nuclei and scant, GFAP-negative, cytoplasm. In two of these seven cases we observed cells that were stained with the GFAP antibody but were not associated with blood vessels, being instead randomly located among tumour cells whose neuronal differentiation was demonstrated in adjacent sections immunostained with NSE antibody. In the intracraneal tissue of the metastasized case large numbers of GFAP-containing stellate cells interspersed with undifferentiated tumour cells lacking GFAP were observed.

The S-100 results parallelled the GFAP data, the same seven tumours being positive for both proteins. The predominantly perivascular stellate immunostained cells were interspersed among a majority of S-100-negative cells and showed nuclear and diffuse cytoplasmic staining.

When serial sections were compared, cross-reactivity was observed neither between glial markers and anti-neuron-marker antibodies, nor between neuron markers and anti-glial-marker antibodies.

Tumour cells lacked detectable CEA and AFP in all 13 cases studied. This result cannot be attributed to problems with the ABC immunoperoxidase technique, because the results with positive and negative controls were quite satisfactory.

The retinoblastoma that was examinated at ultra-

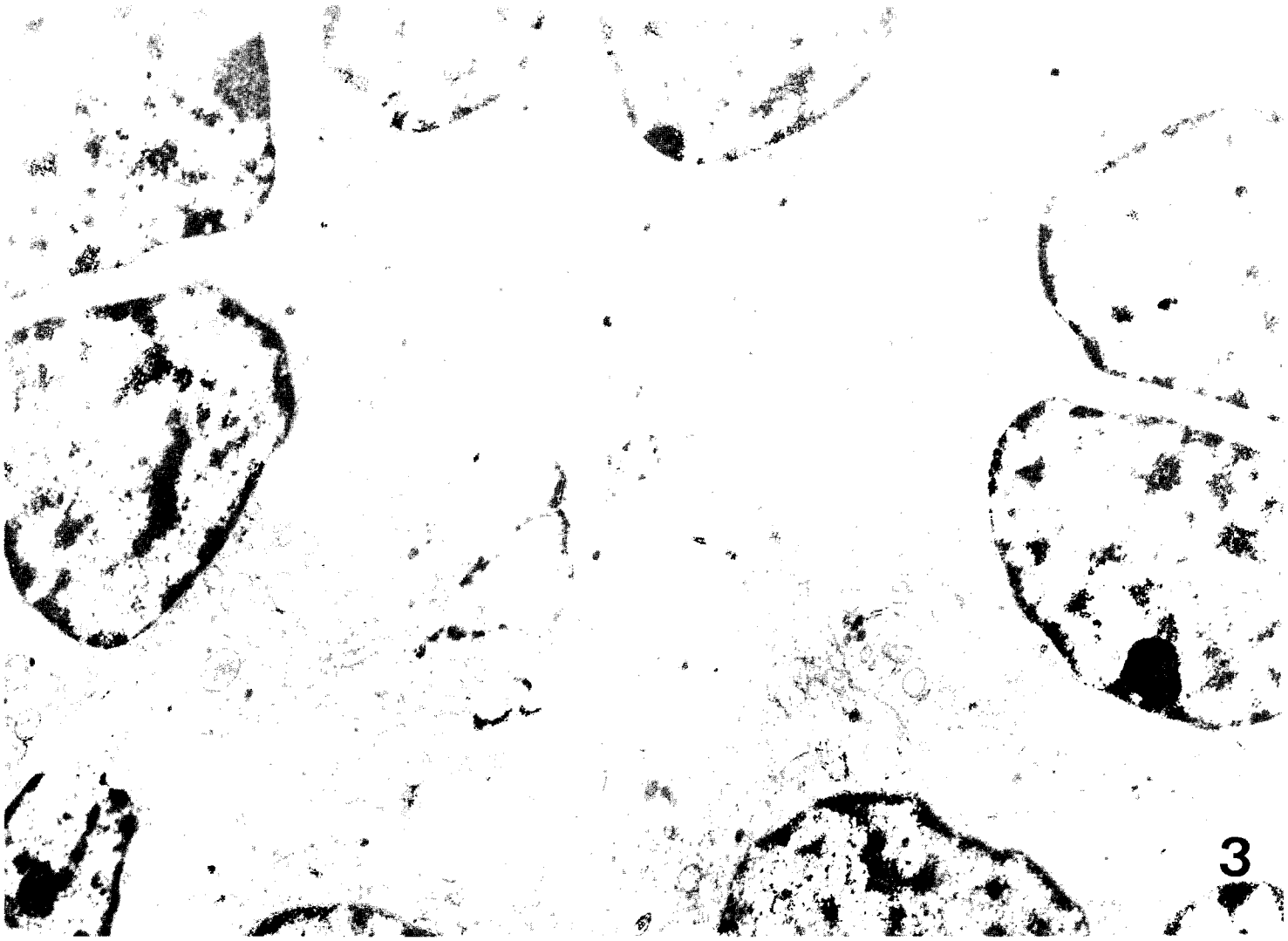

Fig. 3. Ultrastructural appearance of retinoblastoma, showing abundant nuclear chromatin, sections of elongated cell processes, intercellular junctions and a number of neurosecretory granules. × 8,500

structural level consisted predominantly of primitive embryonal cells with abundant nuclear euchromatin and a large number of cytoplasmic ribosomes. Occasionally, the cells formed rudimentary rosettes. Elongated cell processes were observed, containing numerous microtubules and clustered neurosecretory granules. The granules were also present in the cell body (Fig. 3).

Discussion

In the present study, as in a number of previous studies (Messmer et al., 1985; Kivelä, 1986; Rodrigues et al., 1986; Perentes et al., 1987; Sawa et al., 1987), all the retinoblastomas investigated were strongly positive for NSE. Though it should be borne in mind that NSE is not always indicative of neural differentiation, it is well known that is never expressed by glial cells. None of our tumours appeared to express 70 Kd or 200 Kd neurofilament polypeptides. Reported immunoreactivity for NF antibody in retinoblastomas (Perentes et al., 1987; Russell and Rubinstein, 1989) has generally been slight and limited to isolated cells. Most authors report negative results for the 200 Kd subunit (Kivelä et al., 1986) although others have obtained positive responses in isolated cells or, occasionally, in small groups of cells forming Flexner-Wintersteiner rosettes (Perentes et al., 1987). Sawa (1987) saw positivity against 68 Kd NF antiserum and slight positive response for the 180 Kd subunit, but no cells immunostained with antiserum against the 210 Kd protein.

The finding that our retinoblastomas immunostained positively for GFAP and S-100 protein is in agreement with several other reports (Lane and Klintworth, 1983; Molnar et al., 1984; Terenghi et al., 1984; Messmer et al., 1985). The positive cells were predominantly perivascular, as in studies by Lane and Klintworth (1983), Kivelä et al. (1986) and Perentes et al. (1987).

who suggested that as a secondary response, glial elements may have proliferated and migrated into or around the tumour from the retina or (in the case of metastasis to the central nervous system, as in one of our cases) from the brain or spinal cord. The finding of Kivelä et al. (1986) that double indirect immunofluorescence staining and immunohistochemistry showed colocalization of vimentin and GFAP supports the opinion that all the neurologlial elements seen in neuroblastomas are reactive like the neoplastic elements as do the results of Kivelä et al. (1986) and Russell and Rubinstein (1989) on the distribution of reactivity to anti Leu-7 monoclonal antibody. Against this hypothesis it has been argued that glial cells are often found in retinoblastomas too far from normal astrocyte locations for migration to be plaustible (Russell, and Rubinstein 1989); and since the cytological features of these glial elements are indistinguishable from those of cells with neuronal differentiation some authors consider the former to be neoplastic as well (Molnar et al., 1984; Terenghi et al., 1984; Craft et al., 1985; Messmer et al., 1985; Rodriguez et al., 1986), suggesting that in the course of the development of the tumours primitive retina glial elements have given rise to both GFAP-positive cells and to other that have lost the capacity for synthesizing GFAP. Unfortunately since normal, reactive and neoplastic astrocytes all contain GFAP (Russell and Rubinstein, 1989) they cannot be distinguished immunohistochemically using this marker. In some cases, they have been stated to be neoplastic when they may have been stromal (Russell and Rubinstein, 1989). Only in a case described by Messmer et al. (1985) was the tumour so predominantly composed of astrocytes as to be classifiable as an astrocytoma.

The ultrastructural appearance of retinoblastomas varies with their degree of differentiation. In well-differentiated tumours with rosettes, photoreceptor differentiation and apical cilia in 9 + 10 configuration are evident. In poorly differentiated tumours distinctive neural features such as euchromatic nuclei, numerous cytoplasmic ribosomes and neurosecretory granules are observed (Tsó et al., 1970; Popoff and Ellsworth, 1971; Dickson et al., 1976; Ramsay et al., 1979). Pure photoreceptor differentiation is questioned by some electron microscopic studies of medulloblastoma (a tumour of similar origin) which have found glial and neuronal elements) (Scherenberg and Liss, 1969; Ermel and Bruche, 1974; Barnard and Pambakian, 1980).

The significance of the high levels of oncofoetal antigens that have been detected in the sera of some retinoblastoma patients has yet to be assessed (Sang and Albert, 1982; Das et al., 1984). We failed to find CEA or AFP in any of the tumours we studied. Unfortunately, the serum CEA and AFP levels of these patients were not determined.

In conclusion, our immunoperoxidase study using antibodies to NSE, GFAP and NF confirmed previous observations by other researches that significant numbers of glial cells are common in retinoblastomas. On the basis of their predominantly perivascular distribution and characteristic stellate shape, we conclude that they are probably reactive. We believe, however, that further progress on the question of the possible multipotentially of retinoblastoma cells will require studies using the retinoblastoma cell cultures that have been established (Reid et al., 1974; McFall et al., 1977; Kyritsis et al., 1984; Kyritsis et al., 1984, 1986; Tsokos et al., 1986).

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