An Immunohistopathologic Study of Trilateral Retinoblastoma

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We examined retinal and midline pineal tumors from four patients with trilateral retinoblastoma who had antibodies against neural-associated antigens including neuron-specific enolase, photoreceptor cell proteins (S-antigen and rhodopsin), and glial fibrillary acidic protein. Expression of neuron-specific enolase was observed in all four patients. S-antigen immunoreactivity was present in three of four ocular tumors and two of four pineal tumors examined, whereas results of labeling with rhodopsin and glial fibrillary acidic protein were negative in each of the ocular and pineal tumors.

Since trilateral retinoblastoma was described by Bader and associates,1 other investigators have confirmed the association of bilateral retinoblastoma with a midline intracranial tumor usually in the pineal region.2-12 These tumors may originate from photoreceptor-like cells in ocular tumors and from vestigial photoreceptor cells of the pineal organ.1 The pineal neoplasm displays histologic features similar to retinoblastoma, including photoreceptor differentiation, and probably represents a primary tumor rather than a metastatic focus.1 More recently, unilateral retinoblastoma and a pineal malignant neoplasm have been reported, suggesting a form fruste of this entity.13,14 The interval between the appearance of ocular tumors and the development of the pineal neoplasm may vary from a simultaneous onset to several years.12,19 Current treatment of the pineal tumor, including surgery and radiotherapy, has essentially been unsuccessful with no long-term survivors. A recent report, however, described clinical tumor regression in two patients treated with vincristine and cyclophosphamide.15

Immunohistologic studies have shown that photoreceptor-specific markers including S-antigen, rhodopsin, and interphotoreceptor retinoid-binding protein16-20 are expressed in retinoblastoma. However, the demonstration of immunoreactivity of only one of these markers, S-antigen, in trilateral retinoblastoma is limited to one other published report.21 Because of the histologic similarity of the retinal and pineal tumors, we refined and expanded our previous observations to investigate their possible common antigenic relationship using antibodies directed against neural-associated antigens, photoreceptor cell-associated antigens (S-antigen and rhodopsin), and glial fibrillary acidic protein in four patients with trilateral retinoblastoma.

Material and Methods

Antibodies—Monoclonal antibody MAbA9-C6 was prepared from the fusion of mouse myeloma cells with spleen cells immunized with purified retinal S-antigen, as previously described.20 Polyclonal antibodies against neuron-specific enolase (Dako) and glial fibrillary acidic protein were used. Additionally, purified MAbA9-C6 ascites fluid (2.84 mg/ml) in a 1:50 to 1:100 dilution, neuron-specific enolase antibody in a 1:200 to 1:500 dilution, rhodopsin antibody in a 1:50 dilution, and glial
fibrillary acidic protein antibody in a 1:100 dilution were used.

**Tissues**—Unstained paraffin sections from four cases of trilateral retinoblastoma were obtained: one from a previously reported patient from Children's Hospital, Washington, D.C., 8 one from the Registry of Ophthalmic Pathology, 12 Armed Forces Institute of Pathology, and two (one eye and one brain tumor) from patients previously described by Brownstein, Chadarrevian, and Little. 2 However, these previous reports described only routine light microscopy of hematoxylin and eosin stains, except for S-antigen immunoreactivity reported in two cases by Brownstein, Chadarrevian, and Little. 2

In the eye, the retinoblastomas in all cases were moderately to well differentiated with scattered areas of necrosis and calcification. Well-differentiated areas showed Flexner-Wintersteiner rosettes. The optic nerve and choroid were free of tumor.

The four pineal tumors consisted of poorly differentiated small round cells with areas of necrosis and calcification in three of four cases and focal areas of Flexner-Wintersteiner rosettes in one case.

**Immunohistochemistry**—The slides were deparaffinized and hydrated, followed by incubation with hydrogen peroxide to inactivate endogenous peroxidase. The slides were then rinsed in phosphate buffered saline and incubated in the primary antibody, biotin-labeled goat anti-mouse immunoglobulin (for S-antigen and rhodopsin), or goat anti-rabbit immunoglobulin (for neuron-specific enolase and glial fibrillary acidic protein; 1:400) for 30 minutes. This was followed by incubation with avidin-biotin-horseradish peroxidase complex for 30 minutes. After rinses in phosphate buffered saline, 3-3'-diaminobenzidine tetrahydrochloride was used for visualization for five minutes, followed by washes in phosphate buffered saline and hematoxylin counterstain, dehydration through xylene, and mounting in Permount.

**Results**

**Ocular tumors**—All retinoblastomas showed cytoplasmic labeling with anti-neuron-specific enolase antibodies, with reactivity of approximately 75% to 80% of tumor cells; this was observed in areas of more anaplastic diffusely arranged tumor as well as in Flexner-Wintersteiner rosettes (Fig. 1). Labeling with S-antigen antibody (MAB9-C6) was observed in the retinoblastomas from three of the four eyes (Table). In these cases, 15% to 20% of tumor cells stained, particularly those in Flexner-Wintersteiner rosettes (Fig. 2). Reactivity was noted in the cytoplasm of tumor cells as well as in slender tapering cytoplasmic processes in the Flexner-Wintersteiner rosettes (Fig. 2). The photoreceptor cell layer of the adjacent normal retina also bound MAB9-C6, which served as an internal positive control. Results of labeling with anti-rhodopsin and anti-glial fibrillary acidic protein antibodies were negative in all the retinoblastomas examined. Binding with rhodopsin was noted in the outer segments of the photoreceptor cell layer of normal retina. Anti-glial fibrillary acidic protein antibody labeled the glial elements in the optic nerve and nerve fiber layer of the normal retina adjacent to the tumor as expected, but was scant or absent in neoplastic cells (Fig. 3).

**Pineal tumors**—All four pineal tumors showed reactivity with anti-neuron-specific enolase (Fig. 4) (Table). MAB9-C6 bound tumor cells focally within two tumors (Fig. 5); however, the immunoreactivity for S-antigen was less pronounced than that noted in the retinal tumors. No labeling of tumor cells was noted with antibodies directed against rhodopsin or with glial fibrillary acidic protein (Fig. 6).

**Discussion**

In lower vertebrates, the pineal organ has a primary photoreceptor function, which diminishes in phylogeny. 22,23 Morphologic evidence of pineal photoreceptor differentiation has been observed in the neonatal rat as well as in the human embryo. 24 Photoreceptor differentiation has also been noted in some pineoblastomas. 25,26 An immunologic relationship between the pineal organ and the retina is supported by the localization of S-antigen in fetal, neonatal, and adult retina and pineal glands. 21,22,24 S-antigen has also been described in human pineal tumors. 7,23 Additionally, interphotoreceptor retinoid-binding protein, a photoreceptor-specific marker, has been localized in primate retina and pineal organ. 22 Recently, Donoso and associates 21 investigat-
Fig. 1 (Rodrigues and associates). Well differentiated retinoblastoma with numerous Flexner-Wintersteiner rosettes showing diffuse staining of tumor cells with anti-neuron-specific enolase antibody (×500).

Fig. 2 (Rodrigues and associates). MAbA9-C6 (S-antigen) binding to Flexner-Wintersteiner rosettes in well-differentiated retinoblastoma (×500).

Fig. 3 (Rodrigues and associates). Retinoblastoma showing lack of labeling of tumor cells with glial fibrillary acidic protein. Adjacent normal retina shows labeling only of the nerve fiber layer (×80).

Fig. 4 (Rodrigues and associates). Diffuse staining of pineal tumor with anti-neuron-specific enolase antibody (×330).

Fig. 5 (Rodrigues and associates). Pineal tumor showing labeling with anti-S-antigen antibody (×500).

Fig. 6 (Rodrigues and associates). Absence of glial fibrillary acidic protein immunoreactivity in pineal tumor (×330).
ed S-antigen immunoreactivity in four cases of trilateral retinoblastoma and observed labeling in all the eyes and two of four brain tumors. In retinoblastoma, the expression of rod outer segment proteins that participate in the visual process, such as S-antigen and rhodopsin, suggests that the tumor cells show photoreceptor cell differentiation. Although rhodopsin immunoreactivity has been associated with retinoblastoma, none of the ocular or brain tumors expressed this antigen in our study. This antigen may be more susceptible to the effects of fixation than S-antigen, or these findings could be related to the differentiation of the tumor, as rhodopsin has been localized only in a few highly differentiated retinoblastomas. 17,19

These data substantiate the immunologic relationship between the retina and pineal organ in the neoplastic state. Our study confirms and expands these findings. Furthermore, we noted labeling for neuron-specific enolase in all of the ocular tumors and pineal tumors. Tumor cell expression of neuron-specific enolase varied from 75% to 80% in the ocular tumors to 50% to 80% in the pineal tumors. Most of the enolase in neurons appears in the form of the \( \gamma \)-subunit, particularly in the pineal organ, which contains approximately 60% of the brain content of \( \gamma \)-enolase. In general, labeling was more pronounced in the ocular tumors compared with the pineal tumors. The difference in the amount and extent of labeling, which was generally more pronounced in the ocular tumors, could be related to a greater degree of denaturation of proteins; when the pineal organ is removed with the brain it is fixed in formalin for four to six weeks before processing, whereas tissue fixation of ocular tumors usually varies from two to seven days. This relatively prolonged fixation could account in part for the difficulty in labeling photoreceptor-specific proteins such as rhodopsin and interphotoreceptor retinoid-binding protein, which appear to be more labile than neuron-specific enolase and S-antigen. An alternative explanation could be the greater degree of differentiation in the ocular tumors compared with those involving the pineal gland.

Our data indicate that the neoplastic cells in both components of trilateral retinoblastoma are predominantly neuronal in antigenic expression, since labeling was observed with antibodies against neuronal antigens and photoreceptor cell proteins but not with glial associated antigens. These results are similar to those observed in the more common form of retinoblastoma unassociated with pineal tumors. 18,19 Additionally, the expression of photoreceptor-specific proteins, interphotoreceptor retinoid-binding protein and rhodopsin, has been observed in retinoblastoma 18,19 but has not yet been reported in trilateral tumors. This could be related to the optimal binding of certain photoreceptor cell-specific markers (rhodopsin, S-antigen, and interphotoreceptor retinoid-binding protein) in fresh unfixed tumor, in contrast to masking of some of these proteins, particularly interphotoreceptor retinoid-binding protein, in the paraffin sections that were available for immunohistochemical staining of trilateral retinoblastoma in the present study. Our data further substantiate the immunologic relationship between the retina and pineal gland and tumors originating in these tissues. Trilateral retinoblastoma is now considered to be another form of expression of the retinoblastoma gene. Recently the retinoblastoma gene was tentatively localized to a 70-kilobase DNA fragment corresponding to the human chromosome band 13q14 in patients with retinoblastoma. 38 The possibility of a similar locus in pineal tumors has yet to be determined.

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**TABLE**

**IMMUNOHISTOLOGIC STAINING IN FOUR CASES OF TRILATERAL RETINOBLASTOMA***

<table>
<thead>
<tr>
<th>TUMOR TYPE</th>
<th>HISTOLOGIC DIFFERENTIATION</th>
<th>NEURON-SPECIFIC ENOLASE</th>
<th>S-ANTIGEN</th>
<th>RHODOPSIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma</td>
<td>Moderately to well differentiated with Flexner-Wintersteiner rosettes</td>
<td>4/4</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Pineal</td>
<td>Poorly differentiated; rosettes in one case</td>
<td>4/4</td>
<td>2/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*Number of cases staining positive/total number of cases.
Acknowledgment
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References


