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Expression of the HNK-1 carbohydrate epitope in human retina and retinoblastoma*

An immunohistochemical study with the anti-Leu-7 monoclonal antibody

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Summary. Fifty formalin-fixed and paraffin-embedded retinoblastoma specimens and five normal human eyes were studied with the monoclonal anti-Leu-7 antibody, directed against the HNK-1 carbohydrate epitope that is shared by human natural killer cells and many neuronal, glial and neuroectodermal cells. The laboratory method was a sensitive immunohistochemical staining procedure, and neuroectodermal tumours that usually express this epitope were used as positive controls. In the human retina, Müller cell membranes were positively stained, but additional staining of neuronal cells was not excluded at the light microscopical level. A positive cytoplasmic reaction was also seen in ciliary and retinal pigment epithelial cells. All but one intraocular retinoblastomata studied contained cells staining positively for the HNK-1 epitope, but these cells were probably not neoplastic. Although positive reaction has previously been reported in three retinoblastomata, the present results suggest that positive cells are derived from entrapped and infiltrated retina. Staining of adjacent sections against leukocyte common antigen suggested that the positively staining cells were not natural killer cells.

Key words: Retina – Retinoblastoma – Immunohistochemistry – HNK-1 epitope – Leukocyte common antigen

Introduction

The monoclonal antibody HNK-1 was originally raised against a membrane fraction of the HSB-2 human T-lymphoblastoid cell line and selected because it specifically labelled a subset of lymphocytes enriched in natural killer and killer cells (Abo and Balch 1981). It detects a carbohydrate epitope shared by many macromolecules. This epitope has been demonstrated on myelin-associated glycoprotein (McGarry et al. 1983; Nobile-Orazio et al. 1983; Sato et al. 1983), on murine neural cell adhesion molecules N-CAM, L1 and J1 (Kruse et al. 1984, 1985; Wernecke et al. 1985; McGarry et al. 1985), on a glycosphingolipid of human peripheral nerves (Ilyas et al. 1984; Chou et al. 1985), as well as on several as yet uncharacterized glycoproteins of central and peripheral nervous tissue and neuroendocrine secretory granules (Nobile-Orazio et al. 1983; O'Shannessy et al. 1985; Tischler et al. 1986). It is also shared by two membrane proteins on large granular lymphocytes of human peripheral blood (Kubagawa et al. 1983).

The HNK-1 epitope is resistant to fixation in formalin and embedding in paraffin (Lipinski et al. 1983). In addition to large granular lymphocytes of lymphoid organs and myelin sheaths of central and peripheral nerves (Mori et al. 1983; Schuller-Petrovic et al. 1983; Ando and Tamaki 1985) HNK-1 antibody stains many normal and malignant neuronal, glial and neuroectodermal cells and cells of the amine precursor uptake and decarboxylation (APUD) system (Lipinski et al. 1983; Tsutsumi 1984; Caillaud et al. 1984; Shioda et al. 1984; Wahab and Wright 1985; Bunn et al. 1985; Motoi et al. 1985; Smolle et al. 1985; Perentes and Rubinstein 1985, 1986). During embryogenesis, the epitope is expressed on multipotential glial and neuronal precursor cells (Kruse et al. 1984, 1985; Tucker et al. 1984; McGarry et al. 1985; Wernecke et al. 1985; Bronner-Fraser 1986) and it is thought to be important in cell-cell and cell-substratum interactions during differentiation. Consequently, its distribution in embryonal tumours has received attention (Caillaud et al. 1984; Perentes and Rubinstein 1986).

Retinoblastoma is a malignant tumour of the

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neural retina, but its precise cell of origin and lines of differentiation are disputed (Tso 1980). Generally it has been considered a neuronal or neuroectodermal tumour without glial differentiation. Recent immunohistochemical evidence has suggested, however, that an embryonal multipotential precursor cell might give raise to retinoblastoma (Kyritsis et al. 1984; Jiang et al. 1984; Molnar et al. 1984). The present study was undertaken to clarify the distribution of the HNK-1 carbohydrate epitope in human retina and retinoblastoma to help to understand the genesis of this tumour. Antibodies to leukocyte common antigen were used to help to differentiate positively staining cells from natural killer cells.

Materials and methods

Histological specimens. During the years 1962–1985 a total of 66 retinoblastoma specimens from 57 patients have been examined in the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital. In 50 cases from 46 patients the formalin-fixed and paraffin-embedded tumour was still available. These retinoblastomata were reported to be unilateral in 29 cases and bilateral in 20 cases. One tumour was an orbital recurrence.

In addition to the light microscopically normal appearing parts of the retinas present in the retinoblastoma specimens, retinas from five formalin-fixed and paraffin-embedded normal human eyeglobes enucleated because of a retrobulbar tumour were studied.

Formalin-fixed and paraffin-embedded specimens from two oligodendrogliomas, pheochromocytomas, ileal carcinoid tumours, pulmonary oat cell carcinomas and prostatic adenocarcinomas were kindly provided by docent Markku Miettinen from the Department of Pathology, University of Helsinki, to serve as positive control material. Two orbital lymphomas were used as positive controls in stainings against leukocyte common antigen.

Sections (5 µm thick) were cut from the specimens and mounted on chromium-gelatin-treated glass slides to ensure adherence (0.05 g potassium chromium(III)sulphate dodecahydrate and 0.5 g gelatin in 100 ml distilled water).

Immunohistochemical staining. Immunohistochemical staining to demonstrate the HNK-1 epitope was carried out using a commercial version (Vectastain ABC Kits for Mouse IgG and IgM, Vector Laboratories, Burlingame, CA) of the avidin-biotinylated peroxidase method (Hsu and Fanger 1981).

In brief, the sections were routinely deparaffinized in xylene and hydrated in an ethanol series. Endogenous peroxidase activity was destroyed as previously described (Kivelä and Tarkkanen 1983). Non-immunological binding of antibodies was blocked by incubation with normal serum (Vectastain ABC Kits, horse for anti-IgG and goat for anti-IgM secondary antisera) diluted 1:50 with phosphate buffered saline (PBS, pH 7.4) containing 2% bovine serum albumin (E. Merck, Darmstadt, FRG) for 30 min at room temperature. In preliminary stainings, delipidization in acetone for 60 min (Schuller-Petrovic et al. 1983) or treatment with 0.4% pepsin (E. Merck, Darmstadt, FRG) in 0.01 N hydrochloric acid at 37° C for 15 to 60 min was found not to have any beneficial effect on the positive staining reaction.

The primary HNK-1 mouse monoclonal IgM-kappa antibody was obtained commercially (anti-Leu-7, Cat. No. 7390, Lot H0707, Becton Dickinson Monoclonal Center, Mountain View, CA). The optimal dilution for the antibody as determined in preliminary stainings was found to be 1:100 (diluted with PBS-albumin). An irrelevant mouse monoclonal IgM-kappa antibody, directed against human dendritic reticulum cells (DAKO-DRC1, Code No. M709, Lot 025, Dakopatts a/s, Denmark), diluted 1:5 was used as a control primary antibody (Naiem et al. 1983).

Commercially available monoclonal antibodies to human leukocyte common antigen (DAKO-LC, Code No. M701, Lot 124, Dakopatts a/s, Denmark) diluted 1:10 in PBS-albumin were used to detect lymphocytes (Warnke et al. 1983). This product is a mixture of two mouse monoclonal IgG1 antibodies recognizing different epitopes on leukocyte common antigen, a group of membrane glycoproteins shared by most human leukocytes, including natural killer cells. Incubation with all primary antibodies was carried out in a moist chamber at 37° C for 60 min.

Two secondary antisera were used to detect the anti-Leu-7 primary antibody. In one series, it was detected with horse anti-mouse-IgG (whole molecule) antiserum (Vectastain ABC Kit) which binds to the kappa light chain of the anti-Leu-7 antibody. This antiserum was also used to detect DAKO-DRC1 and DAKO-LC primary antibodies. In the other series, goat anti-mouse-IgM (mu-chain specific) antiserum was used (Vectastain ABC Kit) which binds to the heavy chain of the anti-Leu-7 antibody. Incubation with the secondary antiserum (diluted 1:200 with PBS-albumin) was carried out in a moist chamber at 37° C for 30 min.

Subsequent application of the avidin-biotinylated peroxidase complex (Vectastain ABC Kit) and treatment with 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, Mo) to generate the specific colour reaction were carried out as previously described (Kivelä and Tarkkanen 1983). A duplicate series stained with anti-mouse-IgG secondary antiserum was counterstained for 2 min in dilute Mayer's haematoxylin to facilitate interpretation of staining results. All sections were mounted in Aquamount (BDH Chemicals Ltd, Poole, England).

Results

Light microscopy

The histopathological characteristics of the 50 retinoblastomata examined are described in Table 1. In addition to the five normal eyeglobes, there were parts of light microscopically normal appearing retina left for study in 31 retinoblastoma specimens. The control tumours demonstrated typical morphology under the light microscope.

Immunohistochemical staining

With the monoclonal anti-Leu-7 antibody, directed against the HNK-1 epitope, myelin sheaths of extraocular nerves (Fig. 1 A) and Schwann cells along ciliary nerves were positively stained in every ocular specimen. In the optic nerve, positive reaction accompanied nerve sheaths but astrocytes were negatively stained. Neoplastic cells in two oligo-

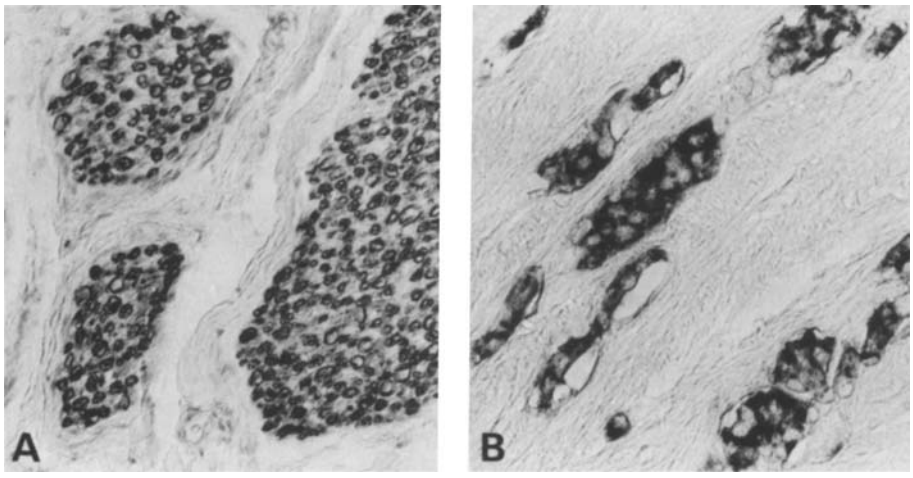

Fig. 1 A, B. Positive controls in the immunohistochemical staining for the HNK-1 epitope. **A** Myelin sheaths of orbital nerves stain intensely (×330). **B** Neoplastic cells of an ileal carcinoid tumour that infiltrate between negative smooth muscle cells demonstrate intense cytoplasmic staining (×330)

Table 1. Distribution of the intraocular retinoblastomata studied into different clinicopathological groups

	Unilateral	Bilateral	Total
Flexner-Wintersteiner rosettes	45% ($n=13$)	60% ($n=12$)	51% ($n=25$)
Infiltration into the choroid	24% ($n=7$)	20% ($n=4$)	22% ($n=11$)
Infiltration into the optic nerve	17% ($n=5$)	20% ($n=4$)	18% ($n=9$)
	$n=29$	$n=20$	$n=49$

One additional tumour was an orbital recurrence

dendrogliomas, two pheochromocytomas, one carcinoid tumour and two prostatic adenocarcinomas were positive for the HNK-1 epitope, as were a small number of scattered lymphocytes in two orbital lymphomas (Fig. 1B). Two pulmonary oat cell carcinomas and one carcinoid tumour showed negative staining. These results agree with previous studies (Mori et al. 1983; Tsutsumi 1984; Caillaud et al. 1984; Motoi et al. 1985; Wahab and Wright 1985). Substituting the primary antibody with an irrelevant mouse monoclonal IgM-kappa antibody abolished the positive staining reaction. Omitting the primary antiserum, the secondary antiserum or the ABC complex also resulted in a negative reaction. Identical results were obtained with both secondary antisera.

In normal human eyes, positive reaction for the HNK-1 epitope was seen throughout the retinal layers (Fig. 2). Outer and inner segments of photoreceptor cells showed negative staining. In the region of the outer limiting membrane there was, however, a distinct discontinuous layer of positive staining reaction that seemed to be located between photoreceptor inner segments (Fig. 2A). In both nuclear layers there were radial fibers staining positively for the HNK-1 epitope, and some cell bodies were also apparently surrounded by a continous rim of positive reaction (Fig. 2A). In other specimens cross-sections of such fibers were seen between photoreceptor cell nuclei. Both plexiform layers and the nerve fiber layer showed an extensive positive reaction that was associated with cell membranes (Fig. 2A). Footplates of Müller's cells in the nerve fiber layer were limited by positively staining cell membranes (Fig. 2B). Intraretinal blood vessels were also surrounded by HNK-1 positive cell processes (Fig. 2C). Cytoplasmic staining was not seen in any cell type of the neural retina. There were no differences in staining patterns between morphologically normal appearing retinas from eyeglobes with retinoblastoma and those enucleated because of an orbital tumour.

Positive cytoplasmic staining for the HNK-1 epitope was noted in the ciliary pigment epithelium. Positive reaction was also present in the retinal pigment epithelium, where it was stronger near the ora serrata region as compared to the posterior pole. In the ciliary body area, there was additional positive staining of subepithelial connective tissue matrix.

Cells staining positively for the HNK-1 epitope were seen in all but one intraocular retinoblastomata studied and the orbital recurrence examined was found to be negative (Figs. 3 and 4). The positive cells were, however, usually associated with infiltrated retina, tumour stroma (Fig. 3A) and blood vessels (Fig. 3B) rather than with undifferentiated retinoblastoma cells. However, many other stromal areas were negative for the HNK-1 epitope. The Flexner-Wintersteiner rosettes did not

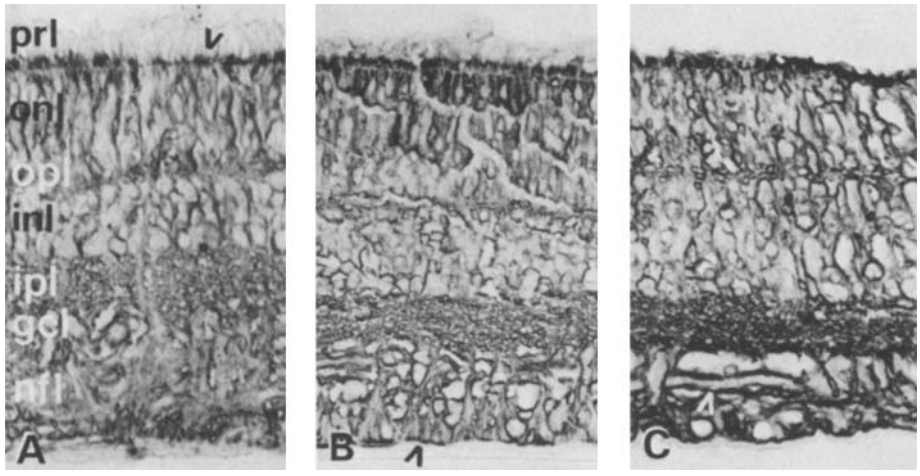

Fig. 2A–C. The HNK-1 epitope in human retina (immunohistochemical staining without counterstain). **A** Slender positively staining fibers are seen between negative photoreceptor cell inner segments (*arrowhead*). Radially oriented cell processes are observed in both nuclear layers, and some cell bodies are also apparently surrounded by a rim of positive reaction. At the light microscopical level it is not possible to say whether this reaction is located on Müller cell or closely apposed neuronal cell membranes. Both plexiform layers and the nerve fiber layer show staining of cell processes (×250). **B** The staining pattern is similar except that several footplates of Müller's cells (*arrowhead*) are seen in the nerve fiber layer, limited by positively staining membranes (×280). **C** An intraretinal blood vessel (*arrowhead*) is surrounded by positively staining cell processes (×250). Prl is photoreceptor, onl and inl outer and inner nuclear, opl and ipl outer and inner plexiform, gcl ganglion cell, and nfl nerve fiber layer

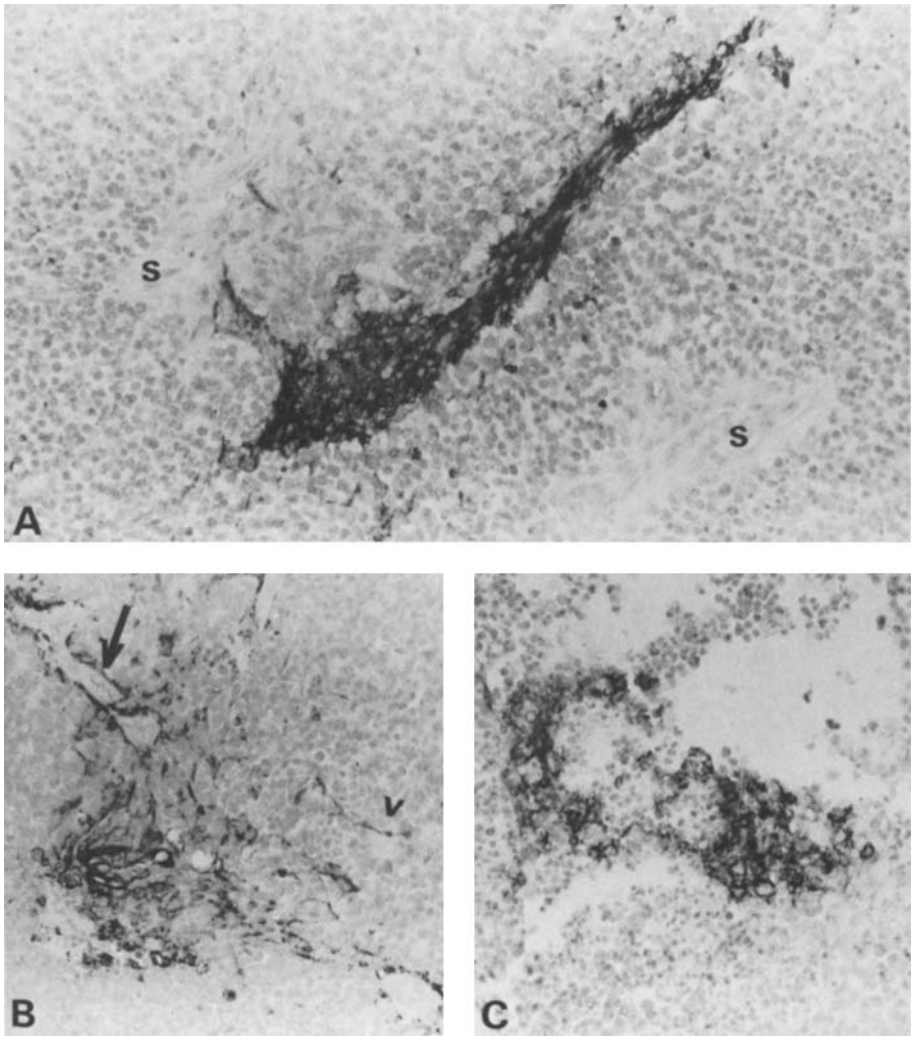

Fig. 3A–C. The HNK-1 epitope in retinoblastoma (immunohistochemical staining with haematoxylin counterstain). **A** An islet of fusiform stromal cells that are surrounded by negative tumour cells show positive staining reaction. Other stromal areas (s) are not stained (×210). **B** Perinuclear and fibrillar positive staining reaction near infiltrated retina. Blood vessels are surrounded by positively staining cells (*arrow*). One cell process (*arrowhead*) coincidentally radiates through a Flexner-Wintersteiner rosette (×210). **C** An isolated focus of small round cells that show positive perinuclear staining reaction in an otherwise negative tumour. These resemble neoplastic cells (×200)

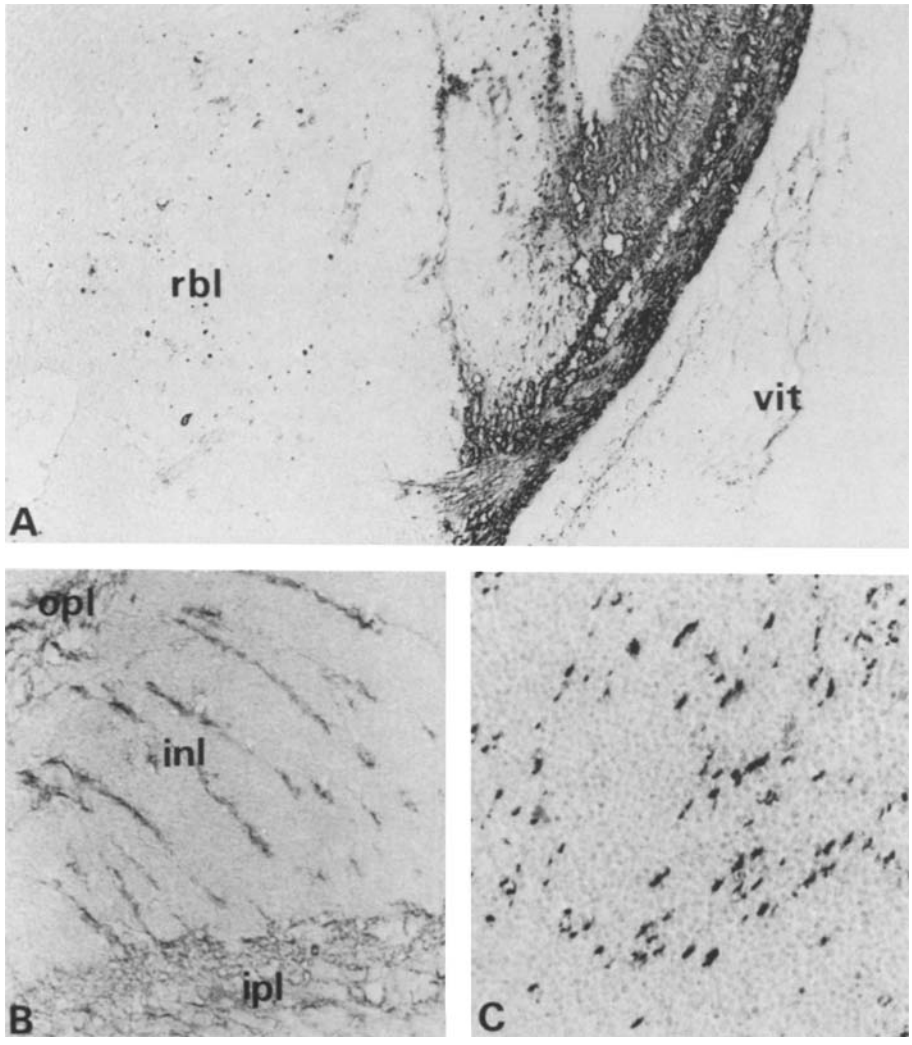

Fig. 4A–C. The HNK-1 epitope in retinoblastoma (immunohistochemical staining without counterstain). **A** The typical positive reaction in normal retina is lost where retinoblastoma (rbl) tumour cells apparently arise from the retina. Vit, vitreous body (×80). **B** At the transition zone, the positive staining reaction in the inner nuclear layer (inl) resolves into slender cell processes, limited by positively staining cell membranes, suggesting their origin from Müller's cells. Opl and ipl are outer and inner plexiform layers (×340). **C** Retinoblastoma cells infiltrate between similar cell processes seen as cross-sections in another plane of section (×190, counterstained with haematoxylin)

stain positively, but occasionally HNK-1 positive cell processes radiated through them (Fig. 3B). Isolated clusters of positively staining small round cells without apparent connection to infiltrated retina were occasionally seen (Fig. 3C). Positive reaction against the HNK-1 epitope quickly disappeared where apparently normal retina was transformed into retinoblastoma tumour tissue (Fig. 4), and HNK-1 positive radial cell processes in such regions provided evidence to support the idea that Müller's cells were stained (Fig. 4B, C). Necrotic areas were often non-specifically stained. There was no correlation between different retinoblastoma types studied (Table 1) and their reactivity with the HNK-1 epitope.

With the monoclonal antibodies against leukocyte common antigen, all lymphocytes were stained in the two lymphoma controls (Fig. 5A). A few positively staining mononuclear cells were seen in chorioidea and iris stroma. The normal retinas were entirely devoid of any positive reaction. Within retinoblastomata, a few infiltrating lymphocytes were seen to stain positively in eleven tumours and small clusters of positive cells were detected in five additional cases (Fig. 5B). These areas did not correspond to areas of cells staining positively with the anti-Leu-7 antibody.

Discussion

Immunohistochemical studies have demonstrated that expression of the HNK-1 epitope is not tissue-type specific. Schwann cells, oligodendrocytes and some neurones can all show positive staining reaction (Schuller-Petrovic et al. 1983; Motoi et al. 1985; Perentes and Rubinstein 1986). Furthermore, outside the nervous system neuroectodermal cells and endodermally derived APUD cells, as well

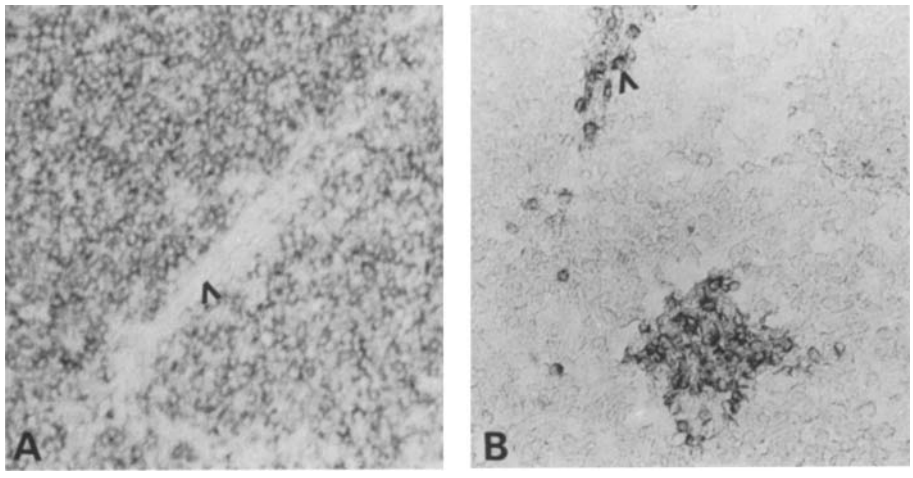

Fig. 5A, B. Immunohistochemical staining for leukocyte common antigen (without counterstain). **A** All lymphocytes in an orbital lymphoma used as a control show positive staining of cell membranes. Blood vessels and associated connective tissue (*arrowhead*) are negative (×180). **B** An accumulation of positively staining mononuclear cells, surrounded by negatively staining retinoblastoma cells. Additional lymphocytes show perivascular location (*arrowhead*) and infiltrate between tumour cells (×200)

as certain unrelated cell types (including natural killer cells) are positively stained (Mori et al. 1983; Caillaud et al. 1984; Wahab and Wright 1985).

The radial fibers observed with the anti-Leu-7 antibody in both nuclear layers of the neural retina and the reaction seen between photoreceptor inner segments, possibly related to fiber baskets, suggests that the Müller cell is one cell type expressing the HNK-1 epitope in human retina. The staining pattern was, however, very different from that previously observed in human Müller's cells with antibodies against vimentin and glial fibrillary acidic protein (Kivelä et al. 1986), and with a monoclonal antibody against this cell type (Chan et al. 1984). These antibodies seem to stain cytoplasmic components of Müller's cells, whereas the HNK-1 epitope is primarily associated with cell membranes. The prominent staining observed in both plexiform layers suggests that some neuronal cell types may also express the HNK-1 epitope. Likewise, in the developing avian neural retina putative ganglion cell bodies and nerve processes in both plexiform layers have been shown to stain positively with anti-HNK-1 antibodies (Tucker et al. 1984). Immunoelectronmicroscopical studies will confirm which cell types express the HNK-1 epitope in human retina.

The staining pattern observed was very similar, even though not identical, to that reported by Stefansson et al. (1984) and Molnar et al. (1986) with monoclonal antibodies against myelin-associated glycoprotein (MAG), one of the macromolecules sharing the HNK-1 epitope (McGarry et al. 1983; Nobile-Orazio et al. 1983; Sato et al. 1983). These antibodies were thought to stain Müller's cells in normal human retina and in one of their seven retinoblastomata (Molnar et al. 1984). Monoclonal antibodies raised against MAG often detect identical or closely related carbohydrate epitopes to that recognized by the HNK-1 antibody (Nobile-Orazio et al. 1983; Dobersen et al. 1985). The monoclonal anti-MAG antibody of Stefansson et al. (1984) detects several higher molecular weight proteins in addition to MAG on immunoblots of adult human retina and may thus also stain other antigens than MAG in tissue sections.

While primitive multipotential precursor cells of the nervous system (Tucker et al. 1984) and many cells of adult human retina express the HNK-1 epitope, neoplastic cells in the fifty retinoblastomata studied were negative for this epitope. Many positive cells that usually derived from infiltrated retina or stromal elements were observed, although the possibility remains that some of these cells might have been neoplastic. Staining of adjacent sections with antibodies against leukocyte common antigen suggested that they were not natural killer cells. Some of these cells may be reactive astrocytes (Perentes and Rubinstein 1986). When compared with results from a previous study of the same retinoblastoma material (Kivelä et al. 1986), there were more stromal cells expressing the HNK-1 epitope than cells positive for glial fibrillary acidic protein (GFAP) but vimentin-containing cells were even more abundant. In addition, no lobular pattern was seen resembling that typical of vimentin distribution in retinoblastoma (Kivelä et al. 1986). This suggests that HNK-1 positive elements were passively entrapped and infiltrated by tumour cells.

One research group has previously reported positive staining for the HNK-1 epitope in three paraffin-embedded retinoblastomata and in cells of the MS.Rb retinoblastoma cell line (Lipinski et al. 1983; Caillaud et al. 1984). They do not provide evidence, however, that the positive cells seen

in histological sections were neoplastic cells and not related to infiltrated retina. Expression of the HNK-1 epitope is more common in cultured cell lines than in corresponding tumour specimens (Lipinski et al. 1983; Cole et al. 1985). GFAP has also been observed in two retinoblastoma cell lines but usually not in tumour tissue (Kyritsis et al. 1984; Jiang et al. 1984; Molnar et al. 1984; Kivelä et al. 1986). Expression of these antigens in retinoblastoma cultures may be a phenomenon related to cell growth in laboratory environment. Interestingly, pineoblastoma, another malignant tumour of supposed embryonal origin, has recently also been shown to be negative for the HNK-1 epitope (Perentes and Rubinstein 1986).

The results of the present study suggest that retinoblastoma cells are either derived from HNK-1 negative cell types of the human retina, or they lose the HNK-1 epitope during malignant transformation. This epitope may be a useful adjunct when the relation of retinoblastoma cells to normal retina is studied. Further studies will also be needed to determine which antigens share the HNK-1 epitope in these tissues.

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