Human retinoblastoma contains clusters of cells immunoreactive for methionine-enkephalin and methionine-enkephalin-arginine-phenylalanine. Some tumour cells also exhibited methionine-enkephalin-arginine-glycine-leucine-like immunoreactivity. The results are in agreement with those obtained with similar testing of neuroblastoma cell cultures. It is concluded that some human retinoblastoma cells are capable of synthesizing preproenkephalin A or parts of this molecule. Invest Ophthalmol Vis Sci 25:1210-1212, 1984

Retinoblastoma is the most frequent, malignant, ocular tumor in children. In many of these cases (particularly bilateral ones), retinoblastoma may have been inherited as an autosomal dominant trait. We recently have shown that the tumor contains both nerve fibres and clusters of tumor cells displaying substance-P (SP)-like immunoreactivity. 1 The majority of the tumour cells were, however, negative. 1 Neuroblastoma-glioma cell cultures have been shown to synthesize both leucine and methionine enkephalins 2 and to contain both somatostatin 3 and opiate receptors. 2 Bearing the close relationship between neuroblastoma and retinoblastoma in mind, it should be noted that immunohistochemical studies have revealed not only SP- 4 but also enkephalin-like immunoreactivity 4-6 and many other classical transmitters and neuropeptides 4-8 in the inner plexiform layer and amacrine cells of the retina in several mammalian species.

Materials and Methods. Our patient is a healthy 14-month-old girl who had developed unilateral squint at the age of 10 months and had lost the vision of both eyes just before examination. At examination, the vitreous cavities of both eyes were observed to be filled by a yellowish vascular mass. No normal retina could be visualized. Both eyes were enucleated and the diagnosis of bilateral retinoblastoma was confirmed.

Following enucleation of the eyes, the retinas were fixed quickly for 1 to 2 hr with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 at 4°C overnight. They were then transferred to 0.1 M sodium phosphate buffer containing 20% sucrose and stored at 4°C overnight. Cryostat sections were cut at 15 μm and processed immediately for indirect immunofluorescence. Sections were collected on gelatin coated slides, air dried for 60 min, rehydrated in phosphate buffered saline (PBS) containing 0.25% Triton X-100. Specific antisera against Met-enkephalin (ME), Met-enkephalin-Arg-Phe (MEAP) and Met-enkephalin-Arg-Gly-Leu (MEAGL) were applied at optimal dilution in PBS-Triton. These were 1:1000 for ME and MEAGL and 1:500 for MEAP. Incubation was carried out at 4°C for 48 hr in a moist chamber. Then the sections were incubated with 20% normal lamb serum (DAKO; Copenhagen, Denmark) in PBS-Triton for 20 min at room temperature to diminish nonspecific adsorption of secondary antiserum. The sections then were washed with PBS-Triton and incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit immunoglobulins diluted 1:40 with PBS-Triton for 40 min. After a final washing with PBS, the slides were covered with coverslips and examined under a Leitz Dialux 20 fluorescence microscope equipped with an epi-illuminator. Control sections were incubated with specific antisera preabsorbed with corresponding and related opioid peptides.

The preparation and specificity of the antisera produced in rabbits have been described in earlier reports. 7-9 In some control experiments, the secondary antibody (FITC-conjugated sheep anti-rabbit immunoglobulin) was omitted.

Results. Haemotoxylin-eosin sections of the tumor revealed a well-differentiated retinoblastoma with rosettes, but these could not be identified reliably enough at fluorescence microscopy. A small portion of tumor cells exhibited either ME- or MEAP-like immunoreactivities in all sections examined (Figs. 1, 2). The central necrotic areas were, however, negative. The ME-like immunoreactivity was seen in round or slightly ovoid cells, 12-20 μM in diameter (Fig. 1). Similar cells exhibited MEAP-like immunoreactivity (Fig. 2). Very few cells with similar appearance also
exhibited MEAGL-like immunoreactivity (Fig. 3). Without statistical analysis, it seemed that the tumor cells showing either ME- or MEAP-like immunoreactivity occurred much more frequently than those with MEAGL-like immunoreactivity, although none of them could be regarded as the predominant cell type in this tumor. The immunoreaction with MEAGL antiserum was abolished completely by preabsorption with 1 μM MEAGL peptide but not by 100 μM ME or MEAP. Similarly, 1 μM MEAP peptide absorbed the MEAP immunoreaction completely, whereas ME and MEAGL failed to diminish the fluorescence at 100 μM. Five μM ME abolished the immunofluorescence with the corresponding antiserum (Fig. 4), whereas MEAP and MEAGL did not affect this immunoreaction.

The specimens that were treated otherwise normally but in the absence of the secondary antibody were negative.

**Discussion.** The results provide immunohistochemical evidence that human retinoblastoma cells synthesize preproenkephalin A or parts of this mole-
The cells appear to be heterogenous as only a small portion of them exhibit immunoreactivities to different preproenkephalin A fragments. It remains possible that the majority of the cells produce small quantities of these peptides that are not detected by our antisera because of limited sensitivity.

The preproenkephalin A molecule contains four copies of ME, one copy of MEAGL and one copy of MEAP that forms the carboxyl terminus of the molecule.8-10 It is possible that the posttranslational processing of the large molecule in retinoblastoma cells is not complete, because ME and MEAP immunoreactive cells appeared to be more numerous than MEAGL immunoreactive cells, despite the fact that the MEAGL antisera is very sensitive in immunocytochemistry and detects the same neuronal structures as ME antisera in the central nervous system.7

The position of the MEAGL in the middle of the preproenkephalin A as compared with the terminal position of MEAP is also a possible explanation to the lower detection sensitivity of MEAGL-like immunoreactivity. All antisera used in this study detect multiple molecular forms of immunoreactivities in the rat brain, spinal cord, and adrenal medulla.7 It remains to be studied with gel filtration techniques coupled to radioimmunoassay whether the immunoreactivities demonstrated in this study are present as pentapeptides, octapeptides, or as larger molecular forms. The possibility that dynorphin- or β-endorphin-related peptides are detected by our antisera is excluded because the antisera have no cross-reactivity with these peptides and in preabsorption tests these peptides failed to abolish the immunofluorescence. So far, both SP and enkephalin-like immunoreactivities have been found in retinoblastoma cells. This does not mean necessarily that the tumor cells produce the entire peptide precursor since fragments of the molecule possibly could be detected by the antisera used. The heterogeneity of the tumor cells suggests that retinoblastoma cells may be composed of several clones or that one clone is capable of producing different peptides.

It has been shown that SP promotes the growth of neuroblasts,11 but the significance of neuropeptides in the retinal cells undergoing malignant change is unclear. The findings may prove to be important not only for diagnosis and follow-up of retinoblastoma but also for understanding the nature of this tumor. In addition to SP and enkephalins, other neuropeptides such as vasoactive intestinal polypeptide, cholecystokinin, neurotensin, thyrotropin releasing hormone, and leuteinizing hormone releasing hormone have been identified in the retinas of experimental animals.4-6,12 Since at least some of these neuropeptides occur in different subpopulations of amacrine cells, a neuropeptide immunohistochemistry might allow for the development of an amacrine cell labelling technique.

Key words: enkephalin, immunoreaction, retinoblastoma, humans

Acknowledgment. The authors thank Dr. H.-Y. Yang for antisera against the opioid peptides.

From the Department of Ophthalmology, University of Helsinki, Haartmaninkatu 4 C, SF-00290 Helsinki, Finland, and the Department of Anatomy, University of Helsinki, Silta-vuorenpenkeri 17, SF-00170 Helsinki, Finland. Supported in part by a grant from the Paulo Foundation To Drs. Tervo. Submitted for publication: March 1, 1984. Reprint requests: Ahti Tarkkanen, MD, Helsinki University Eye Hospital, Haartmaninkatu 4 C, SF-00290, Helsinki, Finland.

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