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Localization of cellular retinol-binding protein and retinol-binding protein in cells comprising the blood–brain barrier of rat and human

(choroid plexus/vitamin A/immunohistochemistry)

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ABSTRACT **Brain is not generally recognized as an organ that requires vitamin A, perhaps because no obvious histologic lesions have been observed in severely vitamin A-deficient animals. However, brain tissue does contain cellular vitamin A-binding proteins and a nuclear receptor protein for retinoic acid. In the present study, immunohistochemical techniques were used to determine the cell-specific location of cellular retinol-binding protein in human and rat brain tissue. Cellular retinol-binding protein was localized specifically within the endothelial cells of the brain microvasculature and within the cuboidal epithelial cells of the choroid plexus, two primary sites of the mammalian blood–brain barrier. In addition, autoradiographic procedures demonstrated binding sites for serum retinol-binding protein in the choroidal epithelium. These observations suggest that a significant movement of retinol across the blood–brain barrier may occur.**

The mammalian brain is secluded in a specialized environment created by a series of selective membranes at the blood–brain interface. The passive entry of many biomolecules into neutral tissue is prevented by tight junctions that exist between the endothelial cells of the cerebral microvasculature. An additional barrier between the blood and the cerebrospinal fluid (CSF) is formed by tight junctions between adjacent epithelial cells of the choroid plexus. Saturable, facilitative transport processes for monosaccharides, amino acids, fatty acids, and various vitamins exist within the cells comprising these barrier sites to control the passage of these nutrients into neural tissue. The presence of a mechanism for the transport of vitamin A across neural capillaries and/or choroidal epithelium is not presently recognized.

Retinol (vitamin A alcohol) is delivered to target tissues as a complex with serum retinol-binding protein (RBP) (1). A putative plasma membrane receptor on target cells binds RBP and internalizes the retinol (1). A cytoplasmic protein termed cellular retinol-binding protein (CRBP) may then transport the internalized retinol through the aqueous milieu of the cytoplasm to intracellular sites of action or metabolism (2). Studies of the retina (3) and testes (4, 5) have demonstrated high levels of CRBP in some of the cells that form the blood–retina and blood–testis barriers, suggesting a role for CRBP in the transcellular movement of retinol across these blood–organ barriers. CRBP is present in human (6) and rat (7, 8) neural tissue. Here, we report that CRBP can be demonstrated in those cells that form the blood–brain barrier of humans and rats, specifically within endothelial cells of the brain microvasculature and in cuboidal epithelial cells of the choroid plexus. Furthermore, binding sites for RBP were observed in epithelium of the choroid plexus of rat. Consequently, translocation of retinol across the blood–brain barrier may occur via RBP uptake from the plasma with subsequent transcellular movement of retinol as a complex with CRBP.

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METHODS

Preparation of Tissues. Adult male rats weighing 300–350 g were anesthetized with pentobarbital and perfused through the heart with 150–200 ml of Tyrode's buffer. Whole-body fixation was accomplished by cardiac perfusion with Perfix (Fisher). Brains were removed and sliced through the lateral and third ventricles into coronal sections ≈3 mm thick. The coronal sections were fixed an additional hour at room temperature by immersion in Perfix. Human tissue was obtained at autopsy and fixed by immersion in 10% buffered formaldehyde. The tissues were dehydrated and embedded in paraffin for sectioning. The examples shown here, in Figs. 1*C* and 2 *C* and *D*, were from a 54-year-old male who died of an acute myocardial infarction.

Immunohistochemical Procedures. CRBP in rat and human tissue was immunolocalized according to the ABC immunoperoxidase method. The primary antibodies were affinity-purified IgG from rabbit against rat liver CRBP or human liver CRBP (4, 6) employing a Vectastain ABC kit obtained from Vector Laboratories. Five-micron sections of tissue were processed as described (4). Control incubations used nonimmune or preimmune rabbit IgG in place of the primary antibody. All slides were lightly counterstained with hematoxylin following peroxidase staining.

Autoradiographic Localization of ^{125}I-Labeled RBP (^{125}I-RBP) in Rat Choroid Plexus. RBP was purified from bovine serum according to methods published previously (9). Eleven micrograms of the protein was iodinated according to the chloramine-T method to a specific activity of 2.94×10^3 Ci/mmol (1 Ci = 37 GBq). A weanling 38-g Sprague–Dawley rat was anesthetized with 30 mg of Nembutal per kg and injected in the right external jugular vein with 1.5 mCi of the ^{125}I-RBP. Ten minutes after injection, the blood was flushed from the animal for 5 min by transcardiac perfusion with Hanks' balanced salt solution. Thereafter, the animal was similarly perfused for 10 min with a mixture of 1% formaldehyde/1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) in order to crosslink bound RBP to its membrane receptor. The choroid plexuses were then removed from the

Abbreviations: RBP, retinol-binding protein; CRBP, cellular retinol-binding protein; CSF, cerebrospinal fluid.

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brain and further fixed for 1 hr in cacodylate-buffered 1% osmium tetroxide, dehydrated, and embedded in Araldite 502 (CIBA Pharmaceutical). Autoradiograms of tissue sections were then prepared as described earlier (10).

RESULTS

Cellular Localization of CRBP by Immunohistochemical Techniques. Light microscopy of the tissue sections treated with the primary antiserum revealed positive staining for CRBP within the microvasculature of human and rat cerebral cortex (Fig. 1 *A–C*). The predominant staining was of the cytoplasm of cells, which, by location and histological characteristics, were judged to be endothelial cells. However, the limitations of light microscopy precluded the exclusion of the possibility that scattered processes of pericytes also contributed to the immunostaining seen in some vessels. Vascular staining was consistently more obvious in the endothelium of rat than in human. Approximately 60% of detectable rat vessels showed obvious staining compared to 20% of human vessels. This staining was dependent on the presence of antibodies to CRBP, as identical procedures using nonimmune rabbit IgG resulted in no apparent specific staining (data not shown). CRBP had not been observed within the vascular endothelium of the testes (4) or the intestine (11) when these tissues were examined in similar immunohistochemical studies. The blood vessels of the latter tissues do not contain tight junctions and allow unrestricted diffusion of most serum constituents.

CRBP was also apparent throughout the cuboidal epithelium of the choroid plexus of human and rat (Fig. 2 *A* and *C*). Particularly evident in human sections was the absence of staining in the vascularized connective tissue matrix of the choroidal stroma (Fig. 2*C*). Sections treated under identical conditions with preimmune rabbit IgG showed no specific staining (Fig. 2 *B* and *D*).

Autoradiographic Localization of ^{125}I-RBP in the Rat Choroid Plexus. Autoradiographic procedures following intravenous injection and aldehyde perfusion revealed binding of ^{125}I-RBP to the basolateral surface of the cuboidal epithelium (Fig. 3) reminiscent of that previously observed in the retinal pigment epithelium (12). However, some ^{125}I-RBP appeared to have been internalized by the epithelial cells of the choroid plexus. No internalization had been evident in the retinal pigment epithelium. It is conceivable that the mode of delivery of retinol might be different for the two epithelial layers. The choroid plexus epithelium might employ the principle of receptor–ligand internalization observed for other systems—e.g., asialoglycoprotein and its receptor (13). The epithelial cells of the choroid plexus, retina, and ciliary body were the only cells examined in this study that contained levels of plasma membrane RBP receptor detectable by autoradiographic methods. Although there was vigorous endocytosis of ^{125}I-RBP from the filtered blood by apical plasma membrane of kidney proximal convoluted tubule cells, the basal surfaces of epithelial cells or cells of epithelial origin in the testis, kidney, liver, trachea, and esophagus were not labeled. The specificity of this binding on retinal pigment epithelium has been demonstrated in isolated cells by competition with nonradioactive RBP (14). Equivalent competition studies were not conducted in the current *in vivo* study, but the similarity in binding observed for the choroid plexus and retinal pigment epithelium, their close developmental kinship, and the abundance of CRBP in their cytoplasm strongly suggest that the observed binding of ^{125}I-RBP was specific.

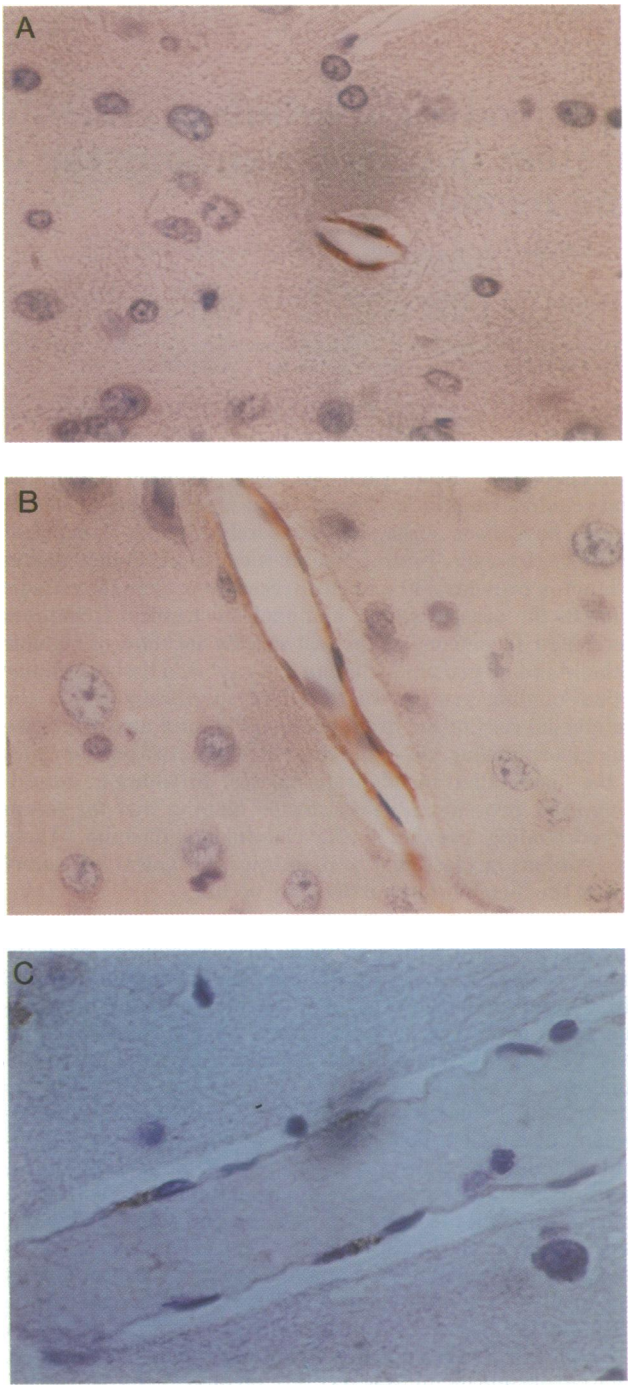

FIG. 1. Demonstration of immunoreactive CRBP in the cerebral microvasculature. Five-micron sections from rat (*A* and *B*) and human (*C*) brain were processed according to the ABC immunoperoxidase method and then lightly counterstained with hematoxylin. The brown reaction product was present in the endothelial cells of rat microvessels seen in cross section (*A*) and in an oblique section (*B*). The human microvessel shown is swollen with blood (*C*) and light brown staining is visible at the thicker parts of the endothelial cells near the nuclei. (×600.)

DISCUSSION

The brain is one of the most metabolically active tissues in the body. Passive diffusion of many nutrients into brain interstitial fluid is restricted to tight junctions that occur between adjacent cells of the vascular endothelium. Consequently, facilitative mechanisms are present that transport a variety of compounds, including monosaccharides, amino acids, pu-

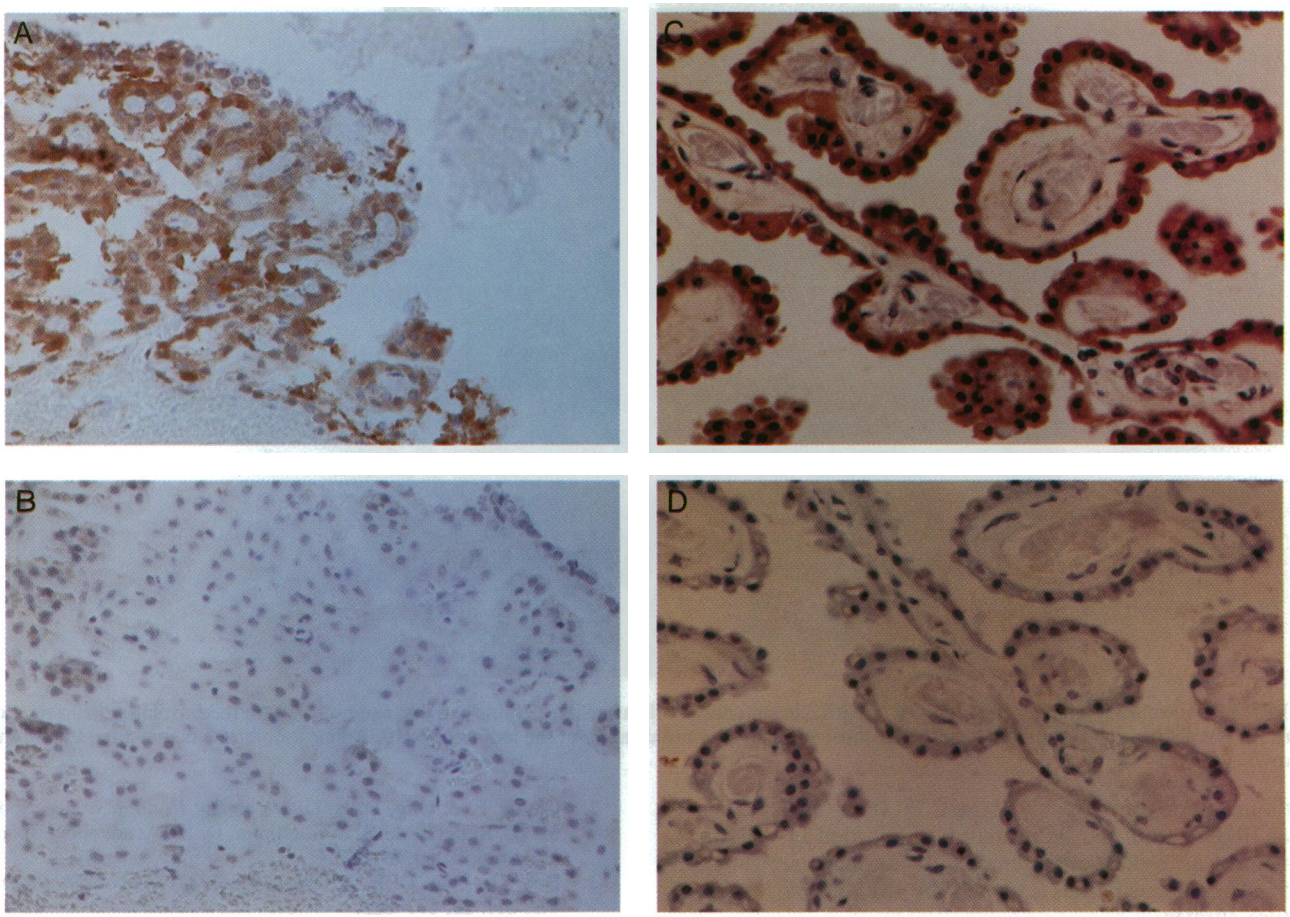

FIG. 2. Demonstration of immunoreactive CRBP in the choroid plexus. Immunoreactive CRBP, indicated by the brown reaction product, is apparent in the cuboidal epithelium of rat (*A*) and human (*C*) choroid plexus. Control sections using preimmune rabbit IgG in place of the primary antibody show no staining in rat (*B*) and human (*D*) sections. All sections were lightly counterstained with hematoxylin. (×240.)

rines, prostaglandins, and ascorbic acid, across these cells and into neural tissue (15). Using immunohistochemical techniques, CRBP was demonstrated in the microvascular endothelium of rat and human brain. It was not observed in the vascular system of other tissues where unrestricted diffusion of plasma serum constituents occurs (4, 11). These observations suggest that a role for CRBP in brain capillary endothelium is to participate in the movement of retinol across this barrier and into the brain interstitial fluid.

The vessels of the choroid plexus are fenestrated and thus many plasma components pass unhindered into the choroidal stroma. However, passive diffusion of most compounds from the choroidal stroma to the CSF is prevented by tight junctions that exist near the apical border of adjacent choroidal epithelial cells. Strong staining for CRBP, particularly intense in human samples, was observed in the choroidal epithelial cells. CRBP was not apparent in the cells of the choroidal stroma. The epithelial cells of the choroid plexus are known to contain specific transport systems for thiamine, ascorbic acid, pyridoxine, folate, and inositol (15). The presence of high levels of CRBP in these cells may indicate a transport system for vitamin A also exists in the choroid plexus.

Interestingly, CRBP has also been observed in the cells of other blood–organ barriers. The Sertoli cells of the testes and the retinal pigment epithelium (RPE) of the eye contain tight junctions between adjacent cells, forming the blood–testes and part of the blood–retina barriers, respectively. Sertoli cells and RPE cells contain high levels of CRBP (3–5). The presence of CRBP within four different cell types comprising separate blood–organ barriers strongly suggests a common functional link for CRBP in these cells.

The presence of RBP binding sites in the epithelial cells of the choroid plexus adds further support to the hypothesis of vitamin A movement across the blood–brain barrier. RBP binding sites have been localized in other tissues—specifically along the basolateral surface of the retinal pigment epithelium (12) and within the interstitial cells of the testes (16). The localization of ^{125}I-RBP along the basolateral surface and within the epithelial cells of the choroid plexus suggests that receptor-mediated uptake of the RBP–retinol complex may occur in these cells. Liver cells have been demonstrated to internalize RBP (17). Although RBP binding was not demonstrable in the microvasculature, uptake at the capillary endothelium could potentially involve RBP receptor activity as well. Precedent for this mechanism of transport was established with the demonstration of transferrin receptors on brain capillaries (18).

Detailed analysis of vitamin A levels in CSF is lacking. To our knowledge, modern sensitive techniques have not been used to determine vitamin A levels in the CSF. However, RBP has been observed in CSF (19), suggesting it as a possible carrier for retinol in this compartment. Several CSF proteins are synthesized and secreted by the choroid plexus, including transthyretin (20, 21), which forms a ternary complex with retinol–RBP in blood. Detectable levels of RBP message in brain (22) suggest the possibility that choroid plexus may also synthesize RBP for export into the CSF. Another possibility might be a retinol-binding protein described as present in neural tissue (23) and subsequently termed interphotoreceptor retinol-binding protein (IRBP). Immunochemical studies (24) have demonstrated detectable levels of IRBP in monkey cerebral cortex homogenates,

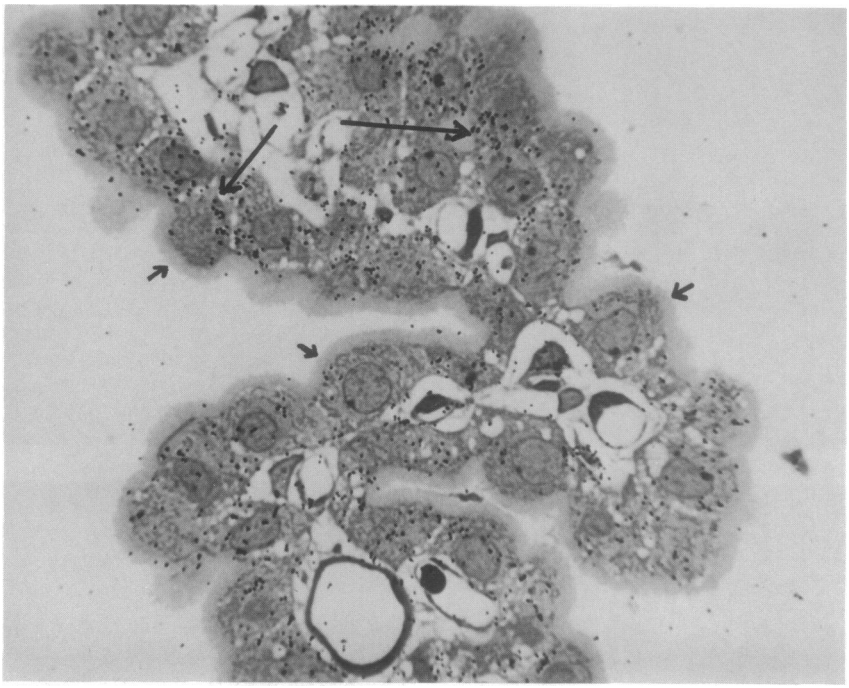

FIG. 3. Autoradiographic localization of ^{125}I-RBP in rat choroid plexus. Silver grains can be seen not only on the basolateral surface (long arrows) but also within the cuboidal epithelial cells, suggesting internalization of the iodinated RBP. Apical membrane is indicated by the short arrows. (×890.)

although none was detected in the CSF. It has been proposed that IRBP within the brain may be important in vitamin A transport as well as maintenance of normal brain function (25). These possibilities can only be decided by results of future studies.

It is becoming clear that vitamin A plays an important role in early development of the neural system. Retinoic acid (all-*trans* or 13-*cis*) can cause malformation of the central nervous system in rodents (e.g., ref. 26) and humans (e.g., ref. 27). Applied exogenously, retinoic acid causes an anteroposterior transformation of the developing central nervous system in *Xenopus laevis*, and it was detected as an endogenous compound (28). Furthermore, cellular retinoic acid-binding protein (29, 30) and CRBP (30), as well as their transcripts (31), have been localized to specific cells of the developing nervous system in mouse and rat. Cellular retinoic acid-binding protein has also been localized in the developing nervous system of chick (32, 33). A possible function for vitamin A in the mature brain has not been postulated but recent studies have identified a nuclear retinoic acid-receptor protein that is expressed in abundant levels in several areas of the adult brain (34–36). This presence of retinoid receptors suggests a continuing role for vitamin A in mature neural tissue. In order for vitamin A to exert its putative effect within the brain, mechanisms must exist for its translocation across the blood–brain barrier. It may be that the observed CRBP in those cells that comprise the primary sites of the blood–brain barrier serves as part of this translocation apparatus.

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