DISTRIBUTION OF AN ENDOGENOUS LECTIN IN THE DEVELOPING CHICK OPTIC TECTUM

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ABSTRACT

We determined the cellular localization of an endogenous lectin at various times during the development of a well-characterized region of chick brain, the optic tectum. This lectin is a carbohydrate-binding protein that interacts with lactose and other saccharides, undergoes striking changes in specific activity with development, and has previously been purified by affinity chromatography from extracts of embryonic chick brain and muscle. Cellular localization in the tectum was done by indirect immunofluorescent staining, using immunoglobulin G derived from an antiserum raised against pure lectin.

No lectin was detectable in the optic tectum examined at 5 days of embryonic development. From ~7 days of development, neuronal cell bodies and fibers were labeled by the antibody; and extracts of tectum contained hemagglutination activity that could be inhibited by lactose or by the antiserum. Lectin remained present in many tectal neuronal layers after hatching; but in 2-month-old chicks it was sparse or absent in most of the tectum except for prominent labeling of fibers in the stratum album centrale. The initial appearance of lectin in the optic tectum was not dependent on innervation by optic nerve fibers since bilateral enucleation during embryogenesis did not affect it. Lectin was detectable on the surface of embryonic optic tectal neurons dissociated with a buffer containing EDTA.

KEY WORDS lectin · optic tectum · brain development · cell surface · cell recognition · cell adhesion

Extracts of embryonic chick tissues, including muscle (5, 12, 14) and brain (7, 8), contain a lectin that can be assayed as an agglutinin of trypsinized rabbit erythrocytes. The hemagglutination activity of this divalent carbohydrate-binding protein is presumably mediated by binding to specific carbohydrate residues on the surface of adjacent erythrocytes. It can be inhibited by specific saccharides, such as lactose and thiodigalactoside (5, 12, 14), but not by many other saccharides, indicating specificity of its binding site.

Lectin activity in extracts of muscle (5, 12) and brain (7) changes substantially with development. In both these tissues, activity is relatively low early in development, rises to maximum levels at around 12 days of incubation, and falls by a factor of 5–10 by ~1 wk after hatching. Striking changes in lectin activity with development of these tissues suggests that this lectin may play a role in their differentiation.

We wish to determine the role of this lectin in brain development. As a starting point, we have examined its distribution by immunohistochemical techniques in a well-studied region of chick brain, the optic tectum. The antibody used was raised against embryonic chick muscle lectin purified to

homogeneity by affinity chromatography followed by isoelectric focusing (13). The muscle lectin is very similar, if not identical, to the brain lectin by both physicochemical and immunological criteria (8). Muscle extracts were used for the purification of the antigen for immunization since, at its peak, the specific activity of lectin in muscle extracts is ~20-fold greater than the peak activity in brain extracts (7).

In the present report, we describe the cellular distribution of the lectin in the developing chick optic tectum as studied by indirect immunofluorescence. We show that: (*a*) lectin is not detectable at very early stages, becomes prominent in differentiating optic tectal neurons, and is absent from most cells in 2-month-old chickens; (*b*) the tectal lectin is prominent in developing neuronal cell bodies and their processes and is also observed in developing ependymal and glial cells; (*c*) lectin is detectable on the surface of some dissociated tectal neurons; and (*d*) the appearance of lectin in tectal neurons is not dependent on innervation by optic nerve fibers; but its distribution in the tectum, like overall tectal development, is influenced by innervation.

MATERIALS AND METHODS

Animals

Fertilized eggs of a White Leghorn strain were obtained from a local hatchery and incubated at 38°C with relative humidity kept at 70%. The developmental age of each embryo was checked according to the criteria of Hamburger and Hamilton (6). Nomenclature of the tectal layers is that of LaVail and Cowan (9).

Assay of Lectin Activity

Extracts of optic tectum were prepared by homogenization in phosphate-buffered saline, pH 7.2 (PBS), containing 0.3 M lactose, as described for embryonic chick muscle (12, 13). Hemagglutination activity was assayed after dialysis of the extracts against PBS by preparing serial twofold dilutions in microtiter V plates (Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.) as described (12, 13). Hemagglutination titer is the highest dilution of extract causing agglutination, e.g., 1:32. Lectin specific activity is defined as the reciprocal of the titer of a sample divided by milligrams of protein per milliliter of the sample.

Immunological Studies

Antibody was raised in rabbits by immunization with embryonic chick muscle lectin that had been purified by affinity chromatography and isoelectric focusing, as described previously (13). The antibody used here was characterized in the past (8, 13). It gave a single band in immunodiffusion plates when tested against purified lectin from muscle (13) and brain (8); and these two antigens showed a reaction of identity (8). Immunodiffusion against crude tectal extract was conducted in agar containing 0.3 M lactose as described previously (8). Inhibition of hemagglutination activity of tectal extracts by antisera was determined as described previously (8). For immunofluorescence studies, we used an immunoglobulin G (IgG) fraction prepared by precipitation with ammonium sulfate followed by chromatography on *O*-(diethylaminoethyl)cellulose (2).

Fixation of the Tissue

Optic lobes were dissected out from embryos or chicks at different stages of development and immediately dropped into 95% ethanol at 4°C. After 60 min, the tissue was cut by hand in pieces 2–4 mm thick. After 15–24 h of fixation at 4°C, the tissue was dehydrated by immersing in four changes of 100% ethanol for 60 min each. It was cleared in xylene by three 60-min immersions, the last at room temperature. Subsequently, the tissue was embedded in paraffin wax and cut into 8-μm sections. Sections were generally taken from the middle of the tectum in the rostro-caudal dimension, but this was not carefully controlled.

Immunofluorescence Studies

After removal of the paraffin, the sections were incubated for 30 min at 4°C with unlabeled goat anti-rabbit IgG (Miles Laboratories Inc., Elkhart, Ind.) to

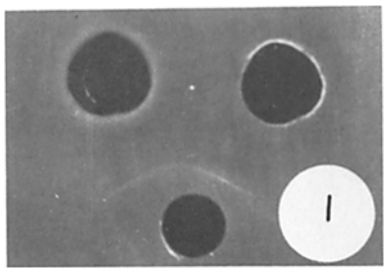

FIGURE 1 Double diffusion of antiserum against tectal extract and purified muscle lectin. The lower well contained 10 μl of immune IgG solution. The upper right well contained 10 μl with ~5 μg of embryonic chick muscle lectin purified by affinity chromatography. The upper left well contained 10 μl of concentrated extract from tectum of 10-day-old chick embryos. The extract had been concentrated 10-fold by ultrafiltration before addition to the well. The agarose was equilibrated with 0.3 M lactose to prevent binding of the lectin to the agarose. A control IgG preparation showed no reaction with this antigen.

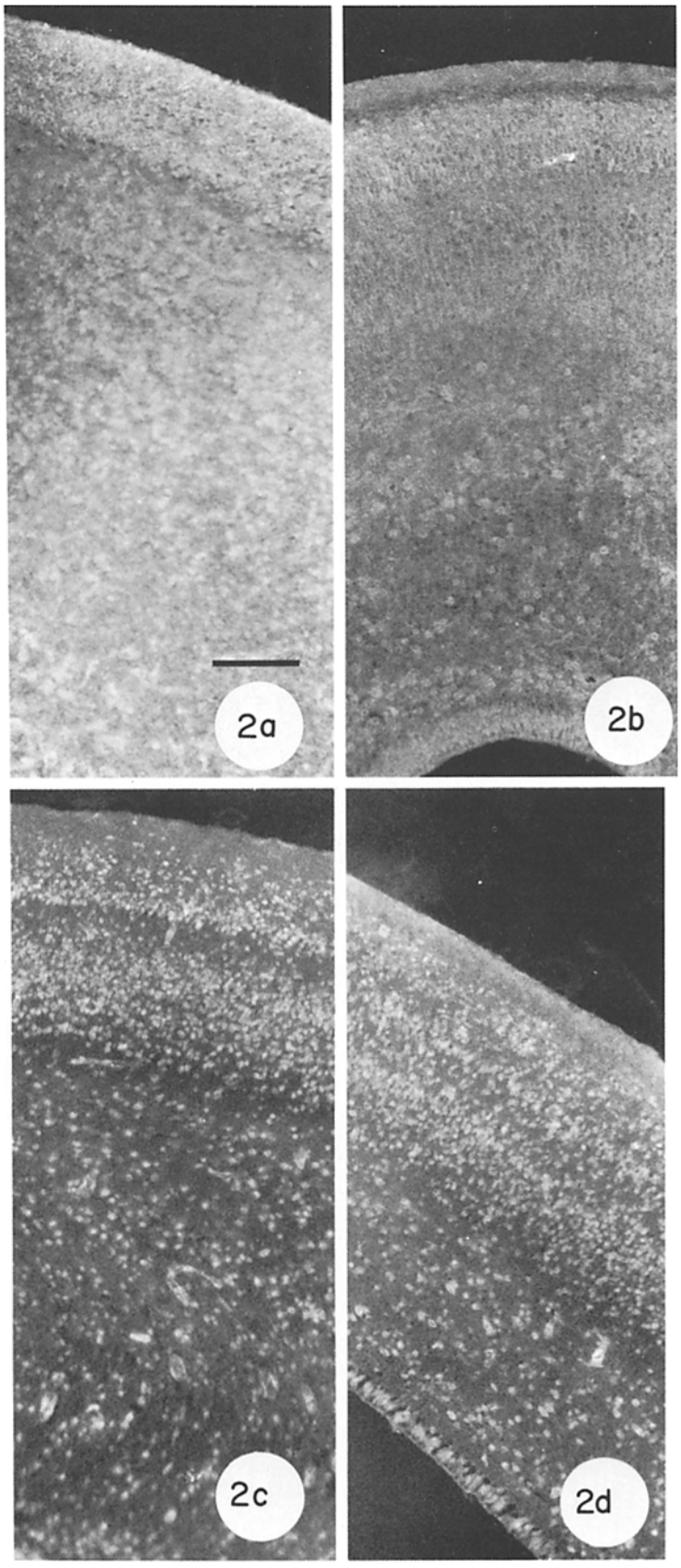

FIGURE 2 Immunofluorescent staining of sections of embryonic chick optic tectum obtained after (*a*) 7 days, (*b*) 10 days, (*c*) 13 days, (*d*) 16 days of incubation. The sections in Fig. 2*b* and *d* were made at the level of the ventricles. Bar, 100 μm.

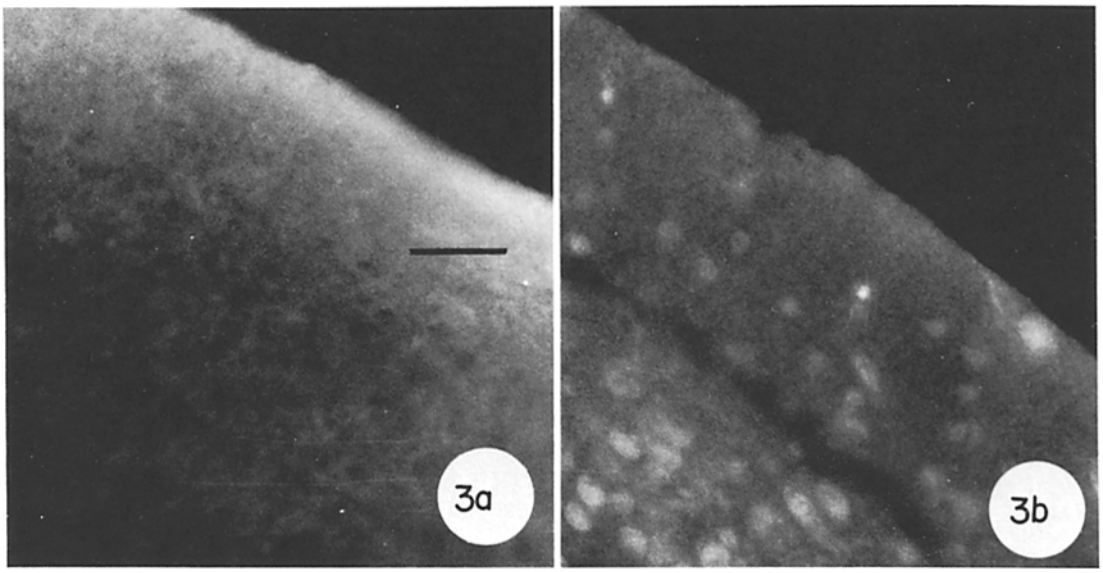

FIGURE 3 Immunofluorescent staining of the outer portion of the optic tectum of (*a*) 7-day, and (*b*) 13-day chick embryos showing the decrease in fluorescence of the outer layers between these times. Bar, 25 μm.

help reduce the nonspecific background. The sections were then washed in PBS and incubated for 30 min at 4°C in appropriate dilutions of preimmune or immune IgG. The samples were washed in PBS and subsequently reacted for 30 min at 4°C with a 1:40 dilution of fluorescein-isothiocyanate-labeled goat anti-rabbit IgG (Microbiological Associates, Bethesda, Md.). After several washes in PBS, the samples were examined with a fluorescence microscope.

Enucleation of Embryos

3½-day-old embryos (stage 21) were exposed through an opening in the shell, and the optic vesicles were removed carefully with a Pasteur pipette. After the opening was sealed, the eggs were replaced in the incubator.

Dissociation and Immunofluorescent Staining of Embryonic Tectal Cells

Tecta from 7-day-old embryos were dissociated in a medium containing EDTA but no proteolytic enzymes, essentially as described by McDonough and Lilien (11). In this method, dissociation is produced by flushing through a Pasteur pipette in appropriate media (11). After dissociation, the cells were centrifuged at 200 *g* for 5 min at 4°C and washed three times with PBS containing 0.1% EDTA. Cells were then washed with HEPES-buffered saline, pH 7.2, containing 1 mg/ml of glucose (this solution is referred to as HBSG). The samples were then incubated at 4°C for 30 min in HBSG containing either immune or normal rabbit IgG, each diluted 1:10 relative to the initial starting concentration of the antiserum. After they were washed three times in PBS, the cells were reacted for 30 min at 4°C with a 1:40 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit IgG. The cells were then washed three times in PBS, suspended in HBSG, and incubated at 37°C for up to 60 min. Samples were studied every 15 min with a fluorescence microscope.

RESULTS

Assay of Lectin Activity in Developing Optic Tectum

We pooled 10–20 optic tecta from groups of chick embryos incubated for various periods of time, and extracted and assayed lectin activity. No lectin activity was detectable in tecta from 5-day-old embryos. Lectin activity was detectable in 7-day-old embryos. The average specific activity in two extracts from tecta of 7-day-old embryos was 13. Specific activity rose to an apparent maximum of 32 in tecta from 10-day-old embryos and declined to 8 in extracts of 16-day-old embryos. Although the general trend was confirmed in several other experiments, the precise titers observed varied with the particular batch of erythrocytes that was used. The average specific activity in the extracts from 10-day embryo tectum was in

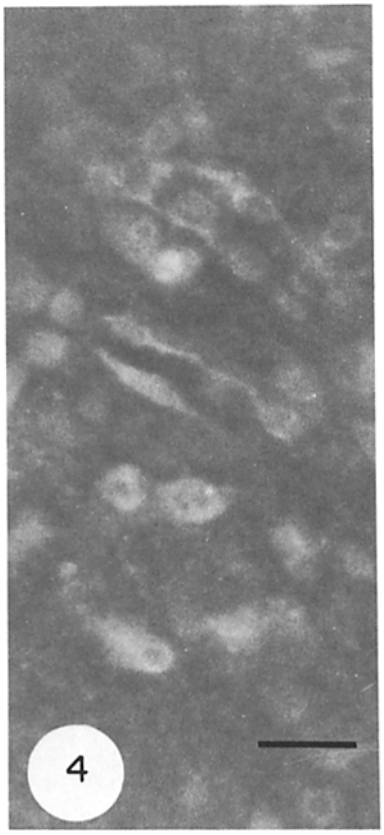

FIGURE 4 Immunofluorescent staining of a portion of the optic tectum of a 13-day-old chick embryo, showing a higher magnification of layer III. Neuronal cell bodies and processes are labeled. Some labeled cells could be glial. Bar, 25 μm.

the same range as that reported previously for whole brain extracts (7). 75 mM lactose completely inhibited lectin activity from 10-day-old embryonic tectum, indicating that this lectin was similar to those previously characterized in muscle and brain (13, 8).

Immunological Studies

Immune serum raised against highly purified lectin from muscle (13) was a potent inhibitor of the hemagglutination activity of extracts from 10-day-old chicks. At a dilution of 1:100, it reduced the hemagglutination titer of these extracts to zero. Identical dilutions of preimmune serum had no detectable effect on the lectin activity. IgG preparations from sera gave identical results. This indicates that the lectin activity from tectum, like that from brain (8), is highly cross-reactive with the purified lectin from muscle.

We also studied cross-reactivity by gel diffusion. Crude tectal extract and purified muscle lectin were placed in adjacent wells and diffused against IgG derived from antibody raised against the purified muscle lectin which was placed in the center well. Both antigens showed a single band which merged without spurring (Fig. 1), indicating immunological identity.

Immunofluorescent Staining

Sections from embryos and chicks of various ages were studied by indirect immunofluorescence. In all cases, sections reacted with normal rabbit IgG showed very faint and diffuse fluorescence.

Tecta from 5-day-old embryos showed no specific immunofluorescent staining, confirming the absence of detectable hemagglutinin activity indicated above. In tecta from 7-day-old embryos, prominent and diffuse staining was observed throughout (Fig. 2*a*), especially in the outermost layer (Fig. 3*a*) called layer IV (9). LaVail and Cowan (9) showed that, at this stage, this region is cell-free and rich in dendrites originating in cells in the lower layers. Although the distribution of staining suggested concentration of antigen in neuronal cell bodies, the neuronal epithelium was not clearly distinguishable. The diffuseness of staining suggested prominent localization in fibers. The lack of distinct cellular detail at this stage may be due, in part, to the relatively poorer fixation generally observed in brain tissue from younger embryos.

By 10 days of incubation (Fig. 2*b*), the outermost zone (layer X) showed relatively diminished labeling. This zone now corresponds to the developing stratum opticum and is largely formed by ingrowing retinal fibers (9). At this stage, we found better definition of neuronal cell bodies and of the neural epithelial cells. At 13 days of incubation (Fig. 2*c*), many layers of tectal neurons showed fluorescence which was found in nerve cell bodies and processes (Fig. 4). However, the outer layers, between layer VIII and X, showed little labeling (Fig. 3*b*). These layers are rich in neurons that are forming synaptic contacts with incoming optic nerve fibers. The overall pattern in 16-day embryo tecta was similar (Fig. 2*d*).

In tecta from two-day post-hatching chicks (Fig. 5*a*), neuronal cell bodies in lamina g (9) were

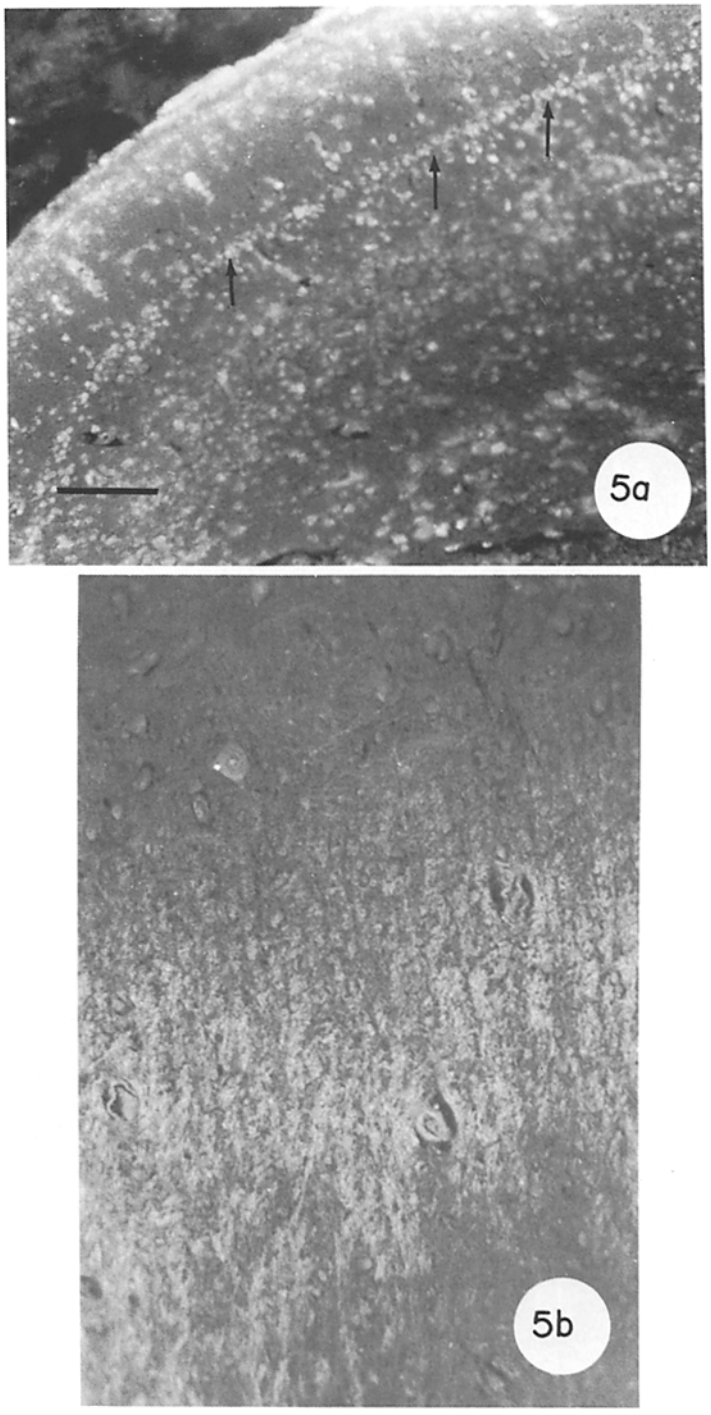

FIGURE 5 Immunofluorescent staining of portions of the tectum of chick (*a*) 2 days after hatching and (*b*) 2 mo after hatching. The relatively sharply defined band of cells (arrows) in Fig. 5*a* is lamina g. In Fig. 5*b*, only a portion of the tectum is shown. The fluorescent fibers are predominantly in the stratum album centrale. The other portions of the tectum not shown in this figure showed little or no fluorescence. Bar, 100 μm.

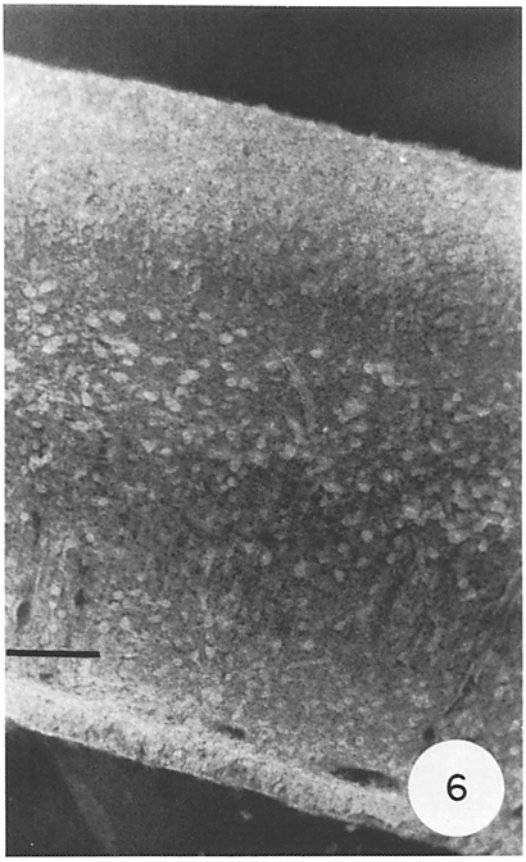

FIGURE 6 Immunofluorescent staining of a section of the optic tectum of a 13-day-old embryo which had been deprived of both optic vesicles at stage 21 ($3^1/_2$ days of incubation). The outermost tectal layers show intense fluorescence in contrast with the normal tecta at this stage of development as shown in Figs. 2*c* and 3*b*. Bar, 100 μm.

prominently labeled and formed an especially noticeable discrete zone. There was relatively little labeling of the cell bodies in the more superficial layers in comparison with the tecta from 16-day-old embryos (Fig. 2*d*). Two months after hatching, there was little or no labeling in most of the tectum, with the prominent exception of fibers concentrated mostly in the stratum album centrale (Fig. 5*b*).

Effect of Denervation

When the eyes were enucleated at embryonic day $3^1/_2$ and tecta were examined at a number of times thereafter, lectin was detected by immunofluorescence techniques. No apparent change in the distribution of the lectin was observed in 8-, 9-, 10-, and 11-day-old embryonic tecta. However, striking changes were found in the tectum from 13-day-old embryos (Fig. 6). The outer tectal layer which, in the normal case (Fig. 3*b*), showed little or no fluorescence, showed intense fluorescence in the denervated state. Apparently, in the normal case there is marked loss or dilution of antigen in this region as a consequence of the maturational events associated with incoming optic nerve fibers; but in the denervated tectum this is not observed. In 18-day-old embryos, we found characteristic maturational abnormalities in denervated tectum, and relatively little labeling was observed compared with normal tectum.

Evidence for Cell Surface Location of Lectin

The immunofluorescence studies of sections of optic tectum showed that the lectin is prominent within nerve cells. In previous studies with muscle, we found that, although there was substantial intracellular lectin, we could also find evidence for lectin activity on the cell surface (13). In an attempt to determine whether or not lectin activity was present on tectal cell surfaces, we dissociated optic tectal cells from 7-day-old embryos. Dissociation was done in a medium containing EDTA. Proteolytic enzymes were not used, to prevent degradation of cell surface molecules. After incubation with antibody and fluorescent goat anti-rabbit IgG, many cells showed diffuse surface fluorescence which subsequently coalesced into discrete patches (Fig. 7*a–d*). Only ~30% of the cells were stained. In general, the large cells were more likely to stain than the smaller cells; but examples of staining of both types are shown.

Since these studies showed that lectin or an immunologically related antigen is detectable on the surface of some dissociated tectal cells, we considered the possibility that the antigen was not normally associated with the cell surface but became spuriously associated because of leakage from some broken cells. To study this, we cultured the dissociated cells for several days, then fixed them before reaction with the immune IgG. The intention was to provide time for lectin that had been bound artifactually to be sloughed off or degraded. Although the cultured cells also showed evidence of surface labeling, there were always some broken cells in the cultures, raising the continued possibility of spurious association with the cell surface.

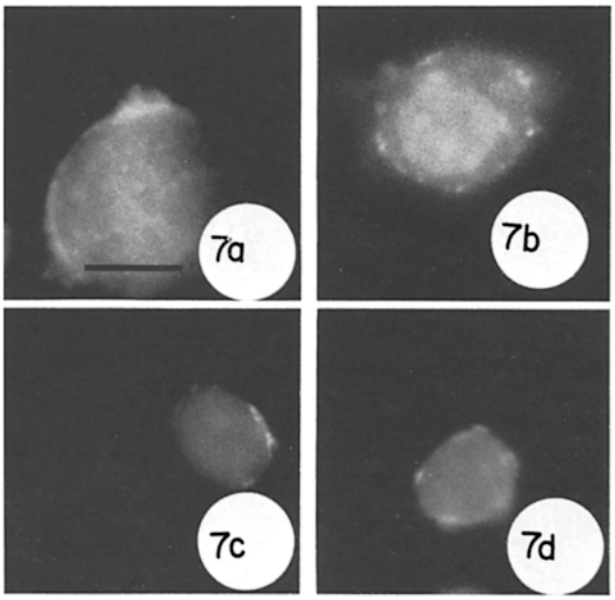

FIGURE 7 Immunofluorescent staining of dissociated tectal cells from 7-day embryos. (*a–d*). Cells of various sizes that had been dissociated mechanically in a medium containing EDTA, washed carefully, then reacted with immune IgG, followed by fluorescent goat anti-rabbit gamma globulin. The photographs were made 30–60 min after incubation at 37°C. Patches of fluorescence appeared only after this incubation. ~70% of the cells in the suspension showed no significant fluorescence. No fluorescent labeling was found when control IgG was used.

DISCUSSION

The results provide evidence that a developmentally regulated lactose-sensitive lectin, previously found in extracts of embryonic chick brain, is associated with neurons at a specific stage in their development. Immunofluorescent staining shows lectin in neuronal cell bodies and their processes. In addition, we have evidence that some lectin may be present on some neuronal cell surfaces.

The histochemical studies indicate that the presence of lectin in tectal neurons is correlated with a specific phase in their maturation; but they do not clarify the specific role that the lectin might play in this process. Lectin is not detectable either as a hemagglutinin or by immunofluorescent staining in 5-day-old chick optic tectum, at which time there is extensive cellular proliferation (3). Therefore, the appearance of lectin does not seem to be correlated with cellular proliferation, as might also be inferred from its very low levels in chick pectoral muscle during cellular proliferation and its appearance at high levels thereafter (5, 12). In tectum, lectin is also apparently not associated with cellular migration, at least in the earliest phase. There is considerable neuronal migration beginning at around 4 days of tectal development and continuing through day 5 and beyond (10). Yet, there is no detectable lectin activity at these very early stages. However, later in development, at a time when neurons are taking up their ultimate position in the tectum, sending out processes and receiving innervation, lectin is prominent.

The precise relationship, if any, between innervation and the presence of lectin in neurons is not presently clear. If there is any relationship at all between these phenomena, our results suggest that lectin becomes prominent before innervation and diminishes after innervation. Some evidence for this comes from our findings on the outer layers of the optic tectum, between 7 and 13 days of development. At 7 days there is considerable staining in these layers whereas at 13 days, after arrival of optic nerve fibers (4), labeling is markedly diminished. The intense fluorescence of these outermost layers in denervated tectum, in which the optic vesicles were removed at 3½ days of incubation, suggests that failure of innervation is correlated with a lack of regression of lectin. However, tecta from older embryos also denervated at 3½ days of incubation showed regression

of lectin. The marked diminution in histochemically detectable lectin from 2-month-old chicks also suggests that when differentiation is completed lectin regresses. A similar conclusion could be drawn from the marked reduction in overall lectin activity in extracts of both muscle and brain of chicks prepared 7 days after hatching (8). The persistence of lectin in specific fibers in two-month-old chicks raises the possibility that it may continue to play a role in special cell types for prolonged periods.

In studies with cellular slime molds, there is evidence that developmentally regulated lectins, which appear on the cell surface, may play a role in cellular associations (1). The fact that tectal lectin changes with development and is detectable on cell surfaces suggests a possible role of this lectin in cellular associations. One possibility is that the carbohydrate-binding site of the lectin binds to appropriate complementary glycolipids and/or glycoproteins on the neurites of cells with which it comes into contact. The fact that lectin is prominent during the period of tectal innervation is consistent with this possibility. However, tectal differentiation is a complex process, and loose correlations such as these are very difficult to interpret. Direct implication of lectin in neuron-neuron interactions, such as synaptogenesis, might be tested by direct ultrastructural studies using immunological techniques.

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