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Immunohistochemical study of the Landolt's club cells in the chicken retina

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ABSTRACT. The immunohistochemical specificity of the Landolt's club cells of the chicken retina were studied by the indirect immunofluorescence and immunoperoxidase bridge methods with specific antiserum against neuronal filament proteins of these cells. In the tissue, the Landolt's club cells were selectively stained with the specific antiserum (antigen : isoelectric point=6.29, molecular weight=69,000). It appeared that the cells were bipolar and that the cell body lay in the superficial part of the internal nuclear layer. However, it was difficult to observe the dendrites like those of typical bipolar cells which synapse with photoreceptors. No reaction was found in ganglion cells, amacrine cells, horizontal cells and other types of bipolar cells which occupy the zone just inside the external plexiform layer.

From the 6th day of tissue culture, the selectively stained bipolar cells appeared on the surface of the monolayer cells. They were small and oval with large, spherical and eccentric nuclei. The cytoplasm was slightly less dense than other neuron cells.

Key words : immunohistochemical specificity — Landolt's club cells — chicken retina — neuronal filament protein — isoelectric point — tissue culture

In our laboratory, we are studying the cellular architecture of the chicken retina with immunohistochemical techniques.[1] The Landolt's club cells have been studied with classical silver techniques[2–6] and with the electron microscope,[7,8] but the functions of the cells have not yet been clarified. In this study we report on the immunohistochemical specificity of Landolt's club cells to antiserum made against specific proteins of these cells *in vitro* and *in vivo* samples, using the indirect immunofluorescence and immunoperoxidase bridge methods.

MATERIALS AND METHODS

Preparation of antisera :

To prepare the specific antiserum, the retina saline-soluble fraction was subjected to flat-bed isoelectric focusing in a granulated gel of Sephadex G-75 superfine. We referred to the LKB Application Note 198[9] in order to prepare the flat-bed gel. Isoelectric focusing was performed at a constant 8 W for 16 hours at 10°C. The zone containing the specific antigen (isoelectric point=6.29 and molecular weight=69,000) was collected from the gel bed and used for the immunization of rabbits (Fig. 1a, b).

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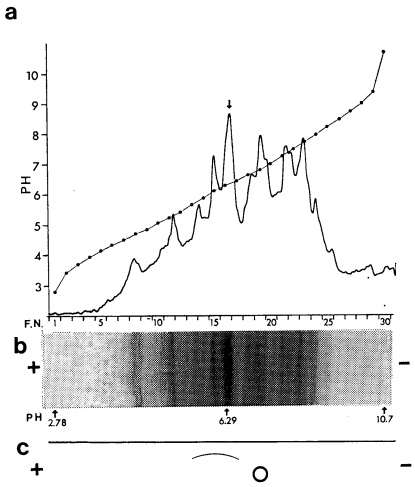

Fig. 1 a, b and c Chicken retina was isofocused by a technique of flat-bed isoelectric focusing. The profile of isoelectric focusing pattern was demonstrated in (b), and its densitometric profile was shown in (a). The pH gradient curve (●——●) is linear except for extremed fractions (pH 2.78 or 10.7). Precipitin arc was revealed corresponding to fraction 16 (F.N. 16, pH 6.29) against anti-F.N. 16 serum, absorbed with chicken liver extracts.

In order to reduce possible interference by nonspecific adsorption artifacts, each serum was absorbed prior to use with chicken liver extracts at 37°C for one hour. Serum was collected repeatedly throughout the course of immunization and tested for its specificity by the agarose-gel immunoelectrophoretic technique (Fig. 1c).

Immunoperoxidase staining in tissues (pre-embedding method) :

The retina and other tissues were collected freshly from decapitated, 2-month-old chickens. Small pieces of tissue (about one mm^3) were immediately fixed in 80% methyl alcohol (+4°C) for 2 hrs and hydrated in a phosphate buffer saline solution. After hydration, the tissue blocks were treated with the appropriate dilutions of the antisera for 6 hrs. The tissue blocks were washed five times with phosphate buffer saline solution for 2 days per wash and dehydrated in a successive series of ethanol, cleared in xylene and embedded in paraffin. After hydration with phosphate buffer saline, the paraffin sections (5 and 15 micron thickness) were treated sequentially with the following reagents: (1) anti-rabbit globulin goat serum diluted 5 folds, for 20 min; (2) anti-horseradish peroxidase rabbit serum (prepared in this laboratory against horseradish peroxidase from Sigma) diluted 5 folds, for 20 min; (3) horseradish peroxidase (Sigma, type II, 0.5 mg/100 ml) in phosphate buffer saline containing 0.1% bovine serum albumin, for 20 min, and (4) 50 mg diaminobenzidine with 0.01% H_2O_2 (0.05 M Tris, HCl buffer, pH 7.6), for 30 min to 1 hr. The sections were washed three times with phosphate buffer saline for 10 min per wash between the application of each solution, and all reactions were carried out at room temperature.

Tissue culture technique :

Primary tissue cultures were prepared on glass coverslips from the primitive

retinas of 8–10-day-old chicken embryos in Eagle's medium (Dulbecco modification +10% FCS).[10] The cultures were maintained at 37°C in humidified atmosphere of 95% air and 5% CO_2 for up to 2 weeks, the medium being changed every 3–4 days. Indirect immunofluorescence pictures were taken of the 6th day of tissue culture samples.

Control of the immunohistochemical reaction :

The specificity of immunohistochemical reactions was controlled by exchanging one of the four components of each reaction layer with a specific reactant. In the first step of the immunoreaction, the immune serum was replaced by preimmune serum or by antiserum against glial acidic protein, a method which has been reported by others in our previous paper.[1]

OBSERVATIONS

Landolt's club cells were selectively stained with the specific antiserum, and the cell body was observed to lie in the superficial part of the internal nuclear layer (Fig. 2a). The cells appeared to be bipolar with their thick, straight axon passing deep into the internal prexiform layer, but the synaptic termination could not be visualized. The cells had long dendrites extending into the external plexiform layer in which they branched into relatively simple buquets. The fibers lay in the longitudinal plane of the bipolar cells in the apical processes of the dendrites passed among one or two bands of photoreceptor bodies and terminated in a bulbous or club-shaped enlargement at the level of the external junctional zone. Outside of the external limiting membrane, the process gave rise to a short cilium (Fig. 2b, 3b).

It was difficult to detect a positive reaction as with classical dendrites of bipolar cells originating almost immediately from the apical stalk and ending in synaptic contact with the base of the photoreceptors. No reaction was found in ganglion cells, amacrine cells, horizontal cells and other types of bipolar cells which occupy the zone just inside the external plexiform layer. Müller cells were stained with the glial acidic protein antiserum (Fig. 3a). In the Müller cells, the radial processes arose on both sides of the cell body and fine processes of fibers extended laterally in the external plexiform layer. In the internal and external nuclear layer, the lateral cell processes formed cytoplasmic lamellae around the perikaryon. Neighbouring lamellae fused to form a honeycomb meshwork which enclosed the somata of the cells. Fiber baskets extended from the external limiting membrane and embraced portions of the photoreceptors.

From the 6th day of tissue culture, the selectively stained bipolar cells appeared on the surface of the monolayer cells. They were small and oval with large, spherical and eccentric nuclei containing distinct nucleoli and small chromatin granules. The cytoplasm was slightly less dense than other neuron cells. A very thin process at the poles could be seen clearly. These cells were very mobile and contracted and expanded rhythmically (Fig. 4a, b).

DISCUSSION

Chicken retinas contain three different cell types in the external limiting membrane : the photoreceptors, Müller cells and Landolt's club cells. In 1871,

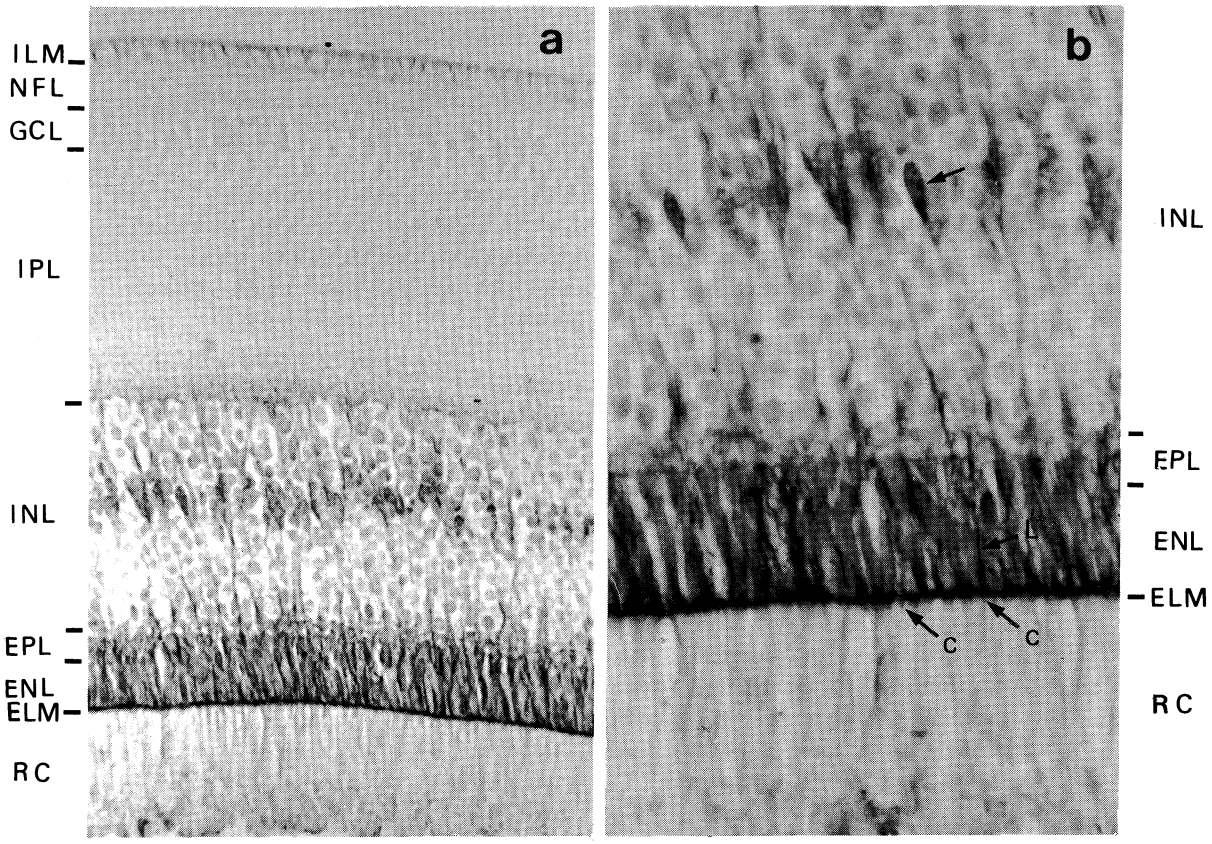

Fig. 2 a and b Landolt's club cells were selectively stained with the specific antiserum and the cell body (arrow) lies in the superficial part of the internal nuclear layer (INL). (a) : straight axon passed deep into the internal plexiform layer (IPL), but its synaptic termination was not visualized. ×270. No reaction was found in the ganglion cells. (b) : same section as (a), the cells had a long dendrites extending into the external plexiform layer (EPL) and it passed among the external nuclear layer (ENL) and terminated in a bulbolus or club-shaped enlargement (L) at the level of the external limiting membrane (ELM). ×540.

(Abbreviation) ILM : internal limiting membrane, NFL : nerve fiber layer, GCL : ganglion cell layer, RC : rod and cone layer, C : short cilium.

Landolt described a club-shaped cell-process which lay between the nuclei of the photoreceptors in newt and frog retinas. On the basis of staining affinities with the use of Golgi impregnation methods, it has been concluded that the club-shaped process is neuronal rather than glial in nature since it stained much more like bipolar dendrites of the external synaptic layer than glial cells.[2,5] More recently, Landolt's clubs have been demonstrated not only in the dogfish, shark, newt, salamander, frog and chicken but also in the retinas of chimpanzees and humans.[4–6] In the chicken retina, there are at least three distinct types of bipolar cells distinguishable with the use of Golgi impregnation methods and many of these cells have Landolt's clubs which extend from the outer plexiform layer to the external limiting membrane.[6]

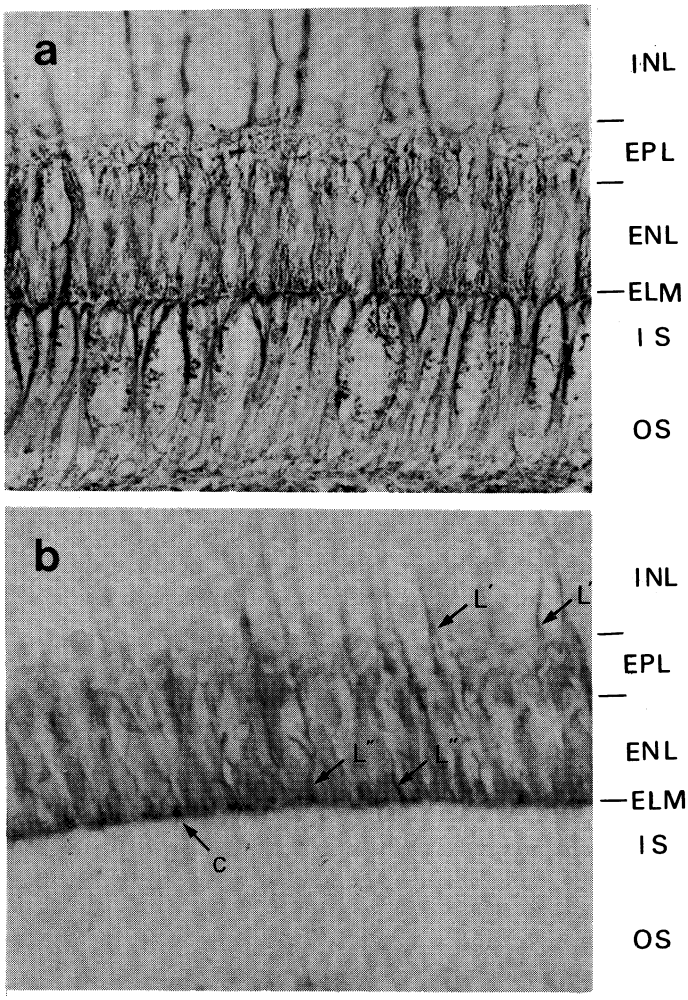

Fig. 3 a and b (a) : Müllser eells were selectively stained with glial acidic protein antiserum. ×540. The radial processes arose on both sides of the cell body and fine processes of the horizontal fibers extended laterally in the external plexiform layer (EPL). Fiber baskets extend from the external limiting membrane (ELM) and embrace portion of the photoreceptors.

(b) : Serious section as seen in Fig. 2. ×540. At the outer border of the internal nuclear layer (INL), a dendrite of Landolt's cell somewhat enlarged (L') and at the external plexiform layer (EPL) it branched into a relatively simple buquet and terminated in a bulbous or club-shaped enlargement (L") at the level of the external limiting membrane (ELM) and outside of the ELM it gave rised to short cilium (C).

(Abbreviation) ENL : external nuclear layer, IS : innersegment, OS : outer segment.

In this study, Landolt's clubs and cell bodies were selectively stained with specific antiserum. The cell bodies appeared to lie in the superficial part of the internal nuclear layer and to be bipolar. However, it was difficult to observe

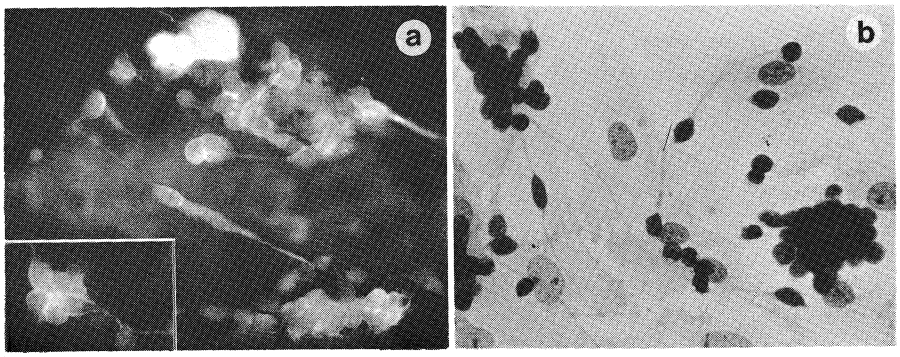

Fig. 4 a and b (a) : Indirect immunofluorescence of the retina of 9-day-old embryo, 6 days in cell culture. Selectively stained bipolar cells appeared on the surface of the monolayer cells. ×800.
(b) : 9-days embryo, 6 days in cell culture, Giemsa stain. ×800.

the dendrites like those of typical bipolar cells which synapse with photoreceptors.

With the fluorescence dye microinjection method, the cell bodies of bipolar cells are observed in the middle of the internal nuclear layer with dendrites branching in the external plexiform layer. Backing the receptor terminals, but these cells do not have dendrites of the Landolt's club type.[11] In addition, electron microscopy reveals that the Landolt's club process morphologically differs from the typical dendrite.[7,8]

We may conclude that the Landolt's club cells are bipolar cells, but not identical to common bipolar cells which play an important part in the visual pathway. Immunohistochemical specificity of the Landolt's club cells suggests that impulses from the intraretinal space are transmitted to the neurons which in turn establish synaptic relations with dendrites or bodies of the multipolar ganglionic cells (Fig. 5).

As we reported in an immunochemical study of the filament proteins of the chicken retina, the Landolt's club antigen belongs to a class of neuronal and glial filament proteins 100 Å in width.[12] These proteins not only have the same molecular weight and a similar peptide map as, but also cross-react, forming a line of complete identity in double immunodiffusion tests, with antisera to glial filament (GFA), common neuronal filament and specific neuronal filament (Landolt's club cell) proteins. Recent comparative studies of GFA and neuronal filament proteins,[13] and the isolation of a GFA-like protein from peripheral nerve where glial fibers are absent, indicated that the protein subunits of glial and nerve filaments are similar.[14] The antisera to neurofilament and glial fibrillary acidic proteins labeled not only neuron but also Bergmann glia and fibrous astrocytes.[15–18] These findings suggest that cytofilaments in other nonneural cells might be composed of similar protein subunits.[19]

From the results of our immunohistochemical study of Landolt's club cells, we must conclude that the specific neuronal filament protein is composed of an immunochemically different protein subunit, even though this conclusion contradicts other assumptions that the structures of the subunits are similar. Recent papers report that monoclonal antibodies can be used to show molecular differences among neurons in the vertebrate nervous system,[20–22] and monoclonal antibodies method are available for use with retinal nerve tissue. At the present

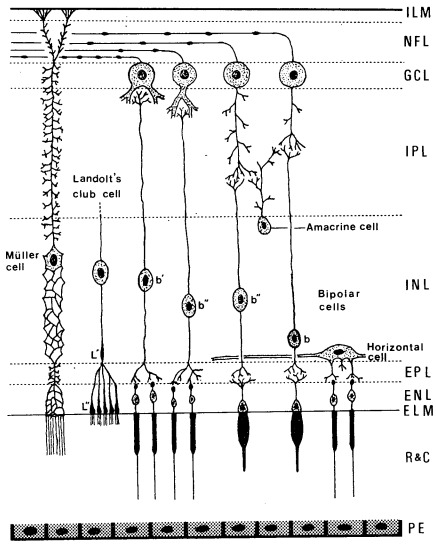

Fig. 5 Diagram of the retinal neurons (modified from Cajal).
(Abbreviation) ILM : internal limiting membrane, NFL : nerve fiber layer, GCL : ganglion cell layer, IPL : internal plexiform layer, INL : internal nuclear layer, ELM : external limiting membrane, R & C : rod and cone layer,PE : pigment epithelium, b', b" : various types of bipolar cells and L', L" : Landolt's club cell, somewhat enlarged (L'), and a bulbous or club-shaped enlargement (L").

time, there are many points which are uncertain, and there is no alternative but to wait for future research and development.

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