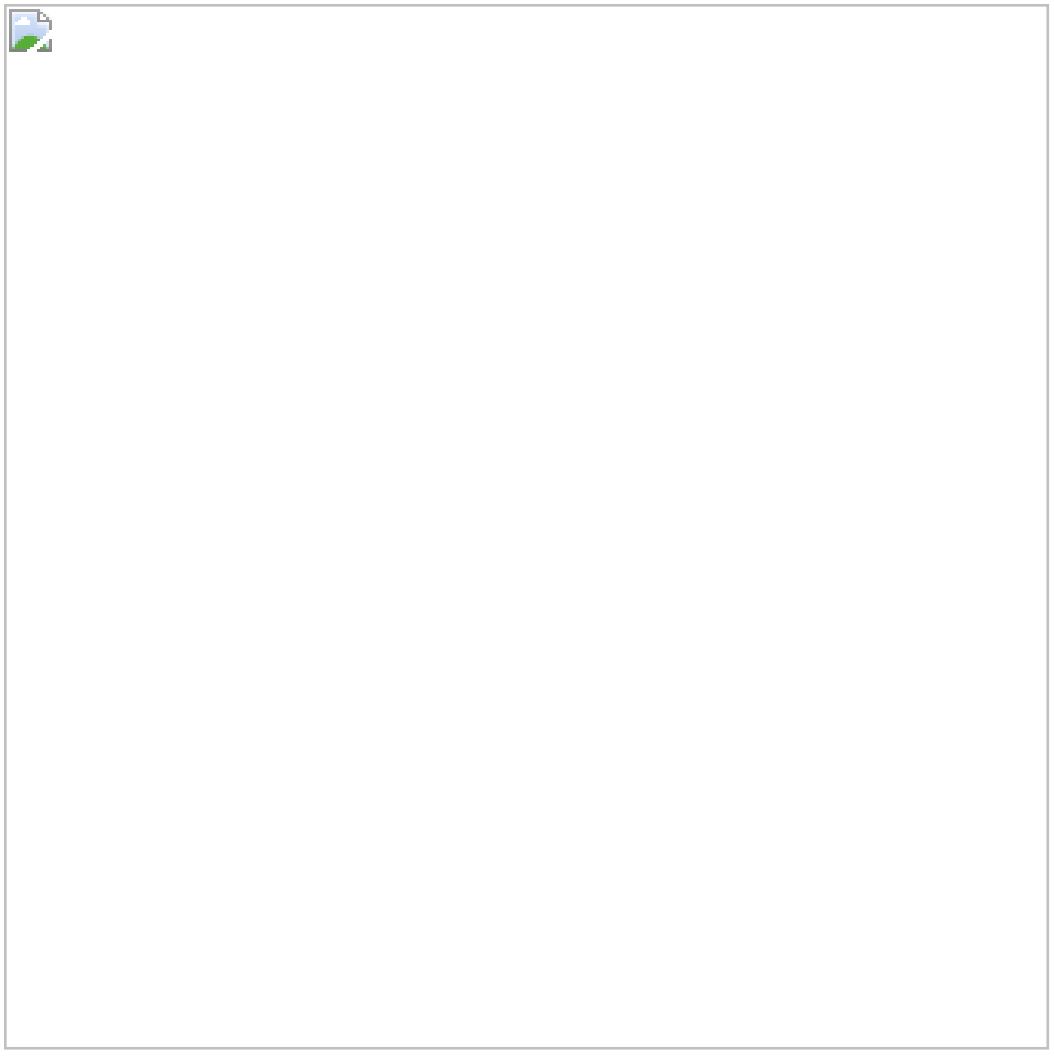

Concanavalin A Promotes Regeneration of Processes of Isolated Ganglion Cells from the Adult Newt Retina

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Concanavalin A Promotes Regeneration of Processes of Isolated Ganglion Cells from the Adult Newt Retina

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ABSTRACT—Retinal cells dissociated from adult newt eyes were seeded onto plastic dishes which were coated with various cell adhesion substances. They were cultured in Leibovitz's L-15 medium with no serum. Concanavalin A (ConA) and poly-L-lysine (PLL) caused relatively good cell attachment, regardless of retinal cell types, in comparison with uncoated dishes. On the other hand, extracellular matrix (ECM) components such as collagen, fibronectin and laminin tended to inhibit the cell attachment.

In both ConA- and PLL-coated dishes, ganglion cells survived for longer periods than other retinal cells and extended neurites with time in culture. The outgrowth of neurites by ganglion cells was found to be promoted to a far greater degree by ConA than PLL. Branching of neurites also appeared to occur more frequently on ConA. From these observations, ConA may be an useful tool for investigating retinal regeneration *in vitro*.

INTRODUCTION

Urodele amphibians possess the ability to regenerate a new functional retina following a complete removal or destruction of the original retina [7, 15, 34]. It is now widely accepted that this regeneration occurs mainly from the transdifferentiation of retinal pigment epithelial (RPE) cells into neurons and partly from intrinsic neuroblasts existing at the retinal margin [16, 22].

How various retinal neurons are differentiated and how functional neural networks are reformed during regeneration remain to be resolved. One strategy to deal with these problems is to study retinal cells in culture, where the cellular and fluid environments as well as the composition of the substrate can be controlled [14, 20, 21, 26].

For trial to seek an optimum culture condition for retinal neurons, we examined here effects of various cell adhesion substances, including a lectin concanavalin A (ConA), poly-L-lysine (PLL) and extracellular matrix (ECM) components such as collagen (COL), laminin (La) and fibronectin (FN), on the attachment and survival of retinal cells dissociated from newt retina. This report demonstrates that ConA and PLL, but not ECM components, enhance the cell attachment, and that ConA promotes regeneration of processes of retinal ganglion cells more potently than PLL does. Parts of this work have appeared in abstract form [30].

MATERIALS AND METHODS

Cell culture

Adult newts (*Cynops pyrrhogaster*) were anesthetized with 0.1% FA100 (4-allyl-2-methoxyphenol; Tanabe). The eyes were excised, and the retina was detached from pigment epithelium. Retinal cells were dissociated with papain (10–15 units/ml) as described previously [13]. Cells were suspended in the culture medium of 80% Leibovitz's L-15 (Flow Laboratories) supplemented with penicillin (400 U/ml) and streptomycin (100 μg/ml) (Meiji Seika Co.). The density of cells in the suspension medium was modified so that cells might adhere to the dish at intervals of more than 50 μm (see the section of "cell counting"). About 2.5 ml of the cell suspension medium was plated onto culture dishes. After the cells were allowed to adhere on the dish for 30 min, the medium containing the nonadherent cells was changed into the fresh one. All cultures were maintained at 25°C in the medium without any growth factors and serum. The cells were examined daily for morphological changes under phase-contrast microscope (Olympus IMT-2). The medium was changed every two or three days during cultivation.

Preparation of substrata

Plastic culture dishes (35 mm in diameter; Falcon, no. 3001) were prepared as control. They were coated with one of the following substrates; ConA (Sigma; 1 mg/ml), PLL (MW>300,000, Sigma; 100 μg/ml), COL.I (Cell Biology Boehringer Mannheim; 1 mg/ml), COL.IV (Nitta Co.; 1 mg/ml), FN (Cell Biology Boehringer Mannheim; 50 μg/ml), and La (Sigma; 50–100 μg/ml). Before plating cells, plastic dishes were incubated with 500 μl of the adhesion substances for 2 hr at room temperature, washed five times with distilled water, and air dried.

Cell counting

After retinal cells in suspension medium were counted in a hemocytometer, the cell suspensions (2.5 ml) were plated onto 35 mm dishes at an initial density of $1–8\times10^4$ cells/ml. At selected time intervals (usually 30 min), nonadherent cells were washed off the dishes by a gentle rinse with culture medium. The total number of adherent cells per dish (962 mm^2) was estimated by summing the number of adherent cells in 10 fields (1×0.65 mm^2/ field) randomly chosen from one dish and multiplying the sum by 148. The rate of

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cell attachment to a substrate was expressed as percentage of the number of cells attached against that initially seeded.

As criteria for determining the survival rate of cells with time in culture, we preliminarily examined correlation between the viability of cells and the change in cell morphology by nigrosin exclusion test. As a result, the living cells which did not take up nigrosin had typically a phase-bright cell body. While, cells either with a dark shrunken cell body or a swelling one with vacuoles took nigrosin, suggesting that they were dead or nonviable. Indeed, these cells typically dettached from substrates and disappeared within a few days thereafter.

Identification of solitary ganglion cells

Retinal ganglion cells were identified by retrograde labelling with horseradish peroxidase (HRP). HRP was injected into the orbit around cut end of the optic nerve of the anesthetized animals according to the protocol of Kaneko and Saito [13]. Ten days were allowed to elapse for HRP to be transported retrogradely to ganglion cell somata. The retinae containing HRP-impregnated ganglion cells were enzymatically dissociated into single cells by the protocol described above. Dissociated cells were dispersed into the culture dishes. Before HRP localization, cells in culture were fixed in ice-cold Zamboni's solution [36]. HRP-positive ganglion cells were visualized using DAB Kit (Vector laboratories).

RESULTS

Attachment of retinal cells on various substrates

Cell suspensions at a density of $1–9\times10^4$ cells/ml were plated onto plastic dishes that had been uncoated or coated with various adhesion substances. After 30 min, the culture medium in each dish was renewed and the number of cells attached to dishes were counted. The rate of attachment of cells to different adhesion substances is compared in Figure 1. The values are presented as mean$\pm$SD of 6 dishes. The attachment of retinal cells onto uncoated dish (UC) was $49\pm8\%$. The rate of cell attachment increased significantly when the dish was coated with either PLL ($64\pm16\%$) or Con A ($70\pm15\%$). On the other hand, the cell attachment to dishes coated with FN was poor ($14\pm9\%$), and that to COL.I-, COL.IV-, or La-coated dishes was extremely poor ($<<1\%$). Longer time intervals after cell plating (>12 hr) did not improve significantly the rate of cell attachment to dish. At lower concentrations of FN ($<5\ \mu$g/ml), COL.I (<0.1 mg/ml), COL.IV (<0.1 mg/ml), or La ($<50\ \mu$g/ml), rather than higher concentrations, the cell attachment tended to be improved. However, such an improvement in cell adhesion may not be effects of these substances, because the rate of the cell attachment to uncoated dish was still higher than that to FN-, COL- or La-coated dish. Although the uncoated dish caused relatively good attachment of retinal cells, more than 80% of the attached cells floated at 24 hr later.

Survival of retinal cells in culture

Because retinal cells adhered effectively onto dishes treated with ConA or PLL, effects of these two substrates on the survival of retinal cells were compared. In freshly plated cells, photoreceptors, bipolar cells and Müller cells could be easily identified on the basis of their morphological resemblance to cells identified *in vivo* [22, 29]. Cells with single long processes were identified as either amacrine or ganglion cells, because these cells consistently displayed action potentials in response to depolarizing current stimulation.

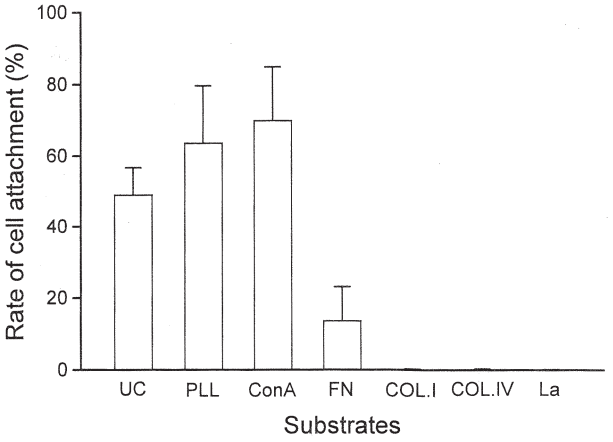


FIG. 1. Rate of attachment of retinal cells to various substrates. Values are expressed as percentage of the number of cells attached to substrates *versus* that initially seeded (see methods for details). Mean$\pm$SD for each substrated was calculated from 6 dishes. UC, uncoated; PLL, poly-L-lysine ($100\ \mu$g/ml); ConA, concanavalin A (1 mg/ml); COL.I, collagen I (1 mg/ml); COL.IV, collagen IV (1 mg/ml); FN, fibronectin ($50\ \mu$g/ml); La, laminin ($100\ \mu$g/ml). Rate of cell attachment was significantly larger with either PLL-coating ($P<0.05$ using one-tailed t-test) or ConA-coating ($P<0.01$) than that with uncoating. Attachment of cells to dishes coated with FN, COL.I, COL.IV, or La was extremely lower as compared to that to uncoated dishes ($P<<0.001$ each).

Retinal cells on ConA-coated dishs decreased in number with time in culture in a manner similar to those on PLL-coated ones. More than 60% of photoreceptor and bipolar cells had deteriorated within 2 weeks, and almost all Müller cells had died within a week. Figure 2 shows changes in morphology of photoreceptors (A), a bipolar cell (B), and a Müller cell (C) with time in culture. Cones (A_1) became spherical soon after plating and extended many thin processes from their somata at least within a day (A_2). After more than 10 days in culture, somata started to darken and thin processes to deteriorate (A_3). While, most rods maintained their morphology until they had collapsed within a week (data not shown). Bipolar cells (B_1) maintained their morphology for a few days, and started to shrink somata and lost their processes within two weeks (B_2 and B_3). Müller cells (C_1) deformed with withdrawal of their apical membrane and swelled with appearance of many vacuoles (C_2), and then collapsed within a few days (C_3).

More than 50% of amacrine/ganglion cells survived for longer than 2 weeks and extended neurites with time in culture. A typical example is shown in Figure 3. This particular cell survived in culture for up to two months. The cultures also contained many unidentified cells with few or no

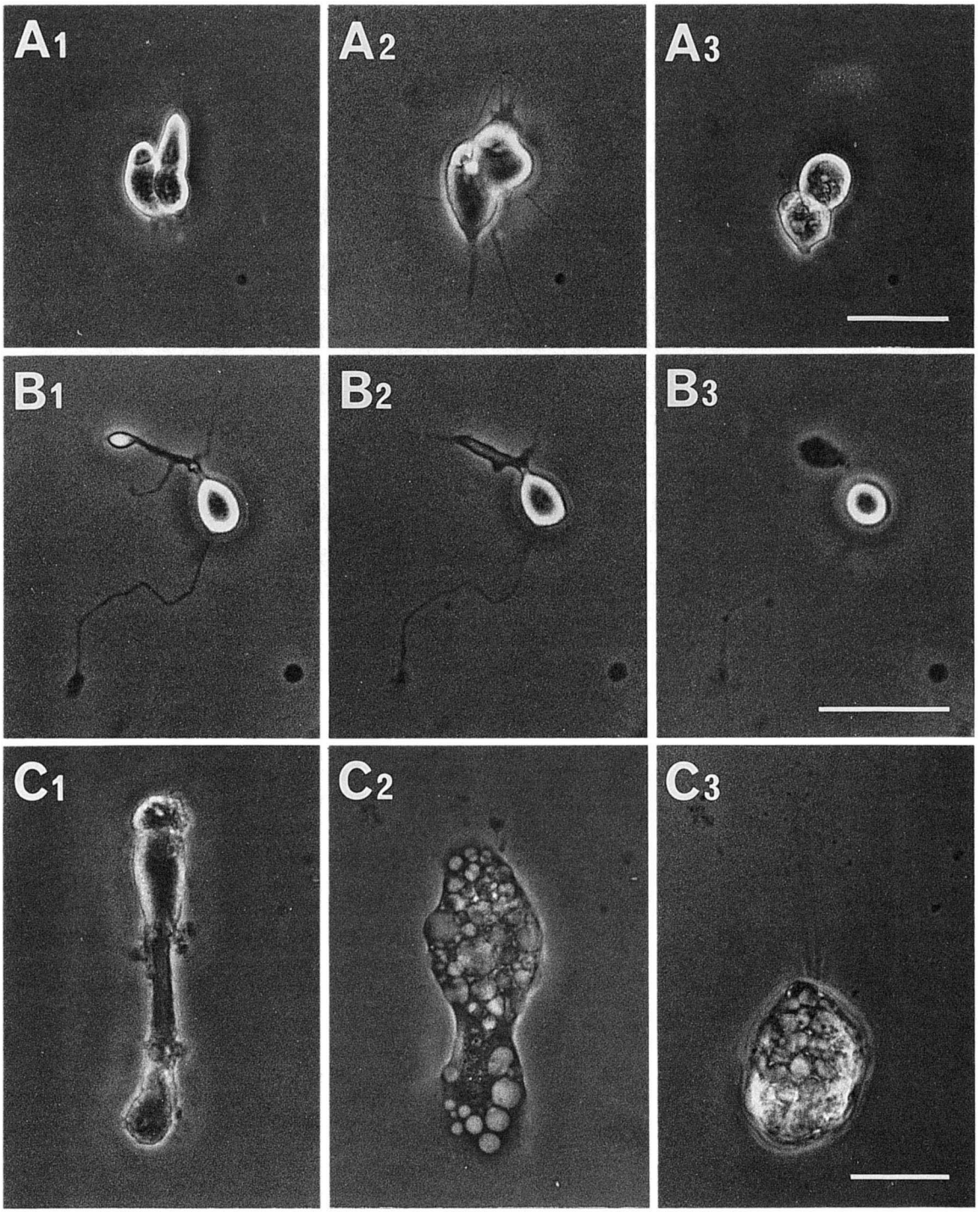


FIG. 2. Morphological changes of retinal cells in culture. A, cone photoreceptor cells. B, bipolar cell. C, Müller cell. The cells were plated in a plastic dish coated with PLL and maintained in 80% Leibovitz's L-15 culture medium without serum at 25°C. The cells were examined daily for their morphological changes and photographed with phase contrast optics at adequate days after plating; A_1, 0 day; A_2, 1 day; A_3, 12 days; B_1, 0 day; B_2, 2 days; B_3, 12 days; C_1, 0 day; C_2, 2 days; C_3, 4 days. Bars: 30 μm.

processes. Some of them survived for longer periods and extended neurites. We speculate that they were amacrine/ganglion cells.

Effects of ConA and PLL on neurite outgrowth of ganglion cells

Since many amacrine/ganglion cells survived in culture more than one month, we examined the growth of neurites from single ganglion cells in cultivations on ConA and PLL.

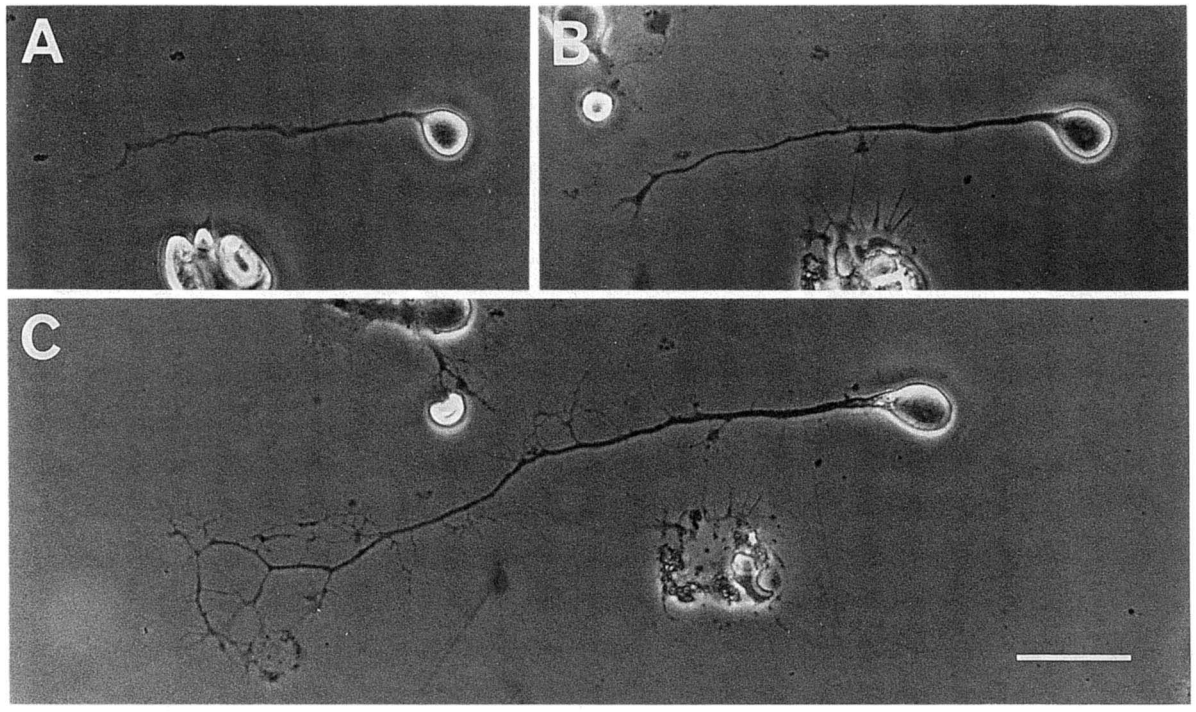
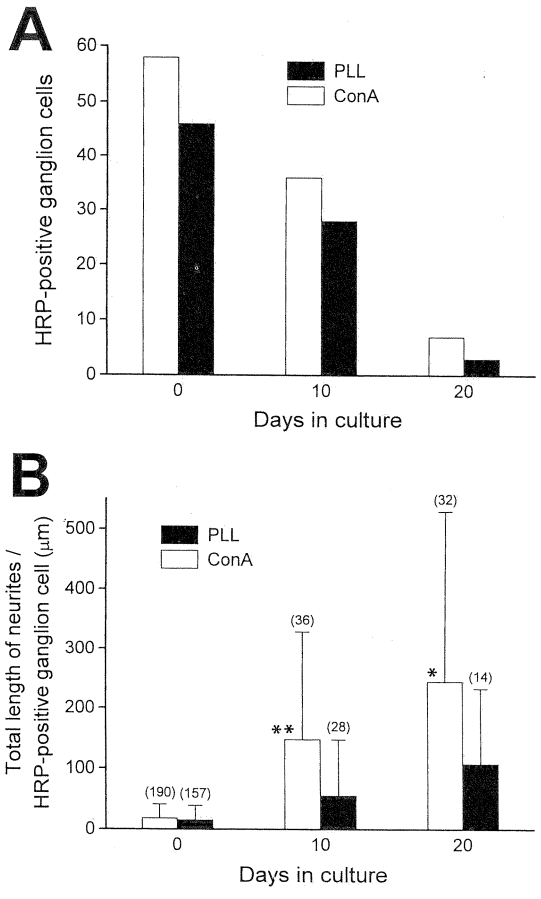


FIG. 3. Growth of a retinal amacrine/ganglion cell in culture. Cells with single long processes, amacrine or ganglion cells, were plated in a plastic dish coated with ConA, and maintained in 80% Leibovitz's L-15 without serum at 25°C. The cell was examined daily for process extension and photographed at 0 day (A), 7 days (B) and 20 days (C) after plating. The cell spread its soma and sprouted and extended neurites with time in culture. Growth cones were observed at ends of many branches and sprouts. Bar: 40 μm.



Ganglion cells were identified as brownish cells impregnated by retrogradely transported HRP.

Figure. 4A shows changes in the number of ganglion cells during cultivations on ConA and PLL. In this particular case, retinal cell suspensions (5×10^4 cells/ml) were seeded on 6 ConA-coated dishes and 6 PLL-coated ones. Retinal cultures in 2 dishes of each substrate were fixed with

FIG. 4. A, Comparison of the number of ganglion cells on PLL- (black bars) and ConA-coated dishes (white bars) after periods in culture. The ganglion cells were labeled by retrograde transport of HRP (see methods for details). Data were obtained from a particular experiment. Retinal cells were cultivated in a total of 12 dishes, of which 6 were coated with PLL and others with ConA. HRP-positive ganglion cells in 2 dishes of each cultivation were counted at 0, 10 and 20 days in culture. Sums of them were plotted against days in culture. B, Comparison of the length of neurites of ganglion cells on PLL- (black bars) and ConA-coated dishes (white bars) after periods in culture. Total length of neurites emerging from the HRP-positive ganglion cell was measured by tracing photographs of cells stained at 0, 10 and 20 days in culture onto graph paper. Data were collected from cells shown in A and those in other 2 similar experiments. Mean$\pm$SD was calculated and plotted against days in culture. Here ganglion cells with no neurite (at 0 days, 98 on ConA, 91 on PLL; at 10 days, 13 on ConA, 13 on PLL; at 20 days, 9 on ConA, 4 on PLL) were taken accounted as those with 0 μm neurite. Total numbers of cells measured were shown in parentheses. * indicates a significant difference between substrates ($P<0.05$ using one-tailed t-test). ** indicates a highly significant difference ($P<0.01$) between substrates.

the fixative at 0, 10 and 20 days after plating cells. The number of the HRP-positive ganglion cells observed at 0 day was 58 in ConA-cultivation, and 46 in PLL-cultivation. These numbers were roughly estimated as 0.03% of the number of retinal cells attached to each substrate. During subsequent days in culture, the number of ganglion cells was 36 on ConA and 28 on PLL at 10 days, and 7 on ConA and 3 on PLL at 20 days. From these results, there seems to be no significant difference in survival rate of ganglion cells between cultivations on ConA and PLL.

Figure 4B compares the total length of neurites emerging from the HRP-positive ganglion cell somata between ConA- and PLL-cultivations. Data were collected from cells shown in Fig. 4A and those in other 2 similar experiments. Length of neurites was plotted against time in culture. At 0 day in culture, about half of the HRP-positive ganglion cells possessed one or two neurites. Mean length of neurites per cell was 19 ± 23 μm (SD, n=190) on ConA-dish, and 16 ± 24 μm (SD, n=157) on PLL-dish. At 10 days in culture, 64% of ganglion cells on ConA (n=36) and 54% of ganglion cells on

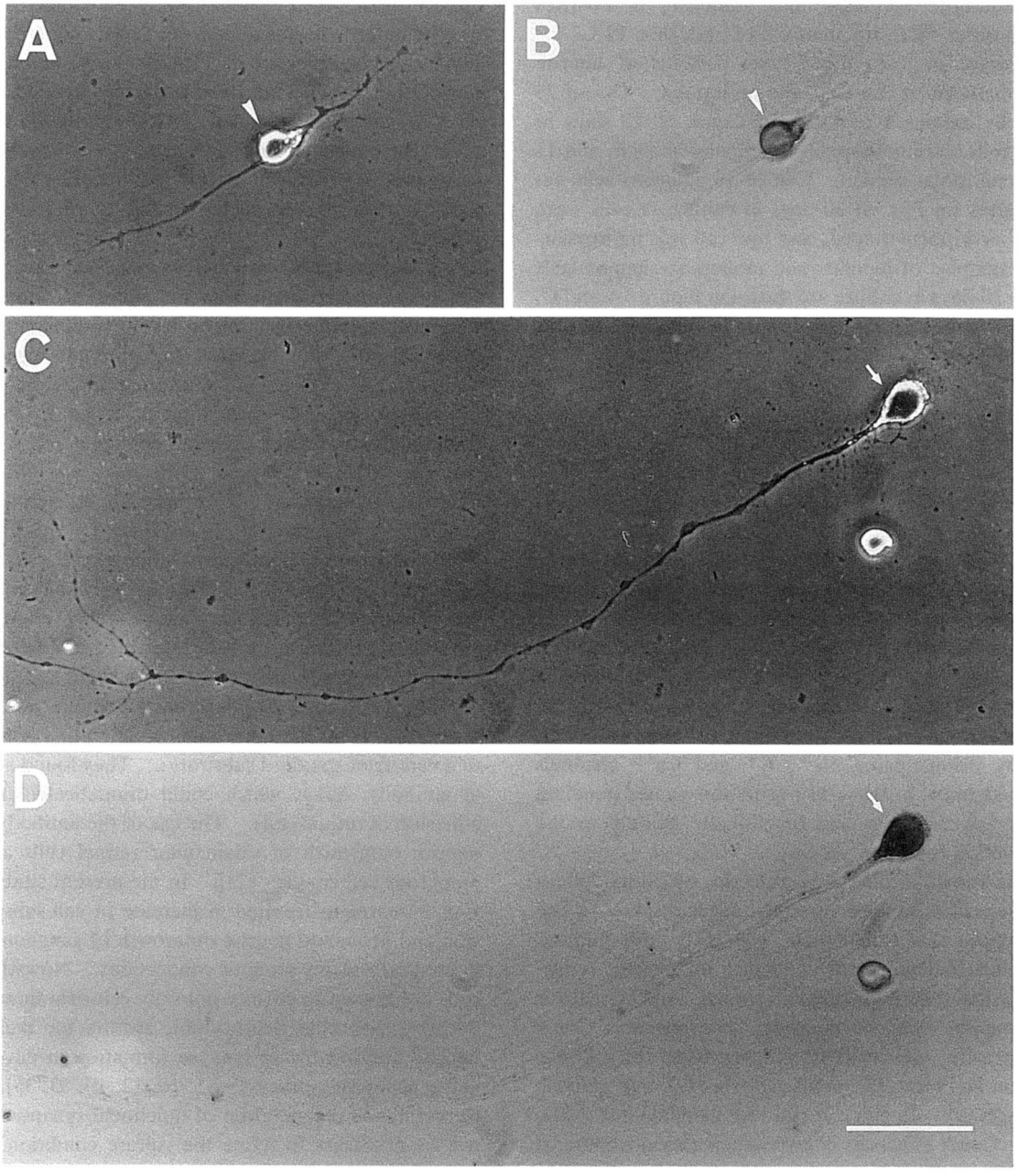


Fig. 5. A, B, Bipolar-shaped ganglion cell at 20 days in PLL-cultivation. C, D, Monopolar-shaped ganglion cell at 20 days in ConA-cultivation. Photographs in A and C were taken with phase contrast optics to show the shapes of ganglion cells clearly, and those in B and D were with normal optics to exhibit a brown reaction product of HRP in the corresponding cells. Arrow heads and arrows show the corresponding cell somata. Bar: 50 μm.

PLL (n=28) possessed neurites. Mean length of neurites per cell was 148±181 μm (mean±SD, n=36) on ConA, and 56±91 μm (mean±SD, n=28) on PLL. At 20 days in culture, 23 of 32 ganglion cells (72%) in ConA-coated dishes and 10 of 14 ganglion cells (71%) in PLL-coated ones possessed neurites. Mean length of neurites per cell on ConA and that on PLL were 245±285 μm (mean±SD, n=32) and 108±125 μm (mean±SD, n=14), respectively. These results indicate that there was a graded increase in the proportion of ganglion cells with neurite outgrowth on both substrates with time in culture, and that the outgrowth of neurites was promoted to a greater degree by ConA than by PLL. In addition, sprouting and branching of neurites appeared to occur more frequently on ConA than PLL.

There were at least three basic patterns of neurite outgrowth; monopolar, bipolar, and multipolar. Out of 23 ganglion cells extended neurites on ConA at 20 days in culture, 12 cells were monopolar- or bipolar-shaped, and 11 cells were multipolar-shaped. Out of 10 ganglion cells extended neurites on PLL at 20 days in culture, 9 cells were monopolar- or bipolar-shaped, and one cell was multipolar-shaped. Examples of bipolar- and monopolar-shaped cells on ConA at 20 days in culture are shown in Figure 5A and C. Evidence that both cells are HRP-impregnated ganglion cells is shown in Figure 5B and D.

DISCUSSION

We examined effects of various cell adhesion substances on isolated cells in a serum free culture medium. The cells adhered well to ConA- or PLL-coated plastic dishes, but not ECM components such as La, FN and COL. In both ConA- and PLL- cultivations, ganglion cells survived much longer than photoreceptors, bipolar cells and Müller cells. They extended neurites with time in culture. The outgrowth of neurites by ganglion cells was promoted to a greater degree by ConA than PLL. In electrophysiological experiments, ganglion cells grown on ConA were initiated action potentials mediated by voltage-gated Na^+, K^+ and Ca^{2+} channels which were identical to those of freshly dissociated ganglion cells, indicating that they are functionally healthy in the ConA-cultivation (data not shown).

ConA is known as one of plant lectins which modulates neuronal properities by binding to the sugar residues of cell surface glycoproteins (for review, see [18]). Modulatory changes include enhancement of neurite outgrowth, reduction of desensitization of glutamate responses, and increase in electrical coupling between neurons. For instance, ConA stimulates neurite outgrowth in neurons from chick dorsal root ganglion [4], leech [3], adult Aplysia [17], and goldfish retina explants [9]. In chick dorsal root ganglion and Aplysia neurons, ConA enhances neurite regeneration whether it is used to coat the dish or is dissolved in the culture medium at nanomolar concentrations. In newt retinal ganglion cells, however, ConA stimulated neurite outgrowth only when it was used as a substrate to be coated on culture dishes but not when it was dissolved in the culture medium. Additon of soluble ConA in the culture medium at less than 10 μM/ml tended to cause the detachment of cells from dishes, and was toxic at more than 100 μM/ml (data not shown). Toxicity of soluble form of ConA has been observed also in culture of developing mouse cerebellar cells [8]. These observations make us speculate that ConA might act by binding to cell adhesion molecules or membrane receptors of newt retinal ganglion cells.

Cell adhesion molecules, such as N-CAM, thy-1, or contactin, are expressed in retinal cells of other species [2, 25, 28]. These molecules are known to be membrane-bound glycoproteins which mediate cell to cell contact and promote neurite outgrowth (for review, see [24]). Of our knowledge, thy-1 and contactin are at least of ConA-binding glycoproteins [25, 32]. Cell adhesion molecules in adult newt retina have not yet been examined. Although we know much less about the molecular mechanisms of ConA action on neurite outgrowth at present, this plant lectin will be a highly useful tool for studying regeneration system of adult newt retina *in vitro*.

It is well known that ECM components such as La and FN play an important role on adhesion, movement and differentiation of cells in many developmental and regenerating processes. For instance, La promotes regeneration of optic nerve in explants of axotomized adult goldfish [11] and rat retinae [6]. An involvement of La in the transdifferentiation of retinal pigment epithelial (RPE) cells into neurons in Rana tadopole retina has been observed [26]. FN plays an important role during limb regeneration [27] and in lens regeneration [5]. In contrast, La and FN inhibited the attachment of retinal cells including RPE cells dissociated from adult newt eyes [31]. This fact may be partly related to the observations that in adult newt (*Notophthalmus viridescens*) retina, La was not detected immunohistochemically, although FN was detected as a slight signal [23].

MacLeish *et al.* [20] have described that retinal neurons dissociated from the adult salamander retina adhered pooly to a variety of standard substrates. They found a monoclonal antibody, Sal-1, which could dramatically enhance the adhesion of retinal cells. The use of the antibody promoted neurite outgrowth of salamander retinal cells and formed numerous cell contacts [21]. In the present study, although ConA treatment resulted in increase in cell-substrate adhesion and promoted neurite outgrowth of ganglion cells, cells did not reform any synaptic connections. Nowadays, retinal cells are known to require not only cell adhesion substances but also diffusible neurotrophic factors for their survival, neurite outgrowth and synapse formation in developmental and regenerating processes [1, 10, 12, 19, 33, 35]. In order to investigate regeneration of functional synapses *in vitro*, it will be necessary to refine the culture condition by supplementation of trophic factors in future study.

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REFERENCES

1 Araujo EG, Linden R (1993) Trophic factors produced by retinal cells increase the survival of retinal ganglion cells *in vitro*. Eur J Neurosci 5: 1181–1188

2 Barnstable CJ, Dräger UC (1984) Thy-1 antigen: A ganglion cell specific marker in rodent retina. Neuroscience 11: 847–855

3 Chiquet M, Acklin SE (1986) Attachment to Con A or extracellular matrix initiates rapid sprouting by cultured leech neurons. Proc Natl Acad Sci USA 83: 6188–6192

4 DeGeorge JJ, Slepecky N, Carbonetto S (1985) Concanavalin A stimulates neuron-substratum adhesion and nerve fiber outgrowth in culture. Dev Biol 111: 335–351

5 Elgert KL, Zalik SE (1989) Fibronectin distribution during cell type conversion in newt lens regeneration. Anat Embryol Berl 180: 131–142

6 Ford-Holevinski T, Hopkins JM, McCoy JP, Agranoff BW (1986) Laminin supports neurite outgrowth from explants of axotomized adult rat retinal neurons. Dev Brain Res 28: 121–126

7 Hasegawa M (1958) Restitution of the eye after removal of the retina and lens in the newt, *Triturus pyrrhogaster*. Embryologia 4: 1–32

8 Hatten ME, Francois AM (1981) Adhesive specificity of developing cerebellar cells on lectin substrata. Dev Biol 87: 102–113

9 Heacock AM (1982) Glycoprotein requirement for neurite outgrowth in goldfish retina explants: Effects of tunicamycin. Brain Res 241: 307–315

10 Hicks D, Courtois Y (1992) Fibroblast growth factor stimulates photoreceptor differentiation *in vitro*. J. Neurosci 12: 2022–2033

11 Hopkins JM, Ford-Holevinski TS, McCoy JP, Agranoff BW (1985) Laminin and optic nerve regeneration in goldfish. J Neurosci 5: 3030–3038

12 Johnson JE, Barde YA, Schwab M, Thoenen H (1986) Brain derived neurotrophic factor supports the survival of cultured rat ganglion cells. J Neurosci 6: 3031–3038

13 Kaneko Y, Saito T (1992) Appearance and maturation of voltage-dependent conductances in solitary spiking cells during retinal regeneration in the adult newt. J Comp Physiol A 170: 411–425

14 Kaneko Y, Sakai H, Chiba C, Saito T (1993) Development of voltage-gated currents in newt retinal neuroblasts in culture. NeuroReport 4: 775–778

15 Keefe JR (1973) An analysis of urodelian retinal regeneration: I. Studies of the cellular source of retinal regeneration in *Notophthalmus viridescens* utilizing ^{3}H-thymidine and colchicine. J Exp zool 184: 185–206

16 Klein LR, MacLeish PR, Wiesel TN (1990) Immunolabelling by a newt retinal pigment epithelium antibody during retinal development and regeneration. J Comp Neurol 293: 331–339

17 Lin SS, Levitan IB (1987) Concanavalin A alters synaptic specificity between cultured *Aplysia* neurons. Science 237: 648–650

18 Lin SS, Levitan IB (1991) Concanavalin A: a tool to investigate neuronal plasticity. Trends Neurosci 14: 273–277

19 Lipton SA, Wagner JA, Madison RD, D'Amore PA (1988) Acidic fibroblast growth factor enhances regeneration of processes by postnatal, mammalian retinal ganglion cells in culture. Proc Natl Acad Sci USA 85: 2388–2392

20 MacLeish PR, Barnstable CJ, Townes-Anderson E (1983) Use of a monoclonal antibody as a substrate for mature neurons *in vitro*. Proc Natl Acad Sci USA 80: 7014–7018

21 MacLeish PR, Townes-Anderson E (1988) Growth and synapse formation among major classes of adult salamander retinal neurons in vitro. Neuron 1: 751–760

22 Negishi N, Shinagawa S, Ushijima M, Kaneko Y, Saito T (1992) An immunohistochemical study of regenerating newt retinas. Dev Brain Res 68: 255–264

23 Ortiz JR, Vigny M, Courtois Y, Jeanny JC (1992) Immunocytochemical study of extracellular matrix components during lens and neural retina regeneration in the adult newt. Exp Eye Res 54: 861–870

24 Patterson PH (1992) Process outgrowth and the specificity of connections. In "An Introduction to Molecular Neurobiology" Ed by ZW Hall, Sinauer Associates, Sunderland, Massachusetts, pp 388–427

25 Ranscht B, Dours MT (1988) Sequence of contactin, a 130-kD glycoprotein concentrated in areas of interneuronal contact, defines a new member of the immunoglobulin supergene family in the nervous system. J Cell Biol 107: 1561–1573

26 Reh TA, Nagy T, Gretton H (1987) Retinal pigmented epithelial cells induced to transdifferentiation to neurons by laminin. Nature 330: 68–71

27 Repesh LA, Fitzgerald TJ, Furcht LT (1982) Changes in the distribution of fibronectin during limb regeneration in newts using immunocytochemistry. Differentiation 22: 125–131

28 Rutishauser U, Hoffman S, Edelman GM (1982) Binding properties of a cell adhesion molecule from neural tissue. Proc Natl Acad Sci USA 79: 685–689

29 Saito T, Kaneko Y, Maruo F, Niino M, Sakaki Y (1994) Study of the regenerating newt retina by electrophysioloy and immunohistochemistry (Bipolar- and Cone-specific antigen localization). J Exp Zool 270: 491–500

30 Sakai H, Kaneko Y, Saito T (1991) Growth of single retinal ganglion cells isolated from newt in culture medium containing various cell adhesion substances. Zool Sci Supl 8: 1031

31 Sakai H, Saito T (1994) Development of voltage-dependent inward currents in dissociated newt retinal pigment epithelial cells in culture. NeuroReport 5: 933–936

32 Saleh M, Bartlett PF (1989) Isolation of a thy-1-like glycoprotein from cat brain: Distribution in retina and brain defined by monoclonal antibodies. J Neurosci Res 23: 152–161

33 Spoerri PE (1988) Neurotrophic effects of GABA in culture of embryonic chick brain and retina. Synapse 2: 11–22

34 Stone LS (1950) The role of retinal pigment cells in regenerating neural retinae of adult salamander eyes. J Exp Zool 113: 9–32

35 Turner JE, Glaze KA (1977) Regenerative repair in the severed optic nerve of the newt (*Triturus viridescens*): effects of nerve growth factor. Exp Neurol 57: 687–697

36 Zamboni L, Demartino C (1967) Buffered picric acid-formaldehyde: a new, rapid fixative for electron microscopy. J Cell Biol 35: 148A