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Embriologia e morfogenesi. — *Lens-forming transformation in the outer cornea of larval* Xenopus laevis *induced by spinal ganglia implanted in the enucleated orbit.* Nota (*) di CARLA CIONI (**), SERGIO FILONI (***) e LUIGI BOSCO (**), presentata (****) dal Socio A. STEFANELLI.

RIASSUNTO. — Gangli spinali innervanti arti normali e rigeneranti sono stati asportati da larve di *Xenopus laevis* allo stadio 56-57 (secondo Nieuwkoop e Faber, 1956) e impiantati al di sotto della cornea esterna nell'orbita enucleata di larve della stessa specie allo stadio 50.

I risultati dimostrano che sia i gangli innervanti arti normali che quelli innervanti arti rigeneranti sono in grado di indurre evidenti trasformazioni lentogene nella cornea esterna. Tuttavia, la percentuale dei casi positivi ottenuti dopo l'impianto dei gangli normali è considerevolmente più bassa di quella ottenuta in seguito all'impianto dei gangli innervanti arti rigeneranti e, in tutti i casi, il processo di trasformazione lentogena della cornea si arresta più precocemente.

Tali dati sono stati attribuiti all'azione di un fattore neurotrofico, prodotto in quantità differenti dai gangli normali e dai gangli innervanti arti rigeneranti, e in grado di vicariare l'azione esercitata dalla retina nel processo di trasformazione della cornea anche in assenza delle sostanze nutritive fornite dall'occhio.

INTRODUCTION

It is well known that, *in vivo*, the eye is important not only in triggering the process of lens-forming transformation of the cornea of larval *Xenopus laevis*, but also in allowing the subsequent development and conservation of the new-forming lens structure (Freeman, 1963; Filoni *et al.*, 1978b). However, Campbell and Jones (1968) have obtained lenses from fragments of outer cornea explanted *in vitro*.

According to these authors the difference between the *in vivo* ad *in vitro* results is due to the fact that whereas enucleation of the eye deprives the cornea of necessary nutritional factors supplied by the aqueous humor, thus inhibiting its intrinsic lens-forming capacity, in the *in vitro* cases, the nutritional factors

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supplied by the culture medium allow the cornea to be completely transformed into a lens.

In experiments involving lentectomy from the dorsal part of the eye in which the inner cornea is left intact, it has been observed that although the outer cornea received all the nutritional factors normally supplied by the aqueous humor, the outer cornea was never transformed into a lens. The lens-forming transformation of the outer cornea occurred only when the inner cornea was incised and direct communication was thus set up with the vitreous chamber. It was thus deduced that for the outer cornea to be transformed into a lens it was not sufficient that it be in optimal nutritional condition, but it was also necessary that it be supplied with one or more factors present in the vitreous chamber (Filoni *et al.*, 1978a; Reeve and Wild, 1978; Bosco *et al.*, 1979; 1980).

Results of various experiments indicate the neural retina as the tissue producing the inducing factor(s) present in the vitreous chamber (retinal factor(s) (Filoni *et al.*, 1981, 1982; Reeve and Wild, 1981).

However, this inductive capacity is not exclusive to the neural retina (Reeve and Wild, 1981). We have recently shown that lumbar spinal ganglia grafted between the two corneas of normal eyes are capable of inducing lens-forming transformations of the outer cornea and that ganglia innervating regenerating hindlimbs have greater inducing capacity than normal ganglia (Filoni *et al.*, in press). However, under the experimental conditions used, the outer cornea could count on the nutritional factors supplied by the aqueous humor as well as on the factors supplied by the implanted ganglion.

The aim of the present work was to establish whether the lens-forming transformations of the outer cornea caused by the implanting of spinal ganglia could also be achieved in the absence of eye nutritional factors.

Materials and methods

The experiments were performed on larvae of *Xenopus laevis* reared from eggs obtained by injecting adults with Pregnyl (Organon), as described by Nieuwkoop and Faber (1956).

Removal of implant tissue from donor tadpoles (Pl. I, fig. 1).

Spinal ganglia innervating regenerating hindlimbs and spinal ganglia innervating normal hindlimbs were removed from stage 56-57 (sec. Nieuwkoop and Faber, 1956) tadpoles that had been anesthetized in 1:3000 MS 222 (Sandoz) in 10% Holtfreter's solution.

In order to obtain spinal ganglia innervating regenerating hindlimbs, hindlimbs of donor tadpoles were amputated at knee level. Six days later, when, according to Dent (1962), the blastema had begun to form, the 8th and 9th spinal ganglia were removed for the purpose of implantation beneath the outer cornea of host tadpoles prepared in the manner described below.

Spinal ganglia innervating normal hindlimbs were also removed and implanted in the same manner.

Before implantation an incision was made in ganglion capsule.

Tissue implants in host tadpoles (Pl. I, fig. 1).

Tadpoles at stage 50 were used as recipients. They were anesthetized in 1:3000 MS 222 (Sandoz) in 10% Holtfreter's solution and operated in full-strength Holtfreter's solution.

Two different kinds of implant were made beneath the outer cornea in the orbit from which the eye-cup had been removed:

Experiment I. Implant of lumbar ganglion from tadpole with regenerating limb. Operations were performed on 55 larvae.

Experiment II. Implant of lumbar ganglion from normal tadpole. Operations were performed on 65 larvae.

In both experiments, five larvae were killed immediately after the operation to serve as controls. The other recipient tadpoles were allowed to recover in full-strength Hotfreter's solution for 24 hours. They were then gradually transferred to dechlorinated tap water and fed on nettle powder.

The surviving animals were killed at 5-7-10 days after operation, fixed in Bouin's solution and embedded in paraffin. Seven μm serial sections were cut and stained with hematoxylin-eosin or treated by PAS reaction.

RESULTS

The staging of lens-forming transformations of the outer cornea was carried out according to Freeman (1963).

Experiment I. Implant of lumbar ganglion from tadpole with regenerating limb (Table I).

New-forming lenses were observed in 15 out of 47 cases examined.

Five days after the operation the outer cornea facing the implanted ganglion was found to have undergone lens-forming transformation in 4 out of the 8 cases examined. In 3 cases there was a small cell aggregate, which was partially delimited by the surrounding cells of the inner layer of the outer cornea (early stage 3), while in the other case the cell aggregate had developed into a lens-forming vesicle (middle stage 3). At day 7 after the operation, 3 out of 19 cases examined showed a lens vesicle at middle stage 3, while in the remaining 3 the lens-forming vesicle was considerably larger (late stage 3) (Pl. I, fig. 2). At day 10 after the operation, 5 out of the 20 cases examined displayed new-forming lenses. In one case the lens-forming vesicle was at late stage 3 (Pl. II, fig. 3), while in 2 the formation of the primary nucleus of the lens fibers had begun (stage 4). In two other cases a secondary fiber had differentiated around the primary nucleus (stage 5) (Pl. II, fig. 4).

Experiment II. Implant of lumbar ganglion from normal tadpole.

New-forming lenses were found in 9 out of the 59 cases examined. Five lenses were found at day 5 after the operation and were at early stage 3. The other 4 were observed at day 7 after the operation and were at middle stage 3 (2 cases) (Pl. III, fig. 5) or late stage 3 (2 cases) (Pl. III, fig. 6).

In both experiments, new-forming lenses gradually grew towards the implanted ganglion and in many cases remained attached to the outer cornea, thus clearly revealing their corneal origin (Pl. I-III, figs. 2, 3, 5, 6). However, only occasionally did the new-forming lenses penetrate the implanted tissue (Pl. II and III, figs. 4, 6). In most cases they were separated from the ganglion cells by the exstensive proliferation of connective tissue from the ganglion capsule, which reconstitutes in the days after implanting.

In all implants there was little evidence of graft reaction. In a few cases, lymphocytes had accumulated around the implanted ganglion.

Discussion

Previous experiments involving the implantation of spinal ganglia innervating both normal and regenerating hindlimbs between the outer and the inner cornea of normal eyes of host larvae (Filoni *et al.*, in press) have shown that both normal and innervating regenerating hindlimbs ganglia can induce visible lens-forming transformations in the outer cornea up to the stage of the differentiation of lens fibers. However, the percentage of positive cases obtained as a result of implanting normal ganglia was found to be much lower than that of innervating regenerating limbs ganglia. These results are assumed to be due to the action of a neurotrophic factor capable of replacing the action of the retinal factor(s) and produced by innervating regenerating limbs ganglia in higher quantity than that produced by normal ganglia.

The results obtained in the present work confirm the previously advanced quantitative hypothesis. These data show that normal and innervating regenerating limbs ganglia implanted in the enucleated orbit can trigger visible lens-forming transformations of the outer cornea. Nevertheless, while the ganglia innervating regenerating limbs can occasionally promote the complete transformation of corneal cells into lenses, when normal ganglia are implanted the lens-forming transformations of the outer cornea reach a certain stage (late stage 3) after which, in none of the cases examined, does the lens-forming vesicle succeed in initiating fibrogenesis.

Furthermore, the percentage of positive cases obtained after implanting normal ganglia is found to be about half that obtained using innervating regenerating limbs ganglia and to undergo a much greater reduction in the days after the operation.

This higher decrement may be explained assuming that, in the days after operation, the onset of phenomena limiting the production and spread of the neurotrophic factor (i.e. gradual decrease in synthetic activity of the implanted

ganglion cells, gradual increase in proliferation of connective tissue around the ganglion cells) played an important role in ensuring that on in a large percentage of cases the concentration of the factor produced by normal ganglia drops below the minimum value required for the differentiation and conservation of the new-forming lens structure.

When compared with those obtained after implanting spinal ganglia between the two corneas of normal eyes (Filoni *et al.*, in press) the present data allow an evaluation to be made of the role played by nutritional substances supplied by the eye during the process of lens-forming transformation of the outer cornea. This comparison shows that in the absence of the eye the percentage of positive cases obtained by implanting both normal and innervating regenerating limbs ganglia is much lower than that obtained in the presence of the eye.

Campbell and Jones (1968) have postulated that the outer cornea of *xenopus* larvae has intrinsic lens-forming competence which it displays whenever, in the absence of the old lens, it receives the nutritional factors that are normally supplied by the aqueous humor. It was subsequently shown in several experiments that the nutritional factors supplied by the lensectomized eye are not enough by themselves to trigger the lens-forming transformation of the outer cornea. For this to occur the cornea must receive some specific factor(s) spreading from the vitreous chamber, also in the presence of the old lens (Bosco *et al.*, 1980; Cioni *et al.*, 1982).

Although the lens-forming inducing factor(s) is normally produced by the neural retina, other larval tissues, such as the bud of the hindlimb, its blastema, pituitary and spinal ganglia, grafted between the outer and the inner cornea

TABLE I.

Summary of the results obtained following implantation of spinal ganglia

Experiment	Days after operation	N° of case operated (*)	N° of cases dead/discarded	N° of cases examined	N° of new-forming lenses	Lens regenerating stage			% of lens formation (Total)
						3	4	5	
I	5	10	2	8	4	4	—	—	
	7	20	1	19	6	6	—	—	
	10	20	—	20	5	1	2	2	
									32
II	5	10	—	10	5	5	—	—	
	7	25	—	25	4	4	—	—	
	10	25	1	24	—	—	—	—	
									15

(*) Moreover in each experiment five larvae were operated and killed immediately after the operation.

of normal eyes can produce lens-forming inducing factors (Reeve and Wild, 1981; Filoni *et al.*, 1983; Filoni *et al.*, in press).

The results of the present work show that the outer cornea of larval *Xenopus laevis* can undergo lens-forming transformations even in the absence of eye nutritional substances when it is reached by lens-inducing factors produced by spinal ganglia. This does not mean, however, that the nutritional factors of the eye have no role to play, since their absence determines a strong decrease in percentage of lens-forming transformations of the outer cornea.

It is thus likely that a constant, adequate supply of non-specific nutritional substances to the corneal cells by the eye allows the outer cornea under the most favourable metabolic conditions, to respond more effectively to specific lens-inducing factors.

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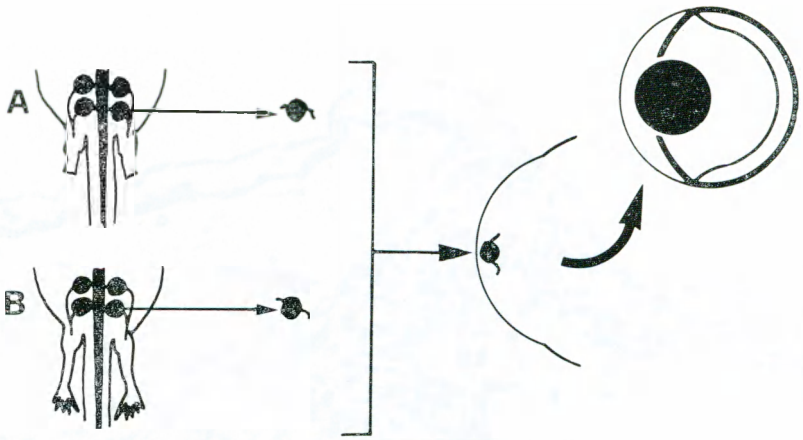
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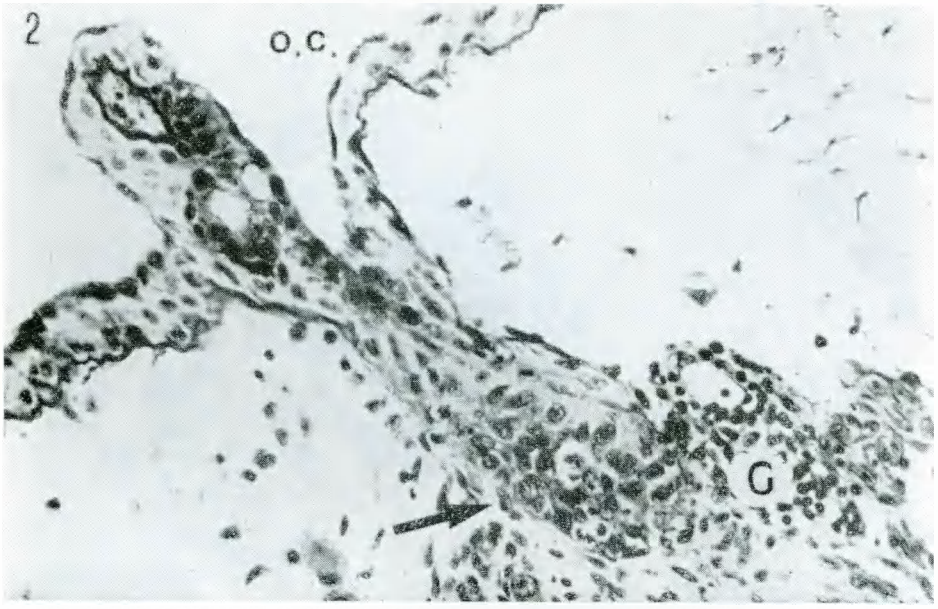
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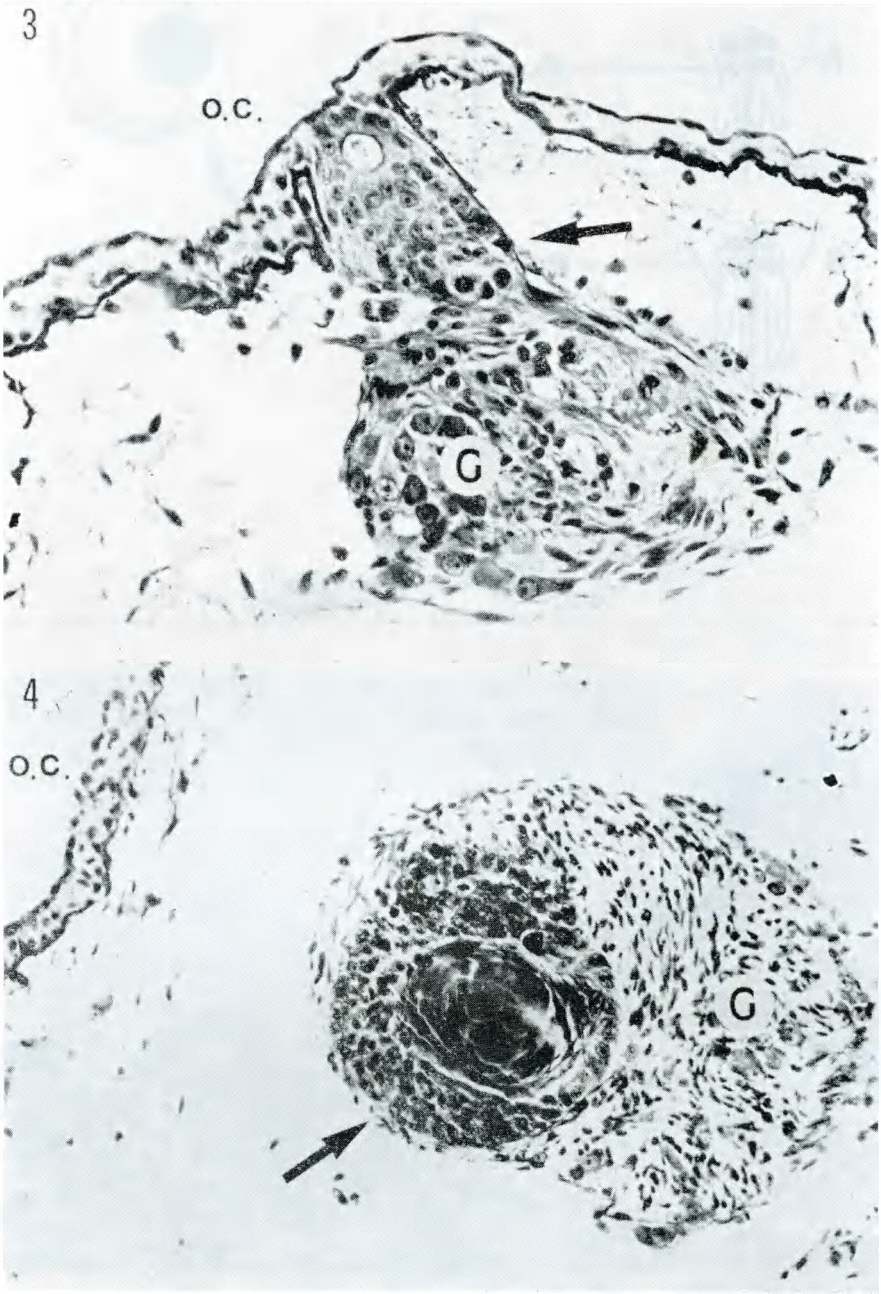
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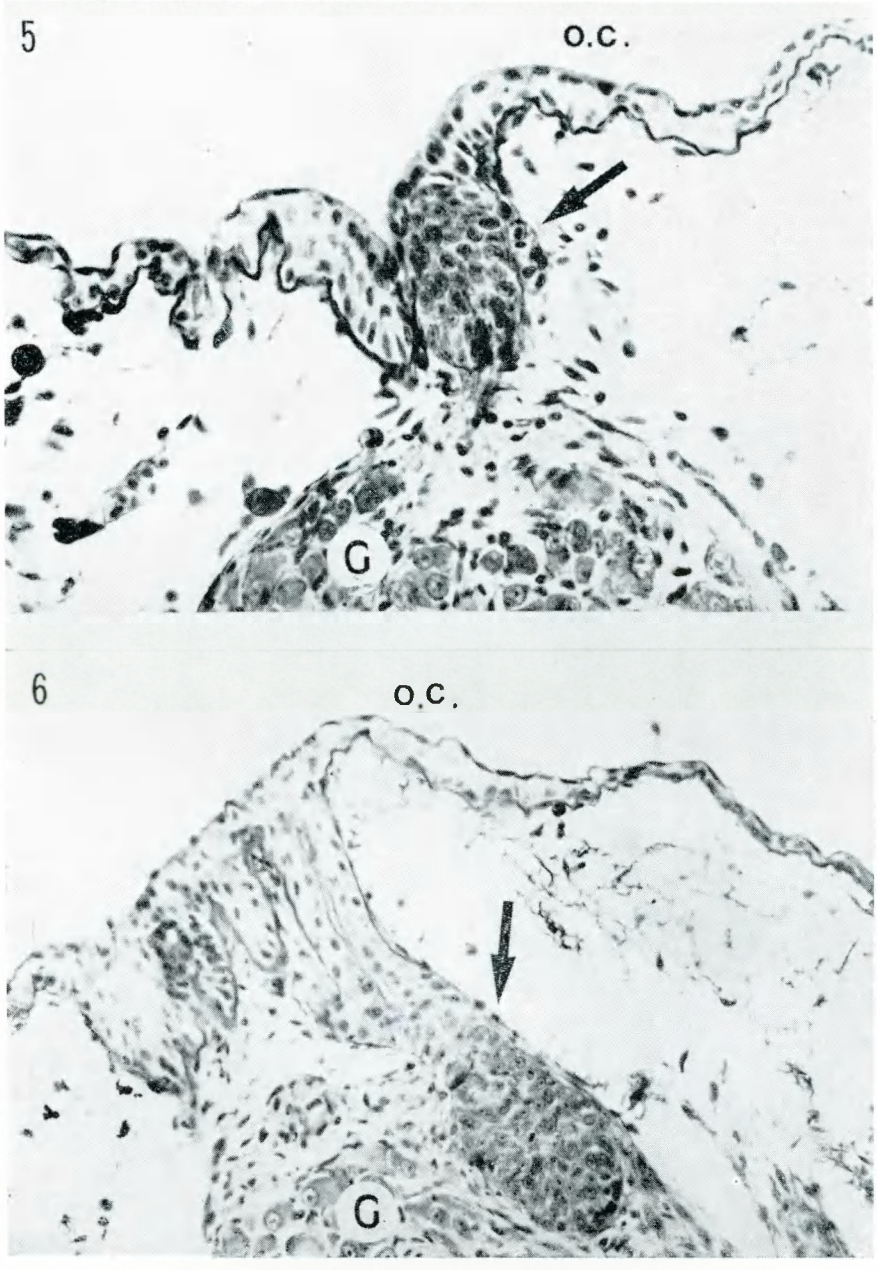
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EXPLANATION OF PLATE I-III

PLATE I

Fig. 1. – Diagram showing two types of implants beneath the outer cornea in an enucleated orbit. A) Experiment I: Implant of a lumbar ganglion from tadpole with regenerating hindlimb. B) Experiment II: Implant of a lumbar ganglion from a normal tadpole.

Fig. 2. – Implant of a lumbar ganglion from tadpole with regenerating limb (Experiment I). *Seven days after implanting*. Note lens vesicle at late stage 3 (arrow) still attached to outer cornea. G: ganglion; o.c.: outer cornea. Stained with hematoxylin-eosin and photographed at a magnification of 350×.

PLATE II

Figs. 3-4. – Implant of a lumbar ganglion from tadpole with regenerating limb (Experiment I) *Ten days after implanting*. Fig. 3. Note lens vesicle at late stage 3 (arrow) still incorporated in the outer cornea. Fig. 4. In the body of the ganglion it is still possible to see a lens at stage 5 (arrow) separated from the outer cornea. G: ganglion; o.c.: outer cornea. Stained with hematoxylin-eosin and photographed at a magnification of 350×.

PLATE III

Figs. 5-6. – Implant of a lumbar ganglion from normal tadpole (Experiment II). *Seven days after implanting*. Fig. 5. Note lens vesicle at middle stage 3 (arrow) which has formed from the inner layer of the outer cornea. Fig. 6. Although the lens vesicle (at late stage 3, arrow) has penetrated the ganglion it is still attached to the outer cornea by means of epithelial clusters. G: ganglion; o.c.: outer cornea. Stained with hematoxylin-eosin and photographed at a magnification of 350×.