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Indirect evidence for neoblasts migration and for gametogonia dedifferentiation in ex-fissiparous specimens of Dugesia gonocephala s.l.

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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Zoologia. — *Indirect evidence for neoblasts migration and for gametogonia dedifferentiation in ex-fissiparous specimens of* Dugesia gonocephala s.l. (*). Nota (**) di ROSALBA BANCHETTI e VITTORIO GREMIGNI, presentata dal Socio M. BENAZZI.

RIASSUNTO. — L'origine delle cellule che costituiscono il blastema è stata studiata al microscopio ottico ed elettronico in varie popolazioni di planarie ex-scissipare appartenenti a *Dugesia gonocephala s.l.*. A tale scopo sono state comparate le distribuzioni dei neoblasti in individui ex-scissipari ad ovari iperplasici e in individui ad ovari normali, sia prima del taglio sia dopo tagli consecutivi effettuati nelle regioni pre- e postfaringee. Negli individui ad ovari iperplasici non sezionati numerosissimi neoblasti con tipica struttura di cellule indifferenziate di tipo embrionale sono sparsi in tutto il corpo in particolare nella regione faringea. 48 h dopo il primo taglio il blastema con tipica organizzazione cellulare si costituisce per opera sia di neoblasti sia di giovani cellule germinali. Dopo i tagli successivi la rigenerazione avviene prevalentemente ad opera di neoblasti che migrano da porzioni del corpo più distali e che costituiscono dei cordoni cellulari pre-, peri- e postfaringei orientati verso la ferita. Questi neoblasti risultano allungati nel senso perpendicolare alla ferita (senso di migrazione) e presentano numerosi microtubuli disposti prevalentemente in senso parallelo all'asse maggiore della cellula. Prolungando il tempo di rigenerazione a 7 giorni, dopo il secondo, terzo taglio, i cordoni cellulari costituiti dai neoblasti in migrazione verso la ferita non sono più visibili probabilmente perché tale lasso di tempo è stato sufficiente ai neoblasti per raggiungere il blastema e riorganizzare le parti asportate. Da questi risultati gli Autori concludono che alla formazione del blastema rigenerativo concorrono soprattutto i neoblasti capaci di migrare da tutte le regioni del corpo, ed in parte elementi ancora in fase di differenziamento, e probabilmente capaci di sdifferenziarsi: in particolare ovogoni e spermatogoni.

Planarian regeneration is a widely reviewed topic in literature, however, the essential aspect of this phenomenon, i.e. the origin of the blastema, is as yet not entirely solved. In fact there are still at least two controversial theories on this problem.

Wolff and Dubois (1947); Dubois (1949); Pasquini, Ghirardelli and Lesi-Massari (1955); Brøndsted (1955, 1969); McWhinnie and Gleason (1957); Grasso (1959); Kolmayer and Stéphan-Dubois (1960); Lender and Gabriel (1960, 1961, 1965); Wolff (1962); Lender (1962, 1965); Fedecka-Bruner (1961, 1964); Cecere, Grasso, Urbani and Vannini (1964); Stéphan-Dubois (1965); Ghirardelli (1965); Le Moigne, Sauzin, Lender and Delavault (1965); Benazzi

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(1966); Stéphan-Dubois and Gusse (1970); Gabriel (1970) and others believe that there exists in the parenchyma of the fully developed animals a source of persistent embryonic totipotent cells called " neoblasts " (Randolph, 1897; Buchanan, 1933) capable of proliferation and migration towards the region of cut, and of formation of the regenerative blastema. This hypothesis is supported by the ultrastructural descriptions of neoblasts of Pedersen (1959); Klima (1962); Skaer (1965); Sauzin (1966); Le Moigne (1967); Morita, Best and Noel (1969) which are in agreement with those of embryonic cells of Le Moigne (1966) (also see Le Moigne, Sauzin and Lender, 1966).

Others, among them Steinmann (1908); Lang (1912); Hyman (1951), Chandebois (1962, 1965); Woodruff and Burnett (1965); Rose and Shostak (1968); Hay (1968); Coward (1969) maintain an alternative theory; they do not accept that neoblasts exist or take part in regeneration phenomena and they believe that the blastema originates from a process of dedifferentiation, proliferation and successively redifferentiation of cells from parenchyma, intestine, gonads, glands etc.

Chandebois, in particular, has formulated a theory based on a cell transformation system in which free cells (cells of type 1 by Prenant, 1922) corresponding to neoblasts of the other Authors have exclusively the function, by the process of cytolysis, of supplying RNA to a permanent totipotent syncytium. This syncytium formed by dedifferentiated cells then forms the regenerative blastema which also has a syncytial structure.

However, whereas Hyman, Hay and Coward believe that the blastema originates from a cooperation between near and distant cells, Chandebois, and Flickinger (1964) deny this concept of migration.

Finally, others suggest an intermediate solution, according to which both dedifferentiated cells and neoblasts participate in the formation of the blastema (Bandier, 1936; Kido, 1958, 1959; Bondi, 1959; Ichikawa and Ishii, 1961; Teshirogi, 1962; Betchaku, 1970).

Previously (Gremigni and Banchetti, 1972), we demonstrated that exfissiparous specimens with hyperplasic ovaries belonging to some populations of *D. gonocephala s.l.* had exceptionally high regenerative power. We attempted an explanation of this phenomenon by hypothesising the presence throughout the whole body of the animals of an enormous number of free cells with thin basophilic cytoplasm, these cells being interpreted as neoblasts (also see Grasso and Benazzi, 1973). The observation, that regeneration takes place more slowly if several cuts are performed at intervals of 5 or 10 days, has been interpreted as being due to a progressive depletion of the reserve of totipotent cells. The results obtained using this material, which we believe to be particularly suited to the study of neoblasts and of regenerative phenomena, have led us to continue our research on the problems of blastema formation.

The hypothesis we started our research with was the following: if the blastema were reconstituted by non differentiated cells migrating even from areas of the body distant from the wound, we could progressively exhaust the reserve of neoblasts in the animal by repeated cuts performed at short

intervals. If on the contrary the blastema were formed by fixed parenchymal cells which dedifferentiate *in situ*, no depletion should take place.

We then studied the fine structure of the basophilic cells of whole ex-fissiparous specimens with hyperplasic ovaries to compare it with the findings of various Authors on neoblasts and embryonic cells. Finally we studied the ultrastructure of the regenerative blastema in an attempt to solve the question of its organization i.e. whether it is syncytial or cellular.

MATERIALS AND METHODS

The specimens used belong to the following populations:

A) Ex-fissiparous specimens with hyperplasic ovaries collected in the River Jordan.

B) Ex–fissiparous specimens with normal ovaries collected at Castello Pino.

C) Specimens whose reproduction is exclusively sexual collected at Paradojo (1).

All the specimens were kept at the same temperature (19–20° C), and for the two months previous to the experiments they were fed with *Tubifex* twice a week in order for them to reach maximum growth.

We then carried out two series of experiments involving cuts. In the first series, thirty specimens belonging to population A, twenty belonging to B and twenty to C were used. Two thirds of the specimens of each population were cut 3–4 mm above the pharynx, the other third 4–5 mm below the pharynx, in order to have both anteriorly and posteriorly regenerating specimens. Successively, other four cuts were performed at intervals of 48 hrs, each cut being 1 mm lower than the preceding one.

In the second series of experiments, twenty specimens from population A were repeatedly cut once every seven days.

In order to study the variations in the way neoblasts are distributed in whole animals, five whole specimens and three which were in the phase of regeneration were taken from each population, they were then fixed for histological examination and sections 8 μ thick were coloured with 0.5 % toluidine blue. Other whole and regenerating specimens were then taken and their neoblast-containing parenchyma and their blastema were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer (pH = 7.3) and postfixed in identically buffered 1% osmium tetroxide. All the samples were embedded in Araldite-Epon. Ultra–thin sections were stained with uranyl acetate and lead citrate; micrographs were taken with a Siemens Elmiskop 101.

(1) Taxonomically the population from the River Jordan (Israel) belongs to the species *D. biblica* Benazzi and Banchetti; the ones from Castello Pino and Paradojo (both in Corsica) belong to *D. benazzii* Lepori; all of them belong to the "*D. gonocephala* group".

Results

Whole specimens.

Neoblasts are distributed throughout the whole parenchyma but they are concentrated particularly in the areas just below the epithelium. This arrangement is common to specimens of the populations A, B and C, which however differ in the density of their neoblasts, this being greater in the strain Jordan than in the others. Apart from this general localization, in specimens with hyperplasic ovaries, most of neoblasts of the prepharyngeal region are near the ovaries and they are in the process of differentiating to oogonia; furthermore, the prepharyngeal parenchyma and in particular the dorsal parenchyma contain a dense field of neoblasts which merge with the ones among the intestinal branches (Plate I, fig. 1). These neoblasts are elongated in a thick cord behind the pharynx. This type of neoblast localization is peculiar to specimens with hyperplasic ovaries and is in contrast with the situation normally found in planarians with agamic or sexual reproduction (Plate I, fig. 2) (see e.g. Lender and Gabriel, 1960 and Lange, 1967 in *D. lugubris*; A. and H. V. Brøndsted, 1961 in *Dendrocoelum lacteum*).

Neoblasts generally have an oval shape with short cytoplasmic protrusions at the two cellular extremities. Their nucleus, of almost regular round shape, contains densely stained chromatin agglomerates; the nucleolus is always present, and the cytoplasm, intensely basophilic, is reduced to a thin layer surrounding the nucleus (Plate I, fig. 3).

On electron microscopic examination, most neoblasts show non-differentiated characteristics as do embryonic cells; the nucleus has a regular contour, the nuclear envelope has very few pores, the nucleolus is small and with a predominantly granular structure, and often lies adjacent to the nuclear membrane. The cytoplasm has few small nuclear extrusions near the nuclear membrane; it contains few roundish mitochondria and has a great number of ribosomes free or in cluster formation, few cisternae or vesicles of the smooth endoplasmic reticulum, and few short microtubules (Plate IV, fig. 12).

First series of experiments involving cuts.

After performing the first cut on specimen from population A, the neoblasts are found to be concentrated in the area of cut and in the prepharyngeal region (Plate II, fig. 4), where they are seen to be elongated in shape. No mitosis was observed in the blastema. In anteriorly regenerating specimens young germinal cells belonging to the ovary seem to be migrating towards the wound, in fact they form small continuous cords which reach the blastema (Plate II, fig. 5). The same situation, in this case regarding the testes, can be seen in the postpharyngeal blastema of specimens with hyperplasic ovaries and in ex-fissiparous specimens with normal ovaries (Plate II, fig. 6). In the latter specimens, 48 hrs after the first cut, numerous neoblasts are still dispersed throughout the body of the animal while instead the blastema is just beginning to form.

After the second cut, ex-fissiparous specimens with hyperplasic ovaries have their neoblasts predominantly grouped into two cords positioned along the sides of the pharynx and continuing either anteriorly or posteriorly towards the region of cut (Plate II, fig. 7). Young germinal cells still numerous at the periphery of the gonads contribute to the formation of such cords.

This same arrangement can be seen after the third cut. In continuity with the lateral and anterior pharyngeal cords, the neoblasts are predominantly concentrated in column-form at the pharyngeal extremity opposite the point of cut. The free neoblasts which were observed in the whole animal in the pre-and post-pharyngeal parenchyma and among the intestinal branches have practically disappeared, having evidently migrated into the cords.

It was observed after the fourth cut on the specimens with hyperplasic ovaries that the blastema was still present, but was reduced and had a thin line of neoblasts merging into it (Plate III, fig. 8). In the distal region, with respect to the cut, the neoblasts are still numerous and are localized in a cord, as was similarly observed after the third cut, and they are still in continuity with the lateral and proximal cords (Plate III, fig. 9).

After the second, third and fourth cuts, in populations B and C, the density of the neoblasts, before the cuts very low with respect to specimen from population A, does not appear to be greatly decreased; so much so that the blastema, though very small, is already in the initial stages of formation 48 hrs after the fourth cut.

Second series of experiments involving cuts.

Seven days after performing cuts on specimens of population A, a large number of the severed regions had begun to regenerate. Thin cords of neoblasts which were observed at the sides of the pharynx disappeared completely after the third and fourth cuts (Plate III, fig. 10). At this stage even the rest of the body of the animal is rather poor in neoblasts with the exception of the areas adjacent to the cut in which are visible free cells migrating to the blastema.

On electron microscopic examination, the blastema shows no syncytial organization and is composed of very distinct, rounded cells, separated by large intercellular spaces (Plate IV, fig. 11); the only contacts between the cells are formed by desmosomes some of which are rather long; the nucleus of considerable dimensions has rather diffuse chromatin. There is always present at least one nucleolus which consists of mixed granular and fibrillar components. The nuclear membrane shows numerous pores and many nuclear extrusions surrounded by mitochondria can be found in the cytoplasm. Many free and clustered ribosomes are present; some cisternae of rough endoplasmic reticulum are visible. In general, a well-defined Golgi apparatus is missing, but there can often be seen numerous concentrations of small, smooth vesicles.

The tissues below the blastema (muscles, nephridia, intestine etc.) show no signs of dedifferentiation.

The neoblasts found in the cords are elongated rather than rounded (Plate IV, fig. 13). The nucleus is clearly ovoid, with dense aggregates of chromatin and a nucleolus of a predominantly granular composition.

The cytoplasm, scarser than in the cells of the blastema, shows few small nuclear extrusions. There can be seen a moderate amount of smooth endoplasmic reticulum. These elongated neoblasts are almost entirely lacking in rough reticulum but instead they have many free and clustered ribosomes; the mitochondria, which are fewer in number than in the blastema, are small, ovoid and, in general, grouped together at the broader pole of the cell.

Microtubules, isolated or in whorl-formation, arranged in various directions, can be seen in all the neoblasts and particularly in the elongated ones. The longest microtubules are positioned parallel to the widest diameter of the cell (Plate IV, fig. 14).

Discussion

Ultrastructural examination shows that whole animals with hyperplasic ovaries possess no totipotent syncytium and that the very numerous cells dispersed throughout the parenchyma have typical characteristics of non-differentiation rather similar to those of embryonic cells and can therefore be safely classed as neoblasts.

Not even the blastema has a syncytial structure, in agreement with the findings of Le Moigne *et al.* (1965); Sauzin (1966); Morita, Best and Noel (1969); Pedersen (1972); Spiegelman and Dudley (1973); and the general morphology of the cells of which it is composed (the structure of the nucleolus, the presence of numerous nuclear extrusions, the development of the granular endoplasmic reticulum, the initial organization of the Golgi apparatus etc.) shows these cells to be activated neoblasts.

We believe that the results obtained from the first series of experiments involving cuts show the blastema to be composed both of free neoblasts distributed in various areas of the body, and of young germinal elements. In fact, in the peripheral areas of the gonads of whole animals, there can be seen numerous cells in phases of differentiation (oogonia and spermatogonia), which determine the ovarian hyperplasia and the enormous number of testes that develop. It is our opinion that even the neoblasts present in the prepharyngeal parenchyma are migrating towards the anterior zones and that a large number of these are destined to differentiate into germinal cells. We believe that in the regenerating animal young germinal cells (above all oogonia and spermatogonia) interrupt the cytodifferentiation and migrate towards the wound to constitute the blastema. This hypothesis is in agreement with that of Steinmann (1908) and with findings of Manelli and Contoli-Amante (1966), who however believe that the peripheral cells of the gonads still are neoblasts.

The blastema, after repeated removal at 48 hr intervals, is constituted more slowly each time by the neoblasts which are situated initially further from the wound, and which after every cut form cords directed towards the

region of cut. The neoblasts constituting these cords are elongated with scarse cytoplasm and with numerous microtubules positioned parallel to the greater axis of the cell. It should be noted that even after the third and fourth cuts a continuity is maintained among these pre-, peri- and post-pharyngeal cords and they remain conspicuous most probably because the cuts are performed so soon one after the other that not all the migrating neoblasts have time to reach the blastema between one cut and the next.

In the ex-fissiparous specimens with normal ovaries, in which the number of neoblasts in the whole animal is notably smaller than in the animals with hyperplasic ovaries, the regenerative processes are slower but the morphology of the neoblasts and the organization of the blastema are identical.

The second series of experiments performed on ex–fissiparous specimens with hyperplasic ovaries at interval of seven days between cuts shows that the majority of the neoblast population diminishes after only a few consecutive cuts.

To us this shows that an increase in the regeneration time results in the migration of the neoblasts, constituting the central and distal cords, into the area of cut in order to complete the reorganization of the excised structures, and this is in accordance with the hypothesis of a previous work. This latter stated that only three days after performing a cut 1 mm above the pharynx the eyes reappeared, and after ten days the anterior region had been completely reorganized (Gremigni and Banchetti, 1972).

From our observations we can conclude that fundamentally to the formation of the regenerative blastema the following elements participate:

1) Numerous neoblasts distributed throughout the body of the animal; first those nearer the wound and successively those situated in areas further away but capable of migrating as far as the wound and thus forming continuous cords.

2) To a lesser extent, but very evident in the animals with hyperplasic ovaries, the young germinal cells which are found at the periphery of the gonads and which should therefore be capable of interrupting the process of differentiation taking place and becoming once again totipotent cells.

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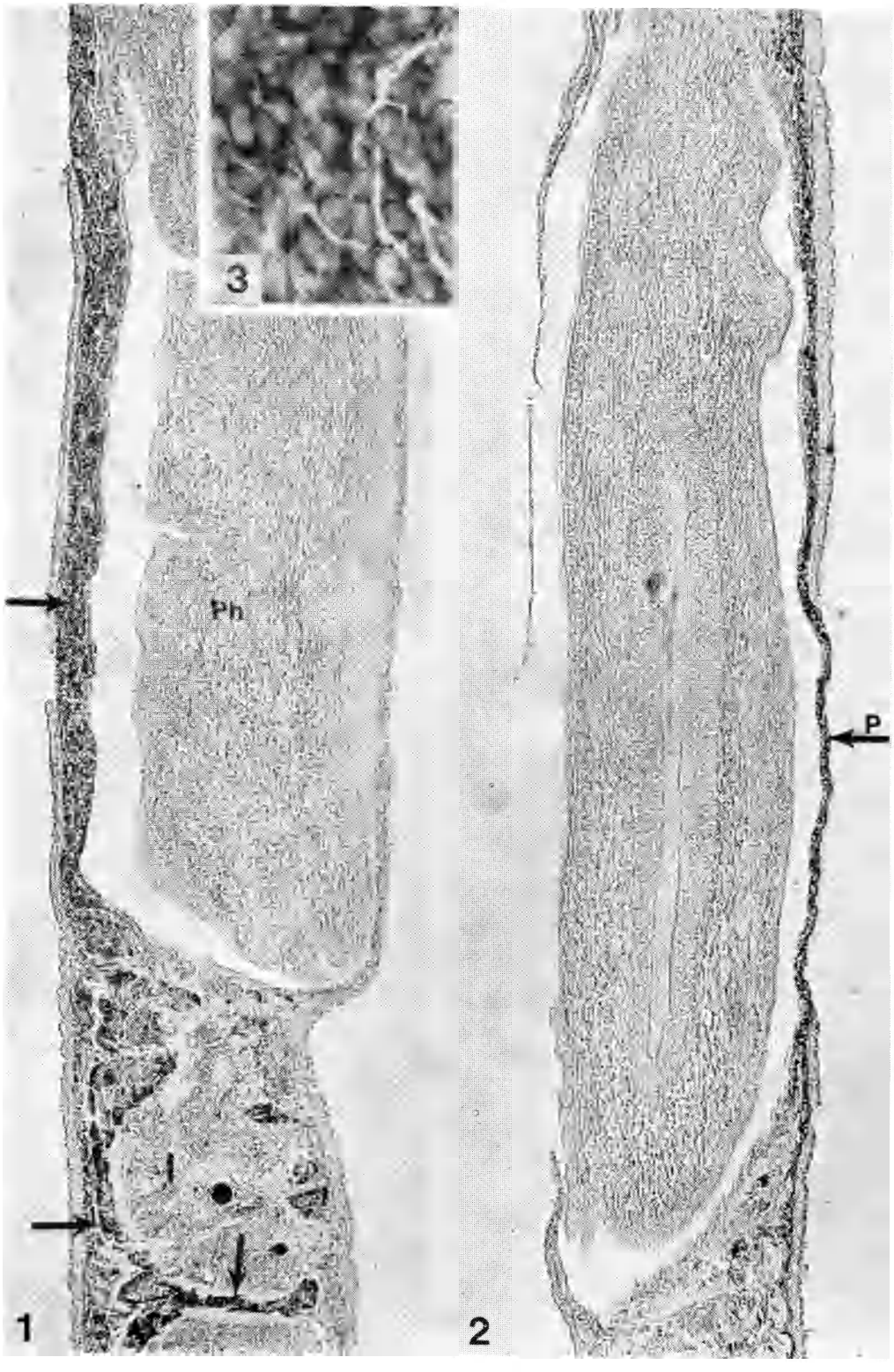
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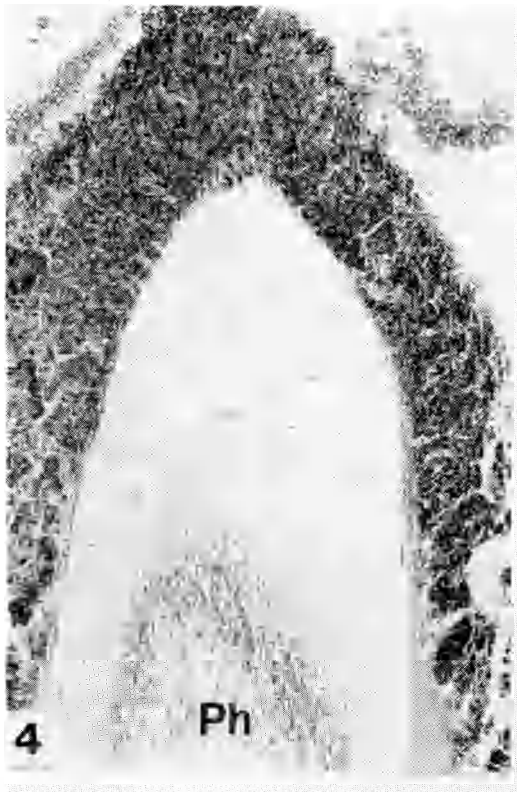
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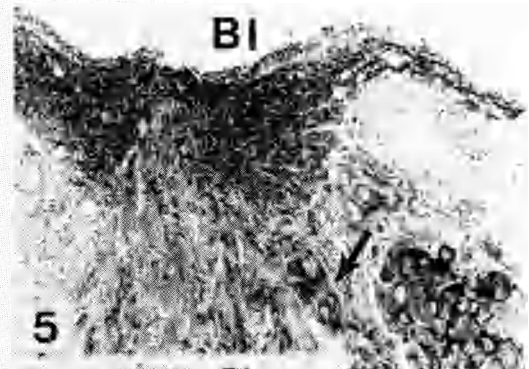
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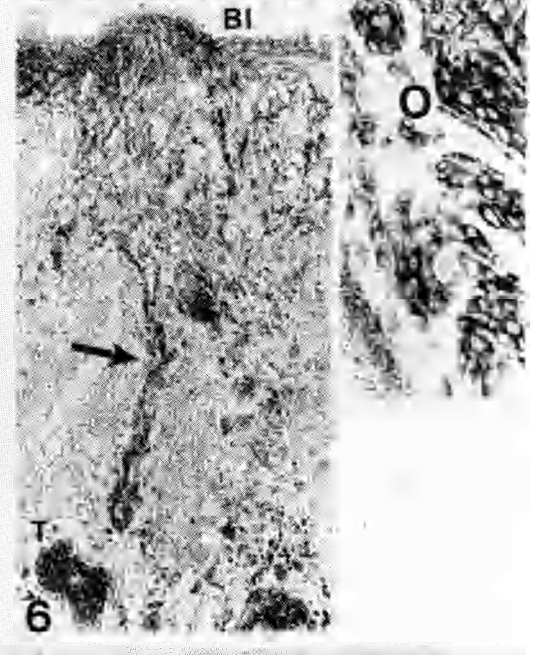
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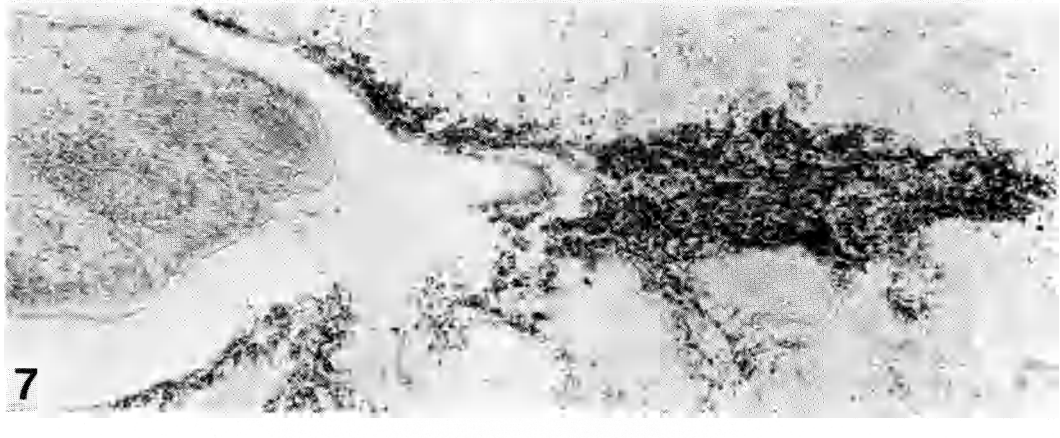
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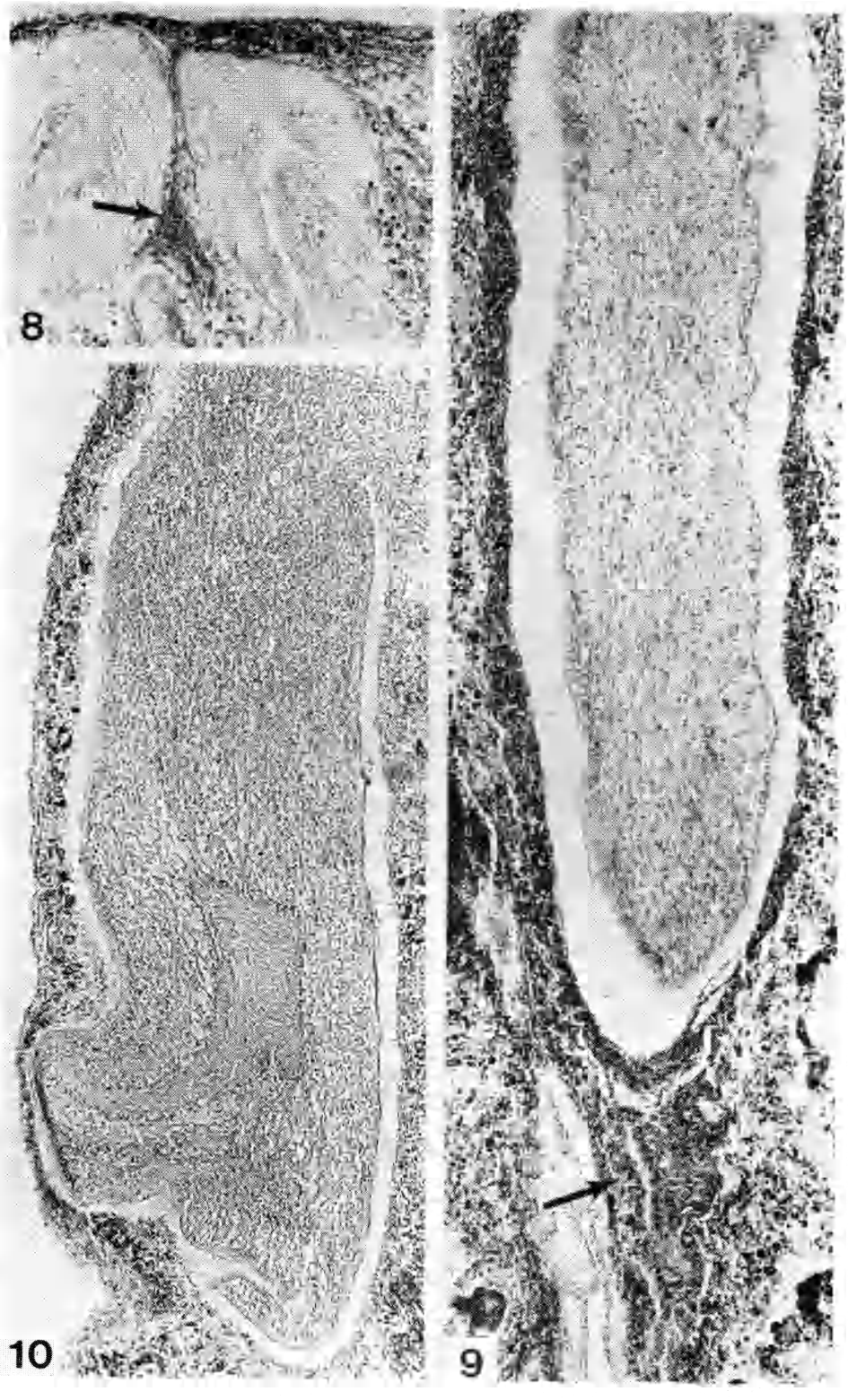
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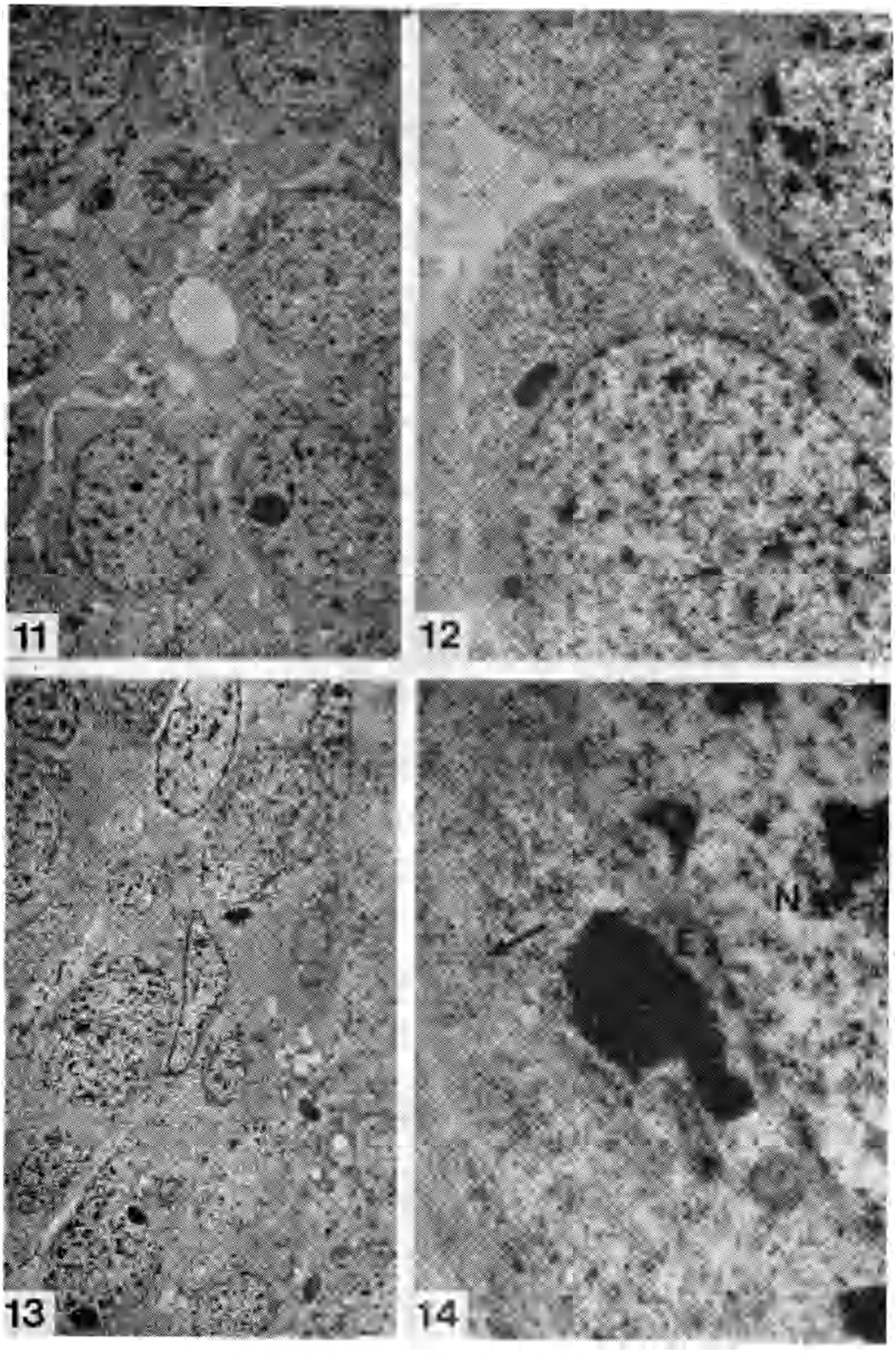
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EXPLANATION OF PLATES I–IV

PLATE I

Fig. 1. – Central region of a whole ex-fissiparous specimen from population A. The image shows the distribution of neoblasts in the pharyngeal parenchyma and among the intestinal branches (→). Ph = pharynx. I = intestine (×40).

Fig. 2. – The same section from a specimen of population C shows no neoblasts in the peripharyngeal region, but only granules of pigment ($\xrightarrow{P}$) (×40).

Fig. 3. – High magnification of the pharyngeal parenchyma of fig. 1, showing the morphology of neoblasts. The nucleolus is often present (×240).

PLATE II

Fig. 4. – Frontal section of the pharyngeal parenchyma of an anterior regenerant from population A 48 hrs after the first cut. Note the condensation of neoblasts in the peri- and pre-pharyngeal parenchyma. Ph = pharynx. (×37).

Fig. 5. – Frontal section of the blastema (Bl) of an anterior regenerant from population A 48 hrs after the first cut. Several oogonia are migrating to the wound (→). O = Hyperplasic ovaries (×53).

Fig. 6. – Frontal section of the blastema (Bl) of an anterior regenerant from population B 48 hrs after the first cut. Thin cords of spermatogonia migrating to the wound are evident (→). T = testes (×40).

Fig. 7. – Middle region of a posterior regenerant from population A 48 hrs after the second cut. The postpharyngeal cord of neoblasts is migrating to the wound. The neoblasts around the pharynx are very scanty (×30).

PLATE III

Fig. 8. – Reduced blastema of a posterior regenerant from population A 48 hrs after the fourth cut in continuity with a thin cord of migrating neoblasts (→) (×45).

Fig. 9. – Frontal section of an anterior regenerant from population A 48 hrs after the fourth cut. The postpharyngeal cord of neoblasts (→) opposite to the wound is continuous with the lateral cords (×34).

Fig. 10. – Frontal section of an anterior specimen from population A seven days after the third cut. No peripharyngeal cord of neoblasts is visible (×34).

PLATE IV

Fig. 11. – Portion of the blastema of a specimen from population A 48 hrs after the first cut. (×3.750). Glut. 3 %–OsO_4 1 %.

Fig. 12. – Neoblasts of the parenchyma of a whole specimen from population A (×16.000). Glut. 3%–OsO_4 1%.

Fig. 13. – Low magnification micrograph showing a cord of migrating neoblasts in a specimen from population A 48 hrs after the second cut (×3.600). Glut. 3%–OsO_4 1%.

Fig. 14. – Microtubules with course parallel (→) to the main axis in a migrating neoblast of fig. 13. E = nuclear extrusion; N = nucleus (×36.000). Glut. 3%–OsO_4 1%.