On the role of germ cells in planarian regeneration

I. A karyological investigation

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SUMMARY

Specimens from a polyploid biotype of Dugesia lugubris s.l. were used to clarify the role and fate of germ cells during planarian regeneration. These specimens provide a useful karyological marker because embryonic and somatic cells (3n = 12) can be easily distinguished from male (2n = 8) and female (6n = 24) germ cells by their chromosome number. We succeed in demonstrating how primordial germ cells participate in blastema formation and take part in rebuilding somatic tissues. This evidence was obtained by cutting each planarian specimen twice at appropriate levels. The first aimed to induce primordial germ cells to migrate to the wound. The second cut was performed after complete regeneration and aimed to obtain a blastema from a cephalic or caudal area devoid of gonads. A karyological analysis of mitotic cells present in each blastema obtained after the second cut provided evidence that cells, originally belonging to the germ lines, are still present in somatic tissues even months after complete regeneration. The role of primordial germ cells in planarian regeneration was finally discussed in relation to the phenomenon of metaplasia or transdifferentiation.

INTRODUCTION

Several lines of evidence obtained by our group substantiate the view that undifferentiated cells play an important role in blastema formation in planarians (Banchetti & Gremigni, 1973; Gremigni & Puccinelli, 1977; Puccinelli & Gremigni, 1977; Gremigni & Picano, 1977). Cells showing ultrastructural and cytochemical characteristics common to embryonic cells have been described by many authors to be scattered throughout parenchyma in a number of planarians (see for reviews on the subject Brøndsted, 1969; Pedersen, 1972; Gremigni, 1974; Chandebois, 1976). These cells, usually referred to as 'neoblasts' are thought to behave as reserve cells during physiological or traumatic regeneration: 'Neoblast theory' (Wolff & Dubois, 1947a, b; Dubois, 1949; Wolff, 1962; Lender, 1962).

On the other hand, our previous investigations provided evidence that following transection, primordial germ cells too may take part in blastema formation; however, germ cells undergoing meiosis and mature gametes degenerate. These findings are in line with earlier histological observations (Vandel, 1921; 1

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Teshirogi, 1962; Fedecka-Bruner, 1965-1967) and give further support to the 'dedifferentiation theory' as proffered among others by Lang (1912), Steinmann (1925), Hyman (1951), Hay (1968) and Coward (1969). According to this theory, undifferentiated reserve cells should not be involved in planarian regeneration, which instead should occur by contribution of specialized or specializing cells.

Since our findings seem to support both theories, we were led to formulate a conciliatory view in which both the so-called neoblasts and de-differentiated cells can participate in blastema formation (Gremigni, 1974; Gremigni & Puccinelli, 1977).

Indeed, if germ cells may migrate into the blastema, then it would be important to know whether they may transform into a cell type which is identical or different from the original one, during rebuilding of injured or removed tissues. The latter possibility would be referred to as 'metaplasia' or 'transdifferentiation'.

This question is still unresolved in planarians, although some evidence favoring metaplasia has been obtained (Woodruff & Burnett, 1965; Rose & Shostak, 1968).

In this study we provide additional data on this question by using specimens from a polyploid biotype of *Dugesia lugubris* s.l. in which male (2n = 8) and female (6n = 24) germ cells may be easily distinguished from embryonic and somatic cells (3n = 12) on the basis of their chromosome number. In particular we aim to establish whether germ cells are capable of taking part in rebuilding somatic tissues, after migrating into the blastema.

For this purpose we induced germ cells to participate in the formation of a blastema which would regenerate an area devoid of gonads. The evidence obtained showed that the karyological analysis of the regenerated area was an adequate tool to establish the fate of germ cells during regeneration.

**MATERIAL AND METHODS**

The planarian used in this study belongs to the triplo-hexaploid biotype of *Dugesia lugubris* s.l. The nomenclature s.l. (= sensu lato) was introduced by Benazzi, Puccinelli & Del Papa (1970) and is referred to the two allied species *D. lugubris* and *D. polychroa* which form a unique group with the taxonomic value of super-species (or subgenus *Schmidtea* according to Ball, 1974). The specimens were originally collected from Lake Iseo (Italy) and then maintained in our laboratory. All specimens of this population have a triploid somatic line (3n = 12), a hexaploid female germ line (6n = 24) and a diploid male germ line (2n = 8). The haploid set (n = 4) comprises one large metacentric chromosome and three acrocentric chromosomes of different length. Meiosis occurs normally in both male and female lines, but embryonic development is pseudogamic, so that all embryonic cells are triploid (see Benazzi, 1957; Benazzi & Benazzi-Lentati, 1976; Gremigni & Puccinelli, 1977 for details on the karyology and reproductive biology of this and of other biotypes of the genus *Dugesia*).
Figs. 1–6. Schemes of the operations carried out in regeneration experiments (see text for explanations). o, ovaries; t, testes; ph, pharynx. The cross indicates the area removed after the cuts. The stippled area indicates the tissue regenerated after the first cut.

In these specimens terminal cephalic and caudal areas are devoid of gonads (Fig. 1), which cannot be rebuilt there even following transection. This is so because there are two ovaries located ventrally behind the eyes, and numerous testes widely spread in the lateral-dorsal area of the organism extending from behind the ovaries to almost the end of the body.

All specimens were cultured individually and when they reached a stage of sexual maturity they were used for experiments. Frequent controls were done
Figs. 3-6. For legend see p. 55.
to the cultures to avoid the use of specimens which could have undergone some regeneration.

Sexually mature specimens were then divided into four groups of ten organisms each. Each specimen was cut twice; the first cut aimed to allow germ cells to move from the gonads which remained in the stump into the blastema. When the removed region (cephalic or caudal area) was completely regenerated, a second cut was made in order to obtain a new blastema from the respective cephalic or caudal stumps, both of which were devoid of gonads. The karyological analysis of cells present in the blastema, which was formed after the second cut, allowed us to establish the nature of cells which had previously participated in the regeneration of somatic tissues.

**Group A.** Each specimen was first cut anterior to the ovaries. A second cut was then made at a level identical to the first one, after the posterior stump (p.s.) had completely regenerated a cephalic area. The posterior fragment was then removed, while the cephalic stump (ce.s.) was allowed to form a blastema which will be cited hereafter as ce.s.b. (Fig. 1).

**Group B.** Each specimen was first cut just posterior to the ovaries. A second cut was then made just posterior to the testes after the anterior stump (a.s.) had completely regenerated a posterior area. The anterior fragment was then removed while the caudal stump (c.s.) was allowed to form a blastema which will be cited hereafter as ca.s.b. (Fig. 2).

**Group C.** Each specimen was first cut anterior to the pharynx. A second cut was then made just posterior to the eyes after the posterior stump (p.s.) had completely regenerated an anterior area. The posterior fragment was then removed while the cephalic stump (ce.s.) was allowed to form a blastema (ce.s.b.). (Fig. 3).

**Group D.** Each specimen was first cut just posterior to the testes. A second cut was then made at the same level as the first one after the anterior stump (a.s.) had completely regenerated a posterior area. The anterior fragment was then removed, while the caudal stump (ca.s.) was allowed to form a blastema (ca.s.b.) (Fig. 4).

Samples of blastemas obtained in each of the four groups were prepared for karyological observations after the second cut.

Two more groups of ten organisms each were used as controls. These specimens were transected only once either at a cephalic (Fig. 5) or caudal (Fig. 6) level and in each case the blastema which formed from the terminal stump was studied.

**Karyological technique**

Experimental stumps were prepared 60 h after the second cut and control stumps 60 h after the unique cut. The terminal stumps were treated for 6 h with a 0·3% aqueous solution of colchicine. Each blastema was then removed
Fig. 7. Cephalic stump blastema (ce.s.b.) from group A specimens. Four c-metaphasic cells are visible. Two of these are triploid (3n) and two are hexaploid (6n).

Fig. 8. Caudal stump blastema (ca.s.b.) group B specimens. One triploid (3n) and one hexaploid cell (6n) are visible.

Fig. 9. Cephalic stump blastema (ce.s.b.) from group C specimens. One diploid (2n) and one triploid cell (3n) are visible.

Fig. 10. Caudal stump blastema (ca.s.b.) from group D specimens. One diploid (2n) and one triploid cell (3n) are visible.
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from the stump with a razor-blade, stained for 20 minutes in a lactic–acetic solution of orcein and finally squashed on slides.

OBSERVATIONS

The time needed to obtain complete regeneration following the first cut was found to vary in the four groups of specimens studied and depended on the extent of the area removed. Thus the small cephalic area of specimens in group A was completely rebuilt in about 15–17 days, the large posterior areas of specimens in groups B and C were rebuilt in about 45–50 days, and the small caudal area of specimens in group D was rebuilt in about 20–22 days. Regeneration was considered to be completed when the area rebuilt appeared pigmented as normal.

The number of metaphase cells per blastema was found to vary slightly from one squash to another in the same group. From 80 to more than 100 well-preserved metaphase cells were observed in ce.s.b., while from 60 to 80 well-preserved metaphase cells were observed in ca.s.b.

To verify whether hexaploid or diploid cells were still present in the tissues of the cephalic or caudal area after regeneration, specimens of groups A and B, or of groups C and D respectively were analyzed for their chromosome number.

Cephalic stump blastema (ce.s.b.) of group A

Four of the ten ce.s.b. samples exhibited only triploid cells. The other six samples had also a small number of hexaploid cells (2, 1, 1, 3, 2, 1 respectively) (Fig. 7). Moreover, diploid cells (3, 1, 1, 2 respectively) were observed in four blastemas.

Caudal stump blastema (ca.s.b.) of group B

Five of the ca.s.b. samples exhibited only triploid cells. The other five samples had also hexaploid cells (2, 2, 1, 1, 1 respectively) (Fig. 8). No diploid cells were observed.

Cephalic stump blastema (ce.s.b.) of group C

Every ce.s.b. sample exhibited triploid (97–98 %) and diploid (2–3 %) metaphase cells (Fig. 9). No hexaploid cells were observed.

Caudal stump blastema (ca.s.b.) of group D

One ca.s.b. sample exhibited only triploid cells. The other nine samples had both triploid (97–98 %) and diploid (2–3 %) metaphase cells (Fig. 10). No hexaploid cells were observed.
**Control blastema**

All the cephalic and caudal stump blastemas obtained after the unique cut exhibited only triploid metaphase cells.

**DISCUSSION**

The present karyological study demonstrated that every blastema formed by a cephalic or caudal stump after a second cut consisted mainly of triploid cells. In addition some ce.s.b. or ca.s.b. obtained from specimens of the groups A and B exhibited a small number of hexaploid cells. Moreover, some diploid cells were observed in four of the ce.s.b. of group A, but not in the ca.s.b. of group B. In interpreting these data we suggest that primordial female germ cells migrated to the blastema after the first cut. The second cut then induced the tissue which had regenerated in the terminal cephalic or caudal area to form a new blastema. In these conditions some hexaploid cells have also participated in the blastema formation after the second cut. This demonstrates that dedifferentiated cells of female origin took part in rebuilding the somatic tissue.

Since some testes were still present in the posterior stump obtained after the first cut, male germ cells from specimens of group A may migrate to the blastema and then take part in the formation of the ce.s.b. after the second cut. In contrast with this possibility one should take note that no male germ cells were present in the ca.s.b. of group-B specimens after the second cut because testes were absent in the anterior stump which was obtained after the first cut.

Specimens of groups C and D cut twice presented a small percentage (2-3 %) of diploid cells besides triploid cells in both ce.s.b. and ca.s.b. To account for these observations we suggest that primordial male germ cells migrated to the blastema after the first cut and subsequently took part in the ce.s.b. or ca.s.b. after the second cut. This finding shows that dedifferentiated cells of male origin may actually take part in the regeneration of somatic tissues in the terminal area, which is devoid of gonads.

Our interpretation is also supported by control experiments. These showed that hexaploid or diploid cells never participate in ce.s.b. or ca.s.b. obtained from specimens cut only once, because gonads are not present in the stump.

The possibility that the hexaploid or diploid cells observed in ce.s.b. or ca.s.b. obtained after the second cut could be female or male germ cells differentiated during regeneration, is ruled out by the observation that in neither cephalic nor caudal stumps were gonads rebuilt. In fact, the influence of female and male fields, which according to the 'sexual gradient theory' (Vannini, 1965; 1974) causes the differentiation of totipotent cells into germ cells, is absent in the cephalic and caudal terminal area. It is worth noting that in planarians the sexual field influence seems to be mediated by specific neurosecretory products (Lender, 1964; Grasso & Quaglia, 1970a, b; Grasso, 1974) and in addition in our biotype
it has a macroscopic expression because undifferentiated cells undergo chromosome doubling in the female territory and haploid elimination in the male territory (Benazzi, 1957).

The present karyological data demonstrate that most of the cells which come to form the blastema in our biotype are triploid. These cells could be considered either as embryonic, reserve cells or as somatic dedifferentiated cells. In addition, by taking into account the evidence that cells originally belonging to the female or male germ lines can take part in the regeneration of somatic tissues, we suggest that germ cells can transdifferentiate into another cell type depending on the field influence they meet during regeneration.

Another way of looking at regeneration would be to assume that all germ cells present in areas devoid of gonads do not redifferentiate. However, according to this view, these cells should remain undifferentiated and inactive until a regenerative stimulus causes them to migrate towards areas containing gonads. This way of thinking could, however, lead to unlikely conclusions. In fact, if one assumes that specialized somatic cells too can dedifferentiate in a manner similar to germ cells and redifferentiate only into the original cell type, the dedifferentiated cells present in the blastema would be unipotent rather than pluripotent cells as widely believed. Moreover, this line of reasoning could lead one to conclude that in any region of the planarian body, even a small one, stem cells for every cell type should be present. This hypothesis is not presently supported by any experimental evidence and would contrast the ‘physiological gradient theory’ (Child, 1920; Wolff, Lender & Ziller-Sengel, 1964) according to which somatic or germ cells are not predetermined nor even precociously segregated in planarians.

To conclude, the present karyological data, along with other cytophotometric data (Gremigni, Miceli & Picano, 1979) support the view that primordial germ cells can participate in the regeneration of tissues devoid of gonads by first dedifferentiating at the blastema level, and then transdifferentiating into somatic cells, although a direct proof is as yet not available to substantiate the latter phenomenon.

We wish to thank Dr F. Giorgi for useful discussion and advice. We are also grateful to Mr R. Vaselli for technical help and to Mr I. Kaufman for help in translating the paper. The C.N.R. of Italy supported this work.

REFERENCES


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(Received 30 March 1979, revised 20 June 1979)