Regeneration and pattern formation in planarians

III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells

JAUME BAGUÑÀ¹, EMILI SALÓ¹ and CARME AULADELL²

¹Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain
²Unitat de Biologia Cellular, Departament de Bioquímica i Fisiologia, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

Summary

In most regenerating systems, blastema cells arise by dedifferentiation of functional tissue cells. In planarians, though, it is still debatable whether dedifferentiated cells or a population of undifferentiated cells, the neoblasts, are the main source of blastema cells. Moreover, it is unclear whether in the intact organisms neoblasts are quiescent cells ‘reserved’ for regeneration or if they serve as functional stem cells of all differentiated cell types. Both uncertainties partly stem from the failure to distinguish by conventional labelling methods neoblasts from differentiated cells.

Here we describe a new approach to these problems based on testing the regenerative and stem cell capabilities of purified neoblasts and differentiated cells when introduced, separately, into irradiated hosts. Introduction of neoblasts led to resumed mitotic activity, blastema formation, and extended or complete survival of the host; differentiated cells, in contrast, never did so.

Therefore, planarian neoblasts can be qualified as totipotent stem cells and the main source of blastema cells, while dedifferentiation does not seem to operate either in intact or regenerating organisms. In addition, these results strengthen the idea that different types of regeneration and blastema formation, linked to the tissular complexity of the organisms, are present in the animal kingdom.

Key words: neoblast, planarians, regeneration, blastema, pattern formation, stem cells, totipotency.

Introduction

Although many studies have been published on the mechanisms of blastema formation and differentiation in planarians and the cell types that engage in it or do not (see Brønsted, 1969, and Chandebois, 1976, for general references) a main question remains still unanswered: do blastema cells arise from a stock of undifferentiated cells (neoblasts) already present in the intact organism or by dedifferentiation of differentiated functional cells? In the last 50 years, this issue has been polarized into what is called the ‘neoblast versus dedifferentiation’ controversy, confronting the so-called ‘neoblast theory’ and the ‘dedifferentiation theory’ (Slack, 1980; Baguñà, 1981).

The ‘neoblast theory’ stems from Wolff and Dubois' demonstration that the lack of regeneration after X-irradiation in planarians was caused by the progressive disappearance of neoblasts and lack of cell turnover, and from the observation that an organism with an unirradiated region (either a shielded region or an unirradiated graft) formed a blastema after a delay proportional to the distance between the wound and the healthy unirradiated tissue (Wolff & Dubois, 1948). This suggested that a blastema can be formed by migratory undifferentiated cells (neoblasts) that had migrated throughout the irradiated region from the unirradiated area. Hence, neoblasts were qualified as totipotent and migratory cells capable of forming all the tissues of the regenerate (Dubois, 1949; Leder, 1962; Gabriel, 1970).

The ‘dedifferentiation theory’, mainly based on histological and electron microscopic data (Flickinger, 1964; Woodruff & Burnett, 1965; Hay, 1966; Rose & Shostak, 1968; Coward & Hay, 1972; Chandebois, 1976), suggests that neoblasts do not exist at all, or at least are not involved in regeneration, and that dedifferentiation of specific cells near the wound is the main way of recruiting cells to form the blastema. This is analogous to the situation found in regeneration of vertebrates and higher invertebrates.

As presently stated, both theories are incomplete and open to criticism. The neoblast theory does not exclude the possibility that the migratory neoblasts making the blastema may have resulted from the dedifferentiation of differentiated cells in the unirradiated region before their migration to the wound. Moreover, it is also possible that in normal regeneration differentiated cells near the wound can dedifferentiate and contribute, albeit slightly, to making the blastema. Finally, it has
been shown that the so-called migration of neoblasts is not a true cell migration but the result of the slow and progressive spreading of neoblasts mainly caused by random movements linked to cell proliferation (Saló & Baguña, 1985). Therefore, the idea of neoblasts as totipotent and migratory cells making exclusively the blastema is still unsettled.

The evidence for dedifferentiation in planarian regeneration is also not well founded. Three main criticisms can be raised against it. First, evidence for dedifferentiation of gastrodermal cells to neoblasts claimed using vital dyes as markers (Rose & Shostak, 1968) is hardly acceptable as the markers used diffuse from cell to cell and proper controls were not done. Second, recent EM studies have not found, despite a careful search, any evidence of, or role for, dedifferentiation in the formation of the regenerative blastema in several species of planarians (Bowen et al. 1982; Hori, 1983, 1986; Morita & Best, 1984). Finally, mitotic studies have shown that the early blastema is formed from very early dividing (1–8 h of regeneration) G2 neoblasts, a period too short to make dedifferentiation likely (Saló & Baguña, 1984).

A case for dedifferentiation in planarians was brought up some years ago by Gremigni and co-workers in a very interesting series of experiments (Gremigni & Miceli, 1980; Gremigni et al. 1980, 1982). Using a strain of planarians that are naturally occurring mosaics: the somatic cells are triploids, the male germ diploids, and the female germ hexaploid; they showed that regeneration from a cut surface through the gonadal region gave rise to blastemas and regenerates that contained mainly triploid cells, but also diploid and/or hexaploid cells from which somatic cells (e.g. pharyngeal muscle cells) originated. This suggested that dedifferentiation and transdifferentiation (and hence, metaplasia), however limited, occurred during planarian regeneration.

These results, held as conclusive evidence for dedifferentiation in planarians and, hence, for similar mechanisms of blastema formation in most animal groups (Slack, 1980), can be criticized on the grounds that they do not demonstrate the occurrence of dedifferentiation and metaplasia but, at the most, suggest the existence of dedetermination (or transdetermination) (Baguña, 1981). This is because the loss of a haploid complement during spermiogenesis and its doubling during oogenesis, though one of the first steps from neoblasts to germ cells, is only a small step in cell determination and occurs in undifferentiated cells of the germinative epithelium, which are undistinguishable from somatic neoblasts. Moreover, it is known that differentiating and differentiated germ cells like spermatocytes, spermatids and spermatooza, and its counterparts in the female germ line, degenerate and lyse after transection (Fedecka-Brunner, 1967; Bowen et al. 1982) and, therefore, cannot dedifferentiate to give blastema cells.

Overall, the whole issue is clearly unsettled and needs clarification. A way out from this riddle would be to have clear cell markers to distinguish neoblasts from differentiated cells, making it possible to follow their fates during blastema formation. As neoblasts are the only known mitotic cells in planarians, several attempts have been made to label them with tritiated thymidine to determine whether blastema cells are made of labelled cells (coming from neoblasts) or unlabelled cells (coming from dedifferentiated cells). However, except for the reported incorporation of this precursor into neoblasts in the small turbellarian Microstomum lineare (Palmberg & Reuter, 1983) and Convoluta sp. (Drobyshева, 1986), nobody has succeeded so far in labelling planarian neoblasts with thymidine or other precursor (for a discussion, see Coward et al. 1970). On the other hand, the very few attempts made to label differentiated cells specifically have also been unsuccessful so far (Saló, 1984).

To overcome these difficulties, we describe here a new approach based on purifying, from total cell suspensions, neoblasts and differentiated cells, and testing their regenerative and stem cell capabilities when introduced, separately, into the parenchyma of irradiated hosts which have neither functional neoblasts nor mitotic activity. The rationale of this experimental approach is that if neoblasts are the stem cells of all (or most) differentiated cell types and the main cell type making the blastema, their introduction into a dying nonregenerating irradiated host will lead to its survival and regeneration, whereas introduction of differentiated cells will neither rescue the host nor make it regenerate. Conversely, if dedifferentiation does occur, introduction of differentiated cells will, eventually, result in their dedifferentiation and, therefore, in the rescue of the host and/or in its regeneration.

Whatever the outcome, the results obtained will be of general significance as they would support either the existence in all animal groups of a general mechanism to obtain undifferentiated regenerative cells (e.g. dedifferentiation), or the presence, in different animal groups, of different mechanisms that may be linked to certain structural or functional characteristics.

Materials and methods

Species

Planarians used in this work belong to the fissiparous strain of the species Dugesia(G) tigrina, and to the sexual and asexual races of Dugesia(G) mediterranea (Saló & Baguña, 1985). They were maintained in Petri dishes in the dark at 17 ± 1°C in planarian saline (PS; Saló, 1984) and fed with Tubifex sp. In all experiments, one-week-starved organisms were used and the temperature kept at 17 ± 1°C.

Enrichment of neoblasts and differentiated cells

Enriched fractions of neoblasts and differentiated cells were prepared, from initial total cell suspensions, by serial filtration and Ficoll density gradients, respectively. Suspensions of total cells were obtained from one-week-starved donor organisms, 8–10 mm in length. Briefly, donors were cut into pieces and dissociated by gently pipetting into single cells in a modified Holtfreter solution (MHS; Betchaku, 1970) that had kanamycin sulphate (Sigma, London) at 10 μg ml⁻¹. Cells were pelleted at 300 g for 5 min and resuspended in MHS at 10⁶ cells ml⁻¹, this being considered the total cell fraction. The percentages of neoblasts and differentiated cells were esti-
Neoblasts in planarian regeneration

Neoblasts were enriched following a serial sieving procedure that separates cells by size. 5 ml samples of the total cell fraction were serially filtered through nylon meshes of decreasing pore sizes (180, 50, 20 and 8 μm). Being the smallest cell type (mean diameter, 7.0 ± 2.2 μm; Auladell and Bagufa, unpublished data), neoblast is the main cell type passing through the last filter. Enriched neoblasts were centrifuged at 400 g for 10 min, washed and resuspended in MHS.

Differentiated cells were enriched by Ficoll density gradients (Collet et al. 1984; Salé, 1984). 1 ml samples of total cells (10⁶ cells ml⁻¹) were placed on top of a discontinuous Ficoll density gradient (Ficoll 400, Pharmacia; 10 ml fractions of 3, 6, 9 and 12 % Ficoll in MHS) and spun at 1000 g for 1 h at 4°C. Differentiated cells were mainly recovered at the 3–6% interphase. Cells were collected, washed twice and resuspended in MHS.

The degree of enrichment was determined comparing the percentage of each type of cell between the initial and the enriched fraction. Viability was assessed by the Trypan blue exclusion test. Yield (in percentage) indicates the number of neoblasts and differentiated cells present in the enriched fraction as compared to those present in the initial total cell fraction, all measured with a hemocytometer under phase-contrast microscopy (Bagufa & Romero, 1981).

Host organisms
Organisms to be used as hosts were fully grown (10–12 mm long) adults of the same species kept under identical conditions to donor organisms.

Irradiation
Organisms to be used as irradiated hosts were exposed to 8000 rads (1000 rads min⁻¹) using a HT-100 Philips X-ray machine (1.7 mm Al filter; 100 kV, 8 mA) and kept in PS with kanamycin sulphate.

Number of neoblasts in nonirradiated and irradiated organisms
At different intervals after irradiation, organisms were macerated into single cells, and the percentage of neoblasts to total cells quantified by phase-contrast microscopy (Bagufa & Romero, 1981). As controls, nonirradiated intact and regenerating organisms were macerated and the percentage of neoblasts estimated.

Introduction of donor cells into irradiated hosts.

Experimental procedure
The experimental and control groups and the injection procedure employed are depicted in Figs 1 and 2. From the initial group of intact organisms, 10–12 individuals were kept aside to serve as external nonirradiated controls (a, Fig. 1). The rest (b, Fig. 1; 50 individuals) were irradiated at 8000 rads

Fig. 1. Diagrammatic representation of the experimental procedure employed in this work (for a detailed explanation, see text). a: nonirradiated controls; b: irradiated organisms; b₁: irradiated controls; b₂: organisms to be injected; b₂₁: sham (saline)-injected organisms; b₂₂: cell-injected organisms. Hatched: irradiated (X-rays) organisms. Cross-hatching: host body regions where donor cells were injected.
and, one day after, split in two groups; one (b1, Fig. 1; 10 individuals) to serve as noninjected irradiated controls, and the other (b2, Fig. 1; approx. 40 individuals) to be sham-injected with MHS (b2i, 10 individuals) or to be used as hosts to injected donor cells (b22, 30 individuals; 10 organisms for each cell fraction).

Cell fractions (total cells, enriched neoblasts, and enriched differentiated cells) were introduced, separately, into the parenchyma of irradiated hosts (cross-hatching, Fig. 1) by a microinjection procedure (see below). 2 days after injection, experimental and control groups were transversally cut 1 mm in front and below the injected areas (or at similar levels along the anteroposterior axis in control groups) to stimulate proliferation and regeneration, and kept at 17°C in PS. All groups were monitored for a period of 60 days and the percentage of surviving and regenerating organisms recorded.

Prior to injection, cell fractions were centrifuged for 15 min at 400 g and resuspended in 100 μl of MHS (final cell concentration 5000 cells μl⁻¹). After immobilizing the irradiated host by cold (1 h at 4°C), a triangular piece (1 mm side) was cut out from the postcephalic area of the host and kept aside (Fig. 2).

Using a 80-100 μm (inner diameter) glass micropipette, connected via a polyethylene tubing to a screw-drive-controlled Hamilton microsyringe, 7—8 μl of cell suspension (approx. 35—40×10³ cells in MHS, see below) (b22, Fig. 1) were carefully introduced into the parenchyma of the lateral and rear sides wound area. After the injection, when the needle was withdrawn, the triangular piece was placed again in the postcephalic area of the injected host. Injected organisms were kept, wrapped in cigarette paper and placed on top of saline moistened Whatman no. 1 paper, in Petri dishes in the dark for 12 h at 4°C and for 24 h at 12°C before being transferred to PS at 17°C. As controls, a group of irradiated organisms (b2i, Fig. 1) was injected with 7—8 μl of MHS using an identical protocol as in cell-injected groups. The results presented are the mean of three different experiments.

**Estimates of the number and viability of injected cells**

The actual number and viability of injected cells was estimated by an indirect labelling method. Briefly, donor organisms were fed an artificial food mixture containing fluorescent latex beads (Fluoresbrite carboxylated, Polysciences; green fluorescence, 1-0 μm in diameter) that are incorporated specifically into the cytoplasm of differentiated cells (Saló & Baguña, 1985), mainly gastrodermal and fixed parenchyma cells (Baguña and Romero, unpublished data). One day after feeding, organisms were dissociated into single cells, differentiated cells enriched by ficoll density gradients (see above) and resuspended at 5000 cells μl⁻¹ in MHS. The number of differentiated cells labelled with fluorescent latex beads in a volume identical to the injection volume (7—8 μl, having 35—40×10³ differentiated cells) was estimated by epifluorescence (Leitz Dialux 20). The average value found (~7—400±1—600 labelled cells; that is, one out of every five cells) was taken as a control zero value.

2 h after injecting 7—8 μl of enriched, partially labelled, differentiated cells (~35—40×10³ cells), irradiated hosts were dissociated into single cells and the total number of live labelled cells estimated and compared to control zero values.
Simultaneous measurements were made also at different periods (2, 5, 10, 15, 20 and 40 days) after injection. As controls, identical experiments were made using as hosts intact nonirradiated organisms.

Taking into account that labelled cells represent between 18 and 22% of the total differentiated cells injected, and assuming similar losses between labelled and unlabelled cells during injection and once within the host, the number of labelled cells recovered from the host at different times after injection serves both to estimate the number of cells actually introduced and kept for different periods of time within the host, as well as their viability. Besides, though this labelling methods works only for differentiated cells, it is a reasonable assumption that similar results would apply for total cells and enriched neoblast fractions.

Criteria for mortality, survival and regenerative capabilities in noninjected and injected organisms

Lange & Gilbert (1968) showed that supralethal X-ray doses (8000–10000 rads) given to several species of planarians sterilizes all the neoblasts and leads invariably to the death of the organism within 20–40 days after irradiation in a species-specific time course. The criterion for mortality was that the entire animal be lysed with no piece left intact. Following this criterion, injected planarians are here considered survivors if they are alive and fully functional at 60 days postinjection. The regenerative capability of irradiated injected organisms is considered to be restored when an anterior blastema bearing eyes appears before 20 days after injection and cutting.

Chromosomal marker

To distinguish injected from host cells we have made use of a chromosomal method (a heteromorphic chromosome pair; Baguñá, 1973) between the sexual and asexual races of Dugesia(S)mediterranea (for more details, see Saló & Baguñá, 1985).

Mitotic index

Control and experimental organisms were fixed in 1 N-HCl, stained by a modified Gomori technique and mounted whole (Saló & Baguñá, 1984). Mitotic figures and nuclei were counted, with the aid of an ocular grid divided into 100 squares, in a 2 mm strip of tissue along the anteroposterior (cephalocaudal) axis, above and below the wound (see Fig. 2). The number of mitoses were recorded from 20 of the squares and the mitotic index (number of mitoses/100 nuclei) calculated.

Results

The pattern of mortality in irradiated organisms

After irradiating at 8000 rads, planarian cells do not proliferate, cell renewal slows down and organisms die within 3–5 weeks depending on species, temperature, age and nutritional conditions (Lange, 1968; Chandedois, 1976). At 17°C and in the culture conditions used in this work, mean survival times (period in days when half of the irradiated population is still alive) for Dugesia(G)tigrina and Dugesia(S)mediterranea were 21.8±2.6 (n = 30) and 38.6±5.3 (n = 30) days, respectively. The observed pattern of lysis starts in the cephalic and caudal regions and progresses proximally to the center until the organism vanishes.

As shown by Lange (1988), death of irradiated organisms is due to lack of cell renewal, the latter being a consequence of lack of proliferation and subsequent death of undifferentiated cells (neoblasts). It should be expected, therefore, that the number of neoblasts will decrease after irradiation. The results found in both species (see Fig. 3 for Dugesia(G)tigrina) clearly shows, for both intact and regenerating irradiated and nonirradiated organisms, that the percentage of neoblasts decreases steadily after irradiation to values less than 1% of total cells at two weeks postirradiation. Mitotic figures were never seen in irradiated organisms.

Isolation and partial purification of neoblasts and differentiated cells

Table 1 summarizes the main results and Fig. 4 (A–C) illustrates the appearance of the initial and enriched cell fractions.

Although pure populations of neoblasts and differentiated cells were not obtained, differences in percentage for each class of cells between enriched and initial (total cells) fractions indicates that a considerable enrichment has been achieved, this being particularly relevant for neoblasts (30 to 88%). The viability of enriched cells was found to be high for all fractions, a fact also supported by their appearance under electron microscopy (Auladell & Baguñá, unpublished data). The yield was, however, rather poor for all fractions, mainly for differentiated cells (only 18% of the initial cells being recovered).
Fig. 4. Phase contrast micrographs of initial and enriched fractions from the planarian *Dugesia*(*G*) *tigrina*. (A) total cells (initial cell population); (B) enriched neoblasts; (C) enriched differentiated cells. gc: gastrodermal cells; mc: muscle cells; nb: neoblasts; pc: parenchyma cells. Bar, 20 μm.

Injection of cells into irradiated hosts. Number and survival of injected cells

Two main problems are encountered when trying to introduce cells by injection into the parenchyma of organisms, like planarians, made of a solid mass of tissue: (1) to have a reliable estimate of the actual number of cells introduced; (2) to check if these cells, mainly differentiated cells, survive for long periods once within the host tissues.

To answer both questions, differentiated cells labelled with fluorescent latex beads were used (see Methods). From the number of labelled differentiated cells recovered 2 h after injection, we estimate that 20–24×10^3 cells, out of 35–40×10^3 cells injected, were actually introduced within the host. This amounts to 60–70% of injected cells. To check the long-term viability of injected differentiated cells once within the host, similar measurements were made at different periods after injection. The results (Fig. 5) show that differentiated cells stay alive and maintain their numbers at least up to 20 days after injection.

Table 1. Enrichment, viability and yield of the different cell fractions obtained from *Dugesia*(*G*) *tigrina*

<table>
<thead>
<tr>
<th>Cell types (%) ± s.d.</th>
<th>Viability (%) ± s.d.</th>
<th>Yield (%) ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells</td>
<td>70 ± 5</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Enriched neoblasts</td>
<td>9 ± 4</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Enriched different.</td>
<td>87 ± 7</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

*Numbers (in %) are mean values ± S.D. (n = 4; up to 1000 cells for each measurement). For the sake of clarity average values and standard deviations have been rounded.

† Percentages of differentiated (Differ) and undifferentiated (Undiffer) cells in each fraction were determined by phase-contrast microscopy according to the morphological criteria set in Baguñà and Romero (1981).

†† Viability as assessed by the trypan blue exclusion test.

‡‡ Yield (in %) indicates the number of neoblasts and differentiated cells present in the enriched fractions are compared to its number in the initial total cell fraction, both measured with a hemocytometer under phase-contrast microscopy.

Fig. 5. Number of fluorescent-labelled differentiated cells recovered from irradiated hosts of *Dugesia*(*G*) *tigrina* at several periods after injection. The results are means ± S.D. (n = 3; 5–6 individuals for each experiment). For more details, see text.
Neoblasts in planarian regeneration

Table 2. Survival and regenerative performances of control and injected groups of Dugesia(G)tigrina

<table>
<thead>
<tr>
<th>Group*</th>
<th>Symbol*</th>
<th>Number of organisms</th>
<th>Mean survival time† (days ± s.d.)</th>
<th>Survivorstt (60 days)</th>
<th>MIt‡ (×10²)</th>
<th>Regeneration‡‡ (20 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (non irradiated)</td>
<td>a</td>
<td>45</td>
<td>22.4 ± 3.2</td>
<td>45</td>
<td>7.8 ± 2.2</td>
<td>+ (45)</td>
</tr>
<tr>
<td>Control (irradiated)</td>
<td>b₁</td>
<td>41</td>
<td>21.6 ± 4.5</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Injected (sham-injected)</td>
<td>b₂₁</td>
<td>30</td>
<td>32.6 ± 4.6</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>Injected (total cells)</td>
<td>b₂₂</td>
<td>34</td>
<td>24.2 ± 2.1</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Injected (differ. cells)</td>
<td>b₂₂</td>
<td>35</td>
<td>34.7 ± 3.8</td>
<td>6</td>
<td>2.0 ± 0.4</td>
<td>+ (6)</td>
</tr>
<tr>
<td>Injected (neoblasts)</td>
<td>b₂₂</td>
<td>32</td>
<td>32-6 ± 4.6</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

*See legend of Fig. 1.
†Mean survival time is the period (in days) when half of the irradiated population is still alive.
‡‡Survival at 60 days, taken as a sign of complete recovery, indicates the number of organisms staying alive and fully functional 60 days after irradiation.
‡Mitotic index (MI; number of metaphases/100 cells), determined 12 days after injection using standard procedures (see Methods).
‡‡Regeneration at 20 days indicates the presence (+) or absence (−) of blastemal structures 20 days after cutting; in parentheses, number of positive individuals.

organisms. Total cell fractions, where neoblasts represent 30% of total cells, increased by 10 days the mean survival time, though all organisms finally die. In contrast, enriched neoblast fractions, where these cells represent almost 90% of the cell population, increased the mean survival time by 12 days and led in 20% of the organisms (6 out of 32) to complete recovery of the irradiated hosts.

The regenerative capacity of injected hosts parallels the results found for survival (Table 2). Like control and sham-injected irradiated organisms that do not regenerate, organisms injected with differentiated cells neither regenerate nor build a blastema. Instead, those injected with total cells gave very small transient blastemata that regressed 8–10 days after injection, whereas most organisms injected with enriched neoblasts gave blastemata of almost normal size, some bearing new eyes, appearing at later periods (c. 12–15 days after cutting) than in control nonirradiated groups (c. 4–6 days after cutting).

As these results suggested that survival and regenerative capacity of irradiated hosts relies on the actual number of neoblasts introduced and since these cells are known to be the only cell type in planarians endowed with mitotic capacity, it should be expected that differential survival and regenerative capacity stems from different rates of cell proliferation. Mitotic analyses in injected hosts showed, accordingly, that only those injected with total cells and neoblasts had significant levels of mitoses. Instead, hosts injected with differentiated cells never had any mitotic figure (Table 2).

Evidence that neoblasts are the stem cells of all differentiated cells in planarians

To establish that donor neoblasts, and not revitalized host cells (either neoblasts or differentiated cells), lead to increased or complete survival of the host, injection of enriched neoblasts into irradiated hosts between the sexual and asexual races of Dugesia(S)mediterranea, which differ in a chromosomal marker (Saló & Baguñà, 1985), were performed following an identical experimental procedure (see Figs 1 & 2). This experiment also had an important additional interest: to ascertain if injected neoblasts from one race may replace all the differentiated cells of the host and, hence, 'transform' one race into another and vice versa. If this were so, neoblasts could unambiguously qualify as the stem cells of all differentiated cell types in planarians.

The results found showed, in all combinations tested, that mitotic figures within the host belong always to donor cells and not to revitalized host cells (data not shown). These results agree with earlier reports, using tissue grafting procedures that had pigmentation, ploidy or chromosomal differences as cytological markers (see Saló, 1984; and Saló & Baguñà, 1985, for general references), in ruling out a possible revitalization of host cells either by the injection procedure or by the injected cells.

As expected, injection of neoblasts from the asexual race of Dugesia(S)mediterranea to irradiated hosts of the sexual race rescued the host and transformed it into an asexual individual able to reproduce by fission but unable to reproduce sexually. Conversely, the introduction of neoblasts from the sexual race into irradiated asexual hosts transformed the latter into individuals unable to reproduce asexually and capable, after developing germ cells and the copulatory complex, to mate and lay cocoons (Saló, 1984; data not shown).

Discussion

We have analyzed the capabilities of neoblasts and differentiated cells to give blastema cells during regeneration and to be the stem cells of all differentiated cell types during the daily cell renewal in intact organisms. The analysis has been based on the differential survival and regenerative capacity of irradiated hosts when injected with different cell fractions. Our main conclusion is that the ease of recovery and regeneration of irradiated hosts is proportional to the number of neoblasts introduced and that, at least under the experimental conditions employed, differentiated cells are not able to rescue the host or make it regenerate. Taking into account that neoblasts are the only planar-
ian cell type known to divide (Saló & Baguñà, 1984; Morita & Best, 1984), these results strongly suggest that these cells are totipotent (or at least pluripotent) stem cells in the intact organism and the main source of blastema cells in regenerating organisms. In turn, these results argue against the role of cell dedifferentiation as a means to recruit undifferentiated cells both in intact and regenerating planarians.

Of the fractions tested, enriched neoblasts and total cells were the only ones giving increased survival times, resumed mitotic activity and, in the former, complete survival. However, it is at first surprising that neoblast injection gave a low percentage of total rescue, the percentage falling to zero when total cells were injected. These results could be understood if the number of neoblasts actually introduced is considered. Assuming that $25 \times 10^3$ donor cells are introduced at each injection (Fig. 5), it follows that $18-22 \times 10^3$ and $7-8 \times 10^3$ neoblasts are introduced, respectively, from enriched neoblasts and total cell fractions. If the area where cells are injected is estimated to span 2 mm in length along the anteroposterior axis (see Fig. 1), the number of neoblasts introduced is 3-4 times (neoblast fraction) or 8-10 times (total cell fraction) lower than the number present in a similar area of a control nonirradiated organism ($\sim 35 \times 10^3$ neoblasts mm$^{-1}$ in length in an 11 mm long organism; Baguñà & Romero, 1981; Romero, 1987). Interestingly, the ratio of the number of neoblasts in experimental and controls is fairly close to the ratio calculated, from the data of Table 2, for their respective mitotic indices. This supports again the correlation found between neoblast density and mitotic activity and suggests that only fairly high rates of mitotic activity (as seen after injection of enriched neoblasts) can produce rates of cell replacement compatible with extended and complete survival. An additional test backing this proposal, based on increasing the number of neoblasts injected from total cells and neoblast fractions by increasing the volume of cell suspension injected, failed because planarian parenchyma, being a solid mass of cells with little intercellular spaces, did not take up more cells (unpublished data).

A similar argument could apply to explain both the presence of permanent blastemal structures only in those hosts injected with enriched neoblasts as well as their delayed appearance when compared to regenerating nonirradiated controls (15-20 days vs 6-8 days). Since rates of blastema formation and differentiation are closely correlated with rates of mitotic activity (Saló & Baguñà, 1984, 1988), it may be expected that only mitotic rates close to (though lower than) controls, as seen after neoblast injection (Table 2), guarantee a supply of neoblasts compatible with blastema formation. However, their numbers will not be enough to keep pace with normal (control) regenerative rates; hence, the delayed appearance of differentiated structures in the blastemata.

Which is the main role of neoblasts in planarians?
The 'transformation' of sexual to asexual races, and vice versa, in Dugesia(S) mediterranea via introduction of neoblasts from one race into irradiated hosts of the other demonstrates the main role of neoblasts in planarians: namely as the stem cells of all (or most) differentiated cell types in the intact organisms. This 'transformation' can be envisaged as due to the slow but continuous replacement of host neoblasts and differentiated cells, unable to divide, by nonirradiated donor neoblasts capable of division and differentiation. This process will last until no host cells are left and all neoblasts and differentiated cells are of donor genotype. In other words, injected neoblasts would use the irradiated host as a sort of three-dimensional 'feeder-layer' where slowly turning over irradiated host cells are replaced by proliferating neoblasts that probably use host positional cues and signals to differentiate.

It must be pointed out, however, that earlier studies using grafts of nonirradiated donor tissue pieces into whole irradiated hosts had already showed the 'transformation' of hosts into donors (Lender & Gabriel, 1965; Teshirogi, 1976; Chandebois, 1976), this being interpreted as due to the slow and progressive replacement of host cells by proliferating and differentiating donor neoblasts. In these experiments, however, the possibility that dedifferentiated graft cells and not neoblasts were the actual source of new cells could not be ruled out. The results presented here make this possibility very unlikely, suggesting in turn that planarian neoblasts are true totipotent stem cells.

Cell dedifferentiation in planarians: does it still have a role?
Functionality of injected differentiated cells within the host shown by fluorescent latex beads (Fig. 5), lack of revitalization of host differentiated cells shown using chromosomal and ploidy markers, jointly with the lack of recovery, mitotic activity and regenerative capacity of irradiated organisms injected with enriched differentiated cells (Table 2), and the criticisms raised in the Introduction, makes it very unlikely that cell dedifferentiation plays a substantial role either in intact or regenerating planarians. This is in line with recent data on thymidine incorporation in other Turbellaria (acoela, Drobysheva, 1986; polyclads, Drobysheva, 1988; rhabdocoela, Palmberg & Reuter, 1983) and Cestoda (Wikgren et al. 1971) where intact and regenerating organisms show specific incorporation of undifferentiated cells (neoblasts) whereas there are no signs of dedifferentiation.

Before cell dedifferentiation in planarians can be ruled out, however, a last argument for it can be considered. This is to think of blastema and postblasta cells appearing after neoblast injection as arising by dedifferentiation of differentiated cells produced from injected neoblasts and not directly from the latter. This argument can also be extended to intact organisms if neoblasts are considered as transient proliferating cells arising continuously by dedifferentiation from some differentiated cell types (e.g. gastrodermal cells). Although this is the strongest argument we can think of against our interpretation, as a clear answer cannot be
presently given, we consider it unlikely mainly because injected differentiated cells, despite being fully functional, never produced any undifferentiated mitotic cells.

We are fully aware, however, that a definite proof of the exclusive (or even a main) role of neoblasts in planarians must be based on marking differentially, and permanently, neoblasts and differentiated cells and tracing, in both intact and regenerating organisms, the lineage of these cells. Since freshwater triclads seem to be impervious to exogenous labelled DNA precursors, other labelling methods such as neoblast-specific monoclonal antibodies and introduction of gene markers into neoblasts by retrovirus infection (see Price, 1987, for general references) are presently being tried (Romero, Burgaya, Bueno, Sumoy & Baguña, work in progress).

**General implications**

Our results also have two interesting afterthoughts. First, they argue strongly against the long-standing tradition of considering planarian neoblasts as cells mainly 'reserved' for regeneration (Wolff, 1962; Slack, 1980). Since most planarian species reproduce sexually and very rarely regenerate in nature, it is hardly understandable, either on economic or evolutionary grounds, to maintain a population of undifferentiated 'reserve cells', amounting to 25–30% of total cells (Baguña & Romero, 1981), unless they serve a more important function in the adult: to be the stem cell of all (or most) differentiated cell types (Baguña, 1981; Lange, 1983), as has also been demonstrated in other groups of Turbellarians (Palmberg & Reuter, 1983; Drobyshova, 1986, 1988) and Cestoda (Wikgren et al. 1971). Besides, in most animal groups the so-called 'reserve cells' have been found to be either nonexistent (witness the so-called 'neoblasts' in several groups of Annelida, mainly Polychaetae; Hill, 1970) or to be the source of several (e.g. the interstitial cells in hydra; David & Gierer, 1974) or unique (e.g. satellite cells of muscle cells in Vertebrata; Cameron et al. 1986) differentiated cell types during daily tissue renewal.

Second, and to us more importantly, our results seem to contradict the recent trend to extrapolate to all animal groups, including planarians, the phenomenon of cell dedifferentiation as a unique mechanism to recruit undifferentiated blastema cells (Slack, 1980). In our view, this trend results from overlooking the present diversity of modalities of regeneration (witness the morphallactic processes in some lower groups like Coelenterata (Cummings & Bode, 1984) and lower Turbellarians (Palmer & Reuter, 1983), as well as the particular role of some specific cell types, like satellite cells, as a source of regenerative myoblasts in some Vertebrates (Cameron et al. 1986)) and the possible relationship between the actual mechanism of regeneration and the tissular complexity of the species. Indeed, it has been argued (Baguña, 1981) that the planarian neoblast system, based on a unique and totipotential self-renewing stem cell present everywhere, though appropriate to the low level tissular complexity of planarians, is inadequate for epimorphic regenerating organisms like Insecta or Amphibia with static tissues wholly made of non-renewing terminal differentiated cells and renewing tissues with different determined stem cells placed in different body regions. Therefore, the dedifferentiation process, necessary for the latter, is not necessary for organisms like planarians (as well as for hydram) in a total and continuous state of rapid cell turnover (Baguña & Romero, 1981; Lange, 1983; Romero, 1987).

Although the present day modalities of regeneration (morphallaxis, epimorphosis, and mixed types combining aspects of both) and the diverse mechanisms to recruit undifferentiated blastema cells may be thought to be connected to the structural complexity (coarsely measured in number of cell types and their organization into definite tissues and organs; Kauffman, 1969) and the phyyletic position of these animal groups (Field et al. 1988) via the extant mechanisms of cell renewal in the adult, it is nonetheless highly probable that under these formally diverse mechanisms a general, unique, modality of pattern restoration during regeneration, based mainly on short-range cell–cell interactions and where cell origins will be less important, will be uncovered.

We would like to thank Professor Peter Lawrence and two anonymous referees for improving the first draft of the manuscript. This work was supported by grants from the University of Barcelona and Comisión Asesora de Investigación Científica y Técnica (CAICYT) to JB.

**References**


Cummings, S. G. & Bode, H. R. (1984). Head regeneration and
polarity reversal in Hydra attenuata can occur in absence of DNA synthesis. Wilhelm Roux's Arch. devl Biol. 194, 79–86.


(accepted 9 June 1989)