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Regeneration and pattern formation in planarians

I. The pattern of mitosis in anterior and posterior regeneration in *Dugesia (G) tigrina*, and a new proposal for blastema formation

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SUMMARY

Mitotic activity during regeneration in the planarian *Dugesia (G) tigrina* shows a biphasic pattern, with a first maximum at 4–12 h, a second and higher maximum at 2–4 days, and a relative minimum in between. The first peak is mainly due to pre-existing G2 cells entering mitosis shortly after cutting, whereas the second maximum is due to cells that divide after going through the S period from the onset of regeneration.

From a spatial point of view, the highest mitotic values are found in stump (postblastema) regions near the wound (0–300 μm), though regions far from it also show increased mitotic values but always lower overall values. As regeneration continues the postblastema maximum shifts slightly to more proximal regions. In contrast, no mitosis has been found within the blastema, even though the number of blastema cells increases steadily during regeneration. These results suggest that blastema in planarians forms through an early accumulation of undifferentiated cells at the wound boundary, and grows by the continuous local migration of new undifferentiated cells from the stump to the base of blastema.

The results obtained demonstrate that blastema formation in planarians occurs through mechanisms somewhat different to those shown to occur in the classical epimorphic models of regeneration (Annelida, Insecta, Amphibia), and suggest that planarian regeneration could represent an intermediate stage between morphallactic and epimorphic modalities of regeneration.

INTRODUCTION

Planarian regeneration has been classically described in terms of an early process of epimorphosis leading to blastema formation, and a later, and ill-defined, process of morphallaxis that results in pattern restitution through a sequential anteroposterior chain of inductions and inhibitions (Wolff, Lender & Ziller-Sengel, 1964). One of the main concerns about these processes has been to determine the temporal and spatial pattern of mitotic activity and to correlate this pattern with cellular activities taking place during blastema formation and the later stages of regeneration (e.g. cell migration, cell differentiation and dedifferentiation, pattern formation...).

In the planarian *Dugesia (S) mediterranea*, data on temporal pattern of mitosis in regions near the wound area show a first mitotic maximum during the very first hours (4–12 h) of regeneration, a second and higher maximum at 2–4 days, and

a relative minimum in between (Baguñà, 1976*b*). Other authors, studying different species, found similar patterns except that they failed to detect the first mitotic maximum (Best, Hand & Rosenvold, 1968; Coward, Hirsh & Taylor, 1970; Gabriel, 1970). The finding of a high and sudden burst of mitosis shortly after cutting in *Dugesia (S) mediterranea*, prompted us to suggest that the barely visible 1-day blastema was the result of a local proliferation of pre-existing G2 cells (Baguñà, 1976*b*). On the other hand, studies on mitotic activity in regions far from the wound gave conflicting results. Some authors (Coward *et al.* 1970; Gabriel, 1970) found increasing mitotic activity from 24 h on, suggesting that this was due to a kind of compensatory mechanism to replenish cells which had migrated to the wound to form the blastema. Instead, other authors found no increase (Lindh, 1957*a*) or a lesser increase (Baguñà, 1976*b*) from the very first hours of regeneration.

However, the most important problem linking mitotic activity and pattern formation in planarian regeneration refers to mitotic distribution along the anteroposterior (cephalocaudal) axis and to their changes through time. Two main problems arise: 1) does blastema growth occur mainly through cell proliferation?; 2) how do the changes in mitotic distribution in blastema and postblastema regions relate to pattern re-specification?

The data obtained so far are highly variable. As regards to mitotic activity in the blastema and its relationship with blastema growth, some authors found mitotic values in blastema which were similar to the stump region (Best *et al.* 1968), or lower levels (Coward *et al.* 1970), or no mitosis at all (Chandebois, 1976). If mitosis in the blastema does occur, it is important to know if new cells produced by mitosis are enough to explain blastema growth. If mitosis does not occur, then does the blastema form and grow by 1) cell proliferation of pre-existing undifferentiated cells (neoblasts) near the wound followed by local cell migration; or 2) local cell dedifferentiation and migration.

As regards the changes in the spatial pattern of mitotic activity in stump regions, Coward *et al.* (1970) reported maximal mitotic activity in regions far from the wound in the early hours of regeneration, shifting later to regions near it (blastema and postblastema). Instead, Lindh (1957*a*) and Baguñà (1976*b*) found a first and rapid mitotic maximum in regions close to the wound, shifting later to regions far from it.

It is evident that the whole issue needs clarification. So, the purpose of the present study was to examine the spatial and temporal patterns of mitotic activity in anterior and posterior regenerating *Dugesia (G) tigrina*, and compare them to data found by others in different species. Special care was taken to measure mitotic activity in the blastema. Also, a detailed study of mitotic dynamics in regions near and far from the wound was undertaken to assess the extent of mitotic activity and its possible relationship to pattern re-specification. Finally, several techniques were used to ascertain the existence of a population of G2 cells and determine its role during regeneration.

The results obtained show that mitotic dynamics during regeneration in *Dugesia (G) tigrina* are similar to the published data for *Dugesia (S) mediterranea* (Baguñà, 1976*b*; Saló, 1978); that a population of G2 exists in the intact organism which quickly divide to give the first mitotic maximum; that the blastema does not have mitotic activity; that in postblastema regions the maximum in mitotic activity shifts slightly from regions near the wound to regions further away from it as regeneration progresses; and that a substantial increase in mitotic activity in regions far from the wound occurs from the very beginning of regeneration. In subsequent papers (Saló & Baguñà, in preparation), blastema kinetics, evidence against extensive cell migration during regeneration, the origin of blastema cells, and models of pattern formation will be discussed.

MATERIALS AND METHODS

The planarians used in this work belong to the species *Dugesia (G) tigrina* (Girard) collected near Barcelona (Spain). They were reared in Petri dishes with planarian saline and kept at 17 °C in the dark. The animals were fed weekly on raw beef liver and the water changed. In all experiments one-week starved organisms were used.

Terminology

To assist the reader, Fig. 1A shows a diagram of an intact planarian with the main axes, and the body levels where, as mentioned in the text, cuts were made.

Figure 1B shows the main body regions in regenerating individuals. By 'blastema' we mean the small, unpigmented mound of tissue made of small undifferentiated cells, which forms and grows above the wound (*w*). Stump refers to the pigmented old body below the wound, and operationally it is convenient to consider it as made by a small region near the wound (postblastema, *pb*), where most mitotic activity occurs, and a larger region that covers the rest (*r*) of the regenerant.

Counting of nuclei and mitotic cells

To count mitotic figures and interphasic nuclei the Gomori technique, slightly modified, was used. Animals, fixed in Carnoy for 15 min were placed for 2 min in 1 N HCl at 20 °C and then in 1 N HCl at 60 °C for 6 min. They were then stained in acetic orcein for 5–15 min (depending on body size) at room temperature, differentiated in 5 % acetic acid for 20 min and mounted *in toto* (Baguñà, 1974). To make a semipermanent preparation (lasting up to 1–2 months at 4–6 °C) they could be mounted in 25 % glycerol and sealed with nail varnish. Using this technique, nuclei and mitotic figures are easily seen and counted.

Mitotic index in intact organisms

Intact organisms of 5 and 8 mm in length were used. For each group, five

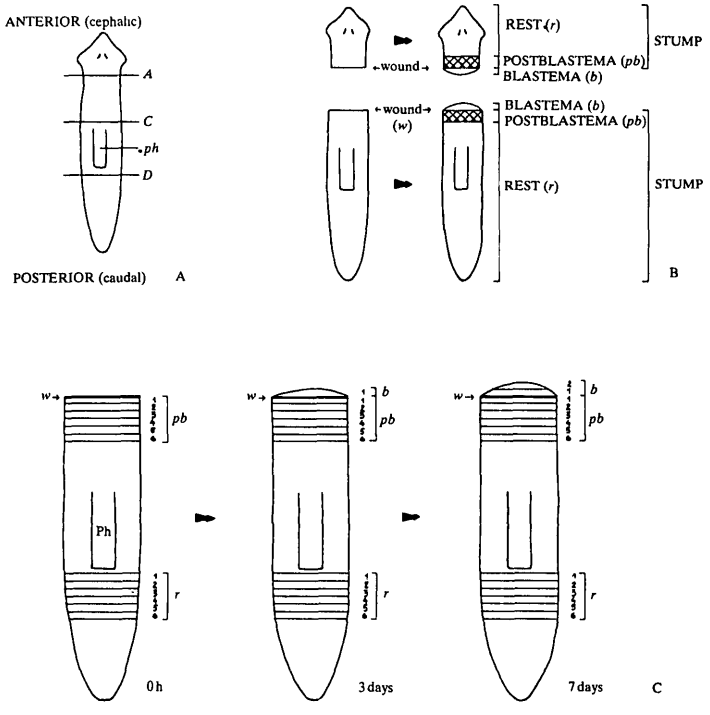

Fig. 1. (A) Diagrammatic dorsal view of *Dugesia (G) tigrina*, and body levels (*A*, *C* and *D*) where cuts were made. *ph*: pharynx. (B) Diagrammatic representation of different body regions in anterior and posterior regenerating *Dugesia (G) tigrina*. (C) Diagrammatic representation of the successive transverse anteroposterior strips of tissue in blastema (*b1*, *b2*), post-blastema (*pb*) (1,. . ,6) and regions far from the wound (*r*) (1,. . ,6), where mitosis were counted along the process of regeneration. *w*: wound; *ph*: pharynx.

animals were used, each result being the mean of three different experiments. Mitotic figures and nuclei were counted along the anteroposterior axis with the aid of an ocular grid divided into 100 squares. Nuclei and mitotic figures were recorded in 20 % of the squares (one every five lines of transversel squares along the anteroposterior axis). To allow for animals of different lengths, the anteroposterior axis of the animal was divided into tenths, and the mitotic index (number of mitosis/100 nuclei) calculated for each 10 % interval.

Mitotic index in regenerating organisms

Animals of 5, 8, and 11 mm in length, cut at the postcephalic (*A*), prepharyngeal (*C*), and postpharyngeal (*D*) levels (Fig. 1A), were used. To analyse the changes in mitotic index in blastema and postblastema, the mitotic index was measured in six successive transversal strips along the anteroposterior axis (Fig. 1C, strips 1 to 6) in both anterior and posterior regeneration. To allow for organisms of different length, the strips of 5, 8 and 11 mm long animals had lengths of 70, 110 and 150 μm respectively. Since at 1–2 days of regeneration the

blastema is easily distinguishable, mitoses occurring inside it were counted separately and the mitotic index measured (strips b1 and b2, Fig. 1C).

Mitotic changes in regions far from the wound serve as an internal control in regenerating organisms. Therefore, we measured the mitotic index in six successive strips of postpharyngeal and caudal regions of anteriorly regenerating organisms cut at levels *A* and *C*, and at level *D*, respectively (Fig. 1C). Equivalent regions were used to measure mitotic changes in regions far from the wound in posteriorly regenerating organisms. As a general control, the number of mitoses in strips of the same length of similar body regions of intact organisms was measured. Mitotic counts in regenerating organisms were made at 1, 2, 4, 8, 12 and 24 h, and at 2, 3, 5, 7, 9 and 14 days of regeneration. For each group, five animals were used, each result being the mean of three different experiments.

DNA determination in different cell types

Determination of the amount of DNA per nucleus was performed using Feulgen staining and quantitative absorption measurements (Leitz MPV1 microspectrophotometer). Animals were macerated in a solution of methanol: glacial acetic acid: glycerol: distilled water (2: 1: 1: 12) (Baguñà, 1976*a*; Baguñà & Romero, 1981), and dissociated cells mounted on slides. Cells were treated for 10 min in 5 N HCl at 37 °C, rinsed briefly in distilled water, stained for 40 min with Schiff's reagent, washed in sodium bisulphite solution and mounted with DPX.

Neoblasts and four differentiated cell types (nerve, epidermal, gastrodermal and fixed parenchyma cells) were identified in phase contrast due to its peculiar morphology (Baguñà & Romero, 1981) and their DNA measured. DNA values are expressed in arbitrary units.

It should be pointed out that, as in most european *Dugesia (G) tigrina* studied so far, the specimens used in this study are mosaics of diploid ($2n = 16$) and triploid ($3n = 24$) cells (approx. 50 % belonging to each class; Collet & Saló, unpublished data). Therefore, S (3C) and G2 (4C) diploid cells and G1 (3C) and S (4C–5C) triploid cells are indistinguishable, an important point when trying to calculate the percentage of cells in each of the different periods of the cell cycle. Nevertheless, since triploid cells in G2 (6C cells) or in S (5C cells) can easily be distinguished from diploid G2 cells (4C) and from cells with lesser DNA content, a fair estimation of cells in G2 can be made.

Hydroxyurea (HU) experiments

Three types of experiments were done. In the first (HU-1), intact animals were placed in 2×10^{-2} M solution of hydroxyurea (HU) and their mitotic index measured after 1, 2, 3, 4 and 5 days of exposure. As controls, the mitotic index of untreated intact animals was measured. In a second experiment (HU-2), groups of animals were exposed 15 h before cutting and from then onwards to HU (2×10^{-2} M) in sterile saline. Mitotic index was measured at 0, 4, 8, 24, 48,

72 and 96 h after cutting and compared to mitotic indexes of regenerating untreated animals. In the third class of experiment (HU-3), groups of animals were exposed to HU (2×10^{-2} M) for different periods of time (1, 2, 3, 4 and 5 days) before cutting. After being cut they were kept in the same HU solution and their mitotic index measured at 0 and 8 h of regeneration. The values obtained were compared to the ones obtained in untreated organism at 0 and 8 h of regeneration. For each group, five animals were used, each result being the mean of three different experiments.

This set of experiments was done to ascertain: 1) if a population of G2 cells do exist in the intact organism ready to enter into mitosis after cutting; and 2) if the second mitotic maximum seen during regeneration results from a late entry of pre-existing G2 cells into mitosis or is due to G1 cells that go through the S-period after cutting. Hydroxyurea, which kill cells at the S phase of the cell cycle, should preclude any mitotic increase due to cells that must pass through the S-period to divide. So, if despite a prolonged exposure to HU, a mitotic increase is seen after cutting, a population of G2 cells may still be present within the intact organism.

RESULTS

Mitosis in intact organisms

The anteroposterior mitotic index in each 10 % interval in length in fed and unfed (1-week starved) animals is shown in Fig. 2. The intact unfed organism has, excluding the small region anterior to the eyes and the pharynx, a continuous population of proliferating cells scattered uniformly throughout the parenchyma; so, there is no 'growth zone' in any region of the organism. Fed animals

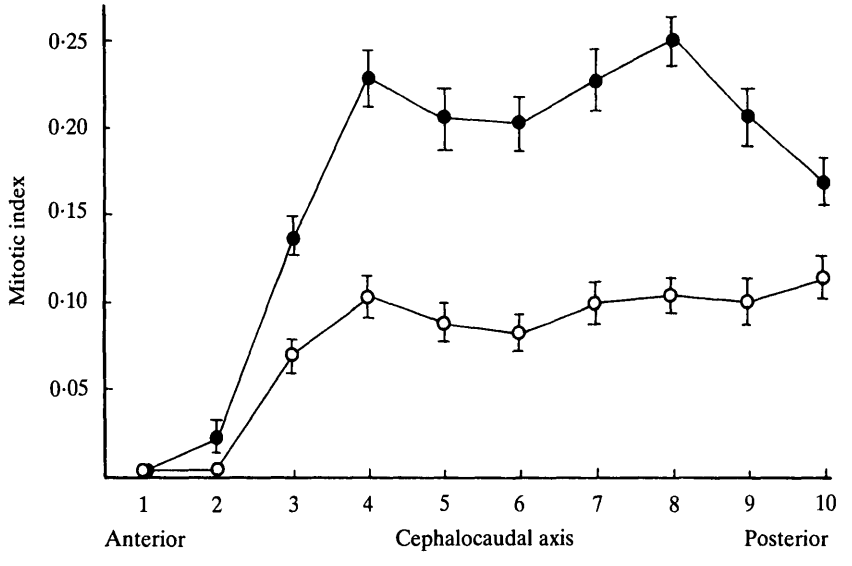

Fig. 2. Mitotic index in 10 % intervals in length along the anteroposterior (cephalocaudal) axis in fed (●) and unfed (○) intact organisms.

show a sustained increase in mitotic index for several days, though the antero-posterior distribution remains unchanged when compared to unfed control animals. This agrees with the reported increase in mitosis seen after feeding in *Dugesia (S) mediterranea* (Baguñà, 1974, 1976*a*; Baguñà & Romero, 1981) and *Dugesia (G) dorotocephala* (Betchaku, 1975). Moreover, the mitotic increase seen after feeding is very rapid; this suggests in turn that a substantial proportion of neoblasts in the intact worm must be in the G2 phase of the cell cycle ready to enter mitosis. This result is substantiated by studies of neoblasts DNA content determination (Betchaku, 1975; this work, Results).

Temporal mitotic dynamics during anterior and posterior regeneration

a) Blastema (b)

The blastema appears at 1 day of regeneration as a thin strand of unpigmented tissue above the wound. From then on, it grows in size and cell number following a sigmoidal pattern (Saló, 1984). If cell proliferation was the cause of this growth, mitosis should be easy to detect. However, despite a careful search, we could not find any mitotic figures in the regenerating area. Occasionally some mitotic figures appeared at the blastema/stump boundary, but could not be assigned unequivocally to either area.

We must conclude that the blastema does not have any mitotic activity during planarian regeneration, and that its growth should be explained by mechanisms other than cell proliferation.

b) Stump regions near the wound (postblastema, pb)

The mitotic index values for postblastema of anterior regenerating organisms of different sizes cut at body levels *A*, *C* and *D* are shown in Fig. 3A. The values shown are the mean of the values for the six successive strips below the wound (see Fig. 1C), which span a length of 400 μm to 900 μm depending on body size.

The overall mitotic curve found is bimodal with a first and low maximum at 4–8 h, a relative minimum around 1 day, and a second, higher, and long-lasting maximum at 2–3 days of regeneration. Some differences depend on body length and body level. Of particular interest is the higher mitotic density found per *A*-cut 5 mm-long organisms as compared to *D*-cut 5 mm animals ($\varepsilon = 0{\cdot}01$) and the opposite result found for similar body levels in 11 mm-long animals ($\varepsilon = 0{\cdot}001$). As expected, this correlates with the axial gradient of regeneration rate for small *Dugesia (G) tigrina* and its reversal for 11 mm-long organisms (Saló, 1978). For posteriorly regenerating organisms the temporal pattern of mitosis is similar to the one found for anterior regeneration.

In both anterior and posterior regeneration, the mitotic increase giving rise to the first mitotic maximum is already seen, as a burst of prophases, at 1–2 h of regeneration. This suggests, as was first pointed out for regenerating *Dugesia (S) mediterranea* (Baguñà, 1976*b*), that a substantial proportion of neoblasts should be in the G2 period ready to enter in mitosis.

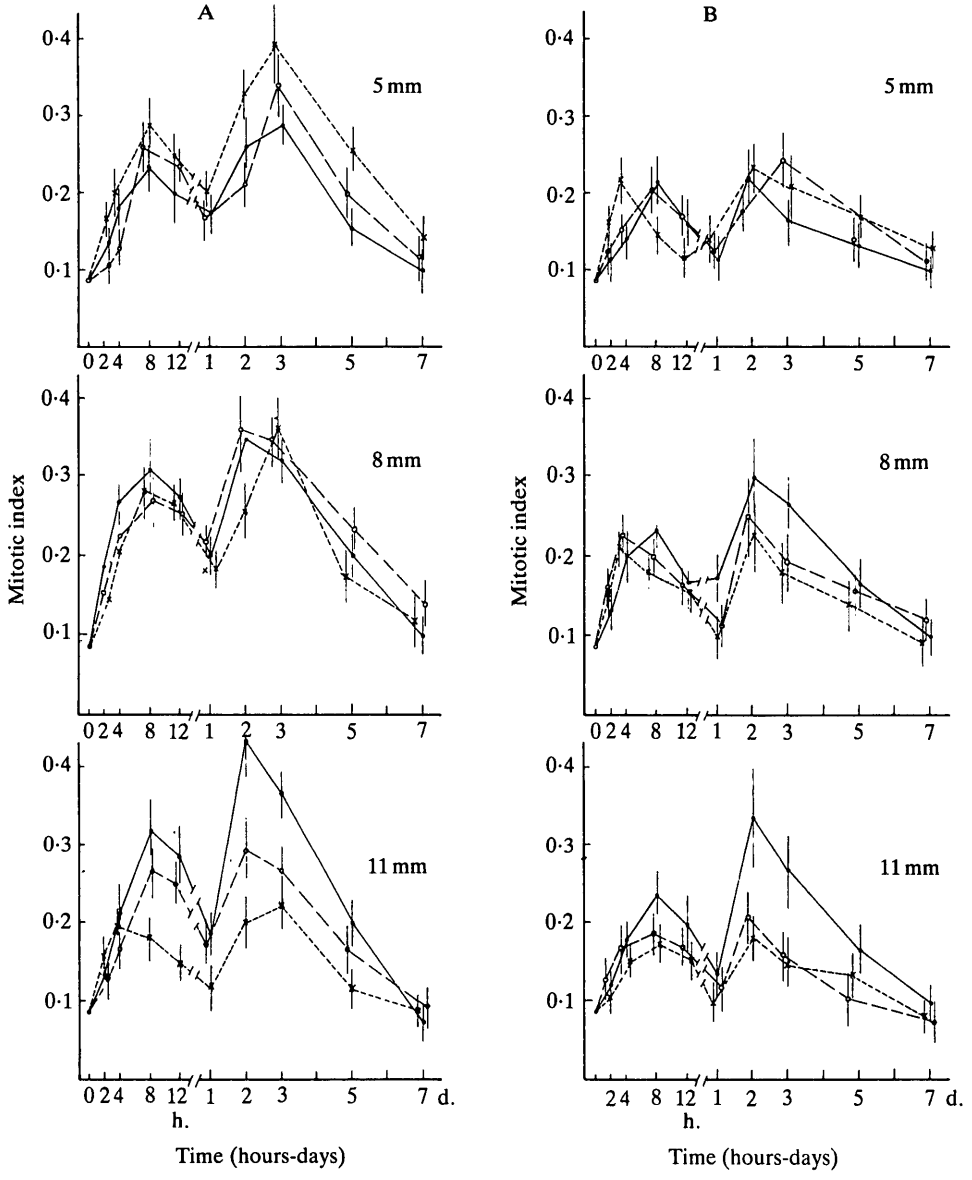

Fig. 3. Temporal mitotic index values in (A) blastema (*b*)/post-blastema (*pb*) areas, and (B) regions far from the wound (rest, *r*) of anteriorly regenerating 5, 8 and 11 mm long organisms cut at body levels *A* (×----×), *C* (○– –○), and *D* (●——●). Ordinate: mitotic index; Abscissa: hours and days of regeneration.

c) Stump regions far from the wound (r)

The mitotic index found for stump regions far from the wound for anterior regenerating organisms of different body size cut at levels *A*, *C* and *D*, are shown in Fig. 3B. The mitotic response is similar to the one found for postblastema regions. The curve obtained is bimodal with a first maximum at 4–8 h, a second maximum at 2–3 days, and a relative minimum around 1 day of regeneration.

However, the response, mainly the second maximum, is lower than the one found behind the wound region. For posteriorly regenerating animals, the results obtained (not shown) reveal a similar pattern to anterior regeneration.

On the other hand, the mitotic response is very rapid and suggests that, as in the postblastemal regions, the first mitotic maximum results mainly from the division of cells in the G2 period of the cell cycle.

Overall, these results suggest that the mitotic response in regenerating *Dugesia (G) tigrina* is not a local phenomenon restricted to regions near the wound but a general response occurring throughout the body. This may indicate the existence of an intercellular communication mechanism very sensitive to traumatic stimuli.

Spatial mitotic dynamics during anterior and posterior regeneration, and a mechanism of blastema formation

Figure 4 shows the mitotic index values for each of the six transverse strips along the anteroposterior axis near the wound area of anteriorly regenerating animals. For the sake of clarity only data from anterior regenerating 5 mm long animals are shown, other body lengths giving similar results.

For each body level, and in both anterior and posterior regeneration, the maxima in mitotic activity during the early phases of regeneration occur in regions close the wound. Later, the maxima shift away from the wound area slightly. As already stated, as soon as blastema becomes visible (e.g. at 1–2 days of regeneration), no mitotic activity could be detected within it throughout the process of regeneration; instead, postblastema areas always show high mitotic values. Since blastema size and the number of blastema cells increases as regeneration progresses (Saló, 1984), a mechanism of blastema formation and growth mediated through an early accumulation of undifferentiated cells at the wound boundary, followed later by the continuous crossing of undifferentiated cells (produced by cell proliferation and/or dedifferentiation) from stump to the base of blastema, seems the most reasonable hypothesis. As regards the spatial pattern of mitosis found in regions far from the wound (r), no significative change was found between the six strips measured along the anteroposterior axis.

Evidence for the existence in the intact organisms of cells with a prolonged G2 period, and its role during regeneration

Early evidence for a G2 population of cells in the intact animal came from the quick and massive cell division seen after feeding and regeneration in *Dugesia (S) mediterranea* (Baguñà, 1974, 1976*a*) and *Dugesia (G) dorotocephala* (Betchaku, 1975). Both phenomena have been detected too in *Dugesia (G) tigrina* (Baguñà & Romero, 1981, in feeding animals; this work for regenerating animals).

Further evidence, obtained in this work, is as follows: a) Feulgen cytospectrophotometry of total cell population from intact organisms show the presence of

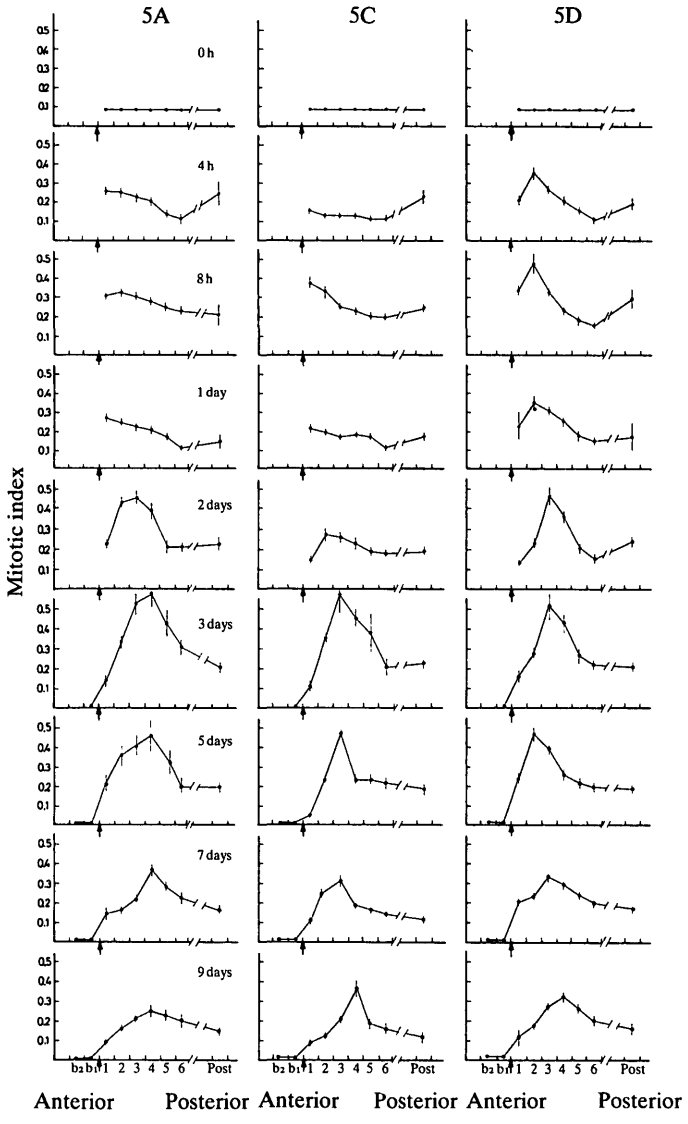

Fig. 4. Mitotic index along the anteroposterior axis (strips *b1* and *b2* of blastema, strips 1 to 6 of postblastema, and regions far from the wound (Post)) in anteriorly regenerating 5 mm long organisms cut at levels *A*, *C* and *D*. The vertical arrow on the abscissa mark the region (strip) where the stump/blastema boundary is located. Post: mean mitotic index for regions far from the wound (*r*, in Fig. 1C).

cells with 5C and 6C values which should correspond to S and G2 triploid cells (Fig. 5A). Identification of individual cell types by phase contrast showed that S and G2 triploid cells were all neoblasts (Fig. 5C); instead, all the differentiated cell types studied had DNA values around 2C–3C (Fig. 5B,D,E,F). These results, make clear that G2 neoblasts are the only G2 cells in intact

planarians and represent a fairly important percentage of total neoblasts.

b) Intact organisms exposed continuously to hydroxyurea (HU-1 experiment) show a steady decrease in mitosis, reaching near zero values at 4–5 days of incubation (Fig. 6A). This mitotic decrease occurs from 8 h of incubation, being very steep during the first day of exposure, and reaching 20 % of normal mitosis at 24 h. After, the decrease is much slower, 5 days being needed to block mitosis completely. As shown by the zero values found for ^{32}P incorporation into DNA at 24 h of treatment (Collet, Saló & Baguñà, unpublished data), the HU concentration used blocks (and eventually kills) all S cells by one day of exposure. Therefore, the existence of mitosis in the second, third and fourth day of exposure to HU suggest that a slow-cycling population of G2 cells exists in the intact organism entering at a low pace into mitosis during HU treatment. Since cytospectrophotometric data (Fig. 5) clearly shows that G2 cells are neoblasts, the slow-cycling cells should belong to this type of cells.

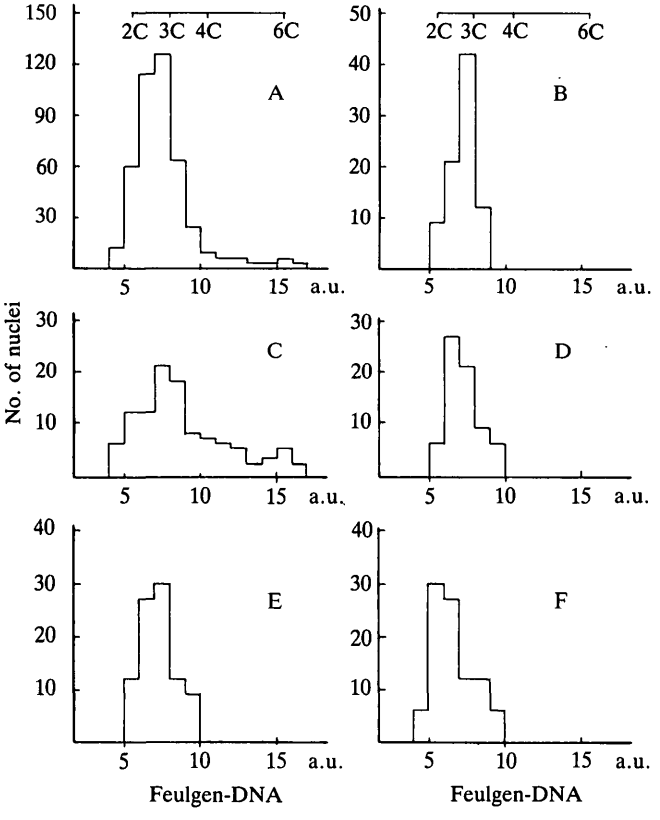

Fig. 5. Nuclear DNA content of: (A) total cells; (B) epidermal cells; (C) neoblasts; (D) gastrodermal cells; (E) fixed parenchyma cells; and (F) nerve cells. Planarians were dissociated to single cells by maceration, and the nuclear DNA content of Feulgen-stained cells determined microspectrophotometrically. Standard 2n and 4n, and 3n and 6n DNA values were determined from mitotic figures (telophase and metaphase) of diploid and triploid cells respectively (see text). Ordinate: number of nuclei. Abscissa: nuclear DNA content in relative units of absorption.

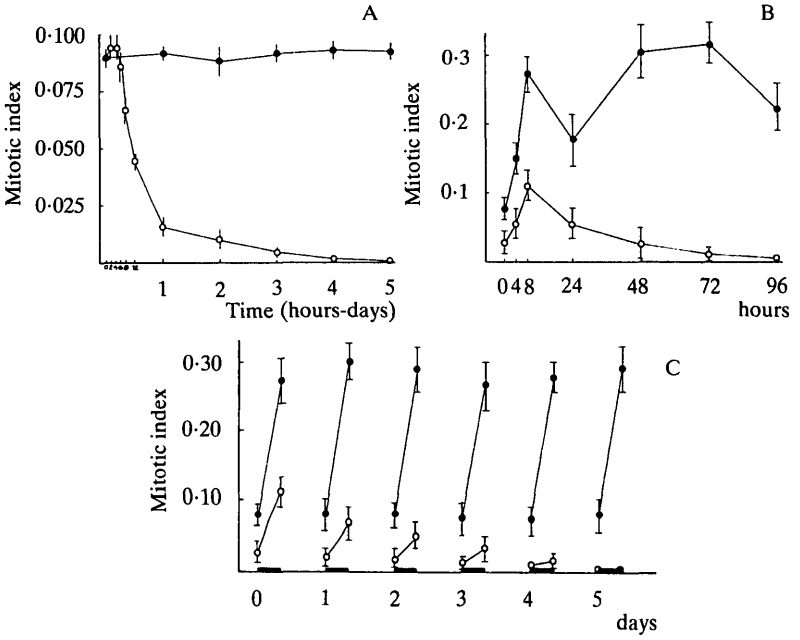

Fig. 6. Hydroxyurea (HU) experiments. (A) HU-1 experiment. Mitotic index of 2×10^{-2} M HU-treated (○——○) and untreated control (●——●) intact organisms. Ordinate: mitotic index; Abscissa: hours and days of incubation in HU. (B) HU-2 experiment. Temporal pattern of mitosis during regeneration of 2×10^{-2} M HU treated (○——○) and untreated control (●——●) organisms. HU-treated organisms were preincubated in HU for 15 h before cutting. Ordinate: mitotic index; Abscissa: hours of regeneration. (C) HU-3 experiment. Mitotic index at 0 and 8 h of regeneration of 2×10^{-2} M HU-treated (○——○) and untreated control (●——●) organisms. Experimental uncut organisms were placed in HU for 15 h and from then on incubated in the same solution for different periods of time (0 to 5 days), cut, and their mitotic index measured at 0 and 8 h of regeneration. Ordinate: mitotic index; Abscissa: period of incubation in HU before cutting (in days). Black bar: interval between 0 and 8 h of regeneration.

c) As in feeding and regenerating *Dugesia (S) mediterranea* (Baguñà, 1974, 1976*b*), data on changes in the proportion of mitotic phases during anterior regeneration in *Dugesia (G) tigrina* show a sharp increase of prophases and to a lesser extent of metaphases as early as 1 h after cutting (results not shown). Such mitotic increase could be explained assuming the existence of a population of G2 cells entering mitosis shortly after cutting. According to (a) and (b), such cells are neoblasts.

However, some further questions still remain. Thus, is the first mitotic maximum entirely due to division of pre-existing G2 cells? And, is the second mitotic peak due to pre-existing G2 cells that enter mitosis at 2–4 days of regeneration, or due to cells entering mitosis after going through the S period from the onset of regeneration? To answer these questions, the mitotic index of regenerating organisms exposed to hydroxyurea was studied (HU-2 experiment; Fig. 6B).

The results show that organisms regenerating in HU have a first mitotic maximum around 8 h of regeneration with values 40 % of normal, a marked mitotic decrease at 24 h, and the absence of the second mitotic maximum. First, this suggests that only half of the first mitotic maximum cells are pre-existing G2 cells; the other half are probably cells that happen to be at the end of the S period when HU is applied. On the other hand, the absence of the second maximum strongly suggest that this maximum is not due to G2 cells that enter mitosis at late stages of regeneration but to G1 cells that enter mitosis after replicating their DNA from the onset of regeneration.

A last piece of evidence suggesting a role for pre-existing G2 cells during the early stages of regeneration comes from the study of the mitotic index of regenerating organisms after long pretreatments in HU (HU-3 experiment; Fig. 6C). Thus, organisms cut after two days in HU show a mitotic increase at 8 h of regeneration with values 20 % of normal. Similar measurements after 3 and 4 days of exposure show increases of 12 and 3 % of normal values, whereas organisms cut after 5 days in HU show no mitotic response. In all cases, the second mitotic maximum does not appear (results not shown). These results, jointly with those from the HU-1 experiment, demonstrate that the mitotic increase seen in organisms after a long pretreatment in HU is due to slow-cycling neoblasts which are still in the G2 period.

Overall, the HU-experiments suggest: 1) that a population of G2 neoblasts exists in the intact worm, being quickly mobilized after cutting to give the first mitotic maximum; 2) that the second mitotic maximum is not due to a later entry into mitosis of pre-existing G2 neoblasts but to cells that pass through a round of DNA synthesis after the onset of regeneration; and 3) that the G2 neoblasts that go into mitosis belong mainly to the slow-cycling subpopulation of these cells that spend most of their time in G2.

DISCUSSION

Planarian regeneration: the intact organism

The spectrophotometric data on DNA content in the intact organism show that 3–4 % of the total cells are in G2; all of them neoblasts (Fig. 5A,C). Since neoblasts represent 20–25 % of total cells (Hay & Coward, 1975; Baguñà, 1976*a*; Baguñà & Romero, 1981) we should expect 12–20 % of neoblasts to be in G2, a range that includes the value found (approx. 16 %) for G2 neoblasts after Feulgen staining (Fig. 5C). On the other hand, HU experiments in intact organisms show both the need of long incubations (up to 5 days) to suppress mitosis completely, and the existence of a very steep mitotic decrease during the first 24 h of incubation (Fig. 6A). Taking into account that DNA synthesis stops after 1 day of HU treatment, both results could be best understood assuming that in intact organisms proliferative cells (neoblasts) are functionally divided in two main compartments: a) a small one ($\simeq$20 % of total neoblasts), made of slow-cycling cells that

spend most of their time in G2; and b) a bigger one (≃80 % of total neoblasts), made of rapidly cycling cells, most of them at G1 and S periods.

So, intact organisms could be considered as systems comprising: a) 12–14 non-dividing differentiated cell types turning over continuously and being replaced by differentiating neoblasts (Baguñà & Romero, 1981); and b) a single type of proliferative cells (neoblasts), probably divided into two functional subpopulations, of which 15 % are in G2, 15 % engaged in DNA synthesis, and the rest (≃70 % of total neoblasts) in the G1 period.

Temporal pattern of mitosis during regeneration

The temporal pattern of mitosis during regeneration in *Dugesia (G) tigrina* is basically similar to the one found for *Dugesia (S) mediterranea* (Baguñà, 1976*b*): a first maximum at 4–12 h, a relative minimum around 1 day, and a second and long-lasting maximum at 2–3 days of regeneration. These results contrast with published data for *Dugesia (S) lugubris* (Dubois, 1949; Gabriel, 1970) and *Dugesia (G) dorotocephala* (Best *et al.* 1968) where mitosis remained unchanged up to 24 h, a maximum appearing at 2–4 days. However, mitotic activity at the very beginning of regeneration (0–12 h) were not studied by these authors; therefore, their suggestion that blastema cells appearing during the first 24 h were the result of neoblast migration from regions far from the wound or the result of cell dedifferentiation should be questioned. On the other hand, Lindh (1957) found in *Dugesia (S) polychroa* an early proliferative response at 6 h of regeneration, a relative minimum at 24 h, and a second maximum at 2–3 days. Unpublished data obtained by us in different sexual reproducing species (sexual race of *Dugesia (S) mediterranea, Dugesia (S) polychroa* biotype D, biotype E of *Dugesia (S) lugubris*, and *Dugesia (D) gonocephala*) show too a similar bimodal pattern (Baguñà & Saló, in preparation).

Taken together, it seems that in most planarians species, regeneration results in two proliferative maxima: an early one at 4–12 h, and a second at 2–3 days, with a relative minimum in between.

The first mitotic maximum: the role of G2 cells

In most regenerating systems the first mitotic increase is seen from 24 h on or in later periods of regeneration (Marilley & Thouveny, 1978; Karlsson, 1981; Wallace, 1981). Since most eukaryotes have S periods lasting on average from 6 to 10–12 h, and since autoradiographic studies of labelled mitosis show that the early ones are already labelled, it follows that the first cells going into mitosis are G1 (or G0) cells that, after a period of dedifferentiation or a 'pre-replicative phase', go into the S period, and then to divide.

Therefore, any mitotic increase seen during the early hours (0–8 h) of regeneration could only be understood assuming that pre-existing cells in G2 enter mitosis shortly after cutting. That this is so in planarians is borne out from: a) the sudden increase in prophases 1 h after cutting; b) the increase in mitotic

figures seen at 2–4 h after cutting; and c) the appearance of a mitotic increase at 8 h in organisms incubated for long periods in hydroxyurea (HU) (Fig. 6C). Since HU blocks the cells at the S phase of the cell cycle, a sudden mitotic response after cutting in organisms incubated for long periods with HU clearly means that early dividing cells come mainly from G2 cells already present in the intact organism.

Two further points deserve comment. First of all, it is necessary to point out that though pre-existing G2 cells represent a fair percentage (≃40–50 %) of cells making the first mitotic maximum, other cells, not in the G2 period when the organism is cut (e.g. at the end of the S period), could go into mitosis after a brief stay in G2. This is suggested by the data shown in Fig. 6B. On the other hand, the G2 cells that divide during the first mitotic peak belong most probably to the slow-cycling subpopulation of neoblasts. The fact that a lesser increase in mitotic activity is seen when the organism is cut after longer pretreatments in HU (Fig. 6C) could be explained by the ever decreasing number of cells left in G2 in the intact organism (Fig. 6A).

The second mitotic maximum: the role of DNA synthesis

The results obtained in regenerating organisms incubated with hydroxyurea (HU-2 experiment; Fig. 6B) show that the second mitotic maximum is due to cells that enter mitosis after going through the S period after the onset of regeneration, and not to pre-existing G2 cells entering into mitosis at 2–3 days. This means that new DNA synthesis is needed to give the second maximum and to complete regeneration. Indeed, a biphasic pattern of DNA synthesis during regeneration in the planarian *Polycelis tenuis* has been found with a first maximum at 10–18 h, a second and long-lasting maximum from 28 h to 3–4 days, and a relative minimum in between (Martelly & Le Moigne, 1980). This increase in DNA synthesis precedes the second mitotic maximum, a result in agreement with the HU-results mentioned above.

However, it is still an open question whether cells going through the S period after the onset of regeneration are: a) G1 neoblasts arising from the division of pre-existing G2 neoblasts during the first mitotic peak; b) pre-existing G1 neoblasts not related to the first mitotic maximum; or c) G1 cells produced by cell dedifferentiation during the first hours of regeneration. This very important problem awaits further investigation.

Spatial pattern of mitosis during regeneration: mitotic pattern in blastema and postblastema

Despite a careful search, no mitoses have been found within the blastema throughout the process of regeneration. The absence of mitosis within the blastema is rather surprising as most workers agreed that cell proliferation occurred within it (Lindh, 1957; Best *et al.* 1968; Coward *et al.* 1970; Gabriel, 1970; Baguñà, 1976*b*), although Chandebois (1976) disputed this.

In contrast to blastemal regions, postblastema (stump) regions show high levels of mitosis from the onset and throughout regeneration. In early regeneration (0–48 h) the maximum in mitotic activity occur in regions close to the wound. As regeneration progresses, the maximum in mitosis shifts slowly to regions away from it (Fig. 4), both in anterior and posterior regeneration. The significance of this displacement is uncertain.

The occurrence and significance of mitosis in regions far from the wound

The results obtained from *Dugesia (G) tigrina* clearly show that mitosis in regions far from the wound show a pattern similar to the postblastema: a first maximum at 4–8 h, a second maximum at 2–4 days and a relative minimum around 1–2 days. Therefore the mitotic response in those regions, being so early, do not seem to be directly related to the suggested replenishment of cells caused by a previous cell migration as suggested by Dubois (1949). On the other hand the mitotic increase, being so rapid and occurring throughout the worm, suggest that some kind of intracellular communication mechanism, very sensitive to traumatic stimuli, should exist. In this context, it is interesting to point out the striking similarity between this phenomenon and the fast and general mitotic response seen after feeding (Baguñà, 1974), where a kind of humoral or neurohumoral mechanism was postulated to account for it.

Since an increase in cell division and DNA synthesis is needed in regions far from the wound, an interesting question arises: what are these new cells for? It is suggested that the new cells produced might have some role in the process of pattern respecification and body remodelling occurring in the late stages of regeneration.

The mechanism of blastema formation in planarians: a new proposal

Blastema formation and growth during regeneration in Amphibia (Wallace, 1981), Insecta (Karlsson, 1981), and Annelida (Marilley & Thouveny, 1978), occur mainly through a first stage of cell dedifferentiation, a middle stage of blastema cell proliferation, and a late stage of redifferentiation. Data so far available suggest that planarians follow a different scheme to build their blastema. The results obtained in *Dugesia (G) tigrina* (this work), and unpublished data gathered for different species (*Dugesia (S) mediterranea, Dugesia (S) lugubris* and *Dugesia (D) gonocephala*, Saló & Baguñà, in preparation), show that during regeneration mitoses do not occur within the blastema. Moreover, using a histoenzymological method to detect DNA polymerase active cells in the planarian *Polycelis tenuis*, Martelly, Rey & Le Moigne (1981) showed that positive-reacting cells occur just behind the blastema/stump boundary and never inside the former.

If blastemata do not undergo mitoses nor DNA polymerase activity, and since kinetic studies show that blastemata mainly grow by increasing their number of cells (Saló, 1984), a mechanism of blastema formation and growth based on an

early accumulation of cells at the distal end of the wound followed by a continuous crossing of undifferentiated cells from stump to the base of blastema seems the most reasonable hypothesis. However, where and how blastema cells originate and how they accumulate and move to form the blastema are still open questions. It could be that a massive dedifferentiation of cells near the wound takes place (Hay, 1968; Hay & Coward, 1975; Chandebois, 1976; Gremigni & Miceli, 1980), giving cells to initiate blastema formation; further cells, produced later on by cell dedifferentiation and/or proliferation in the stump, may be added to the blastema through local short migrations.

On the other hand, if the role of neoblasts in regeneration is considered (Dubois, 1949; Pedersen, 1972; Betchaku, 1975; Baguñà, 1976*b*, 1981; Bowen, den Hollander & Lewis, 1982), the increasing number of cells within the blastema could be explained as resulting from proliferation of neoblasts in a rather narrow (200–300 μm) strip of tissue behind wound and blastema followed by their local migration to the base of blastema. The fact that neoblasts placed far from the wound move slowly (40–50 μm/day) and at random, whereas when placed near it move faster (100–150 μm/day) and directionally, jointly with stathmokinetic data which shows that cells produced by mitosis within a 200–300 μm long strip of tissue near the wound can, after local migrations, account for the increasing number of blastemal cells during regeneration (Saló & Baguñà, 1983), support this proposal.

Whatever the origin of blastema cells, it is clear that blastema formation and growth in planarians follows a rather different mechanism to most epimorphically regenerating organisms. Besides, this modality of blastema formation could be considered as an intermediate stage between regeneration by morphallaxis (e.g. *Hydra*) where blastemata do not exist, and regeneration by epimorphosis (Annelida, Insecta, Amphibia...) where blastemata form and grow mainly by internal proliferation. Moreover, this modality in blastema formation could have in turn a deep influence on pattern formation mechanisms in planarians.

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