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DAVID SCHLESSINGER

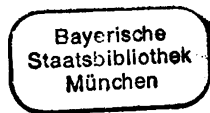


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Stem Cell Differentiation in Hydra

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INTRODUCTION

Two obvious features distinguish multicellular organisms from most microbial systems: the variety of different cell types they contain and the ordered spatial array in which these cells occur. Both features arise during embryonic development by virtue of cell differentiation and cell communication—the two topics of this symposium. Although development of some (mosaic) embryos is highly determinate, the majority of embryos remain remarkably undetermined with regard to cell differentiation until well into development. Such embryos have a high capacity to regenerate excised or missing parts, indicating that the determination of cells for particular pathways of differentiation occurs late and is strongly influenced by the environment, in particular, by signals emanating from neighboring cells. Despite a long tradition in embryology, little is known about these signals. At the least, however, they appear to transmit information about the presence or absence of neighboring cells and thus a cell's position in a larger cell mass (the embryo). Still less is known about the relationship between such "positional" signals and the choice of particular pathways of cellular differentiation.

In seeking to investigate such problems, several laboratories in recent years have turned to the simple metazoan hydra. As a model system, hydra has several advantages. It has a simple, cylindrical body column consisting of two cell layers and only five basic cell types. Furthermore, hydra regenerate well, which is presumptive evidence for the occurrence of intercellular communication. Finally, as I hope to show here, hydra possess undetermined stem cells capable of differentiating into either of two major classes of cell—nerve cells and nematocytes. The type of differentiation depends on the position of the stem cell in the animal in direct analogy to the situation in embryos. It should, however, be clearly understood that this work involves adult hydra propagated asexually in the laboratory, not hydra embryos.

In the following, I will concentrate on interstitial cells and their differentiation into nerve

cells and nematocytes. The differentiation pathways *in vivo* have been worked out in detail (9–11, 16, 17, 20, 23), and recent experiments using cell culturing techniques have added extensive information about the stem cells and their differentiation potential (C. N. David and S. Murphy, in preparation). I shall also review the relationship between the control of stem cell differentiation and the control of morphogenesis. Although I shall consider morphogenetic controls only briefly, recent progress in this area has been remarkable (13). In particular, evidence for the involvement of low-molecular-weight, diffusible substances in the morphogenetic "gradients" of hydra is accumulating rapidly, including direct isolation of putative morphogens (21, 22). An explicit theory for the establishment and maintenance of morphogen gradients has also been presented (15). Together with extensive data gathered from transplantation and regeneration experiments (18, 19, 25–28), these results promise, for the first time, an adequate molecular explanation for the control of morphogenesis in hydra.

HYDRA GROWTH AND MORPHOGENESIS

Hydra is a small (5 to 10 mm) freshwater polyp. It consists of two concentric cylinders of epithelial cells (endoderm and ectoderm) enclosing a central gastric cavity. The tissue is specialized at the proximal end to form a basal disk (hold-fast) and at the distal end to form a hypostome (mouth structure), surrounded by a whorl of six tentacles. The gastric region, which makes up most of the body column, is relatively unspecialized.

Hydra grow asexually by budding off young animals from the proximal gastric region. Although hydra tissue grows constantly, mature animals do not increase in size since tissue is lost in the form of buds and, to a lesser extent, lost from the tips of tentacles and the basal disk (3, 4). Both morphogenesis of buds and continuous renewal of hypostome and basal disk result from localized cell differentiation and tissue movement and not from localized cell proliferation (2, 7).

CELL PROLIFERATION AND DIFFERENTIATION

Mature hydra contain about 120,000 cells (1). About 20% of these are epithelial and gland cells, which constitute the principal structural element of hydra tissue. Most of the remaining cells are interstitial cells and their differentiated derivatives, nerves and nematocytes (Fig. 1 and 2). Interstitial cells are cytologically undifferentiated cells occurring singly, in pairs, or in larger clusters of 4, 8, or 16 cells. Clusters arise by a series of synchronous divisions (16) with incomplete cytokinesis, leaving daughter cells connected by cytoplasmic bridges (23).

Nematocyte differentiation involves proliferation of interstitial cells to form clusters of 8 and 16 cells, followed by differentiation of a nematocyst capsule in each cell (8, 16, 20). After capsule differentiation, cell clusters separate to single cells, which migrate to the tentacles from the site of their differentiation in the body column. By comparison, nerve cell differentiation appears to occur from the pool of single or paired interstitial cells. The time course of nerve and nematocyte differentiation has been determined by labeling interstitial cell precursors with [^3H]thymidine and following, by autoradiography, the appearance of labeled, newly differentiated cells (9). Nematoblasts,

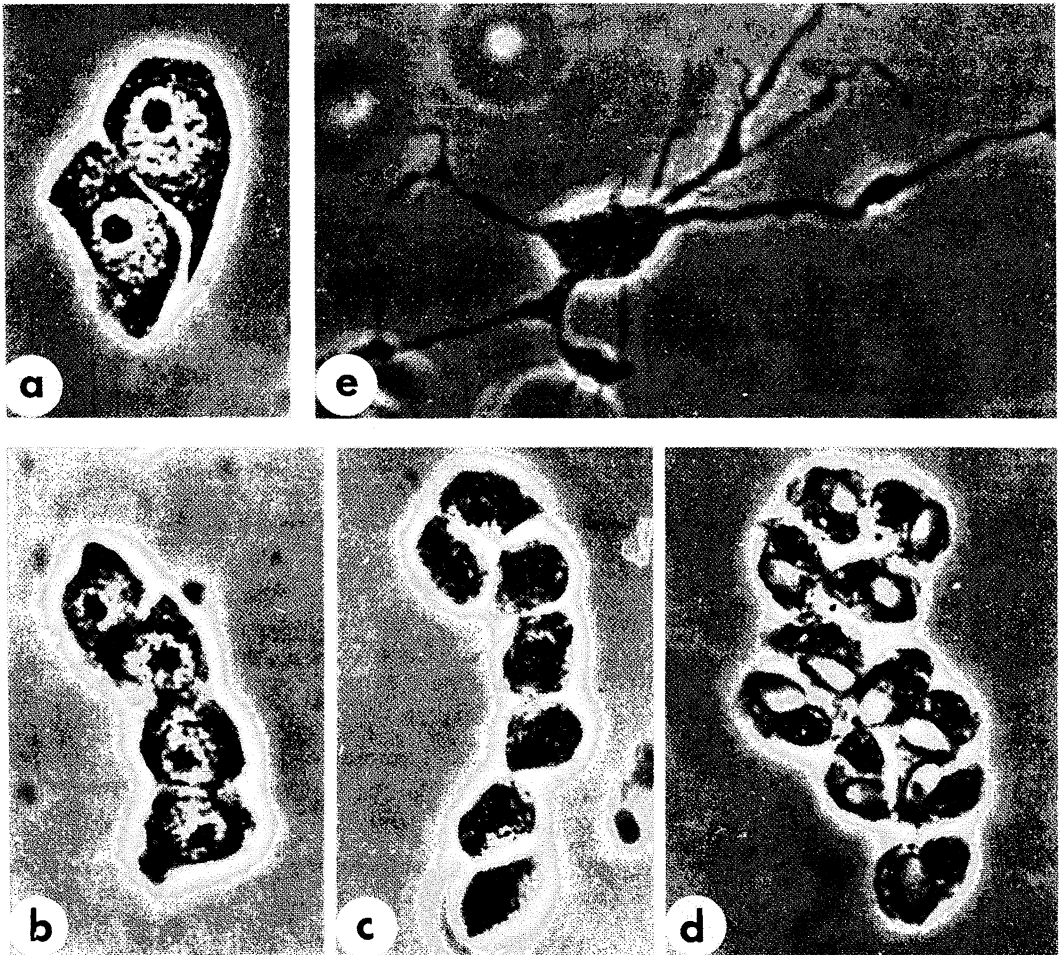


FIG. 1. Interstitial cells, nerve cells, and nematocytes. (a) Pair of large interstitial cells typical of the population containing stem cells. (b and c) Clusters of four and eight interstitial cell precursors to differentiating nematoblasts. (d) Desmoneme nematocytes at a late stage of differentiation (just prior to splitting of clusters) in which capsules are almost completed and nuclei are pycnotic. (e) Nerve cell. All micrographs are of dissociated (macерated) cell preparations; interstitial cells are held together by cytoplasmic bridges. Dissociation procedure simultaneously fixes cells, thus maintaining *in vivo* morphology and facilitating identification. Phase contrast. $\times 1,180$. Modified from David (6).

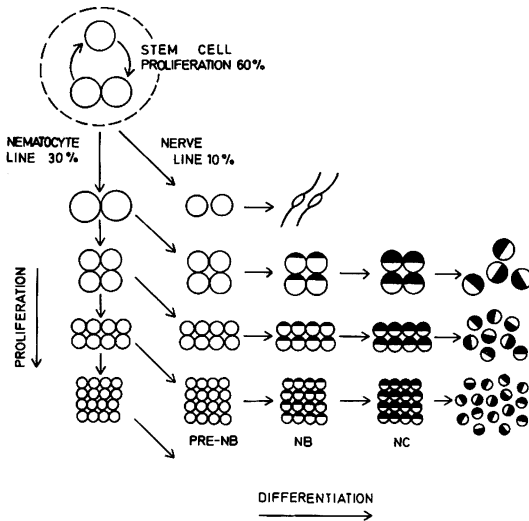


FIG. 2. Schematic representation of stem cell proliferation and nerve and nematocyte differentiation in hydra. Stem cells occur as single or paired interstitial cells. In each stem cell generation, 60% of daughter cells remain stem cells, 30% initiate proliferation of a cluster of nematocyte precursors, and 10% initiate nerve differentiation. In the nematocyte line, differentiation of nematocyst capsules commences after proliferation ceases. Pre-nematoblasts (Pre-NB) are morphologically identical to interstitial cells. Later, a vacuole develops in the cytoplasm (NB) which differentiates into mature capsule (NC) before the cluster breaks up and single nematocytes migrate from body column to tentacles. Reproduced from David and Gierer (9).

the first detectable stage of differentiation, appear 12 h after labeling and fully differentiated nematocytes appear 72 to 96 h after labeling. Fully differentiated nerve cells appear by 18 h after labeling. Since both nerves and nematocytes have $2n$ postmitotic nuclear deoxyribonucleic acid content, the lag following [^3H] thymidine labeling must include cell division as well as differentiation.

The pathways of nerve and nematocyte differentiation are summarized schematically in Fig. 2. Continuous differentiation of nerves and nematocytes in hydra requires the existence of a self-renewing population of interstitial stem cells to maintain a supply of precursors. From a detailed kinetic analysis of the rates of differentiation and the turnover of intermediate pools, David and Gierer (9) calculated that 1,760 stem cells per hydra per day enter the nematocyte line and 550 enter the nerve line. The number of stem cells per hydra required to support this flow of differentiated cells, as well as growth of the stem cell popu-

lation, is about 3,600 (based on an average stem cell cycle time of 24 h [5] and a doubling time for the population of about 3.5 days [7]). This estimate is in rough agreement with the abundance of single and paired interstitial cells in hydra, suggesting that these populations may contain the stem cells. However, there are no cytological criteria to distinguish stem cells from other interstitial cells.

A CLONAL ASSAY FOR STEM CELLS

To develop a direct assay for the stem cell, we have modified a technique introduced by Till and McCulloch (24) to characterize stem cells of the hemopoietic system. Stem cells have the capacity to proliferate more stem cells (self-renewal) and the capacity to give rise to daughter cells for differentiation. Thus, given the proper environment, single stem cells can found clones containing daughter stem cells and differentiated products. Till and McCulloch used lethally X-irradiated mice as hosts to provide a suitable environment for cloning injected hemopoietic stem cells. The injected stem cells settled in the spleen and other lymphatic tissues where they gave rise to easily detectable nodules of growing cells after 1 to 2 weeks. In an analogous experiment, we have attempted to clone hydra stem cells in inactivated host animals. We have used nitrogen mustard treatment instead of X-irradiation. Like X-irradiation, nitrogen mustard kills actively proliferating cells but does not seriously impair the metabolism and function of non-proliferating or slowly proliferating cells. Hydra survive for several weeks after nitrogen mustard treatment, although the rapidly proliferating stem cells are inactivated within several days and phagocytized by the tissue. Thus, 1 week after treatment, hydra consist of a shell of epithelial cells devoid of interstitial cells and differentiating nematoblasts (12).

To introduce live cells into such host tissue, we have taken advantage of the fact that suspensions of hydra cells reaggregate to produce solid masses of cells which regenerate normal hydra structures under appropriate conditions (14). Cells prepared from nitrogen mustard-treated hydra also reaggregate and regenerate, since extensive cell division is not required. However, like nitrogen mustard-treated hydra, such regenerates die after 2 to 3 weeks. If live cells are mixed into suspensions of nitrogen mustard-treated cells, mixed regenerates are formed in which the live cells continue to proliferate and differentiate. Since nitrogen mustard-treated (host) interstitial cells are eliminated after about 1 week, simple staining

of such mixed regenerates with toluidine blue reveals only interstitial cells derived from the added live cells. If limiting numbers of live cells are added, then individual clones of interstitial cells appear randomly scattered through the ectoderm of the host regenerate (Fig. 3). Control regenerates, to which no live cells are added, contain no clones; increasing numbers of live cells per regenerate lead to increasing numbers of clones. An average of one clone (= one stem cell) per regenerate is obtained when 150 live cells are added. Since 50% of all cells are lost at various stages before regenerates are firmly established, an estimate of 1 stem cell per 75 total cells (1.3%) is more nearly correct. This estimate approaches the value of 3.5% (3,600 stem cells per hydra) derived from population studies on whole animals (9).

DIFFERENTIATION POTENTIAL OF STEM CELLS

Are stem cells multipotent, i.e., capable of differentiating both nerves and nematocytes, as suggested in Fig. 2, or are there distinct populations of stem cells determined for one or another type of differentiation? To answer this question, we have used the clone assay to analyze the differentiated products of individual stem cells. Nerves and nematocytes differentiated in clones were distinguished from residual nerves and nematocytes of the nitrogen mustard-treated host by labeling with [³H]thymidine at 8 to 10 days of growth. After labeling, regenerates were incubated for 2 days to allow time for differentiation of nerves and nematocytes. Cell preparations were made of each regenerate for autoradiography and then quantitatively scored for labeled interstitial cells, differentiating nematocytes and nerves. To be certain that the regenerates examined contained only single clones, we conducted the experiment under conditions where fewer than 20% of all regenerates had clones. The results of one such experiment in Table 1 show that each clone contained nematocyte precursors, differentiated nematocytes, and nerve cells, as well as stem cells. Thus, individual stem cells are capable of throwing off both major classes of differentiated products. This result suggests that the stem cell population is homogeneous in terms of differentiation potential and that spatial patterns of differentiation in hydra must be explained in terms of "positional information" influencing the determination of individual stem cells for the nerve or nematocyte pathway.

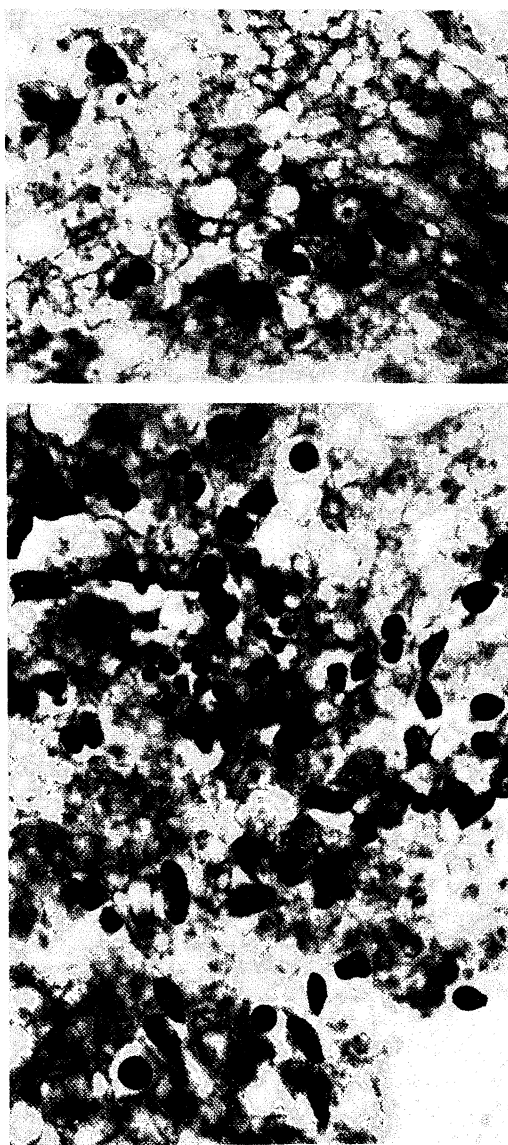


FIG. 3. Two stages in growth of a stem cell clone. Top: 4-day clone containing 16 cells—primarily large interstitial cells in pairs. Bottom: 10-day clone containing about 150 cells including a variety of interstitial cell clusters and differentiating nematoblasts. Host interstitial cells were eliminated by pretreatments with nitrogen mustard. Whole mount; fixed with alcohol and stained with toluidine blue.

REGIONAL CONTROL OF NERVE AND NEMATOCYTE DIFFERENTIATION

Nerve and nematocyte differentiation in hydra do not occur uniformly throughout the body column, but are localized in specific regions: nematocyte differentiation in the gastric

TABLE 1. *Composition of stem cell clones*^a

Clone no.	Interstitial cell clusters ^b					Nematocytes	Nerves
	"Stem cells"		"Nematocyte precursors"				
	1	2	4	8	16		
1a	12	24	14	11	2	2	20
3a	8	28	5	3	5	45	21
8a	9	8	6	2	1	3	17
9b	4	36	10	1	2	38	39
18 regenerates containing no stem cell clone	0	0	0	0	0	0	0

^a Stem cell clones were prepared, labeled with [³H]thymidine to distinguish clone cells from host cells, and analyzed for labeled cells by autoradiography as described in the text. Four of 22 regenerates were found to contain clones. All interstitial cells in each clone were labeled and are scored in the table along with labeled nematocytes and nerve cells. No interstitial cells, labeled or unlabeled, were found in the other 18 regenerates. These regenerates also contained no labeled nematocytes or nerves cells. Control regenerates to which no live cells had been added contained no interstitial cells and no labeled nematocytes or nerve cells.

^b The assignment of function, i.e., "stem cells" and "nematocyte precursors," to particular classes of interstitial cells is described in the text and has been justified in detail elsewhere (9).

region, and nerve differentiation primarily in the hypostome and basal disk. In the case of nematocyte differentiation, this has been demonstrated by use of a staining procedure which is highly specific for cells at a late stage of differentiation (8). This stain demonstrates the presence of clusters of differentiating nematocytes throughout the gastric region, but not in the hypostome or basal disk and the immediately subjacent tissue (Fig. 4b). The boundary between these "clear zones" and regions of nematocyte differentiation is remarkably sharp.

The interstitial cell precursors to differentiating nematocytes are also localized exclusively in the gastric region. The hypostome and basal disk contain few interstitial cells and no clusters of 4 or more cells, whereas clusters of 4, 8, and 16 interstitial cells are abundant in the gastric region. Figure 4a shows the hypostome and distal gastric region stained for interstitial cells with toluidine blue. There is a clear boundary between the regions of high and low interstitial cell density. A similar situation obtains near the basal disk as well. These results indicate that the regional control of nematocyte differentiation certainly occurs before the four-cell stage and probably at the level of stem cell determination.

A specific stain is not available to identify differentiating nerve cells. However, newly differentiated nerves can be distinguished from pre-existing cells by [³H]thymidine labeling. Using this technique, David and Gierer (9) have shown that new nerve cells appear at a rate two to six times greater in the hypostome and basal disk than in the gastric region (Table 2). Thus, the distribution of nerve cell differen-

tiation is essentially the inverse of the regional distribution of nematocyte differentiation.

Together with the finding that interstitial stem cells are multipotent, the above results suggest that control leading to regional localization of nerve cell and nematocyte differentiation in the hydra body column is exercised at the level of stem cell determination. Table 2 summarizes quantitatively the number of stem cells determined for nerve and nematocyte differentiation per day in the hypostome, gastric region, and basal disk of standard hydra.

ROLE OF MORPHOGENETIC CONTROL MECHANISMS IN STEM CELL DETERMINATION

Hypostome and basal disk are dominant regions which control the morphogenesis and differentiation of tissue in the body column. The essential properties of these regions, summarized below for hypostomes, have been deduced from a variety of regeneration and tissue transplantation experiments (18, 19, 25-28). Basal disks display analogous properties.

1. *Activation.* Small pieces of hypostome transplanted to sites in gastric regions activate host tissue surrounding the implant to participate in the differentiation of a "lateral" hypostome at that site. The activation is restricted to tissue immediately surrounding the hypostomal transplant.

2. *Inhibition.* Hypostomes inhibit the differentiation of hypostomes in adjacent tissue. Certain types of transplants form lateral hypostomes in hosts without hypostomes (decapi-

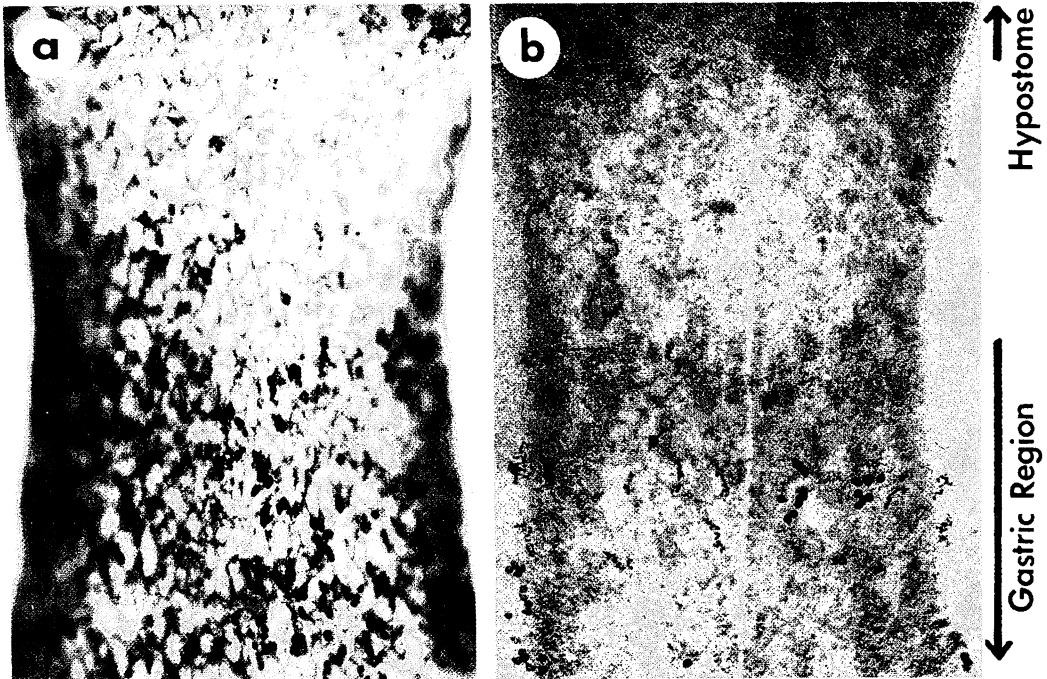


FIG. 4. Localized control of nematocyte differentiation in region subjacent to hypostome. (a) Toluidine blue staining of interstitial cells. (b) Thiolacetic acid- Pb^{2+} staining specific for a late stage of nematocyte capsule differentiation. Clusters of 8 and 16 stained capsules stand out as dark beads against weakly stained background tissue. Micrographs show about one-fifth of body column starting at base of tentacles. Hypostome and adjacent areas contain few interstitial cells and no differentiating nematocytes. Although the size of "clear zones" varies from animal to animal, the boundary between the two regions is usually quite sharp.

tated hydra), but are inhibited from doing so if the host hypostome is present. The inhibition extends from hypostomes far down the body column.

Levels of activation and inhibition can

TABLE 2. Stem cell determination in hypostome, gastric region, and basal disk"

Region	No. of stem cells determined	
	Nerve line	Nematocyte line
Hypostome	90	0
Gastric region . . .	16	100
Basal disk	30	0

" Values are expressed per 1,000 epithelial cells to normalize for differing sizes of the three regions. Large "standard" hydra contain about 22,000 epithelial cells in the body column: 2,400 in the hypostome, 17,600 in the gastric region, and 1,800 in the basal disk (including proximal peduncle) (1). The results in the table were calculated on the basis of 1,760 stem cells per day initiating nematocyte differentiation and 550 stem cells per day initiating nerve differentiation in a standard hydra (9).

change rapidly and are independent of cell differentiation and morphogenesis. By 4 h, a regenerating hypostome has the same level of activation and inhibition as a normal hypostome (26), although cell differentiation and morphogenesis of the new hypostome require 24 to 48 h. This temporal sequence of events suggests that levels of activation and inhibition may, in fact, be the signals effecting changes in cell differentiation and morphogenesis.

Although the mechanisms of activation and inhibition are still largely unknown, it appears likely that they are mediated by activating and inhibitory molecules ("morphogens") which are expected to be of low molecular weight and freely diffusible in tissue. Both the kinetics of inhibition decay (18) and the "diffusion" of inhibition through tissue (28) are consistent with the hypothesis that inhibition is mediated by a low-molecular-weight molecule. Furthermore, Schaller (21, 22) has recently isolated and purified from hydra a small peptide (molecular weight, 900) that significantly accelerates hypostome regeneration when added to regenerating pieces and appears to be the

postulated activator involved in hypostome morphogenesis.

The localization of nerve cell differentiation in hypostome and basal disk and of nematocyte differentiation in the body column closely parallels the localization of morphogenetically dominant regions in hydra. This suggests that the same control mechanisms may be involved, namely, activating and/or inhibitory morphogens. Further parallels between these processes occur during regeneration. As noted above, changes in levels of activation and inhibition occur rapidly at regenerating sites. If these changes are, in fact, responsible for increasing stem cell determination to nerve cells, a rapid increase in newly differentiated nerve cells is expected during hypostome regeneration. Bode et al. (1) have measured such an increase starting 24 h after cutting. Inhibition of nematocyte determination at the regenerating site is also expected. However, as a result of the 4- to 6-day lag between determination of stem cells and completion of nematocyte differentiation (Fig. 2), changes in nematocyte differentiation might be correspondingly delayed. Recently, we have shown the persistence of clusters of differentiating nematocytes in regenerating hypostomes for 5 to 6 days after cutting (C. N. David and S. Murphy, unpublished data). Thus, there are marked parallels in the spatial distribution, as well as temporal changes, during regeneration which strongly implicate identical or closely related systems in the control of morphogenesis and the control of stem cell determination.

CONCLUSION

Stem cells in hydra appear to be an excellent model system for elucidating in detail the relationship between morphogenetic gradients and cell determination. However, not all cell determination is the result of such extracellular environmental factors. There is reason to believe that certain determinative events in embryogenesis are mediated by factors present in the cytoplasm of fertilized eggs and are probably mechanistically quite different from the determination of stem cells in the adult hydra. In hydra there is also evidence for such a class of cell determination. Stem cells, although capable of differentiating a variety of cell types, including germ cells in sexual animals, do not appear to give rise to all cell types, e.g., epithelial and gland cells. One suspects that events or factors unique to the fertilized egg may lead to determination of epithelial and gland cells, as well as interstitial stem cells, early in embryonic development and that these

events are not reproduced in the environment of the adult hydra. In experimental terms, this suggests that it may be impossible to grow entire hydra from the progeny of a single stem cell.

The technique outlined in this paper for culturing hydra cells in inactivated (nitrogen mustard-treated) host tissue—essentially a feeder layer technique—promises to be extremely useful. It now makes possible a variety of experiments on (i) the effects of isolated morphogens on stem cell determination *in vitro*, (ii) the timing of cell determination relative to phases of the stem cell cycle, and (iii) the effects of unusual combinations of cell types in host aggregates on the determination of stem cells. Such experiments will significantly enhance our understanding of the mechanisms controlling stem cell determination. It even appears possible that a true cell culture could be developed by successive selection steps from culture medium to nitrogen mustard hosts (to rescue and propagate survivors) and back to culture medium.

ACKNOWLEDGMENTS

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