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Regeneration of Hydra from Reaggregated Cells

A. GIERER, S. BERKING, H. BODE*, C. N. DAVID, K. FLICK, G. HANSMANN, H. SCHALLER & E. TRENKNER

Max-Planck-Institut für Virusforschung, 74 Tübingen, Spemannstrasse 35

Separated cells of hydra reaggregate and develop into normal animals. The regeneration of serially grafted aggregates derived from different parts of hydra tissue demonstrates that the polarity of morphogenesis in hydra is the result of the cellular composition of the tissue, not cellular orientation.

TISSUE reconstitution by reaggregation *in vitro* of single cells has been a useful method for the investigation of tissue organization and differentiation. In several cases organ-like structures have been obtained (for example, Weiss and Moscona[1], De Long[2]) and in the case of sponges[3,4] it has been possible to reconstitute an entire animal. We have succeeded in reaggregating cells of the freshwater coelenterate *Hydra attenuata* and regenerating normal animals from the aggregates. This is in agreement with a recent report by Noda[5] who obtained regenerates from cell aggregates of *Pelmatohydra robusta*. A number of authors[6–14] have already shown successful regeneration of aggregates made by combining small fragments of hydra or the partially disintegrated tissue obtained by pressing hydra through a fine mesh net.

Here we describe the process of reaggregation and regeneration and the use of aggregates to show that it is the composition of hydra tissue, and not cellular orientation, that is responsible for polarity of morphogenesis.

Disaggregation, Reaggregation and Regeneration

Suspensions of hydra cells were produced by mechanical disruption of hydra tissue in a modified cell culture medium (Table 1). The distribution of cell types in such suspensions is similar to that of hydra tissue. Between 70% and 80% of the cells are interstitial cells (I-cells), nematoblasts, nerve cells, and gland cells all of which occur almost exclusively as single cells. The remaining 20–30% are epithelial cells (epithelio-muscular cells of ectoderm and endoderm) of which one-third are single, one-third in clusters of two to four cells and one-third in clusters of five to fifteen cells.

Reaggregation of hydra cells occurs spontaneously in dense cell suspensions ($>10^6$ ml.$^{-1}$) but routinely, aggregates were obtained by centrifuging cells together. After formation of aggregates the cell culture medium was diluted stepwise to hydra medium during the next 20–30 h (Table 1). Regeneration of tentacles, hypostomes, and basal disks in such aggregates requires three to five days (Fig. 1*d*.) For regeneration of hydra from reaggregated cells, two requirements must be fulfilled. (1) Aggregates must contain more than 15% epithelial cells. If tissue disruption was too severe, epithelial cells were selectively destroyed and no aggregates formed. (2) Aggregates must contain a minimum number of cells. Aggregates containing $5–30\times10^4$ cells regenerated in all cases. Aggregates containing 2×10^4 cells regenerated in 10–50% of cases; smaller aggregates formed only hollow spheres. For comparison a mature hydra has about 10^5 cells[18]. Very large aggregates ($>10^7$ cells) did not regenerate well although long aggregates (0.5×50 mm) made in thin tubes did regenerate.

During reaggregation and regeneration considerable cell destruction and/or loss occurs, leaving only about one-third of the original cells in aggregates after two days (Table 1). It is therefore possible that the epithelial cells surviving in aggregates are mainly derived from larger clumps of 5–15 cells. Table 2 shows the recovery of cells in aggregates formed from a cell suspension enriched in single epithelial cells. The 33,000 epithelial cells in the final regenerate cannot be derived from a minor fraction of the original cell suspension such as the larger clumps because no significant epithelial cell mitosis occurred during this period. Rather they must be mainly derived from single cells and/or very small clumps (two to four cells).

The initially irregular cell mass forms a firm aggregate with a smooth surface within 6 h. At this stage the aggregate begins to develop a clear outer layer surrounding an orange core—a separation reminiscent of normal ectoderm and endoderm tissue in hydra. After 20–30 h the aggregate develops into a hollow sphere during which process cells are lost to the medium and/or digested. By 40–48 h tentacle buds begin to appear, usually spread irregularly over the surface of the aggregate. After about 2.5–3 days hypostomes appear and the tentacles begin to collect around the hypostomes or are resorbed (Fig. 1*d*). At 5–6 days the regenerate is able to feed on *Artemia*. Eventually the hypostomes divide up the tissue and several normal animals develop. Later the animals are capable of budding.

In histological sections taken immediately after formation of aggregates, the distribution of cells seems to be random. Subsequently cells reorganize so that interstitial cells and nematoblasts appear nearer the periphery and epithelial cells more toward the centre of the aggregate. By 20 h the core of epithelial cells appears to break up and ectoderm and

* Present address: Developmental and Cell Biology, University of California, Irvine, California 92664.

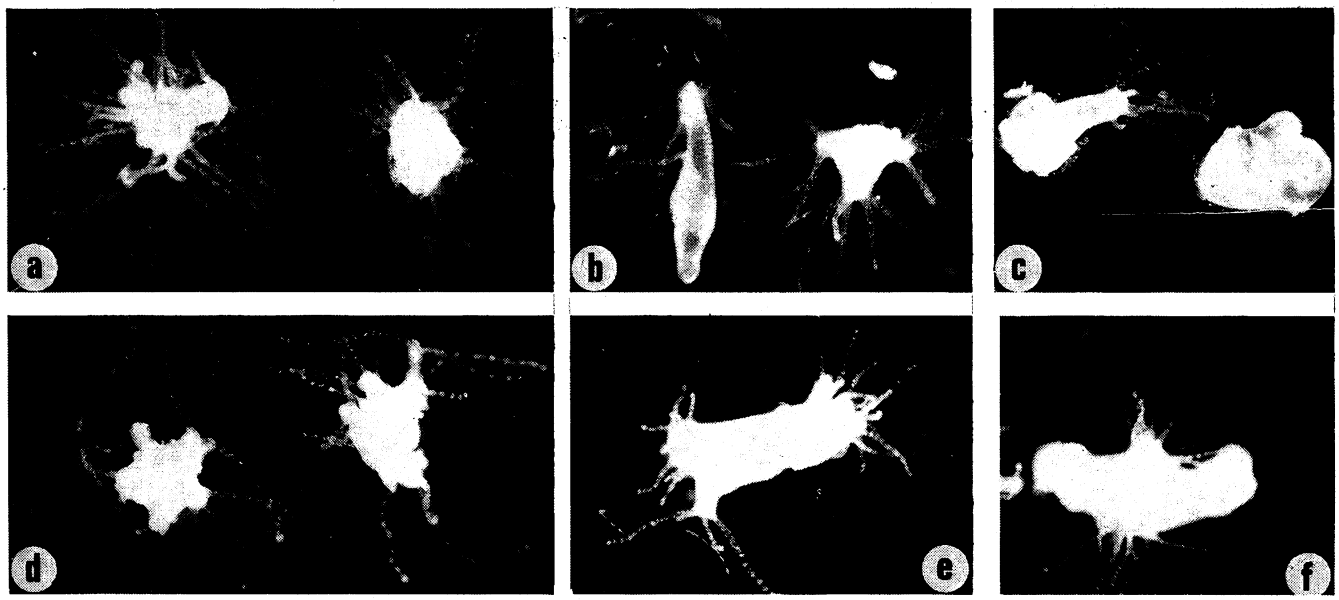

Fig. 1 7-day regenerates from reaggregated cells of *Hydra attenuata*. Cells were prepared from whole hydra or from selected parts of hydra tissue and aggregates formed as described in Table 1. The aggregates regenerated hypostomes, tentacles and basal disks after 3–5 days and normal animals after several weeks. *a*, Regenerate from aggregate containing head cells (cells from hypostome and tentacles). *b*, Regenerate from aggregate containing cells of gastric region only. *c*, Regenerate from aggregate of foot cells (peduncle and basal disk). *d*, Regenerate from aggregate containing cells of whole hydra. *e* and *f*, 4-day regenerates from linear combinations of three aggregates of cells from distal and proximal gastric region (*e*) DPD, (*f*) PDP (see text). (×10.)

endoderm cells begin to form a structure similar to the hydra body wall: two opposed layers with a mesoglea between. I-cells occur in this two layered structure almost exclusively in the ectoderm.

The different cell types of hydra are present initially in aggregates in approximately the same proportion as in intact hydra tissue[18] (Table 1). The most conspicuous change in cell composition during the first 24 h is the loss of about 75% of the nematoblasts, little I-cells, and nerve cells. Epithelial cells, big I-cells, and gland cells appear to be selectively retained in regenerating aggregates during the initial stages of hollow sphere and tissue layer formation, but undergo major losses between 24 and 48 h. During the first 24 h big I-cells almost always occur singly, in contrast with the clusters of two and four common in normal tissue[17].

During the first 20 h of aggregate regeneration—before tissue layer formation has occurred—interstitial cell mitotic activity is virtually absent. The average mitotic index from 0–18 h is <0.5% compared with about 3% in intact tissue (David and Campbell, to be published) and 2.5% at later stages of aggregate regeneration (Fig. 2). Following the increase in I-cell mitosis there is an increase in the abundance

Table 1 Distribution of Cell Types in Regenerating Aggregates of Hydra Cells

Time (h after aggregate formation)	Epithelial cells	Interstitial cells		Nematoblasts	Nerve cells	Gland cells	Total cell No.
		Big	Little				
0	30×10^3	41×10^3	35×10^3	25×10^3	8×10^3	6×10^3	145×10^3
2	27×10^3	44×10^3	29×10^3	26×10^3	6×10^3	8×10^3	140×10^3
6	35×10^3	40×10^3	23×10^3	20×10^3	4×10^3	5×10^3	127×10^3
18	42×10^3	34×10^3	12×10^3	11×10^3	3×10^3	6×10^3	102×10^3
24	30×10^3	30×10^3	9×10^3	9×10^3	3×10^3	6×10^3	87×10^3
33	25×10^3	23×10^3	8×10^3	6×10^3	4×10^3	5×10^3	70×10^3
45	16×10^3	18×10^3	7×10^3	3×10^3	5×10^3	2×10^3	51×10^3
65	19×10^3	18×10^3	9×10^3	5×10^3	4×10^3	2×10^3	57×10^3
99	15×10^3	15×10^3	14×10^3	8×10^3	4×10^3	3×10^3	59×10^3
188	15×10^3	10×10^3	13×10^3	25×10^3	4×10^3	3×10^3	70×10^3

Hydra attenuata, cultured according to the method of Loomis and Lenhoff[15] in hydra medium (10^{-3} M $CaCl_2$, 10^{-5} M EDTA, in tap water), were used for the experiments. Animals (100 $ml.^{-1}$) were incubated for about 30 min at room temperature in 70 mOsm cell culture medium (E. T., H. B., K. F., and G. H., unpublished) (KCl 3.6 mM, $CaCl_2$ 6 mM, $MgSO_4$ 1.2 mM, Na-citrate 6 mM, Na-pyruvate 6 mM, glucose 6 mM, TES buffer 12.5 mM, phenol red 100 mg $l.^{-1}$, rifampicin 50 mg $l.^{-1}$, adjusted to *p*H 6.9) before shearing them 10–20 times through the short, narrow orifice (0.7–1.0 mm diameter) of a pipette. A considerable fraction of the animals were disrupted into cells by this process; non-disrupted pieces were sedimented and sheared again. Cell preparations were combined and diluted (to approximately 10^6 cells $ml.^{-1}$) to give a 5–10 cm liquid column in a test tube. After sedimentation at 1*g* for 10–20 min to remove cell clumps the upper two-thirds were used as cell preparation. The cells were collected by centrifugation (3 min, 200*g*) and resuspended in fresh medium by gentle pipetting. Disruption in Haynes–Burnett[16] medium, or 0.07 M sucrose or 0.035 M NaCl also yielded cell preparations suitable for reaggregation experiments.

Two methods were used to form aggregates: (1) to make large aggregates an aliquot of cells ($1–2\times10^5$) was centrifuged (3 min, 100*g*) in a small conical Eppendorf tube and the cell pellet transferred to a Petri dish containing fresh medium; (2) to make small aggregates ($2–5\times10^4$ cells) 3–5 μl. of concentrated cell suspension was taken up into thin (0.5 mm diameter) polyethylene tubing, the solution clamped off at one end and the cells centrifuged (3 min, 100*g*) on the free minuscus at the opposite end of the liquid column. The cell pellet was left to stabilize for 10 min in the tubing and then gently ejected into a Petri dish containing fresh medium.

Aggregates formed in 70 mOsm medium were transferred to 35 mOsm medium (2-fold diluted cell culture medium) after 4 h, to 17 mOsm medium after another 6–12 h and finally to hydra medium (5 mOsm) after a further 8–16 h.

The quantitative distribution of cell types was determined on three to five aggregates at each time shown using maceration preparations[17,18]. In such preparations all hydra cell types (including mitotic cells) can be recognized and counted.

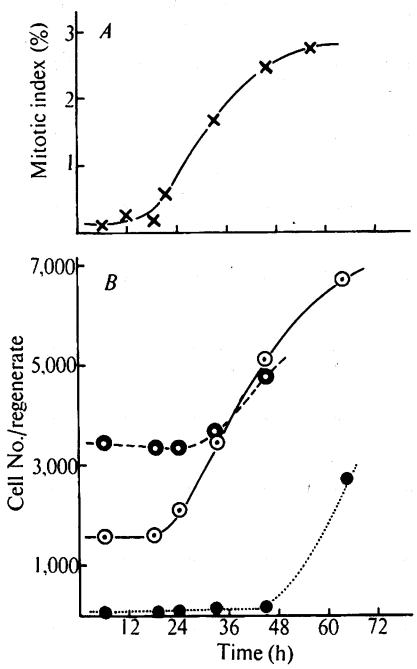

Fig. 2 Mitotic index, number of big interstitial cells in nests, and number of nerve cells during aggregate regeneration. The counts were done on the same maceration preparations used for Table 1. The cell numbers in *B* are expressed per aggregate. *A*, Mitotic index of big I-cells. *B*, Number of big I-cells in nests of two (⊙—⊙) and nests of four (● ... ●); ○ - - - ○, number of nerve cells.

of nerve cells and nests of 2 and 4 big I-cells (Fig. 2). Their appearance is followed by an increase in little I-cells (nests of 8 and 16 cells) between 3 and 4 days which differentiate finally into nematoblasts (Table 1) as in normal animals[19]. The results suggest that nematoblasts and nerve cells differentiate from I-cells after the I-cells have undergone mitosis in agreement with results *in vivo* (C. N. D., to be published).

The differentiation of I-cells to nerve cells in aggregates has been directly followed by adding cells from hydra labelled with ^{3}H thymidine, disaggregated immediately after labelling, to suspensions of reaggregating cells. In normal animals, labelled nerve cells appear about 24 h after pulse labelling with ^{3}H-thymidine (C. N. D., to be published). In aggregates no labelled nerve cells were found at 24 h, but after 48 h, about 16% of the nerve cells were labelled, while the big I-cells were labelled to about 25%. This indicates that somewhat more than half the nerve cells at this stage are newly differentiated from I-cells in agreement with the counting data (Table 1) showing that nerve cells have approximately doubled between 24 and 48 h.

Morphogenesis and Polarity

Regenerating aggregates are a useful tool for studying the morphogenetic capacity and polarity of hydra tissue. In particular it can be shown that the origin of cells in an aggregate influences its morphogenetic capacity. Aggregates containing only head cells (hypostome and tentacles) formed regenerates covered with numerous tentacles visible after 2 days (Fig. 1*a*). Hypostomes developed in such aggregates only after 5–6 days. Aggregates of cells from the gastric region formed mono-, bi- or tripolar regenerates in which hypostomes developed first (2 days) and tentacles shortly thereafter (2.5 days) (Fig. 1*b*). Cells from the basal region gave rise to aggregates which regenerated basal disks after 2 days and head structures after 6–7 days or not at all (Fig. 1*c*).

In normal hydra the regeneration of sections of body column from which both head and foot have been excised occurs such that the original axial polarity is rigidly maintained[20–22]. Furthermore, Wilby and Webster[22] have recently shown that sections of body column inserted with reversed polarity into a second hydra maintain their polarity for several days. Polarity, therefore, seems to be a fairly stable property—a conclusion which is supported by the regeneration of aggregates of cells derived from different parts of hydra tissue. Several theories have been proposed to explain polarity[23]. It could be due either to a graded distribution of cells or substances[18,24,25] or to a stable unidirectional polarization of the individual cells, in which case stable regional differences are unnecessary. In the first case, aggregates of cells derived from distal regions, if combined with aggregates of cells derived from proximal regions, should regenerate a head in the distally derived portion. In the latter case, head formation by distally or proximally derived cells should be equally probable.

Table 2 Participation of Single Cells in Aggregate Regeneration

	Epithelial cells		All other cells	
Original cell suspension *	70,000		130,000	
Cells in clusters †	1	51%	1	100%
	2–4	40%	>1	0%
	5–10	9%		
	>10	0%		
Aggregate after 1.5 h	45,000		55,000	
Regenerate after 48 h	33,000		38,000	

* Aliquots (5 µl.) containing 200,000 cells were centrifuged (100*g*, 2 min) to make aggregates. The cell numbers in regenerating aggregates were determined as in Table 1.

† An aliquot of the cell suspension was seeded in a Petri dish and the distribution of cells in clusters determined in an inverted phase contrast microscope.

Cells were prepared as in Table 1 except that a second 1*g* sedimentation step was added and the middle third of the liquid column used as cell preparation.

Aggregates were produced from approximately 100,000 cells derived from the head region (H) or the gastric region (G) of animals without buds. Three such aggregates were centrifuged on top of each other in polyethylene tubing (0.5 mm) to obtain the sequences HGH and GHG. HGH always produces tentacles at both ends, but no head structures in the middle; GHG produces tentacles in the middle, but no head structures at the ends. To show that the location of heads is not merely defined by cells derived from previous heads, but by tissue polarity, the gastric region itself was subdivided into two equal zones, one proximal (P) and one distal (D). Aggregates were made from cells derived from each of these zones and the triplet combinations DPD and PDP were produced. DPD produced hypostomes and tentacles at the ends in all cases (Fig. 1*e*) and only occasional (2/11) single tentacles in the middle. PDP produced hypostomes and tentacles always in the middle (Fig. 1*f*) and in some cases (9/20) additional single tentacles at the ends.

Thus cells derived from the more distal regions consistently form the head regions in the triple aggregate combinations. This is in agreement with results obtained from tissue grafting experiments[25] and shows furthermore that the cell types, or tissue components, responsible for determining polarity are not irreversibly destroyed or lost during disaggregation and reaggregation. The results strongly support the idea that tissue polarity is primarily due to a graded distribution of cell types or cellular constituents and not to cellular orientation. More complex theories involving a stabilizing interaction between a gradient of substance and the orientation of polar cells[22,26] would be consistent with these experiments only if the gradient, and thus a regional difference, is the more stable property primarily responsible for polarity.

Further support for these conclusions comes from experiments in which sections of gastric tissue were grafted in series, either with normal or reversed polarity. For these experiments a body column without buds was cut into ten

sections. When regrafted in the original order with the original polarity, a head regenerated in the distal section as expected. If the original order was maintained but the polarity of each piece reversed, the result was the same (E. T., unpublished results). Again source, not orientation, of tissue in the body column controls polarity of the regenerate.

In summary the aggregation technique permits new approaches to the study of cell differentiation and tissue morphogenesis in hydra. In particular cell differentiation can be assayed by the mixing of labelled cells of various origins and pretreatments into aggregates. Morphogenesis at the cellular level can be studied by combining various cell types in controlled spatial distributions. Two simple applications along these lines have been presented. In the first, it was shown that differentiated nerve cells are incorporated into regenerating aggregates and that cells introduced into aggregates as precursors subsequently differentiate to nerve cells at the stage of tentacle formation. Second, it was shown that those components which determine tissue polarity survive disaggregation and reaggregation, and that the stability of tissue polarity is due to the graded composition of cell types, or cellular constituents, and not to their orientation.

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