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Genetic analysis of developmental mechanisms in hydra

XIX. Stimulation of regeneration by injury in the regeneration-deficient mutant strain, reg-16

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Summary

A mutant strain of *Hydra magnipapillata*, reg-16, has a very low regenerative capacity. After head removal, it usually restores 10–20 % of the original number of tentacles in 7 days.

A procedure was found to markedly improve tentacle regeneration in this strain. The closed wound located at the apical regenerating tip of the decapitated polyp was gently reopened using a pair of forceps. Reg-16 polyps treated in this way at 24 and 48 h after head removal restored nearly all of the original number of tentacles in 7 days.

A lateral tissue transplantation procedure was employed to examine the effect of wound reopening on the morphogenetic potential of decapitated reg-16 polyps. Wound reopening produced a significant increase in head activation level without producing a preceding decrease in head inhibition level. This and other observations suggest that the coupled activation–inhibition changes that normally occur after head removal from the wild-type hydra do not occur in this strain.

Mechanisms responsible for the wound reopening effect and the absence of activation–inhibition coupling in the mutant strain reg-16 are discussed.

Key words: hydra, regeneration, injury effect, genetic analysis, mutant reg-16.

Introduction

Hydra has a strong regenerative capacity. After head and foot removal, it regenerates a new head from the original apical end and a new foot from the original basal end of the body column within several days.

Mutant strains of hydra have been isolated that show various types of regenerative abnormalities (Sugiyama & Fujisawa, 1977*a*). One strain, reg-16, shows a very reduced ability to regenerate a head after removal of its original head. However, it regenerates a foot normally, and also buds normally. This suggests that the defect(s) of this strain resides specifically in its head regeneration mechanism (Sugiyama & Fujisawa, 1977*b*).

Previous studies have shown the following characteristics of this strain. Examination of 'morphogen' levels in reg-16 tissue by Kemmner & Schaller (1981) showed that this strain contained significantly less head activator and significantly more head inhibitor than the wild-type strain. In addition, the same authors also showed that inhibitor release from the tissue of this strain after head removal was much slower than that of the wild-type hydra. Examination of 'morphogenetic potentials' by lateral tissue transplantation by Achermann & Sugiyama (1985) showed that the intact reg-16 polyp had a significantly lower head activation gradient and a significantly higher and steeper head inhibition gradient than the wild-type polyp. These authors also showed that activation and inhibition level changes occurring after head removal from this strain were very different from those in the wild-type hydra. It was suggested that these morphogen and morphogenetic potential abnormalities in reg-16 were pleiotropic expressions of the same genetic defect, and that they were directly responsible for the reduced regenerative capacity of this strain (Achermann & Sugiyama, 1985).

Hydra tissue consists of three self-proliferating cell lineages: ectodermal epithelial, endodermal epithelial and interstitial cell lineages. Six different types of chimaeric strains were constructed which consisted of different combinations of the three cell lineages of strain reg-16 and a wild-type strain (105). Analyses of these strains provided evidence suggesting that the defect(s) responsible for reduced regenerative capacity and low head activation level in reg-16 resides primarily in the ectodermal and endodermal epithelial cell lin-

eages, whereas the defect responsible for the high head inhibition level resides primarily in the endodermal epithelial and interstitial cell lineages (Wanek *et al.* 1986; Nishimiya *et al.* 1986).

Kemmner & Schaller (1981) made an interesting observation on the regeneration behaviour of strain reg-16. The head was initially removed from reg-16 in a normal manner. Several hours later a small amount of tissue was reamputated from the apical end of the decapitated polyp, and subsequently this was repeated twice. Polyps treated in this manner regenerated a significantly higher number of tentacles than normally decapitated animals. This observation was later confirmed by Achermann & Sugiyama (1985).

The present study was initiated to find out why reamputating the apical tip tissue from the decapitated reg-16 polyp improved tentacle regeneration. Two types of experiments were carried out. In the first experiment, different types of stimuli were applied to the wounded tissue at the apical tip of the decapitated reg-16 polyp and their effects on regeneration were examined. It was found that, of various stimuli tried, reopening of the healed tissue without removing any tissue produced the most significant effect. In the second type of experiment, lateral tissue transplantation was used to examine the effect of wound reopening on head activation and inhibition levels. Wound reopening produced an increase in activation level without producing a preceding decrease in inhibition level. This indicates that the coupled activation–inhibition changes that normally occur in the wild-type hydra (Wolpert *et al.* 1974) do not occur in strain reg-16.

The similarity of the wound reopening effect to the 'injury effect' previously observed in transplantation phenomena in the wild-type hydra by MacWilliams (1983*b*) will be discussed. In addition, the absence of activation–inhibition coupling in strain reg-16 will be discussed in the light of current reaction–diffusion models for hydra pattern formation (Gierer & Meinhardt, 1972; Meinhardt, 1982; MacWilliams, 1982).

Materials and methods

Strains

Two strains of *Hydra magnipapillata* were used. Strain 105 is a normal wild-type strain used as the standard strain. Strain reg-16 is a mutant strain which has a reduced head regenerative capacity. The origin and various properties of this strain are described in previous publications (Sugiyama & Fujisawa, 1977*a*,*b*; Kemmner & Schaller, 1981; Achermann & Sugiyama, 1985; Wanek *et al.* 1986; Nishimiya *et al.* 1986).

Culture

Stock cultures of 105 and reg-16 animals were maintained under the rigorously controlled mass culture conditions described by Takano & Sugiyama (1983). Relatively young animals showing their first bud protrusion were collected daily from stock cultures, and used in all experiments. Polyps of strain 105 vitally stained with Evans blue were produced by the procedure previously described by Nishimiya *et al.* (1986).

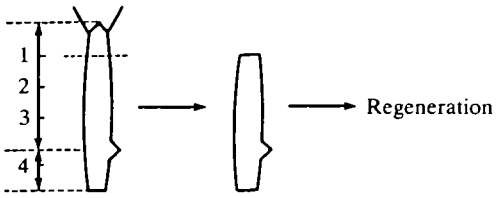

Fig. 1. Schematic representation of the procedure for standard regeneration assay (see Materials and methods).

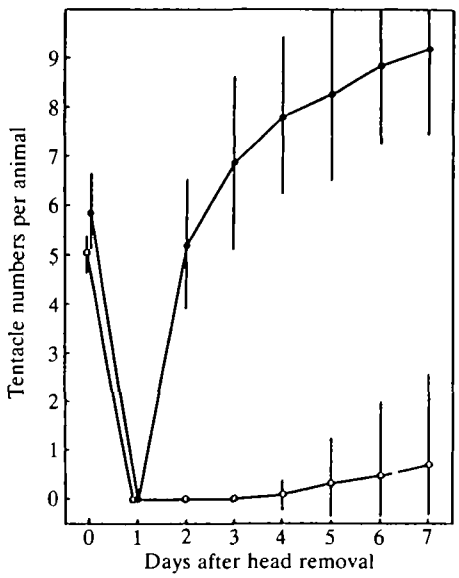

Fig. 2. Tentacle regeneration after head removal from normal strain 105 (closed circles) and mutant strain reg-16 (open circles). The values for day 0 indicate the tentacle numbers on original intact animals before head removal. Sample size was 27 for strain 105 and 46 for strain reg-16. The vertical bars represent standard deviation (also in Figs 3 and 4).

Standard regeneration assay

Fig. 1 shows the standard procedure used for regeneration assay. The body column of a well-stretched animal, from the hypostome to the bud protrusion, was divided into four equal lengths, the column from the protrusion to the basal disk was divided into a ratio of 1:2, and the four positions thus obtained were numbered from 1 to 4 according to Sugiyama (1982) as shown on the left in Fig. 1.

For standard head regeneration assay, the head was removed from polyps by amputation at position 1. The decapitated animals were individually placed in a small plastic Petri dish (50 mm in diameter) containing approximately 10 ml of the culture solution, and kept for 7 days without feeding. The number of tentacles regenerated by these animals was recorded daily under a dissecting microscope, and used as an index of head regeneration.

Reinjury

The head was removed from reg-16 polyps by amputation at position 1 as in the standard regeneration assay. 24 and 48 h later, four types of stimuli were applied to the wounded tissue at the apical end of the decapitated polyps. This is schematically shown on the left side in Fig. 3. In **reamputation**, a

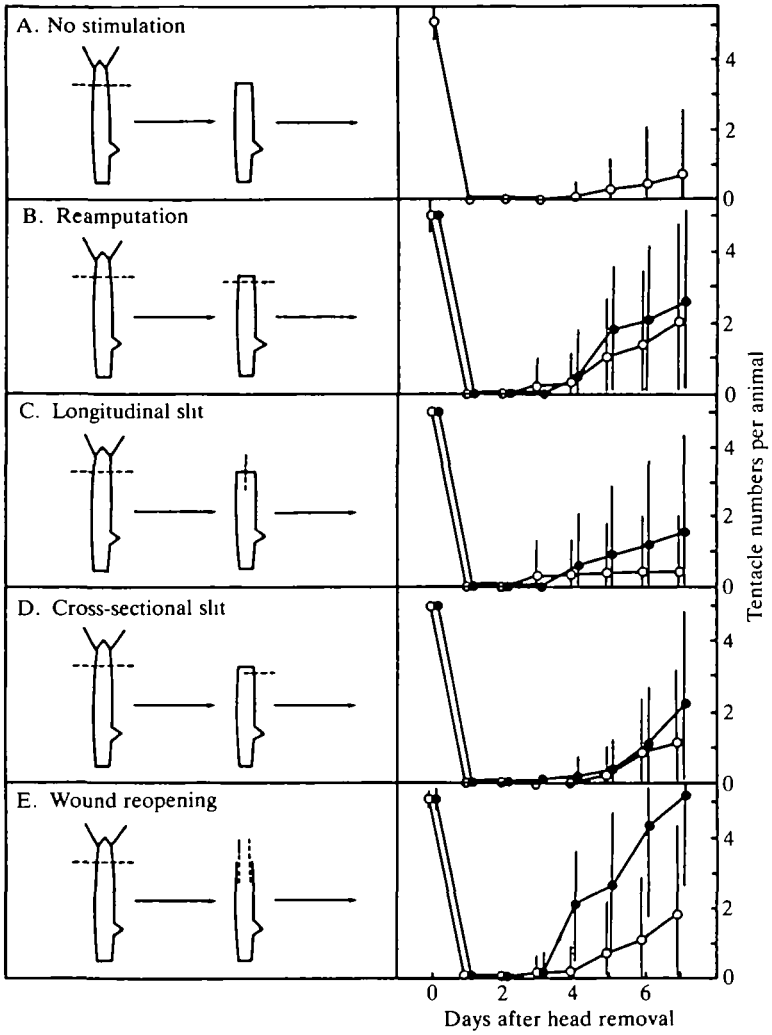

Fig. 3. Tentacle regeneration by decapitated reg-16 polyps stimulated by reinjury once at 24 h (open circles) or twice at 24 and 48 h (closed circles) after the original head removal. Four types of reinjury applied to the healed tissue are schematically shown on the left side (see Materials and methods for details). Minimum sample size was 18.

small amount of tissue was amputated from the apical end by a cut which was made immediately below the original amputation site (Fig. 3B). In the second type of stimulus, *longitudinal slit*, a slit was made along the body axis from the apical end. The length of the slit was about the same as the width of the body column (Fig. 3C). With *cross-sectional slit*, a slit was made perpendicular to the body axis immediately below the original amputation site. The length of the slit was approximately two-thirds of the width of the body column (Fig. 3D). In the fourth case, *wound reopening*, the healed tissue at the apical end was reopened using a pair of forceps with very fine tips. Initially the tip of the forceps was gently pushed through the centre of the healed wound to make a small hole. This hole was made gradually larger by pushing out the tissue with forceps from inside until the diameter of the hole became the same size as the gastric cavity (Fig. 3E).

Tissue transplantation

Changes in head activation and inhibition levels in decapitated reg-16 animals were assayed using the lateral tissue transplantation procedure previously described by Achermann & Sugiyama (1985) (see Fig. 5). To follow the change in head activation levels, heads were removed from reg-16 polyps by amputation at position 1. The apical tips were then excised from these animals at different times, and grafted onto standard host animals. Based on the results of a preliminary study, position 3 on the 105 polyp vitally stained with Evans blue was used as the recipient site on the standard host (upper part of Fig. 5).

To follow the change in head inhibition levels, heads were similarly removed from reg-16 animals. After various periods of time, the standard donor tissue was grafted onto the decapitated polyps at a site immediately below the original amputation site corresponding to position 2 of the original animal before amputation. Based on the results of a preliminary study, position 3 of the intact 105 polyp vitally stained with Evans blue was used as the source of the standard donor tissue (lower part of Fig. 5).

Grafted animals were kept individually in small plastic dishes (50 mm in diameter) containing about 10 ml of culture solution for 5–6 days without feeding, and examined for secondary head formation by the transplanted tissue.

All cultures and experiments were carried out in a constant temperature culture room maintained at 18 ± 0·5 °C.

Results

Fig. 2 shows the tentacle regenerative capacity of strains 105 and reg-16 as assayed by the standard regeneration procedure described in Materials and methods (also see Fig. 1). The wild-type strain 105 originally had an average of 5·9 tentacles per polyp.

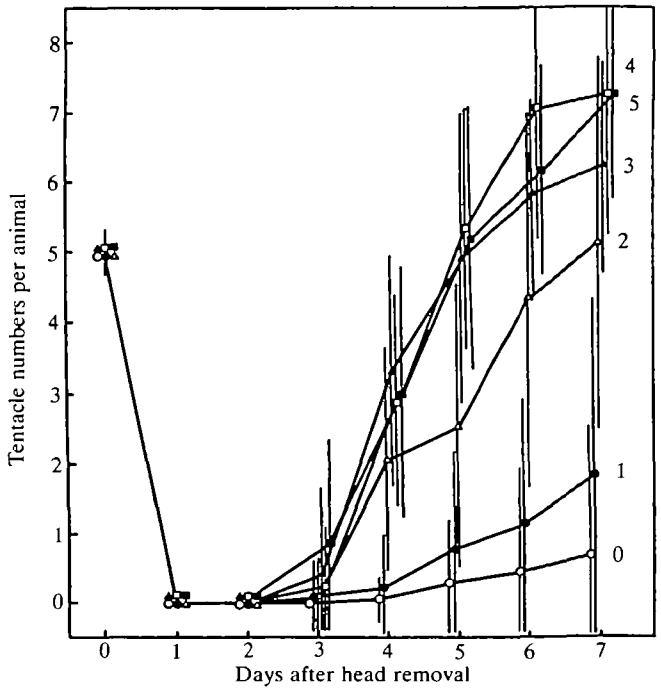

Fig. 4. Effect of repeated wound reopening on tentacle regeneration in reg-16. The first wound reopening was made 24 h after head removal, and the following wound reopenings were made at 24 h intervals. The numbers in the figure indicate the total number of wound reopenings applied. Sample sizes were 46, 54, 35, 12, 12 and 12 for wound reopenings of 0, 1, 2, 3, 4 and 5 times, respectively.

After head removal, 105 polyps restored almost all of the original number of tentacles by day 2, and about 60 % more by day 7. In contrast, strain reg-16 originally had an average of 5·0 tentacles per polyp, and restored none by day 2 and only about 15 % of the original number by day 7. This result confirmed previous observations that reg-16 had a significantly lower tentacle regenerative capacity after head removal than 105 (Sugiyama & Fujisawa, 1977*b*; Wanek *et al.* 1986).

Stimulation of regeneration

Kemmner & Schaller (1981) and Achermann & Sugiyama (1985) reported that tentacle regeneration by reg-16 after head removal was significantly improved by repeated reamputation of a small amount of tissue from the apical end of the decapitated polyps. In the present study, the same reamputation procedure and three other procedures described in Materials and methods were used to stimulate the apical tip of decapitated reg-16 polyps, and their effects on tentacle regeneration were examined.

The results, presented in Fig. 3, show that all four types of procedures used produced some positive effects. The most significant effect was produced by the wound reopening procedure (Fig. 3E). In this procedure, the healed tissue at the apical end of the body column of a decapitated polyp was gently reopened without removing any tissue. When treated in this way twice, at 24 and 48 h after original head removal, the decapitated reg-16 polyps restored nearly all the original number of tentacles in 7 days. This effect was reproducibly observed whenever the healed tissue was widely opened and the diameter of the opening made was nearly equal to the diameter of the gastric cavity. The effect, however, was not fully observed when the opening was smaller. Wound reopening had virtually no effect on the vigorous tentacle regeneration of strain 105 (data not shown). In order to examine further the effects of wound reopening on reg-16, the following experiments were carried out.

Fig. 4 shows the result of an experiment in which wound reopening was repeatedly applied. In this experiment, the initial wound reopening was made 1 day (24 h) after the original head removal, with subsequent wound reopenings made at one day intervals. The total number of wound reopenings varied from 0 to 5. The figure shows that a significant increase in tentacle regeneration was achieved when the number of wound reopenings was increased from 1 to 2. The first wound reopening alone, or each wound reopening after the second one, had relatively smaller effects.

Table 1 shows the result of an experiment in which

Table 1. *Average number of tentacles regenerated per polyp by decapitated reg-16 polyps stimulated twice by wound reopening**

Interval between head removal and 1st wound reopening (hours)	Interval between 1st and 2nd wound reopening (hours)					
	0	12	24	36	48	60
0	0·7					
6	0·8	1·6	2·7		3·9	1·9
12	0·4	2·8	3·4	4·3		2·6
24	1·9	5·9	5·2	4·3	4·1	
36	3·8	5·5	5·3	5·7	3·9	
48	3·9	4·7	5·7	4·9	4·5	
60	1·7	3·3				
72	1·5		1·4			

* Tentacle numbers per polyp at day 7 after original head removal are shown. Values higher than 5·0 are indicated by the dotted lines surrounding them. Sample sizes ranged from 14 to 54.

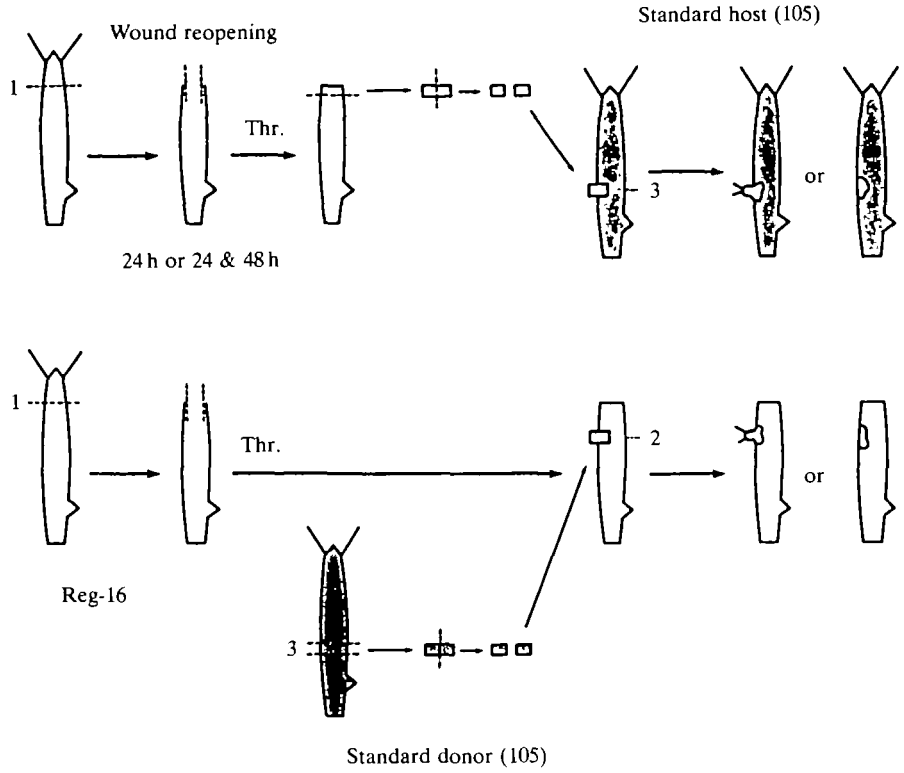

Fig. 5. Schematic representation of procedures used to examine head activation and inhibition level changes after head removal (see Materials and methods for details).

the number of wound reopenings was 2, but the timing varied. It can be seen that the timing of both the first and the second wound reopening was very important. Significant numbers (more than 5) of tentacles were regenerated only when the first reopening was made during a period 24–48 h after head removal and the second reopening was made 12–36 h later. Wound reopening made at other times produced less significant effects. These results indicated that the decapitated reg-16 polyps gradually acquired responsiveness to the effect of wound reopening, and that they were in the most responsive state from about 1 to 3 days after head removal (see Discussion).

Lateral tissue transplantation

The lateral tissue transplantation procedure described in Materials and methods (also see Fig. 5) was employed to examine the effects of wound reopening on activation and inhibition levels in the decapitated reg-16.

Reg-16 polyps were decapitated at day 0, and divided into two groups. The animals in the first group were left undisturbed (control). The animals in the second group were subjected to wound reopening at 24 and 48 h after head removal. To examine the head activation levels in these animals, the apical tip was excised from them at various times after decapitation, transplanted to the standard host (position 3 of intact 105), and its capacity to form a secondary head on the host was determined. The results, presented in Fig. 6, show that the donor tissues obtained from the control animals (not subjected to wound reopening) 0–4 days after head removal all induced heads at similarly low rates (3–33%). This

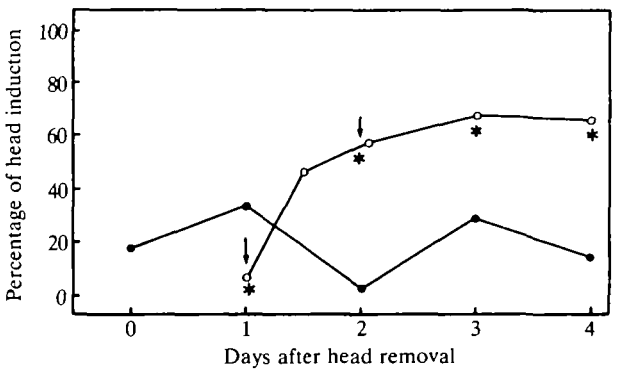

Fig. 6. Effect of wound reopening on head activation level changes in reg-16. Abscissa represents time after head removal. Ordinate shows percentages of secondary head formation obtained by transplanting apical tip of decapitated polyps to standard host. A high percentage value represents a high head activation level (see main text). Closed circles represent normally decapitated animals (control) and open circles represent animals subjected to wound reopening at 24 and 48 h after head removal (indicated by arrows). Asterisks attached next to symbols indicate that these results were statistically significantly different ($P < 0{\cdot}01$) when compared with control animals at the corresponding times.

agreed well with previous observations by Achermann & Sugiyama (1985) that head activation level changed little after head removal from reg-16. In comparison, donor tissues obtained from animals subjected to wound reopening (second group) formed secondary heads at significantly higher rates than those from the first group. The only exception was donor tissue ob-

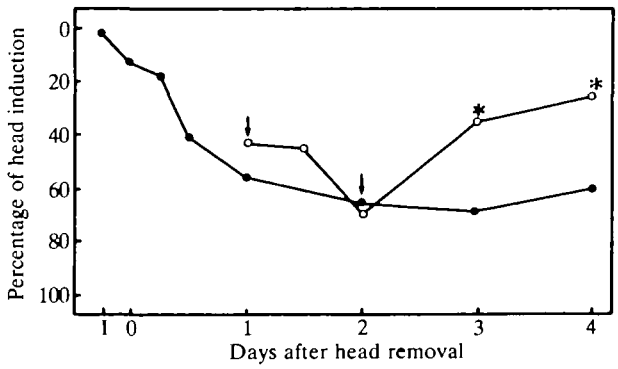

Fig. 7. Effect of wound reopening on head inhibition level changes in reg-16. The abscissa represents the time after head removal. I represents intact polyp before head removal. The ordinate shows percentages of secondary head formation obtained by transplanting the standard donor tissue to decapitated polyps. A high percentage value represents a low head inhibition level (see main text). The same symbol marks used in Fig. 6 are used.

tained immediately after the first wound reopening (24 h after head removal). It induced heads at a lower rate than that obtained from control animals at the corresponding time. This difference, however, was probably caused by the extensive damage in the apical tip by the first wound reopening operation. (The second wound reopening produced much less damage to the tissue.) These results indicated that wound reopening produced an increase in head activation level in the decapitated reg-16 polyps.

To examine head inhibition level changes, the standard donor tissue (position 3 of intact 105 polyp) was transplanted to the decapitated polyps at various times after decapitation, and the capacity of the recipient animals to resist the formation of a secondary head by the transplanted tissue was examined. The data in Fig. 7 show that the standard donor tissue induced heads at relatively low rates when transplanted to control animals soon after decapitation, and at gradually higher rates when transplanted later. This observation showed that the head inhibition level in the decapitated reg-16 animals was initially relatively high, decreased gradually for the first day or so, and thereafter remained at a low level. This agreed well with previous observations made by Achermann & Sugiyama (1985). The standard donor tissue, when transplanted to animals subjected to wound reopening, induced heads initially at about the same rates as when transplanted to control animals (from day 1 to 2). Later, however, it induced heads at significantly lower rates on animals subjected to wound reopening than on control animals. This indicated that wound reopening produced no immediate change but a delayed increase in inhibition level.

Discussion

Wound reopening effect

Strain reg-16 regenerated tentacles very poorly under standard conditions (Fig. 2). Its tentacle regeneration, however, was significantly improved when the healed tissue at the apical tip of the decapitated polyp was stimulated by reinjury (Fig. 3). The most marked effect was observed when the entire part of the original wounded tissue was directly injured again by the wound reopening procedure (Fig. 3E). Other procedures making an injury of similar extent to a nearby tissue had much smaller effects (Fig. 3B–D).

Injury effect

The wound reopening effect observed in reg-16 is probably identical to the 'injury effect' proposed by MacWilliams (1982). Two factors appear to play important roles in determining head regeneration. One is the relative head activation and head inhibition levels in the tissue. Determination of head regeneration takes place when the head inhibition level (S value) falls more than a threshold value below the head activation level (P value) (Wolpert *et al.* 1974).

The other factor is the 'injury effect' which was proposed on the basis of the following observation by MacWilliams (1983*b*). Using a normal transplantation procedure, a donor tissue was transplanted to the intact host polyp in the middle of its body column. In this process, donor tissue was selected that had little capacity to produce a secondary head on the host under normal conditions. After waiting for the transplanted tissue to heal (24–48 h), the host's head was amputated. This produced a rapid inhibition decrease in the host tissue, creating a large difference between the activation level of the transplanted tissue and the inhibition level of the host tissue. However, secondary head formation did not occur under this condition. Finally, the transplanted tissue was injured again, which resulted in a significant increase in secondary head formation. This indicated that a fresh injury was also an essential factor in determining a secondary head formation.

The wound reopening effect in strain reg-16 can be readily explained by this injury effect. When the head is removed from the wild-type polyp, the head inhibition level decreases rapidly (Wolpert *et al.* 1974; MacWilliams, 1983*a*,*b*; Achermann & Sugiyama, 1985). This produces a large activation–inhibition difference while the initial injury made by head removal is still fresh. Therefore, a single operation of head removal produces a condition that satisfies both the first and the second factors required for head determination in the wild-type hydra. The situation, however, is different after head removal from reg-16. Since the head inhibition level decreases very slowly in this strain, a large activation–inhibition difference is not produced until 18–24 h after head removal (Figs 6 and 7). During this period, the injury made by head removal has presumably healed. Therefore, head determination cannot occur in the decapitated reg-16 polyp unless a new injury is introduced by wound reopening. Thus, two operations are needed to satisfy the two factors for head regeneration in reg-16; the initial head removal produces the activa-

tion–inhibition difference, and the subsequent wound reopening produces the injury effect.

The observation that repeated wound reopenings were needed to obtain a significant effect (Table 1, Fig. 4) suggests that the injury effect is weak in reg-16, and that additive effects of repeated injury are needed to compensate for this defect.

Head activation increase by the injury effect

MacWilliams postulated that injury has a direct effect in producing an activation level increase. This is based on the observation that an extensive injury made to the tissue of an intact polyp produced little inhibition decrease in the injured tissue (MacWilliams, 1983*b*). This view is supported by direct observation in the present study. Wound reopening produced an activation increase without producing a preceding inhibition decrease in reg-16 (Figs 6 and 7).

It is important, however, to note here that wound reopening applied after the inhibition level decrease (24 h after head removal) was effective, but applied earlier was not effective, in enhancing tentacle regeneration (Table 1). One way to interpret this observation is that the injury effect can initiate an activation increase in tissue with a relatively low inhibition level, but not in tissue with a relatively high inhibition level.

An alternative interpretation is that an injury initiates a relatively small activation rise in any tissue, regardless of its inhibition level. In tissue with a relatively low inhibition level, this small rise leads to a further and more significant activation increase by some type of autocatalytic amplification mechanism. In contrast, the initial small rise is suppressed and the activation level is brought back to the original level in tissue with a relatively high inhibition level. Which interpretations are correct cannot be decided from available evidence.

Uncoupling of activation and inhibition level changes in reg-16

According to the reaction–diffusion model, the gradual activation increase occurring after head removal is initiated by the preceding rapid inhibition decrease in normal hydra (Gierer & Meinhardt, 1972; Meinhardt, 1982). Interestingly, these coupled changes of activation and inhibition levels were not observed in reg-16. The inhibition level decreased slowly after head removal, but the activation level remained virtually unchanged in this strain (Figs 6 and 7; also see Achermann & Sugiyama, 1985).

This apparent absence of activation–inhibition coupling in reg-16 can be explained in three ways. The first explanation is that this mutant strain has a genetic defect in the coupling mechanism, and that this defect is responsible for the absence of the activation increase in this strain.

The second explanation is that the coupling mechanism is intact in reg-16, and that an activation increase is initiated normally after decapitation in this strain. This increase and the injury-initiated increase, when combined together, cause the activation level to rise above a threshold level, which leads to a further and more significant activation increase by some type of autoamplification mechanism. However, this increase alone is insufficient to bring the activation level above this threshold, and thereby fails to produce the subsequent large increase.

The third explanation is as follows. Presently there is no direct evidence for the existence of the coupling mechanism in hydra. Therefore, the possibility exists that this mechanism is actually absent in hydra, and that the activation increase is initiated by the injury effect alone after head removal. Low inhibition level created by the inhibition decrease is a necessary condition for the injury-initiated increase to lead to the subsequent significant increase by some type of amplification mechanism as already discussed. However, the inhibition decrease is not a sufficient condition for producing the activation increase.

Which, or whether any, of the three explanations given above is correct cannot be determined from presently available evidence.

Conclusion

According to MacWilliams (1982), injury has the capacity to initiate an activation increase in normal hydra. This view is strongly supported by the present study. Wound reopening produced an activation increase without producing a preceding inhibition decrease in decapitated reg-16 (Figs 6 and 7).

According to Gierer & Meinhardt (1972) and MacWilliams (1982), an inhibition decrease also initiates an activation increase. This process, however, was not observed in the present study. There was no activation increase in response to the slow inhibition decrease after head removal from reg-16 (Figs 6 and 7). The reason for this is not clear at present.

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