Early steps in the regeneration of the musculature in *Macrostomum* sp. (Macrostomorpha)

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ABSTRACT. *Macrostomum* sp., a microturbellarian from the Northern Adriatic, is able to regenerate only its posterior end after artificial surgery. Restoration of muscle tissue is one of the early events in regeneration. Morphallactic and epimorphotic processes occur simultaneously and consecutively. Shortly after surgery, rearrangement of muscles near the wound was observed. Six to eight hours later a faint transient network of muscle fibres was visible in the parenchyma at the site of the future blastema. One day after surgery an outgrowth of existing longitudinal fibres beneath the epidermis surrounded the developing blastema. At the caudal end of these fibres excessive forking was observed. New myocytes for circular musculature perpendicular to the growing longitudinal muscles were visible around the blastema. One week after surgery the adhesive plate and all muscles of the male copulatory organ were completely regenerated, after another week the regenerate had reached the length recorded prior to surgery.

KEY WORDS: muscle regeneration, Platyhelminthes, Macrostomorpha, epimorphosis, morphallaxis.

INTRODUCTION

Reorganisation and regeneration of lost musculature after artificial surgery as well as the restoration of the nervous system are some of the early processes in regeneration of Platyhelminthes (BAGUÑÀ, 1998; HORI et al., 1999). A layer of intact musculature at the regenerating end is necessary (SCHÜRMANN & PETER, 1998) for wound closure and subsequent blastema formation. While a large amount of literature on regeneration is available for the Tricladida, and regeneration of muscles has been studied in detail by electron microscopy (e.g. HORI, 1983; MORITA & BEST, 1984a,b) and with a monoclonal antibody (CEBRIÀ et al., 1997; CEBRIÀ & ROMERO, this volume), only few data are available on this topic for "microturbellarians" (see PALMBERG, 1990, 1991). We have traced the early regeneration of the musculature in *Macrostomum* sp. (a member of the *Macrostomum tuba* clade), which only regenerates its posterior end.

In this paper we have focused on distinguishing morphallactic and epimorphotic processes in regeneration of muscle tissue, that is the restoration of missing parts by pre-existing cells versus by proliferation and differentiation of stem cells, using fluorescence labelled Phalloidin (RIEGER & SALVENMOSER, 1991; and RIEGER et al., 1994).

MATERIAL AND METHODS

Animals were collected originally at Lignano (Northern Adriatic, Italy). Specimens used for this study were taken from laboratory cultures (details of cultures described in Rieger et al. 1988). Prior to experiments the animals were fed with diatoms for one week and starved for two days to minimise autofluorescence. Animals were cut in half and regeneration was observed of both the posterior and anterior parts (n=19). Surgery was carried out in artificial seawater. In order to avoid toxic effects, $MgCl_2$ was not used as an anaesthetic at this point. After the appropriate regeneration time worms were relaxed in seawater-isotonic $MgCl_2$ and fixed with 4% paraformaldehyde for one hour. Subsequently animals were rinsed several times in 0.1M phosphate buffered saline (PBS) and treated with 0.2 % Triton X-100 in PBS. The whole mount fluorescence technique was used, applying Phalloidin – Alexa (Molecular Probes, Oregon, USA, solution 1:70) to stain muscle F-actin filaments (RIEGER & SALVENMOSER, 1991; RIEGER et al., 1994), mounting in Vectashield (Vecta) and examination with a Polyvar epifluorescence microscope

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(Reichert, Austria) and a confocal laser scanning microscope (Zeiss, LSM 510 Germany).

RESULTS

Immediately after surgery constriction of the circular musculature occurred at the caudal end of the anterior regenerate (Fig. 1). The wound was closed after one to
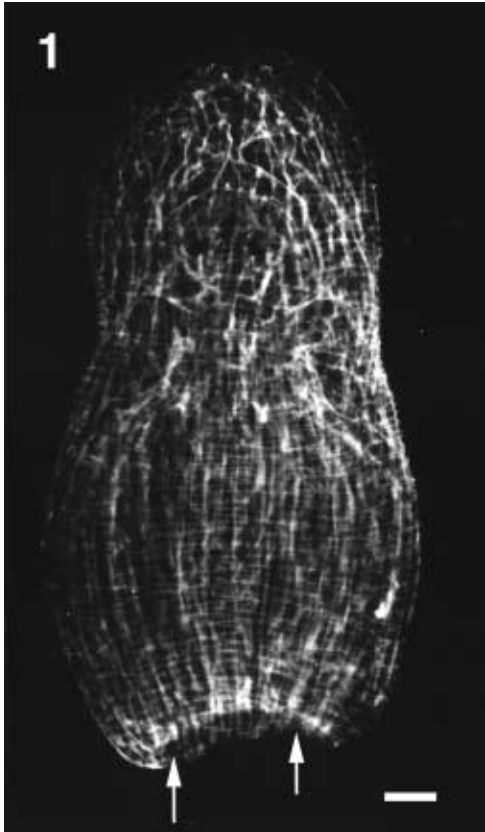

Fig. 1. – Phalloidin staining of *Macrostomum* sp., after dissection, dorsal view. Severed and contracted muscles are seen in the wound region. Scale bar: 25 μm.

two hours. Phalloidin staining at this time showed severed and contracted longitudinal and diagonal muscles and constricted circular muscle fibres. After two to eight hours the regenerating end appeared round and morphallactic processes became visible: The ends of muscle fibres in the caudal-most circular muscle bands were bent in caudal or diagonal direction. Some of the dorsal longitudinal fibres of the body-wall musculature appeared to be rearranged and showed outgrowth in caudal and medial directions. Other fibres at the lateral sides in the animal were bent diagonally (Fig. 2). The growth process started later or was slower in the case of the ventral longitudinal fibres, or these fibres remained contracted for a longer period of time. Consequently, the wound appeared shifted to a more ventral position (Fig. 2). In addition, a network of delicate muscle fibres was observed after 8 hours in the

region of the future blastema at this point. All muscles of the body-wall as well as some fibres from the gut musculature seemed to be involved in the formation of this network (Fig. 3).

In most animals the blastema became visible after 17 to 19 hours. Phalloidin staining after 30 (±6) hours of regeneration showed that the muscle network had completely disappeared. Instead, one could now observe caudal outgrowth and crossing of pre-existing longitudinal fibres, with extensive forking of these fibres at their connections to the basal matrix of the body wall (Fig. 4A). Faintly stained developing myocytes also became obvious, in perpendicular orientation to the longitudinal muscles (Fig. 4B). In contrast to the longitudinal musculature, circular fibres of the body-wall always developed from new myocytes. A few diagonal fibres could be seen at this time; they seemed to be derived from the longitudinal layer (Fig. 4B).

After two days, regeneration of the caudal end had started (Fig. 5 shows the stage after three days) and after seven days all anatomical elements of the caudal part appeared to be regenerated . The adhesive plate with its complex dorso-ventral musculature was rebuilt as well as the false seminal vesicle and the musculature of the male copulatory organ. After another week the regenerate had reached the same size as the posterior half in control animals.

The posterior fragments of the bisected animals did not survive, all of them died off within one to two weeks. We never found regenerated muscles in posterior fragments. Contraction of the circular muscles and wound closure were the only signs of regeneration. No further processes such as the formation of the network of muscles, outgrowth of longitudinal fibres etc., could be observed.

DISCUSSION

Wound closure is the first step in regeneration (see lit. in SÁNCHEZ ALVARADO, 2000). It is crucial for further survival to prevent exposure of the internal organs to the environment. In *Macrostomum* sp. wound closure is achieved by a contraction of circular musculature that leads to a reduction of the wound surface. Flattening of epithelial cells and rearrangements of junctional complexes occur within one to two hours (unpublished observation, D. Riedl). This phenomenon is also well known from freshwater triclads (BAGUÑÀ, 1998 and literature therein), from *Stenostomum leucops* and *Microstomum lineare* (PALMBERG, 1990, 1991) and is likely a common morphallactic process.

Two steps can be distinguished in the early muscle regeneration in *Macrostomum*. First, morphallactic processes take place whereby the musculature in the wound region is repaired and rearranged. Secondly, epimorphosis occurs in the form of differentiation of neoblasts into new muscle cells.
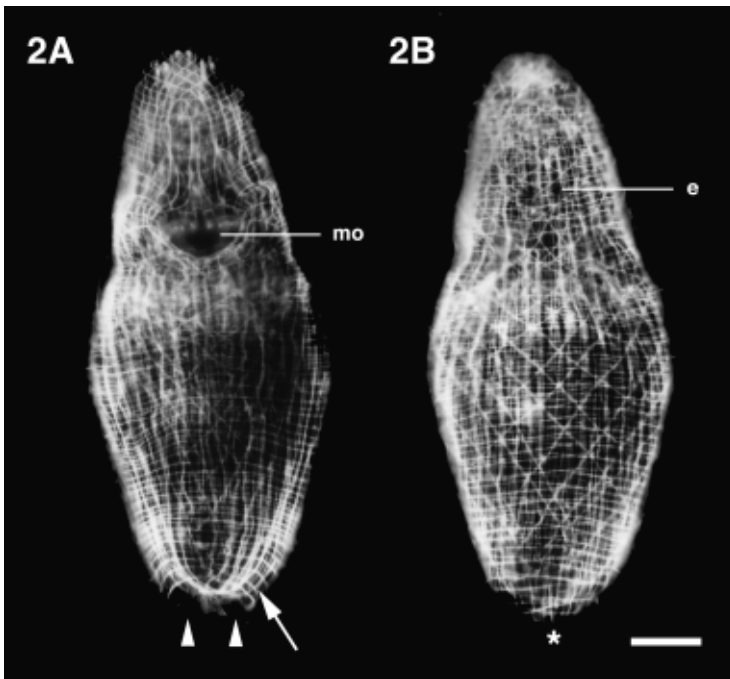
Fig. 2. – A: Ventral view of *Macrostomum* sp. after 2 h of regeneration. Wound is shifted slightly to ventral side (arrowheads). Some longitudinal fibres are bent medially (arrow). B: dorsal view of a specimen with intact body-wall musculature to the very caudal end (asterisk). Scale bar: 50 µm, mo = mouth opening, e = eye.
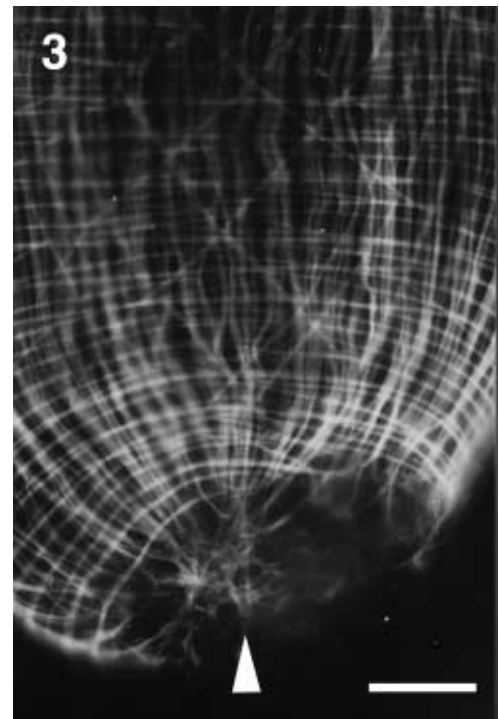

Fig. 3. – Phalloidin staining after 8 h of regeneration, ventral view. Note faint network of muscles in region of future blastema (arrowhead). Scale bar 15 µm.
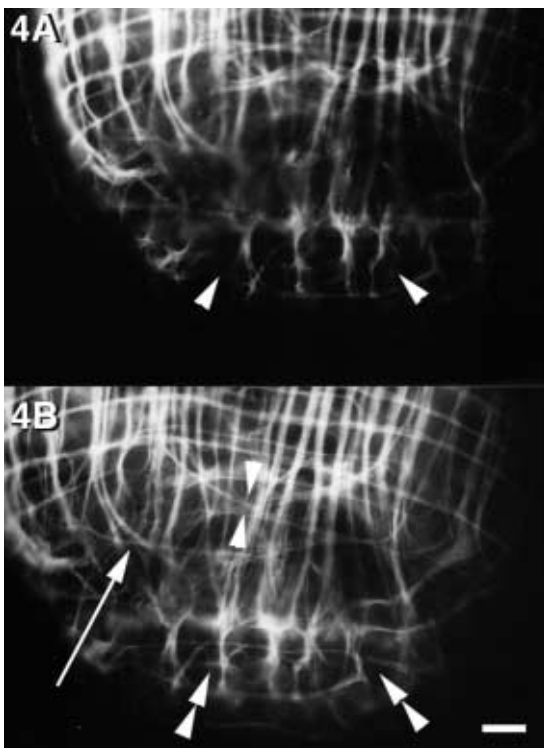

Fig. 4. – Ventral view of *Macrostomum* sp. after 24 h of regeneration. A: Outgrowth and forking of pre-existing longitudinal fibres (arrowhead). B: bending (arrow) and crossing (arrowhead) of longitudinal fibres. Note developing circular fibres (double arrowheads). Scale bar 15 µm.
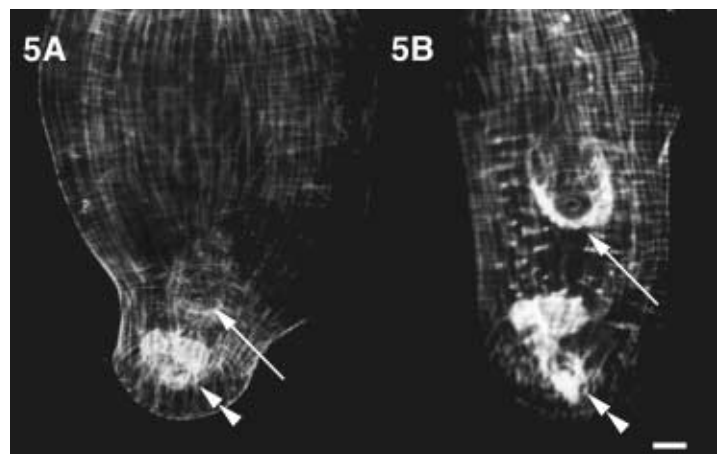

Fig. 5. – A: Phalloidin staining after 3 days of regeneration, ventral view. Note developing musculature of male copulatory organ (double arrowhead) and vagina (arrow) in the regenerating caudal adhesive plate. B: Caudal end with musculature of male and female copulatory system in mature control animal. Male copulatory organ (double arrowhead), vagina (arrow). Scale bar 25 µm.

The latter process is, however, different for circular and longitudinal muscles. While all fibres of the circular layer must be formed anew during regeneration, most fibres of the longitudinal layer result from outgrowth of pre-existing longitudinal fibres.

One hour after surgery the cut and contracted longitudinal fibres are clearly visible. During the following few hours outgrowth and bending of these pre-existing fibres in the body-wall musculature can be observed. The ventral displacement of the wound during restoration of the posterior end of *Macrostomum* sp. corresponds to the shift of the wound during restoration of the anterior end of planarians (CHANDEBOIS, 1980, CEBRIÀ, personal communication). This first step of regeneration does not require neoblasts; it is a purely morphallactic process.

The formation of the transient muscle network beneath the epidermis of the wound area, where the future blastema will be formed, may function in stabilizing the wound region. Its formation and later break-up are also morphallactic.

In *Macrostomum* a small blastema becomes visible after one day. In Platyhelminthes it is generally accepted that the blastema is formed both by undifferentiated neoblasts and by differentiating cells (HORI, 1983; MORITA & BEST, 1984a,b; SALO & BAGUÑA, 1984). At that point morphallactic and epimorphic processes can be observed simultaneously also in the regeneration of the body-wall musculature.

The formation of myocytes in rings perpendicular to longitudinal muscles at the periphery of the blastema is the first sign of muscle cell differentiation from neoblasts. The ultrastructural features of neoblasts in *Macrostomum* and their role in growth and regeneration have been demonstrated (RIEGER et al., 1999; LADURNER et al., 1998; LADURNER et al., 2000). Neoblasts are concentrated in the blastemal area (unpublished observation D. Riedl, W. Salvenmoser). NEWMARK & SÁNCHEZ ALVARADO (2000) have reported the distribution of S-phase cells and migration and differentiation of neoblasts in whole-mounts of *Schmidtea mediterranea*. PALMBERG (1990, 1991) has shown, in *Microstomum lineare* and in *Stenostomum leucops* with tritiated thymidine and autoradiography, that not all muscle cells in the regeneration area were labelled. Taken together, these findings indicate the simultaneous occurrence of epimorphic and morphallactic processes are common in early regeneration.

In *Macrostomum* sp. the nervous system also regenerates (unpublished results D. Riedl), and new muscle-nerve connections as well as specialized gap junctions (see RIEGER et al., 1991) must be formed to keep the musculature functioning. It was observed that as early as eight hours after surgery the main nerve cords had united and formed the caudal loop (data not shown). The involvement of neuroactive substances in activating neoblast differentiation during regeneration has been discussed previously (e.g. BAGUÑA et al., 1989). We assume that the lack of serotonergic neurons associated with the caudal portion of the central nervous system in the genus *Macrostomum* may in part be responsible for the incapacity of posterior fragments to regenerate anterior parts of the body (see LADURNER et al., 1997). The regeneration phenomenon cannot only be explained by the presence of neoblasts because S-phase cells are also present in the posterior fragment. Further investigations are necessary to elucidate this problem.

Later regeneration of the musculature in *Macrostomum* sp. is achieved primarily by epimorphosis, and the musculature of the male copulatory organ as well as the musculature in the adhesive plate must be formed from differentiating neoblasts. The growth of the regenerate to the final size is a process similar to cell maintenance (LADURNER et al., 2000, see also GREMIGNI, 1988 for differences between physiological and traumatic regeneration).

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