Cell interactions and regeneration control

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ABSTRACT This paper is a review of the main findings of our laboratory on the control of regeneration by cell interactions. These include results related to the role of both cell contact and local soluble factors in regeneration of the legs of insects and newts and of the parapodia and segments of nereis. The pattern of these structures is considered to be defined by positional information distributed as longitudinal and transverse positional value sequences carried by epidermal (insect) or mesenchymal (newt) cells. By associating tissues to create transverse and longitudinal discontinuities in these sequences, single or multiple regenerating structures were obtained. These structures are formed by the intercalation of cells characterized by intermediate positional values which fill the gap between the tissues in contact. Positional information may also be changed during regeneration by the nerve cord in nereis and retinoids in the newts. We describe additional cases where morphogenesis occurs without any overt discontinuity in positional information, such as from a locally injured or non-injured insect trochanter, or after deflection of nerves in nereis and newt. Regeneration following an amputation may be considered as a special case of intercalary regeneration, the first stage being the juxtaposition of normally non-contiguous cells resulting in a longitudinal or/and a transverse gap. We also report studies on local factors produced by nerves and the blastema during newt limb regeneration.The nerve factor is necessary for the division of blastemal cells. After denervation, mesenchyme differentiates in an abnormal way. The mitogenic signal from the nerves is mediated by the PKC pathway. Its production is enhanced by regeneration of cut nerve fibers. The blastema also produces growth factors. We show that the epidermal cap and mesenchyme contain acidic FGF-like factor, and that the proliferating mesenchyme stimulates nerve fibers to regrow into the blastema.

KEY WORDS: *regeneration, insect, newt, worm, positional information, growth factors*

Introduction

As is the case during embryogenesis, cellular interactions play a key role in regeneration. They involve cellular contact and soluble substances that are responsible for pattern formation and growth. During the last two decades we have studied the interactions involved during the regeneration of the organs of different animals including the annelid *Nereis*, the stick insect *Carausius*, the spider *Tegenaria* and the urodeles *Pleurodeles* and *Ambystoma*. Initially, the interactions involved were explored by juxtaposing normally non-adjacent cells (Bart, 1965a, b). Our results lead us to conclude that the cells involved in morphogenesis have a differentiation depending on their location. In other words, they have positional information (Wolpert, 1969).The first part of this paper presents our main results in this field. Furthermore, potential morphogenetic centers need systemic and local stimulating growth substances to develop. In this field, we studied the control of proliferation by trying to understand how locally produced factors, namely nerve factors and blastemal factors, exert their mutual influence on either the nervous system or the epidermal and mesenchymal cells of the blastema. These results are considered in the second part of this paper.

The role of cell contact in regeneration

Regenerates of the appendages originate from the stump tissues lying close to the the cut surface. In the urodele limb regeneration blastema, the apparently undifferentiated blastemal

Abbreviations used in this paper: a-p, antero-posterior; c-AMP, adenosine 3', 5'-cyclic monophosphate; d-v, dorso-ventral; FGF, fibroblast grwoth factor; GAG, glycosaminoglycan; NdBGF, nerve derived blastema growth factor; p-d, proximo distal; PC12, rat adrenal pheochromocytoma cells; PKC, protein kinase C; TPA, 12-0-tetradecanoylphorbol-13-acetate.

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cells derive from the stump tissues and their morphogenetic potency can be expressed when the blastema is grafted to foreign territories (reviewed by Stocum, 1984). A narrow apical zone of stump tissue is involved in the regeneration processes of the *Pleurodeles* limb (Lheureux, 1972). Similarly, if the trochanter of a stick insect leg is transplanted onto the coxa of another segment leg or in place of a leg, it develops the parts that should lie distal to it (Bart, 1970a). In annelids, by local X irradiation (Boilly, 1969a, b) or intracoelomic injection of thorium oxide (Boilly, 1969c), we demonstrated that the tissues at the body amputation plane are the only tissues to be involved in the segment regeneration.

Legs of insects or spiders, parapodia and segments of nereids, and limbs of urodeles are organized in a spatial pattern defined with reference to spatial coordinates corresponding to the classical proximo-distal (p-d), antero-posterior (a-p) and dorso-ventral (d-v) axes. The a-p axis of an entire animal is called the cephalo-caudal axis.

Transverse discontinuity

Insects

Supernumerary structures in insects were reported long ago (Bateson, 1894). To explain how they form, Przibram (1921) supposed that the two surfaces exposed by a partial break of a leg were capable of giving rise to supernumerary limbs. Later, Bodenstein (1937), Furukawa (1937) and Balazuc (1947) experimentally obtained similar structures by introducing a non concordance in the orientation between a stump and a graft. They proposed that the non concordance caused the subdivision of the initial morphogenetic field.

Initially, we showed that when a right leg of a stick insect is grafted onto the stump of a left leg, either the d-v or a-p axis of the graft is reversed with respect to the corresponding transverse axis of the stump, and two supernumerary legs usually developed (Bart 1965a). The supernumerary legs arose from the junction between the stump and the graft, on opposite sides of the operated leg, either anterior and posterior or dorsal and ventral, according to the axis that had been reversed. Similar results have been obtained in the spider *Tegenaria saeva* (Lheureux, 1970). By grafting an epidermal area from the coxa into a window cut out of the opposite face of the coxa, we obtained one or two supernumerary formations developed from the contact between the associated epidermis (Fig. 1). When the coxal graft was replaced by a sternal or tergal epidermal graft, no supernumerary outgrowth formed. This result pointed out the main role of the leg epidermis in insect leg regeneration (Bart 1965b, 1966, 1971b). Considering that wound healing of an amputated or autotomized limb promoted the contact between epidermal cells from opposite sides, we proposed that this contact is the causal factor triggering regeneration (Bart 1965b). Usually, the symmetry relationships between supernumerary and axial legs are those described by Bateson (1894). For example, following the grafting of a dorsal epidermal area of the coxa into a ventral window of the same segment, the dorsal side of the new structure is continuous with the dorsal epidermis of the graft, whereas the ventral side is continuous with the ventral epidermis of the recipient. The new structure is a mirror image of the operated leg (Bart 1970a). By associating prothoracic and metathoracic leg tissues differing in morphology and pigmentation, and by grafting an antennal epidermal area into a window in the leg, we obtained chimera formations (Bart 1971b). For example, the leg and antennal part of the regenerate were directly continuous with the initially associated leg and antenna epidermis. However, different results were obtained in *Tegenaria*, by grafting a pedipalp onto a leg stump. Two supernumerary appendages developed, one originating from the stump and the other one from the graft (Lheureux, 1971). This result is similar to some results obtained by Bullière (1970) and Bohn (1972) in cockroaches. Compared with the above *Carausius* experiments, stump and graft were transversally complete and associated end to end. It seems that a physiological isolation occurred between the stump and the graft and allowed each of them to regenerate from most of their amputation surface.

On the other hand, we established that the transverse positional information was present along the entire leg, from the coxa to the tarsus, and that any association of opposite positional information, even between epidermis of different segments, provokes outgrowth formation.

When only two opposite epidermal sectors are juxtaposed, the resulting supernumerary regenerate, which is formed without direct contribution of epidermis from the other sides, is nevertheless transversally complete. Therefore, the confronted regions of epidermis maintain their differentiation, but they are also able to form new positional values, those of the adjacent sides. We also performed interspecies associations of leg segments between the stick insects *Carausius*, *Sipyloïdea*, and *Clitumnus* (Bart 1974). In spite of the apparently complete elimination of the grafts as early as the first post-operative molt, we observed normal regenerates originating only from the epidermis of the recipient, showing that only one species provided a complete set of positional values from a partial set of values. Thus, the stability of cellular determination of a given sector is not irreversibly determined. This result is closely connected to that obtained by French and Bullière (1975a, b) on *Blabera craniifer*. The latter suggested a distribution of transverse positional values and pointed out the importance of transverse intercalary regeneration in morphogenetic center formation (French, 1976; French *et al.*, 1976). However, conditions of intercalary regeneration in *Blabera* are different from those of *Carausius* where, in the case of a discontinuity caused by the removal of one of the four sides of a leg, the larval epidermis heals without intercalating a regenerate corresponding to the missing side. As long as the epidermal discontinuity is not maximal, cells from the dedifferentiated epidermal edges in contact are not able to yield the missing positional values.

The supernumerary legs usually show the parts that lie distal to the level of the junction between the stump and the graft. Sometimes, the more proximal structures may be missing and in some cases the regenerate consists of an apical tarsus directly associated with coxal or femoral tissues. Obviously, in these cases, regeneration does not obey the progressive distalization rule proposed by Bryant *et al.* (1981).

Amphibians

Supernumerary limbs have also been produced in amphibians, either in embryos (Harrison, 1918 among others) or in larval and adult stages (reviewed by Tank and Holder, 1981, Wallace, 1981). In the newt *Pleurodeles*, we tried to induce supernumerary morphogenetic centers to appear by juxtaposing tissues from opposite sides. Several experimental processes were used. The first one consisted in modifying the relative location and orientation of skin

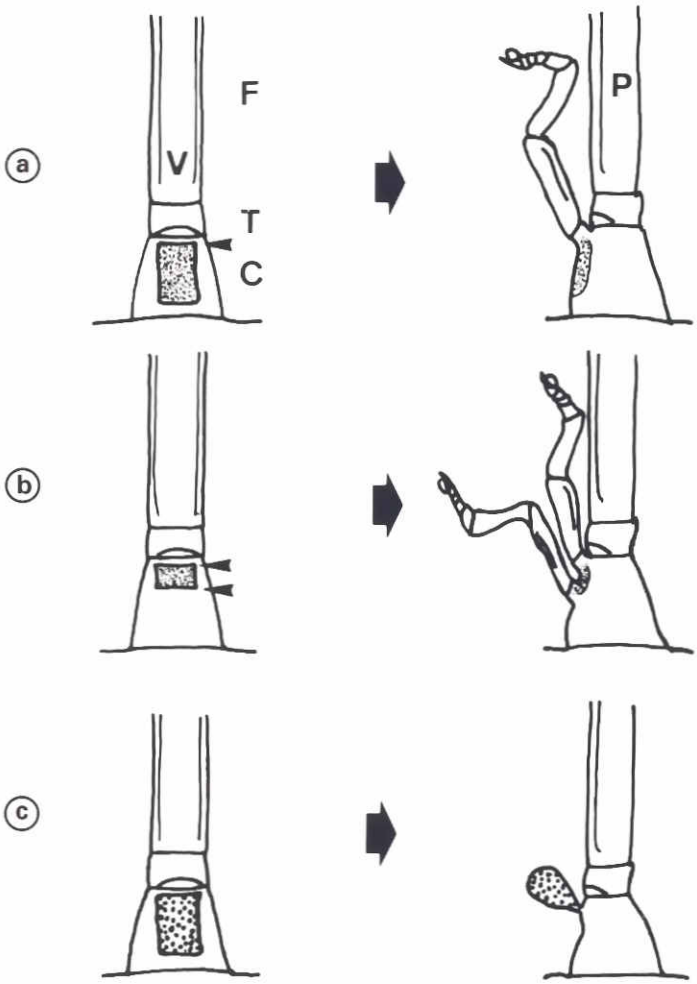

Fig. 1. Transverse epidermal differentiation and morphogenesis in the *Carausius* leg. *Three different cases of grafts are presented, viewed from the ventral side (V) of the leg base, immediately after grafting. In* **(a)** *and* **(b)** *dorsal coxa areas of different sizes have been grafted, whereas in* **(c)**, *the graft is a sternal or a tergal piece. The corresponding more interesting results at the second post-operative moult are seen from the posterior side (P).* **(a)** *A supernumerary leg has been formed at the distal junction of the graft and the recipient coxa (arrowhead). Its d-v orientation is opposite to that of the recipient leg.* **(b)** *When the graft has a short proximo-distal length, two symmetrical supernumerary legs may be formed respectively at the proximal and the distal junctions (arrowheads).* **(c)** *The coxa rejects the graft and heals. No supernumerary leg morphogenesis occurs. C: coxa; T: trochanter; F: femur*

or muscle sectors of the stump (Lheureux, 1972, 1975a, b). The second technique of induction consisted of deflecting the brachial nerve tip to the surface of the limb where tissue fragments from opposite sides had been juxtaposed (Lheureux, 1977). The third one consisted of associating left blastema grafts and right stumps, so that one of the transverse axes of the graft, d-v or a-p axis, was reversed with respect to the transverse axis of the stump (Lheureux, 1978). All these techniques provided many supernumerary structures varying in location, orientation and development, and we concluded that a juxtaposition of tissues from opposite sides is necessary for the formation of a supernumerary limb with a normal pattern (Lheureux, 1972, 1975a, 1981). Transverse intercalary regeneration probably occurs in supernumerary limb formation as proposed by French *et al.* (1976).

The triploid marker was also used to study the contribution of stump and blastema cells to the supernumerary regenerates. It was established that there is a more or less equal contribution of the tissues from the blastema and the stump to the supernumerary limbs in *Pleurodeles* (Fig. 2a) (Lheureux, 1988) as also occurs in the axolotl (Muneoka and Bryant, 1984). A sharp limit was usually established between diploid cells from the stump and triploid cells from the blastema. In that way, the intercalary transverse regeneration corresponds to a set of cells bearing the missing positional values which are gradually added from both experimentally joined tissues.

In special cases, it was observed in *Pleurodeles*, as we showed in the stick insect, that the cells of the supernumerary limb originated from only one of the mismatched tissues grafted together. This is the case when a healthy triploid blastema is transplanted onto an irradiated diploid limb stump. Multiple limbs form as previously shown in other species (Adler and Bryant, 1977; Maden, 1979; Holder *et al.*, 1979), proving that irradiated tissues can keep their positional information. An analysis of the ploidy of all the skeletal elements of these limbs reveals they are composed of non-irradiated triploid cells only (Fig. 2b) (unpublished data). During these events, the positional information of the irradiated tissues only served as boundary values for the non-irradiated tissues that were in contact with them. Since a gap of transverse positional values was experimentally introduced, the healthy tissues dedifferentiated and the resulting cells divided and intercalated the missing values to constitute a complete sequence of transverse positional values, a requisite for a normal supernumerary limb to develop.

Another question is whether all tissues bear positional values. Epidermis is not morphogenetic (Carlson, 1975; Lheureux, 1976). Indeed, a cuff of limb dermis behaves in the same way when it is covered with a sheet of epidermis with a normal or reverse

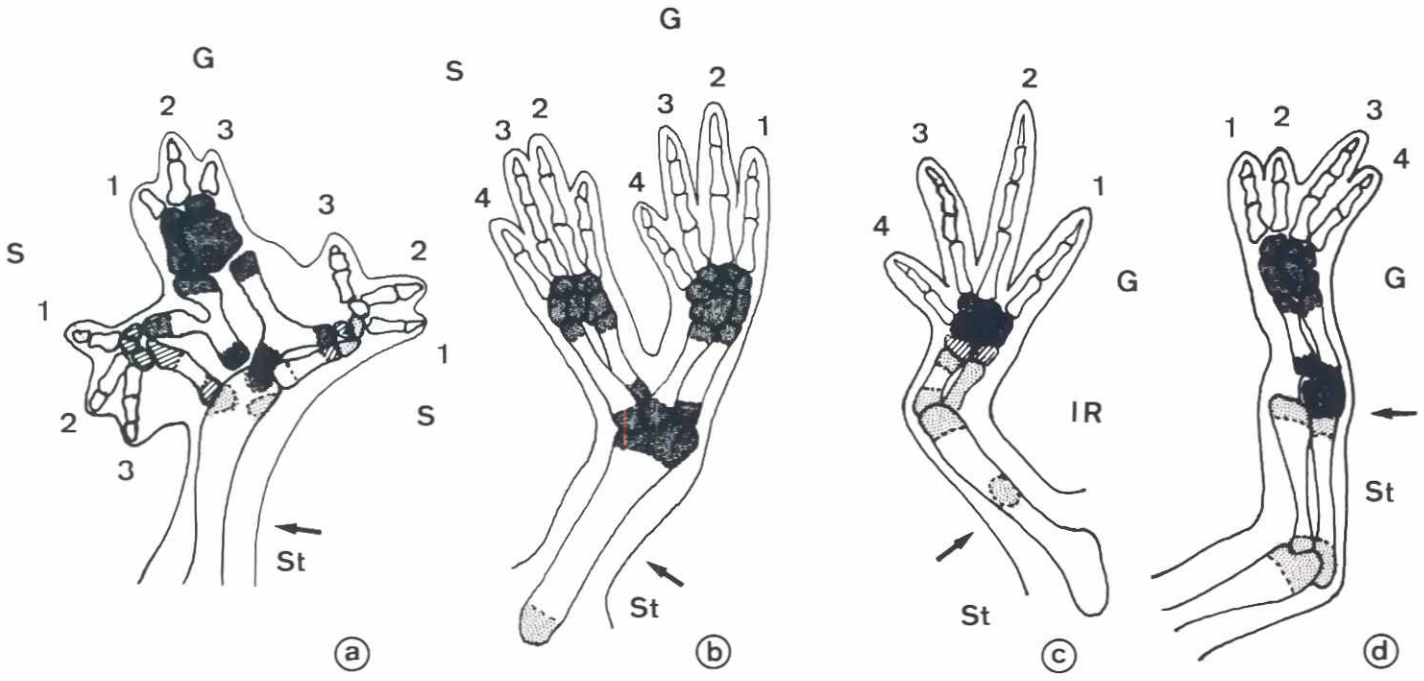

Fig. 2. Contribution of triploid blastema and diploid stump cells in *Pleurodeles* in the skeleton of duplicated limb regenerates resulting from different experiments. *Triploid skeletal elements are shown by heavily stippled areas; diploid skeletal elements are shown by lightly stippled areas; mixed skeletal elements are shown by hatched areas.* **(a and b)** *Transverse duplications formed at the elbow after transplantation of a triploid proximal stylopod blastema onto a diploid contralateral stump at the same p-d level. The a-p axis of the graft was reversed with respect to the a-p axis of the stump.* **(a)** *Blastema and stump are non-irradiated. Supernumerary outgrowths consist of blastema and stump cells.* **(b)** *The stump is irradiated and the blastema is non-irradiated. The entire skeleton of the regenerate derived from non-irradiated tissues.* **(c)** *Left limb regenerate following ipsilateral transplantation of a triploid wrist blastema onto a mid-stylopodium stump. The normal limb shows an intercalary regenerate originating from the stump tissues.* **(d)** *Right limb regenerate following ipsilateral transplantation of a triploid mid-stylopodium blastema onto a diploid wrist stump. The entire regenerate originated from the graft.*

orientation. The mesodermal tissues such as muscle and dermis bear positional values since a variety of experimental juxtapositions between muscle, skin or dermis from opposite sides resulted in extra limbs (Lheureux, 1972, 1975a, b). No convincing experiment suggests that cartilage is morphogenetic but this possibility cannot be rejected (Lheureux, 1983a).

Annelids

Parapodia show clear d-v polarity, having dorsal and ventral cirri, a bilobed dorsal notopodium and a bilobed ventral neuropodium. The data concerning both parapodial regeneration and the induction of parapodia by deflecting the nerve cord only to the lateral part of the body wall, suggest that parapodial morphogenesis relies on interactions between the dorsal and the ventral body wall. The results of experimental transplantations of parapodia (Boilly-Marer, 1969), or body wall sectors cut off from dorsal or ventral sides (Boilly-Marer, 1971a, b), clearly show that the juxtaposition between the dorsal and the ventral body walls induces supernumerary parapodium formation (Figs. 3, 4). These parapodia develop at the junction of dorsal and ventral territories if the parapodial nerve is present at this place (Boilly-Marer, 1971b). Thus the grafting of parapodia results in the formation of one supernumerary parapodium per segment, since only one side of the grafted parapodium is placed in contact with an opposite side of the recipient segment. On the contrary, a body wall graft generates two supernumerary parapodia since two edges of the graft confront the body wall of the recipient. In all cases, the orientation of the supernumerary parapodia is such that their dorsal side is continuous with the dorsal recipient segment - or dorsal graft - and similarly, their ventral side is continuous with the ventral side of the recipient segment. These results suggest that parapodia are lateral transition elements between the dorsal and the ventral sides of the segments (Boilly-Marer, 1969).

D-v positional information was also demonstrated in the peristomium, the first segment where the parapodia have regressed during the larval period, except for their dorsal and ventral cirri which lengthen and give the long peristomial tentacles. The grafting of either dorsal or ventral body wall areas from peristomium onto the opposite side of another segment did not induce typical parapodia but only gave rise to hypomorphic parapodia or only cephalic appendages like tentacular cirri or antennae. In contrast, no morphogenesis occurred when a dorsal or ventral peristomial body wall area was grafted onto the homologous side of another segment (Boilly-Marer, 1974).

Although we do not know which tissue carries the d-v polarity, we showed that the ventral nerve cord controlled d-v polarity during caudal regeneration (Boilly and Combaz, 1970; Combaz and Boilly, 1974). In the absence of the nerve cord, the regenerate is devoid of parapodium and anal segment cirrus (Fig. 5) (Boilly and Combaz, 1970; Combaz and Boilly, 1974). Moreover, in these regenerates

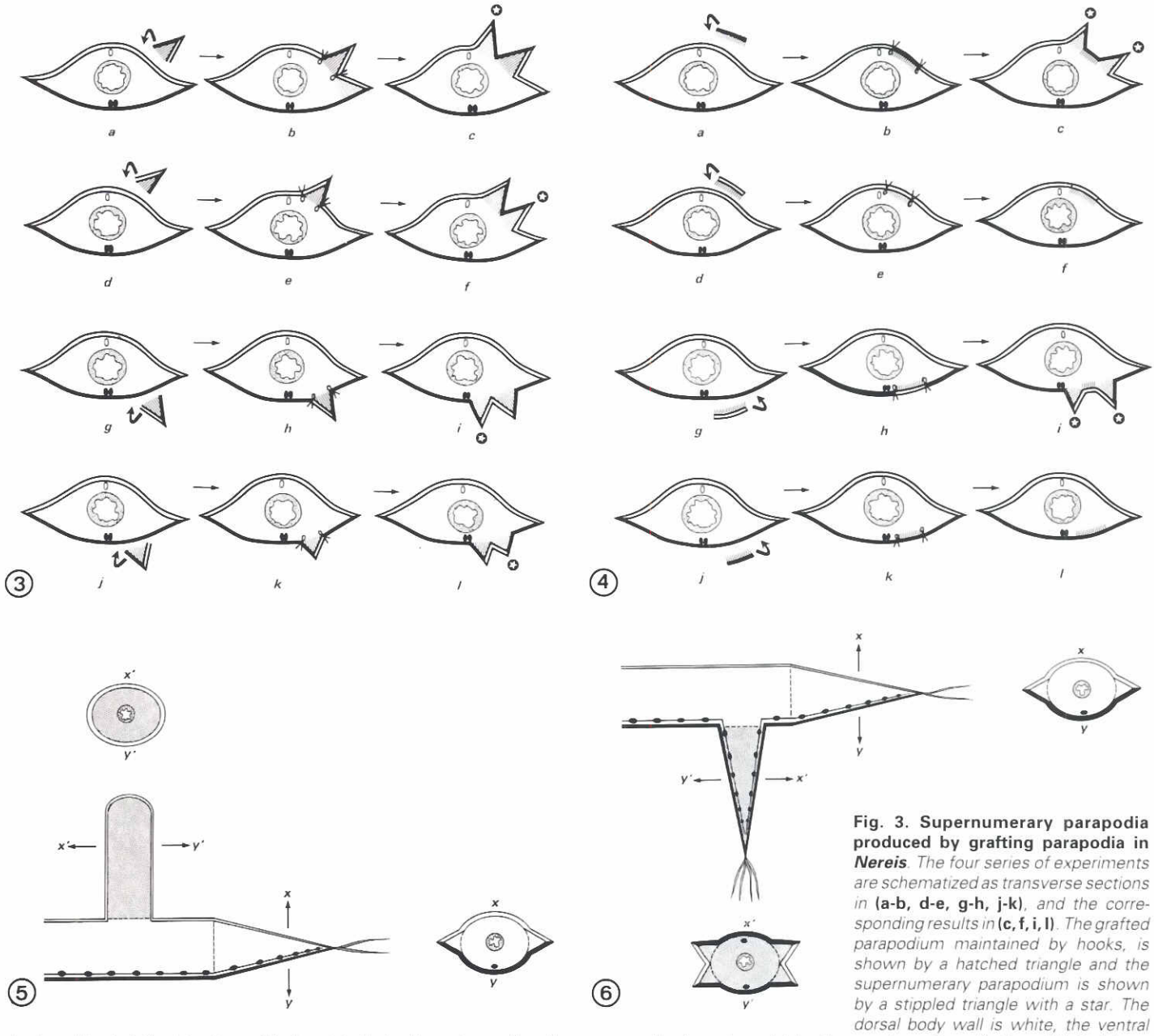

Fig. 3. Supernumerary parapodia produced by grafting parapodia in *Nereis*. *The four series of experiments are schematized as transverse sections in* **(a-b, d-e, g-h, j-k)**, *and the corresponding results in* **(c, f, i, l)**. *The grafted parapodium maintained by hooks, is shown by a hatched triangle and the supernumerary parapodium is shown by a stippled triangle with a star. The dorsal body wall is white, the ventral body wall is dark, the intestine epithelium is indicated by a sinuous line; the nerve cord is shown by solid double egg-shape. From Boilly and Boilly-Marer, 1972, with authorization*

Fig. 4. Supernumerary parapodia obtained by grafting a body wall sector. *The four series of experiments are schematized as transverse sections in* **(a-b, d-e, g-h, j-k)**, *and the corresponding results in* **(c, f, i, l)**. *The grafted body wall sector maintained by hooks, is hatched and each supernumerary parapodium is shown by a stippled triangle with a star. The dorsal body wall is white; the ventral body wall is dark; the nerve cord is shown by solid double egg-shape. From Boilly and Boilly-Marer, 1972, with authorization*

Fig. 5. Supernumerary caudal extremity formed in the absence of the nerve cord, on the dorsal side of a worm after a deviation of the intestine. *This induced dorsal tail looks like a cylinder because of the equal sizes of its segments. The absence of a gradual decrease in segment size suggests that there is no cephalo-caudal polarity. The induced dorsal tail formed neither parapodia nor anal cirri. Thus, its body wall is supposed to be only dorsal (X' Y'= transverse section of this regenerate) compared to the normal tail regenerated in a caudal direction (X Y= transverse section of this regenerate) which presents a normal cephalo-caudal polarity and a typical dorso-ventral structure. The nerve cord is shown by a solid line with solid circles and, in transverse sections, it is shown by double egg-shape. The intestine has only been drawn on the transverse sections. From Boilly* et al., *1975, with authorization.*

Fig. 6. Supernumerary caudal extremity formed in the presence of the nerve cord, on the ventral side of a worm after deviation of the intestine. *The induced ventral tail has a typical cephalo-caudal polarity, two nerve cords, two dorsal sectors, two ventral sectors, four parapodia per segment and four anal cirri. (X' Y'= transverse section of this regenerate). The distal tail regenerate is normal. (X Y= transverse section of this regenerate). The nerve cord is shown by a solid line with solid circles and, in transverse sections, it is shown by double egg-shape. The intestine has only been drawn on the transverse sections. From Boilly* et al., *1975, with authorization.*

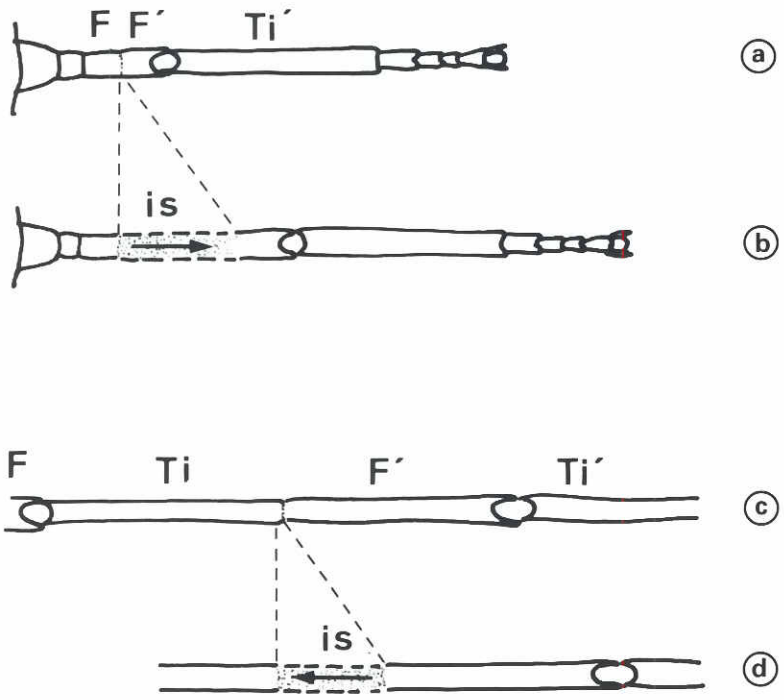

Fig. 7. Longitudinal discontinuity and intercalary morphogenesis in the *Carausius* leg. (a) *A distal femur part is grafted onto a proximal femur stump.* **(b)** *After several moults, the distal part has formed an intercalary structure (i s) with proximal features (proximalization) and a normal proximo-distal orientation (arrow).* **(c)** *A proximal femur (F') level is grafted onto a distal tibia level.* **(d)** *The intercalary structure observed after several moults has been formed by the distal end of the tibia. It is tibial and has reverse proximo-distal polarity with respect to the rest of the leg (arrow). F: recipient femur; F': grafted femur; Ti: recipient tibia; Ti': tibia of the graft.*

there is no d-v differentiation of the papillae around the anus in the heteronereis forms (Boilly-Marer and Combaz, 1972). By grafting dorsal or ventral body wall areas from these regenerates onto normal segments, supernumerary parapodia were induced on the ventral side only, whatever the origin of grafts. Thus, in the absence of the nerve cord, the body wall of the regenerates bears only dorsal positional values (Combaz and Boilly-Marer, 1976). During normal regeneration, the nerve cord would be responsible for the ventral positional values. Actually when a worm on which an additional nerve cord was grafted in a dorsal location is amputated through the graft, each regenerated segment forms four parapodia (Combaz, 1975). These results suggest that such regenerates have two ventral sides in opposite locations, each of them facing a nerve cord and that they are separated by two dorsal sides replacing the lateral sides. Furthermore, a similar state was observed in *Nereis* when the nerve cord was accidently split into two parts (Fig. 6) (Boilly-Marer, 1970; Boilly *et al*, 1975).

Longitudinal discontinuity

Proximo-distal axis of the limbs of arthropods and urodeles

Insects

Bohn (1967, 1970a, 1971) was the first to establish that a proximo-distal gradient of epidermal differentiation exists in cockroach larval legs. This gradient does not extend over the full length of the leg but spans each segment and is repeated in all segments. When cut surfaces from the same level of one segment or from the same level of two different segments, such as femur and tibia, are experimentally joined, no morphogenesis occurs. On the other hand, the association of surfaces from different levels of either one segment or two different segments results in an intercalary regeneration that removes the initial discontinuity. Any intercalary regenerate is polarized proximo-distally in agreement with the initial levels of the associated surfaces. If the mismatched parts correspond to a distal level of the stump and a proximal level of the graft, the proximo-distal polarity of the intercalary regenerate is reversed with respect to the rest of the leg. In contravention of the general rule of distalization, the intercalary regenerate originates, in most cases, from the distal level which therefore forms more proximal structures (Bohn, 1970a, b, 1971, 1976).

We obtained results similar to those of Bohn (Bart, 1969, 1972) without, however, obtaining undeniable proximalizations. By the use of regenerated femur or tibia, we recently showed that the epidermis of the distal femur was able to proximalize when it was joined to proximal femur or tibia. Moreover, when a proximal femur and a distal tibia were grafted together, the intercalary regenerate was only tibial (Fig. 7) (Bart, 1988). This result proves that the femur of a *Carausius* leg does not exert any "dominance" on the tibia as hypothesized by Bullière (1971) and Bullière and Bullière (1985), who suppose that the leg is characterized by only one p-d sequence and that the tissues of a segment "dominate" the more distal segment tissues. However, when distal and proximal cells are joined, proximalization usually occurs, because proximalization of distal cells can happen more rapidly than distalization of proximal cells. This could be linked with the fact that the distal parts of the femur and the tibia grow faster than the proximal ones (Bart, 1988).

Amphibians

We first tested the p-d potencies of limb tissues from different levels of the p-d axis. We used X-irradiated limbs as recipients for grafts of healthy skin from different levels (Lheureux, 1975b). A proximal non-irradiated skin cuff grafted on the stump of an irradiated limb at the level of distal zeugopodium allowed the development of all the structures distal to the graft level, and conversely, a skin cuff from a non-irradiated wrist grafted on a stylopodium of an irradiated limb allowed the development of only

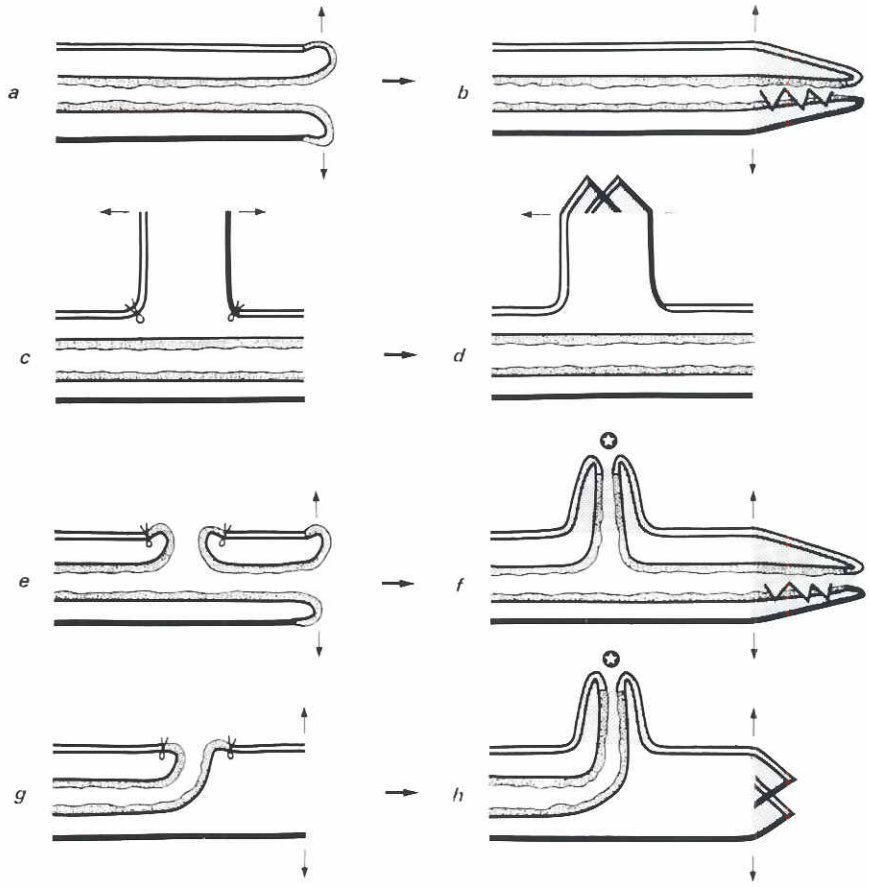

Fig. 8. Supernumerary segments induced by deviating the tip of the cut intestine through the dorsal body wall. *The experiments are schematized in* **(a, c, e, g)**, *and the results obtained in* **(b, d, f, h)**. *The arrows indicate the plane of amputation. Caudal regeneration from a stump devoid of intestine induces the formation of parapodia instead of segments* **(d, h)**. *Normal regeneration is shown in b and f in the caudal direction; in these cases, parapodia (shown by three triangles) differentiate on the new segments while they are never observed on the caudal regenerates (stars in f, h) formed from the deflected intestinal tip. The nerve cord has not been drawn. From Boilly and Boilly-Marer, 1972, with authorization*

a hand. It was concluded that each level of the healthy skin controlled the distal development of the regenerate.

Stocum (1975) on the axolotl and Iten and Bryant (1975) on *Notophthalmus viridescens* grafted together limb blastema and limb stump from different p-d axis levels, to introduce either a potential lack or excess of structure between the juxtaposed surfaces. They obtained a normal limb in the regenerates of the first series and excess of structure in the regenerates of the second series. Similar experiments were performed on *Pleurodeles* limb and gave similar results (Lheureux, 1978). When a blastema from a proximal stylopod level is grafted onto the distal wrist stump, it develops all the distal parts which should lie distal to its level of origin. Since a nearly complete limb develops beyond the wrist, thus giving a regenerate with excessive limb structures, the distal limb stump does not exert any control on more proximal level tissues. On the other hand, when a wrist blastema is transplanted onto a proximal limb stump, the missing part located between the levels of the associated graft regenerates.

Compared to similar associations in the insect segments, some results are different. An amphibian intercalary regenerate only forms when intercalary structure is removed. It never appears in the case of extra structure, and reverse p-d segments never develop. One question was whether the intercalary regenerate of the amphibian limb originated from grafted blastema or stump tissues. In other words, is a given tissue able to produce more proximal cells as observed in the insect segment? To answer this question, Pescitelli and Stocum (1980) used triploid tissues of axolotl as a marker. A triploid blastema from the wrist was transplanted onto a proximal limb stump of a diploid animal. The resulting intercalary regenerate was diploid, proving that the distal positional values are produced only from the more proximal cells. In *Pleurodeles*, by analysing the ploidy of cartilaginous elements, we confirmed that the intercalary structure originated only from more proximal structures (Fig. 2c). As expected, a triploid proximal blastema transplanted distally provided all the cells of the regenerated part (Fig.2d) (unpublished data).

However, by the use of retinoids during amphibian limb regeneration, a proximalization of the distal tissues of the limb has been obtained (Niazi and Saxena, 1978; Maden, 1982; Lheureux *et al.*, 1986). The particular feature of the response of *Pleurodeles* regenerating limbs treated by retinol palmitate consists of a proximalization of the tissues when the treatment was applied to old larvae, and a transverse duplication when it is applied to regenerating limb buds of newly hatched larvae.

Cephalocaudal axis in Nereis

In Nereids, the parapodia are short appendages showing no clear markers that may be used to study their proximo-distal regeneration by means of experimental manipulations. Taking the whole body of the worm into consideration, we observe a cephalocaudal gradient concerning the size of the regenerating segments, the smallest ones being caudal. Cutting of the worm body between

two segments leads at first to an extrusion of the intestine and then to a close healing of the wounded integument and the intestine epithelium. This process results in the formation of a new anal segment and a new growing zone that allows new segments to regenerate. The regeneration of the anal segment seems to be initiated by the contact established between the intestine and the body wall of the worm (Boilly, 1969a, 1974). To investigate the importance of such a contact, we amputated a worm and removed its intestine at the level of the wound. No anal segment formed and no segmental regeneration occurred. Only new parapodia appeared at the stump where the dorsal wall and the ventral wall of the body healed together (Fig. 8). Conversely, an experimental (Boilly, 1973) or accidental contact (Boilly *et al.*, 1975) between the deviated intestine and either the dorsal or the ventral body wall resulted in regeneration of segments as during the normal caudal regeneration (Figs. 5, 7, 8). Although our results prove that the ventral nerve cord is not necessary for segment regeneration because such a regeneration is possible on the dorsal side of the worm, it seems that the nerve cord controls the cephalo-caudal differentiation of the regenerates. Thus, segments regenerated on the dorsal side were all the same size. Likewise, the reamputation of such regenerates did not allow a segment regeneration (Combaz, 1972). Thus, it is possible to consider that the anal segment is regenerated to insure a normal transition between the intestine wall and the body wall just as we consider the parapodium as a transition between the dorsal and the ventral body wall of the worm. In addition, the juxtaposition of the anal segment and the stump will induce the intercalation of additional segments until the cephalocaudal gap is filled.

Regeneration without overt discontinuity

Conditions that differ from contact between cells with different positional values can also induce morphogenetic processes.

Insects

In the stick insect, local injuries involving one sector of either the proximal or distal trochanter can trigger an outgrowth close to the injury site without modifying the rest of the leg. When local removal of a femoral sector is performed next to the trochanter, a supernumerary structure is induced at the level of the injury. This structure shows only one overtly differentiated side. It may be composed of femur, tibia and tarsus. An equivalent result is obtained when an area of femur contiguous with the trochanter is cut off and immediately replaced at the same location and with the same orientation (Fig. 9). In the above experiments, triggering of morphogenesis is most efficient when the epidermis is broken between the trochanter and the femur. Since the autotomy occurs between the trochanter and the femur, these results suggested that regeneration following an autotomy is the sum of local injury responses, each injury being considered as partial autotomy (Bart, 1970b). Nevertheless, the association of opposite positional values in triggering regeneration following an autotomy cannot be excluded.

A morphogenetic process may occur in a non injured trochanter. The limit between the trochanter and the femur is a fracture zone where autotomy occurs when the leg is injured in the femur. From the trochanter stump, regeneration occurs faster than at any other level. When regeneration proceeds from a short femoral stump with a length similar to the trochanter, morphogenetic processes occurred in both femur and trochanter (Bart, 1971a). On the one hand, the femur stump regenerated tarsus, sometimes tarsus plus tibia, and in most cases tarsus plus tibia plus femur. Apparently, regeneration from femur stump is disto-proximal. On the other hand, the trochanter regenerated one or several segments in a proximo-distal direction. The resulting legs are therefore composite. They are nearly normal in about three-quarters of the cases, though the parts produced by the femur and the trochanter are variable. In the other cases, the regenerates have extra parts.

Amphibians

To test the necessity of contact between two tissues from opposite sides, we have limited the contact to cells bearing very closely-related positional values. This was carried out by cutting off a skin flap from the p-d axis of the limb and grafting it after 90° rotation as a cuff around the stump of an X-irradiated limb, in place of the irradiated skin. This resulted in hypomorphic regenerates (Lheureux, 1975b). On the other hand, when a healthy skin cuff from the contralateral limb was transplanted without p-d rotation in place of the corresponding skin of an amputated irradiated limb, a normal regenerate developed, since the graft contained an entire sequence of positional values. The skin flap from the p-d axis of the limb only bore a few neighboring positional values, which were not sufficient to give rise to a complete set of transverse positional values.

Another experimental manipulation in *Pleurodeles* consisted of the induction of a local outgrowth by deflecting the apical brachial nerve tip through a skin slit, without creating a positional gap (Lheureux, 1977). In this case, regenerates corresponded at best to a two-digit structure, without a clear skeletal pattern.

Annelids

In annelids it is also possible to obtain supernumerary structures by deflecting nerves through the body wall. Parapodium morphogenesis was induced when the nerve cord was deflected through the intersegmentary lateral body wall only (Combaz, 1974). Since such a structure was not obtained when the nerve cord was deflected through the ventral or the dorsal body wall, we consider that the lateral body wall corresponds to the place where the dorsal and the ventral sides of the body meet. In some cases, supernumerary parapodia developed ventrally between the stump and the regenerate when caudal regeneration occurred without the nerve cord (Combaz and Boilly, 1971). Their occurrence can be interpreted as the result of the juxtaposition of the ventral body wall of the stump and the ventral sector of the regenerate. We considered that the ventral body wall of this regenerate differentiated dorsal positional values because of the absence of the nerve cord (Combaz and Boilly, 1971).

The role of local soluble factors in regeneration

Regeneration also depends on local factors that may be produced by nerve and blastema.

Factors from nerves

The nervous system seems not to be concerned in insect leg regeneration (Bart, 1970a) but it is an essential factor in annelid and amphibian regeneration.

In the annelids, histological data suggest that the tip of the severed nerve cord releases a cell stimulating factor (Boilly, 1967, 1968). However, since an actual denervation of the worm remains impossible because nerve cell bodies lie along many nerves, the

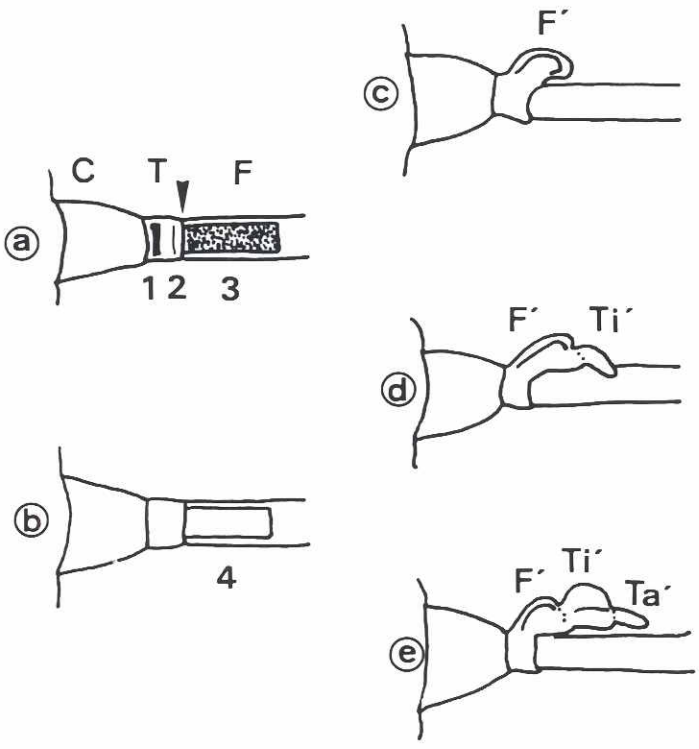

Fig. 9. Experiments resulting from injuries to the *Carausius* trochanter. *Trochanter injuries are numbered 1, 2, 3, 4 in a and b: 1. severe proximal injury; 2. light distal injury; 3. removal of a femur area contiguous with the autotomy level; 4. removal of the same femur area followed by an orthotopic reimplantation. The different structures observed are shown in c, d, e. Following 1,3,4, outgrowths are frequently formed (about 3/4 of the cases) in the region of the trochanter injuries. They are less frequent following 2 (1/4 of the cases). After 1, a femur outgrowth (F') is usually formed. After 3 and 4, the outgrowth sometimes presents femur + tibia (Ti') + an atypical tarsus without claws (Ta'). More often, it consists of femur and tibia. The autotomy level is shown by an arrowhead. C: coxa; F: femur; T: trochanter.*

local role played by the nervous system in regeneration could not be experimentally demonstrated.

However it is possible to completely denervate an amphibian limb by cutting the nerves and thus to show their role in limb regeneration (Todd, 1823). Through numerous experimental manipulations, many workers have clearly established how nerves act (Singer,1974,1978). Nerves release a protein factor named neurotrophic factor by Singer (1974). The role of nerves in cell proliferation was demonstrated by denervation of the amputated limb before the blastema had reached the state of cell differentiation. In *Pleurodeles*, this operation caused a significant decrease in cellular proliferation. This decrease reached 70% in the mid-bud blastema analyzed four days after the denervation (Boilly *et al.*, 1985a). Nerve extracts that are able to stimulate blastemal cell proliferation both *in vivo* and *in vitro* (Boilly and Albert, 1988a) contain a growth factor, and we proposed replacing the term "neurotrophic factor" with the term nerve-derived blastema growth factor (NdBGF), which is not ambiguous. The chemical nature of this growth factor is still unknown (review by Carlone and Mescher, 1985). Although NdBGF has not yet been purified to homogeneity from nerve extracts, some molecules have been shown to stimulate blastemal cell proliferation. These include growth promoting substances such as insulin (Globus and Vethamany-Globus, 1985, Albert and Boilly, 1986), transferrin (Munaim and Mescher, 1986; Albert and Boilly, 1988), neuropeptides such as substance P (Globus *et al*, 1983), ß-endorphin (Vethamany-Globus *et al.*, 1984) or growth factors such as chicken brain growth factor (Carlone and Rathbone, 1985) glial growth factor (Brockes and Kintner, 1986) or fibroblast growth factor (FGF) (Albert *et al.*, 1987). Recently, we have focused our study on the mode of action of NdBGF at the cell level.

Blastemal cell kinetics

In vivo studies using three different methods for cell proliferation analysis, namely, cytophotometry (Oudkhir *et al.*, 1985), autoradiography (Boilly *et al.*, 1985b) and microscopic image analysis (Boilly *et al.*, 1986) led us to conclude that the G1 phase of blastemal cells was lengthened while some of the mesenchyme cells left the cell-cycle prematurely in G0-1. These data concerned mesenchymal and epidermal cells of denervated mid-bud stage blastemata. Denervation affects successively the mesenchymal and the epidermal cells (Boilly *et al.*, 1985a), a result similar to those of *in vitro* experiments (Lassalle *et al.*, 1985; Oudkhir *et al.*, 1986). Since denervation causes resorption of blastemata and fibrosis, we attempted to determine the fate of G0-1 cells induced by denervation. Thus,we studied two activities involved in these processes, namely, phosphatase activity and extracellular matrix synthesis in denervated and innervated blastemata. Four days after limb denervation, we observed a 57% increase in phosphatase activity in the blastemal mesenchyme (unpublished data). These results suggest that the autolytic activity of the mesenchyme is stimulated in denervated blastemata, probably in connection with cell resorption. Denervation affects neither the glycosaminoglycan (GAG) sulfate synthesis nor the types of these GAGs in both blastemal components. However, the size of GAGs may be altered by denervation. In mesenchyme, large chondroitin sulfates appeared although they are missing in differentiated innervated blastemata (unpublished data). Thus, denervation induces an early entrance of the mesenchymal cells into G0 and their differentiation in a special way characterized by stimulation of the acid phosphatase activity and increase of the size of some GAGs. Other extracellular matrix elements of the mesenchyme also change when the blastema is denervated (Smith *et al.*, 1975; Mescher and Munaim, 1986). Recently, it has been shown that denervated blastema mesenchyme accumulated a great amount of collagen (Vanrapenbusch and Lassalle, 1989), a result in agreement with the occurrence of fibrosis in the denervated blastema.

NdBGF transduction

To know which second messenger is used in blastema cells when NdBGF is released, we measured the amount of c-AMP by radioimmunoassay and the activity of protein kinase C (PKC) using the histone phosphorylation method (Couturier *et al.*, 1984) in both denervated and innervated blastemata.

We found that the amount of c-AMP varied in the opposite direction to DNA synthesis. Non-amputated limbs are characterized by low DNA synthesis and the highest level of c-AMP, while in blastemata, the lower the level of c-AMP, the higher the cell proliferation (unpublished data). Moreover, 96 hours after denervation, the cone stage blastema cell proliferation is reduced by 70%,

and c-AMP concentration is 2.3 times higher than in the control. These results show that c-AMP is not the second messenger of the nerve factor signaling molecules that stimulate the cell growth in blastemata.

When regenerating limbs are compared with normal limbs for PKC activity, one notes a steady increase during regeneration. In mid-bud stage regenerates, PKC activity reaches a maximal level that is four times higher than in a non-amputated limb. Moreover, a significant translocation of PKC activity from cytosol to membrane is observed. PKC translocation is known to be closely connected with its activation (Woodgett *et al.*, 1987), which is maximal in mid-bud stage blastemata, the latter being clearly defined by a high level of cell proliferation and nerve dependence (Oudkhir *et al.*, 1989).

Four days after the denervation of the regenerating limb at cone stage, membrane PKC activity decreases compared with the innervated blastema. *In vitro* results confirm these data. In the blastema cultured with a dorsal root ganglion that stimulates cell proliferation, the membrane PKC activity level is higher than the level in blastema cultured alone (Oudkhir *et al.*, 1989).

The nerve dependence of the membrane PKC activity suggests that the mitogenic signal from the nerves is mediated by the PKC activation pathway that is known to be involved in cell growth (Woodgett *et al.*, 1987). This interpretation is reinforced by the fact that a phorbol-ester (TPA) stimulates both PKC translocation towards the blastemal cell membrane and DNA synthesis. Finally, PKC translocation only occurs in mesenchymal tissue that is more specially sensitive to the nerve influence, as shown in the above results (unpublished data).

Regulation of NdBGF release

During axolotl limb regeneration, a significant increase in protein synthesis in the whole spinal cord has been established (Boilly and Scaps, 1988). Thus, the mitogenic effects of spinal cord extracts from either amputated or non-amputated animals have been tested *in vitro* on dissociated mesenchymal cells. Spinal cord extracts from axolotls that had regenerated a cone stage blastema following limb amputation 14 days previously stimulated cell proliferation twice as much as spinal cord extracts from non-amputated animals. Among the regenerating animals, spinal cord extracts from the oldest animals are less efficient than extracts from younger animals (Boilly and Albert, 1988b). This increase of mitogenicity in the spinal cord seems to be correlated with nerve fiber regeneration. This correlation has been confirmed by the following experiments. Sections of spinal cord were first cultured *in vitro* until they formed neurites. A blastema was then co-cultured with each spinal cord section and its proliferation index was compared with that of a blastema cultured alone. The proliferation index increased by 20% to 30% when co-cultured with a spinal cord section that had regenerated a few neurites, while it increased by about 65% when it was associated with a spinal cord fragment that had previously regenerated many fibers (Boilly and Bauduin, 1988).

Factors from the blastema

Through the use of an X-irradiated limb as a recipient for non-irradiated tissue grafts (Lheureux, 1983a), we observed that both epidermal and mesodermal tissues have to be healthy to ensure cell proliferation in the blastema. In the irradiated limb, epidermis was soon replaced by healthy epidermis that migrated from non-irradiated regions (Lheureux, 1983b), and allowed the non-irradiated mesodermal tissue grafts to grow and form a blastema. Conversely, blastema formation and cell growth were soon arrested when the non-irradiated mesodermal tissue grafts were covered with irradiated epidermis (Lheureux and Carey, 1988), suggesting that healthy epidermis is necessary for underlying mesenchyme growth. The epidermal cap was thought to be involved in the limb regeneration by releasing a mitogenic factor (Globus *et al.*, 1980). Actually, we showed that epidermal cap extracts are particularly active on *in vitro* blastemal cell proliferation, since they are twice as efficient as spinal cord extracts used at the same protein concentration (Boilly and Albert, 1989). By affinity chromatography on heparin, we recently detected acidic FGF-like factor in epidermal cap extracts (Boilly, 1989). Mesenchymal blastema extracts are also endowed with mitogenic properties (Boilly and Albert, 1989) and contain acidic FGF-like factor (Boilly, 1989). Nevertheless, mitogenic activity of mesenchymal extracts is half that of epidermal cap extracts. We do not know the origin of mesenchymal FGF-like factor and we wonder whether the acidic FGF-like factor is transferred from the epidermal cap to the underlying mesenchyme. Nevertheless, our results account for the stimulation of proliferation of mesenchymal cells by acidic FGF (Albert *et al.*,1987) or heparin (Boilly, 1989) and also for the stimulation of cell proliferation in blastemata under the action of FGF (review by Albert *et al.*, 1987).

The limb blastema contains not only mitogenic factors but also neuronotrophic factors (Richmond and Pollack, 1983; Boilly, 1989). A limb blastema exerts a stimulatory effect on *in vitro* spinal fiber outgrowth and neuron survival. The neuronotrophic influence of the blastema originates from its mesenchymal component and is correlated with mesenchymal proliferation (Boilly, 1989). In short, it appears that mesenchymal cells and spinal cord neurons act mutually so that spinal neurons produce a mitogen (NdBGF) which stimulates mesenchyme proliferation that enhances, in its turn, the production of NdBGF by stimulating spinal fiber regeneration. Although we do not know the nature of the blastemal neuronotrophic factor, we think it is not FGF, a growth factor with both neuronotrophic and mitogenic properties, because the epidermal cap that contains a high level of FGF-like factor and allows PC12 cells to differentiate does not stimulate neurite outgrowth from a spinal cord explant (Boilly, 1989).

Conclusion

Loss of leg or body parts by amputation or autotomy, together with various juxtapositions of normally non-adjacent differentiated cells can trigger morphogenesis. We concluded from these results that cells contain positional information. We investigated how this information is distributed and how it may change along the longitudinal and transverse axes of the structures. In *Carausius*, a gap in the epidermal positional information may be the only local factor required for triggering morphogenesis, whereas, in addition, a nerve is necessary in *Nereis* and *Pleurodeles* regeneration. In some cases morphogenesis results in an overt intercalation of structures between the juxtaposed differentiated tissues which were mismatched either transversally or longitudinally. Thus, following an amputation or an autotomy, we consider that regeneration needs the establishment of a longitudinal discontinuity. This would occur because a distal boundary appears at the amputation level. In most cases, this distalization would be the result of either the disappearance of some distal tissue inhibition or stimulation from cell

contact within the stump.

The second stage in the process of regeneration consists of filling the gap with cells originating from the division of stump dedifferentiated cells, under the influence of systemic or local stimulating factors. In the amphibian limb blastema, local factors are produced by nerves and blastemata. The nerve factor is particularly abundant when the nervous system is itself stimulated by a neuronotrophic factor from the growing blastema cells. The blastema also contains acidic FGF-like factor which may contribute to blastema growth. Cell proliferation would stop when cells are surrounded by cells identified as neighbors.

Many questions such as the nature and the stability of positional information remain unanswered. In particular, it would be interesting to know the importance of dedifferentiation in the modulation of positional information.The fact that retinoids in the newt and the nerve cord in nereis may change positional information of the regenerating cells should eventually help to answer these questions. How cells estimate a positional value from neighboring cells and why and how they respond to it are other major problems.

We are also concerned with another field of investigation related to the nature of the growth factors involved in cell proliferation of the blastema, and the regulation of their production and release. More precisely, it will be interesting to focus investigations on the bioavailability of corresponding growth factors and on the expression of their blastemal cell receptors.

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