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THE NERVE DEPENDENCE OF AMPHIBIAN LIMB REGENERATION

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SUMMARY

The regeneration of limbs in urodele amphibians is a context where the developing and regenerating peripheral nervous system interacts with the mechanisms of epimorphic regeneration. After amputation of a limb, there arise at the amputation plane the blastemal cells which are the progenitor cells of the regenerate. These cells divide rapidly and subsequently differentiate to give rise to the internal tissues (cartilage, muscle and connective tissue) of the regenerate. Division of the blastemal cells requires the presence of nerve axons at the amputation plane, at least during the initial stages of regeneration. This requirement can be circumvented by allowing a limb to develop in the absence of a nerve supply (the 'aneurogenic limb'), but the underlying mechanisms have been unclear. We have derived a monoclonal antibody called 22/18 that has provided new information about these issues. It is specific for blastemal cells *versus* normal tissue in the limb, specific for regeneration *versus* development, and specific for blastemal cells that arise after amputation in the presence of the nervous system *versus* its absence (in either development or the aneurogenic limb). The antibody reactivity appears to mark a cell transition involved in the imposition of nerve-dependent growth control.

INTRODUCTION

This chapter is concerned with interactions between the peripheral nervous system and the vertebrate limb in both development and regeneration. Many adult urodele amphibians, such as newts and axolotls, are able to regenerate their appendages after amputation. Quite apart from its medical significance, limb regeneration may be studied for its intrinsic interest, and also as an accessible context for investigating the traditional issues of embryonic development. The peripheral nervous system plays a central role in the early events of limb regeneration because it is responsible for controlling division of the progenitor cells of the regenerate. I shall be concerned not only with this relationship between the nerve supply and the regenerate, but also with how the relationship is established as peripheral axons and Schwann cells enter the developing limb bud. Some new insights into these issues have come from the derivation of a monoclonal antibody that marks an early event in the formation of the progenitor cells. This reagent also provides direct evidence about a distinction between limb development and regeneration, and illustrates the value of monoclonal antibodies in tackling such complex issues.

Key words: limb regeneration, growth factors, aneurogenic limb.

THE EVENTS OF LIMB REGENERATION

This subject has been extensively reviewed (Wallace, 1981; Sicard, 1986) and will only be considered in outline in this account. After amputation, epidermal cells migrate rapidly to close the wound surface within 12 h in an adult newt (Repesh & Oberpriller, 1980). The wound epidermis subsequently increases in thickness at the centre of the amputation surface to form the apical epidermal cap. A critical early event in regeneration is the genesis of the progenitor cells, which arise locally within approximately 0·5 mm from the amputation plane. These are referred to as blastemal cells and they divide rapidly over the next 2 or 3 weeks to form a protruding conical blastema. The term 'bud' will be reserved for limb development. There is little or no overt cytodifferentiation within the blastema over the first 2 weeks, but then the cells progressively reconstruct the internal tissues of the regenerate, giving rise to cartilage, muscle and connective tissue. The blastema does not contribute significantly to the epidermis of the regenerate, nor does the epidermis give rise to blastemal cells (reviewed by Wallace, 1981; Stocum, 1986). The formation of the limb regenerate takes about 5 weeks in an adult newt and is independent of the position of amputation along the proximal–distal axis. It is clear, therefore, that a shoulder blastema must initially grow faster than a wrist blastema (Iten & Bryant, 1973), since the new arm or hand is formed at approximately the same end point.

This last case is an example of what might be called the position-dependent properties of the blastema. Other examples would be the difference between a forelimb and hindlimb blastema, or between a limb and tail blastema. These properties have been investigated indirectly by a wealth of experiments involving grafting and transplantation of urodele limbs and blastemas. Although such experiments have suggested general rules for deploying positional values (for example Bryant, French & Bryant, 1981), there is no information about the molecular basis of these differences and this remains a critical problem for future investigation. This issue will not be further considered in this account, except to suggest that some of the approaches used to investigate the nerve-dependence or the origin of the blastemal cells could profitably be employed in future to tackle the positional properties.

The mode of regeneration of the urodele limb by formation of a local growth zone or blastema is termed 'epimorphic' (Morgan, 1901). This basic strategy is found in a number of invertebrate phyla, for example the regeneration of segments in annelids, and in many of these cases there is suggestive evidence of nerve involvement in growth of the blastema (Goss, 1969). The organization of the vertebrate peripheral nervous system is particularly suitable for investigating nerve–target interactions, and the urodele has provided the clearest case for study of these issues.

THE 22/18 ANTIBODY

The regeneration of the limb obviously proceeds on a significantly larger scale than its development. There are probably at least ten-fold more blastemal cells than early bud mesenchymal precursors. Furthermore, the blastema is a renewable resource

since it is rapidly replaced after removal. These circumstances make it possible to obtain significant quantities of progenitor cells – a relatively rare situation in vertebrates. We exploited this opportunity to immunize mice with the early blastema of the newt and screen the antibody-secreting clones by immunofluorescence on sections of the early regenerate (for details, see Kintner & Brockes, 1985). The 22/18 antibody was the sole isolate that makes a clear distinction between the blastemal cells and the cells of the normal (unamputated) limb. The properties of this reagent have made it a valuable probe for events in development and regeneration, and these are discussed in detail in several publications (Kintner & Brockes, 1984, 1985; Fekete & Brockes, 1987; H. Gordon & J. P. Brockes, in preparation; Fekete, Ferretti, Gordon & Brockes, 1987). In this account I shall mention briefly the contribution that 22/18 has made to the different issues under discussion.

The 22/18 antigen is an intracellular determinant that is present on an intermediate filament subunit or possibly an intermediate filament-associated protein (Fekete *et al.* 1987). As early as 36–48 h after amputation, it appears in Schwann cells of the nerve sheath, and fibroblasts or interstitial cells of the connective tissue (H. Gordon & J. P. Brockes, in preparation). About 80 % of the cells in the early blastema react with the antibody but this reactivity decreases during days 14–21 after amputation, leaving a minority of positive cells (Kintner & Brockes, 1985). As these cells differentiate they lose reactivity and the regenerate, like the original limb, is essentially negative. The appearance of reactivity does not represent a transient response to injury, since expression is stable if regeneration is arrested *in vivo*, or if the early blastema is dissociated and the cells maintained *in vitro* (Fekete *et al.* 1987). The induction of 22/18 is provoked by injury but does not require the presence of the wound epidermis or that cells enter S phase (H. Gordon & J. P. Brockes, in preparation).

NERVE-DEPENDENCE OF REGENERATION

The forelimb of the adult newt is innervated by the third, fourth and fifth spinal nerves with their motor and sensory components, and by a quantitatively minor sympathetic input (Singer, 1942). The innervation to the limb can be conveniently varied by cutting the axons at the brachial plexus or elsewhere. The distal axons rapidly degenerate, leaving behind the tubes of denervated Schwann cells. As shown in Fig. 1, there are several protocols for investigating the effect of denervation on regeneration. The limb may be denervated before amputation, in which case the wound epidermis forms and the initial population of blastemal cells arise normally (Mescher & Tassava, 1975; H. Gordon & J. P. Brockes, in preparation). Whereas the blastemal cells begin to divide at day 4 or 5 after amputation in the innervated case, division does not begin after prior denervation (Mescher & Tassava, 1975; Maden, 1978). If the early blastema is denervated up to about day 13 after amputation in the adult newt, then division of the blastemal cells falls precipitously and regeneration stops (Singer & Craven, 1948). Older blastemas respond differently in that regeneration proceeds to give a limb that is well-formed but smaller than control

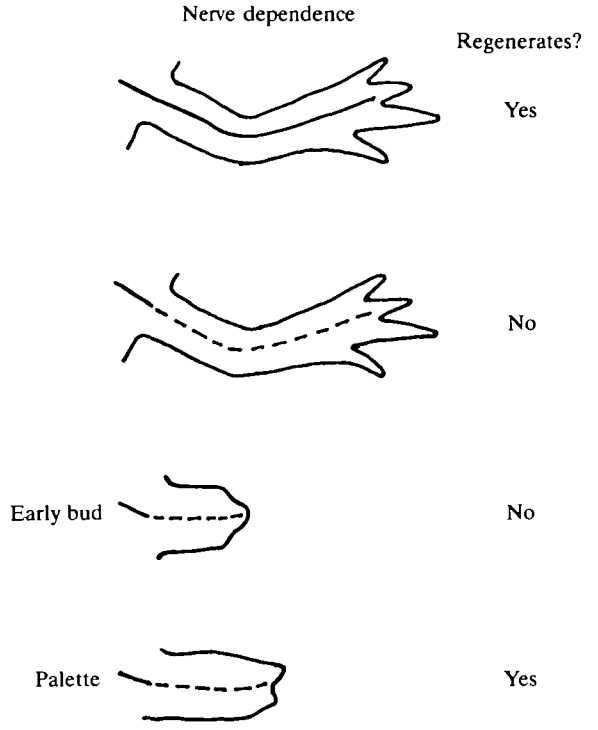

Fig. 1. The nerve-dependence of regeneration. After denervation at the brachial plexus the remaining Schwann cells in the distal stump are represented as a dotted line. This convention is also followed in Figs 2 and 3. The early bud and palette stages are before and after the critical juncture at which denervation no longer arrests regeneration (see text).

regenerates (see Fig. 1; Singer & Craven, 1948). The importance of the nerve was first reported by Todd (1823) and significant contributions to our understanding have come from the classical studies of Schotté, Butler & Singer. I should like to consider the role of the nerve in relation to formation of blastemal cells, the control of division and the different consequences of denervation at the various times (Fig. 1; see also Tassava & Olsen, 1986).

It is important to recognize that the denervation experiments demonstrate that axons do not have to be present at the amputation plane for generation of the initial complement of blastemal cells. This has been demonstrated both by using classical histological stains for blastemal cells (Mescher & Tassava, 1975) and by use of the 22/18 antibody (H. Gordon & J. P. Brockes, in preparation). In the latter case the reactivity in Schwann cells and in interstitial cells under the wound epidermis occurs on amputation of a denervated limb, indeed the reactivity in Schwann cells is already present after cutting the axons (Kintner & Brockes, 1985; H. Gordon & J. P. Brockes, in preparation). There are certain caveats about these experiments which should be considered. First, it is hard to rule out some long-term effect of axons that is retained on denervation and manifest after amputation in the appearance of blastemal cells. Second, it is possible that the denervated Schwann cells that persist

in the limb are responsible for some important 'signalling' in the initial appearance of blastemal cells on amputation. The results with 22/18 and the aneurogenic limb (discussed later) are quite consistent with such a possibility, although only in relation to the induction of 22/18 and not necessarily in relation to other aspects of blastemal cell identity. Third, it is possible that later contributors to the blastema (for example muscle) *are* dependent on the presence of axons to do so, since it would be difficult with the present methods and uncertainties to detect such an effect. Nonetheless, it is important that axons, and also the wound epidermis (Mescher & Tassava, 1975; H. Gordon & J. P. Brockes, in preparation), do not need to be present for the initial events of blastemal cell formation. It is likely that both agents affect the blastema only through effects on cell division, and it is misleading to say that either is 'required' for blastemal cell formation or dedifferentiation.

The axonal control of cell division in the blastema was examined in an admirably detailed and quantitative series of studies by Singer that have been reviewed elsewhere (Singer, 1952, 1974; Wallace, 1981). The most important finding was to establish quantitative relationships between the density of innervation at the amputation plane and the occurrence of regeneration. Although it is possible to dispute certain aspects of interpretation, for example the existence of a 'threshold' innervation (Wallace, 1981), the data are so extensive as to establish the validity of the phenomenon (nerve-dependence) beyond doubt. Second, Singer and his colleagues demonstrated that limbs with either an exclusively sensory (Singer, 1943) or an exclusively motor innervation (Singer, 1946; Sidman & Singer, 1960) were capable of regeneration. Although the experiments do not rule out some quantitative difference in efficacy between the two populations, it is clear that both are capable of sustaining division in the blastema. In the case of the sensory population, this occurs even when the central process to the spinal cord is cut, thus disrupting any reflex circuitry. As a result of his studies Singer proposed that axons release a neurotrophic factor that sustains division in the blastema (Singer, 1952, 1974). In a sense the terminology is somewhat unfortunate today in that most trophic effects of nerves, for example on muscle fibres, are exerted quite independently of cell division. We would tend now to refer to Singer's activity as a mitogenic growth factor. The search for activities of this sort has occupied a number of laboratories but in no case are the data so compelling that one can confidently attribute a role in nerve-dependent proliferation to a particular molecule (see Brockes, 1984; Carlone & Mescher, 1986). Rather than reviewing these studies here, I shall make a number of general points. Although Singer understandably proposed only one such factor, it is certainly possible that there are more (Globus & Vethamany-Globus, 1986), although it is important to distinguish the instructive 'competence-inducing' molecules from the permissive 'progression' factors that are needed for transit through the cell cycle (Pledger, Estes, Howe & Leof, 1984). Furthermore, it is possible that the proximal signal for proliferation is not made by the neurone, but is made in the blastema as a result of some interaction with axons. It is thought, for example, that the synthesis of peripheral myelin proteins by Schwann cells depends on a contact interaction with axons (Mirsky *et al.* 1980; Brockes, Fryxell & Lemke, 1981), and it has been

demonstrated that axons deliver a mitogenic signal to Schwann cells on contact (Salzer, Williams, Glaser & Bunge, 1980).

My final point concerns the criteria used to evaluate the relevance of a molecule for nerve-dependent proliferation in the blastema. We have suggested (Brockes & Kintner, 1986) that any candidate should satisfy at least four points. It must be present in the blastema, be lost on denervation, and must stimulate that population of blastemal cells that are dependent on the nerve *in vivo*. Finally, and perhaps most crucially, a monospecific antibody that blocks its biological activity should abrogate the effect of the nerve when introduced into the normal blastema. It should, therefore, turn off division of the relevant population in the blastema. The identity of this population has been clarified by the derivation of 22/18, which is an excellent marker for nerve-dependent blastemal cells. When newts were injected with tritiated thymidine at 2 days after denervation of early (13-day) blastemas, the labelling index of 22/18-positive cells decreased seven-fold relative to the control side. At later stages (25 days) the minority (see above) of 22/18-positive cells showed the same quantitative dependence as before. The 22/18-negative population was less affected, particularly at the later stage (Kintner & Brockes, 1985). The existence of cells at later stages with the same dependence as those at earlier ones makes it most unlikely that the nerve acts on division in the blastema by an indirect mechanism, such as establishing the vasculature (Smith & Wolpert, 1975). It is clear that any candidate molecules must act to stimulate division of the 22/18-positive population. This criterion has been met in the case of glial growth factor, and the methodology used to establish this point is detailed by Brockes & Kintner (1986).

The change in proportion of 22/18-positive cells is a contributing factor to the different consequences of early and late denervation (Schotté & Butler, 1944; Singer & Craven, 1948). At early stages most of the cells are positive and division is rapidly arrested after denervation, whereas at later stages most of the cells are negative and their labelling index changes by only 20% on denervation (Kintner & Brockes, 1984). It seems plausible that a critical number of cells is required in the blastema to initiate morphogenesis and that until this number is attained, at day 13–15 in the adult newt, the effect of denervation is to arrest regeneration completely. After this point, morphogenesis and patterning occur to give a limb which is somewhat smaller because of the consequences of late denervation.

The effects of denervation on the two populations in the blastema suggest that there must be two 'systems' of growth control, one dependent on the nerve axons for division and one that is not. The second could reflect, for example, the action of a circulating growth factor, although there are a number of other possibilities. The distinctive attribute of 22/18-positive cells is that they do not respond to the alternative system and hence cease to divide on denervation. The 22/18-negative population responds to the nerve system, but they must in addition respond to the alternative system since their division is less affected by denervation, particularly at later times (Kintner & Brockes, 1985). The hypothesis of a second system will recur in discussions of normal development and regeneration of the aneurogenic limb.

THE ONTOGENY OF NERVE-DEPENDENCE

At a time when the neurotrophic hypothesis had recently been proposed, a remarkable series of experiments was reported that seemed to challenge some aspects of it. These issues are outlined schematically in Fig. 2.

By using methods of experimental amphibian embryology it is possible to derive a limb that develops in the near absence or total absence of a nerve supply. Such aneurogenic limbs become depleted in muscle as a secondary consequence of the lack of innervation but are otherwise relatively normal in appearance. Nonetheless, they will regenerate when amputated despite the absence of nerve fibres in the resulting blastema (Yntema, 1959; Thornton & Steen, 1962; Steen & Thornton, 1963; Thornton & Tassava, 1969). In a second series of experiments, the aneurogenic limbs were transplanted to a normal larva in place of the forelimb. When the transplanted limbs are innervated from the host brachial nerves, they quite abruptly become dependent on an intact nerve supply for regeneration (Thornton & Thornton, 1970). The phenomena of the aneurogenic limb pose an intriguing puzzle

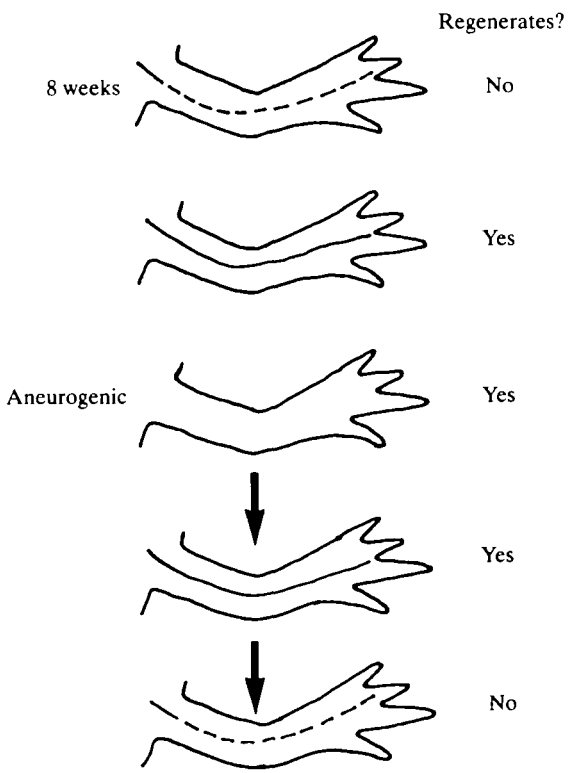

Fig. 2. The aneurogenic limb. The top two limbs are the innervated and denervated cases shown in Fig. 1. The bottom two represent the experiment of Thornton & Thornton (1970) in which the aneurogenic limb is transplanted and becomes nerve-dependent for regeneration. The 8 weeks refers to the minimum time that the denervated case is 'held' denervated, and yet is still found to be nerve-dependent for regeneration after amputation.

(Fig. 2). We are presented with a system that depends on an interaction between the axons and the blastemal cells for its regeneration, yet if it develops in isolation it is quite independent. It should be stressed that even if the normal limb is maintained in a denervated state for 7–8 weeks by repeated operations, it does not 'forget' its nerve-dependence on amputation (Liversage & McLaughlin, 1983; Scadding, 1984).

In considering explanations for the aneurogenic limb, it is necessary to understand first why it is not nerve-dependent for regeneration, and second why it becomes so after innervation. The earlier attempts to explain the results have focused on the availability of the putative mitogenic factor since this seemed an obvious possibility for regulation (for a review, see chapter 2 of Wallace, 1981). For example, it is possible that the cells of the developing limb make growth factors capable of sustaining division of blastemal cells in regeneration, but after the nerve supply has entered it turns off such synthesis and makes division nerve-dependent. Alternatively, the interaction of the nerve-derived factor with cells could shut off their responsiveness to other factors. In the absence of any molecular information about the factors involved, it has not been possible to test any of these possibilities. The present explanation is somewhat different in that it focuses on the cellular heterogeneity in the blastema that is disclosed by 22/18, and the different responses of the two populations to denervation. It predicts that the aneurogenic blastemal cells are different from those that arise in the innervated case. Before considering this I should like to analyse what happens when the limb bud is innervated. It is likely that the interaction between the brachial nerves and the aneurogenic limb in the above experiments (Thornton & Thornton, 1970) is a normal feature of limb development, and most attempts to explain the results tacitly acknowledge this fact.

I have mentioned above that the appearance of 22/18 after amputation is provoked by injury and identifies cells whose proliferation is nerve-dependent. Since neither of these aspects is considered to be part of normal limb development, it was of obvious interest to examine the distribution of 22/18 in newt embryos of limb bud stages. The antigen was found to be expressed transiently in several locations, including the epidermis, glial cells in the neural tube, the aorta and the lens. It was expressed by less than 1 % of the mesenchymal cells in the limb bud, and was not expressed by the flank mesenchyme at the stage before outgrowth of the bud (Fekete & Brockes, 1987). It appears, therefore, to be specific for mesenchymal cells of the regeneration blastema as compared to those of the developing limb bud. This is consistent with 22/18 being a marker for nerve-dependent proliferation, since the urodele limb undergoes considerable growth before nerves can be detected among the mesenchymal cells (Fekete & Brockes, 1987).

In view of the low expression of 22/18 during normal development, it is possible to amputate the bud at different stages and observe the reactivity around the amputation plane (Fekete & Brockes, 1987). It should be stressed that at all stages, the amputated bud or limb regenerates. Nonetheless, amputation of either fore- or hindlimbs at early stages does not provoke a 22/18 response when compared with the contralateral control (Fekete & Brockes, 1987). In these cases the nerve fibres, as detected by an antiserum to the low molecular weight neurofilament subunit, had not

reached the plane of amputation. Amputation after forelimbs had reached the three-digit stage provoked a strong 22/18 response. This response occurred in blastemal cells that were often located close to the nerve as detected in double-labelled sections. The appearance of the 22/18 response is a function of the stage of limb development as shown by the amputation of fore- and hindlimb buds at a larval stage where development of the forelimb is greatly advanced relative to the hindlimb. In short, while regeneration occurs at all stages the blastema changes from a 22/18-negative to a 22/18-positive one, and this change is correlated with the appearance of axons and probably Schwann cells at the amputation plane. A more detailed account of the transition is given in Fekete & Brockes (1987).

This result is shown in Fig. 3 (compare Fig. 2) – the 'pre-innervated' limb regenerates with a 22/18-negative blastema. The hypothesis is that, as with the later stages of adult regeneration discussed above, there is a second system of growth

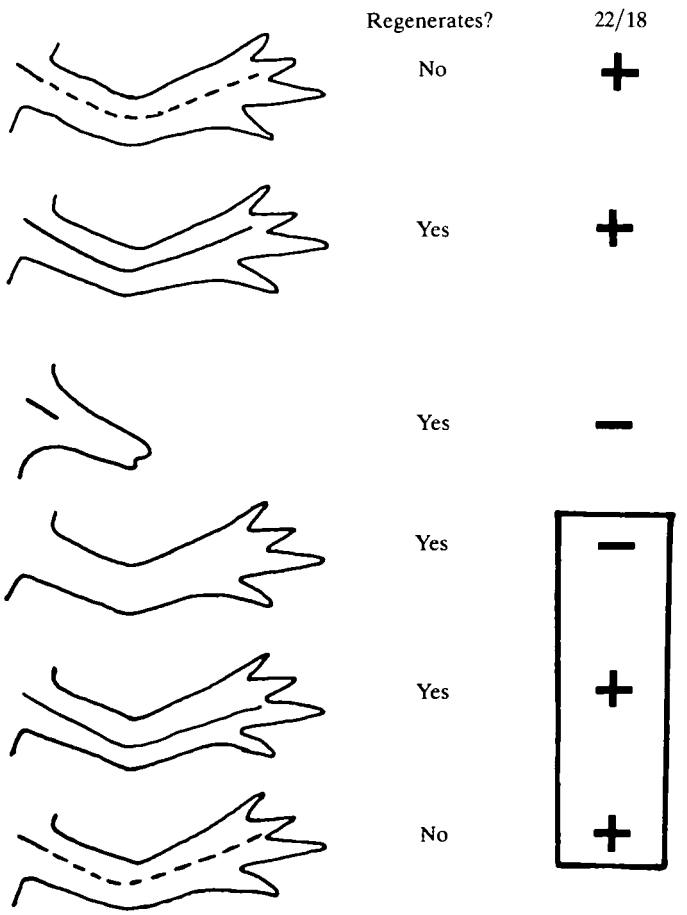

Fig. 3. The 22/18 transition. The third limb from the top is the 'pre-innervated' case in development that incorporates the results of Fekete & Brockes (1987). The box encloses predicted results for the aneurogenic limb, and its innervated derivatives (see Fig. 2).

control that stimulates division of 22/18-negative limb precursors in normal development and regeneration of the 'pre-innervated' and aneurogenic limbs. When the limb is innervated, the cells at the amputation plane undergo a transition that leaves them refractory to this system, and hence dependent on the nerve axons for division (Fekete *et al.* 1987). At least one aspect of this transition involves a change in the cytoskeleton that is detected by the 22/18 antibody. This is a hypothesis that does not necessarily depend on any long-term effect that the nerve may exert on the developing or aneurogenic limb. The control is exerted at the time of amputation, which in one case gives rise to progenitor cells dependent on the nerve, and in the other does not. The key prediction (Fig. 3) is that the aneurogenic blastema is 22/18-negative.

In order to make aneurogenic forelimbs, a pair of urodele embryos is fused in parabiosis at a stage before the forelimbs emerge. The following day an incision is made in one partner and most of the neural tube and ectodermal placodes are removed, after which the embryo heals and is sustained by its partner (Yntema, 1959). In favourable cases the forelimb of the operated embryo will develop normally in the virtual absence of a nerve supply. The pair is left until a stage where the first digits are forming and a clear 22/18 response is expected (see Fekete & Brockes, 1987) after amputation of the forelimb on the innervated partner. In such experiments the aneurogeic limb gives little or no 22/18 response (D. M. Fekete & J. P. Brockes, unpublished observations), thus verifying the prediction of Fig. 3. The absence of nerves in the aneurogenic limb has been checked with the neurofilament antiserum. These results extend the correlations noted in the developing bud between the 22/18 response and the presence of innervation, and demonstrate experimentally the importance of the nervous system. It appears, therefore, that 22/18 is specific not only for blastemal cells *versus* normal tissue in the limb, but also for blastemal cells *versus* limb bud cells, and for blastemal cells that arise after amputation in the presence of axons and Schwann cells (see later) *versus* their absence in either normal development or the aneurogenic limb. The 22/18 transition appears to be involved in the imposition of nerve-dependent growth control.

CONCLUSIONS

I have discussed three different influences that the peripheral nervous system exerts on regeneration in the urodele limb. (1) The mitogenic effect of nerve axons on blastemal cells that is arrested by denervation. (2) A contribution of 22/18-positive Schwann cells to the early blastema (Brockes, 1984; Kintner & Brockes, 1985; H. Gordon & J. P. Brockes, in preparation). The quantitative extent of this contribution is unknown, as is the eventual fate of the cells. (3) The imposition of nerve-dependent growth control by virtue of the fact that the immediate precursors of blastemal cells undergo a transition leaving them dependent on influence 1 for division. This transition, or one aspect of it, is detected by the 22/18 antibody.

Since the phenomena indicate the existence of an alternative system of growth control, most clearly manifest by the regeneration of the aneurogenic limb, the control by the nervous system has the quality of an 'intervention'. I should like to consider the third influence in more detail. Inspection of Figs 1–3 indicates that it does not require the presence of axons at the amputation plane, as shown most clearly in the case where the limb is denervated for 7–8 weeks before amputation. Even in the normally innervated case (Fig. 1), the axons transiently retract from the amputation plane. It is therefore amputation in the presence of denervated Schwann cells that leads to a 22/18-positive nerve-dependent blastema. One aspect of the effect is the contribution of Schwann cells to the blastema, but it is clearly not so simple because the connective tissue cells also become 22/18-positive after amputation (H. Gordon & J. P. Brockes, in preparation). Although we favour the hypothesis that a single population of such cells exists with respect to the 22/18 transition, it could be that there are two populations, one recruited for the aneurogenic or 'pre-innervated' blastema, and one for the normally innervated case. In either event, the role of the Schwann cells is of great interest and could be tested by deriving larval limbs that are devoid of Schwann cells by virtue of early neural crest removal (Harrison, 1924). It would be striking if limbs containing axons but not Schwann cells were to give 22/18-negative blastemas on amputation, since this would indicate that the Schwann cells are required for signalling the transition in the connective tissue cells.

The nerve-dependence of limb regeneration offers an interesting perspective on more general issues of growth control. Although there is increasing information about the molecular diversity and mechanism of action of mitogenic growth factors, there is rather little understanding of how they may act *in vivo* to regulate cell division. A central concern of this paper has been to suggest that a key point of regulation is the responsiveness of the progenitor population rather than the availability of growth factors. The ability to manipulate the neural input in development and in the adult has obviously been critical in elucidating the cellular mechanisms involved. In one sense this is just a step towards defining the molecular basis of the regenerative response, but experience indicates that a certain level of definition is required before this is possible. Only after the detailed studies on nerve-dependence by Singer was it realistic to design assays for the nerve-derived mitogen. As detailed here, the monoclonal antibody approach has identified an early cell transition that appears to be involved in the imposition of nerve-dependent proliferation. In order to approach the molecular basis of this transition, we have established culture conditions in which dissociated cells from the early blastema, or cells that migrate from explants of the normal limb, will stably express 22/18. We are also attempting to clone the gene for 22/18, to express it in cultured cells from the limb, and to identify the flanking sequences that control its expression. It may be possible to determine if the antigen is functionally important in the early transition, and to use its control mechanism to express other genes of interest in cells in culture, or after introducing the cells into the blastema. Such approaches offer possibilities

for studying several problems related to regeneration, including that of the position-dependent properties of the blastema considered earlier.

I should like to thank D. Fekete, P. Ferretti, H. Gordon and C. Kintner for their contributions to the experimental work from my laboratory that is discussed here.

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