Muscle nerve branches do not develop in chick wings devoid of muscle

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SUMMARY

If the somitic mesoderm of a 2-day chick embryo is destroyed by X-irradiation, the adjacent limb develops with a normal pattern of connective tissues, but is devoid of muscle. The innervation of muscleless wings produced in this way was examined in silver-stained whole mounts, fixed 3 to 8 days later. The main nerve trunks and their cutaneous branches developed normally; but the nerve branches which in a normal limb would lead to individual muscles were generally absent. In almost all those exceptional cases where muscle nerve branches were present, muscle was found to be present also, despite the X-irradiation. Where there was no muscle, the muscle nerve branches apparently did not even begin to form. As a control for side effects of the X-irradiation, wing buds were grafted from normal to irradiated embryos and vice-versa, and again analysed for their innervation. The results confirmed that the absence of muscle nerve branches was due to the absence of muscle cells in the limb. Thus (1) the routes taken through a limb by the main mixed nerve trunks and by their cutaneous branches are determined by the connective tissues, and not by any mechanisms requiring muscle cells; but (2) muscle cells are necessary to provoke the formation of the side branches leading to the sites of individual muscles.

INTRODUCTIONS

As nerve fibres grow into a developing limb, they follow routes defined by the prior pattern of the limb itself (Braus, 1905; Harrison, 1907; Hamburger, 1939; Piatt, 1956; Straznicky, 1963; Narayanam, 1964). The limb is a composite of several different tissues, and one may ask specifically which of these tissues govern which features of the pattern of innervation. What part, for example, do the muscle cells play in guiding axons along their proper paths? A recent

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discovery has made it possible to tackle this question directly. It has been shown that myoblasts are originally separate from the other cells of the limb: they derive from the somitic mesoderm, and migrate from it at an early stage into the somatopleural mesoderm from which the rest of the limb bud will be formed (Christ, Jacob & Jacob, 1974, 1977; Chevallier, Kieny & Mauger, 1976, 1977). Thus by destroying the somitic mesoderm by X-irradiation at an early stage, before the emigration of myoblasts, it is possible to deprive the limb bud of myoblasts, and so to create a limb which is devoid of muscle, and yet has an otherwise normal pattern of connective tissues (Chevallier, Kieny & Mauger, 1978). Even the tendons will begin their development in the usual fashion in such a muscleless limb (Kieny & Chevallier, 1979).

In the normal chick wing bud, the first nerves to be seen are the rudiments of the main mixed trunks (Roncali, 1970; Bennett, Davey & Uebel, 1980). The nerve branches innervating individual muscles become visible slightly later (Lewis, in preparation), just as myotubes are beginning to differentiate and the dorsal and ventral muscle masses are splitting to form the definitive named muscles (Shellswell & Wolpert, 1977). The nerve branches consist of axons which turn aside from the main nerve trunk where it passes close to a muscle rudiment (Tello, 1917). Since the distance to the target muscle is at first very short, it is possible that axons may turn aside from the main nerve trunk because the target cells themselves – the myoblasts or myotubes, rather than the intervening connective tissues – act directly on the growth cones to cause a diversion. If so, muscle nerve branches should not develop, even transiently, in a limb that contains no muscle cells. Bennett et al. (1980) have gone a step further, and suggested that some ‘nerve growth factor’ synthesized by the cells of the developing muscles may direct the initial growth of axons into the limb bud. If that were the case, one should expect to find, in a muscleless limb, that not even the main nerve trunks take their normal routes.

We have made chick embryos with wings devoid of muscle, by X-irradiating the somites at an early stage, and have examined the pattern of innervation of the resulting limbs, looking for an answer to the following question: in the absence of muscle cells, will the connective tissues of the limb serve to guide the nerves along their normal routes; and in particular, will muscle nerve branches be formed? We find that the main nerve trunks and all their cutaneous branches do indeed develop according to the normal pattern, but that the muscle nerve branches are missing from the outset.

**MATERIALS AND METHODS**

The experiments were performed on chick embryos (Wyandotte × Rhode Island Red).
Fig. 1. An embryo at the stage of 20 pairs of somites, 5 h after X-irradiation of a portion of the right somitic mesoderm posterior to the 11th somite (arrows). Dotted lines: cephalocaudal limits of the wing field. Irradiation performed at the stage of 17 pairs of somites; neutral red stain. Scale bar = 1 mm.

Irradiation

The somitic mesoderm was destroyed on the right side of 2-day embryos [stages 12 to 19 (mostly 12 to 17) pairs of somites] by local X-irradiation, which was carried out under the same conditions as reported in previous papers (Chevallier et al. 1978; Kieny & Chevallier, 1979), namely at 20 kV and 30 mA for a period of 10 min. The embryo was placed at a distance of 37 cm from the anticathode, and was irradiated (approximately 1000 rad) through a slot (1.6 mm long x 0.1 mm wide) cut in a shield of tantalum foil 0.1 mm thick, which protected the rest of the embryo and part of the extraembryonic area. The
slotted shield was placed in such a way that a region 1-6 mm long was irradiated posterior to the 11th somite. The somitic mesoderm at the level of the wing (somites 15–20) was always included, but the extent to the rear of the wing level varied, according to the stage of the embryo, from 2 to 6 presumptive somites. The damage due to the X-irradiation can be visualized by staining with the vital dye neutral red: this is used initially to colour the embryo so as to facilitate the localization of the zone to be destroyed, and is then retained in the damaged zone preferentially and for a longer time than in the other regions of the embryo. We checked in this way the accuracy of the localization of the X-irradiation in most of the embryos, 3 to 5 h after the exposure (Fig. 1).

**Grafting**

At stages 19 to 20 (Hamburger & Hamilton, 1951), the right wing buds of some of the chick embryos irradiated at 2 days were exchanged with those of normal chick embryos. The wing buds were cut off at the shoulder level, transposed, and fixed onto the shoulder stumps with 2 to 3 silver pins.

**Fixation and staining**

Embryos were fixed 3–8 days after irradiation, in a mixture of ethanol, distilled water, formalin, and glacial acetic acid, in the ratio 75:15:5:5 (Bodian, 1937). The head, viscera and posterior half of the body were discarded, leaving the wings attached to their portion of the trunk. The specimens were processed as whole mounts to reveal the pattern of innervation, according to the following schedule (Lewis, 1978, adapted from Bodian, 1936):

1. Fix for 1–3 days.
2. Rinse for 1–7 days in 70 % alcohol (4 changes).
3. Rinse for 1 h in distilled water.
4. Incubate for 17–40 h at 37 °C in the dark, with agitation, in a 1 % aqueous solution of protargol (‘Strong Silver Protein’, Établissements Roques), together with a piece of clean copper wire (0·5–2 g per 100 ml, according to the time of incubation and the mode of agitation, using wire of diameter 0·91 mm from BDH). To dissolve the protargol, sprinkle it on the surface of the water at 37 °C and leave it to pass into solution without stirring.
5. Reduce for 1 h at 4 °C, with agitation, in an aqueous solution of hydroquinone 1 % plus anhydrous sodium sulphite 7·5 %.
6. Rinse for 2 h at 4 °C, with agitation, in distilled water (three changes).
7. Tone for 1 h at 4 °C, with agitation, in 1 % yellow gold chloride, acidulated with acetic acid (3 drops/100 ml).
8. Rinse for 1 h at 4 °C, with agitation, in distilled water (2 changes).
9. Bleach for 2 h, or more or less according to darkness of specimen, in fresh aqueous 5 % potassium ferricyanide.
10. Rinse for 1 h in water (2–3 changes).
Nerve development in wings without muscle

(11) Soak for 0.5 h in an aqueous solution of sodium thiosulphate 5% plus potassium hydroxide 2%.
(12) Rinse for 1 h in water (2 changes).
(13) Dehydrate in alcohol and clear in methyl salicylate.

This procedure stains the nerves in whole mounts black against a transparent yellow or red background. The specimens were examined under a Zeiss Stereomicroscope IVB, equipped with a substage condenser for Köhler illumination. A central stop in the plane of the condenser aperture diaphragm gave dark-ground illumination when required: this was useful for photography. To check whether or not muscle was present, the specimens were examined between crossed polaroid filters; muscle, being birefringent, then appears bright against a dark background. To search more carefully for muscle and to inspect the state of the spinal cord, some of the specimens that had been silver-stained and examined as whole mounts were afterwards embedded in paraffin, serially sectioned, and stained with haematoxylin and eosin.

Embryos were staged according to the normal series of Hamburger & Hamilton (1951). We follow Yasuda (1960) and Roncali (1970) in the nomenclature of nerves, and Sullivan (1962) in the nomenclature of muscles. We abbreviate the names of muscles as follows: Dorsal series: Tric, triceps; EMR, extensor metacarpi radialis; Sup, supinator; Anc, anconaeus; EDC, extensor digitorum communis; EMU, extensor metacarpi ulnaris; EML, extensor metacarpi longus; EIL, extensor indicis longus; EIB, extensor indicis brevis; AdI, adductor indicis; EMB, extensor metacarpi brevis; IOD, interosseus dorsalis; UMD, ulnmetacarpalis dorsalis. Ventral series: Bic, biceps; Brach, brachialis; PS, pronator superficialis; PP, pronator profundus; EECU, entepicondyloularis; FCU, flexor carpi ulnaris; FDP, flexor digitorum profundus; UMV, ulnmetacarpalis ventralis; FDS, flexor digitorum superficialis; AbI, abductor indicis; FI, flexor indicis; AbM, abductor medius; IOP, interosseus palmaris; FDQ, flexor digiti quarti.

RESULTS

(I) Embryos subjected simply to X-irradiation of somitic mesoderm

Altogether 71 embryos were X-irradiated at 2 days of incubation so as to destroy the somitic mesoderm on one side of the body, and then, after a further 3–8 days of incubation, were fixed and silver stained as whole mounts to reveal the pattern of wing innervation. Seventeen of these specimens were excluded from the analysis because the X-irradiation had been misdirected or ineffectual (so that the embryo appeared normal), or because the wing skeleton was defective, or because the staining was inadequate. This left 54 specimens for analysis, as listed in Table 1.

X-irradiation of the early somitic mesoderm destroys the tissue that lies between the spinal cord and the limb primordium; the effect can be clearly seen 24 h after irradiation (Fig. 2). Thus in all our specimens, the wing lay closer than
Table 1. Summary of material analysed

<table>
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<tr>
<th>Stage</th>
<th>Number of specimens analysed</th>
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<td>25-26</td>
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<td>—</td>
</tr>
<tr>
<td>27</td>
<td>3</td>
<td>Tric; Bic, FDP</td>
</tr>
<tr>
<td>28</td>
<td>11</td>
<td>Tric, EMR; Bic, FDP</td>
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<td>7</td>
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<td>Bic, Brach, PS, PP, EECU, FCU, FDP</td>
</tr>
<tr>
<td>30-31</td>
<td>5</td>
<td>Tric, EMR, Sup, Anc, EDC, EMU,</td>
</tr>
<tr>
<td>32-33</td>
<td>5</td>
<td>EIL, EML, EMB, IOD, UMD;</td>
</tr>
<tr>
<td>34-35</td>
<td>11</td>
<td>Bic, Brach, PS, PP, EECU, FCU, FDP,</td>
</tr>
<tr>
<td>36-37</td>
<td>5</td>
<td>UMV, FDS, AbM, IOP, FDQ</td>
</tr>
</tbody>
</table>

Fig. 2. An embryo 24 h after X-irradiation, cross-sectioned in the brachial region. The somitic tissue on the right hand side has been destroyed, while the neural tube appears unharmed. Haematoxylin and eosin stain. Scale bar = 100 μm.

usual to the spinal cord, and was generally somewhat tilted and displaced in a dorsoventral direction relative to it. In most cases, the dorsal root ganglia and the spinal roots were not segmental as they would normally be; this was especially clear in specimens fixed at early stages (Fig. 3). Presumably the usual segmentation is a secondary consequence of the segmentation of the somitic mesoderm, and does not reflect any intrinsic periodicity in the spinal cord or dorsal root ganglia (Detwiler, 1934).

**Time course of wing innervation**

In all cases, nerve fibres had entered the wing on the irradiated side. At early stages (up to about stage 26 or 27), the innervation of the normal wing has not developed to the point where muscular and cutaneous nerve branches can be separately distinguished, and muscle fibres have not differentiated sufficiently
Fig. 3. Dorsal midline view of an embryo fixed and silver stained at stage 26, 3 days after X-irradiation of somitic mesoderm on the right hand side. Note that on the irradiated side the spinal ganglia and roots are not segmented, and the limb lies abnormally close to the spinal cord. Scale bar = 0.5 mm.

to be easily visible by polarized light. The pattern of innervation in the wing on the irradiated side at these early stages looked roughly normal dorsally, though often somewhat reduced ventrally.

The subsequent development of the pattern of innervation was assessed by comparison with the wings on the control, unirradiated side of our embryos. In these, as in the wings of a normal embryo (Lewis, in preparation), the earliest muscle nerve branches to become visible are relatively proximal. Separate branches destined for individual muscles can be distinguished even before those muscles themselves have split off as separate entities from the rest of the dorsal and ventral muscle masses. Thus the nerves to triceps, biceps and FDP can first be seen at stage 27 or 28, to EMR at about stage 28, to the other muscles of the forearm at about stage 29, and to the intrinsic muscles of the hand at about stage 30. The timing is difficult to pinpoint exactly, because the earliest rudiments of the muscle nerve branches are ill-defined and hard to see. Most of them appear first as short, unfasciculated tufts or fringes of weakly stained fibres at the sides of the main mixed nerve trunks (Fig. 4). It takes about one stage (or about half a day) for clearly defined and tidily fasciculated muscle nerve branches
to develop from these beginnings (Fig. 5). From stage 30 onwards, the nerve branches to all the muscles of the wing are as a rule plainly distinguishable (Lewis, 1978), with the exception of the intrinsic muscles of digit II and expansor secundariorum, which develop somewhat later. The muscle nerve branches that we scored are listed for each stage in Table 1. After stage 30, these muscle nerve branches simply become longer and more widely ramified. We did not examine systematically the innervation of the shoulder muscles, since the staining of this region was unreliable.

Fig. 4. The radialis profundus nerve in the region of the elbow, in wings fixed and silver stained at stage 28. (a) Muscleless wing. (b) Contralateral control wing. The beginnings of the nerve branch to the muscle EMR are visible in the control wing, but absent in the muscleless wing. Scale bar = 100 μm.

Fig. 5. Dorsal view of wings fixed and silver-stained at stage 29. (a) Muscleless wing. (b) Contralateral control wing. Nerve branches to Tric, to EMR and to EMU and EDC can be seen in the control wing, but are absent in the muscleless wing. Scale bar = 0.5 mm.
Fig. 7. The radialis profundus nerve and its branches in the region of the elbow, in wings fixed and silver stained at stage 36. The pattern of cutaneous innervation is essentially the same in the muscleless wing (a) and in the control wing (b); but the muscle nerve branches visible in the control wing (those to EMR, Sup and Anc are in focus here) are absent in the muscleless wing. Scale bar = 0·5 mm.
Abnormalities of wing nerve pattern: the general rule

In each of the 54 specimens analysed, the wing on the irradiated side was scored for the presence or absence of each of the muscle nerve branches visible on the control, unirradiated side. The vast majority of the branches looked for was absent (Figs. 4–7); and, with a very few exceptions, wherever a muscle nerve branch was seen, the corresponding muscle was found to be present also, implying that the X-irradiation had not been fully effective in depriving the wing of myoblasts (see below).

By contrast with the system of muscle nerve branches, and as illustrated in Figs. 4–7, main nerve trunks and cutaneous branches deriving from them were always present in the experimental wings. Dorsally, this sensory component of the innervation was usually more or less normal in both pattern and quantity. Ventrally, it was usually reduced: the median and interosseus nerves were often missing or petered out in the forearm.

The detailed data on muscle nerve branches are set out in Table 2. They can be summarized as follows, if we combine the results from all stages. The total number of muscle nerve branches looked for was 735. No judgement could be made on 57 of these, because of poor staining. Of the remaining 678, 595 (88%) were absent; 67 (10%) were present, with the corresponding muscle present also; and 16 (2%) were present, with no corresponding muscle visible.

From stage 30 onwards, the results are more or less independent of the stage at fixation: we noticed no significant systematic differences (except possibly with regard to the muscle UMD, discussed below). The earlier stages, up to and including stage 29, are of special interest, because they represent the period in which muscle nerve branches would normally be just beginning to develop. The figures for these earlier stages, from 27 to 29 inclusive, are as follows: The total number of muscle nerve branches looked for was 137. No judgement could be made on 33 of these, because of feeble staining. Of the remaining 104, 61 (59%) were absent; 38 (36%) were present, with the corresponding muscle present also (though often only as an ill-defined part of a still undivided muscle mass, and usually less dense than on the control side); and 5 (5%) were present, with no corresponding muscle visible. Thus the proportion of cases in which muscle nerve branches were present is larger than at the later stages. This fact, however, probably does not have much significance. Out of the 21 specimens fixed at stages 27–29, 15 belonged to a batch in which the most medial part of the somitic mesoderm was not irradiated, so as to be more sure of avoiding direct damage to the neural tube. In this batch of specimens, parts of the vertebrae and of the spinal musculature were often present on the irradiated side, and it is to be expected therefore that the wings should more commonly have contained some muscle. It is to be stressed that at the early stages, just as at the late stages, the majority of the muscle nerve branches present in the wing on the control side were absent in the wing on the irradiated side, whereas the
Table 2(a). *Results of X-irradiation of somitic mesoderm, for embryos fixed at stages 27 to 29, showing the number of cases in each category for each muscle nerve branch assessed*

<table>
<thead>
<tr>
<th>Nerve absent</th>
<th>Tric</th>
<th>EMR</th>
<th>Anc</th>
<th>EDC</th>
<th>EMU</th>
<th>Bic</th>
<th>Brach</th>
<th>PS</th>
<th>PP</th>
<th>EECU</th>
<th>ECU</th>
<th>FDP</th>
<th>Total</th>
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<td>4</td>
<td>3</td>
<td>3</td>
<td>8</td>
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<td>7</td>
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<td>7</td>
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<td>7</td>
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<td>7</td>
<td>7</td>
<td>7</td>
<td>21</td>
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The set of nerves assessed depended on the stage of the embryo: see Table 1. Nerves were scored as 'reduced' if they had not more than about half the normal bulk of axons, on a rough visual estimate. Nerves listed as 'not scored' represent cases where the staining was inadequate. Where a nerve branch was absent, the corresponding muscle was never seen by polarized light; in such cases, no check was made for the absence of muscle in histological sections. Innervated muscles, where present, were often of subnormal bulk.
Table 2(b). Results of X-irradiation of somitic mesoderm, for embryos fixed at stages 30 to 37, showing the number of cases in each category for each dorsal muscle nerve branch assessed.

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<th>Nerve absent</th>
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See notes to Table 2(a).

Table 2(c). Results of X-irradiation of somitic mesoderm, for embryos fixed at stages 30 to 37, showing the number of cases in each category for each ventral muscle nerve branch assessed.

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<th>Nerve present but reduced</th>
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See notes to Table 2(a).
Table 3. The nerve to the site of the muscle UMD, after X-irradiation of the somitic mesoderm

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<td>4*</td>
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<td>32-33</td>
<td>2</td>
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<td>34-35</td>
<td>6</td>
<td>5</td>
<td>11</td>
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<td>36-37</td>
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</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>10</td>
<td>25</td>
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</table>

* There were altogether 5 specimens at this stage, but only 4 could be scored for the UMD nerve branch.

main nerve trunks and their cutaneous branches were present there, and followed the normal paths.

Qualifications to the general rule

There are some minor qualifications to the general rule, however. The experimental wings sometimes contained nerves taking aberrant paths. Specifically, at both early and late stages, a thin fascicle or small group of fascicles could often be seen to diverge from the main proximal ventral nerve trunk (n. brachialis longus inferior) at about the level at which the nerves to biceps and brachialis normally arise, and then to travel in parallel with that main nerve trunk as far as the elbow; there, in the crook of the elbow, the aberrant fibres generally rejoined the main nerve trunk of the forearm (n. interosseus). In a very few cases, a similar phenomenon could be seen elsewhere: fibres diverging from n. brachialis longus superior, at about the level where the branch to triceps or where the branch to EMR normally originates, continued beyond the site where the muscle should have been but was not, and finally rejoined n. radialis profundus. We emphasize that such cases were exceptional, and we do not know whether the aberrant fibres were sensory or motor.

Another exception to the general rule concerns the nerve to the muscle UMD. This nerve seems to belong in a class by itself. It was present in 10 out of the 25 specimens that were scored for it (stages 30-37; see Table 3), and in 8 of those 10 specimens the muscle UMD itself appeared to be absent. In the 8 cases where the nerve was present but unaccompanied by muscle, it was abnormally thin and lacked the usual ramifications in the region where the muscle should normally be (Fig. 6). It was not seen in any of the 5 specimens fixed at stages 36-37. The muscle UMD lies at the very end of the main deep nerve of the dorsal side of the wing (n. radialis profundus), however, and it is unclear whether one should classify the nerve fibres that go in its direction as a motor nerve branch, or simply as the terminal part of a main mixed nerve trunk. Again
Fig. 8. Wings fixed and stained at stage 35, viewed between crossed polaroid filters. Ventral aspect. The brightly birefringent muscles are clearly visible in the control wing (b), but are absent from the wing on the irradiated side (a). The thin bright lines in the experimental wing (a) are not muscle, but the fibrous periosteum of the long bones, which is also birefringent. Scale bar = 1 mm.

we do not know whether the fibres seen taking this route towards the site of UMD were motor or sensory. Though we did not study the shoulder systematically, it is worth mentioning here that we did occasionally notice nerve branches taking the routes that normally lead to the big muscles of the shoulder (e.g. pectoralis) and then ending blindly without terminal ramification, in striking contrast to the control side. It is, however, possible that some shoulder muscle may have been present in these cases.

Muscle

The wings were all examined as whole mounts by polarized light to detect muscle (see Fig. 8). The X-irradiation seemed in general to have done its job: no muscle was seen except in the minority of cases where muscle nerve branches were also visible. Conversely, in most instances where a muscle nerve branch other than that to UMD was visible, so also was the corresponding muscle. The polarized-light test for muscle, however, is not very sensitive, especially at early stages and when the background staining of the tissue is dark; small amounts could easily be missed. We therefore embedded and sectioned wings in which muscle nerve branches had been seen, and checked for the presence of muscle by ordinary histological criteria. The muscle UMD was usually not seen, even when its nerve branch had been seen; but in almost all other instances where a muscle nerve branch had been visible, the corresponding muscle turned out to be present, though often reduced in size (Fig. 9). Setting aside UMD, the total number of exceptional cases in which we saw a muscle nerve branch, but no muscle, was only 8 (out of a possible 624). Three of these were specimens at relatively late stages (30, 34 and 35, respectively), in which a very thin fascicle appeared to be heading towards an absent brachialis, triceps or EMR muscle. The other five exceptional cases were all at early stages (29 or before). At such stages, muscle differentiation has not normally progressed very far, the dorsal and ventral muscle masses have not yet completed their splitting, and it can be
Fig. 9(a). A wing from the irradiated side of an embryo fixed and silver-stained at stage 32. Two attenuated nerve branches can be seen going in the direction of EMR and EML. Scale bar = 0-5 mm. (b). A section of the wing shown in (a), cut at the level of EML, and stained with haematoxylin and eosin. The nerve branch to EML (arrow) can be seen to innervate a group of muscle fibres: the X-irradiation has not after all deprived the wing entirely of muscle. Scale bar = 50 μm.
difficult even in sections to identify the tissue; this was especially true of our specimens, which had already been through the silver-staining procedure before sectioning, and so did not present as clear a histological picture as they would otherwise have done. Thus our failure to see muscle at these stages does not prove that it was absent.

**(II) X-irradiation of somitic mesoderm combined with grafting of wing buds**

In principle, abnormalities of the pattern of innervation in our irradiated embryos might be due either to abnormalities (i.e. absence of myoblasts) in the wing itself, or to abnormalities of the spinal cord or of the tissues that lie between it and the wing. To distinguish between these possibilities, we did a series of grafting experiments, in which wing buds of normal embryos were exchanged with wing buds of embryos whose somites had been irradiated. The buds were cut off and transferred at an early stage (19–20), before they had begun to be innervated (Roncali, 1970). We thus produced two types of specimen: those in which a normal wing bud had been grafted onto an irradiated host in place of its own, and those in which a presumptively muscleless wing bud from an irradiated embryo was grafted onto a normal embryo in place of its own. The specimens were fixed after 5–6 days, at stages 30–34.

Many of the embryos died as a consequence of the grafting operation combined with X-irradiation, and so the number that survived for us to analyse was rather small. In both sets of cases, the innervation of the grafted wing was often distinctly subnormal, lacking main nerve trunks and cutaneous nerve branches as well as muscle nerve branches. This is probably to be explained simply as a consequence of the dislocation of the normal nerve pathways at the graft-host junction: such failures of innervation are commonplace when limb buds are interchanged or grafted from one place to another (Hamburger, 1939; Hollyday, Hamburger & Farris, 1977; Morris, 1978; Lewis, 1978).

We consider first the cases in which a normal wing bud was grafted onto an irradiated host. Of the 10 surviving specimens, we discarded 4 because even the sensory component of their innervation appeared very scanty and abnormal. We analysed the remaining six specimens as before. The data can be summarized as follows: A total of 138 muscle nerve branches were looked for. No judgment could be made on 20 of these, because of poor staining. Of the remaining 118, 60 (51%) were absent, and 58 (49%) were present. By contrast, in the original series of experiments described above, in which the wings of irradiated embryos were studied in situ, only 13% of muscle nerve branches were present.

In the reciprocal series of grafts from irradiated embryos onto normal hosts, we examined again 10 specimens. We discarded two of these, one because it was almost entirely devoid of innervation, the other because it appeared practically normal, with almost all muscles and nerve branches present. The data for the remaining 8 specimens can be summarized as follows: A total of 184 muscle
nerve branches were looked for. No judgement could be made on 18 of these, because of poor staining. Of the remaining 166, 137 (83%) were absent and 29 (17%) were present, with the corresponding muscle present also. These figures are not significantly different from those for the original series of experiments, in which the wings of irradiated embryos were left in situ.

In short, though the number of specimens is rather small, the grafting experiments indicate that it is the constitution of the wing itself that determines the presence or absence of muscle nerve branches in the wing.

**DISCUSSION**

X-irradiation of the somitic mesoderm gives rise to limbs in which there is no muscle; and in such limbs muscle nerve branches do not develop. They do not even put in a transient appearance, so far as we could tell. Nerves do, however, grow out along the routes normally taken by the mixed nerve trunks, and a practically normal pattern of cutaneous nerve branches is formed. In almost all of those few exceptional cases in which we did see muscle nerve branches, muscle also was present despite the X-irradiation. We do not know for certain that the muscle nerve branches that we saw consisted chiefly of axons from motoneurons, but it seems a reasonable supposition, to some extent supported by HRP studies of normal development (Landmesser, 1978), and by studies of embryos lacking dorsal root ganglia (Lewis, unpublished).

The fact that muscle nerve branches did not develop in the wings devoid of muscle is open to several interpretations. First, it is conceivable that scattered or misdirected X-rays might have destroyed the motoneurons in the spinal cord at the outset. In a subsequent paper we plan to examine the fate of the motoneurons in these experiments, and to give details of the histology of the spinal cord after irradiation of the somites. Suffice it to say here that the irradiation in some cases did seem to have done direct damage to the spinal cord, and that, in cases where the cord was otherwise normal, a deficit of brachial motoneurons could be seen as early as stage 28. But this cannot be the general explanation of the absence of motor nerve branches. For in the wings where scraps of muscle were present, and just in those wings, the corresponding muscle nerve branches were present also. Furthermore, in normal wings grafted onto irradiated hosts, muscle nerve branches commonly did form; whereas in the reciprocal grafts, where the wing lacked muscle cells, the muscle nerve branches were lacking, even though the spinal cord and trunk had not been tampered with. From all this it follows that in the irradiated embryos motoneurons are generally able to survive in sufficient numbers to innervate any muscle that may be present in the wing.

By much the same argument, we can exclude another interpretation. It might be suggested that, since the tissue between the somites and the spinal cord has been drastically disturbed, the axons growing out from the spinal cord are
Nerve development in wings without muscle

misdirected and for that reason never find their way into the limb – and would not do so even if there were muscle in it. Lance-Jones & Landmesser (1980b) have indeed argued that the somitic tissues play an important part in guiding axons selectively to specific targets in the chick limb. But it is most unlikely that our results are to be explained simply in terms of faulty guidance from the spinal cord to the base of the limb. Sensory fibres find their way into the limb, so why should not motor fibres also? Why should motor fibres fail to find their way through this disturbance, if they can find their way into an ectopically grafted limb in an otherwise normal embryo (Hollyday et al. 1977; Morris, 1978)? And again, most tellingly, why in the limbs of our irradiated series that contained muscles were nerve branches to those muscles present? And why in normal limbs grafted onto irradiated embryos did muscle nerve branches commonly develop? Evidently axons in an irradiated embryo can reach muscles if muscles are present: the disturbed tissue between the spinal cord and the base of the limb presents no barrier. Thus we conclude that when muscle nerve branches are missing after somite irradiation, their absence is due to the absence of muscle in the wing. The absence of muscle must be the cause, and not the effect, of the absence of muscle nerves; for it is known that muscles themselves can differentiate autonomously in a limb bud deprived of innervation (Shellswell, 1977; Lance-Jones & Landmesser, 1980a; Lewis, 1981).

This still leaves several possibilities open. (A) It could be that motor axons will not enter a limb at all unless it contains muscle cells. (B) Alternatively, it could be that motor axons will enter the limb without muscle cells and follow along the main nerve trunks, but will not branch off from them to the sites of individual muscles unless the rudiments of those muscles are present. (C) Lastly, it could be that motor axons enter the limb without muscle cells and grow right out along the normal paths to the sites where muscles should be, but then, finding none, withdraw or die. The evidence as yet is not adequate to decide between these possibilities, but there are some comments to be made.

First of all, with regard to (C), we did not observe any transient muscle nerve branches (except that to UMD). Even at the very earliest stages at which muscle nerve branches were visible in the control wings, they appeared to be absent in the wings on the irradiated side. If axons were growing right out to the sites where muscles should normally have been, they must have been retreating or dying very promptly – say within half a day or less – on finding that there was no muscle there.

The behaviour of motoneurons deprived of their targets has been studied previously in the chick by simply extirpating the wing or leg primordium at an early stage (Hamburger, 1958; Oppenheim, Chu-Wang & Maderdrut, 1978). In embryos treated in this way, the motor axons grow out and form a neuroma at the base of the amputated limb, but this neuroma is a transient structure. The deprived motoneurons begin to die in large numbers at about stage 29 (in the case of wing extirpation), and by stage 36 only about 10% of them are left in
the ventral horn. The death of brachial motoneurons due to absence of a wing thus occurs promptly, at about the time when muscle nerve branches should be beginning to form, and earlier than the death of brachial motoneurons seen during normal development, which has its peak at about stage 35 (Oppenheim & Majors-Willard, 1978). Such studies, however, do not answer the questions which preoccupy us here: they do not help us to disentangle the role of the connective tissue of the limb from that of the muscle cells themselves in controlling the outgrowth of axons and the survival of the nerve cells.

In our muscleless wings, though the muscle nerve branches were absent, the pattern of sensory innervation was almost normal. This shows that the routes taken through the limb by the main mixed nerve trunks, and by their cutaneous branches, are not determined by any mechanism requiring muscle cells. It must be the connective tissues proper that define the courses of the mixed nerves, and so, in normal development, guide motor as well as sensory axons most of the way to their targets. We would thus argue strongly against the suggestion that it is some chemotactic effect exerted by developing muscle that directs axons as they first grow into the limb bud (Bennett et al. 1980). Our results allow us to draw a distinction between the parts of the pattern of wing innervation which do depend on muscle cells for their establishment, and those which do not: it is only the side branches leading to individual muscles that depend on the presence of muscle cells. From this point of view, it is perhaps not surprising that the nerve to muscle UMD was often present even though UMD itself was absent; for this nerve, from its mode of development, can fairly be considered to be the terminal segment of the mixed trunk n. radialis profundus, rather than a side branch from it.

Motor and sensory axons must respond differently to the system of guiding cues that the limb provides. In the experiments we have reported here, we could not distinguish between the two classes of fibre: we simply saw nerves, or an absence of nerves. To clarify further the rules governing nerve outgrowth and the choice of path, it will be necessary to examine each component of the innervation in the absence of the other.

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