

Growth of Nervous Tissue in the Regenerated Rabbit Ear Chamber

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ABSTRACT

Highly specific nerve staining using thiocholine techniques has been applied to a study of nerve regeneration in titanium rabbit ear chambers. Early and extensive ingrowth of nerves rich in true cholinesterase activity was demonstrated.

INTRODUCTION

Since its description (10), the regenerated rabbit ear chamber preparation has been extensively used in studies of vascular and cellular behaviour in the microcirculatory area of conscious animals. Despite widespread use of the method, the question of innervation of the regenerated tissue has received scant attention. We considered it of interest, therefore, to briefly report our experience in applying modern histochemical techniques to a study of nerve ingrowth in chamber tissue.

MATERIALS AND METHODS

Titanium ear chambers were aseptically inserted into Sandy-Lop rabbits according to the method previously described by Arfors et al. (1).

Cholinesterase activity was displayed histochemically by Gomori's (6) modification of Koelle & Friedenwald's (8) method using ear chamber tissues of three weeks, two and four months of age. Normal rabbit ear perichondrium from the same rabbits treated in identical fashion to the chamber tissue served as control.

The tissues stained by the cholinesterase method were used fresh and were sufficiently thin (about 100 μm) to obviate the need for sectioning. After excision of the ear chamber the top cover glass was removed and the regenerated tissue stained in situ on the bottom plate. Incubation with acetyl thiocholine iodide as substrate was continued at 37°C for 18-24 hours. To inhibit pseudo-cholinesterase and thus demonstrate true cholinesterase activity, a portion of ear chamber tissue was pretreated with 1×10^{-5} M tetraisopropylpyrophosphoramidate (ISO-OMPA, 3.42 mg/l of 40% sodium sulphate) for 30 min. The same concentration of ISO-OMPA was included in

the incubation medium. During dehydration and clearing procedures, the ear chamber and perichondrial tissues were kept under a cover glass to minimise tissue distortion. Nerve staining by silver impregnation was also done using a modification (5) of Bielschowsky's (2) method. In this instance prior fixation in 10% buffered formalin (pH 7.0) was required.

The mounted preparations were viewed using transmitted or phase contrast light. Photographs were taken with a Reichert automatic camera using Kodak Ektachrome film.

RESULTS

Since the tissues used in this study were not sectioned, ingrowing nerves could be followed as uninterrupted dark brown lines. Occasional blood vessels could be faintly distinguished due to the presence of small amounts of pseudo-cholinesterase in the smooth muscle of the vascular wall.

In the three week old chamber, a single nerve trunk could be seen entering the chamber tissue. After giving off a small branch it extended some 2 mm towards the centre of the chamber. This regenerated nerve was shown to contain true cholinesterase; enzymatic activity persisted after inhibition of pseudo-cholinesterase by ISO-OMPA.

Nerve regeneration followed a similar pattern and was developed to a similar extent in the two- and four-month-old ear chambers. At both ages, the regenerated tissue was completely interwoven by a meshwork of nerve fibres with a maximal diameter of around 5 μm (Fig. 1). The larger nerves were seen to be made up of several neurofibrils and in this respect they resembled the nerves seen in perichondrial tissue. Not surprisingly, however, no thick nerve bundle entered the narrow chamber space, most being single fibrils which seemed to pursue a random course to end blindly in the chamber tissue or more often in relation to a blood vessel. The nerve fibres were

apparently unmyelinated but had an investing neurolemmal sheath. Schwann cell nuclei could be readily identified as local deviations of the neurofibrils but nodes of Ranvier were not observed.

Silver impregnation of ear chamber tissue showed the striking development of the perivascular nerve plexus particularly well (Fig. 2). Excessive and uneven staining with silver were, however, troublesome.

DISCUSSION

Using intravital staining with methylene blue dye, Clark et al. (3) demonstrated the ingrowth of an unmyelinated nerve accompanying an arteriole three weeks after insertion of a rabbit ear chamber. The present study using specific histochemical techniques confirms and extends these findings. The thiocholine method gives highly selective nerve staining, the only other deeply staining elements being fat cells, sebaceous glands and hair roots, none of which are found in regenerated chamber tissue. Arterial smooth muscle also shows some activity due to the presence of pseudocholinesterase (4), but counterstaining is required to display the vascular wall clearly. Our results show that differentiation of the capillary loops into definitive arterioles and venules is quickly followed by innervation of these structures. By two months a meshwork of ramifying nerve fibres has developed. True cholinesterase activity appears to be present from the outset and enzyme activity was evenly distributed along the nerve fibre. The staining features of the nerves suggest that they are postganglionic fibres of the autonomic nervous system. Most fibres terminate in relation to blood vessels but some nerve strands appear to end blindly, an observation which was also made by Grant & Thompson (7) using normal rabbit ear preparations.

Silver impregnation techniques suffer the disadvantage when applied to ear chamber tissue that the abundance of reticular fibres may lead to staining of non-nervous tissue with consequent difficulty in precise interpretation. However, as shown by Richardson (9) using rabbit intestinal tissue, silver staining was particularly useful in demonstrating the richness of the perivascular nervous network which quickly develops in ear chamber tissue (Fig. 2).

As a final point it may be mentioned that the histological appearances of the derivatives of the different germ layers found in regenerated chamber tissue do not differ from that described in unspecialised connective tissue found elsewhere in the body. There seems, therefore, no a priori reason to presume that the physiological responses observed in fully vascularised and stable ear chamber tissues should be atypical.

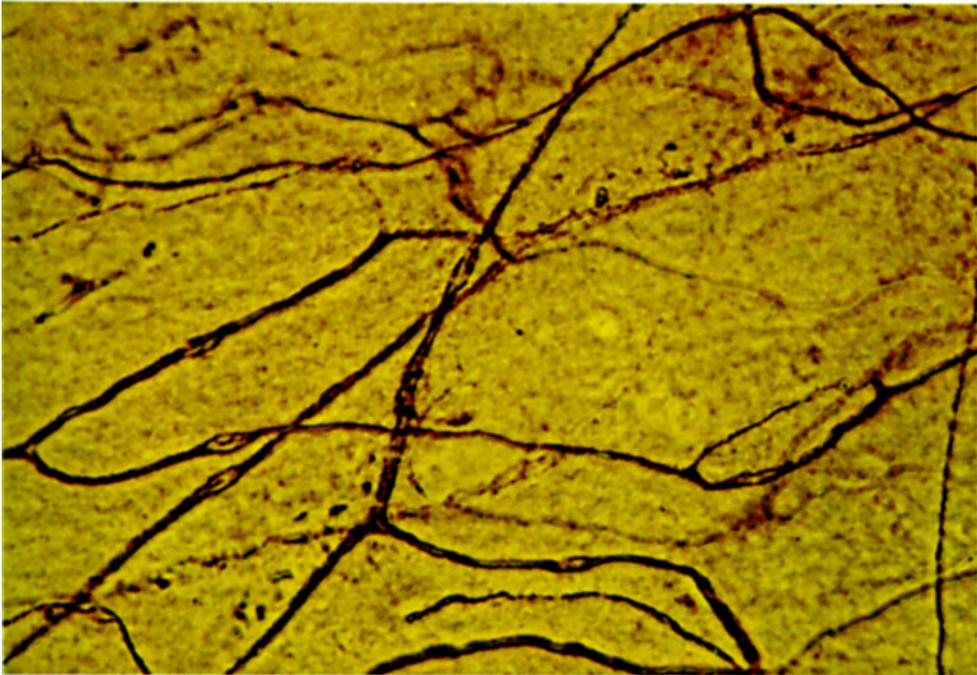
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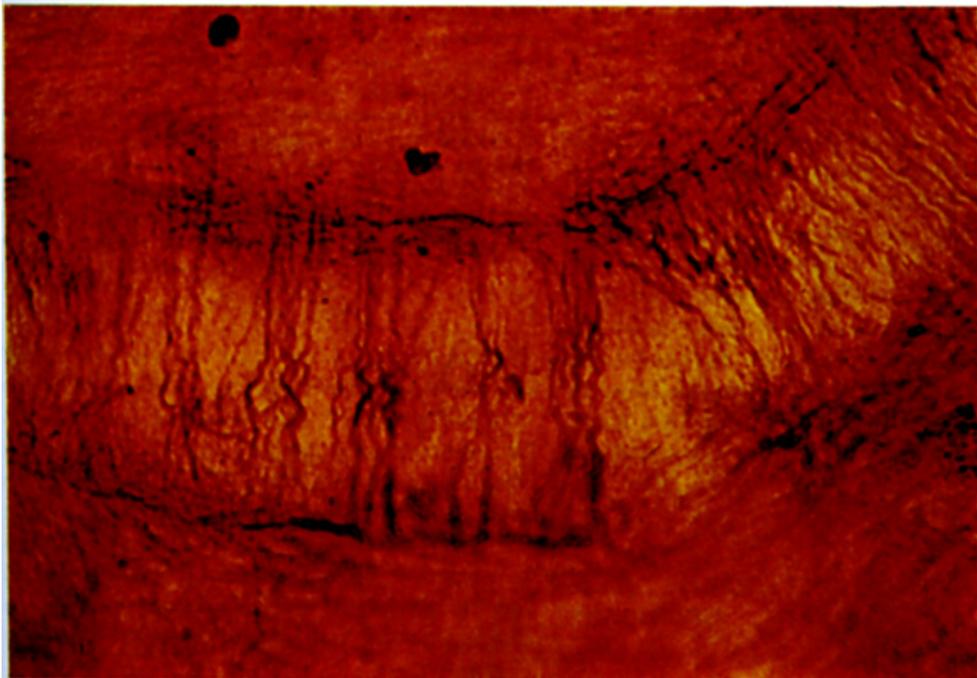
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Fig. 1. Thiocholine stain of four month old ear chamber tissue showing the rich meshwork of regenerated nerve fibres which develop. Phase contrast light linear magnification $\times 275$.

Fig. 2. Silver impregnation of four month old ear chamber tissue showing the complex perivascular network of nerve fibrils surrounding a $40 \mu\text{m}$ diameter venule. Transmitted light, linear magnification $\times 700$.