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RT97- and calcitonin gene-related peptide-like immunoreactivity in lumbar intervertebral discs and adjacent tissue from the rat

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ABSTRACT

The innervation of rat intervertebral disc and adjacent ligamentous tissue has been investigated using 2 antibodies, RT97 and anti-calcitonin gene-related peptide. Immunoreactivity to the peptide was found in many fibres throughout the long ligaments around the intervertebral discs and in the periosteum, especially associated with vascular channels entering the vertebral bodies. Few of the immunoreactive fibres entered the annular lamellae of the disc tissue. Most of those which terminated did so as fine fibres which lay close to, or in, the interlamellar spaces of the outer annulus fibrosus. Calcitonin gene-related peptide-like immunoreactivity was also found in more complex endings in the longitudinal ligaments and rarely within the annulus fibrosus. RT97-immunoreactivity was also present in the complex endings and associated fibres. Conversely, RT97-immunoreactivity was apparent only in a few fine filamentous fibre endings. This suggested that the majority of fine filamentous, or free, nerve endings were of an unmyelinated sensory origin. Alternatively, those endings of a more complex nature, which were RT97-immunoreactive, were of a myelinated sensory origin. No immunoreactivity to either antibody was seen in the inner annular or nuclear tissue. It was therefore concluded that the sensory innervation of the rat intervertebral disc has both myelinated and unmyelinated components, the latter being more extensive. Both types of innervation appear to be restricted to the outermost rings of the annulus fibrosus.

INTRODUCTION

It has long been assumed that the innervation of the intervertebral disc is essentially sensory in origin (Roofe, 1940; Malinsky, 1959; Nachemson, 1975; Bogduk et al. 1981; Bogduk, 1983). This assumption has formed the basis for the hypothesis that damage to the intervertebral disc can directly produce pain (Nachemson, 1975). However, based on the anatomy, the innervation of the intervertebral disc and adjacent ligamentous structures could have a sympathetic efferent and/or a sensory afferent origin (Bogduk et al. 1981; Bogduk, 1983). The majority of the previous investigations into the innervation have used histological techniques with varying specificity. The main problem with techniques such as heavy metal (silver) deposition (Roofe, 1940; Malinsky, 1959; Jackson et al. 1966; Yoshizawa et al. 1980; Bogduk et al. 1981; Guillot et al. 1988; Yahia et al. 1988) or acetylcholinesterase staining (Jackson et al. 1966; Kojima et al. 1990*a*, *b*) is that they lack the ability to differentiate types of neurons clearly. The question of origin has therefore not previously been resolved. It has been possible, using the above techniques, to reveal the position and structure of nerve fibres and the larger nerve endings. However, there have been varying levels of success with respect to the finer nerve fibres.

Immunohistochemical techniques are now available which selectively label neuronal tissue and therefore may be used to differentiate the components of an innervation. The most popular markers for sensory nerve fibres have been antisera raised to neuropeptides, the most commonly used ones being calcitonin gene-related peptide (CGRP) and substance P (SP), both of which have been implicated in neurogenic vascular changes (Holzer, 1988). It has been possible, using antisera to these neuropeptides, to determine the innervation pattern of sensory neurons in a variety of tissues such as the nervous

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system (Lee et al. 1985), bone (Bjurholm et al. 1988), periosteum (Hill & Elde, 1988), skin (Silverman & Kruger, 1989) and joints (Ichikawa et al. 1989; Grönblad et al. 1990; Kidd et al. 1990; Mapp et al. 1990). Antibodies to neurofilament subunits have also been used in the study of innervation patterns (Dalsgaard et al. 1985, 1989). One such antibody is a monoclonal (RT97), which reacts with the 200 kDa subunit of neurofilament and appears to differentiate unmyelinated from myelinated sensory neurons (Lawson et al. 1984; Lawson & Waddell, 1985; McCarthy & Lawson, 1990).

Previous applications of this technique to the intervertebral disc initially suggested there was no innervation by SP-like immunoreactive fibres (Korkala et al. 1985). In contrast, it was reported more recently that there may indeed be SP-like immunoreactivity present in the intervertebral disc (Weinstein et al 1988; Coppes et al. 1990). Nevertheless, when looking for an innervation it would be more advantageous to use the marker present in the largest proportion of those nerve cells under study. CGRP-like immunoreactivity (CGRP-LI) is present in the vast majority of sensory cells originating from a variety of nonepidermal tissues in the rat (Molander et al. 1987; Perry & Lawson, 1990). In addition, CGRP-like immunoreactivity has been used to study the innervation of many tissues, including articular cartilage (Ichikawa et al. 1989; Grönblad et al. 1990; Kidd et al. 1990; Mapp et al. 1990). A further advantage is that CGRP-like immunoreactivity does not appear in postganglionic sympathetic neurons or their fibres, outside the gastrointestinal tract (Lee et al. 1985; Hill & Elde, 1988), at least in the rat. The latter is an important consideration when attempting to resolve the sensory component of a tissue such as the intervertebral disc, where there is the possibility of a sympathetic motor component. The large amount of available data concerned with the immunoreactivity profiles for sensory and sympathetic nerves made the study of rat intervertebral discs an obvious choice. A further benefit was the size, which allowed for a sequential study through all of the tissue. The presence of CGRP-like immunoreactivity, along with SP- and vasoactive intestinal peptide-like immunoreactivity in the intervertebral disc of the rat has been reported previously (Weinstein et al. 1988). However, as few experimental details were present in that report, a more complete study of this subject was justified. CGRP-like immunoreactivity was chosen to give an assessment of a large component of the sensory innervation to this tissue. An estimate of the proportion of this which was of a myelinated fibre origin was to be determined using the monoclonal antineurofilament antibody, RT97.

MATERIALS AND METHODS

Tissue from a total of 8 normal female Wistar rats aged 8–15 wk was used in this study. Intervertebral discs were excised from 2 levels to compare the overall distribution of immunoreactivity in each. The L2/3 and L4/5 intervertebral discs were dissected free from the majority of vertebral bone after cutting through adjacent tissue, e.g. posterior longitudinal ligament and anterior longitudinal ligament, with a scalpel blade. The main emphasis at all times was to ensure the annulus fibrosus was not damaged. Care was also taken not to expose the nucleus pulposus; some end-plate/vertebral body bone therefore remained adhering to the intervertebral disc. This could also be used to maintain the shape of the intervertebral disc during the initial processing. The excised material was fixed by immersion in Zamboni's fixative (Stefanini et al. 1967) for 2 h at room temperature and then cryoprotected by a 10–15 h immersion in a solution of 30% sucrose in 0.1 M phosphate buffer, pH 7.4, at a temperature of 4 °C. The material was sectioned on a cryostat, where serial sections were taken with a thickness of 20 μm on microscope slides coated with gelatin. Between 80 and 150 adjacent sections could be made from each intervertebral disc. Adjacent sections in any series had a gap of 40 μm between them. The sections were taken transversely from 12 intervertebral discs and sagittally from 4 intervertebral discs.

Endogenous tissue peroxidase activity was quenched by soaking the sections for 30 min in 0.3% hydrogen peroxide solution in distilled water. Adjacent series from each intervertebral disc were processed with one of the two antibodies described below. All dilutions of antibody were made in isotonic phosphate-buffered saline (pH 7.4). The antiserum to CGRP was a rabbit polyclonal IgG diluted 1:800 (donated by Professor J. Polak, Hammersmith Hospital or obtained from Amersham Ltd). The antibody reaction site was revealed using the indirect peroxidase technique (goat antirabbit IgG conjugated with horseradish peroxidase enzyme obtained from Dako) using diaminobenzidine (Lawson et al. 1984).

The RT97 antibody was a mouse monoclonal IgG used at a dilution of 1:800 (donated by S. Lawson, Bristol University). The antibody reaction site was revealed as described for the antisera to CGRP, the horseradish peroxidase being localised on a goat antimouse IgG (Dako) for this antibody.

The sections of immunohistochemically processed tissue were viewed under a light microscope (Olympus). Details of the use and specificity of both these antibodies have been published previously (Gibson et al. 1984; Lawson et al. 1984). Controls of 2 types were performed: (1) the second layer, horseradish peroxidase-conjugated antibody, was applied to sections without prior application of the primary antibody, or (2) primary antisera to CGRP which had been preincubated with 10^{-5} M CGRP were applied to the sections. Positive controls included application of the antisera to 7 μm thick sections of dorsal root ganglia extracted from the rat lumbar spine (McCarthy & Lawson, 1990). Both tissues were removed at the same time and prepared for immunohistochemistry in the same way.

Each section under study was drawn and the position of any immunoreactive structure labelled. This allowed approximate reconstructions of the whole intervertebral disc to be made. Furthermore, a reasonable estimate of the extent of its innervation was also possible using this method.

RESULTS

Control sections, treated as outlined above, were clear of reaction product, except for occasional blood-related peroxidase activity. No fibres or other structures which could be mistaken for the innervation were present on the negative controls, i.e. without active primary antiserum. Studies of the antibody reactivity on sections of rat lumbar dorsal root ganglia showed qualitatively similar staining patterns to those reported previously (McCarthy & Lawson, 1990).

The immunoreactivity found in L2/3 intervertebral discs was qualitatively similar to that found in L4/5 intervertebral discs. For that reason the results will be described as being from lumbar intervertebral discs and no further distinction between level of origin will be made.

General morphology

The normal rat lumbar intervertebral disc has a similar gross morphology to that of other mammals such as man. In the present material the outer fibres of the annulus fibrosus were seen to form lamellae which were thicker in the anterior than the posterior aspects. There were approximately 30–35 distinct lamellae, of which between 2 and 4 appeared continuous with the adjacent ligaments. The rest fused with the end-plate cartilage. Towards the centre of the intervertebral disc the lamellae became less distinct and the inner annulus eventually merged with the contents of the nucleus pulposus. The nucleus comprised an oval structure whose craniocaudal diameter was between 0.7 and 1.2 mm in the sectioned tissue, i.e. not under load. The anteroposterior diameter of the nucleus was between 1.5 and 2 mm, with a distinct outward distortion and compression of the posterior annulus fibres being present in all specimens.

It must be noted here that photomicrography of the intervertebral disc is hampered by the arrangement of the tissue. The lamellae in the annulus negate the use of interference contrast methods to enhance detail of staining. The thickness of the tissue compounded the problem, but this was the only way possible to follow the finer elements of the innervation.

Intervertebral disc

Immunoreactivity was present as fibres of varying length and width, mainly within the outermost 4 or 5 lamellae of the annulus fibrosus (Fig. 1). Very few (4 fibres in the whole study) were found deeper than this, the deepest being as far as 8 laminae from the outside. All the deeper fibres were CGRP-like immunoreactive. RT97-immunoreactive fibres tended to be thicker and were much rarer than those with CGRP-like immunoreactivity. Immunoreactive fibres were of 2 types: thin and difficult to trace microscopically, or thick, short and relatively easy to follow. RT97-immunoreactivity was mainly of the latter type and terminated as a complex fluorescence (Fig. 2). The majority of fibres were found in the anterolateral intervertebral disc close to the vertebral body. Only one such fibre entered the disc to any depth; this kept a very close association with the end-plate and eventually terminated in the adjacent annular tissue. It was possible to determine, on serial sections, that these endings contained both CGRP-like immunoreactivity and RT97-immunoreactivity (Fig. 2). In contrast, CGRP-like immunoreactivity was predominantly found as thin fibres which were longer and, following their entry into the annulus fibrosus, tended to run between the lamellae of collagen bundles. Inside the intervertebral disc, as well as outside, the thin fibres were more abundant than the thicker ones. Both types of immunoreactivity were present on the anterior as well as the posterior aspects of the intervertebral disc. Few immunoreactive fibres were found penetrating the lateral edges of the intervertebral disc; those which did enter the annulus tended to return to the adjacent ligaments. There was no obvious relationship between vascular tissue and immunoreactivity either inside the intervertebral disc

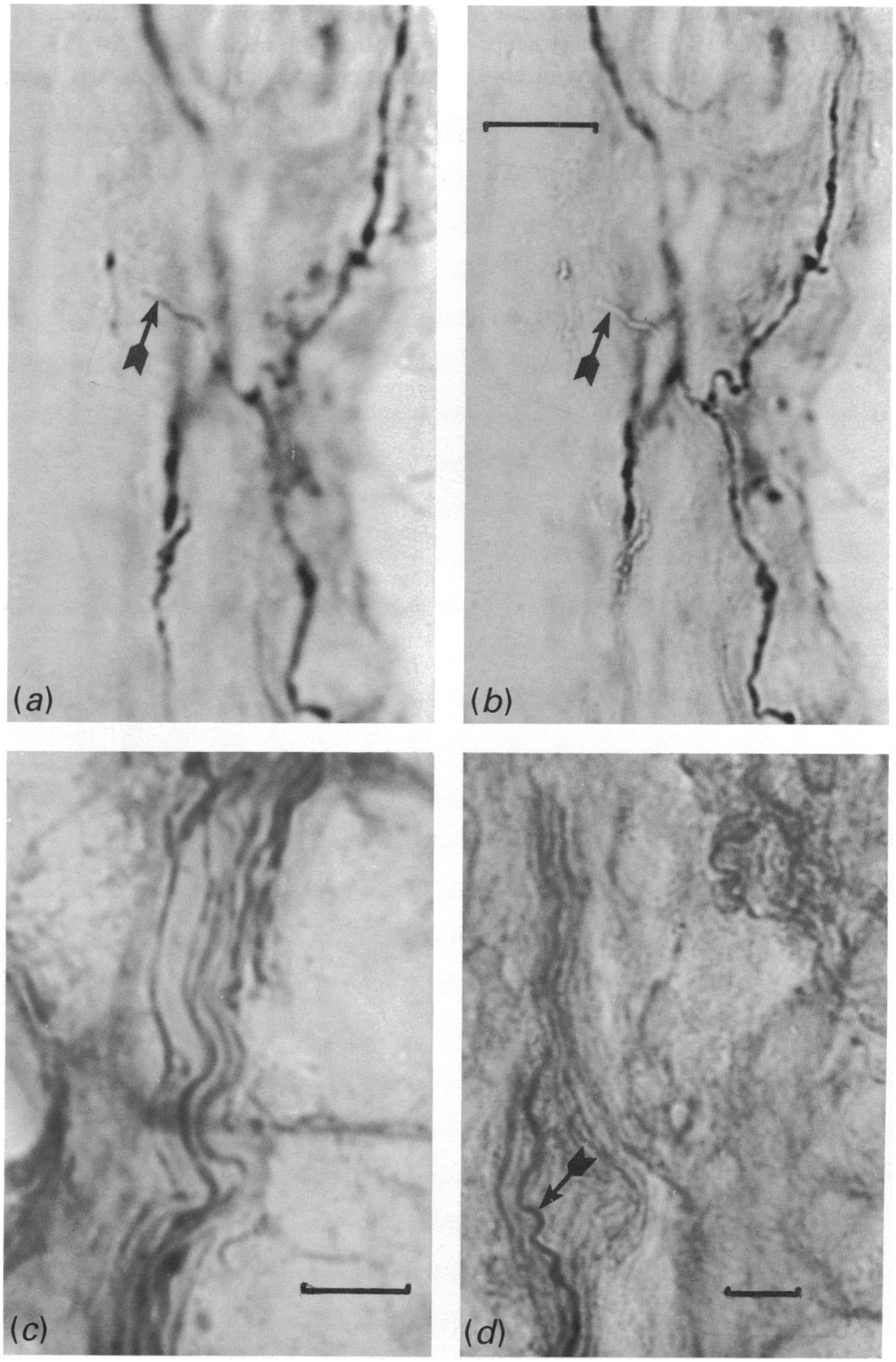

Fig. 1. Immunoreactive nerve fibres in intervertebral disc. (*a*, *b*) Adjacent longitudinal ligaments and adipose tissue (*c*, *d*). (*a*) CGRP-like immunoreactivity in the outer annulus fibrosus. The thicker fibres travel parallel to the lamellae whereas occasional fine structures (arrows) appear perpendicular to these. This can be seen more clearly in (*b*), which is the same field at a different focal plane. (*c*) A bundle of CGRP-like immunoreactive fibres in the adipose tissue lying between the anterior longitudinal ligament and annulus fibrosus. (*d*) RT97-immunoreactivity in a bundle of fibres travelling in the posterior longitudinal ligament. Bars, 10 μm.

or at the site of entry of either into the intervertebral disc. However, both RT97 and CGRP-like immunoreactivity were found in areas of vascularity outside the intervertebral disc. No CGRP-like immunoreactivity or RT97-immunoreactivity was found in the nucleus pulposus or the inner annulus fibrosus. The

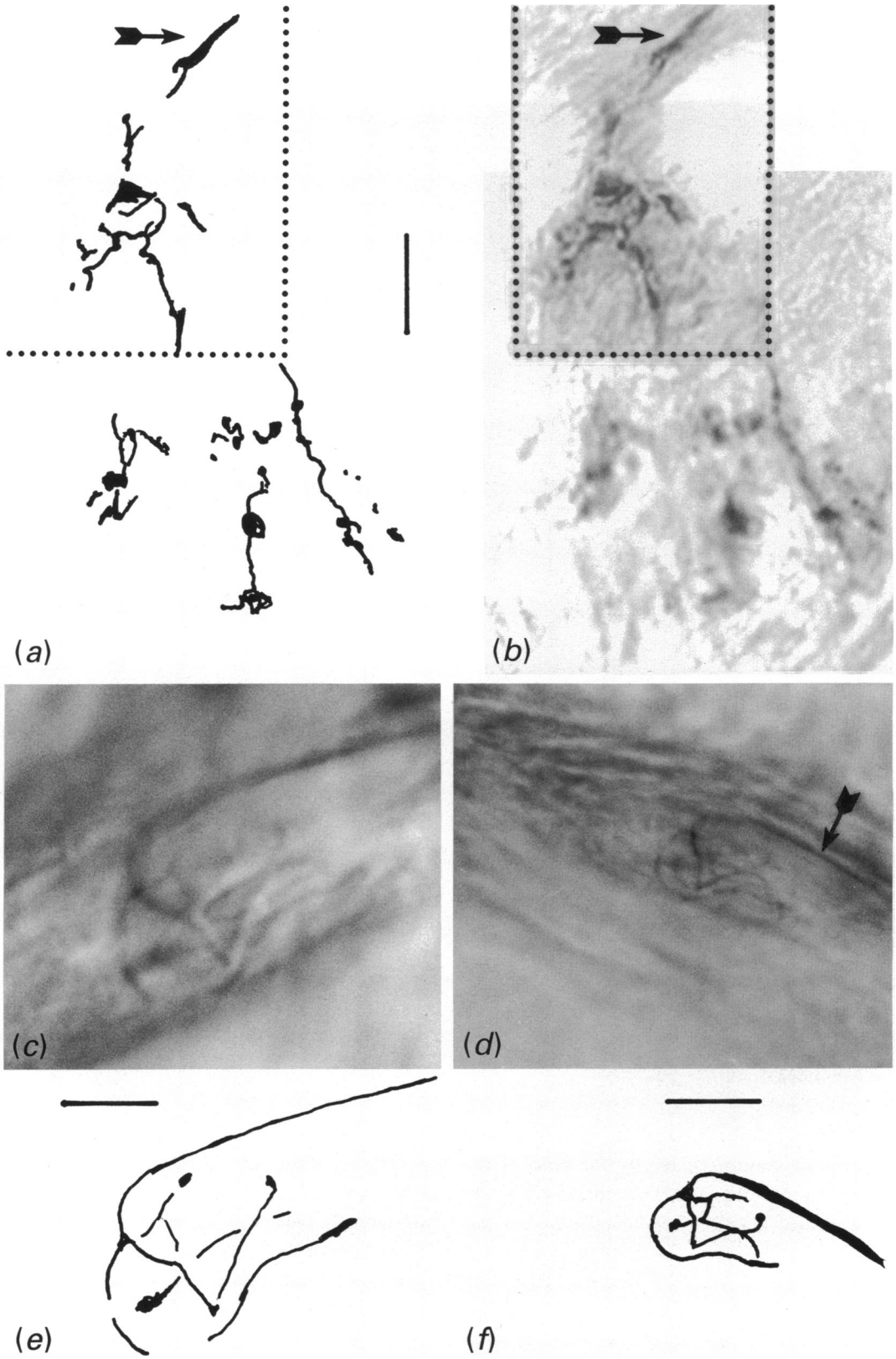

Fig. 2. Immunoreactive complex nerve terminal structures found within annular tissue. (*a*, *b*) Composite nerve ending made from 2 adjacent sections. The upper boxed area shows the CGRP-like immunoreactivity section, whereas the lower area shows the RT97-immunoreactivity in the same orientation. (*a*) is the schematic representation of (*b*), the ending being located on the anterior edge of an L4/5 intervertebral disc, 4 lamellae from the anterior longitudinal ligament. (*c*, *d*) Photomicrographs of a whole nerve ending stained for CGRP-like immunoreactivity. (*e*, *f*) Schematic representations of (*c*) and (*d*). The thickness of the tissue and alignment of the collagen fibres compounded the difficulty in resolving such fine immunoreactivity. It is possible to see bulb-like terminations at the end of some finer fibres. (*c*) used a higher power [× 100] objective with bright field, whereas (*d*) used an intermediate objective [× 40] with bright field and the condenser aperture shut down to increase depth of field. Any use of interference contrast greatly emphasised the background with respect to the immunoreactivity. Bars: (*a*), (*b*), 20 μm; (*c*), (*e*), 10 μm; (*d*), (*f*), 20 μm.

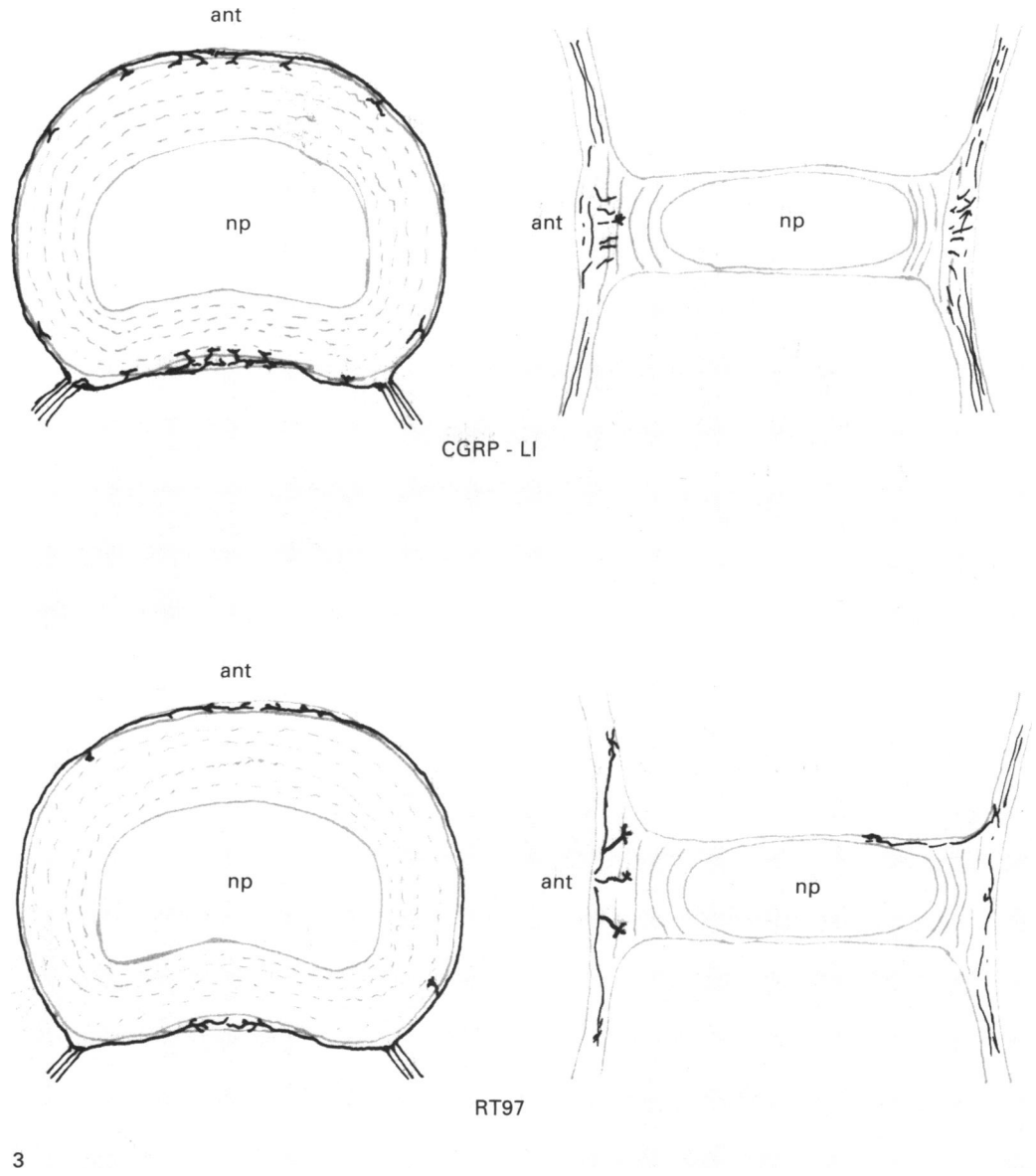

Fig. 3. Schematic representations of RT97-immunoreactivity (lower) and CGRP-like immunoreactivity (upper) in the intervertebral disc and adjacent ligamentous tissue. Transverse (right) and sagittal (left) sections through a hypothetical midline are outlined faintly with the innervation represented by bold lines. The anterior annulus (*ant*) was wider than the posterior annulus. No immunoreactivity was found in the nucleus pulposus (*np*).

overall patterns of immunoreactivity for both of the antibodies in the lumbar intervertebral discs are illustrated in Figure 3.

Adjacent tissue

The periosteum and vascular channels which entered the vertebral bodies, as well as adjacent longitudinal ligamentous tissue, contained a variety of RT97-immunoreactive and CGRP-like immunoreactive fibres and endings (Fig. 4). Again, only a small proportion of the immunoreactivity was to RT97 and, as stated above, these were the thicker fibres.

The majority of the CGRP-like immunoreactive fibres were thin, and terminated as fine filaments. Few apparently encapsulated endings were found. These were similar to those shown in Figure 2, and were mainly located close to the junction between the end-plate, intervertebral disc and ligaments, especially on the anterior or anterolateral aspects. In all cases where it was possible to resolve both immunoreactivities it was determined that these structures were also RT97-immunoreactive. On initial entry into the ligamentous tissue many of the immunoreactive fibres travelled antero- or posterolaterally. Offshoots could then be found leaving the bundle to travel caudally or

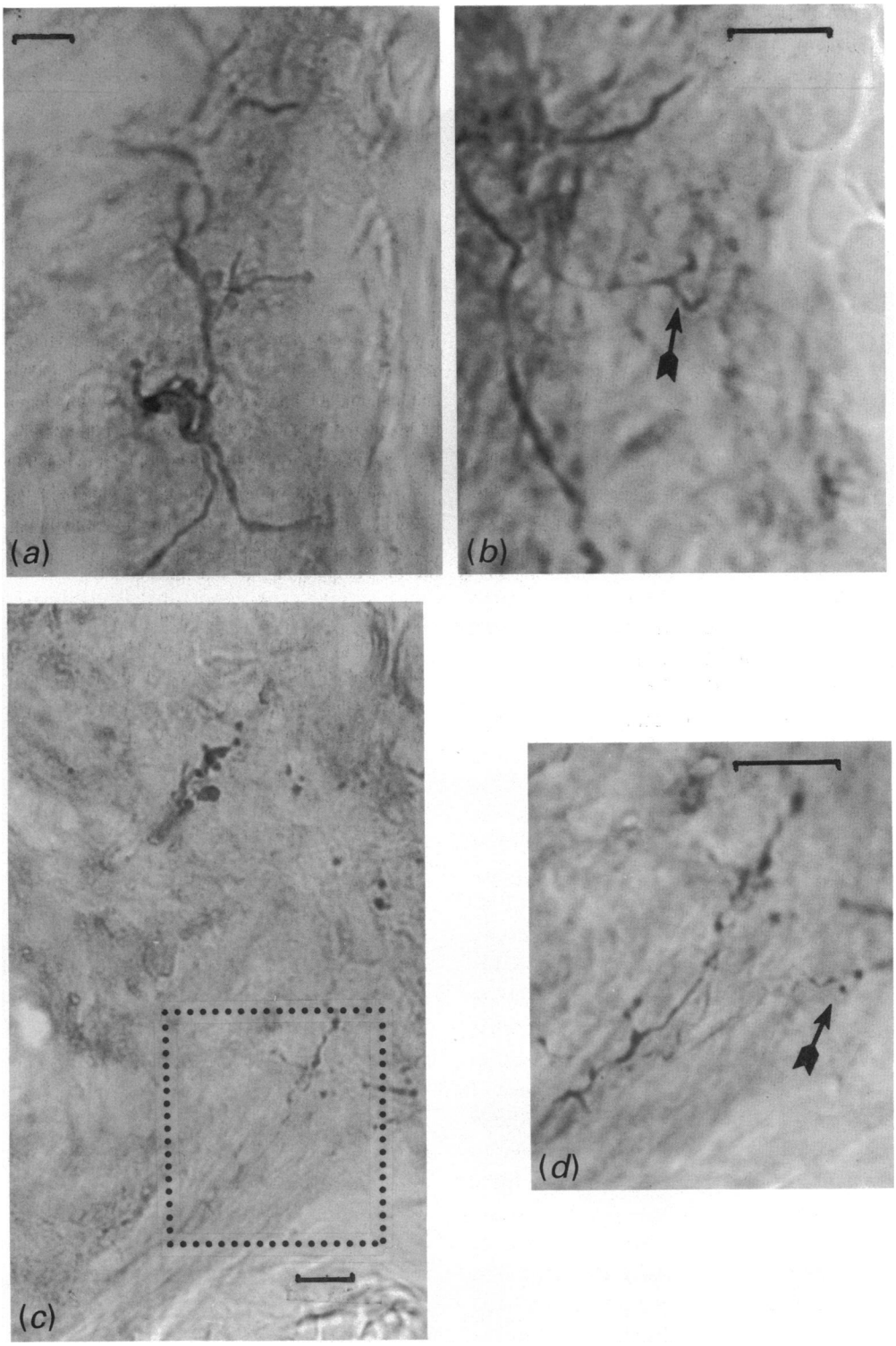

Fig. 4. Immunoreactive nerve terminations in ligaments and vascular tissue related to the vertebral body. (*a*) RT97-immunoreactivity at the junction between anterior end-plate and anterior longitudinal ligament. (*b*) CGRP-like immunoreactivity (arrow) in ligamentous tissue lateral to the anterior longitudinal ligament, adjacent to the end-plate. (*c*) Vascular channel containing CGRP-like immunoreactive fibres, some of which appear to be entering the vertebral body. One of these structures has been magnified (*d*) to illustrate the fine detail and possible terminal structures (arrow). Bars, 10 μm.

cranially; the actual arrangement around the intervertebral disc in the longitudinal ligaments is very complex. A high density of CGRP-like immunoreactive fibres was usually associated with the entry of vascular channels into the bone. This was not as obvious with respect to RT97-immunoreactivity; however, some of these fibres entered the vertebral body and penetrated deep enough to be visible in the

vascular compartments inside the end-plate. No terminal structures were found inside the vertebral body.

DISCUSSION

A large proportion of the CGRP-like immunoreactive fibres found in this study were best described as fine and were apparently not immunoreactive to the RT97 antibody. It may be assumed that these axons were unmyelinated or C-fibres, a proposal which is supported by their negative RT97-immunoreactivity (Lawson & Waddell, 1985; McCarthy & Lawson, 1990). There were many CGRP-like immunoreactive fibres, of various thicknesses, in both anterior and posterior regions of the intervertebral disc. This confirms the results of a previous study of the posterior ligaments and annulus of rat intervertebral disc (Kojima et al. 1990*a*, *b*). The general layout of the acetylcholinesterase-containing innervation appeared very similar to that found in this study. However, these previous studies limited themselves to the larger fibres. Many of the finer CGRP-like immunoreactive fibres which entered the intervertebral disc remained in close apposition to the longitudinal ligaments. On entry into the intervertebral disc, the fine fibres eventually disappeared amongst the networks of annular fibres. In such instances it is not possible to be entirely certain as to overlap between the 2 immunoreactivities. However, the disparity between RT97-immunoreactivity and CGRP-like immunoreactivity in fine fibres would indicate that many were probably unmyelinated. Occasionally, it was possible to see very small (10 μm long) branches at the site of what was assumed to be the nerve ending; in these cases no evidence of encapsulation was found. The presence of such free nerve endings would support any hypothesis which suggested that the annular tissue is sensitive to noxious stimuli. It would also appear that these observations exclude the obvious, yet previously understated, possibility of the fine fibre innervation of the intervertebral disc being primarily of an efferent sympathetic origin. The possibility of a sympathetic innervation to the intervertebral disc is based on the presence early in an animal's life of a vascular supply to the intervertebral disc (Peacock, 1952). The animals in this study were sexually mature, and well above the critical age at which the great reduction in vascularisation occurs (Peacock, 1952; Brunner & Frewein, 1989). The persistence of a remnant of the original vascularisation to the outer annular fibres has been reported (Jamiolkowska, 1981). This would give a potential role and route of entry into the annulus for any nerve supply. As reported here, however, there was no obvious relationship between immunoreactive fibres and the entry into the intervertebral disc of vascular channels.

There was a relatively small number of fibres and terminal structures immunoreactive to both RT97 and CGRP antisera. Such fibres were thicker, and predominantly found on or in the anterior aspects of the intervertebral disc. The predomination of complex endings in the anterior/anterolateral aspects of the intervertebral disc in association with the vertebral end-plate confirms the results of a previous study on canine intervertebral discs (Guillot et al. 1988). This would also support the suggestion that these fibres, and the anterior annulus, have a role in mechanoception, and may even indicate that this role exists throughout the animal kingdom. The finding of RT97-, as well as CGRP-immunoreactive fibres entering the vertebral body via the vascular channels both confirms and extends the report by Bjurholm et al. (1988), implying that a part of the innervation to that tissue may be of a myelinated sensory origin. No terminal structures were found within the vascular channels of the vertebral body, so that the functional significance of this finding remains open to speculation.

Although these results are interesting, it must be recognised that they are from a quadruped and not a biped. Such changes may produce differences in the biomechanics of the system and, therefore, probably also in the sensory innervation pattern. The proportions of each innervation reported here may therefore be different from what is found in man. This does not detract from the relevance of this study, however, as both systems would be under load and experience similar forms of distortive stresses. The consequences of having endings from both myelinated and unmyelinated sensory axons in relation to the normal functioning of the spine would probably be similar in all mammals, including man.

The role of the sensory nerve supply to the intervertebral disc has previously been restricted to the reception of painful stimuli. Supporting the concept that neuropeptides may have a role in the intervertebral disc, especially in the 'painful' disc, is evidence such as that of Weinstein et al. (1988), who showed a changed neuropeptide content in the sensory ganglia related to the damaged segment. It is possible that the changes seen by this group, however, were a result of the effects of trauma on the tissue adjacent to the intervertebral disc, which has a greater innervation. Therefore, with respect to the role of this innervation, pain perception cannot be ruled out. From the position and type of the innervation

described here it may also be concluded that the nerve fibres also have other functions. It may be postulated that responses to positional change, such as stretch, torsion and distension, might be a plausible alternative to pain perception. A further consideration is that of the potential efferent role for sensory neurons (Holzer, 1988). CGRP has a variety of actions which could form part of this role, such as cutaneous vascular (Brain et al. 1985) and osteogenic effects (Bernard & Shih, 1990). In addition, it must be considered a possible candidate in the sensitisation of the so-called 'silent' C fibres as postulated by McMahon & Koltzenburg (1990). It would appear from this list of possible actions that the involvement of the neuropeptide CGRP in a tissue such as the intervertebral disc may be on a variety of levels: from everyday feedback supporting growth to extremes where it causes vascular changes, exacerbating the effects of inflammation, sensitisation of extra 'pain-sensitive' fibres and even stimulation of osteogenesis within the tissue. Such effects would obviously lead to the exacerbation of any acute symptomatology which had initially stimulated the sensory fibres.

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