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Innervation of the human cardiac conduction system at birth

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Abstract

***Objective*—To study the pattern of innervation of the conduction system of the neonatal heart in humans.**

***Design*—A prospective analysis based on immunohistochemical and enzyme histochemical examination of newborn human hearts.**

***Setting*—A general district hospital.**

***Main outcome measures*—Fresh necropsy tissue.**

***Material*—Hearts of three neonatal humans with no cardiac anomaly, freshly taken at necropsy.**

***Methods*—Serial sectioning to obtain a three dimensional reconstruction of the cardiac conduction system, followed by identification of the pattern of innervation by immunohistochemical and enzyme histochemical techniques; with a panel of antisera against protein gene product (PGP) 9·5 as a general neural indicator; dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) as indicators for sympathetic neural tissue; and selected neuropeptides—namely, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calcitonin gene related peptide (CGRP), and substance P (SP). Gomori's technique was used for locating cholinesterase activity.**

***Results*—PGP immunoreactive (PGP-IR) nerves were present in large numbers in the sinus node, atrioventricular (AV) node, and penetrating atrioventricular bundle; in moderate numbers in the branching bundle; and occasionally in the bundle branches. Small numbers of DBH-IR and TH-IR nerves were seen in the sinus and AV nodes, mainly perivascularly; there were few in the penetrating and branching bundles and none in the bundle branches. A few perivascular NPY-IR nerves were seen only in the sinus node. VIP-IR, CGRP-IR, and SP-IR nerves were not seen. Pseudocholinesterase activity was found in the conduction tissue, whereas occasional acetylcholinesterase positive nerves were found only in the sinus and AV nodes.**

***Conclusion*—A considerable innervation of the human cardiac conduction system is present at birth, although, by comparison with the results of other studies on adult tissue, the mature pattern has not yet been established. Thus it is still in the process of maturation, especially with regard to the acquisition of various neurotransmitters, including the more recently described neuropeptides.**

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Knowledge of the anatomy of the autonomic innervation of the cardiac conduction system is crucial to elucidate mechanisms of neural control of cardiac function, particularly the genesis of arrhythmias. Innervation has been extensively studied in various mammalian species, with not only silver stains for neural tissue in general, but also histochemical methods for cholinergic nerves and histofluorescent techniques for adrenergic nerves.[1–3] More recently, sensitive immunohistochemical techniques have allowed more specific and precise anatomical resolution;[4–6] and have identified peptidergic neurotransmitters in cardiac ganglion cells and nerves that supply the conduction system of the heart.[7] Considerable variation exists between species in the density and distribution of subtypes of nerves in mammalian hearts.[8] It is thus potentially inaccurate to base concepts of the patterns of innervation in the human on findings in other species.

There have been few studies that have used enzyme histochemical techniques to examine the innervation of the human heart,[1 9 10] and no immunohistochemical studies on the innervation of the human cardiac conduction system have been previously reported, to the best of our knowledge. Techniques have shown the cholinesterase positive innervation of the conducting tissue of adult[1 11] but not neonatal human hearts. We, therefore, set out to delineate the pattern of innervation of the cardiac conduction system in the newborn human heart, by a combination of enzyme and immunohistochemical techniques.

Patients and methods

TISSUE PREPARATION

Hearts were taken at necropsy within 18 hours of death from three full term newborn infants with no congenital cardiac anomalies. Table 1 shows the relevant clinical information.

Tissue blocks containing the sinus node, atrioventricular node, penetrating bundle, branching bundle, and bundle branches were prepared by the method described by Davies *et al.*[12] These were immediately frozen in isopentane previously cooled in liquid nitrogen. Each tissue block was then serially

Table 1 Clinical summaries of the three newborn infants at birth

Patient	*Sex*	*Age (time from death to necropsy)*	*Heart weight*	*Cause of death*
1	M	Day 2 (18 h)*	16·7 g	Short limbed dwarfism (thanatophoric dysplasia)
2	F	Still birth (15 h)*	18·0 g	Cord round neck twice
3	F	Day 10 (6 h)*	17·1 g	Severe asphyxia neonatorum (undiagnosed breach presentation)

*Time intervals between death and necropsy in hours are indicated in parentheses.

sectioned in a cryostat to a thickness of 10 μm, mounted on gelatin subbed glass slides, and stored at −70°C. Initially, every 20th section was stained with Masson's trichrome technique (fig 1A), and these were used to evaluate the morphology of the specialised conduction tissue. Thus we made a three dimensional reconstruction of the conduction tissue of the heart. In the interesting areas, the intermediate sections were studied by enzyme and immunohistochemical methods.

IMMUNOFLUORESCENCE REACTIVITY

We used an indirect immunofluorescence technique with avidin-biotin. Table 2 shows the details of the primary antisera. Cryostat sections were air dried at room temperature for one hour and then fixed in 0·4% parabenzoquinone for three minutes. They were then sequentially incubated at room temperature with the diluted primary antisera for 16 hours, biotinylated antirabbit immunoglobulin for 30 minutes, then fluorescein avidin D for 30 minutes, with thorough washing in phosphate buffered saline between the steps. The sections were mounted in glycerol mixed 1:1 with buffered saline and examined with an epi-illuminated microscope. Appropriate positive and negative controls were used for all the antisera.

The relative number of immunostained nerves was assessed visually in a semiquantitative manner and graded from 0 to + + + +. Examples of this grading are given in the figures.

CHOLINESTERASE ACTIVITY

Tissue sections were fixed in formol calcium at 4°C for 20 minutes. They were washed in tap water and then incubated in Gomori's stock solution,[13] containing 2 mg/ml of acetyl thiocholine iodide as substrate, at 37°C, with media at pH 6·0 for 16 hours. After washing in tap water, they were developed for 60 seconds in freshly prepared 1% ammonium sulphide solution at 20°C, and counterstained in haematoxylin for two minutes. The sections were dehydrated, cleared, and mounted in Canada balsam dissolved in tetrachlorethylene. Control sections were incubated in the absence of substrate.

As appropriate, sections were preincubated at 20°C with tetra-isopropyl-pyrophosphoramide (Sigma Chemicals, USA) as an inhibitor of pseudocholinesterase. Other sections were incubated in the presence of 1,5-bis (4-allyldimethyl-ammoniumphenyl)-pentan-3-one-dibromide (Sigma Chemicals, USA) as an inhibitor of acetylcholinesterase.

The intensity of the staining reaction was also assessed semiquantitatively and graded from 0 to + + + +, with examples given in the figures.

Results

The patterns of immunofluorescence and enzyme histochemistry of the innervation of the cardiac conduction system of the three hearts were similar (tables 3 and 4).

IMMUNOFLUORESCENCE

Table 3 shows that abundant protein gene product 9·5 immunoreactive nerve fascicles and fibres were present in the sinus node, both in the perivascular regions and among the nodal cells (fig 1B). On high power magnification, many of the nerve fibres were seen close to the nodal cells (fig 1C). Positively staining nerve fascicles and fibres were also seen among the cells of the atrioventricular node, penetrating bundle (of His), and the branching bundle, the density of fibres being slightly less than in the sinus node. In the bundle branches, however, positively staining nerve fibres were only seen focally, concentrated in their proximal portions among and close to the conduction fibres (fig 2). Many nerves marked with reaction product were also present in the right atrial and interventricular septal myocardium adjacent to the conduction tissue, and were more numerous in the atrial myocardium.

Dopamine β-hydroxylase immunoreactive nerves were present in the sinus and atrioventricular nodes, but were sparse. They were seen mainly in the perivascular regions with extensions among the adjacent nodal cells (fig

Table 2 Characterisation of antisera for immunohistochemistry

Antisera	*Dilution*	*Source*
Protein gene product 9·5—polyclonal (rabbit)	1:400	Ultraclone
Dopamine β-hydroxylase	1:200	Eugene Tech International, USA
Tyrosine hydroxylase	1:500	INCSTAR, USA
Neuropeptide Y	1:800	UCB Bioproducts, Belgium
Vasoactive intestinal polypeptide	1:500	ICN Immunobiologicals, USA
Calcitonin gene related peptide	1:30	UCB Bioproducts, Belgium
Substance P	1:500	Amersham

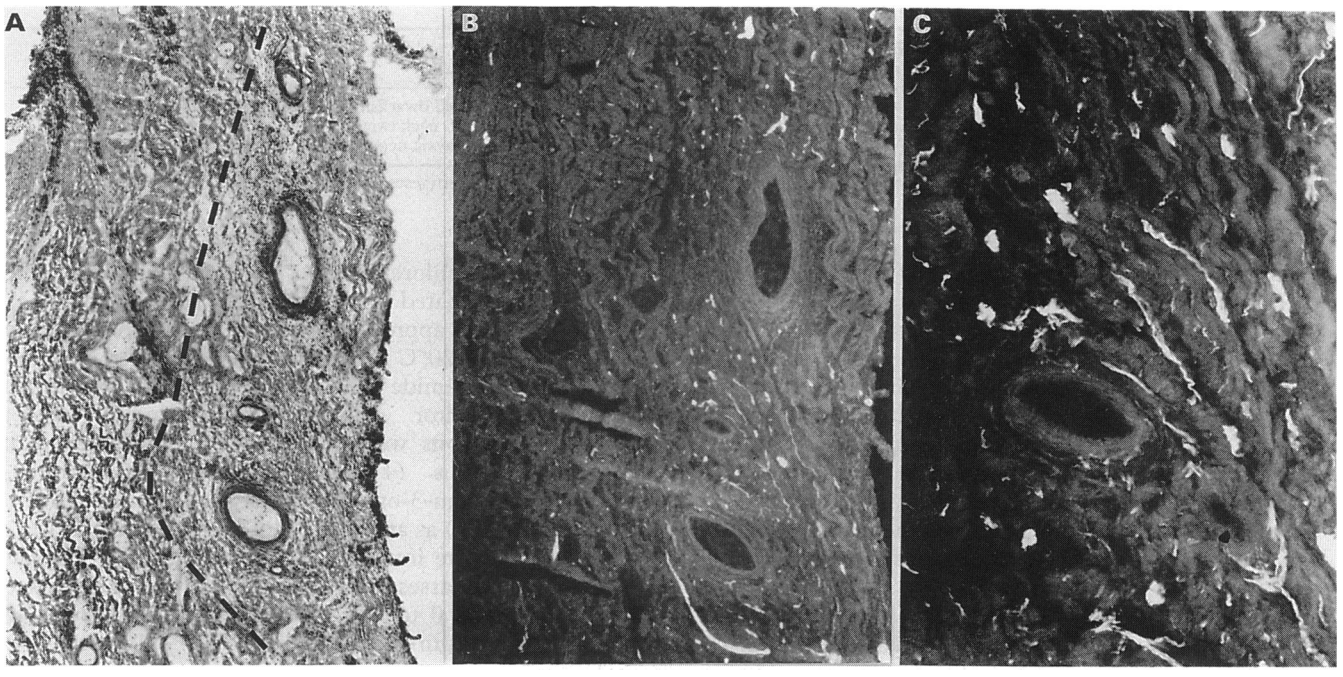

Figure 1 (A) Section of the sinus node stained with Masson's trichrome technique, showing the network of nodal cells grouped around branches of the nodal artery (outlined with dotted lines). Original magnification, × 5. (B) Immunofluorescence micrograph of an adjacent section of the sinus node. The immunofluorescent nerves appear as white wavy lines. There is an abundant network of nerves (+ + + +) displaying positive immunostaining for protein gene product 9·5. Original magnification, × 5. (C) High power magnification to show the close apposition of nerve fibres to nodal cells (+ + + +). Original magnification, × 20.

3). In the penetrating and branching bundles, fluorescent nerves were scarce, occurring only in perivascular locations. Nerves were absent from the bundle branches but occasional perivascular nerves were found in the fibrous sheath surrounding the bundle branches. Few perivascular nerves positive for dopamine β-hydroxylase were seen in the atrial and ventricular myocardium. The pattern of distribution of the nerves reacting to tyrosine hydroxylase was similar, but they were even fewer in number.

Only occasional perivascular nerves reacting to neuropeptide Y were found in the conduction tissue or the atrial and ventricular myocardium. No nerves were found reacting to vasoactive intestinal polypeptide, calcitonin gene related peptide, and substance P.

CHOLINESTERASE ACTIVITY

Table 4 shows that the cells of the sinus node showed prominent pseudocholinesterase but weak acetylcholinesterase activity (fig 4A). Also, nerve fibres with a positive pseudocholinesterase reaction were seen among the nodal cells (fig 4B). Few nerve fibres had acetylcholinesterase activity, but they could still be identified amongst the nodal cells. By contrast, the adjacent atrial myocardial fibres were unstained (fig 4A).

A similar pattern of reaction was seen in the atrioventricular node, penetrating and branching bundles, and bundle branches (fig 5). Occasional nerve fibres showing positive cholinesterase staining were noted only in the atrioventricular node. The adjacent ventricular myocardium was devoid of cholinesterase activity (fig 5).

Table 3 Pattern of innervation by immunofluorescence

Region	*PGP 9·5*	*DBH*	*TH*	*NPY*	*VIP*	*CGRP*	*SP*
Sinus node	+ + + +	+ +	+	+	0	0	0
Atrioventricular node	+ + +	+ +	+	±	0	0	0
Penetrating bundle	+ + +	+	±	±	0	0	0
Branching bundle	+ +	+	0	0	0	0	0
Bundle branches	+	±	0	0	0	0	0

Relative number of immunoreactive nerves graded: 0, no immunoreactivity detected; ±, immunoreactive nerves rarely detected; +, scattered individual nerve fibres; + +, moderate number of fibres; + + +, large number of fibres; + + + +, very abundant nerve fibres. PGP 9·5, protein gene product 9·5; DBH, dopamine β-hydroxylase; TH, tyrosine hydroxylase; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide; CGRP, calcitonin gene related peptide; SP, substance P.

Discussion

The innervation of the conduction system in the neonatal human heart has not previously been studied with immunohistochemical techniques. One of the reasons for this has been the difficulty in obtaining sufficiently fresh human cardiac tissue for such studies. The validity of studying fresh postmortem material was justified by those studies that showed that surgical and relatively fresh postmortem cardiac tissue from human hearts gave comparable results on enzymohistochemical and immunohistochemical analysis.[1 10] This view is endorsed by our findings, as the results from each specimen were virtually identical, even though the hearts were taken at different times after death (six, 15 and 18 hours). Thus, with a combination of histological, enzymohistochemical and immunohistochemical techniques, we have been able to delineate the pattern, and type of innervation of the human cardiac conduction system at birth. It is, perhaps, worth emphasising that the conduction tissues are specialised myocardial cells, and that it is their intimate association with nerves which controls and coordinates conduction of the cardiac impulse.

Table 4 Pattern of cholinesterase activity

Region	Uninhibited		Acetylcholinesterase inhibitor		Pseudochinesterase inhibitor	
	Conduction fibres	Nerves	Conduction fibres	Nerves	Conduction fibres	Nerves
Sinus node	+ + + +	+ +	+ + + +	+ +	+ +	+
Atrioventricular node	+ + + +	+	+ + +	+	+	+
Penetrating bundle	+ + +	0	+ +	0	+	0
Branching bundle	+ + +	0	+ +	0	+	0
Bundle branches	+ +	0	+ +	0	±	0

Relative intensity of the staining reaction rated: 0, no staining; ±, focal equivocal staining; +, weak staining; + +, mild staining; + + +, moderate staining; + + + +, intense staining.

Protein gene product is a specific indicator of nervous tissue,[14] and is therefore useful to show the general pattern of innervation. Use of this indicator has shown that nerve fascicles and fibres are abundant throughout the specialised conduction tissue of the neonate, with the notable exception of the bundle branches and their ramifications. The fact that many nerves lie close to nodal cells (fig 1C), suggests a direct neuroeffector relation. Many nerves were also shown in the adjacent atrial and ventricular myocardium, being more numerous in the atrium. The distribution of those fibres is undergoing further analysis.

As dopamine β-hydroxylase and tyrosine hydroxylase are cathecholamine synthesising enzymes, they have been regarded as useful specific indicators for the location of sympathetic neural tissue.[15] Whereas this is valid in most instances, it is important to note the recent findings of Hardebo *et al.*[16] They have shown that several neuropeptides, together with dopamine β-hydroxylase and tyrosine hydroxylase, coexist with vasoactive intestinal polypeptide and choline acetyltransferase in subpopulations of neurons in the cranial parasympathetic ganglia of the rat. Thus the presence of both dopamine β-hydroxylase and neuropeptide Y, or the presence of tyrosine hydroxylase, is not necessarily an unequivocal indication of peripheral adrenergic neurons.

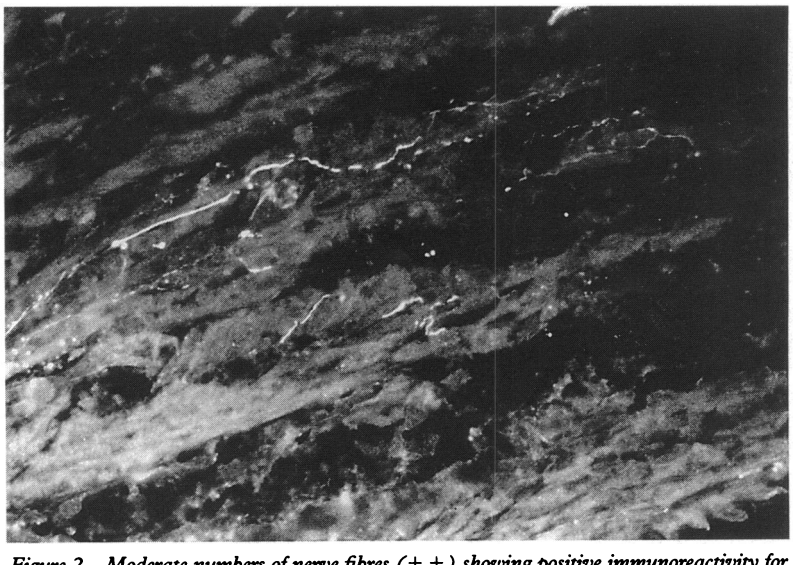

Figure 2 Moderate numbers of nerve fibres (+ +) showing positive immunoreactivity for protein gene product 9·5 in the initial portion of the left bundle branch. They are seen in close apposition to the conduction tissue. Original magnification, × 20.

In our study, nerve fibres reactive to dopamine β-hydroxylase were found mostly in the sinus and atrioventricular nodes, albeit in small numbers, around blood vessels (fig 3). There were even fewer in the penetrating and branching components of the ventricular axis, and they were absent from the bundle branches themselves. Their perivascular concentration supports the concept that the paraarterial route constitutes the most important pathway for sympathetic innervation, both for the conduction tissues and the myocardium.[3]

These findings in human neonates accord with those recently described by Ursell *et al*[4] who studied the anatomical distribution of sympathetic nerves in the developing dog heart. They showed that sympathetic neural tissue first appeared at mid-gestation in the atrium, including the sinus and atrioventricular nodes, and in the ventricular epicardium. At birth, the sympathetic nerves supplying the conduction tissue increased in number, but were still found mainly in the sinus and atrioventricular nodes. The adult pattern was not reached until two months after birth. Then sympathetic nerves were present throughout the conduction tissues with a decreasing gradient in innervation from the sinus and atrioventricular nodes to the penetrating bundle. Although the adult pattern of sympathetic innervation of the human cardiac conduction system is not yet known, it is particularly interesting to compare our findings with those of a recent study by Wharton *et al* on the general innervation of adult human hearts.[10] They showed moderate numbers of nerves positive for tyrosine hydroxylase, mainly perivascular, in both the atrial and ventricular myocardium. We found such nerves positive for tyrosine hydroxylase only rarely in our newborn hearts. Thus it seems reasonable to postulate that the sympathetic innervation of the human heart may follow a similar process of maturation as that found in the dog heart. This hypothesis has to be confirmed by more detailed studies.

Neuropeptide Y is a peptide known to coexist with noradrenaline in certain sympathetic nerves.[17–19] Its actions include inhibition of release of neurotransmitters and direct vasoconstriction of vascular beds.[19–21] In the study of Wharton *et al*, nerves positive for neuropeptide Y formed the densest population of peptide containing nerves in the adult human heart. Their pattern of distribution corresponded to the nerves positive for tyrosine hydroxylase.[10] We also found patterns of distribution of these nerves to be similar. Such nerves were the only nerves containing the peptide in our neonatal human tissues.

Vasoactive intestinal polypeptide, found in the postganglionic neurons of exocrine glands, is released with acetylcholine on parasympathetic stimulation.[22 23] In the heart, this peptide has been shown to have a powerful direct positive chronotropic effect, producing coronary vasodilation as well as dromotrophic changes in the specialised conduction tissue.[24 25] Although nerves that

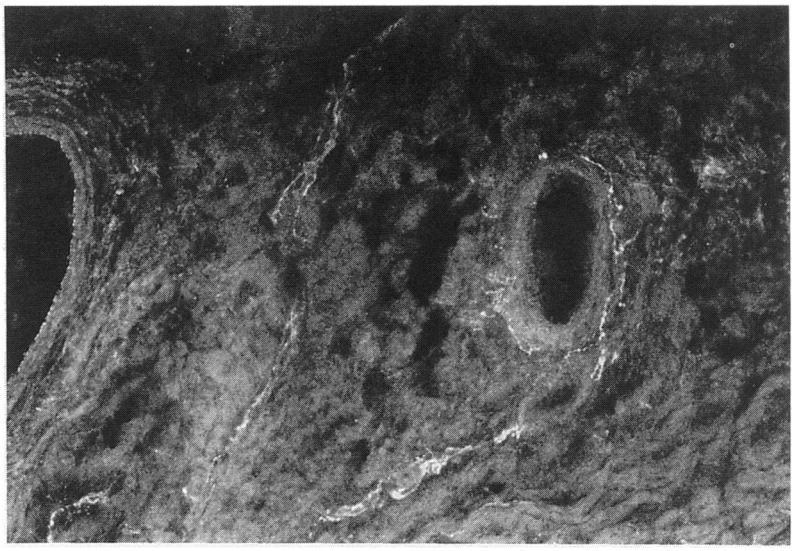

Figure 3 Sinus node containing moderate numbers of nerve fibres (+ +) showing positive immunostaining for dopamine β-hydroxylase. Original magnification, × 20.

display immunoreactivity to vasoactive intestinal polypeptide have been found in the adult human heart, mainly in the atrium,[10] we were unable to show their presence in the newborn heart.

Calcitonin gene related peptide and substance P are present in afferent nerves supplying the mammalian cardiovascular system, and have been shown to play an important part in the neurogenic inflammation syndrome.[26-28] Although found in other species such as the guinea pig[6 29 30] and in adult human hearts,[10] such nerves were not found in our newborn human hearts. Ursell *et al* found only rare varicose nerves that react to calcitonin gene related peptide in the canine heart during late gestation, but the neonatal dog heart contained abundant neural tissue reacting in this way.[5] Thereafter, the density of nerves decreased progressively to reach the adult pattern in which nerves reactive to this peptide were sparse. The area of the sinus node was the primary focus of innervation at all stages, although the atrioventricular nodal region was also preferentially innervated. Further studies on the distribution of these peptides in human hearts of varying ages would thus be of considerable interest.

Corresponding to the findings of previous workers,[1 11] we found moderate cholinesterase activity in the conduction tissue of the human heart at birth (figs 4 and 5). Most of this represents pseudocholinesterase, as the intensity of staining declined considerably after pre-incubation with inhibitors of pseudocholinesterase. A few acetylcholinesterase positive nerves were seen among the nodal cells in the sinus and atrioventricular nodes, but not in the bundle branches. The absence of positive nerves in the bundle branches is no more than expected, as only occasional nerve fibres were shown in these regions by the general nerve indicator, protein gene product. These results contrast with those of Kent *et al*[1] who showed a rich supply of acetylcholinesterase positive nerves not only in the sinus and atrioventricular nodes of adult human hearts, but also in the bundle branches. Furthermore, they found little or no pseudocholinesterase in the conduction cells. If their findings are correct, it seems that, as the human cardiac conduction system matures, it gradually loses its content of pseudocholinesterase, but acquires a rich supply of acetylcholinesterase positive nerves, presumably cholinergic in nature. Thus although at birth, the human cardiac con-

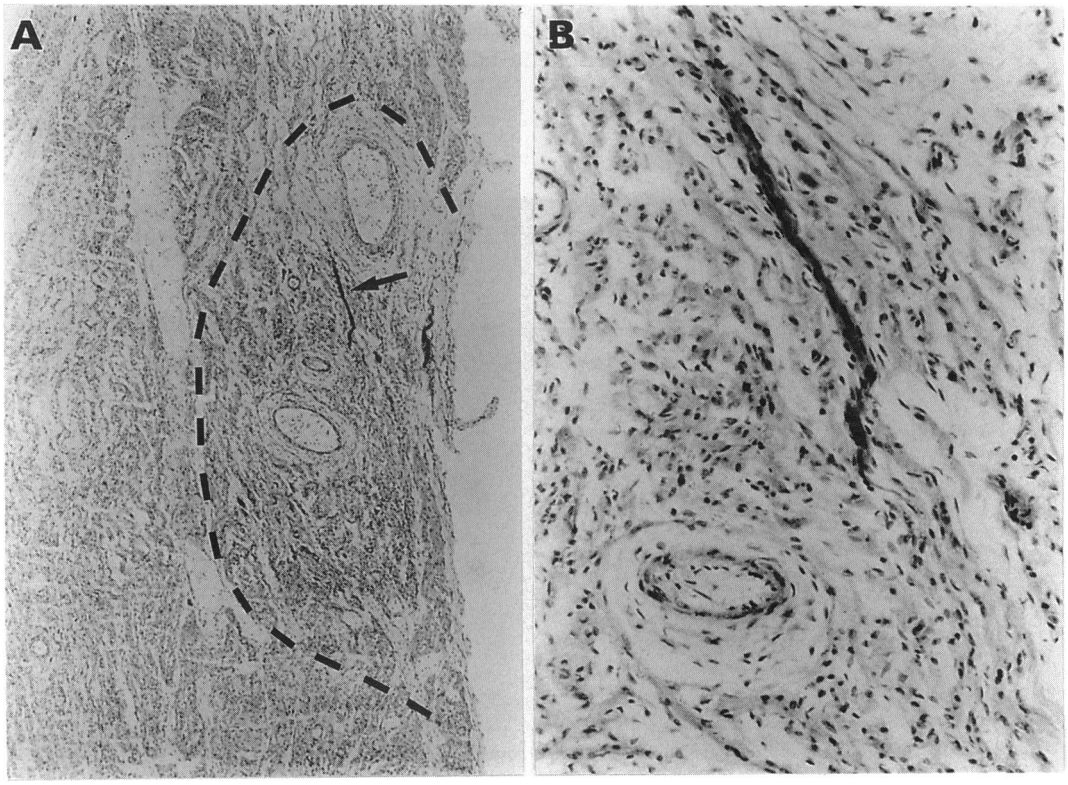

Figure 4 A) The nodal cells show intense cholinesterase staining (+ + + +) (outlined with dotted lines). Moderate numbers of nerve fascicles (+ +) (arrow) with positive cholinesterase staining are seen among the nodal cells. The adjacent atrial myocardium shows negative staining. Cholinesterase staining, original magnification, × 5. (B) Higher magnification to show the positive staining of the nerve fascicle and nodal cells. Cholinesterase staining, original magnification, × 20.

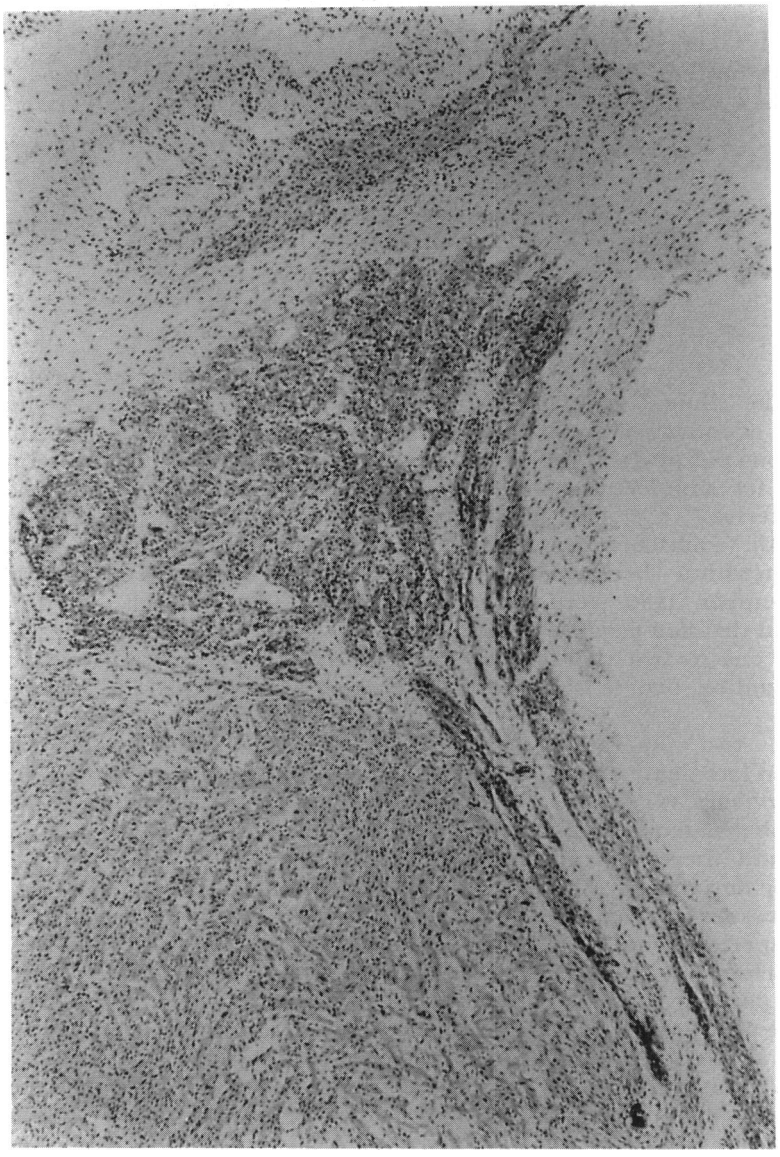

Figure 5 The branching bundle and the initial portion of the left bundle branch show intense cholinesterase staining (+ + + +). Nerves with positive cholinesterase staining are not identified in these regions. The adjacent ventricular myocardium shows negative staining. Cholinesterase staining, original magnification, × 5.

duction system is associated with numerous autonomic nerves, their pattern differs from that seen in other species and with what is known so far of the adult human conduction system. Further detailed studies are required to elucidate fully the maturation that occurs during childhood.

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