Innervation of the Human Cardiac Conduction System
A Quantitative Immunohistochemical and Histochemical Study

Simon J. Crick, BSc; John Wharton, PhD; Mary N. Sheppard, MD, MRCP; Dervil Royston, MD, MRCP; Magdi H. Yacoub, MD, FRCS; Robert H. Anderson, MD, BSc, FRCP; Julia M. Polak, DSc, MD, FRCP

Background Cardiac conduction is influenced by peptidergic mechanisms as well as classic neurotransmitters. The distribution of peptide-containing nerves has not been well defined.

Methods and Results Immunofluorescence and histochemical techniques were used to visualize the innervation of the human conduction system and to distinguish nerve subpopulations according to their peptide and enzyme content. Nerve fibers and fascicles displaying immunoreactivity for protein gene product 9.5 (PGP 9.5) were more numerous in the sinus and atrioventricular nodes than in the penetrating bundle, bundle branches, and adjacent myocardium. The relative density of innervation was greater in the central region of the sinus node than in the peripheral regions. Nerve densities were also higher in the transitional region of the atrioventricular node compared with its compact region. Acetylcholinesterase (AChE)-positive nerves were the main subtype identified in the sinus and atrioventricular nodes, representing half to two thirds of the stained area occupied by PGP 9.5-immunoreactive nerves. Neuropeptide Y-immunoreactive nerves represented the main peptide-containing subpopulation and occurred throughout the conduction system, displaying a similar pattern of distribution and relative density to those demonstrating tyrosine hydroxylase immunoreactivity. Nerve fibers showing immunoreactivity for vasoactive intestinal polypeptide, somatostatin, substance P, or calcitonin gene–related peptide exhibited distinct patterns of distribution and comprised a relatively minor component of the innervation, the percentage of stained area being 10– to 40-fold lower than that occupied by neuropeptide Y– and PGP 9.5–immunoreactive nerves, respectively.

Conclusions The innervation of human conduction tissues exhibits significant regional variation and comprises putative parasympathetic nerves and intrinsic neurons (AChE positive), sympathetic efferent nerves (neuropeptide Y– and tyrosine hydroxylase–immunoreactive nerves), and other peptide-containing nerves, some of which (substance P and calcitonin gene–related peptide containing) are considered to represent afferent nerves. Locally released peptides may be involved in the neural modulation of the human conduction system. (Circulation. 1994;89:1697-1708.)

Key Words • conduction • innervation • neuropeptides • immunohistochemistry

The cardiac conduction system initiates, conducts, and controls the heart beat. It comprises the sinus node, atrioventricular node, penetrating bundle, and ventricular bundle branches. Detailed morphological studies of the system have been undertaken both in animals1–5 and in humans.6–9 While the innervation of the mammalian conduction system has been extensively studied,1,2,4,10 much less is known about the innervation of these tissues in humans. Studies in different animal species have shown marked variation in patterns of innervation. The guinea pig and rat, for instance, have a dense acetylcholinesterase (AChE)-positive innervation of ventricular conduction tissues, whereas this is lacking in the rabbit heart.1,3,8 Furthermore, recent studies in animals have shown that the organization of extrinsic and intrinsic nerves may be more complex than is generally recognized.2,10,11 For example, studies on the canine sinus and atrioventricular nodes, using surgical ablation or pharmacological interventions, have demonstrated a differential distribution of both sympathetic and parasympathetic inputs.12–14 It is also now recognized that, in addition to the classic neurotransmitters noradrenaline and acetylcholine, subpopulations of cardiac nerves contain other putative neurotransmitters or neuromodulators, including several neuropeptides that have potent effects on the heart.15,16 It also has been shown that sensitive and well-developed histochemical and immunohistochemical techniques may be used to demonstrate cardiac innervation as a whole17–19 and to identify the subpopulations of afferent and efferent nerves on the basis of their enzymatic and/or peptidergic content.20,21 We have therefore used these techniques to investigate the distribution of neuronal subtypes in the human conduction system, determining the relative density of specific subpopulations in different regions of the conduction system, using computer-assisted image processing.

Methods

Tissue Processing

Samples from the sinus node, atrioventricular node, penetrating bundle, and bundle branches (Fig 1) were obtained at

Received October 28, 1993; revision accepted December 28, 1993.
From the Department of Histochemistry (S.J.C., J.W., J.M.P.), Royal Postgraduate Medical School, London; the Departments of Pathology (S.J.C., M.N.S.), Cardiothoracic Surgery (M.H.Y.), and Paediatrics (R.H.A.), National Heart and Lung Institute, London; and the Department of Histopathology (D.R.), Beaumont Hospital, Dublin.

Correspondence to Dr John Wharton, Department of Histochemistry, Royal Postgraduate Medical School, DuCane Road, London, W12 ONN, UK.
postmortem from five normal human hearts (mean age, 27 years; range, 17 to 45) (Table 1). Tissues were collected within 24 hours of death, following the ethical standards of the institutions in which they were obtained. Immediately after dissection (Fig 1), the samples were fixed by immersion in modified Bouin’s solution for 24 to 48 hours at 4°C, as previously described. Fixed tissues were subsequently rinsed in several changes of phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.2) containing 15% (wt/vol) sucrose and 0.1% (wt/vol) sodium azide. Samples also were obtained at surgery from the recipient heart of a female patient aged 6 years who underwent cardiac transplantation because of dilated cardiomyopathy, thus permitting comparison of fresh tissues with postmortem samples. Cryostat blocks were prepared by orienting the tissue on a cork mat, surrounding it in mounting medium (Tissue-Tek, Miles Inc, Elkhart, Ind), and rapidly freezing in dichlorodifluoromethane (Arkton-12, ICIF, Cheshire, England) suspended in liquid nitrogen. Serial cryostat sections (15 µm thick) were cut through the entire sinus and atrioventricular nodes (Fig 1) in each heart and processed for immunofluorescence and AChE staining. This entailed the collection of approximately 240 sections from the sinus node, 170 sections from the compact and transitional regions of the atrioventricular node, 80 sections from the penetrating bundle, and 80 sections from the ventricular conduction tissues.

**TABLE 1. Clinical Features of Postmortem Cases**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age, y</th>
<th>Cause of Death</th>
<th>Delay Time, h*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>17</td>
<td>Craniocerebral trauma</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>25</td>
<td>Asphyxiation</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>25</td>
<td>Craniocerebral trauma</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>42</td>
<td>Craniocerebral trauma</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>45</td>
<td>Craniocerebral trauma</td>
<td>10</td>
</tr>
</tbody>
</table>

*Delay between death and autopsy.

**Fig 1.** Schematic diagram illustrating the dissection of human conduction tissues. A strip of tissue was removed from the junction of the superior caval vein and the right atrial appendage, with the sinus node located around the region of the crest of the terminal groove (as shown in the boxed area). The atrioventricular and ventricular conduction tissues were dissected using the landmarks of the membranous septum, tricuspid valve, and the tendon of Todaro (as shown by the large boxed area). The entire region was removed and cut into a continuous series of blocks labeled as posterior and anterior according to their position relative to the membranous septum. Following this protocol, the AV node was situated in the posterior tissue blocks, whereas the ventricular conduction tissues were present in the anterior blocks. As shown, the AV node is positioned above the annulus fibrosus.

**Immunostaining**

An indirect immunofluorescence technique was used together with specific primary antisera to the general neuronal marker protein gene product 9.5, tyrosine hydroxylase, neuropeptide Y, somatostatin, vasoactive intestinal polypeptide, substance P, and calcitonin gene-related peptide (Table 2). Cryostat sections were collected on Vectabond-treated slides (Vector Laboratories, Ltd, Peterborough, England) and air-dried for 1 hour at room temperature. After immersion in buffered saline containing 0.2% Triton X-100 for 30 minutes and washing in buffer, the sections were stained with Pontamine sky blue (BDH, Poole, England) for 30 minutes to reduce background fluorescence and to counterstain elastic tissue. Sections then were washed in buffer and incubated for 16 to 24 hours at 4°C in diluted primary antisera. After incubation, the sections were washed in buffer and incubated for 1 hour at room temperature with fluorescein isothiocyanate–labeled goat anti-rabbit IgG (TAGO, Calif) diluted 1:100. After further washes, the preparations were mounted in either glycerol mixed 2:1 with buffered saline or Vectashield mounting medium (Vector Laboratories, Ltd) and examined with an Olympus AH-2 microscope equipped for epi-illumination.

In control experiments, no immunofluorescence staining was obtained when the primary antisera were omitted, replaced with preimmune serum, or preabsorbed with their corresponding antigens (10⁻³ to 10⁻⁶ mol/L for 16 to 24 hours at 4°C).

**Acetylcholinesterase Staining**

The localization of AChE activity was investigated by means of a modified technique as previously described. Sections were immersed in the incubation solution at a 20-fold higher dilution than originally described. After rinsing in distilled water, the AChE activity was visualized by immersion of the sections for 5 minutes in Tris hydrochloride buffer (50 mmol/L, pH 7.6) containing 0.04% 3,3'-diaminobenzidine tetrahydrochloride and 0.3% nickel ammonium sulfate and...
TABLE 2. Source and Characterization of the Primary Antisera

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Code</th>
<th>Dilution</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP 9.5</td>
<td>Human</td>
<td>2292(RA95)</td>
<td>1/2000</td>
<td>Ultralclone, UK</td>
<td>17</td>
</tr>
<tr>
<td>TH</td>
<td>Rat</td>
<td>2009(KD1-1)</td>
<td>1/400</td>
<td>D.M. Kuhn, USA</td>
<td>51</td>
</tr>
<tr>
<td>C-PON</td>
<td>Human</td>
<td>2005(JN7)</td>
<td>1/600</td>
<td>Hammersmith Hospital, UK</td>
<td>52</td>
</tr>
<tr>
<td>VIP</td>
<td>Porcine</td>
<td>2268(V128)</td>
<td>1/2000</td>
<td>Hammersmith Hospital, UK</td>
<td>53</td>
</tr>
<tr>
<td>SOM-28</td>
<td>Mammalian</td>
<td>1082(70H2T)</td>
<td>1/1000</td>
<td>Immuno-Nuclear, USA</td>
<td>35, 45</td>
</tr>
<tr>
<td>SP</td>
<td>Mammalian</td>
<td>1651(SP95)</td>
<td>1/500</td>
<td>Hammersmith Hospital, UK</td>
<td>46</td>
</tr>
<tr>
<td>CGRP</td>
<td>Rat</td>
<td>1204(CGRP-7)</td>
<td>1/200</td>
<td>Hammersmith Hospital, UK</td>
<td>46</td>
</tr>
</tbody>
</table>

PGP 9.5 indicates protein gene product 9.5; TH, tyrosine hydroxylase; C-PON, C-terminal peptide sequence in the neuropeptide tyrosine (NPY) precursor molecule with an identical distribution to NPY; VIP, vasoactive intestinal polypeptide; SOM-28, somatostatin; SP, substance P; and CGRP, calcitonin gene-related polypeptide.

then for a further 5 to 10 minutes with the addition of 0.003% hydrogen peroxide.

In control experiments, AChE activity could not be detected after incubation of sections without acetylthiocholine iodide and after the addition of 10^-4 mol/L eserine (physostigmine, Sigma Chemical Co, St Louis, Mo) to the incubating solution.

Double Staining Experiments

Double immunostaining could not be achieved in this study because the antisera used were all raised in rabbits. Differential localization of AChE activity and immunofluorescence staining, nonetheless, were demonstrated by performing immunofluorescence and AChE staining on the same tissue sections. This was achieved by immersing immunostained sections in hydrogen peroxide solution and staining for AChE activity as described above. Sections were examined both before and after AChE staining, and images of nerves in the same fields were compared.

Quantification of Immunostained Nerve Density

The relative density of specific nerve subpopulations was determined by computer-assisted image analysis of immunofluorescent- and AChE-stained preparations using a Kontron VIDAS image processing system (Kontron Electronics, Ltd, Watford, England). Immunofluorescence images were captured with a Panasonic low-light video camera (model WV 1900/C) mounted on an Olympus AH2 research microscope; images of AChE-stained preparations were captured with a Hitachi CCTV camera (model HV-720K) mounted on an Olympus BH2-transmitted light microscope. Background auto-fluorescence was kept to a minimum by using optimally diluted antisera and by counterstaining the sections with Pontamine sky blue. Image contrast for immunofluorescent preparations was further improved by using a cutoff filter at 560 nm, which was placed between the microscope and video camera to eliminate red light. A similar procedure was adopted for AChE-stained preparations by placing a green filter between the microscope and the video camera, thus increasing the discrimination of the stained nerves. Intramyocardial blood vessels, nodal arteries, and associated perivascular nerves were edited from the images before discrimination to prevent variation in the distribution of these structures influencing the measurement of stained nerves in conduction tissues and myocardium. Comparative measurements, therefore, were restricted to specific regions of the conduction system and adjacent myocardium, which were outlined manually. Images were digitized into a 512x512-pixel array, with each pixel representing one of 256 gray levels. The captured image then was segmented by setting the upper threshold to 255 so that the stained nerve fibers represented the brightest regions of the image. AChE-positive preparations were interactively discriminated in a similar manner, setting the lower threshold to zero, since the stained nerves represent the darker regions of the image. Measurements of the total field area (conduction tissue or myocardium) and the area occupied by stained nerves were obtained for each field. The percentage of stained area then was derived for each field. All morphological nerve types were quantified, including nerve trunks, nerve fascicles, and nerve fibers that often showed swellings (varicosities). Ganglia and paraganglion cells were excluded from these measurements. The relative distribution of stained nerve fibers and nerve trunks also was assessed by means of a further parameter, intercept density, corresponding to the number of intersections of nerve fibers with fixed horizontal lines across a defined image. Variation in immunostaining between sections was assessed by serially sectioning (15 μm thick) and staining all the sections from one case (a 25-year-old man) and measuring the specific stained nerve area. Subsequent cases also were serially sectioned, but measurements were made only on every 16th (atrioventricular node), 24th (sinus node), and 8th section (penetrating bundle and ventricular conduction tissues). Ten sections, stained for each particular antigen and for AChE activity, were analyzed from each specific region of the conduction system in each case.

Pilot studies involving repeated measurements of immunostained sections demonstrated that there was no significant reduction in the discriminated nerve area after storage at 4°C for up to 3 weeks. The degree of fading of fluorescent preparations upon storage was, however, found to be less when the sections were mounted in Vectashield rather than PBS and glycerol.

Statistics

All data are presented as mean±SEM or mean and 95% confidence interval (95% CI). Variation in the proportion of stained nerve subtypes in different regions of the conduction system was compared after logit transformation of the data using ANOVA or Student’s t test as appropriate. Differences with a P value of <.05 were considered significant.

Results

The overall pattern of innervation was investigated using an antiserum raised to the general neural marker protein gene product 9.5 (PGP 9.5). Immunoactive varicose nerve fibers, nerve fascicles, and nerve trunks were observed throughout the conduction system. Immunoactive cell bodies and clusters of paraganglion cells were observed subepicardially near the sinus node, in the periphery of the node, and occasionally, posterior to the atrioventricular node. The relative density of innervation varied in different regions, the rank order of nerve density being sinus node>atrioventricular...
node>penetrating bundle and bundle branches. All regions of the conduction system displayed a significantly higher nerve density (P<.0001) than the adjacent atrial or ventricular myocardium. Comparison of the measured variables (percentage of stained nerve area and intercept density) indicated that there was a close correlation (r=.9300, P<.0001) between the two when comparing regional variation and relative nerve density. The data have therefore been presented only as measurements of percentage of stained nerve area.

Sinus Node

*Morphology*

The node in sagittal section (that is, in the plane of section perpendicular to the terminal crest) is a wedge-shaped structure situated at the junction of the superior caval vein with the musculature of the terminal crest (Fig 1). In all the cases examined, the node was arranged about a central artery. Nodal cells were fascicular in nature and embedded in a prominent fibrous tissue matrix (Fig 2). The cells around the nodal artery were more densely packed and less eosinophilic than those at the nodal borders. Nodal cells were smaller than the adjacent atrial myocardial cells, and a distinct border was observed between the node and adjacent atrial myocardium (Fig 2).

*Innervation*

The sinus node was the most densely innervated region of the conduction system. PGP 9.5-immunoreactive nerve fibers and fascicles were distributed between nodal cells in the fibrous tissue matrix (Fig 2). Significantly more immunostained nerves (P<.0001) were found in the central region of the node (20.0%, 19.1 to 20.8, mean and 95% CI percentage of stained nerve area) surrounding the nodal artery than in the nodal periphery (11.5%, 10.6 to 12.4) (Fig 3). Whereas immunoreactive nerve trunks accompanied the nodal artery (Fig 2), the perivascular innervation appeared relatively sparse when compared with that observed around small arteries and arterioles in the atrial myocardium. Numerous PGP 9.5-immunoreactive ganglia and nerve trunks were observed in the epicardium overlying the sinus node, with most ganglia occurring on the superior caval venous side of the node (Fig 2). AChE-positive nerves represented the predominant subpopulation identified in the sinus node (Fig 4). The relative density of AChE-stained nerve fibers and fascicles was approximately twofold greater in the central region of the node than in the periphery (P<.0001), corresponding with the regional variation observed in the PGP 9.5-immunoreactive innervation (Figs 5 and 6a). Neuropeptide Y-immunoreactive nerves represented the main peptide-containing subpopulation, exhibiting a similar distribution pattern and immunostained nerve area (P=.1450) to nerves displaying tyrosine hydroxylase immunoreactivity (Figs 4 and 6b). The relative density of neuropeptide Y-immunoreactive nerves was again significantly greater (P<.0001) in the central region of the node compared with the periphery (Fig 5), although the difference was proportionately less than that observed for AChE-positive and PGP 9.5-immunoreactive nerves (Figs 3 and 5). Most of the nerve trunks surrounding the nodal artery were immunoreactive for tyrosine hydroxylase. Examination of double-stained sections indicated that nerves displaying AChE activity represented between half and two thirds of the total PGP 9.5-immunoreactive innervation and were distinct from those displaying neuropeptide Y and tyrosine hydroxylase immunoreactivity.

Scattered nerve fibers were identified displaying immunoreactivity for either vasoactive intestinal polypeptide, somatostatin, substance P, and calcitonin gene-related peptide (Fig 4). These nerves appeared to represent a relatively minor component of the sinus nodal innervation, exhibiting a percentage of stained area 10- to 40-fold less than that of neuropeptide Y- and PGP 9.5-immunoreactive nerves, respectively. In contrast to the PGP 9.5-immunoreactive nerves, they did not display significant regional variation within the node (Figs 5 and 6). Individual vasoactive intestinal polypeptide- and somatostatin-immunoreactive nerve fibers were observed scattered among nodal cells, whereas substance P- and calcitonin gene-related peptide-immunoreactive nerve fibers occurred mainly in nerve trunks and were also found surrounding cell bodies in cardiac ganglia. No difference was found between the area of substance P- and calcitonin gene-related peptide–stained nerves (P=.5068).
Atrioventricular Node

Morphology

The atrioventricular node is located within the atrial component of the atrioventricular muscular septum, being separated from the ventricular myocardium by the annulus fibrosus. In cross section, the node consists of an elongated half oval, with a superficial transitional layer arranged in circumferential fashion around a compact region (Figs 1 and 7). The transitional layer crosses the compact node throughout its length to terminate in the base of the tricuspid valve, while additional transitional fibers enter the node from the floor of the coronary sinus. The transitional cells are larger than those of the compact node and are arranged in parallel strands separated by fibrous tissue. The compact node comprises interconnecting fascicles of cells of similar appearance to those in the sinus node. In some cases, branches of nodal cells (archipelagos) were occasionally seen to project into the annulus fibrosus (Fig 7). These were traced in an anterior direction and found to reenter the compact part of the node.

Innervation

The transitional and compact regions of the node contained numerous PGP 9.5-immunoreactive nerve fibers and nerve trunks, the relative density being greater (P < .0001) in the transitional region (15.3%, 14.4 to 16.1) than in the compact node (9.3%, 8.4 to 10.2) (Figs 6a through 8). PGP 9.5 immunoreactivity also was localized to neuronal cell bodies in ganglia in the atrioventricular groove, lying below the coronary sinus, these being less numerous compared with the number of ganglia associated with the sinus node. AChE-positive nerve fibers and fascicles were distributed throughout the node and also were found to be more numerous in the transitional region than in the compact node (Fig 6a). The AChE-stained nerve area in the node was three- to fourfold greater than in the atrial overlay tissue (Fig 8). In the transitional region, AChE-positive nerves represented the main subpopulation. In the compact region, in contrast, the proportion of AChE-positive nerves was similar (P = .5837) to that of neuropeptide Y-immunoreactive nerves (Fig 6b). Neuropeptide Y- and tyrosine hydroxylase-immunoreactive nerve fibers and fascicles were distributed throughout the node and exhibited a similar immunostained nerve area in the compact (P = .1978) and transitional regions of the node (P = .7378) as well as in the atrial overlay tissue (Fig 6b).

A subpopulation of vasoactive intestinal polypeptide-immunoreactive nerve fibers was demonstrated in the
compact region of the node, often in close proximity to blood vessels. These fibers were not detected in the transitional region (Fig 6c). Somatostatin-immunoreactive nerve fibers occurred less frequently and were demonstrated in the transitional region of the node (Fig 6c). Scattered substance P- and calcitonin gene–related peptide–immunoreactive nerve fibers were detected at low density in the compact node (Fig 6d). Archipelagos of conduction cells, which extend from the nodal tissue into the fibrous annulus, were accompanied by sparse fine varicose nerve fibers exhibiting PGP 9.5, neuropeptide Y, and tyrosine hydroxylase immunoreactivity. No significant difference (P=.7654) was found between the density of PGP 9.5–immunoreactive nerves in the atrioventricular node of the explanted infant heart and that found in adult postmortem hearts (Fig 9).

**Atrioventricular (Penetrating) Bundle and Bundle Branches**

**Morphology**

Cells in the distal end of the node were identical to those in the proximal penetrating bundle, the distinction between the two being made on the basis of penetration of the conduction axis into the annulus fibrosus (Fig 1). On passing further toward the ventricles, the cells were orientated in a more parallel fashion and remained relatively small in size. After traversing the central fibrous body to reach the left ventricular outflow tract, the bundle immediately divides into the left and right bundle branches. The left bundle branch then descends as a sheet of cells within the subendocardium of the ventricular septum, whereas the right bundle branch descends intramyocardially as a discrete cord. The cells of the bundle branches are of similar size to those in the underlying ventricular myocardium but are separated from it by a fibrous sheath.

**Innervation**

Compared with the tissues in the atrioventricular node, the penetrating bundle and bundle branches displayed a less dense innervation, although still greater (P<.0001) than that found in the adjacent ventricular myocardium (Fig 6a). In addition to PGP 9.5 immunoreactivity, nerve fibers in the penetrating bundle and bundle branches displayed immunoreactivity for neuropeptide Y and tyrosine hydroxylase (Fig 6b). Only isolated and sparse nerve fibers in the penetrating bundle and bundle branches displayed AChE staining, vasoactive intestinal polypeptide, substance P, and calcitonin gene–related peptide. Somatostatin immunoreactivity was not observed (Fig 6, a through d).

**Discussion**

This study demonstrates that, as in other species,1-3,9 the components of the human conduction system are more densely innervated than the myocardium in other parts of the heart. This is no more than expected in view of the central role of the autonomic nervous system in initiating and regulating the cardiac impulse. Previous histochemical studies have been unable to delineate the full extent of the innervation and to distinguish its component nerve subpopulations. The early techniques for the visualization of nerves using methylene blue and impregnation of silver were believed to demonstrate all nerves.25-26 These techniques, however, also stained the surrounding fibrous tissues, making it difficult to judge whether apparent differences in nerve density reflected problems with technique or true variation between species.25,26 AChE staining techniques also were used to visualize the innervation of the conduction system, demonstrating a rich distribution of cholinesterase activity within the sinus node, atrioventricular node, and the penetrating bundle of the human heart but were not without their technical problems.27,28 Our present studies, using immunohistochemical as well as histochemical techniques, have now permitted us not only to describe the overall innervation of the human conduction tissues but also to distinguish the component nerve subpopulations.

**Overall Innervation (PGP 9.5 Immunoreactivity)**

As visualized by the general neural marker enzyme PGP 9.5, the conduction tissues exhibited a higher density of nerve fibers, fascicles, and intrinsic ganglion cell bodies than the adjacent myocardium. There was also variation in relative density of innervation within the sinus and atrioventricular nodes. Thus, the percentage area of PGP 9.5–immunoreactive nerves was greater in the central region than in the periphery of the node. This could represent a "hot spot" for pacemaker activity, similar to the well-innervated clusters of conduction cells observed in the rabbit.3,29,30 The differential distribution in the atrioventricular node was between the transitional and compact regions. Transitiona l tissues received a more extensive nerve supply comparable to that observed in the sinus node, whereas the innervation of the compact region of the atrioventricular node was less dense. This may be important in the neural modulation of the conduction delay in the atrioventricular node.10 The density of PGP 9.5–immunoreactive nerves in the penetrating bundle and the
bundle branches was significantly higher than that of the adjacent ventricular myocardium but much lower in comparison to the atrioventricular node. This finding also has been observed in the canine ventricular conduction system but, unlike this previous study, we found very few of these nerves to be AChE positive (see below).

Nerve Subpopulations

The presence of AChE activity in many neuronal cell bodies in epicardial ganglia and intracardiac nerves supplying the conduction system is in agreement with previous studies on the rabbit, guinea pig, calf, primate, and human heart. The predominance of AChE-positive nerves in the sinus node and transitional region of the atrioventricular node supports the physiological phenomenon of vagal inhibitory dominance of the human heart. The artery to the sinus node was devoid of AChE-positive nerves, this finding again endorsing earlier studies using AChE staining techniques. As already mentioned, our results showed that the ventricular conduction tissues received a relatively sparse AChE-positive innervation. In particular, we could not find AChE-positive nerves in the bundle branches. This finding is in contrast to a previous study that claimed to show a dense innervation of the ventricular specialized tissues. The specificity of the AChE staining technique used may well have been responsible for this difference, since the surrounding tissues were also stained in the earlier study. Another study that used fetal hearts found, as have we, a distinct lack of AChE-positive nerves in the ventricular conduction tissues. It is possible, of course, that the lack of nerves in the fetal stage may reflect the failure of maturation, but our present findings indicate that the fetal arrangement for AChE-positive nerves persists in adult life.

The presumptive sympathetic component of the autonomic nervous system has been demonstrated previously using formaldehyde-induced fluorescence. This has yielded negative results in the human conduction system in marked contrast to the guinea pig, in which many fluorescent nerves were found in the sinus and atrioventricular nodes. We have identified the presumptive sympathetic human cardiac innervation immuno-
 histochemically using antisera to tyrosine hydroxylase and neuropeptide Y. Using this technique, our studies have shown that tyrosine hydroxylase–immunoreactive nerves made up a significant but not predominant subpopulation of nerves in the sinus node. In the atrioventricular node, tyrosine hydroxylase–immunoreactive nerves again represented a significant proportion of the total innervation, particularly in the compact region, where they were of similar proportions to AChE-positive nerves. This finding has functional implications in view of the known slowing of the cardiac impulse in the atrioventricular node. Neuropeptide Y–immunoreactive nerves had an extensive distribution throughout the conduction system that was shown by image analysis to be similar to that of nerves containing tyrosine hydroxylase. The majority of nerves in the archipelagos of the atrioventricular node were also immunoreactive for neuropeptide Y and tyrosine hydroxylase.

The dominant subpopulation of nerves in the ventricular conduction tissues also were immunoreactive for both neuropeptide Y and tyrosine hydroxylase. The apparent absence of tyrosine hydroxylase– and neuropeptide Y–immunoreactive ganglion cells in the epi-
Vasoactive intestinal polypeptide-immunoreactive varicose nerves have been described in high density in the sinus node in several species but to a lesser extent in the atrioventricular node. We found these nerve fibers mostly in relation to small blood vessels. Immunoreactive cell bodies have been reported in the canine sinus node, and nerves containing the peptide were occasionally associated with unreactive ganglion cells. The distribution of these nerves was closely associated to regions possessing a high density of AChE-positive nerves. The origin of these nerves, nonetheless, remains elusive, since no immunoreactive ganglion cell bodies have been observed in humans. Somatostatin-immunoreactive nerves were relatively abundant in the sinus node but were very sparse in the atrioventricular node and ventricular conduction system. They had a pattern of distribution distinct from nerves displaying neuropeptide tyrosine and vasoactive intestinal polypeptide immunoreactivity. Somatostatin-immunoreactive neurons have been described in the developing and adult human heart, leading to the suggestion that they represent intrinsic or postganglionic parasympathetic neurons.

Substance P- and calcitonin gene-related peptide-immunoreactive nerves were relatively sparse in the conduction tissues. When found, they were localized in nerve trunks and in close proximity to intrinsic neuronal cell bodies, especially in the epicardial regions of the sinus node. This is in marked contrast to animals such as the guinea pig, in which there is a rich supply of these nerves in both the conduction system and the myocardium. Substance P- and calcitonin gene-related peptide-immunoreactive nerves are colocalized in afferent nerves and are partially depleted by bilateral stellate ganglionectomy and by capsaicin treatment of the vagus nerve and are absent in cardiac allografts. These findings are consistent with an extrinsic origin for these nerves from primary sensory spinal and vagal neurons.

Limitations of the Study

Morphological investigations of human cardiac innervation are limited by the availability of suitable tissue samples. We and others have previously observed that comparable immunofluorescence and histochemical staining of cardiac tissues may be obtained using surgical and fresh postmortem specimens collected within 12 hours of death. The present study provides further support for the use of postmortem material and indicates that the relative density of the total innervation is indistinguishable in explanted surgical and postmortem tissues. In addition to postmortem delay, a number of other factors may have influenced the immunohistochemical and histochemical demonstration of nerves. Tissue sampling, fixation, storage, staining, and image processing were, however, standardized and controlled as far as possible to minimize possible variations arising from preparative procedures and fading of fluorescent preparations. We have previously validated the quantitative morphological assessment of cardiac innervation using a bovine model of the innervated and denervated heart. This approach, nonetheless, remains limited by the resolution of the light microscope as well as the sensitivity and specificity of the immunohistochemical and histochemical techniques used.
Summary

Our study has demonstrated that the human conduction system possesses a rich and complex innervation. It has shown that the sinus and atrioventricular nodes, together with the ventricular conduction axis, exhibit a differential distribution of nerves composed of both autonomic and sensory subpopulations. This knowledge concerning the regional distribution of these nerve subpopulations can now be used to make realistic comparisons with functional studies.

Acknowledgments

This work was funded by a grant from the British Heart Foundation. Antisera raised to regulatory peptides at Hammersmith Hospital were produced in conjunction with Prof S.R. Bloom; antisera to tyrosine hydroxylase were provided by Dr D. Kuhn. We thank the staff of the Beaumont Hospital, Dublin, for their assistance in the collection of tissue samples and members of the cardiac transplant team, Hospital for Sick Children, Great Ormond Street, London. The authors wish to thank Kevin Marron for assistance in the collection of tissue samples and Dr Siew Yen Ho for producing Fig 1.

References


21. Hassall CJS, Burnstock G. Immunohistochemical localisation of neuropeptide Y and 5-hydroxytryptamine in a subpopulation of amine-handling intracardiac neurons that do not contain dopamine hydroxylase in tissue culture. Brain Res. 1987;422:74-82.


Innervation of the human cardiac conduction system. A quantitative immunohistochemical and histochemical study.
S J Crick, J Wharton, M N Sheppard, D Royston, M H Yacoub, R H Anderson and J M Polak

Circulation. 1994;89:1697-1708
doi: 10.1161/01.CIR.89.4.1697

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/89/4/1697

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/