Histochemical and Chemical Studies of the Localization of Adrenergic and Cholinergic Nerves in Normal and Denervated Cat Hearts

By David Jacobowitz, Ph.D., Theodore Cooper, M.D., Ph.D., and Hendrick B. Barner, M.D.

ABSTRACT

The localization of adrenergic and cholinergic nerves in normal and denervated cat hearts was studied histochemically. The norepinephrine content of atria and ventricles was chemically determined by a spectrofluorometric method. In hearts denervated 9 to 42 days, little or no norepinephrine was detected. Histochemically, many catecholamine-containing fibers were present in the atria and ventricles of normal cats, whereas in denervated cats there were none in one and very few in four. There were many cholinergic nerves in the atria and a small to moderate number in the ventricles. The left atria of denervated hearts showed a marked reduction in cholinergic nerve fibers. It is concluded that cardiac denervation by mediastinal neural ablation is often incomplete. When norepinephrine is not detectable by chemical analysis, individual nerve fibers not sectioned can still be histochemically identified.

ADDITIONAL KEY WORDS

atrium catecholamines
acetylcholinesterase ganglia ventricles interventricular septum
interatrial septum papillary muscle aorta norepinephrine

Since extensive cardiac denervation results in loss of much of the capacity of the heart to store norepinephrine (NE) (1), surgical preparations have been used to clarify the role of NE stores in the intrinsic contractile state of the heart (2) and in the response of the heart to drugs that may depend on the release of local NE stores for their cardiac action (3, 4). Physiologic (5), pharmacologic and chemical (6) studies have been relied upon for assessment of completeness of cardiac denervation.

A morphologic study of the effects of cardiac denervation on the distribution of catecholamine-containing nerve elements would aid in assessing the significance of the functional studies in denervated hearts, since it is clear that a totally extrinsically denervated heart is not a heart without intrinsic neural elements (7, 8). Hence, the distribution of adrenergic and cholinergic neural elements in normal and surgically denervated cat hearts was studied by histochemical as well as by chemical methods for measuring catecholamines.

Methods

Hearts were obtained from 9 normal cats and 5 cats that had undergone surgical cardiac denervation 9 to 42 days previously. Extrinsic cardiac denervation was accomplished by mediastinal neural ablation, a surgical procedure described previously by Cooper et al. (8). The soft tissues that presumably contain the cardiac neural plexuses and all nerves that enter or leave the heart were excised. The base of the pericardium was excised and the segments of the great vessels adjacent to the heart were stripped of their adventitia.

All animals were anesthetized with sodium pentobarbital (30 to 35 mg/kg ip). For catecholamine
histochemical observations, the atria, ventricles, S-A and A-V nodes, interatrial and interventricular septa, papillary muscle, and aorta were studied. The tissues were rapidly removed and immediately frozen in isopentane cooled by liquid nitrogen. The specimens were freeze-dried in a vacuum at —35°C for 3 to 5 days and then treated with paraformaldehyde gas at 80°C for 1 hour according to the method of Falck and Hillarp (9-11). Paraffin sections were cut at 15 μ and examined and photographed for monoamine fluorescence using a fluorescence microscope.

Myocardial NE was chemically determined spectrophotofluorometrically by the method of Hogans [quoted by Porter et al. (12)]. Atria and ventricular tissue were frozen in liquid nitrogen and stored on dry ice. Samples of tissues weighing between 100 and 500 mg were taken. Nodal tissue was not chemically analyzed for NE. Tissues were analyzed for NE within 24 hours. Briefly, this method involves homogenizing tissue in 5 ml of butanol and centrifuging; 3 ml of 0.1 M phosphate buffer, pH 6.5, is added to 4-ml of the butanol extract, mixed, and centrifuged. To 1-ml portions of the phosphate buffer layer are added 0.25 ml of 4% versene and 0.2 ml of iodine solution (0.25 g iodine and 0.48 g KI to 100 ml H2O). After exactly 2 min, 0.25 ml of freshly prepared alkaline sulfite is added (0.625 g anhydrous Na2SO3 in 5 ml of H2O and 20 ml of 5 N NaOH). After exactly 2 min, 0.3 ml of 5 N acetic acid is added; the solution is then boiled for 5 min and cooled in an ice water bath for 1 min. The fluorescence activation is set at 390 m/μ, and emission is read at 490 m/μ (uncorrected settings).

Cholinergic nerves were studied by the thiocholine method of Koelle (13) for acetylcholinesterase (AChE). Tissues were removed and frozen with dry ice. Sections of 20 μ were cut with a cryostat at —15°C and placed on slides. The slides were then placed in the appropriate preincubation solutions for 30 min at 37°C and then were incubated for 45 to 90 min. Pseudo-cholinesterase was selectively inhibited by preincubation for 30 min at 37°C with 1.5-2.0 × 10−8 M diisopropylphosphorofluoridate (DFP). All slides were counterstained with eosin.

Results

Adrenergic Innervation. The adrenergic innervation in each region studied showed considerable variation. No attempt to grade the density of the catecholamine-containing fibers was made. In general, the atria and interatrial septa contained a moderate to large number of adrenergic nerves (Fig. 1a), while the ventricles (Fig. 2a), interventricular septa (Fig. 3), and papillary muscles (Fig. 4) usually contained a moderate number, although regions with only a small number were occasionally observed.

The NE content of normal and denervated hearts are tabulated in Table 1. One heart denervated for 9 days had an appreciable amount of NE in both atria and in the right ventricle, probably reflecting incomplete denervation. Cat hearts denervated for 3 to 4 weeks did not contain NE detectable within the limits of sensitivity of the method (0.03 to 0.05 ng NE for cat heart). One heart denervated for 6 weeks had a small quantity of NE in the left atrium. Whether this is a reflection of reinnervation or incomplete denervation is not known. Moreover, when tissues from denervated hearts were examined histochemically for catecholamine-containing nerve fibers, variable numbers of fluorescent nerves were present in several regions of 4 of the 5 denervated hearts. A plus in Table 2 means that a few green-fluorescing catecholamine-containing nerve fibers were present per tissue section of that area. In one heart (24 days), no fluorescent nerve fibers were noted in any of the regions examined. In another

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days denervated</th>
<th>NE Content (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Atrium Right</td>
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<tr>
<td></td>
<td></td>
<td>Atrium Left</td>
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<tr>
<td></td>
<td></td>
<td>Ventricle Right</td>
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<td></td>
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<td>Ventricle Left</td>
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<tr>
<td>Normal</td>
<td></td>
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<td></td>
<td>0.43</td>
<td>0.45</td>
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<tr>
<td></td>
<td>1.70</td>
<td>2.20</td>
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<tr>
<td></td>
<td>2.21</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>1.48</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>2.25</td>
<td>1.37</td>
</tr>
</tbody>
</table>

*Only norepinephrine readings that were twice the magnitude of the blank were taken as significant values.
†, ‡Values corrected for 89% (†) and 55% (‡) norepinephrine recovered (average value of tissue samples of one normal and one denervated heart assayed in one run).
heart, denervated for 26 days, all areas studied contained a few nerve fibers. The left ventricle of the heart denervated for 42 days showed no fluorescent nerve fibers in the apex region but a few fibers near the base of the left ventricle. In addition, the denervated left papillary muscle contained no fluorescent nerve fibers, while the ventricular muscle in the valve region contained a few. This observation might be interpreted as indicating that reinnervation was taking place at this time. Figure 1 shows photomicrographs of the right atrium of normal, denervated, and presumably incompletely denervated cat hearts.
Right ventricle. A, Normal cat. Fluorescent nerve fibers are seen running mostly parallel to the cardiac muscle cells. B, Denervated cat (24 days). No fluorescent nerve fibers present. Lipo-fuscin pigment granules are observed within the cells. C, Presumably incompletely denervated cat (28 days). A large nerve trunk is present in connective tissue between muscle bundles.

The normal atrium contains a high concentration of green-fluorescing varicose nerves coursing between cardiac muscle bundles. From these nerve trunks emanate smaller nerve fibers that come in close contact with the cardiac muscle cells. In the completely denervated heart only autofluorescent lipo-fuscin pigment is seen. The incompletely denervated heart contains only a few nerve fibers. A similar pattern of innervation is seen in the ventricle (Fig. 2). The normal pattern of nerves in the interventricular septum and
the left papillary muscle is shown in Figures 3 and 4.

The ascending aorta contained a thin layer of fluorescent fibers within the tunica adventitia immediately surrounding the tunica media (Fig. 5). A few fibers were seen in close proximity to the vasa vasorum within the tunica media.

When freeze-dried heart tissue is not exposed to paraformaldehyde fumes, no green-
Identification of Catecholamine-Containing Nerve Fibers in Various Regions of Cat Hearts

<table>
<thead>
<tr>
<th>Days denervated</th>
<th>Right Atrium</th>
<th>Left Atrium</th>
<th>Right Ventricle</th>
<th>Left Ventricle</th>
<th>Interatrial septum</th>
<th>Interventricular septum</th>
<th>Papillary muscle</th>
<th>S-A nodal region</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>21</td>
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<td>24</td>
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<td>28</td>
<td>+</td>
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<tr>
<td>42</td>
<td>+</td>
<td>+</td>
<td>(±)</td>
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<td>+</td>
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</tbody>
</table>

Fluorescing varicose nerves are present, but the smooth elastic tissue seen mainly in blood vessels has a green fluorescence of a different shade than that of nerves. In addition, when fluorescent sections are dipped into water, the fluorescence fades in nerves and not in elastic tissue. This was also demonstrated in nerves seen in denervated hearts. When denervated tissue was not exposed to the paraformaldehyde fumes no fluorescent nerves were observed.

Cholinergic Innervation. The cholinergic innervation of the four chambers of the heart was studied using the histochemical stain for AChE. Both atria were comparatively heavily innervated with cholinergic nerves, whereas the ventricles contained a small to moderate number (Fig. 6). After denervation, the number of cholinergic nerves in the left atrium was considerably reduced in all the denervated hearts studied (Fig. 7). A minor reduction was observed in 3 of the 5 denervated right atria studied.

In the interatrial septum and nodal regions, numerous ganglion cells in small clusters frequently lay adjacent to both the A-V and S-A nodal areas. These ganglion cells are nonfluorescent cholinergic cell bodies that presumably make synaptic contact with the vagus nerve (Fig. 8). Frequently seen in these ganglia are intense green to yellow-green-fluorescing cells resembling chromaffin cells of the adrenal medulla. These cells are often in close proximity to ganglion cells, although they are also found outside the ganglion (14).

Discussion

It is apparent that cardiac denervation by mediastinal neural ablation may not be entirely complete. When NE is not detectable within the limits of sensitivity of the chemical method of analysis, a few catecholamine-containing fibers can sometimes be identified microscopically. It is therefore clear that single neurons escaping surgical denervation and chemical detection can be easily identified by the very sensitive method of Falck and Hillarp. These conclusions are consistent with those reached by Peiss et al. (5) after detailed physiologic testing of similar preparations. It is also obvious that the method of cardiac denervation by mediastinal neural ablation cannot necessarily be relied on when evidence of absolute adrenergic denervation is required.

Although the operative procedure is usually employed to effect sympathetic postganglionic denervation, mediastinal neural ablation involves parasympathetic and afferent denervation as well. The parasympathetic denervation is largely preganglionic. After attempts at denervation of sympathetic neurons by mediastinal neural ablation, the number of AChE-staining nerves in the left atrium which might be pre- or postganglionic was reduced by 20 to 50% of the number seen in the control sections. This is probably due at least partly to the extirpation of the cardiac ganglion of Wrisberg, which lies in the concavity of the aortic arch above the pericardium. It is generally considered that the majority of cholinergic postganglionic fibers emanate from ganglia within the heart. Cholinergic fibers of extrinsic origin appear to terminate primarily in the left atrium, although a small decrease in AChE-staining fibers was noted in the right atria. No changes were observed in the ventricles, which contained a small to moderate number of cholinergic nerve fibers. The large
AChe; incubation for 90 min. A, Normal cat right atrium. B, Normal cat left atrium. AChe-staining nerves in both atria follow the muscle cells in a parallel fashion similar to that of the fluorescent nerves. C, Normal cat right ventricle. AChe-staining nerves seen running with a blood vessel (arrow). A large, densely staining nerve trunk is observed above. D, Normal cat left ventricle. Comparatively fewer nerves in both ventricles than in the atria.
number of AChE-staining nerves in both atria suggests that a large number of cardiac cells could be influenced by cholinergic fibers. It would therefore seem that many, if not most, of the atrial cells are innervated by both sympathetic and parasympathetic nerves. Cholinergic elements are also seen in the ventricles, although in smaller numbers. The functional significance of ventricular cholinergic innervation of vagal origin has been a subject of controversy (15). It would appear that morphologic basis for such an innervation is present.

The localization of catecholamine-containing nerves in the cat heart is essentially that...
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described for the rabbit and guinea-pig (16) and the dog (17) in that the adrenergic terminal elements appear to make intimate contact with the cardiac muscle cells. We did not grade the density of adrenergic nerve fibers, but the density of nerve terminals varies from area to area in the same region of the heart (17).

The terminal structure of adrenergic nerves is believed to take the form of a ground plexus (9, 18, 19). The AChE-staining neurons and fibers resemble those described in the beef heart (20). Such fibers in close proximity to the cardiac muscle cells have been designated as a terminal syncytium (21). In view of the high density of nerves from the two autonomic divisions contained within the atria, a close apposition of adrenergic and cholinergic nerves can be considered to take place in the terminal end net. This relationship is consistent with the pharmacologic demonstration that the parasympathetic nervous system could influence sympathetic nervous activity and vice versa in cat atria (22, 23). Such an arrangement of cholinergic and adrenergic terminals in close anatomic proximity is also consistent with a modified version of the Burn and Rand hypothesis (24, 25) of a cholinergic link in adrenergic transmission (26). Convincing evidence of a physiologic significance of a local cholinergic-adrenergic interaction remains to be demonstrated.

These observations also suggest that the intrinsic innervation of the heart is largely cholinergic (7). These findings, however, do not exclude the possibility that some adrenergic fibers may arise from cells originating within the heart.

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DAVID JACOBOWITZ, THEODORE COOPER and HENDRICK B. BARNER

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