Changes in Cholinergic Parameters Associated with Failure of Conotruncal Septation in Embryonic Chick Hearts after Neural Crest Ablation

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SUMMARY. Cells from the neural crest over occipital somites migrate to the heart, where they give rise to parasympathetic postganglionic neurons as well as ectomesenchymal elements which contribute to conotruncal septation. With a microcautery needle, the neural crest over occipital somites was ablated bilaterally in chicken embryos at an early stage of development. Histological examination on incubation day 15 revealed conotruncal malformations, involving malformation or absence of the conotruncal septum in all embryos. Two peaks of embryo mortality were observed. One peak (incubation days 6-8) occurred at the same time as conotruncal septal closure; the second peak (incubation days 11-13) was concurrent with the onset of functional parasympathetic innervation. A disruption of parasympathetic innervation was indicated by: (1) a decrease in acetylcholinesterase staining, (2) a decrease (27%) in the number of ganglion cells in the conotruncus, (3) decreases in the acetylcholine content of atrium (31%) and ventricle (39%), and (4) a decrease (21%) in muscarinic acetylcholine receptor density on incubation day 15. Radiolabeled ligand-binding studies revealed no change in the affinity of cardiac muscarinic receptors for [3H]methylscopolamine (Kd = 0.17-0.21 nM). Agonist-binding affinity and sensitivity to guanine nucleotides were similarly unaffected. The reasons for the limited extent of the parasympathetic lesion are unclear, but may involve recruitment of precursor cells from other regions of the neural crest, partial regeneration of the neural crest following surgical removal, or an alteration in the contribution of incoming sympathetic or preganglionic parasympathetic elements. No such plasticity was associated with neural crest contributions to the structural development of the conotruncus. Malformations were observed in all lesioned embryos.


NEURONS of the peripheral autonomic ganglia and their supporting cells arise from various levels of the neural crest (Horstadius, 1950; LeDouarin and Teillet, 1974). Neural crest is produced from ectoderm in the avian embryo after the neural folds appose each other to form the primitive neural tube. Kirby and Stewart (1983) used orthotopic transplantation of occipital neural crest from quail donors to chick embryo hosts to identify the origin of neural crest cells which provide parasympathetic postganglionic innervation of the heart. The parasympathetic postganglionic neurons, commonly referred to as cardiac ganglion cells, were found to arise bilaterally from the neural crest over somites 1-3 and to migrate toward the heart while it is still a simple tube. Removal of the neural crest over these somites decreased high affinity choline uptake (a marker for cholinergic nerve terminals) by 60% on day 10 of incubation, suggesting a depletion of cardiac ganglion neurons (Kirby and Stewart, 1983). Neural crest removal also produced a variety of conotruncal malformations (Kirby et al., 1983), which is thought to reflect the loss of ectomesenchymal, rather than neuronal, derivatives of the neural crest.

The present study was undertaken to characterize biochemical alterations in cardiac cholinergic systems in response to depletion of parasympathetic innervation. Many trophic interactions occur between motor neuron terminals and skeletal muscle cells during development (Fambrough, 1979; Hollyday, 1980). Comparable interactions of developing autonomic terminals with their end organs have not been demonstrated. It was recently shown that the density of cardiac muscarinic receptors can be altered by atropine treatment on days 12-14 of incubation (Kirby and Aronstam, 1983). This period corresponds to the onset of cholinergic function in the heart (Pappano and Skowronck, 1974). In the present paper, we report the consequences of occipital neural crest ablation: a 20-30% decrease in the number of ganglion cells in the area of the outflow tract associated with a 31-39% decrease in acetylcholine concentration in the atrium and ventricle, as well as a 21% reduction of muscarinic acetylcholine receptors in whole hearts. A second point of interest is the relationship between the neuronal and ectomesenchymal contributions of neural crest to cardiac development. In contrast to the rather modest decreases in biochemical markers of cholinergic systems which suggest compensatory mechanisms in the patterns of cardiac innervation, heart malformations were observed in every embryo in which...
the neural crest over the occipital somites was removed.

Methods

Tissue Preparations and Microsurgery

Fertile Arbor Acre chicken eggs were purchased from Central Soya and stored at 15-20°C. Incubation was initiated in forced-draft incubators maintained at 38°C and 97% relative humidity. The eggs were opened at stages 8-9 (about 30 hours of incubation (Hamburger and Hamilton, 1951)) and prepared for microsurgery according to the method reported by Narayanan (1970). The neural folds over somites 1 and 2, 1 through 3, and 1 through 4 were ablated bilaterally by means of a microcautery unit developed in collaboration with the Biomedical Engineering Department of the Medical College of Georgia. The neural fold consists of the presumptive dorsal part of the neural tube, the neural crest, and some adjacent surface ectoderm (Noden, 1975; Tosney, 1982). Only the neural crest migrates from this region. Ablation of the neural fold was accomplished in a very circumscribed area without damage to the ventral neural tube or underlying mesoderm. The neural crest was ablated in a total of 319 embryos; 123 control embryos were processed in parallel. For control embryos, the eggs were opened, sealed, and returned to the preoperative incubator at the same time as experimental eggs.

After microsurgery, the eggs were sealed and returned to the preoperative incubator until the circulatory system was well-established (about 72 hours of total incubation). The viable eggs were transferred to a second incubator maintained at 37°C and 70% relative humidity. The eggs were monitored for viability daily until day 15 (total incubation). At that time, the hearts were removed and divided into atrial, ventricular, and conotruncal pieces. The atria and ventricles were frozen in liquid nitrogen and divided into atrial, ventricular, and conotruncal pieces. The neural crest was ablated by means of a microcautery unit. The neural crest over the occipital somites was removed.

Histological and Histochemical Examination

The conotruncal regions were fixed in Perfix, embedded in paraffin, serially cross-sectioned, and stained with thionin. The number of cardiac ganglion cells was counted in every 10th section of the conotruncal region between the base of the semilunar valves and the first branches of the aorta. Malformations of the outflow tract were catalogued as the cardiac ganglion neurons were counted. The individual who analyzed these sections did not know whether the embryos were experimental or control.

Acetylcholinesterase histochemistry was used to visualize the cardiac ganglia remaining in the heart after neural crest extirpation. The method of Rickenbacher and Muller (1979) for developing whole hearts was used on the 12th or 15th day of incubation in lesioned and control embryos. Briefly, the embryos were perfused intracardially with cold 10% neutral-buffered formalin. The hearts were removed and postfixed for 2-6 hours at 4°C and washed in distilled water. Cholinesterase was demonstrated by Koelle and Friedewald's modification of Gomori's method as reported by Thompson (166).

[3H]Choline Uptake

[3H]Choline uptake in 15-day atria was measured as described previously (Kirby and Stewart, 1983). The atrial wall was rapidly isolated, minced, and placed in cold Krebs' buffer. Half of the pieces were incubated at 37°C in Krebs' buffer containing 10^-4 M hemicholinium-3. The other half of the tissue was incubated similarly, except that hemicholinium-3 was excluded from the medium. After 5 minutes, the tissue was transferred into Krebs' buffer at 37°C containing 10^-4 M choline chloride and 1 μCi/ml [3H]choline chloride (27 Ci/mmol, Amersham). The tissue was incubated with shaking for a total of 20 minutes, which had been shown previously to represent the time needed for saturation of choline uptake under these conditions. After the 20-minute incubation, the tissue was transferred through two 10-minute washes in Kreb's buffer and was blotted, weighed, placed in scintillation vials containing 1-2 ml ethanol, and shaken vigorously for several seconds. Scintillation cocktail was added and radioactivity was measured, using a Beckman LS 8000 with a tritium-counting efficiency of 38%. Radioactivity was determined in a 10-μl sample of each incubation well. To standardize the results from assay to assay, uptake was corrected for the counts per minute (counts/min) of specific neuronal uptake per million counts available in the incubation well. Neuronal uptake was obtained by subtracting the uptake in the presence of hemicholinium-3 (nonneuronal uptake) from the total uptake.

Acetylcholine Measurements

Atrial and ventricular samples were weighed and homogenized in 1.0 ml (ventricle) or 0.5 ml (atrium) of 1 N formic acid:acetone (15:85, vol/vol). The homogenates were centrifuged at 5000 g for 15 minutes, and 0.2-ml aliquots of the supernatants were evaporated to dryness in a vacuum centrifuge. The extracts were reconstituted in 0.2 ml of 10 mM phosphate-buffered saline, pH 7.4, containing 20 μM physostigmine, and were centrifuged at 12,000 g for 15 minutes. Endogenous acetylcholine was determined in 30-μl (ventricle) or 50-μl (atrium) aliquots of supernatant, by a radioimmunoassay procedure developed by Spector et al. (1978). Heart extracts or serial dilutions of a standard solution of ACh (1.6-100 pmol) were incubated with 100 μl of antiserum (final dilution 1:250) in buffered saline containing 0.125% bovine γ-globulin, 0.5% serum albumin, and 100 μl of [3H]acetylcholine (80 Ci/mmol, Amersham; final dilution 1:4500) in a total volume of 0.5 ml. Antibody-bound [3H]acetylcholine was precipitated by the addition of 0.5 ml of saturated ammonium sulfate. After centrifugation, the pellet was dissolved in water, and aliquots were removed for determination of radioactivity content by liquid scintillation counting. Several samples from atria and ventricles of control and experimental animals were adjusted to pH 11 and heated to 80°C for 15 minutes to hydrolyze endogenous acetylcholine. These hydrolyzed extracts did not displace antibody-bound tritium.

Muscarinic Receptor-Binding Measurements

Cardiac tissues were weighed and homogenized in 10 volumes of 50 mM Tris-HCl, pH 7.4, containing 1 mM MgCl2 and 0.1 mM phenylmethylsulfonyl fluoride (to prevent proteolysis). This homogenate was filtered through cheesecloth to remove undisrupted material, and then centrifuged at 20,000 g for 20 minutes. The pellet was resuspended in fresh buffer and used without further treatment. Protein content was estimated by the method of Lowry et al. (1951).
Muscarinic receptor binding was measured using [3H]-N-methylscopolamine ([3H]MS; 80 Ci/mmol, Amersham). Cardiac tissue (10–15 μg protein/ml) was incubated with [3H]MS for 60 minutes at room temperature in 50 mM Tris-HCl, pH 7.4, containing 1 mM MgCl₂ in a total volume of 5 ml. The suspension was then filtered under vacuum through glass fiber filters (Whatman GF/B). The filters were washed once with 5 ml of buffer and removed for determination of radioactivity content by liquid scintillation counting using a toluene-based cocktail (4 liters of toluene, 1.22 g of 1,4-bis-[4-methyl-5-phenyl-2-oxazolyl] benzene, 180 g of 2.5 diphenyloxazole, 150 ml of BioSolv-3). Nonspecific binding was determined by including 1 μM atropine in a parallel series of incubation media. All experimental points were determined in triplicate.

The number of muscarinic receptors and their affinity for [3H]MS was determined in hearts pooled from 5–6 embryos. [3H]MS binding was measured at nine concentration between 0.032 and 3.2 nM. Binding parameters were determined by nonlinear regression analysis, using a mass action expression for ligand binding to a single population of non-interacting sites, as follows:

\[ B = \frac{B_{\text{max}} \cdot C}{(C + K_{D})}, \]

where \( B \) is binding, \( B_{\text{max}} \) is the concentration of binding sites, \( C \) is the concentration of [3H]MS, and \( K_{D} \) is the dissociation constant. Nonlinear regression was performed using procedures available in the Statistical Package for the Social Sciences. The number of muscarinic binding sites was measured further in 49 different hearts (24 control, 25 experimental). Since there wasn’t enough tissue to obtain complete binding curves from single hearts, receptor concentrations were determined by measuring [3H]MS binding at two near-saturating concentrations of [3H]MS (3.2 and 5.6 nM). Receptor concentration was calculated assuming 95% and 97% receptor occupancy levels at these [3H]MS concentrations. These measurements were performed in quadruplicate, including quadruplicate determinations of nonspecific binding. The validity of this approach to estimating the number of receptors is supported by the close adherence of [3H]MS binding under these conditions to single-site mass action kinetics and by the absence of any significant differences between [3H]MS affinity for muscarinic receptors in hearts from control and experimental animals.

Muscarylcholine binding was inferred from its inhibition of 0.1 nM [3H]MS binding. Binding curves were resolved into high- and low-affinity components by nonlinear regression analysis and a model incorporating two independent binding sites, as follows:

\[ B = \frac{R_{h} \cdot C}{(C + K_{Dh})} + \frac{(1-R_{h}) \cdot C}{(C + K_{Dl})}, \]

where \( B \) is binding, \( C \) is the concentration of carbamylcholine, \( K_{Dh} \) and \( K_{Dl} \) are the carbamylcholine dissociation constants associated with the high- and low-affinity binding sites, and \( R_{h} \) and \( (1-R_{h}) \) are the fractions of receptors displaying high- and low-affinity carbamylcholine binding, respectively. In certain experiments 10 μM 5′-guanylylimidodiphosphate (Gpp(NH)p), a stable analogue of GTP, was included in the binding incubation media.

Statistical Methods

Summary data are presented as mean ± SEM. Statistical significance of ganglion cell counts, [3H]choline uptake, acetylcholine levels, and receptor concentrations and binding parameters were determined by Students’ t-test.

Results

Neural Crest Lesions

A microcautery lesion of the neural crest over somites 1 and 2 is illustrated in Figure 1. The lesion was created at stage 10 (about 35 hours of incubation). The histological examination was made about 5 hours later (i.e., in early stage 11). The neural fold was removed without causing damage to the ventral neural tube or underlying mesoderm (Fig. 1D). The neural tube continued to develop normally both cranial and caudal to the lesion (Figure 1, C and E). Most of the embryos were grossly normal throughout the incubation period, i.e., no external malformations of the beak, eyes, trunk, or limbs were apparent. Approximately 10% of the experimental embryos had defects in the skin and skull over the occiput. Thoracic wall defects were seen in approximately 20% of the experimental embryos. This type of defect is present in higher incidence after any surgical manipulation of the embryo, and does not have a significant correlation with heart defects (Kirby, unpublished data). The experimental embryos were slightly smaller than controls, although the developmental stage of experimental embryos was comparable to that of controls on the day of harvest.

Neural crest lesions over somites 1–2, 1–3, or 1–4 caused conotruncal malformations in every embryo. The only conotruncal abnormality which could be identified in serial sections of the conotruncal region was common outflow (comparable to persistent truncus), indicating a failure of high ventricular and aortico pulmonary septation. Common outflow was seen in 77% on the hearts analyzed from serial sections of the outflow area. If an experimental heart had a less severe form of septal defect (i.e., double outlet right ventricle), it would have appeared essentially normal in our serial sections, since the ventricles below the semilunar valves were removed prior to histological processing for biochemical measurements. It should be noted, however, that all of the hearts from experimental embryos appeared grossly abnormal, as has been described previously (Kirby et al., 1983).

The degree of ganglion cell depletion was the same, whether neural crest over somites 1–2 or 1–3 was removed (Table 1). Removal of neural crest over somites 1–4 resulted in a total ganglion cell count that was not significantly less than control. The outflow tract in embryos with somite 1–4 lesions was significantly longer than outflow tracts from embryos with somite 1–2 or 1–3 lesions or controls. This resulted in a significant decrease in density of ganglion cells following somite 1–4 lesions (Table 1).

A grossly elongated heart taken from a 15-day embryo is shown in Figure 2. Serial section analysis of the entire heart revealed that both of the great vessels arose from the right ventricle. A small
Aorticopulmonary septal defect was also present. Histochemical staining for acetylcholinesterase (Fig. 2) revealed a paucity of cholinesterase-positive nerves and ganglia in the cardiac plexus on both the dorsal and ventral surfaces of the heart, compared with hearts from control embryos.

Two peaks of mortality were observed in the lesioned embryos which survived the immediate postoperative period (Fig. 3). One peak lasted from incubation days 6 through 8, the second from incubation days 11 through 13. Control embryos also displayed a slight increase in mortality on days 7 and 8 of incubation. The survival to day 15 of incubation (at which time all hearts were harvested) was 19% for experimental and 54% for control embryos.

### Ganglion Cell Population

The number of ganglion cells in the conotruncal region of normal embryos rose linearly from 71 ± 7 (n = 5) on incubation day 8 to 980 ± 156 (n = 5) on day 18 (Fig. 4). Regression analysis revealed that the

![Figure 1. A microcautery lesion of the neural folds at the level of somites 1 and 2 in an early stage 11 embryo. The lesion was made in the embryo early in stage 10. After a 5-hour recovery period, the embryo was fixed in ovo (Karnovsky's) for 5 minutes, and then removed from the egg and immersion-fixed for 1 hour. The embryo was photographed, by dark field illumination, and subsequently processed for light microscopy in glycol methacrylate. Panel A shows the whole stage 11 embryo with a total of 12 somites. The outlined area is enlarged (60X) in panel B. The area of the lesion can be seen in the dorsal neural tube adjacent to somites 1 and 2. The notochord is the light structure in the center of the lesioned area. The notochord is clearly visible in this area because the dorsal part of the neural tube is not present. Panels C, D, and E are cross-sections of the same embryo at the levels indicated (140X). Panel C is cranial to the lesion, panel D is at the level of the lesion, and panel E is caudal to the lesion. In panel D, removal of the neural folds caused loss of the dorsal part of the neural tube and some ectoderm; however, the mesoderm lateral to the neural tube was not damaged. The relationship of the developing heart to the position of the lesion can be visualized in panel B.](http://circres.ahajournals.org/)

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Ganglion cells</th>
<th>Length of outflow tract (um)</th>
<th>Ganglion cell density (ganglion cells/unit length)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 1-2</td>
<td>448 ± 30†</td>
<td>1707 ± 80</td>
<td>0.26†</td>
<td>29</td>
</tr>
<tr>
<td>S 1-3</td>
<td>433 ± 61†</td>
<td>1713 ± 120</td>
<td>0.25†</td>
<td>14</td>
</tr>
<tr>
<td>S 1-4</td>
<td>615 ± 51</td>
<td>1941 ± 100†</td>
<td>0.32†</td>
<td>19</td>
</tr>
<tr>
<td>Control</td>
<td>681 ± 34</td>
<td>1643 ± 40</td>
<td>0.41</td>
<td>47</td>
</tr>
</tbody>
</table>

* (Semilunar valve to the base of the first branches of the great vessels).
† Different from control P < 0.025.
mean number of neurons was highly correlated with age between 8 and 18 days of incubation \((r = 1.00)\). On incubation day 20 (just prior to hatching), the number of cells decreased sharply to 663 ± 54 \((n = 8)\).

In embryos with neural crest lesions over somites 1–2 or 1–3, the number of ganglion cells was 34–36% less than in controls on incubation day 15.

As presented above, the total number of ganglion cells after somite 1–4 lesion did not decrease significantly from control; however, the density of ganglion cells in the outflow area was significantly less than control. The depletion of total number of ganglion cells at day 10 after somite 1–3 lesions was about 50%, indicating that some recovery occurred between days 10 and 15.

\[^3\text{H}]\text{Choline Uptake}\]

\[^3\text{H}]\text{Choline uptake was measured in seven atria from 15-day embryos with neural crest lesions over somites 1–3. The counts/min of \[^3\text{H}]\text{choline uptake per mg atrium (corrected for the number of counts available) were 435 ± 114. The mean uptake in eight control atria was 661.5 ± 125.8 counts/min. This represents a 34% decrease from control and correlates quite well with the decrease seen in total number of ganglion cells in the outflow tract at the same age and following the same lesion.}\]

\textbf{Acetylcholine Content}\n
The acetylcholine content of 15-day incubation atrium and ventricle was measured in hearts from 23 control and 26 experimental embryos. Acetylcholine levels ranged from 1.5—4.5 nmol/mg protein, and were 31% lower in atria \((P < 0.02)\) and 39% lower in ventricles \((P < 0.01)\) from experimental animals (Table 2). In the same series of animals, the ganglion cell content was 31% lower in the experimental animals. Lesions over somites 1–2, 1–3, and
1–4 were equally effective in reducing acetylcholine content, and only the grouped data are presented.

Muscarinic Receptors

$[^3H]$MS bound to muscarinic receptors in control and experimental embryos with the same high affinity ($K_D = 0.17 - 0.21$ nm; Fig. 5, Table 3). In three samples of pooled hearts, the number of $[^3H]$MS-binding sites was 22% lower ($P < 0.01$) in the experimental animals with neural crest lesions over somites 1–3.

Muscarinic receptor content of the whole heart (atria and ventricles) was also estimated separately in two series of 24–25 embryos. The ganglion cell content of the conotruncus was 24% lower in embryos with neural crest lesions ($P < 0.01$), whereas the muscarinic receptor content was 21% lower ($P < 0.001$; Table 3). Lesions over somites 1–2, 1–3, and 1–4 were equally effective in reducing muscarinic receptors, and only the grouped data are presented.

Carbamylcholine binding was measured in competition experiments with 0.1 nM $[^3H]$MS (Fig. 6). Carbamylcholine was resolved into high- and low-affinity components by iterative nonlinear regression procedures (Table 3). In experiments with hearts pooled from five to six embryos, neural crest lesions did not alter cardiac muscarinic receptor affinity of carbamylcholine (of either the high- or low-affinity sites) or the fractions of receptors which displayed high- and low-affinity carbamylcholine binding. Moreover, muscarinic receptors in hearts from control and experimental embryos were equally sensitive to inclusion of 10 μM Gpp(NH)p in the binding assay media (Fig. 6; Table 4). Gpp(NH)p decreased the apparent number and affinity of high-affinity binding sites; Gpp(NH)p increased the number of low-affinity binding sites without affecting their affinity for carbamylcholine (Table 3).

Discussion

The purpose of these experiments was to create parasympathetically aneural hearts by removing neural crest anlage of cardiac ganglion cells. Such a preparation would permit the evaluation of trophic
TABLE 2

Acetylcholine Content of Developing Chick Heart after Depletion of Parasympathetic Innervation by Neural Crest Lesions

<table>
<thead>
<tr>
<th></th>
<th>Ganglion cells</th>
<th>Acetylcholine</th>
<th>Atrium</th>
<th>Ventricle</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>680 ± 32</td>
<td>4.04 ± 0.38</td>
<td>3.06 ± 0.25</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>473 ± 41</td>
<td>2.79 ± 0.32</td>
<td>1.89 ± 0.25</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>% Change</td>
<td>−31%</td>
<td>−31%</td>
<td>−39%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-test</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM. All measurements were made in hearts from 15-day embryos. The neural crest over somites 1–2, 1–3, or 1–4 was removed at stage 9 or 10 in the experimental embryos. Acetylcholine content is expressed as nmol/mg protein. Acetylcholine content in atria vs. ventricles is different (P < 0.05) in both control and experimental embryos.

**Effects of Neural Crest Lesions on the Development of Parasympathetic Innervation**

To identify the origin of neural crest cells that provide parasympathetic innervation of the heart, Kirby and Stewart (1983) used orthotopic transplantation of ocipital neural crest from quail donors to chick embryo hosts. The parasympathetic postganglionic neurons (i.e., the cardiac ganglion cells) were found to arise exclusively from the neural crest over somites 1–3.

This raises the crucial question of why the depletion of parasympathetic innervation of the heart obtained in the present study was incomplete. The lesions were designed to remove all anlagen of parasympathetic nerve-cardiac muscle interactions. We were not successful in this attempt, for reasons which are not immediately clear. Our failure suggests some hitherto unappreciated flexibility in the development of the neuronal component of the neural crest that is not shared by ectomesenchymal neural crest elements.

The relationship between the heart and the nerves that innervate it is difficult to study in the adult, where the constituent structures cannot be separated. In the present study, advantage was taken of the natural temporal separation of elements in the developing chick heart in order to produce a heart with diminished parasympathetic innervation. The neural crest which seeds cardiac ganglion cells was removed before the cells migrated to the heart. These lesions resulted in substantial, although incomplete, depletions of molecular components of the cardiac parasympathetic system.

Previously, derenervation of the heart was attempted in two different ways: (1) by transplanting the heart and, thereby, severing the nerves, and (2) by using various drugs which either create lesions in, or disrupt the function of, the cardiac nerves. Neither of the approaches provides an ideal model for the study of neuronal interactions with the heart. In transplanted hearts, both sympathetic and parasympathetic divisions of the autonomic nervous system are disrupted, and the heart itself sustains considerable insult. Moreover, intrinsic parasympathetic ganglion cells remain intact. On the other hand, drugs are likely to produce a variety of nonspecific and/or secondary effects which create difficulties in interpreting results from these studies.

**FIGURE 5.** [3H]MS binding to muscarinic acetylcholine receptors in hearts from 15-day chick embryos. Specific (atropine-sensitive) binding in hearts from control (C) chicks and from chicks in which the neural crest over somites 1–3 was removed at stage 10 (●) is shown. The lines are drawn according to parameters obtained in nonlinear regression analyses using a single binding site model. The dissociation constants were 0.17 ± 0.03 and 0.21 ± 0.04 nM (n = 3) in the control and experimental hearts, respectively. The numbers of binding sites in control and experimental hearts were 524 ± 22 and 407 ± 11 (n = 3) fmol/mg protein, respectively (P < 0.01). Each point is the mean from three measurements performed with hearts pooled from five or six embryos. Inset: Scatchard plot of the binding data. B [3H]MS bound in fmol/mg protein; F concentration of free [3H]MS in nM.
Results are expressed as means ± SEM. All measurements were made in hearts from 15-day embryos. The neural crest over somites 1-2, 1-3, or 1-4 was removed at stage 9 or 10 in the experimental embryos. Muscarinic receptor content is expressed as fmol of \[^{3}H\]MS-binding sites per mg particulate protein.

There are several possible explanations for the apparent persistence of cardiac ganglion cell populations. It is possible that, after creating the lesions, adjacent cranial and caudal areas of the neural crest are recruited to contribute migrating cells (or to replace the cells that were removed). In the chimera experiments, quail cells replaced the chick neural crest cells over the lesioned somites. Therefore, any ability of adjacent areas to contribute to parasympathetic innervation of the heart would be masked.

A second possibility is that the dorsal neural tube is replaced by dividing cells from the surviving ventral neural tube. Another possibility is that, in the absence of normal parasympathetic cardiac innervation, neuronal elements from the neural crest over somites 10-20, which normally give rise to the sympathetic innervation of the heart (Kirby and Stewart, 1984), provide cells morphologically indistinguishable from ganglion cells. A fourth possibility is that a few ganglion cells from the neural crest over somites 1-3 survive the lesion and migrate to the heart. The linear increase in ganglion cells between days 8 and 18 demonstrates the dynamic production of new neurons during this period. Heathcote and Sargent (in press) have shown a very similar pattern with frog atrial ganglion cells. In such a flexible situation, a few cells may be capable of seeding a full complement of ganglion cells. The fact that the somite 1-4 lesion caused a less significant depletion in ganglion cells in the conotruncus than lesions of neural crest over somites 1-2 or 1-3 suggests that the surviving but injured neural tube may be responsible for replacement of cardiac ganglion cells. Further work is required to evaluate these possibilities.

The limited decreases in pre- and postsynaptic biochemical markers of cholinergic synapses are perhaps less unexpected, due to the multiplicity of cholinergic elements within the heart. Acetylcholine content decreased 31-39%, whereas the number of muscarinic receptors decreased 21%. In a previous study (Kirby and Stewart, 1983), a second presynaptic indication of cholinergic neurons, high-affinity \[^{3}H\]choline uptake, was decreased 60% by similar neural crest lesions. However, choline uptake was measured on days 9 and 10 of incubation and, as we have shown here, there is a considerable change between days 10 and 15. Muscarinic cholinergic receptors are located on ganglion cells, myocardial membranes, and on the terminals of postganglionic cholinergic, and, possibly, adrenergic, neurons (Higgins et al., 1973; Nomura et al., 1979; Raiteri et al., 1981). Whereas it is frequently assumed that most muscarinic receptors are located on myocardial cell surfaces, it is not clear which cells contribute to the
changes reported here. It should also be noted that, in mature tissues, a disruption of muscarinic transmission with receptor blockers results in an increase (an up-regulation) of cholinergic receptors (Wise et al., 1980; Westlund et al., 1981).

Ganglion cells are also not the sole source of acetylcholine or choline uptake in the heart. A considerable amount of acetylcholine and choline uptake would be expected to be present in terminals of the vagus. The behavior of preganglionic neurons in the face of a diminished ganglion cell population has not been ascertained. To address these questions, it would be useful to produce a more complete depletion of the cardiac ganglion cell population and to eliminate preganglionic parasympathetic innervation of the heart.

Neural crest lesions did not alter the affinity of cardiac muscarinic receptors for antagonists or agonists. Agonist binding had multiple components; two-thirds of the receptors displayed high-affinity agonist binding in hearts from both control and experimental animals. Cardiac muscarinic receptors are coupled to a guanine nucleotide-dependent regulatory protein which plays a role in the elaboration of postsynaptic muscarinic responses. One indication of this coupling is a decrease in agonist-binding affinity in the presence of guanine nucleotides (Berrie et al., 1979; Hulme et al., 1984). In the present experiments, neural crest lesions did not alter the sensitivity of agonist binding to guanine nucleotides: An increase in the fraction of receptors displaying low-affinity carbamylcholine binding was always observed. A conversion of high- to low-affinity receptors was consistent with the results of others (e.g., Galper et al., 1982; Waelbroeck et al., 1982; Birdsall et al., 1984; Dunlap and Brown, 1984). In addition, the apparent affinity for carbamylcholine of the high-affinity receptor population was markedly reduced. The reason for this alteration is not clear. It is possible that high-affinity receptors are converted to a novel low-affinity state different from that of the low-affinity receptors observed in the absence of guanine nucleotides.

Connective tissue derivatives of the neural crest are necessary for the development of the heart and great vessels, as well as a number of other structures derived from the embryonic pharyngeal apparatus (Kirby and Bockman, 1984). Removal of the neural crest causes a variety of developmental defects in these structures; these defects are analogous to those seen in human babies. The lesions created in the present study produced cardiac malformations in every embryo. Thus, there is a dissociation of the consequences of depletion of the connective tissue vs. neuronal contribution of the neural crest to the developing heart. The possibilities for rearrangement and compensation of the neural elements appears to be much more extensive. In clinical application, this suggests that children with conotruncal abnormalities would not necessarily have abnormal vagal control of the heart.

Many reports have appeared which characterize changes in nicotinic acetylcholine receptors in developing skeletal muscle (Fambrough, 1979). This is the first report showing the influence of parasympathetic postganglionic denervation on muscarinic acetylcholine receptors and acetylcholine content in an end organ.

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