0031-3998/95/3804-0479$03.00/0
PEDIATRIC RESEARCH

Vol. 38, No. 4, 1995
Printed in U.S.A.

Glucocorticoid Stimulation Interacts with Sympathetic Innervation to Affect Cardiac Development *in Oculo*

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ABSTRACT

The effects of chronic glucocorticoid stimulation and sympathetic innervation on myocardium developing in the absence of hemodynamic load were tested by grafting embryonic rat hearts into the anterior eye chamber (*in oculo*) of adult host rats. Myocardial grafts in control rats with normal hormonal milieu were compared with grafts in rats with chronic glucocorticoid stimulation (dexamethasone 40 μg/d) or glucocorticoid receptor type II blockade (RU 38486, 330 μg/d). Unilateral superior cervical ganglionectomy of one eye chamber prevented sympathetic innervation to one graft in each host. Two indices of growth, graft size (projected area) and terminal graft weight, were obtained. Dexamethasone treatment increased both size and weight of sympathetically innervated grafts, whereas RU486 treatment significantly decreased graft weight. Conversely, dexamethasone treatment decreased graft size in denervated eye chambers, whereas RU486 treatment had no effect. No differences in graft beating rate were observed among conditions. Sympathetic innervation modulated the effect of glucocorticoids on developing myocardium, suggesting that growth of sympathetically innervated myocardium is enhanced with glucocorticoid exposure, but growth of noninnervated myocardium (*e.g.* fetal heart) may be compromised by excessive glucocorticoid exposure. (***Pediatr Res* 38: 479–484, 1995**)

Abbreviations

CON, control
DEX, dexamethasone
E-0, -12, and 14, embryonic d 0, 12, and 14
SCGin, superior cervical ganglion intact
SCGx, superior cervical ganglion removed
RU38486, Mefipristone
TGF-β, transforming growth factor-β

The literature provides evidence that glucocorticoid stimulation can either facilitate or impede cardiac growth. Excess glucocorticoid exposure in adult rats reduced body weight without a concomitant reduction in heart weight (1), a phenomenon known as cardiac sparing. In embryonic chick heart, a single high dose of cortisol increased cardiac mass measured 48 h later (2). However, DEX treatment of rats on d 17–19 of gestation decreased cardiac DNA content, possibly reflecting deficits in cell proliferation (3). DEX treatment of neonatal rats decreased left ventricular mass at maturity (*i.e.* 60 d) (4). The present study examined the effects of glucocorticoids on cardiac development using the anterior eye chamber culture system (*i.e. in oculo*) which allows one to manipulate the neurohumoral environment of myocardium developing in the absence of hemodynamic load.

Received May 10, 1994; accepted May 19, 1995.
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Supported by National Heart, Lung, and Blood Institute Grants R29-HL39048 and R01 HL-42258 and by the March of Dimes Birth Defects Foundation. D.C.T. was an Established Investigator of the American Heart Association during data collection period.

The growth of intraocular heart grafts is sensitive to the neural milieu and to corticosteroid levels in the host rat (5). Grafts become vascularized and innervated by collaterals that sprout from the iris. Growth proceeds through cell division in the early weeks to cellular enlargement in the later weeks (6). The adult rat host provides a stable hormonal milieu for the graft and allows manipulations that might compromise the viability of a normally developing fetus. Typically, surgical sympathectomy of the eye chamber decreases graft projected area and weight compared with grafts in intact eye chambers (7). Removing the adrenocortical steroids from the host circulation through adrenal medullectomy or adrenalectomy increased growth of ventricular grafts in sympathetically denervated eye chambers compared with sham-operated hosts (5). Ventricular graft growth in sympathetically denervated eye chambers was again compromised when adrenalectomized hosts received a replacement dose of corticosterone. The data suggested that low to moderate glucocorticoid exposure suppressed growth of ventricular grafts *in oculo* when sympathetic innervation of the myocardium was absent.

In the mammalian heart, sympathetic innervation of the heart is incomplete in the fetus (8). The data on intraocular

ventricular graft growth suggest that prenatal glucocorticoid stimulation may inhibit the growth of the developing heart while innervation is incomplete. Although high levels of 11-dehydrogenase enzymes in the placenta convert approximately 85% of maternal plasma glucocorticoids to an inactive metabolite, the normally low glucocorticoid levels in the fetus can be increased transiently by maternal stress (9) or by treatment with synthetic glucocorticoids that are not inactivated by the placenta (10, 11).

Our previous study did not provide information on whether excessive glucocorticoid stimulation of innervated heart would compromise graft growth or whether whole heart grafts (ventricles with atria attached) would respond in the same way as isolated ventricular grafts. The present study was designed to elucidate these points by using a potent synthetic glucocorticoid, DEX, to provide chronic glucocorticoid stimulation to whole heart grafts. Further, the role of glucocorticoid type II receptors was tested by providing pharmacologic blockade with RU38486. We hypothesized that growth would be most compromised by glucocorticoid exposure when sympathetic innervation to the graft was absent and that these effects would be mediated by the glucocorticoid type II receptor.

METHODS

Design. In all three experimental groups, hosts had intact adrenal glands and one eye chamber that was surgically sympathectomized before grafting. Drugs were administered through slow-release pellets (Innovative Research, Toledo, OH) implanted s.c. at the nape of the neck on the day of grafting. Glucocorticoid stimulation was provided by the administration of DEX (40 μg/d), a synthetic glucocorticoid. Pilot studies in our laboratory indicated that chronic treatment with higher doses of DEX compromised the health of the host rats. Glucocorticoid receptor type II blockade was produced by the administration of RU38486 (330 μg/d), a dose that had been shown to increase ACTH and with marginal increases in corticosterone (12). The RU38486 was a gift from Rousel-Uclaf, France. The CON group had pellets of carrier matrix, but no drugs.

Subjects. Sprague-Dawley hosts (8–9 wk old) and pregnant females were obtained from our breeding colony. The colony stock was originally purchased from Taconic Farms and is supplemented with new stock at regular intervals. Hosts were group-housed with free access to Purina rat chow and tap water in a room maintained on a 12-h light:dark cycle. Housing conditions and surgical manipulations conform to standards set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the research protocols were approved by the University of Alabama at Birmingham Animal Care and Use Committee. The animal facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Surgical sympathectomy. Hosts were anesthetized with ketamine/xylazine (100 mg/kg, 7.5 mg/kg, respectively, intraperitoneally). One eye chamber in each host was permanently sympathetically denervated by superior cervical ganglionectomy (SCGx) performed 1 wk before the grafting of fetal tissue. Successful eye chamber sympathectomy was confirmed by ptosis of the ipsilateral eyelid during measurements.

Fetal heart grafting. Pregnancies were timed by designating day E-0 as the date a sperm plug was found. The whole heart was aseptically dissected from embryos obtained from pregnant female rats at 12 d of gestation (E-12). The pupils of the hosts were dilated with tropicamide. The hearts were then grafted by injecting the tissue into the anterior eye chamber through a small incision made in the cornea of adult hosts. The embryonic heart was positioned over the iris. A triple antibiotic ophthalmic ointment was applied to the eyes after surgery to prevent infection. Pellets were then implanted s.c. at the nape of the neck.

Graft growth and beating rate. The size (projected area) and beating rate (beats/min) of the grafts were measured biweekly through a surgical microscope equipped with a calibrated micrometer while rats were under ether anesthesia. Graft size was derived by measuring the longest width of the graft and its perpendicular axis. The projected area measure allows growth to be monitored during periods of rapid proliferation (approximately the first 4 wk *in oculo* (6, 13). A second index of growth was graft weight, which could be obtained only at the end of the study. The projected area measurement of graft size has been reliable and is sensitive for the effects of denervation (6) and hormonal manipulation (5, 14) of the grafts and correlates well with graft weight (15). Discrepancies between graft size and weight can occur because the projected area reflects only two of the three dimensions of the grafts and weight can be affected by iris tissue and water weight. Grafts are carefully trimmed of iris tissue and blotted before being weighed to reduce discrepancies. We confirmed that iris had been trimmed and that grafts were intact during histologic review. The beating rate was determined by measuring the time required for 20 contractions.

Harvesting grafts. Grafts were harvested from hosts and immersion fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA) overnight. After grafts were weighed, the tissue was embedded in paraffin, sectioned at 4 μm, and stained. The hematoxylin and eosin-stained sections were examined for morphologic evidence of myocardial degeneration to ensure that only healthy grafts contributed data to this study. Two independent raters judged scarring and inflammatory cell infiltration in grafts on a 3-point scale from healthy (<20% of graft area) to grim (>70% of graft area). The rejection rate of grafts during the 8-wk culture period was 50% in CON, 60% in DEX, and 62% in RU3486. This includes grafts that failed to survive the 8-wk study as well as those surviving but eliminated from the data set upon histologic evaluation.

Statistical analyses. Data were analyzed by planned comparisons using the software provided in the Statistical Analysis System (SAS, Cary, NC). Statistical differences between means of projected area were determined from profile analysis comparing CON to DEX and CON to RU3486. Significant effects in the profile were pursued further by separate comparisons of innervated grafts (SCGin) in CON *versus* DEX and noninnervated grafts (SCGx) in CON *versus* DEX. Graft beating rates at 2 wk after grafting were analyzed by separate

comparisons of innervated grafts (SCGin) in CON *versus* DEX and noninnervated grafts (SCGx) in CON *versus* DEX. Graft weight, host body weights and weight gain, host heart weight, host heart to body weight ratios, and combined adrenal weight were analyzed by analysis of variance. Follow-up comparisons were made by Student-Newman-Keuls tests. Differences were considered significant at $p < 0.05$.

RESULTS

Glucocorticoid stimulation affects graft size. As expected, surgical sympathectomy decreased the projected area of whole heart grafts in CON hosts by 8 wk *in oculo* [$F(1,8 = 7.75, p < 0.02$]. However, the effects of glucocorticoid stimulation on graft size depended on sympathetic innervation to the graft [$F(1,15) = 12.40$, $p < 0.003$; Fig. 1]. Differences between DEX and CON during the first 4 wk *in oculo* demonstrated that DEX treatment promoted growth in innervated eye chambers [$F(1,7) = 7.08, p < 0.03$; Fig. 1]. Conversely, DEX treatment restricted growth of whole heart grafts in sympathetically denervated eye chambers [$F(1,8) = 5.78$, $p < 0.04$; Fig. 1], with the largest differences found during the last 4 wk *in oculo*.

Glucocorticoid stimulation affects graft weight. Analysis of graft weights comparing CON to DEX revealed that both DEX [$F(1,14) = 5.45$, $p < 0.04$; Table 1] and intact sympathetic innervation [$F(1,14) = 5.20$, $p < 0.04$; Table 1] produced heavier grafts, falling short of a statistical interaction [$F(1,14) = 3.11$, $p = 0.10$]. This trend partially supports our initial hypothesis that DEX interacts with sympathetic innervation to modulate graft growth. Whole heart grafts in the innervated eye chamber of DEX hosts weighed almost twice as much as innervated grafts in CON hosts [$F(1,7) = 7.19, p < 0.03$, Table 1], indicating that size differences found in projected area at 2 and 4 wk were maintained at the end of the 8-wk study period.

Table 1. *Weight (mg) of whole heart grafts after 8 wk in oculo*

	Innervated	Noninnervated**
CON (*n*)	3.4 ± 0.7 (5)	2.9 ± 0.6 (5)
DEX (*n*)*	7.2 ± 1.4 (4)	3.5 ± 1.2 (4)
RU38486 (*n*)*	1.7 ± 0.6 (4)	1.9 ± 0.4 (4)

Weights (mg wet weight) are means ± SE.
* $p < 0.05$ for main effect of drug treatment *vs* control.
** $p < 0.05$ for main effect of graft innervation. The number of observations in each group is listed in parentheses.

Glucocorticoid receptor type II blockade affects graft growth. Graft growth was also affected by chronic glucocorticoid type II receptor blockade (RU3486). Although no significant differences between whole heart grafts in RU3486 and CON in graft size were revealed by the overall profile analysis of projected area measurements [$F(1,14) = 2.57$, $p < 0.13$; Fig. 2], grafts in RU3486 hosts tended to be smaller after 8 wk *in oculo*, [$F(1,14) = 3.98, p < 0.07$]. Additionally, grafts in the innervated eye chamber of RU3486 hosts weighed half as much as grafts in CON hosts [$F(1,14) = 5.41, p < 0.04$; Table 1], suggesting that the differences in projected area measures were amplified when tissue was weighed.

Beating rate. Glucocorticoid manipulations did not affect the beating rate of grafts at the 2-wk time point (Table 2), suggesting that glucocorticoid-related growth differences are not explained by altered contractile activity. Trends observed

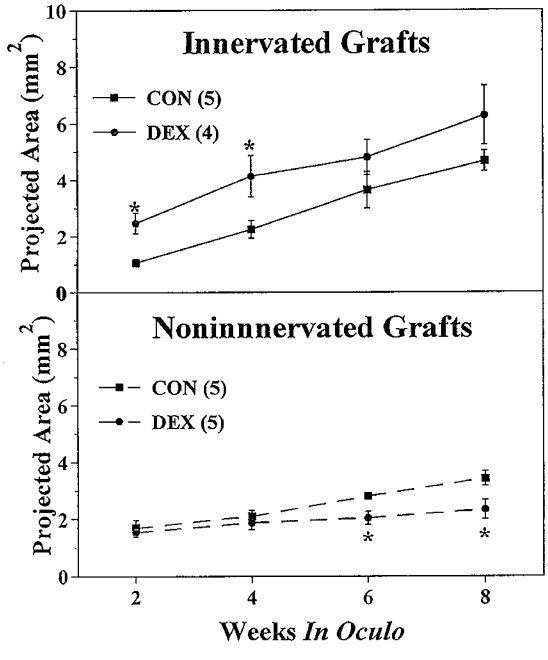

Figure 1. Glucocorticoid stimulation with DEX treatment enhanced growth in innervated grafts (*top panel*) or inhibited growth in noninnervated grafts (*e.g.* the eye chamber was surgically sympathectomized) (*bottom panel*). Values are means ± SE. *$p <$] 0.05 *versus* CON. The number of grafts in each group is listed in parentheses.

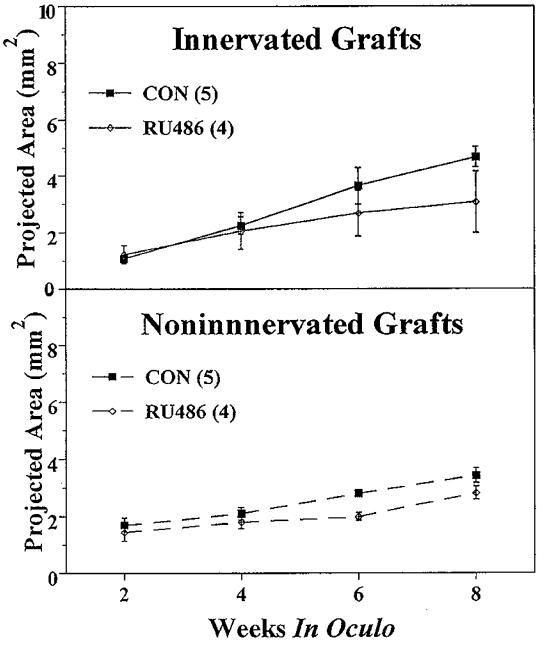

Figure 2. Although grafts in hosts treated with glucocorticoid receptor blockade (RU3486) tended to be smaller than grafts in CON hosts, these differences were not significant in either innervated (*top panel*) or noninnervated (*bottom panel*) CON grafts. Values are means ± SE. The number of grafts in each group is listed in parentheses.

Table 2. *Beating rate of whole heart grafts*

	Innervated			Noninnervated		
Age (wk)	CON (5)	DEX (4)	RU3486 (4)	CON (5)	DEX (5)	RU3486 (4)
2	352 ± 84 [100]	420 ± 134 [100]	232 ± 120 [100]	460 ± 107 [100]	521 ± 52 [100]	423 ± 74 [75]
4	224 ± 68 [100]	302 ± 93 [100]	185 ± 38 [100]	237 ± 29 [100]	361 ± 98 [80]	276 ± 172 [50]
6	270 [20]	223 ± 77 [75]	172 ± 35 [75]	158 ± 31 [100]	385 ± 65 [60]	135 [25]
8	261 [20]	191 ± 66 [75]	132 ± 10 [50]	249 ± 71 [80]	372 ± 137 [60]	199 ± 89 [50]

Values are in beats/min, means ± SE. The number of grafts in the study is listed in parentheses. The percent of grafts beating at a given time is listed in brackets.

in the beating rate data are consistent with previous findings (*e.g.* Ref. 7). Beating rate decreased over the 8-wk culture period, possibly reflecting the increased parasympathetic contol of beating rate as the graft matured (16). In addition, grafts in denervated eye chambers beat somewhat faster than grafts in innervated eye chambers. Inasmuch as the grafts are measured under white light, even beating rate in healthy grafts can be entirely suppressed due largely to parasympathetic inhibition; therefore, a lack of beating does not necessarily designate a dead graft unless the graft is pale in color.

Drug effects on hosts. Initial body weights among groups did not differ. As expected, DEX treatment decreased the amount of weight gained by host rats during the study period [$F(2,20) = 13.02$, $p < 0.0002$; Table 3]. There was a trend for DEX treatment to reduce body weight by the end of the study compared with CONs [$F(2,20) = 2.72$, $p < 0.09$; Table 3]. Neither adrenal weights, heart weights, nor heart weight to body weight ratios differed among conditions at the end of the study.

DISCUSSION

Glucocorticoids and growth of intraocular cardiac grafts. Data from sympathetically innervated whole heart grafts support the role of glucocorticoids in promoting cardiac growth. DEX treatment markedly increased the size of whole heart grafts in innervated eye chambers during the first 4 wk *in oculo*, a period when graft growth is likely to reflect differences in cell division or cell survival. The projected area of sympathetically innervated grafts exposed to DEX was already larger at the time of first measurement, suggesting that DEX had its primary effects on graft size within the first two weeks after grafting. In fact, the growth curves of DEX-treated and CON grafts in innervated eye chambers were parallel after the 2-wk measurement. The increased size of sympathetically innervated grafts exposed to DEX was confirmed by the larger weight observed at 8 wk *in oculo*. A similar increase in cardiac size was obtained when a single high dose of cortisol was administered to embryonic chicks (2). When glucocorticoid receptors were blocked by RU3486 treatment of the host, graft weight was significantly decreased. We conclude that the increased growth of sympathetically innervated grafts treated with DEX was mediated by the glucocorticoid type II receptor.

Table 3. *Effects of DEX and RU38486 on host rats*

	CON (9)	DEX (8)	RU38486 (6)
Body weight (g)1			
Initial	305 ± 8	321 ± 10	312 ± 8
Terminal	466 ± 11	437 ± 15	480 ± 12
Weight gain (g)	161 ± 6	116 ± 9*	168 ± 7
Heart weight (g)	1.40 ± 0.08	1.34 ± 0.06	1.34 ± 0.04
Heart weight/body weight (g/kg)	3.0 ± 0.1	3.1 ± 0.1	2.8 ± 0.1
Total adrenal weight (mg)	50 ± 3	43 ± 4	53 ± 1

Values are means ± SE. The number of hosts in each group is listed in parentheses.

* $p < 0.05$.

In noninnervated grafts, DEX treatment decreased graft size during the last 4 wk *in oculo*, suggesting that increases in cell volume were prevented. Alternatively, early deficits in cell division or cell survival may have been amplified during the cellular enlargement phase of growth or extracellular matrix deposition may have been decreased. Slotkin *et al.* (3) have shown that DEX treatment of fetal rat pups decreased DNA content in neonatal rat hearts, suggesting deficits in cell division or cell survival. Similarly, neonatal DEX treatment of rat pups produced deficits in left ventricular weight in the adult rat (4). Glucocorticoid receptor blockade had no significant effect on the growth or weight of whole heart grafts in denervated eye chambers. This raises the possibility that the dose used was insufficient to block the receptors or that the glucocorticoid suppression of growth in noninnervated myocardium is mediated by another factor.

Taken as a whole, our results suggest an intriguing relationship between glucocorticoid stimulation and sympathetic innervation in affecting the development of cardiac tissue. Glucocorticoid stimulation produced larger myocardial grafts when sympathetic innervation to the graft was intact and produced smaller grafts when sympathetic innervation to the grafted myocardium was prevented. Thus, glucocorticoid stimulation appeared to enhance the growth-promoting effects typically seen with the presence of sympathetic innervation and also amplified the growth compromising effects seen in grafts cultured in denervated eye chambers. We conclude that, when innervation is present, glucocorticoids promote cardiac growth. However, when innervation to cardiac tissue is incomplete, as in the case of cardiac grafts developing in denervated eye

chambers or in the normally developing fetus (8), glucocorticoid stimulation may compromise cardiac growth.

The enhanced growth of ventricular grafts in the sympathetically denervated eye chambers of adrenalectomized hosts found in our previous study was not replicated by pharmacologic blockade of glucocorticoid type II receptors in the present study. It is possible that the atrial component of whole heart grafts responds differently to glucocorticoid manipulations than does the ventricular component. Alternatively, corticosterone replacement in adrenalectomized rats may not have suppressed ventricular graft growth in denervated eye chambers via the glucocorticoid type II receptors. These results suggest that further explanation of the receptor mediation of glucocorticoid influences on developing myocardium and its interaction with sympathetic innervation will be informative.

Glucocorticoids in the developing fetus. Glucocorticoid levels are low in the rat fetus and neonate, with surges during late gestation (17) and parturition (18). Cytosolic glucocorticoid receptors have been identified in the hearts of 8-d chick embryos (2) at a stage of cardiac development equivalent to that of an E-14 rat fetus (19). Glucocorticoid receptors (type II) are present in the rat heart during the perinatal period (20) when the heart is growing primarily by cell division. Circulating glucocorticoid levels rise during the third postnatal week, a period when the heart grows primarily by cellular enlargement (21). In mature hearts, glucocorticoid treatment increased protein synthesis (1). Thus, glucocorticoids may modulate cardiac growth both during the phases of cellular proliferation and hypertrophy. Like the heart developing *in situ*, intraocular cardiac grafts grow primarily through cell division during the first several weeks of development, followed by increases in cell volume (6, 22). The present study suggests that glucocorticoid stimulation and sympathetic innervation interact to affect graft growth during both the hyperplastic and hypertrophic phases of cell growth.

Interaction between glucocorticoids and sympathetic stimulation of the heart. Glucocorticoids can modulate the sympathetic nervous system activity in a variety of ways. The steroids can decrease the norepinephrine content of the heart, with subsequent functional deficits in sympathetic control of heart rate (23, 24) and promote the expression of the differentiated phenotype in sympathetic ganglia (25), seemingly at the expense of elaboration of nerve processes which innervate the heart and other organs. Adrenalectomy of adult rats increases cardiac β-adrenergic receptor number (26), impairs β-receptor coupling (27) and prevents the development of cardiac hypertrophy in rats subjected to chronic isoproterenol treatment (28). Thus, glucocorticoids appear to affect the sympathetic activity at multiple levels which may in turn affect cardiac growth.

Glucocorticoids and growth factors. Glucocorticoids are known to modulate several growth factors which either promote or suppress cell proliferation. In fetal mouse hearts maintained in organ culture, corticosterone had anabolic effects on the hearts when insulin was present in the media and catabolic effects in insulin-free media (29). Glucocorticoids decrease the expression of IGF (30, 31), putative promoters of myocyte proliferation. In cell culture of non-myocytes, glucocorticoids can inhibit proliferation by interfering with IGF-I (32) or by promoting the synthesis of TGF-β, a mitotic inhibitor (33). Conversely, DEX can counteract the inhibition of proliferation produced by TGF-β (34, 35). Whether glucocorticoids also modulate the expression or activity of growth factors such as IGF-I and TGF-β in the developing heart has yet to be examined.

Summary. We demonstrated that DEX treatment interacts with sympathetic innervation to modulate the growth of intraocular embryonic heart grafts. DEX treatment of host rats enhanced the size and weight of whole heart grafts in sympathetically innervated eye chambers. This effect appears to be mediated through glucocorticoid receptor blockade (type II) because treatment with RU38486 produced smaller grafts. Conversely, when sympathetic innervation to the graft was prevented, DEX compromised graft growth. Like the intraocular grafts developing in noninnervated eye chambers, the fetal heart has incomplete innervation (8). One question to investigate thus becomes whether excessive prenatal glucocorticoid stimulation *in situ* would compromise cardiac growth as found in the noninnervated grafts or whether the steroids would promote cardiac growth as we found in the innervated intraocular grafts. The current data suggest the need for studies that evaluate the effects of glucocorticoid stimulation on cardiac development *in situ*. Furthermore, measures of fetal/neonatal heart growth may be prudent in cases of excess maternal stress or when synthetic glucocorticoids are used to promote fetal lung maturation. Although synthetic glucocorticoids are invaluable in increasing the surivival of premature infants, possible iatrogenic problems that affect the developing myocardium should be considered.

Acknowledgments. The authors thank William Belser, Jeanette Bicknell, and Tim Love for their technical assistance in this study. We also thank the people who made the histologic evaluations possible: Dr. Sanford Bishop, Dorothy Madden and Eunice Pickett in the Pathology Department.

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