Calcitonin Gene-related Peptide Expression Is Altered in Pulmonary Neuroendocrine Cells in Developing Lungs of Rats with Congenital Diaphragmatic Hernia

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Congenital diaphragmatic hernia (CDH) is associated with high neonatal mortality from lung hypoplasia and persistent pulmonary hypertension. Pulmonary neuroendocrine cells (PNEC) produce calcitonin gene-related peptide (CGRP), a potent vasodilator. We previously reported altered distribution of CGRP-positive PNEC in full-term rats with CDH, that may lead to an imbalance in vasoactive mediators. In the present study we examined the expression of CGRP-positive PNEC during lung development in rats with CDH induced by 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen). Cesarean sections were performed on Days 16, 18, 20, or 22, and the lungs were immunostained for CGRP and immunoreactive cells were quantitated through image analysis. On Day 16, CGRP-immunoreactive staining was negative; on Day 18, CGRP-immunoreactive cells were found in all controls (not exposed to Nitrofen), whereas in CDH pups, CGRP-positive cells were present in only four of six cases. On Day 20, CGRP immunoreactivity was similar in CDH pups, Nitrofen-exposed pups without CDH, and controls. On Day 22 (term), significantly more CGRP-positive cells (i.e., number of positive cells per surface area [mm^2] or lung volume [mm^3]) were found in ipsilateral lungs of CDH pups than in controls ($P < 0.05$). The difference was even more striking in contralateral lungs of CDH pups ($P < 0.001$), ruling out nonspecific effects of Nitrofen. In CDH lungs, the proportion of immunostained epithelium and the size of the neuroendocrine cell clusters (neuroepithelial bodies [NEB]) were not significantly different from those of controls. On Day 22, supraoptimal dilution immunocytochemistry yielded similar results in CDH pups and controls. We conclude that in CDH, CGRP expression in PNEC and NEB is delayed during early stages of lung development. Because CGRP also exhibits growth factor-like properties for endothelium and epithelial cells, the lack of this factor during a crucial developmental stage (canalicular period) may be causally related to lung hypoplasia. **IJsselstijn, H., N. Hung, J. C. de Jongste, D. Tibboel, and E. Cutz. 1998. Calcitonin gene-related peptide expression is altered in pulmonary neuroendocrine cells in developing lungs of rats with congenital diaphragmatic hernia. Am. J. Respir. Cell Mol. Biol. 19:278–285.**

Amine- and peptide-producing pulmonary neuroendocrine cells (PNEC) are distributed throughout the airway mucosa as solitary cells and as innervated clusters, called neuroepithelial bodies (NEB) (1, 2). PNEC have an important role during lung development (2, 3) and neonatal adaptation (2, 4), particularly in the regulation of pulmonary vascular tone (5). Furthermore, NEB are transducers of the hypoxic stimulus and therefore may function as airway chemoreceptors in the regulation of respiration (6).

One of the peptides produced by PNEC is calcitonin gene-related peptide (CGRP) (7, 8). In humans, CGRP-immunoreactive cells have been identified from 22 wk of gestation onward (9), mostly within the epithelium of distal conducting airways (10). In rat lungs they have been identified from gestational Day 18, with the greatest expression near term (4). Several studies have indicated that CGRP has potent vasodilatory and bronchoconstricting activity (5, 10, 11). Other biologic activities of CGRP include stimulation of growth, with effects on endothelial cells and airway epithelium (12, 13). Increased levels of in-

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Abbreviations: congenital diaphragmatic hernia, CDH; calcitonin gene-related peptide, CGRP; number of CGRP-positive areas (both PNEC and NEB) per mm^2 lung area, CGRP-positive frequency; neuroepithelial bodies, NEB; pulmonary neuroendocrine cells, PNEC; % immunostained airway epithelium, %IMS-epithelium; % immunostained airways, %IMS-airways.

tracellular CGRP have been found in lungs of chronically hypoxic rats (14) and in children with bronchopulmonary dysplasia (BPD) (15).

Infants with congenital diaphragmatic hernia (CDH) have abnormal morphologic development of lungs and intrapulmonary blood vessels (16, 17). The high neonatal mortality and morbidity in these infants is ascribed to the severity of lung hypoplasia and persistent pulmonary hypertension (18). Our previous study in a rat model of CDH revealed that lungs of full-term rat pups contained relatively more CGRP-immunostained NEB than did lungs of age-matched controls (19). This finding suggests that NEB may play a role in the pathogenesis of lung hypoplasia or may lead to an imbalance of vasoactive mediators in CDH. The aim of the present study was to investigate the developmental pattern of pulmonary CGRP-positive cells in lungs of fetal rats with CDH during different stages of lung development.

Materials and Methods

Animal Model

Female Sprague–Dawley rats (Harlan Olac, Wichester, UK) were mated during a period of 1 h (Day 0 of gestation). Eleven of 19 pregnant rats received 100 mg of 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen; Rohm & Haas Co., Philadelphia, PA) in 1 ml of olive oil orogastrically under light ether anesthesia on Day 10 of gestation (20, 21). The remaining eight rats provided control pups. Nitrofen induces a large left-sided diaphragmatic defect with severe lung hypoplasia, sometimes in combination with a small right-sided defect, in up to 80% of offspring of rats treated with this regimen. Food and water were supplied *ad libitum* throughout the period of pregnancy. The pregnant dams were anesthetized by inhalation of diethylether, and a cesarean section was performed on Day 16 (two litters for each group), Day 18 (three Nitrofen-group litters and two control-group litters), Day 20 (three Nitrofen-group litters and two control-group litters), or Day 22 (three Nitrofen-group litters and two control-group litters). The fetuses were removed and killed after cervical intersection with a needle before any breathing occurred. Autopsy revealed a diaphragmatic defect in the Nitrofen-exposed rat pups. The lungs, with trachea attached, were removed for histologic examination. Three groups were studied: rat pups with CDH (Day 16: $n = 8$; Day 18: $n = 6$; Day 20: $n = 10$; Day 22: $n = 7$) and pups without CDH (non-CDH; Day 16: $n = 4$; Day 18: none; Day 20: $n = 7$; Day 22: $n = 5$) in the Nitrofen group, and control pups (Day 16: $n = 5$; Day 18: $n = 8$; Day 20: $n = 4$; Day 22: $n = 4$).

The experimental protocol for the study was approved by the Animal Care and Use Committee of Erasmus University, Rotterdam.

Histologic Examination

The lung tissue from rat pups was processed according to Springall and colleagues (14). The lungs were fixed by immersion in Bouin's fluid, dehydrated in graded ethanols, and embedded in paraffin, and 4-μm sections were cut. Immunostaining for CGRP was performed with specific rabbit polyclonal antibody against rat CGRP (CA-08-220; Cambridge Research Biochemicals Incorporated, Wilmington, DE), using the peroxidase–antiperoxidase method (22). The optimal dilution of 1:400 of the primary antibody was used, according to the manufacturer's instructions. Counterstaining was performed with hematoxylin.

With the aid of a projecting microscope, the total area of the lungs and all CGRP-positive areas (both PNEC [i.e., solitary cells] and NEB [i.e., clusters of three or more cells]) were traced on paper (magnification: ×700) (23). Morphometric analysis included measurements of the size of CGRP-positive areas, the number of CGRP-positive areas per lung, the total surface area of lung sections, the CGRP-positive areas of airway epithelium, and the total epithelial surface area of the immunopositive airways. These measurements were made with a Macintosh computer (Apple, Inc., Cupertino, CA) and the National Institutes of Health (NIH, Bethesda, MD) Image 1.53 program. The resulting data were used to calculate the number of CGRP-positive areas per mm^2 lung (frequency of CGRP-positive cells) (19), and the epithelial CGRP-positive area in relation to the total epithelial area, referred to as the percentage of immunostained epithelium (%IMS-epithelium) (23). The percentage of airways containing CGRP-positive cells, referred to as immunopositive airways (%IMS-airways), was determined by counting (24). The size of CGRP-immunostained NEB, consisting of three or more cells, was determined by counting the number of nuclei. The median number of nuclei of the NEB in each case was used for data analysis.

TABLE 1

CGRP immunoreactivity in rat lungs on gestational Day 18

	Control		CDH	
	Left	Right	Left	Right
Cases with CGRP-positive PNEC	8/8	7/8	4/6	4/6
Cases with CGRP-positive NEB	7/8	5/8	4/6	3/6
Number of CGRP-positive areas	2.5 (1–6)	2.5 (0–6)	1.5 (0–5)	1 (0–2)
Number of CGRP-positive NEB	2 (0–6)	2 (0-6)	1 (0–4)	0.5 (0–1)
NEB size	4 (3–6)	6 (3–9)	3 (3–5.5)	5 (3–6)

Definition of abbreviations: CDH = congenital diaphragmatic hernia; CGRP = calcitonin gene-related peptide; NEB = neuroepithelial bodies; PNEC = pulmonary neuroendocrine cells.

For controls and CDH rat pups, the number of cases in which CGRP-positive PNEC and the number in which CGRP-positive NEB (consisting of three or more cells) were found, the number of CGRP-positive areas (consisting of PNEC and NEB), the number of CGRP-positive NEB, and the size of the NEB (determined by the median number of nuclei) are shown for each lung. For all parameters except the number of CGRP-positive cases, the median and range values are indicated.

Lung sections taken on gestational Days 16 and 18 were used to establish the presence of CGRP-positive cells. The median number of nuclei in NEB in lung sections from Day 18 rat pups was also determined. In addition, the %IMS-epithelium, %IMS-airways, and median number of nuclei of NEB were determined in controls and in rat pups with CDH on gestational Day 22.

Supraoptimal Dilution Immunocytochemistry

Supraoptimal dilution immunocytochemistry was used to compare levels of anti-CGRP immunostaining in the CDH- and the control-group lung sections from gestational Days 20 and 22 (14, 25). The lung sections used for this experiment were adjacent to the lung sections described earlier. They were stained with a supraoptimal concentration of primary antibody of 1:24,000, which is a 60-times dilution of the optimal dilution (25), or with a concentration of 1:60,000, which was the dilution used by Ebina and colleagues (25). Coverslips were then mounted on wet sections, which were immediately examined. The number of CGRP-positive areas per lung and the size of those areas were determined as described previously. Thereafter, these sections were reincubated with primary antibody at the optimal dilution of 1:400. Sections were then washed, dehydrated, and mounted following counterstaining with hematoxylin. All sections were then reexamined. The ratio of cells staining with supraoptimally diluted versus optimally diluted anti-CGRP, referred to as the cell count ratio, was considered an index of the level of intracellular CGRP (25).

Data Analysis

Values were expressed as means ± SEM unless stated otherwise. Differences between groups were tested with one-way analysis of variance (ANOVA), using the Student–Newman–Keuls test for multiple comparisons, or with the nonparametric Kruskal–Wallis test if appropriate. The chi-square test was used for proportions. Paired *t* tests were used to compare differences between left and right lungs in individual cases, and to compare differences in size of CGRP-positive areas following supraoptimal dilution immunocytochemistry. Statistical significance was assumed at the 5% level for the two-tailed condition.

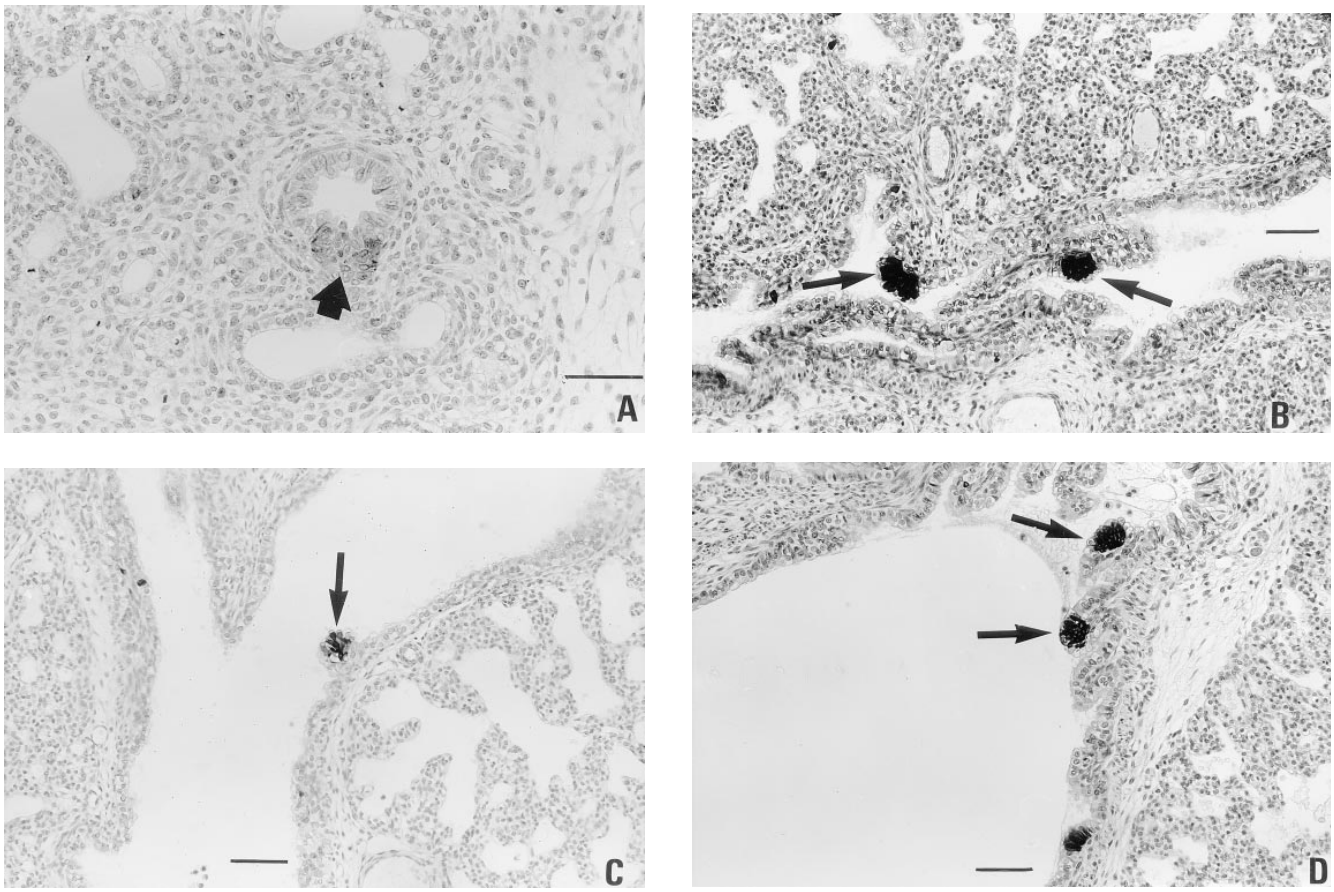
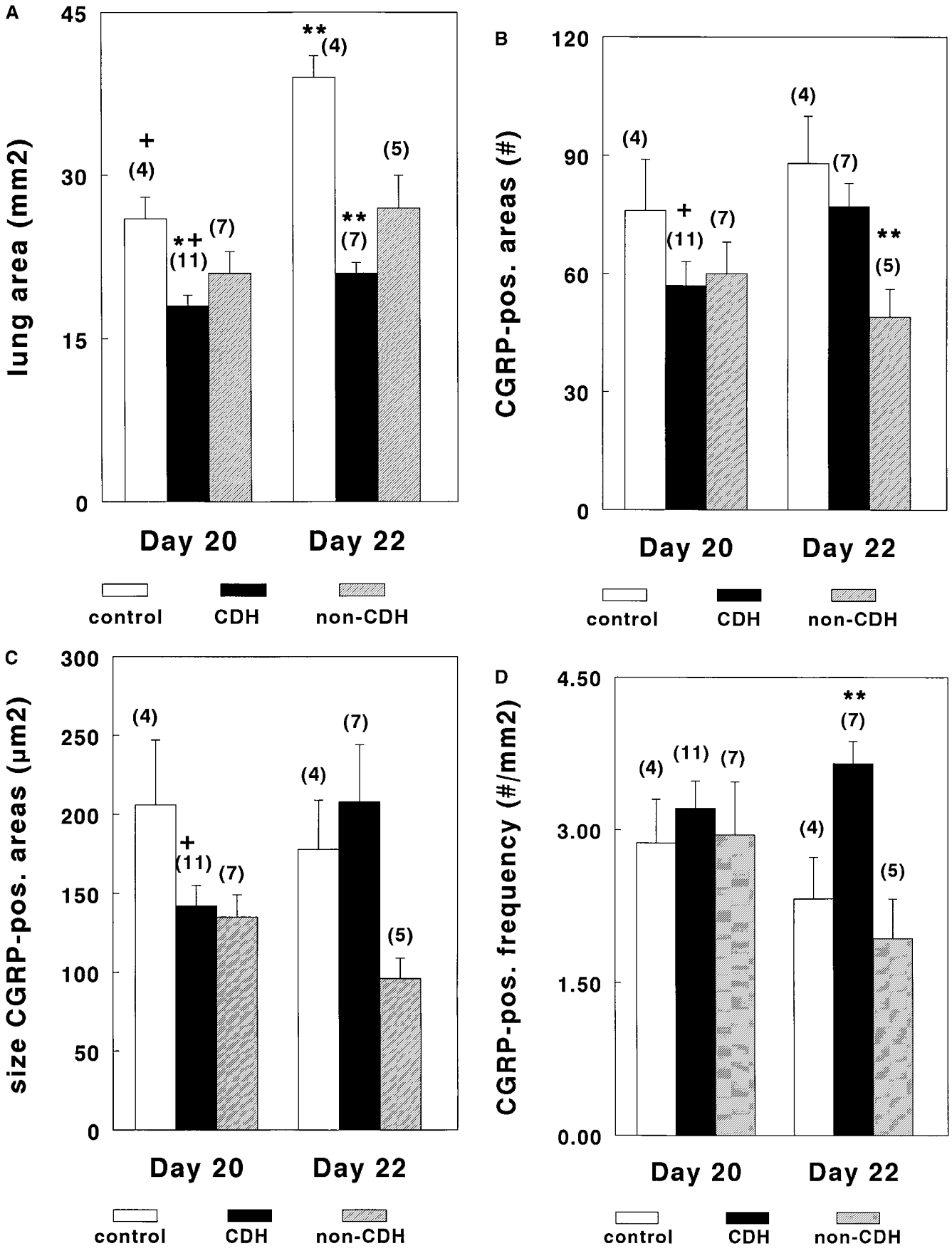


Figure 1. Weak CGRP-positive staining was present in lungs of CDH pups and controls on gestational Day 18, which is indicated here (*arrow*) for the left lung in a CDH rat (*A*). Also shown are representative pictures from left lungs on gestational Day 22 of a CDH rat (*B*), of a Nitrofen-exposed pup without CDH (*C*), and of a control pup (*D*), showing a central airway with some large NEB (*arrows*). The NEB in CDH were bigger and more prominent in the CDH group than in other groups. Counterstaining with hematoxylin. *Scale bars* represent 50 μm.


A
lung area (mm2)
45
30
15
0
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Day 20
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control
CDH
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B
CGRP-pos. areas (#)
120
90
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Day 20
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Day 20
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(7)
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Day 20
Day 22
control
CDH
non-CDH

TABLE 2
CGRP immunoreactivity in rat lungs on gestational Day 22

	Control		CDH	
	Left	Right	Left	Right
%IMS-epithelium	6.5 ± 0.7	7.5 ± 1.1	8.1 ± 0.7	7.5 ± 0.7
%IMS-airways	64 ± 2	63 ± 3	69 ± 1*	68 ± 3
CGRP-positive NEB	11 ± 3	10 ± 2	12 ± 2	9 ± 1
NEB size	4.9 ± 0.4	6.1 ± 0.8	5.4 ± 0.6	4.7 ± 0.3
NEB/mm^3 lung tissue†	3.5 ± 0.8	2.1 ± 0.3	5.0 ± 0.3*‡	3.4 ± 0.3

Definition of abbreviations: CDH = congenital diaphragmatic hernia; CGRP = calcitonin gene-related peptide; IMS = immunostained; NEB = neuroepithelial bodies.

For controls and CDH pups the percentage immunostained airway epithelium, the percentage immunopositive airways, the total number of CGRP-positive NEB (consisting of three or more cells), and the size of the NEB (determined by the median number of nuclei) are shown for each lung. For all parameters the mean ± SEM values are indicated.

*Significantly different from left lung in controls; $P < 0.05$.

†Lung tissue volumes measured by Brandsma and coworkers (26).

‡Significantly different from right lung in CDH; $P < 0.001$.

Results

On gestational Day 16, CGRP immunostaining was negative in lungs of all three groups studied. On Day 18, weak staining was found in all but one lung of controls and in only some lungs of CDH rats (Table 1 and Figure 1A). Both the numbers of cases with positive CGRP immunostaining in the left, ipsilateral lungs, and the numbers of CGRP-positive areas (PNEC and NEB) in the right, contralateral, lungs tended to be lower in CDH than in control pups, but the difference was not significant (Table 1).

The results of morphometric analysis of lungs from the rat pups (the left and right lungs combined) on Days 20 and 22 are shown in Figures 2A through 2D. In pups with CDH, the left lung was significantly smaller than the right lung at both time points (Day 20: 7.7 ± 0.6 versus 9.9 ± 0.5 mm^2; Day 22: 9 ± 0.5 versus 12.5 ± 1 mm^2). On Day 20 the mean total lung area was significantly smaller in CDH pups than in controls (Figure 2A; $P = 0.006$). The mean number of CGRP-positive areas, their mean size, and their frequency were not significantly different (Figures 2B, 2C, and 2D). On both Day 20 and Day 22 there was a tendency toward a difference in size of CGRP-positive areas among the three study groups, but this was not significant ($P = 0.08$ for both gestational ages). On Day 22 the mean frequency of CGRP-positive cells in CDH-pup lungs was significantly higher than in the other groups (Figure 2D; $P = 0.003$).

Additional measurements of lung sections from Day 22 CDH pups and controls revealed that the percentage of immunopositive airways was modestly higher in the left lung in CDH pups than in controls (Table 2; $P = 0.03$). In neither CDH pups nor controls did any of the PNEC/NEB parameters show significant differences in the left versus the right lungs (Table 2).

Histologic examination of lung slides from Day 22 suggested that more prominent and larger NEB were present in CDH (Figure 1B) than in control pups (Figures 1C and 1D). Therefore, we counted the number of NEB and determined the median number of nuclei in NEB for each lung. These parameters yielded no statistical differences between CDH pups and controls (Table 2), but in CDH pups several large NEB with up to 38 nuclei were found, whereas the largest NEB in controls contained not more than 20 nuclei.

Previous studies have shown that between Days 20 and 22, lungs of CDH pups are retarded with respect to the development of future air spaces. Lungs of CDH pups therefore have a very compact aspect on Day 22 (26). We speculated that the higher proportion of CGRP-positive cells in CDH lungs on Day 22 may not have adequately reflected their actual number. Therefore, we corrected the number of CGRP-positive cells for the lung tissue volume, as reported by Brandsma and coworkers (26). The number of CGRP-positive cells per mm^3 lung tissue was slightly but significantly higher in the ipsilateral, left lungs of CDH pups than in those of controls ($P = 0.05$; Table 2). When data for both lungs together were studied, no significant differences between CDH and control lungs were observed (3.42 ± 0.28 cells/mm^3 lung tissue and 2.73 ± 0.38 cells/mm^3 lung tissue, respectively). In CDH lungs, the number of CGRP-positive cells per mm^3 lung tissue was significantly higher in the left lungs than in the right lungs ($P < 0.001$; Table 2).

Supraoptimal dilution immunocytochemistry with the

Figure 2. For controls (*open bar*), CDH pups (*black bar*), and Nitrofen-exposed pups without CDH (*shaded bar*), the means ± SEM are shown for the following parameters on gestational Day 20 and Day 22: total lung surface area (*A*); number of CGRP-positive areas, consisting of PNEC and NEB (*B*); size of CGRP-positive areas (*C*); and numbers of PNEC and NEB per mm^2 lung area (CGRP-positive frequency; *D*). The numbers of animals per group are indicated in parentheses. *Significantly different from controls on the same gestational day, $P < 0.05$; **significantly different from both other groups on the same gestational day, $P < 0.05$; $^+$significantly different from the same group on Day 22, $P < 0.05$.

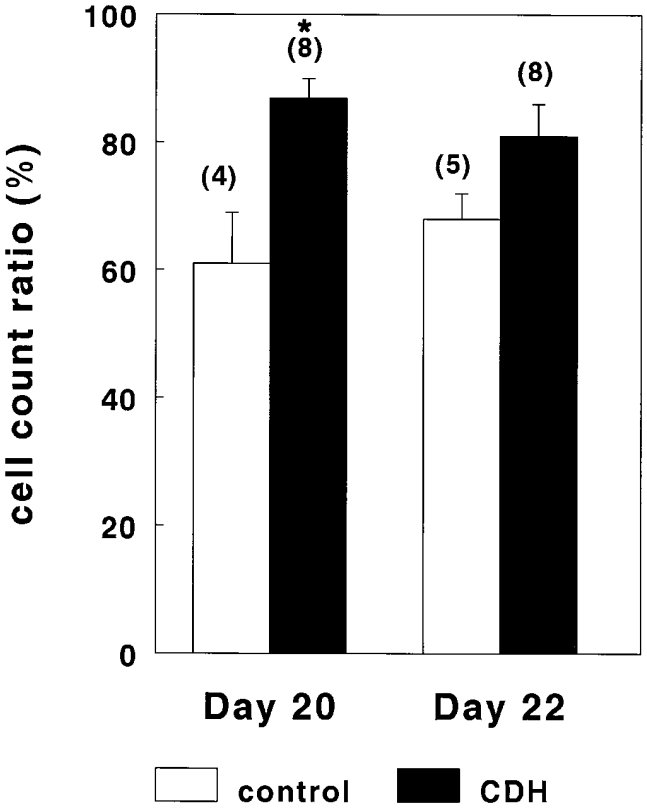

Figure 3. The mean ± SEM percentage of cells that stained following immunostaining with a supraoptimal dilution of the primary antibody of CGRP (1:24,000) compared with optimal staining (1:400), referred to as the cell count ratio, is shown for controls (*open bar*) and CDH pups (*black bar*) on gestational Day 20 and Day 22. The numbers of animals per group are shown in parentheses. *Significantly different from controls; $P = 0.01$.

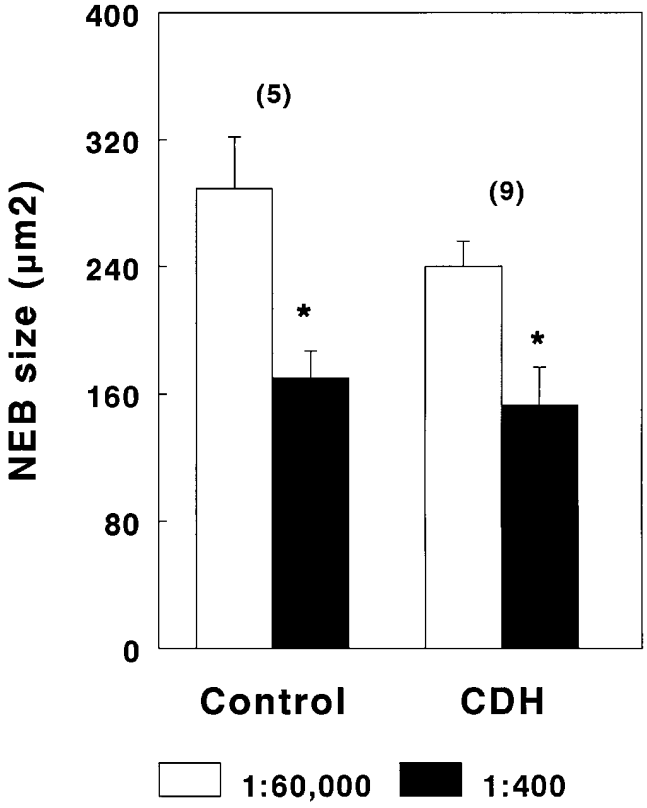

Figure 4. Mean ± SEM sizes of CGRP-positive areas are shown for CDH and control pups on gestational Day 20 after immunostaining with a supraoptimal antibody dilution of 1:60,000 (*open bar*) followed by staining with the optimal dilution of 1:400 (*black bar*). The numbers of animals per group are shown in parentheses. *Significantly different from staining with the supraoptimal dilution in the same group at the same gestational age; $P < 0.01$.

primary antibody diluted 1:24,000 revealed that on gestational Day 20 the cell count ratio was 87 ± 3% in CDH and 61 ± 8% in control pups (Figure 3; $P = 0.01$). On Day 22 no such difference was found. The mean size of the CGRP-positive areas stained with the supraoptimal dilution of antibody was not different from the mean size after restaining with the optimal dilution (data not shown). Experiments with a supraoptimal dilution of 1:60,000 showed a cell count ratio of 25 ± 3% in CDH and 29 ± 3% in control pups on Day 20 (P = NS), and of 26 ± 3% and 23 ± 4%, respectively, on Day 22 (P = NS). Significantly larger CGRP-positive areas were found after staining with a dilution of 1:60,000 than with the optimal dilution in CDH lungs and controls on Day 20 (Figure 4), and in CDH lungs on Day 22 (not shown). Representative photographs show CGRP immunoreactivity in CDH lungs with the supraoptimal antibody dilution of 1:60,000 (Figure 5A), followed by optimal staining (Figure 5B).

Discussion

In the present study we found some delay in the development of CGRP-positive PNEC in lungs of rats with CDH on gestational Day 18, whereas our findings on Days 20 and 22 could indicate that lung development in CDH accelerates toward the end of gestation.

In rats with normal lung development, NEB have been identified from gestational Day 15 onward (8, 27). CGRP immunoreactivity in fetal rats has been observed in cultures starting from Day 15 + 2 (28), and *in vivo* from Day 18 onward (4). Maximal CGRP immunoreactivity is reached on gestational Day 20 (4), and decreases rapidly during the first week after birth (4, 27). Our findings in controls are in accordance with these observations.

In CDH pups, however, both the number and the size of the CGRP-positive areas increased significantly between Day 20 and Day 22. The number of CGRP-positive PNEC and NEB per mm^2 (frequency of CGRP-positive cells) was significantly greater in the CDH group than in both other groups on Day 22. This previously described phenomenon suggests that the PNEC in CDH lungs contain increased levels of CGRP toward the end of gestation (19, 29). However, the lung tissue from CDH pups had a more compact appearance on Day 22 (26). The frequency of CGRP-immunopositive cells therefore may not adequately reflect their actual number. Determination of the CGRP-positive cell number per mm^3 lung tissue, using

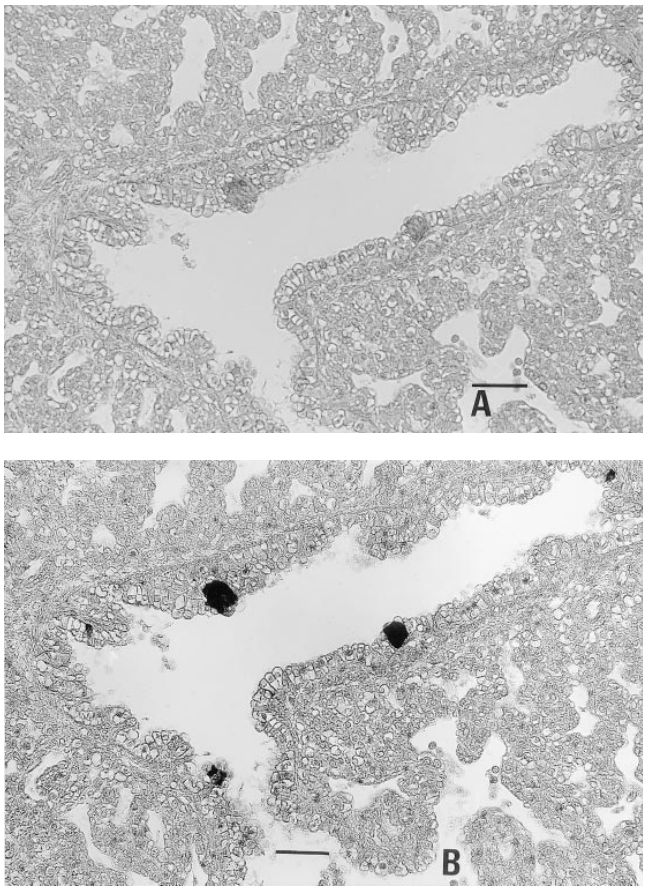

Figure 5. Representative pictures of weak staining in the right lung in CDH pups on Day 22 after immunostaining with the supraoptimal antibody dilution of 1:60,000 (*A*), followed by staining with the optimal dilution of 1:400 (*B*). No counterstaining was done. *Scale bar* represents 50 μm.

previously published data on lung tissue volumes on Day 22 (26), confirmed this assumption.

On Day 22 the left, more hypoplastic lung in CDH pups had a moderately but significantly higher %IMS-airways than that of controls. In both lungs of CDH pups, larger and more prominent NEB were present than in control pups' lungs, although their mean size and the mean number of nuclei per NEB were not significantly different. Interestingly, hyperplasia of bombesin-immunostained NEB has been observed in lungs of human cases of CDH (30).

The higher cell count ratio in CDH than in control pups on gestational Day 20 after immunostaining with a supraoptimal dilution of 1:24,000 suggests that more intracellular CGRP was present in the PNEC of CDH pups. However, the similar cell count ratios at an antibody dilution of 1:60,000 in CDH and control pups indicate no difference in CGRP levels. Yamataka and Puri, using a supraoptimal dilution of 1:20,000, showed that significantly more CGRP-positive cells per mm^2 lung area were present in lungs of CDH pups than in controls on Day 21 (29). This observation is in accordance with our findings on Day 20. That the number of cells staining with supraoptimally diluted versus optimally diluted anti-CGRP antibody (i.e., the cell count ratio) in their study is much lower than in ours may be due to different methodology, such as the use of another CGRP antibody and examination of serial sections.

It remains speculative whether CGRP immunoreactivity in CDH increases toward the end of gestation, as suggested previously (19, 29). In favor of this assumption are the higher frequency of CGRP-immunoreactive cells in CDH pups and the increased numbers of CGRP-positive airways and CGRP-positive cells per mm^3 lung tissue in the ipsilateral, most hypoplastic lungs in CDH pups. Other findings suggest, however, that CGRP immunoreactivity is similar in CDH and control pups, as follows: (*1*) the total number of CGRP-positive cells per mm^3 lung tissue and the size of CGRP-positive areas; (*2*) the proportion of immunostained epithelium; and (*3*) the results of the supraoptimal dilution experiments on Day 22.

Our present data do not allow for conclusions about the direct effect of Nitrofen on CGRP gene expression. Earlier reports suggest that Nitrofen itself has a negative effect on lung development (21, 26), although expression of the proliferation marker proliferating cell nuclear antigen, (PCNA) and of differentiation markers such as collagens III and IV, fibronectin, and laminin did not differ in Nitrofen-exposed and control lungs (A. E. Brandsma, personal communication). Our findings suggest that Nitrofen exposure alone is not sufficient to explain the retarded lung development in this CDH model. This assumption is supported by findings of Brandsma and coworkers (26), who reported delayed development of air spaces only if CDH was present.

Altered expression of neuropeptides may lead to abnormal function of NEB as O_2 sensors in the perinatal period (2, 6). Normally, increased O_2 tension at birth would decrease the stimulation of NEB, whereas continued hypoxia in CDH would increase stimulation in two possible ways (31, 32): Mediators with effects on the pulmonary circulation could be released locally, or signaling via innervation may occur. An imbalance of vasodilatory and vasoconstrictive neuropeptides may play a role in persistent pulmonary hypertension in CDH patients (18). The large CGRP-positive NEB in CDH lungs may contain other peptides, such as bombesinlike peptides, leu-enkephalin, endothelin, or serotonin, which may be involved in pulmonary vasoconstriction (7, 33–35).

In conclusion, the present study shows that the developmental pattern of CGRP immunoreactivity in lungs of CDH rats differs from that of controls. Our data for Day 18 suggest delayed expression at the late pseudoglandular/early canalicular stage of lung development in CDH. During this crucial developmental period, with formation of capillaries and angiogenesis, lack of CGRP may result in decreased vascularization (12), a feature of pulmonary abnormalities in CDH (16, 17). This, and the fact that CGRP has a role in the regulation of airway epithelial-cell proliferation (13), suggest that CGRP could at this stage act as a growth factor, as in the case of expression of bombesinlike peptides in the human lung (36).

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