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Hyperplasia of Alveolar Neuroendocrine Cells in Rat Lung Carcinogenesis by Silica with Selective Expression of Proadrenomedullin-Derived Peptides and Amidating Enzymes

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SUMMARY: Pulmonary neuroendocrine (NE) cells are found as clusters called neuroepithelial bodies (NEBs) or as single cells scattered in the respiratory epithelium. They express a variety of bioactive peptides, and they are thought to be the origin of NE lung tumors. Proadrenomedullin N-terminal 20 peptide (PAMP) is a peptide derived from the same precursor as adrenomedullin (AM). AM and PAMP are C-terminally amidated during their processing by a well-characterized amidating enzyme, peptidylglycine alpha-amidating monooxygenase (PAM). We explored AM, PAMP, and PAM expression as markers for NE hyperplasia in three rodent species (Fischer 344 rats, Syrian golden hamsters, and A/J mice) after a single intratracheal instillation of crystalline silica (quartz), which was previously found to induce different reactions in the three species. Rats developed a marked silicosis, with alveolar and bronchiolar hyperplasia and formation of peripheral lung epithelial tumors. Mice developed a moderate degree of silicosis, but not epithelial hyperplasia or tumors. Hamsters showed dust-storage lesions, but not silicosis or tumors. NE cells were immunolabeled for calcitonin gene-related peptide (CGRP), AM, PAMP, and PAM in serial sections of each lung. The numbers of positive NEBs per lung area and positive cells per NEB were quantified. A marked hyperplastic reaction in the NEBs of silica treated rats occurred only in alveolar NEBs, but not in bronchiolar NEBs. From Month 11 onwards, there were marked differences in the number of alveolar NEBs per section and in the number of cells per alveolar NEB immunoreactive for CGRP. No hyperplastic NE cell reaction was observed in silica-treated mice and hamsters. Significant PAMP and PAM expression was seen only in rat hyperplastic alveolar and in bronchiolar NEBs from Month 11 onwards. In E18, rat fetal lung NEBs were found to be strongly positive for PAMP and PAM. (*Lab Invest 2001, 81:1627–1638*).

The diffuse neuroendocrine (NE) system of the lung is represented in the bronchopulmonary tract by two different elements: solitary pulmonary neuroendocrine cells (PNECs) and clustered neuroendocrine cells, called neuroepithelial bodies (NEBs), which are usually innervated structures (Van Lommel and Lauweryns, 1997).

NE cells of the lung express and secrete several peptides: calcitonin gene-related peptide (CGRP), bombesin or gastrin-releasing peptide (GRP), and calcitonin (CT). Several other immunohistochemical markers label these cells: neuron-specific enolase, chromogranin, PGP 9.5, serotonin, etc. (Scheuermann, 1997). There is a considerable interspecies variation in the pattern of peptides expressed by pulmonary NE cells, but in the three species that we studied (rat, hamster, and mouse) CGRP is the most abundant peptide (Van Lommel et al, 1995) and can be used as a general marker for the endocrine population of the lung.

The roles and possible interplay of pulmonary NE cells in normal lung physiology are not completely clear, but current indications are that NEBs and PNECs act as intrapulmonary chemoreceptors sensitive to hypoxia and hypercapnia and may also exert a paracrine control on the proliferative activity of neighboring cells (Gould et al, 1983; McDowell et al, 1994; Sorokin et al, 1997). A variety of peptides with several proposed biological functions, such as bombesin and CGRP, are produced and secreted by pulmonary NE cells (Van Lommel et al, 1999). Several studies have provided strong support for the relationship between pulmonary NE cells and intrauterine and postnatal pulmonary growth and maturation (Emanuel et al, 1999; Sorokin et al, 1997). Some peptides produced and secreted by the respiratory NE cells strongly stimulate proliferation of lung epithelial cells in vitro (Cuttitta et al, 1985). The relevance of NE cells in

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airway repair after acute lung injury has been studied in several models (Nylen and Becker, 1993). Recent data support the hypothesis that the NEB microenvironment is critical for the maintenance of a "reserve" stem cell population that participates in airway repair upon injury (Reynolds et al, 2000a).

NE hyperplasia has been observed in different mammalian species in relation to a variety of lung conditions or experimental treatments in which regeneration of the chronically injured airways or alveoli is triggered. Either the total number of NEBs, the cell density of each NEB, or both are increased in lung inflammatory, fibrotic, or dysplastic conditions (Aguayo et al, 1990; Willett et al, 1999) or after the exposure to some toxic or carcinogenic products like nicotine or cigarette smoke (Aguayo, 1993), *N*-nitroso compounds (Huntrakoon et al, 1989; Sunday and Willett, 1992), or asbestos (Johnson et al, 1980; Sheppard et al, 1982). Cigarette smokers with NE hyperplasia have a greater risk of developing smoking-related lung disease than smokers without NE hyperplasia (Aguayo, 1993).

Some histological types of human lung tumors show neuroendocrine differentiation: carcinoids, small cell lung cancer, and large cell neuroendocrine carcinoma. These tumor cells express common neuroendocrine markers and share ultrastructural features with normal lung NE cells (Gould et al, 1983; Mosca et al, 1988). PNEC hyperplasia has been proposed as a preneoplastic lesion leading to these neuroendocrine lung tumors (Gould et al, 1983).

NE cell hyperplasia could derive either from proliferation of differentiated NE cells or from the recruitment of nondifferentiated "stem" cells to the endocrine differentiation pathway (Willett et al, 1999). Markers for proliferation have been found in differentiated endocrine cells (Montuenga et al, 1992b; Stevens et al, 1997), but the participation of non-NE progenitor cells in the process has also been shown (Reynolds et al, 2000a, 2000b). Changes in the intracellular levels of peptides that render the cells more detectable by immunocytochemical techniques have also been proposed to explain the rapid increase of endocrine cell numbers found in acute conditions (Bousbaa et al, 1994).

We studied the endocrine cell dynamics in animal models based on a single intratracheal instillation of crystalline silica (quartz) in three species (rats, hamsters, and mice) that markedly differ for their response to silica (Saffiotti et al, 1996). In F344 rats, silica produces a progressive chronic inflammatory and fibrogenic reaction (silicotic lesion), accompanied by hyperplasia of alveolar epithelial type II cells, with the formation of adenomatoid proliferations and, eventually, of peripheral epithelial tumors of different types, mostly adenocarcinomas and adenomas, but also some squamous cell carcinomas, undifferentiated large cell carcinomas, and mixed-type carcinomas. No small cell or other neuroendocrine-like carcinomas were induced in the time frame of the experiment (up to 24 months). Mice of the A/J, BALB/c, and NCr/Nu (athymic nude) strains developed silicotic nodules with necrotic centers, but not persistent alveolar epithelial hyperplasia or lung carcinomas. In Syrian golden hamsters, crystalline silica elicited only a macrophagic storage reaction, and not fibrosis, epithelial reactions, or tumors (Saffiotti, 1993; Saffiotti et al, 1996).

The present study was also aimed at finding possible markers for reactive-hyperplastic NE cells. So far, studies reporting changes in the numbers of NE cells in the lung under various experimental conditions have used general neuroendocrine markers or CGRP/calcitonin. In a proinflammatory environment such as the silicotic lung, extracellular signaling leads to the induction or repression of a variety of genes in the endocrine cells that may become eventually hyperplastic. Other conditions, such as hypoxia, may also affect the pattern of gene expression in these cells. In the present study, we assessed the expression of proadrenomedullin-derived peptides during the silicotic-carcinogenic process. Proadrenomedullin-derived peptides are induced by proinflammatory cytokines (Takahashi et al, 2000) and hypoxia (Garayoa et al, 2000) both in vitro and in vivo. We also studied a new general neuroendocrine marker, the C-terminally amidating enzyme of peptides, peptidylglycine alpha-amidating monooxygenase (PAM), which may also be regulated by cytokines (Scott et al, 1996).

Adrenomedullin (AM) is a peptide with different biological functions (Hinson et al, 2000; Kitamura et al, 1993). Pulmonary AM expression has been previously described in several cell types: bronchiolar epithelium, smooth muscle of both blood vessels and bronchioles, chondrocytes, and alveolar macrophages (Kubo et al, 1998; Martinez et al, 1995). Some nonsmall cell lung carcinomas show increased expression of this peptide (Martinez et al, 1995). Several endocrine cells and organs also express AM. Proadrenomedullin N-terminal 20 peptide (PAMP) is a second peptide derived from the same precursor as AM (Kitamura et al, 1994). Both of these are amidated peptides and PAM is involved during their processing. PAM is the only C-terminal amidating enzyme found in mammals thus far (Prigge et al, 1997); however, it is found in a variety of lung tumors (Saldise et al, 1996) and has been proposed as a candidate for therapeutic intervention leading to autocrine growth factor inhibition in cancer (Iwai et al, 1999). PAMP and PAM can be used as NE markers because PAMP expression is related to endocrine cells in different organs, and PAM takes part in the processing of different peptides, including PAMP, by amidation of the C-terminal end.

Results

Table 1 summarizes the immunohistochemical findings. Table 2 reports the quantitative data obtained with CGRP in treated and control rats.

Fetal Rat Lung

E18 Rat fetal lungs showed a large number of NEBs per section, which were strongly positive for CGRP. Most NEBs were located in the bronchioles. Using

Table 1. Distribution of Peptide Expression in Lung Endocrine Cells of Fetal, Normal, and Silica-Treated Rats, Mice, and Hamsters

	Alveolar	Bronchiolar	
	NEB	PNEC	NEB
Fetal rat	Nd	CGRP− PAMP− PAM− AM−	CGRP+ PAMP+ PAM+ AM−
Normal adult rat	CGRP+ PAMP− PAM− AM−	CGRP− PAMP− PAM− AM+	CGRP+ PAMP− PAM− AM−
Silica rat 0–5 mo	CGRP+ PAMP− PAM− AM−	CGRP− PAMP− PAM− AM+	CGRP+ PAMP− PAM− AM−
Silica rat 11–24 mo	CGRP+ PAMP+[a] PAM+[b] AM−	CGRP− PAMP− PAM− AM+	CGRP+ PAMP+[a] PAM+[b] AM−
Silica-treated mouse	Nd	Nd	CGRP+ PAMP− PAM+ AM−
Silica-treated hamster	Nd	Nd	CGRP+ PAMP− PAM+ AM+

Nd, not determined; NEB, neuroepithelial body; PNEC, pulmonary neuroendocrine cells (isolated); CGRP, calcitonin gene-related peptide; PAMP, proadrenomedullin N-terminal 20 peptide; PAM, peptidylglycine alpha-amidating monooxygenase; AM, adrenomedullin.

[a] Some NEBs are negative. Some cells of positive NEBs are negative.

[b] Some cells are negative.

serial reverse-face sections, we could determine that the same NE cells were also immunoreactive for PAMP and for PAM, but not for AM. Immunostaining was predominantly concentrated at the basal side of the clustered endocrine cells (Fig. 1). No isolated PNEC was immunostained with the antibodies used.

NEBs and PNECs in Normal Adult Rat Lung

NE cells were present in adult rat lungs as clustered alveolar and bronchiolar NEBs or as isolated bronchiolar PNECs. AM was expressed in isolated PNECs, but not in NEBs. In the present study the nonendocrine bronchiolar cells were not immunostained for AM except when the antibody was used in superoptimal concentrations.

NEBs in rats instilled with saline showed the same pattern of peptide expression as untreated adult rats, being positive for CGRP and negative for AM, PAMP, and PAM. These NEBs were not hyperplastic. Saline-instilled rats and untreated rats had similar numbers of alveolar and bronchiolar NEBs per unit area and of cells per alveolar and bronchiolar NEBs (Table 2).

No significant differences were found between the lungs of untreated young rats (8 weeks old) and untreated aged rats (>18 months). The expression pattern of the tested peptides did not change with the age of the rats.

NEB and PNEC Hyperplasia in Rat Lungs after Silica Instillation

Using CGRP immunostaining as general marker, a statistically significant NEB hyperplasia was observed for the first time at Month 11 after silica treatment and was found to progress at later experimental times (Fig. 2, Table 2). Most of the hyperplastic NEBs were located in the alveoli.

In rats killed 17 or more months after treatment, significant differences were found in the number of NEBs per section and in the average number of cells per alveolar NEB, but no significant differences were found in the number of cells per bronchiolar NEB (Fig. 2). Approximately one half of the hyperplastic NEBs were adjacent to areas of nonendocrine epithelial cell hyperplasia.

AM, PAMP, and PAM in Hyperplastic NE Cells

The patterns of expression of proadrenomedullin-derived peptides and of the amidating enzyme were studied (Table 1). During the first 5 months after silica instillation, there was no detectable expression of PAMP and PAM in the NE cells. In rats killed at 11 months or later, most of the NE cells were immunostained for both PAM and PAMP (Fig. 3). The levels of expression of both markers increased with experimental time. There were also differences in the staining intensity among endocrine cells present in the same NEB at a given point; even after long observation times, there was always a subpopulation of CGRP-positive cells that apparently were not expressing either of the other two markers. NE cells within hyperplastic NEBs were consistently negative for AM immunostaining. The specificity of the immunostaining for AM, PAMP, CGRP, and PAM was confirmed by negative results obtained with blocked antibodies.

Significant differences were found in the expression of PAMP and PAM in the silica-treated lungs compared with age-matched untreated rats, both for the number of alveolar NEBs and the number of cells per alveolar NEBs ($p < 0.05$ and < 0.05, respectively for PAM, and $p < 0.01$ and < 0.01, respectively for PAMP). No significant differences were found in the number of bronchiolar NEBs and the number of cells per bronchiolar NEB (Fig. 2).

The NEBs of treated and untreated rats were consistently negative for AM, whereas isolated PNECs present in the bronchiolar epithelium of all groups were immunoreactive with the specific antibody against AM (Fig. 4). PNECs were always negative for CGRP, PAMP, and PAM.

Table 2. Number of NEBs per Section and Average Number of Cells per NEB in Control and Silica-Treated Rats Killed at Different Times[a]

	Alveolar		Bronchiolar	
Time	NEB/cm^2	Cells/NEB	NEB/cm^2	Cells/NEB
Fetal rat			29.131 ± 2.37	5.25 ± 0.42
Aged rats[b]	3.68 ± 2.28	6.37 ± 4.08	6.21 ± 3.32	6.69 ± 2.87
Saline instilled[c]	2.13 ± 2.84	2.44 ± 2.36	3.01 ± 3.25	2.44 ± 2.69
Control[d]	4.05 ± 4.32	2.59 ± 2.36	2.61 ± 2.47	1.96 ± 1.31
1 mo	3.97 ± 1.98	8.1 ± 2.78	4.81 ± 2.24	8.7 ± 3.69
2 mo	1.72 ± 1.79	3.51 ± 3.25	1.16 ± 0.76	4.42 ± 4.24
3 mo	1.13 ± 0.15	2.5 ± 0.36	3.38 ± 0.12	3.66 ± 0.14
4 mo	1.95 ± 1.3	5.27 ± 3.55	1.54 ± 0.75	6.05 ± 1.3
5 mo	1.95 ± 0.22	5.71 ± 1.53	2.91 ± 0.23	5.16 ± 2.11
11 mo	11.7 ± 0.47	9.84 ± 2.61	4.7 ± 1.37	3.47 ± 0.81
17 mo	13.9 ± 4.92*	34.6 ± 9.17*	1.95 ± 1.12	9.64 ± 6.33
23 mo	23.9 ± 6.76*	32.8 ± 7.60*	4.21 ± 0.60	8.25 ± 1.37

[a] Both the number of alveolar NEBs per section and the average number of cells per alveolar NEB, increase significantly with time. Based on CGRP immunostaining.
[b] Aged rats, untreated rats, ages 19 to 28 months (n = 34).
[c] Saline instilled, 8-week-old rats instilled with saline only and killed at different time points: 1 month (n = 2), 2 months (n = 10), 4 months (n = 2), 6 months (n = 4).
[d] Control, untreated 8-week-old rats (n = 15).
* $p < 0.01$. p values were calculated using a Kruskal Wallis test and U-Mann Whitney as post hoc test, comparing each time point with time 0. Bonferroni adjustment was applied.

The hyperplastic alveolar and bronchiolar NE cell populations were compared both in terms of NEB number and the number of cells per NEB by applying a Wilcoxon test. Using CGRP as a general NEB marker, very significant differences were found between the two regions (alveolar vs bronchiolar) in the NEB number ($p < 0.01$) and in the number of cells per NEB ($p < 0.01$). Similar results were found using PAM ($p < 0.01$ and $p < 0.01$, respectively) and PAMP ($p < 0.01$ and $p < 0.01$, respectively) as NE markers.

Proliferative Status of Differentiated Endocrine Cells in the Hyperplastic Lesions

The cellular proliferation that leads to NEB hyperplasia was studied using reverse-face sections immunostained respectively for CGRP and proliferating cell nuclear antigen (PCNA). A very low number of CGRP-positive cells (less than one or two per NEB) were found to be also positive for PCNA immunostaining. Some PCNA-positive cells were observed to be adjacent to (or in proximity of) CGRP-positive neuroendocrine cells (Fig. 5).

Endocrine Cells in Mouse and Hamster Lungs after Silica Instillation

In the lungs of mice and hamsters, using CGRP as general marker, no significant variations were observed in the number of NEBs throughout the experiment and in the number of cells per NEB. In the mouse and hamster, NEBs were immunoreactive for PAM, but not for PAMP at all time points. NEBs were detected in hamster with antibodies against PAM, but not against PAMP at all time points. Some hamster bronchiolar NEBs were also positive for AM, in contrast to mice and rats (Fig. 6).

Isolated bronchiolar PNECs have been previously described in hamster and mouse lungs (Ito et al, 1998; Stevens et al, 1997), but no immunoreactivity was observed in PNEC with our reagents for any of the markers presently studied, including CGRP.

Discussion

The present results demonstrate that NEB hyperplasia is present in rat lungs after intratracheal instillation of crystalline silica. Previous studies reported pulmonary PNEC and NEB hyperplasia in several experimental conditions in which the lung epithelial cells are exposed to inflammatory cytokines, chronic hypoxia, or a variety of toxic compounds. Interestingly, several groups (Johnson et al, 1980; Sheppard et al, 1982) reported that asbestos inhalation produces NE hyperplasia in rats. After quartz instillation in the present model, a very significant hyperplasia of NE cells was found in rat lungs, but it did not occur in mice and hamsters that underwent the same treatment. Carcinogenesis in rat lungs by silica has a long latent period, and tumors were formed in rats killed at 11 months or later. The increase of NE cells is not due to the age of the rats, because untreated old rats did not show NE hyperplasia. The observed species difference in NE cell reaction parallels the reported species differences in silica-induced lung carcinogenesis. In the rat, intratracheal silica induces extensive progressive silicosis, peripheral epithelial hyperplasia, and a marked carcinogenic response, whereas neither hamsters nor mice develop lung carcinomas (Saffiotti, 1993; Saffiotti et al, 1996). The mechanisms responsible for the striking differences in susceptibility to silica-induced carcinogenesis remain to be elucidated, but the role of cellular mediators that may affect

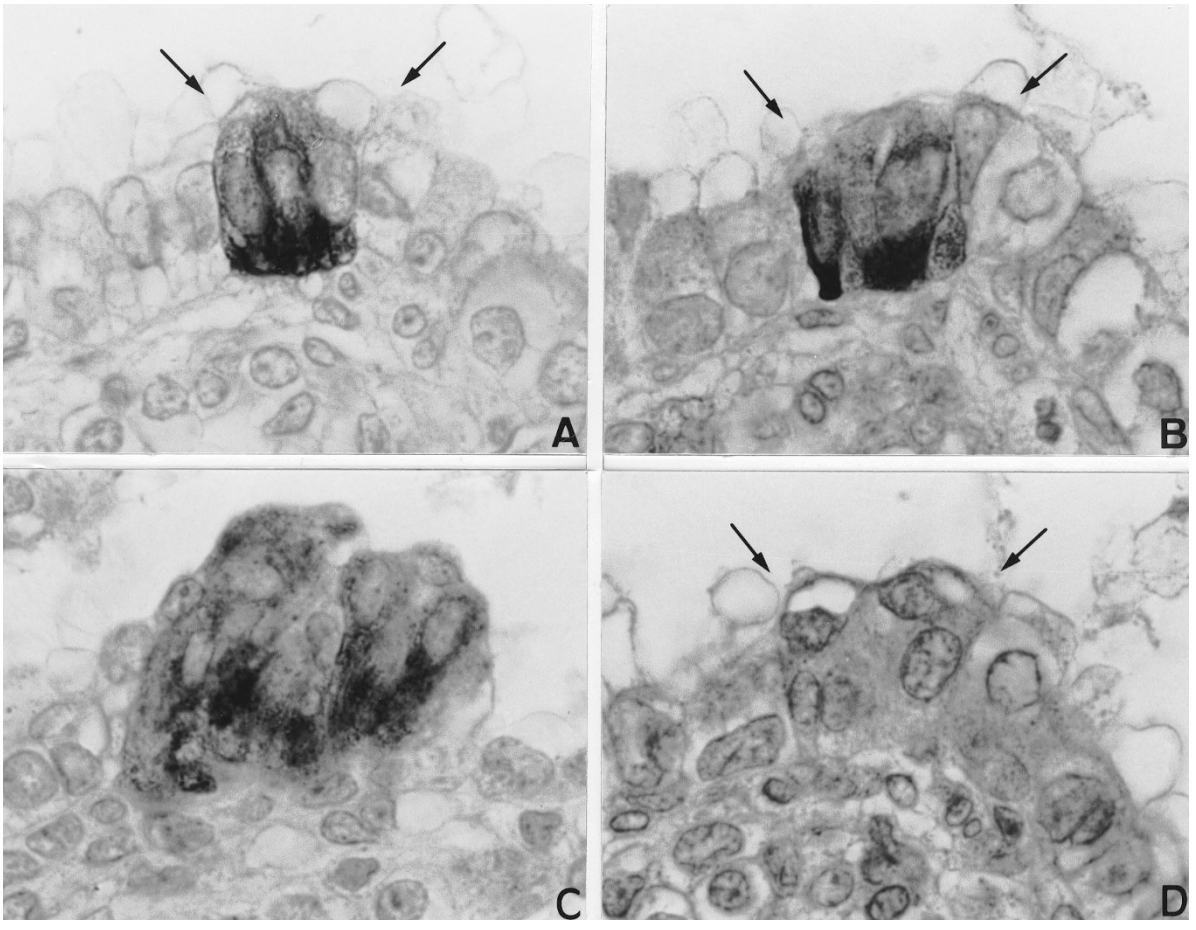

Figure 1.

E18 Rat fetal bronchiolar neuroepithelial bodies (NEBs) marked with different peptides. A, B, and D are perfectly consecutive sections showing the same group of cells (*arrows*) immunostained for calcitonin gene-related peptide (CGRP) (A), proadrenomedullin N-terminal 20 peptide (PAMP) (B), and adrenomedullin (AM) (D). C, A fetal bronchiolar NEB immunostained for peptidylglycine alpha-amidating monooxygenase (PAM). Magnification, ×1250.

alveolar cell proliferation has been considered (Saffiotti, 1993; Seiler et al, 2001). The NE cell hyperplasia in the rat—the species of choice for silica carcinogenesis—and its possible role in stimulating cell proliferation in the adjacent epithelia represents a further hypothetical mechanism. These species differences may be related to other differences in the inflammatory cell response to silica (Driscoll et al, 1996; Schimmelpfeng and Seidel, 1991) and to the role of growth factors such as TGF-β and TNF-α (Saffiotti, 1993; Williams et al, 1996).

In normal rats, bronchiolar and alveolar NEBs are morphologically indistinguishable, and they produce a similar set of peptides. Nevertheless, the observed response of NEBs to silica varies with their localization within the lung. Our quantification protocol shows that two types of NEBs can be distinguished according to their localization, ie, bronchiolar and alveolar. The existence of two subpopulations of NE cells, with different biological properties, had already been suggested for rat lung (Tsutsumi, 1989). Bearing in mind this diversity, we studied separately the response to silica instillation of both subpopulations of NEBs. Only alveolar NEBs react to silica by producing NE hyperplasias. In contrast, no significant differences were seen in the number of bronchiolar NEBs per lung section nor in the number of cells per bronchiolar NEB. Huntrakoon et al (1989) demonstrated that rabbits, injected subcutaneously with diethylnitrosamine (DEN), developed NEB hyperplasia, and most of those NEBs were localized in the alveolar region. In hamsters, DEN injections caused NEB hyperplasia located in bronchioles (Linnoila et al, 1984). In the present rat model, the intratracheally instilled silica particles were concentrated in the peripheral regions of the lung and were found to cause more serious damage to these regions than to the more proximal airways. In fact, intratracheal instillation of crystalline silica in rats provides an animal model for the study of peripheral lung carcinogenesis, in which bronchial tumors were not observed. In addition, bronchiolar and alveolar NE cells may have different biological behavior and sensitivity to certain toxic effects.

Recent literature has paid attention to the question of whether differentiated NE cells are capable of proliferation, and whether hyperplastic reactions are due to neuroendocrine cell division or to the recruitment and differentiation of nearby epithelial stem cells (Reynolds et al, 2000a, 2000b). In the present model, serial PCNA/endocrine marker immunohistochemical

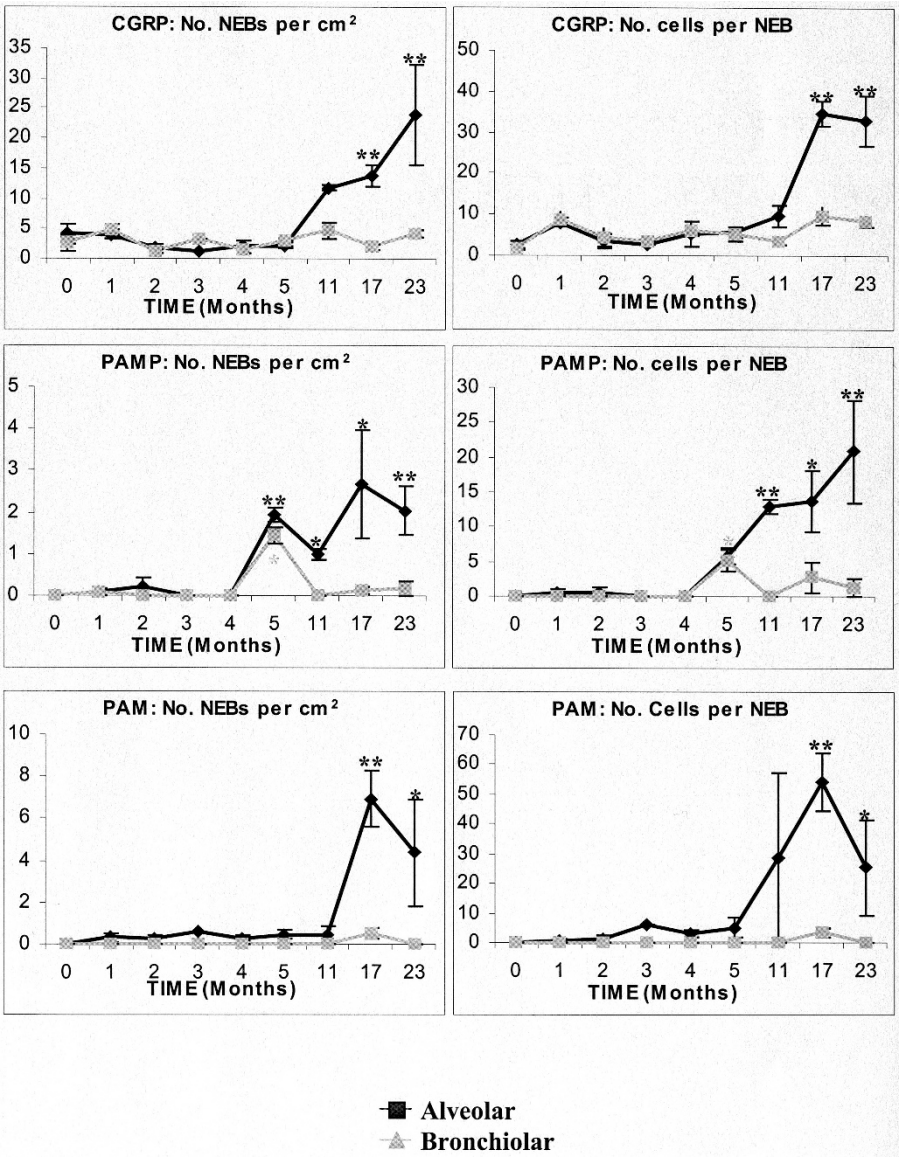

Figure 2.

Change in the number of NEBs in the lungs of silica-instilled rats, studied using CGRP, PAMP, and PAM as neuroendocrine markers. The number of alveolar NEBs and the number of neuroendocrine (NE) cells per alveolar NEB increase significantly after long treatment periods (11–17 months). The number of bronchiolar NEBs and the number of cells per bronchiolar NEB do not change significantly. Very significant differences are found with each marker, when bronchiolar and alveolar data are compared $*p < 0.05$, $**p < 0.01$.

studies were performed to address this question. We found low, but consistent, levels of colocalization of PCNA and CGRP at the time points when the numbers of endocrine cells were sharply increasing. Nonendocrine PCNA-positive epithelial cells were also found in close contact to the NE cells in the alveolar NEBs. Previous studies have shown that the levels of proliferation of lung endocrine cells are very low, although some endocrine cell division may occur (Montuenga et al, 1992a). It has also been shown that nonendocrine cells proliferate in close proximity to the NE cells, suggesting stem cell renewal before endocrine differentiation. It has been recently proposed that NE cells function as a self-renewing progenitor population and that NEB-associated Clara cells are not necessary for NE cell hyperplasia (Reynolds et al, 2000a, 2000b). Although quantification is difficult because of the low number of cells that are double-stained by PCNA and peptides, the present results suggest a mixed model in which NE cell hyperplasia is caused by both endocrine and nonendocrine cell proliferation.

Neuroendocrine cells of the lung produce and secrete a variety of bioactive peptides. So far, none of the peptides reported has been found to be specific for hyperplastic cells. The well-established markers for neuroendocrine cells, such as chromogranin A (Ferrari et al, 1999), neuron-specific enolase (Mosca et al, 1988), bombesin (Emanuel et al, 1999) or, in the case of rodents, CGRP (Johnson et al, 1988), have been used to detect both normal or hyperplastic NE cells. For the first time, the present study found that PAMP, a peptide derived from the same precursor of AM

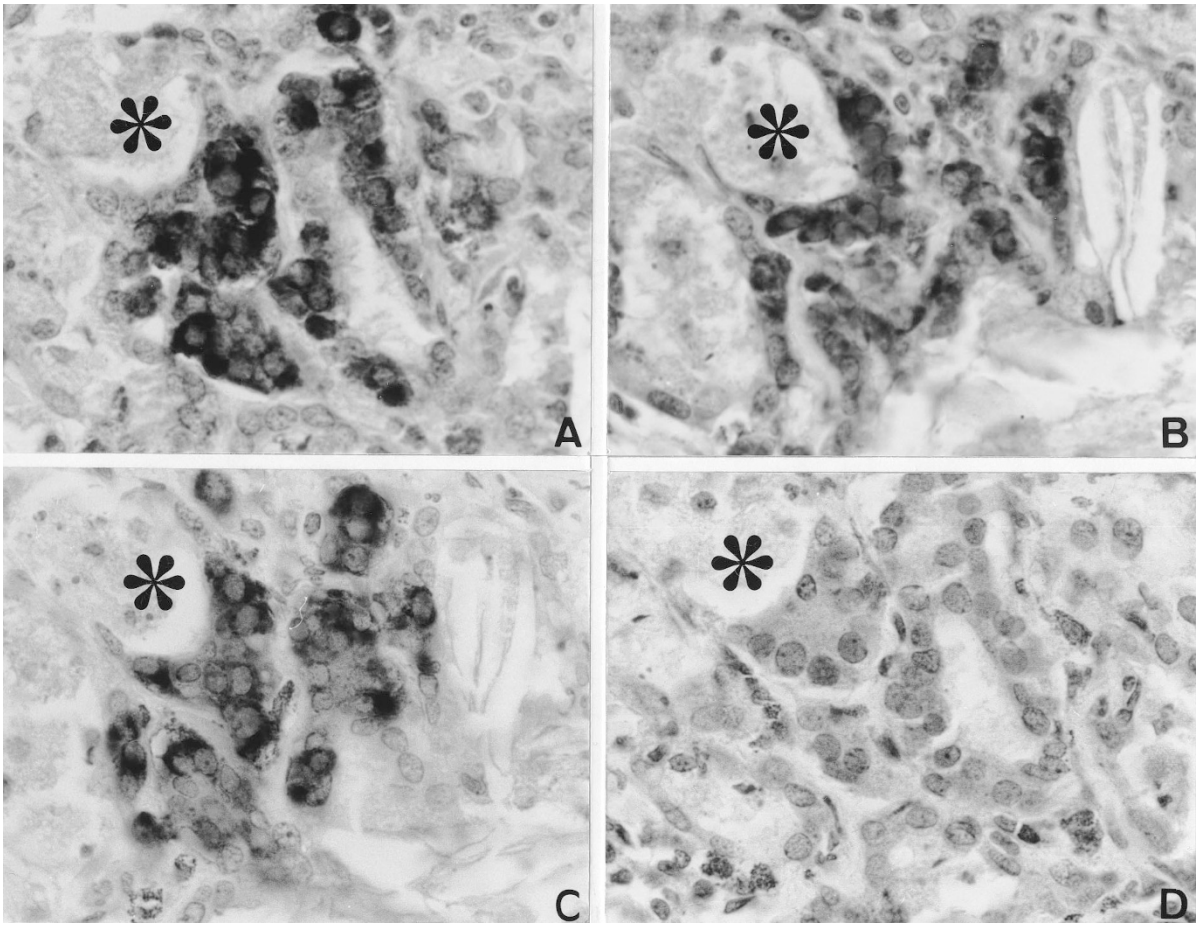

Figure 3.
Serial face-reverse consecutive sections of a hyperplastic alveolar NEB (*asterisks*) of a silica treated rat at 17 months. A, CGRP immunostaining labels the endocrine cells of the hyperplastic NEB. These hyperplastic cells are also positive for PAMP (B) and PAM (C). D, Immunostaining for AM is negative. Magnification, ×500.

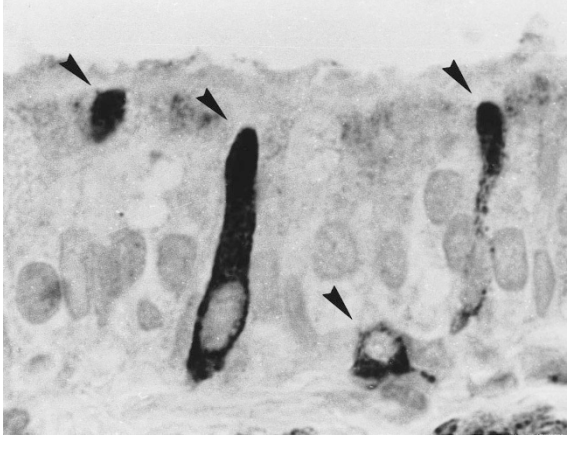

Figure 4.
Bronchiolar epithelium of a silica-treated rat at 17 months showing several pulmonary neuroendocrine cells (PNECs) immunostained for AM (*arrowheads*). Smooth muscle is also positive. Magnification, ×1250.

(Samson, 1998), is expressed at considerably higher levels in hyperplastic endocrine cells than in the cells of normal NEBs and thus could be used as a marker for reactive NE cells, at least in rats. Hyperplastic NEBs of silica-treated rats after the 11th month show PAMP immunoreactivity. Expression of PAMP was also high during the last stages of lung morphogenesis, when the neuroendocrine cells are known to be particularly abundant and functionally relevant (McDowell et al, 1994). NEBs of untreated adult rats are virtually negative for immunoreaction against PAMP. A similar temporal pattern of expression was found for the C-terminal amidating enzyme PAM, which is required for the posttranslational processing of C-terminally amidating peptides like AM or PAMP. We confirmed that this differential expression of PAMP and PAM is due to the treatment, not to the age, of rats. These data collectively suggest that PAMP and PAM overexpression are good markers for activated neuroendocrine cells like those present in embryogenesis and in reactive conditions like the hyperplasia observed in the rat silicotic model. Up-regulation of preproadrenomedullin gene expression by lipopolysaccharide (Zaks-Zilberman et al, 1998), a variety of inflammatory cytokines (Takahashi et al, 2000), and hypoxia (Garayoa et al, 2000) has already been reported. The levels of inflammatory cytokines are significantly increased in silicotic lung lesions (Barrett et al, 1999; Driscoll et al, 1996; Orfila et al, 1998; Rojanasakul et al, 1999). In later stages of human silicosis,

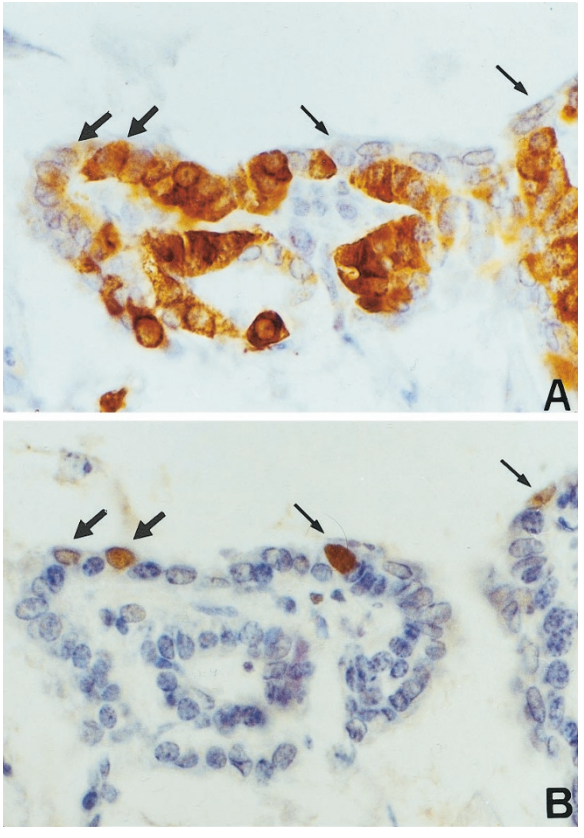

Figure 5.
Proliferative status of NE cells in NEBs. Serial reverse-faced sections showing a hyperplastic alveolar NEB of a silica-treated rat at 17 months immunostained for CGRP (A) and proliferating cell nuclear antigen (PCNA) (B). Only some NE cells are positive for PCNA (*thick arrows*). A very low number of adjacent NE cells (*thin arrows*) is also positive for PCNA. Magnification, ×1250.

hypoxia is also present (Wang and Yano, 1999) and, in the rat model, edema, alveolar proteinosis, and fibrosis would lead to hypoxia. This chronic hypoxia may also explain the increased expression of PAMP found in the reactive NEBs.

We report here for the first time that the isolated PNECs of the bronchiolar epithelium are immunoreactive for AM, the other peptide derived from the same precursor as PAMP. There was an increase in the number of PNECs in the early stage of the silicotic process in rats. Previous animal models of NEB hyperplasia, such as DEN treatment in rabbits (Huntrakoon et al, 1989), did not show any apparent variation in the number of PNECs. Martinez et al (1995) previously reported immunoreactivity for AM in normal human, nonendocrine bronchiolar epithelial cells. In the present study, the nonendocrine rat bronchiolar cells were not immunostained except when the antibody was used in superoptimal concentrations. The reagent used in the present study on rat samples is not the same that was used in the previous study on human tissues, although it was raised against exactly the same epitope. In the present work, we have also shown that bronchiolar PNECs expressed only AM and did not seem to produce PAMP or PAM. In contrast, hyperplastic and embryonic NEBs accumulate PAMP and PAM, but not AM (Table 1). These results clearly support the earlier suggestions (Van Lommel et al, 1999) that PNECs and NEBs are biologically different entities with distinct physiological properties and probably different functional roles.

In summary, a single intratracheal instillation of crystalline silica caused alveolar NEB hyperplasia in rats, but not in hamsters or mice. Two subpopulations of NE cells (bronchiolar and alveolar) were distinguished in relation to their expression of regulatory peptides and their response to silica. Pulmonary neuroendocrine cells showed cell type-specific expression of both proadrenomedullin-derived peptides, AM and PAMP, and of the amidating enzyme, PAM. Finally, the expression of these peptides in rat NEBs changed with time after treatment, and hyperplastic NEBs, observed at late stages after silica treatment, showed the same expression pattern as embryonic NEBs, suggesting that PAMP and PAM may be good markers for reactive neuroendocrine cells. The question remains open whether the activation of NE cells by crystalline silica in the peripheral lung epithelium in rats is involved in the complex mechanisms of carcinogenesis at that target site.

Materials and Methods

Paraffin-embedded tissues from adult rats, hamsters, and mice were obtained from previous experiments carried out at the National Cancer Institute (NCI), Bethesda, Maryland (Saffiotti, 1993; Saffiotti et al, 1996). Tissues from fetal rats came from new experiments carried out at the University of Navarra, Navarra, Spain. All immunohistochemical studies and their quantitation were performed at the University of Navarra. New experiments of silica instillation are under way at the University of Navarra, and the earlier time points (<12 months) gave similar results to the ones obtained with the NCI archival material.

Rats

Fischer F344/NCr female rats from the Animal Production Branch, NCI/Frederick Cancer Research Facility (Frederick, Maryland) were used. The crystalline silica sample was 99% pure α-quartz (min-U-Sil 5, Pennsylvania Glass Sand Co., Pittsburgh, Pennsylvania), with particle size <5 μm (Saffiotti, 1993). Just before instillation, it was autoclaved, resuspended in neutral buffered saline, and briefly sonicated to provide full dispersion. Rats at 8 weeks of age were anesthetized with a mixture of oxygen and methoxyflurane, then were placed on their backs on a metal board slanted at a 60° angle with the mouth kept open, and in this position, at the end of an expiration, they received a single intratracheal instillation of 12 mg quartz in 0.3 ml saline through a 7-cm-long, 19-gauge stainless steel blunt cannula, bent at a 140° angle near the top, connected to a syringe.

The rats were housed in specific-pathogen-free conditions with access to food and water ad libitum. The rats were killed after different time periods since

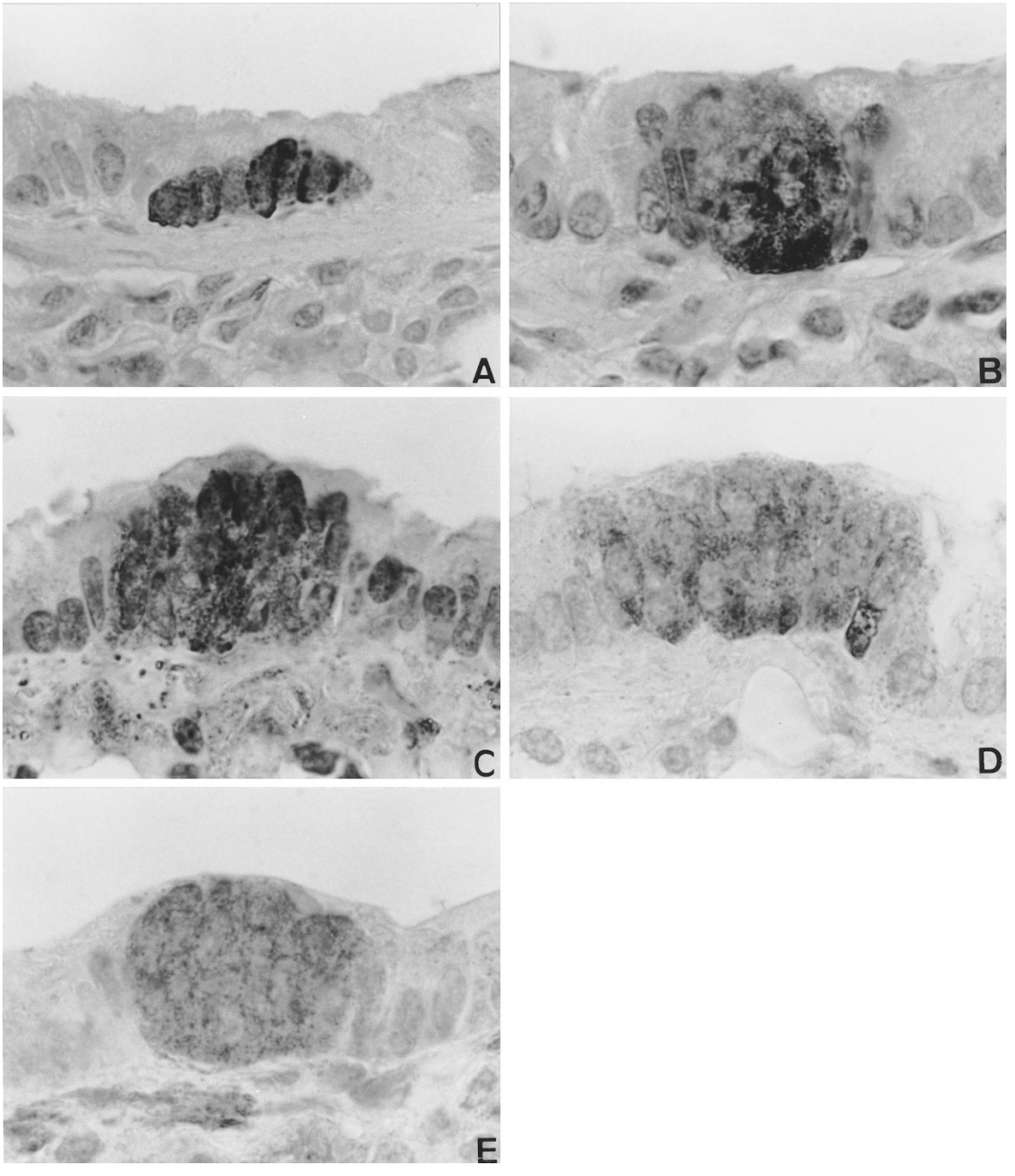

Figure 6.

Bronchiolar NEBs of mouse and hamster immunostained for different peptides. A, Murine NEB immunostained for CGRP. B, Bronchiolar NEB of a hamster immunostained for CGRP. C, Murine bronchiolar NEB immunostained for PAM. D, PAM immunostaining of a hamster NEB. E, AM immunostaining of a hamster NEB. Magnification, ×1250.

the instillation, between Day 0 and two years. The tissues used in the present study were obtained from killings at Months 1 ($n = 9$), 2 ($n = 8$), 3 ($n = 2$), 4 ($n = 4$), 5 ($n = 3$), 11 ($n = 2$), 17 ($n = 15$), and 23 ($n = 5$).

The rats were killed by exsanguination under anesthesia. The trachea was exposed by dissection and ligated during maximal inspiration. The larynx, trachea, bronchi, lungs, lymph nodes, and heart were removed en bloc and fixed by immersion in 4% formaldehyde in a 300-milliosmolar phosphate buffer for 24 hours. Lung lobes were sectioned along their main bronchial axis, embedded in paraffin, and sectioned at 4-μm thickness.

Three different groups of control rats were included in the study. A group of untreated 8-week-old rats were considered as the 0 time point of treatment ($n = 15$). Untreated rats, aged from 19 to 28 months, were included as controls for the aged groups ($n = 34$). A group of 8-week-old rats was instilled with saline only and killed at the following time points: 1 month ($n = 2$),

2 months ($n = 10$), 4 months ($n = 2$), and 6 months ($n = 4$).

Rat Fetuses

Rat fetuses, obtained from F344/NHsd dames at the 18th day of gestation, were studied. Their lungs were removed and fixed in 4% formaldehyde or Bouin's fluid for 17 hours. They were sectioned along their main bronchial axis, embedded in paraffin, and sectioned at 4-μm thickness.

Mice and Hamsters

The lungs of 12 A/JCr mice and 16 Syrian golden hamsters were used. The quartz dose was 10 mg in 0.1 ml saline for mice, and 20 mg in 0.3 ml saline for hamsters. Mice and hamsters were instilled using the methods described for rats, with minor modifications, and their lung tissues were processed in the same way. The lung sections used for the present study came from mice killed at Day 3 and at Months 1, 3, and 6, and from hamsters killed at Day 3 and at Months 1, 3, 6, and 12 ($n = 3$ in each time point).

Immunohistochemistry

A specific polyclonal antiserum anti-CGRP (RPN1842; Amersham, Barcelona, Spain) was used as a general marker for NE cells of the lung at a dilution 1:5000. For the study of AM and PAMP, two polyclonal antisera obtained in rabbit were employed (2469-anti P072 and 2337-anti P070) (Jimenez et al, 1999; Martinez et al, 1995) at a dilution of 1:3000 and 1:1000, respectively. PAM was detected using a mouse monoclonal antibody (2573, clone G8) (Jimenez et al, 2001), diluted 1:2000. The experiments with PCNA were carried out using a commercial monoclonal antibody (MO879, PC10 clone; Dako, Barcelona, Spain), at a dilution of 1:200. Analyses of colocalization of different peptides or markers in the same NEBs were carried out in serial reverse-face sections.

For the immunohistochemical technique, the EnVision® (K4011 and K4007; Dako, Barcelona, Spain) signal enhancement system was used. Slides were deparaffined and their peroxidase blocked with 3% H_2O_2 in water. Tissues were incubated with 5% normal goat serum in TBS (Tris-HCl 0.05M, 0.5M ClNa, pH = 7.36) for 30 minutes at room temperature. After blotting the excess serum from the sections, the diluted primary antiserum was applied and left overnight at 4° C. Tissues were washed in TBS and incubated with the secondary monoclonal or polyclonal EnVision® complex for 30 minutes at room temperature. After washing the slides in TBS, development of peroxidase with diaminobenzidine and H_2O_2 was performed. Tissues were contrasted with Harris hematoxylin and mounted with distyrene, plasticizer, xylene (DPX) mounting medium (BDH, Poole, England). The Histomouse-SP blocking system (95–9541; Zymed Laboratories Inc., San Francisco, California) was employed when mouse monoclonal antibodies were used on mouse lung.

Blocking controls were performed for the peptides we studied. The antibody was preincubated with the peptide overnight at 4° C. The immunohistochemistry was carried out using the blocked antibody.

Quantitative Studies

Sections from each block were immunostained for CGRP, AM, PAMP, and PAM. NEBs were classified in two types according to their localization within the lung. "Bronchiolar" NEBs were those which were surrounded by bronchiolar epithelium; "alveolar" NEBs were those found in contact with alveolar epithelium. The number of positive alveolar NEBs and bronchiolar NEBs in each section after immunostaining with each of the markers was counted. The number of positive cells per NEB was also counted in each case. Only those cells with a visible nucleus were counted. These values were divided by the total area of each lung section, to correct for the probability of finding a NEB per unit area of the section. To get the total sectioned area, the slides were scanned with a Duoscan T1200 AGFA scanner (Madrid, Spain) and the number of square centimeters corresponding to lung tissue (excluding major blood vessels and bronchi) was measured with the help of an image analysis program (Scion; NIH, Bethesda, Maryland). The average NEB frequency was calculated for each experimental group. Proliferative status of the cells was examined by immunostaining with PCNA.

Statistics

The results were statistically analyzed with the SPSS 9.0 program (SPSS Inc., Chicago, Illinois). A Kruskall Wallis test was applied, because not all of the groups had more than 10 individuals. In those cases in which statistical variations were seen, different time points were compared with the corresponding control group using a U-Mann Whitney test with the Bonferroni adjustment. Paired comparisons for alveolar and bronchiolar NEBs were performed using a Wilcoxon test (Chan and Walmsley, 1997).

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