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Induction of EGF receptor and erbB-2 during endotoxin-induced alveolar type II cell proliferation in the rat lung

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Summary. The overall purpose of this study was to produce a model of transient type II cell hyperplasia to enable comparisons of changes in gene expression in the remodelling epithelium with those in carcinogen-induced hyperplastic lesions. Rats instilled with endotoxin had increased numbers of neutrophils in the bronchoalveolar lavage fluid (BALF) by 3 hours that reached maximum levels at 48 hours and returned to background levels 168 hours after instillation. The number of macrophages in the BALF increased throughout the 168 hours following instillation. Epithelial cell hyperplasia was maximum at 96 hours post-instillation in areas of extensive inflammation. The number of alveolar epithelial cells that exhibited bromodeoxyuridine nuclear incorporation reached maximum levels 48 hours after endotoxin treatment and decreased to near background levels at 96 hours. Ultrastructural studies of hyperplastic cells showed the presence of lamellar bodies and condensed chromosomes, characteristics of type II cells in mitosis. At 168 hours after instillation, the hyperplasia regressed to form normal-appearing alveolar structure with few focal lesions. Specific immunostaining for the proto-oncogenes, EGF receptor and erbB-2, on tissue sections increased during the endotoxin-induced hyperplasia. Furthermore, the induction of the 170 kDa and 180 kDa glycoproteins in type II cells isolated from endotoxin-instilled rats was shown by Western analysis. These proto-oncogenes, often thought to be markers of early events during neoplasia, may, therefore, also be associated with wound repair mechanisms after hyperplasia.

Keywords: type II cells, hyperplasia, endotoxin, transient inflammation, EGF receptor, erbB-2

Increased cell proliferation is a common pulmonary response to inhaled irritants, particulates, and physical and chemical carcinogens (Shami *et al.* 1985; Herbert *et al.* 1994). The purpose of this study was to produce a model of transient type II cell hyperplasia to enable comparisons of changes in gene expression in the remodelling epithelium to those exhibited in carcinogen-induced hyperplastic lesions. In humans, many pulmonary adenocarcinomas are believed to originate from type II cells (Edwards 1984).

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Investigations (Harkema & Hotchkiss 1992) have shown that repeated instillation of endotoxin, a lipopolysaccharide that is a major part of the Gram-negative bacterial cell wall, into rat nasal passages results in persistent increased intraepithelial mucosubstances, epithelial cell hyperplasia and secretory cell metaplasia. Furthermore, increased DNA synthesis that was independent of the concomitant inflammatory cell influx (Harkema & Hotchkiss 1993a) has been observed within the transitional cuboidal epithelium of rats. Intratracheal instillation of endotoxin also induces emphysema and a bronchial mucous cell hyperplasia in Syrian hamsters (Stolk *et al.* 1992). Harkema *et al.* (1990) noted that the neutrophilic infiltration induced by endotoxin stimulates increased mucous secretion by the bronchial epithelium of beagle dogs.

Although these effects of endotoxin on bronchial epithelial cells have been repeatedly described, little is known about the immediate proliferative effects of endotoxin on cells in the peripheral lung. In the present study, rats were exposed to a single intratracheal instillation of endotoxin. The hyperplasia of the type II cell was investigated, and changes in the number of neutrophils and alveolar macrophages were quantified. Finally, to differentiate wound healing from premalignant phenomena, changes in the expression of two receptors for growth factors (epithelial growth factor (EGF) receptor and erbB-2) frequently associated with lung cell transformation were monitored.

Materials and methods

Animals and intratracheal instillation

Male F344/N rats (121), 8–10 weeks of age (Taconic Laboratories, Germantown, NY), were housed 2–3 per polycarbonate cage supplied with sterilized hardwood chip bedding and filter tops. Animal rooms were maintained at 20–22°C, with a relative humidity of 20–50% and a 12-hour light/dark cycle starting at 0600 h. Water from a centralized distribution system with sipper tubes and food (Lab Blox, Allied Mills, Chicago, IL) were provided *ad libitum*. Before the experiment, rats were weighed and group assignments were adjusted to result in mean group body weights not significantly different from one another.

Rats were lightly anaesthetized with 5% halothane in oxygen. Fifty-eight rats were intratracheally instilled with 0.5 mg endotoxin (lipopolysaccharide from *E. coli* 0111:B4, Sigma Chemical Co., St Louis, MO) in 0.5 ml pyrogen-free 0.9% NaCl solution; 49 rats were similarly instilled with 0.5 ml pyrogen-free 0.9% NaCl solution; 14 rats were not treated and were kept as shelf controls.

Table 1. Allocation of rats to the various experimental study groups

	Sacrifice period (hours) after endotoxin instillation							
Material	3	6	12	24	48	96	168	Total
Saline	6	6	6	6(3)	6(2)	6(2)	6	49
Endotoxin	6	6	6	6(3)	6(2)	10(3)	10	58
Shelf control				2(3)	4(2)	0(3)		14
						Grand total		121

Numbers in parentheses indicate the group of rats used for electron microscopy.

Necropsy and tissue preparation for histopathology

To label cells undergoing DNA synthesis (Harkema & Hotchkiss 1993b), all rats were injected intraperitoneally (i.p.) with 5-bromo-2′-deoxyuridine (BrdU) (Sigma Chemical Co.) at 50 μg/g body weight in saline 2 hours prior to sacrifice. At least 6 rats were killed at each time point starting at 3, 6, 12, 24, 48, 96 and 168 hours after instillation (Table 1) by i.p. injection of 50 mg sodium pentobarbital (Abbott Laboratories, Chicago, IL). Thoracic contents were exposed and the lungs perfused through the pulmonary artery with phosphate-buffered saline (PBS) without calcium and magnesium ions (GIBCO Laboratories, Grand Island, NY). Thoracic contents were removed and the trachea and lungs isolated. Right lung lobes were tied off distal to the carina, removed, and lavaged five times with 5 ml ice-cold PBS without calcium and magnesium ions; the lavage fluid was collected. The left lung was expanded to inspiratory volume by intratracheal instillation of 10% neutral-buffered formalin (Stephens Scientific, Riverdale, NJ) at 4°C and 25 cm of constant water pressure for 6 hours (Herbert *et al.* 1994). The trachea was ligated and the lung immersed in a large volume of the same fixative overnight.

Fixed lung lobes were cut into cross-sectional slices, each about 0.5 cm thick. Depending on the size of the lung, five or six slices were prepared and numbered from the proximal (slice 1) to distal (slice 5 or 6) end. Slices were then embedded in paraffin and sectioned (5 μm). Sections were used for haematoxylin and eosin and immunohistochemical staining.

Quantification of neutrophils and macrophages

Cells recovered by lavage from the right lung lobes were enumerated using a haemacytometer. Cytological

preparations were prepared and stained with DiffQuick (American Scientific Products, McGraw Park, IL) to determine the different types of cells present in the lavage fluid. One hundred cells were counted from each slide to determine a percentage distribution of macrophages, polymorphonuclear leucocytes (PMNs), and lymphocytes.

Immunohistochemistry

Serial sections adjacent to histologically classified sections of normal lung and hyperplastic regions were placed on Probe-On slides (Fisher Scientific, Pittsburgh, PA) and immunostained. Procedures to identify cells with nuclear BrdU incorporation have been described (Johnson *et al.* 1990). The rehydrated paraffin sections were incubated with anti-BrdU (Beckton Dickinson, Mountain View, CA) after blocking with horse serum (Vector Laboratories, Inc., Burlingame, CA). The reaction of the antibody was detected using a secondary antibody, an avidin–peroxidase complex, and the chromagen diaminobenzidine (DAB). Affinity-purified rabbit polyclonal antibodies raised against synthetic peptides corresponding to amino acid sequences of the human EGF receptor (DVVDADEYLIPQ) and to C-terminal residues of the human erbB-2 glycoprotein 185 (KGTPT VAENPEYGLDVPV) were used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Staining procedures were as described by the manufacturers. Briefly, sections were incubated with antibody solution at 37°C for 1 hour. For the EGF receptor (used at 1:3000 dilution), antibody using 0.5% saponin (Sigma Chemical Co.) to retrieve the antigen was more efficient than microwaving the sections in citrate buffer (BioGenics, San Ramon, CA). For the anti-erbB-2 (used at 1:1000 dilution), however, antigen retrieval by incubating the tissue sections in hot citrate buffer for 15 minutes was most efficient (Krajewski *et al.* 1994). After extensive washes, the specifically bound antibody was visualized using a biotinylated secondary antibody, an avidin–peroxidase complex, and DAB. Reagents used to detect the primary antibody were taken from appropriate Vectastain kits (Vector Laboratories, Inc., Burlingame, CA). The sections were counter-stained with haematoxylin. No immunohistochemical reactions using rabbit IgG showed staining.

Electron microscopy

Lungs to be examined by electron microscopy were removed from the thoracic cavity as described earlier and perfused with PBS. The lungs were inflated to inspiratory volume with air and perfused through the pulmonary artery with Karnovsky's fixative (0.6% glutaraldehyde, 0.45% paraformaldehyde in cacodylate buffer, pH 7.4). The tissue was post-fixed in 1% osmium tetroxide, dehydrated in a graded series of alcohol solutions, infiltrated with propylene oxide solutions, embedded in Epon Araldite, and cut into thin sections with a diamond knife on an Ultracut E ultramicrotome (Reichert-Jung, Cambridge Instruments, Inc., Deerfield, IL). These sections were mounted on Formvar-coated, slotted grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H7000 scanning/TEM (Hitachi Ltd, Tokyo, Japan).

Isolation and characterization of normal and hyperplastic type II cells

To isolate type II cells, the lung vasculature was perfused via the right ventricle with PBS-containing magnesium and calcium cations. The trachea and lungs were removed and the lungs lavaged with ice-cold PBS without magnesium and calcium (5×10 ml). The cell suspension enriched for alveolar type II cells was prepared based on work by Rannels and Rannels (1988). Briefly, the lungs were instilled with 10 ml Joklik's modified essential medium (JMEM) (GIBCO) containing 25 U/ml elastase (ICN Biochemicals, Cleveland, OH) and 0.5% $BaSO_4$ (Sigma Chemical Co.), and incubated for 15 minutes at 37°C. Five ml of the same solution was instilled and the lungs incubated for another 15 minutes at 37°C. The enzymatic reaction was stopped by instilling 15 ml of JMEM that contained 0.08 mg/ml DNase I (Sigma Chemical Co.) and 50% newborn calf serum (NBCS) (Sigma Chemical Co.). The parenchyma was finely minced and vortexed for 1 minute at a medium speed. The dispensed lung tissue was aspirated through a 15-gauge needle and filtered through a 160-μm nylon mesh. The remaining lung tissue on the filter was gently rinsed with 15 ml of JMEM/NBCS.

Dissociated cells were collected by centrifugation (10 minutes at 1000 *g*, 10°C). The cell pellet was resuspended in 10 ml JMEM containing 0.08 mg/ml DNaseI and incubated for 20 minutes at 37°C and 10 minutes at room temperature. Cells were gently resuspended, placed on a discontinuous Percoll gradient (58.5 and 40%), and centrifuged at 1000 *g* for 30 minutes at 10°C. Cells were collected as a 2-ml sample from the 58.5/40% interface, gently mixed with 2 ml JMEM containing 0.08 mg/ml DNase I (Sigma Chemical Co.), and centrifuged for 10 minutes at 1200 *g*, 10°C. The cell pellet was

resuspended in 20 ml Dulbecco's modified essential medium (GIBCO). The cell yield was enumerated using a haemacytometer, and viability was determined by trypan dye exclusion. Fifty to 150 μl of the cell suspension (about 50 000 cells) was used to prepare a cytocentrifuge cytological preparation (Standon, Pittsburgh, PA). Cells were subsequently stained by a modified Papanicolaou stain (Kikkawa & Yoneda 1974). The Percoll fractionated cell suspension was analysed and sorted using a dual laser flow cytometer (Beckton Dickinson, San Jose, CA) linked to a MicroVase IV computer (Digital Equipment Corp., Maryland, MA). Cells were analysed using a 5 W laser operating at 100–200 mW in UV, and a 4-W laser operating at 100–200 mW at a wavelength of 488 nm. Forward and side scatter signals and green (488 nm) and blue (351.1–363.8 nm) signals were collected. Typically 30 000 events were collected and analysed to delineate cell populations contained within the cell suspension. Alveolar type II cells were sorted on the basis of their low green (530–560 nm) and high blue (424 nm) autofluorescence. Alveolar type II cells were sorted into glass tubes, and cytological preparations and staining were completed as above to determine the percentage of type II cells.

Western blot analysis

Type II cells were purified from control and endotoxin-treated rats 3 days post-instillation. Because the purified type II cells were contaminated with inflammatory cells, cells that were collected from the lavage fluid of endotoxin-instilled rats were used to differentiate expression of specific proteins in type II and inflammatory cells. Protein extracts were prepared by lysing cells in 10 mM Tris (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.65% NP-40 using a motor-driven pestle (KONTES Scientific Instruments, Vineland, NJ). The protein concentration was determined using the BCA assay (PIERCE, Rockford, IL). Protein (120 μg) was electrophoresed on a 7.5% sodium dodecyl sulphate polyacrylamide gel (SDS–PAGE) and analysed by Western blotting using antibodies to EGF receptor and erbB-2 at 1:100 dilution as described previously (Tesfaigzi *et al.* 1994).

Statistical analysis

Data were tested for equality of group means using the Tukey Studentized range method (Snedecor & Cochran 1978). The criterion for statistical significance was set at $P \leqslant 0.05$.

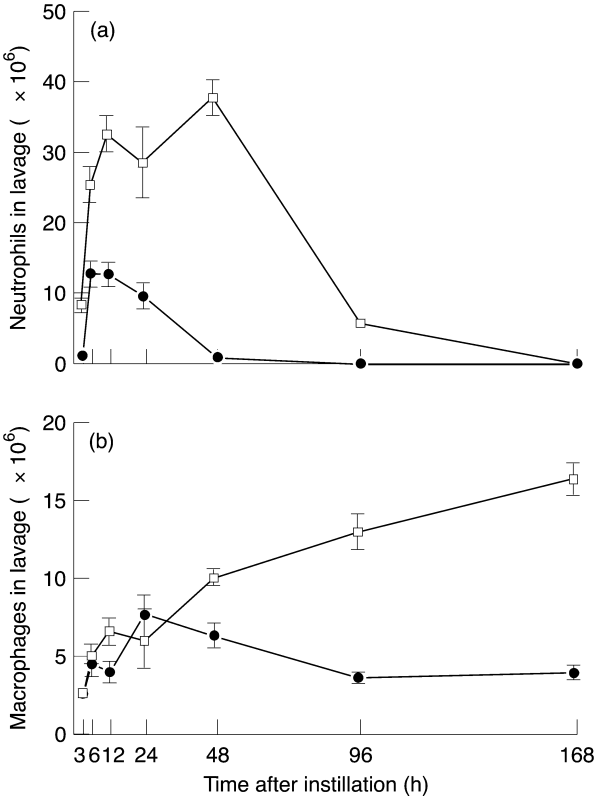

Figure 1. a, The number of neutrophils; b, the number of alveolar macrophages recovered by lavage increased after instillation of ●, saline or □, endotoxin. Each data point represents data from six rats; the error bars represent one standard deviation; and where no error bars are shown, the values were smaller than the size of the symbol.

Results

Bronchoalveolar lavage

The total number of cells recovered by bronchoalveolar lavage increased from 2×10^6 to 10×10^6 within 3–6 hours after instillation in both the saline and endotoxin-instilled rats. These increases were due to increases in the numbers of PMNs (Figure 1a). After 12 hours the number of PMNs from endotoxin-instilled rats was about twice the number from saline-instilled rats. While the number of PMNs in control rats decreased to background values after 48 hours, the elevated numbers of PMNs in endotoxin-treated rats persisted until 48 hours. Ninety-six and 168 hours after endotoxin instillation the numbers of PMNs decreased to baseline values.

The number of macrophages in the bronchoalveolar lavage fluid (BALF) of control rats increased only twofold 24 hours after instillation and returned

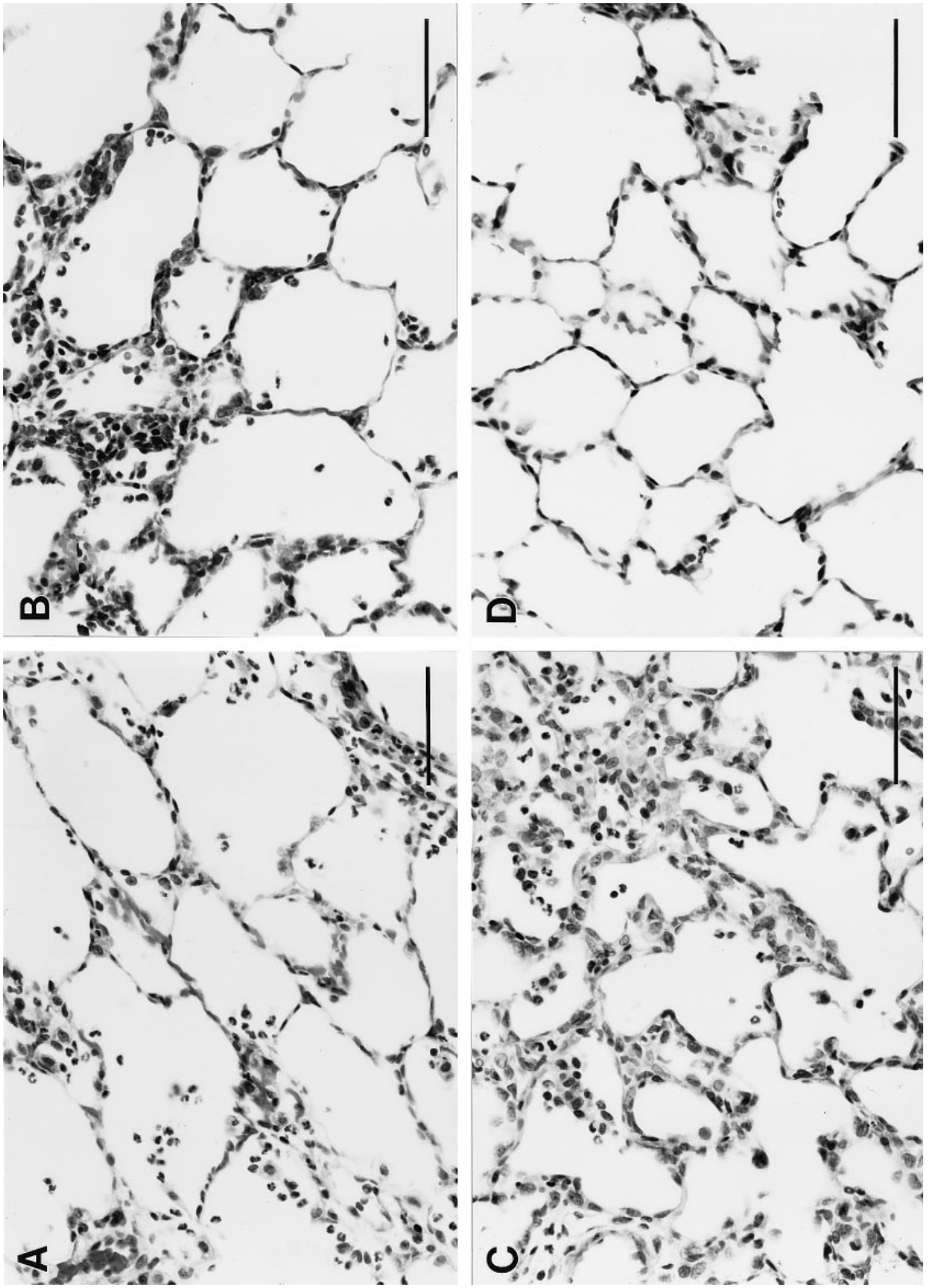

Figure 2. Light micrographs of rat lung A, 24; B, 48; C, 96 and D, 168 hours post-instillation. At 24 hours inflammatory cells have infiltrated the alveoli and airways. At 48 hours alveoli in areas of inflammation are lined by hyperplastic and hypertrophied cells (arrows). At 96 hours the number of epithelial cells is increased due to hyperplasia. At 168 hours alveolar septa are thickened in focal areas and the alveolar spaces contain macrophages. Sections were stained with H&E. The bars represent 20 μm.

to background levels after 96 hours (Figure 1b). In endotoxin-instilled rats, however, the increase of macrophages persisted throughout the 168 hours and reached three times the control values. Essentially no lymphocytes were found in the BALF.

Histopathology

Intratracheal instillitions of endotoxin induced parenchymal changes in the rat lung 3 hours post-instillation, the earliest time point examined. At this time, the influx of cells consisted primarily of PMNs that were found in the alveoli in both the endotoxin-treated and the saline-treated rats. Twelve hours post-instillation, numbers of neutrophils and macrophages appeared to be markedly increased. However, tissue sections from saline-instilled rat lungs showed no inflammatory cells (data not shown). The inflammatory response was evident in rats sacrificed 24 and 48 hours post-instillation (Figure 2A and 2B). Hypertrophy was observed 48 hours after instillation (Figure 2B), and hyperplasia of epithelial cells lining the alveoli was evident in rats sacrificed 96 hours after instillation (Figure 2C). These changes were predominantly found in areas of extensive inflammation, usually surrounding the bronchioles. Hyperplasia was less pronounced in rats sacrificed 168 hours post-instillation where only a few focal lesions were evident (Figure 2D).

The number of cells that had incorporated BrdU into the nuclei was significantly increased in areas surrounding the bronchioles of endotoxin-exposed rats 1–7 days post-instillation. At 48 hours after instillation, the number of BrdU-labelled cells per mm^2 of parenchymal tissue was at least 5 times greater than at 24 or 96 hours (Figure 3). The number of BrdU-labelled cells did not increase in saline-instilled control animals (Figure 3).

Ultrastructure of hyperplastic cells

Cells induced to proliferate by the instilled endotoxin were characterized by electron microscopy. Cells lining the airspace contained numerous lamellar bodies and condensed chromosomes, a sign that cells are in mitosis (Figure 4). None of the type I cells showed any damage due to endotoxin instillation.

Expression of epithelial growth factor receptor and erbB-2 protein

Overexpression of EGF receptor and erbB-2 are known markers of neoplasia (DiFiori *et al.* 1988), and are, therefore, not expected to be induced in transient

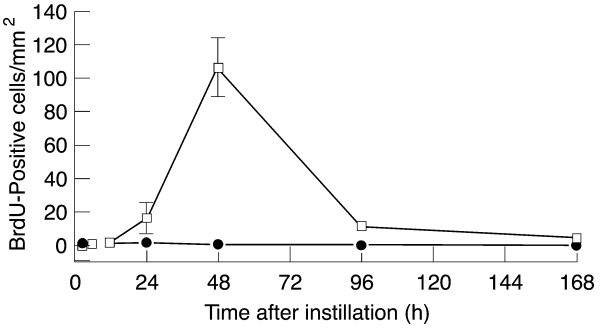

Figure 3. The number of cells per mm^2 of parenchymal lung tissue labelled with BrdU in ●, saline-exposed and □, endotoxin-exposed rats.

hyperplasia.* In saline-instilled control rats, a weak positive staining for the EGF receptor was seen in almost all type II cells 3 days after instillation (Figure 5A). Twenty-four hours after endotoxin instillation, the type II cells exhibited moderate staining. From visual examination of the tissue, it appeared that 90% of the neutrophils and macrophages in the alveoli reacted with this antibody (Figure 5B). After 48 and 96 hours, the hypertrophic and hyperplastic epithelial cells as well as the inflammatory cells showed extensive staining of EGF receptor (Figure 5C and D, respectively). After 168 hours when the epithelial cell hyperplasia had regressed, most epithelial cells still exhibited staining of the EGF receptor (Figure 5E), similar to controls. The immunoreaction was completely inhibited when the peptide to which the antibody was raised was added to the antibody before the reaction (Figure 5F).

The antibody to erbB-2 appeared to stain weakly about 10% of type II cells of the saline-instilled rats (Figure 6A). Twenty-four hours after endotoxin instillation, the intensity of staining for erbB-2 in the alveolar epithelium and inflammatory cells increased dramatically (Figure 6B). Forty-eight hours after endotoxin instillation, alveolar and inflammatory cells retained a marked expression of the erbB-2 protein (Figure 6C). Ninety-six hours after instillation, almost all hyperplastic epithelial and inflammatory cells stained for erbB-2 protein (Figure 6D). Some hyperplastic cells showed the specific membrane staining (Figure 6D). After regression of the hyperplasia, all alveolar epithelial cells still exhibited erbB-2 protein (Figure 6E). The reaction of the erbB-2 antibody was nearly eliminated in the presence of the peptide (Figure 6F); the rest of the staining may be due to a higher affinity of the antibody to secondary structures

* Expression of these receptor proteins in endotoxin-induced cell proliferation was analysed to compare their expression levels in carcinogen-induced hyperplastic lesions.

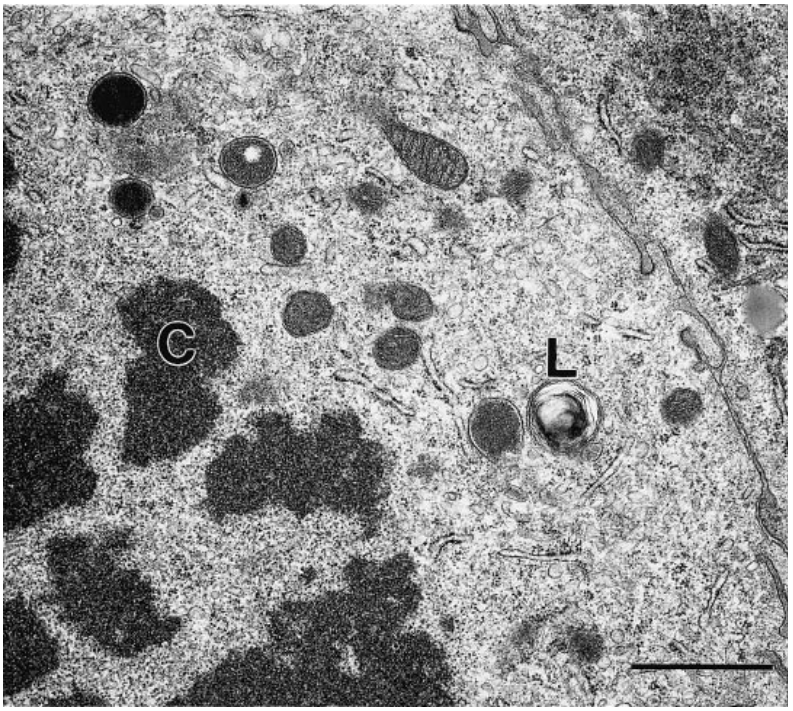

Figure 4. Electron micrograph of hyperplastic alveolar type II cells in the lung of an endotoxin-exposed rat 96 hours post-instillation. Cell shows lamellar bodies and condensed chromosomes. Bar represents 2.0 μm.

within the protein, which would not be formed by the peptide. Tissue sections from all time points after endotoxin instillation were used for the antigen controls; only tissues from 96 hours post-endotoxin instillation are shown to represent obtained results (Figures 5F and 6F).

Characterization of isolated type II cells

Cells isolated by the Percoll gradient flow cytometry method were identified as type II cells by the modified Papanicolaou staining. Highly purified samples (98% pure) of type II cells were obtained by this procedure. Because the number of cells was not sufficient for further biochemical analysis, 68% pure type II cell preparations from both saline and endotoxin-exposed lungs were used for Western blot analysis. Because these type II cells were contaminated with inflammatory cells, the lavaged cells were also subjected to Western analysis. In all three samples a large protein ($\sim$ 220 kDa in size) reacted to the EGF receptor antibody (Figure 7a). Type II cells from endotoxin-treated rats and inflammatory cells from the BALF contained a protein of about 145 kDa that cross-reacted with this protein. However, only the type II cells from endotoxin-treated rats contained the 170 kDa EGF receptor glycoprotein (Figure 7a). Similarly, only the endotoxin-exposed type II cells showed the 180 kDa erbB-2 glycoprotein while the antibody cross-reacted with a 160 kDa protein in all three samples (Figure 7b). The identity of these cross-reacting bands is unknown. These results confirm the results obtained by immunohistochemistry showing that EGF receptor and erbB-2 are induced in type II cells from rats instilled with endotoxin.

Discussion

Non-neoplastic alveolar epithelial hyperplasia can persist in response to repeated exposure of particulates, and some hyperplasias progress to malignant or benign lung tumours (Nikula *et al.* 1995). This study shows that a single intratracheal administration of endotoxin to rats induces proliferation of type II cells and that the proto-oncogenes, EGF receptor and erbB-2, generally known as markers of neoplasia, are overexpressed during this transient hyperplasia. Harkema and Hotchkiss (1991; 1992) have reported that rats instilled intranasally with endotoxin once a day for 3 days show a significant increase in the amount of pulmonary intraepithelial mucosubstances and in the number of bronchial epithelial cells, due primarily to an increase in the number of mucous-secretory cells. Domenici-Lombardo *et al.* (1995) have also shown that hyperplasia of type II pneumocytes and hypertrophy of interstitial fibroblasts occur in rats 48 hours after endotoxin instillation.

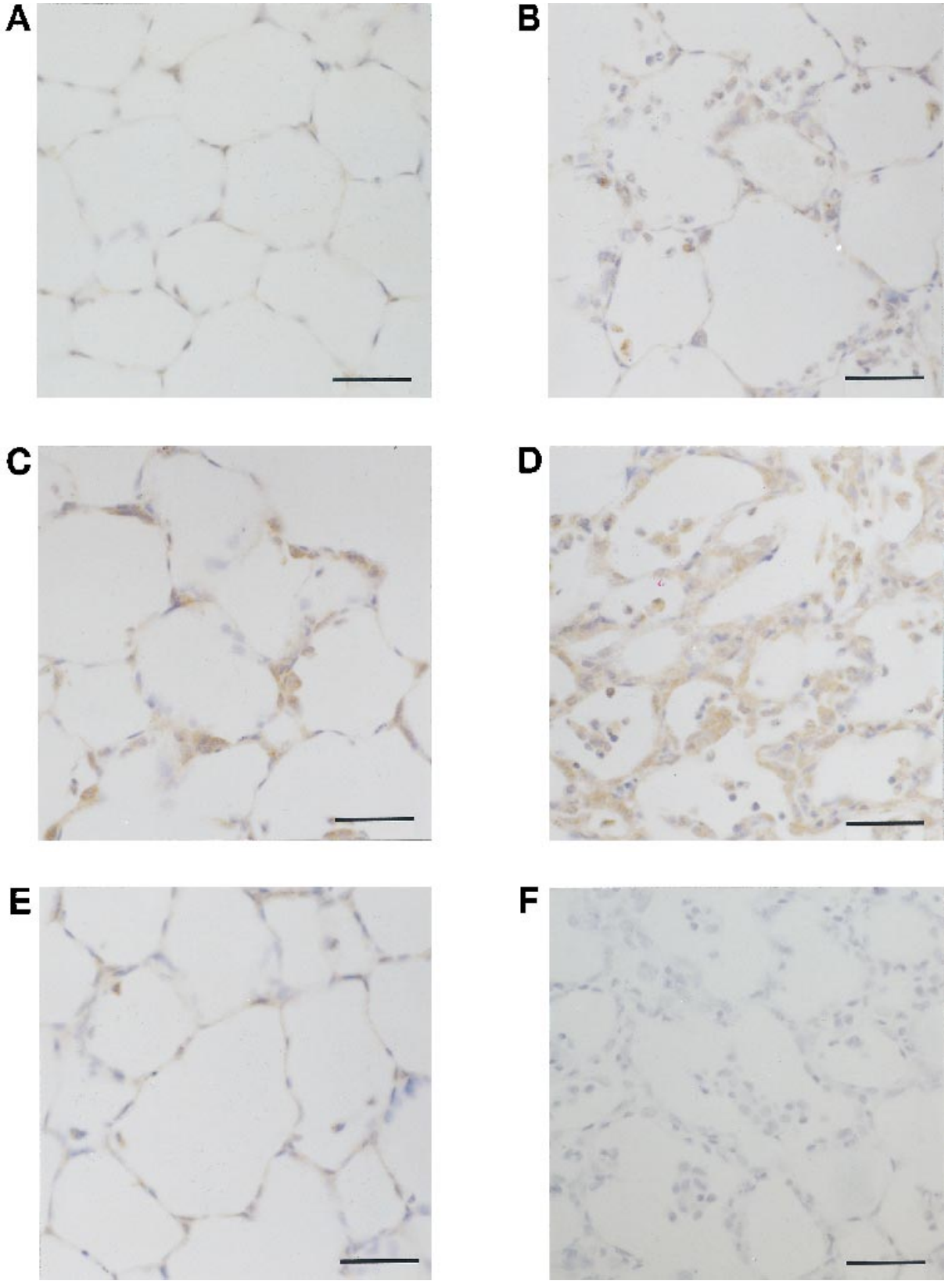

Figure 5. Expression of EGF receptor during the endotoxin-induced hyperplasia. A, The alveolar cells stained weakly for EGF receptor in the lungs of control rats 48 hours after saline instillation. Increased staining was observed B, 24; C, 48 and D, 96 hours post-endotoxin instillation. E, After regression of the hyperplasia, 168 hours post-instillation most of the epithelial cells stained for EGF receptor. F, The reaction of EGF receptor antibody was completely inhibited when the antigen was added to the antibody before the reaction. The polyclonal antibody to EGF receptor was used at 1:3000 dilution. The bars represent 10 μm.

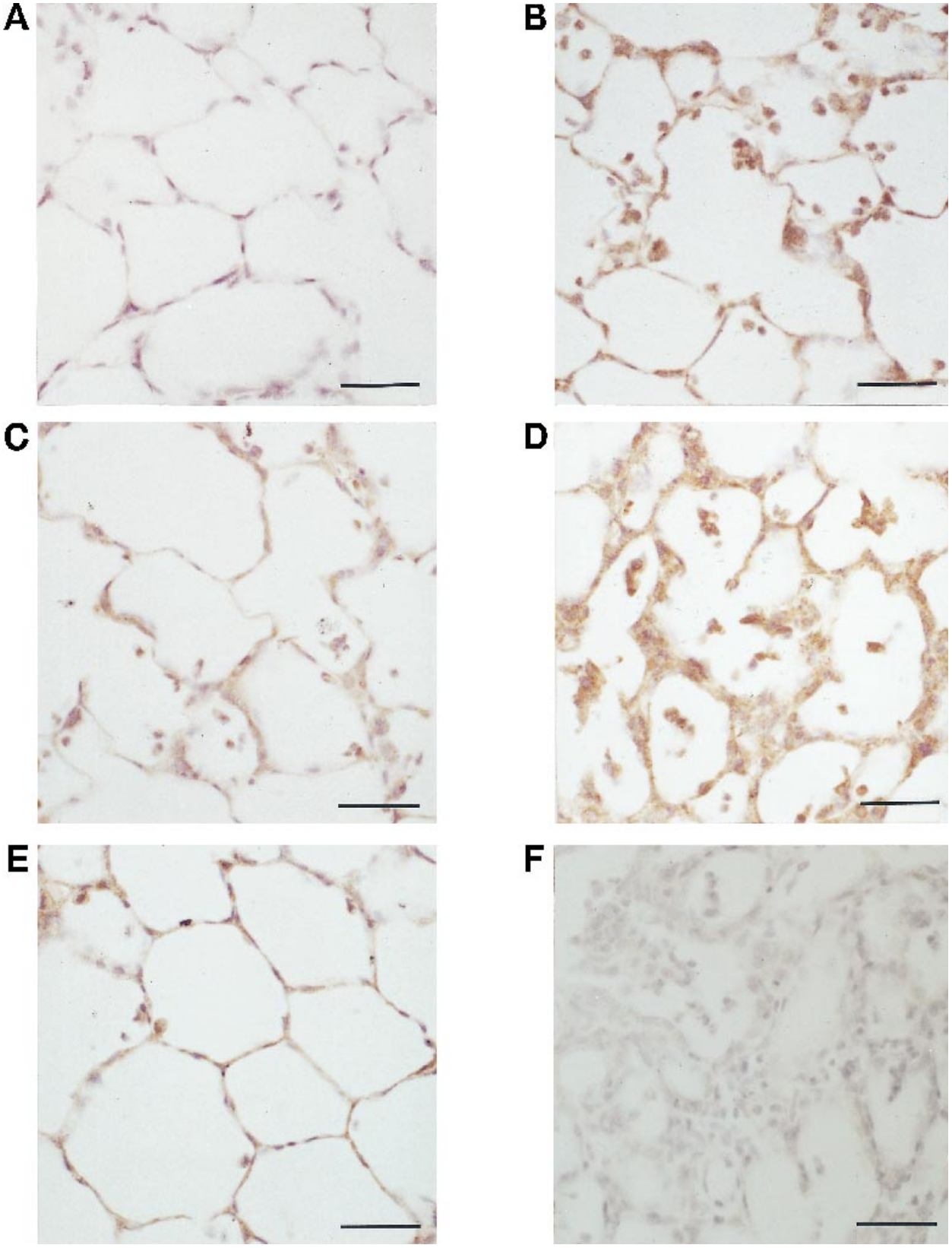

Figure 6. Expression of erbB-2 during endotoxin-induced epithelial cell hyperplasia. A, The alveolar cells stained weakly for erbB-2 in the lungs of control rats 48 hours after saline instillation. Increased staining was observed B, 24; C, 48 and D, 96 hours post–endotoxin instillation. E, After regression of the hyperplasia, 168 hours post-instillation most of the epithelial cells stained for erbB-2 protein. F, The reaction of the erbB-2 antibody was nearly eliminated when the antigen was added to the antibody before the reaction. The polyclonal antibody to erbB-2 was used at 1:1000 dilution. The bars represent 10 μm.

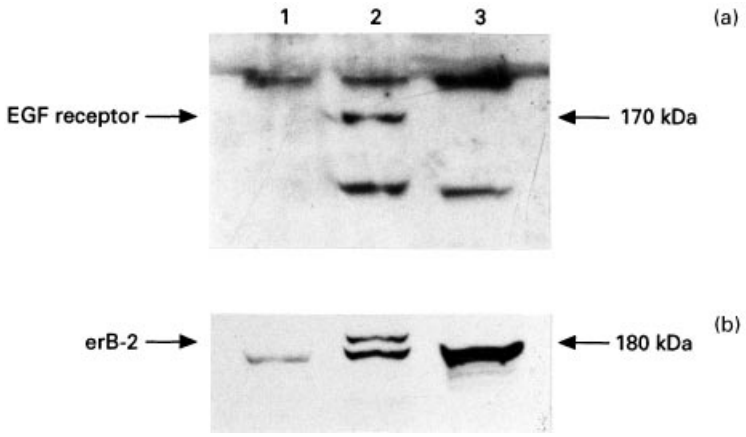

Figure 7. Western blot analysis of proteins extracted from type II cells from control (lane 1) and endotoxin-treated rats (lane 2), and alveolar lavaged cells from endotoxin-instilled rats (lane 3). Proteins that reacted to a, the EGF receptor antibody and b, the erbB-2 antibody are shown. Antibodies were diluted 1:1000.

A strong influx of PMN into the alveolar space was induced by the instilled endotoxin prior to the type II cell hyperplasia. Influx of PMNs due to endotoxin has also been documented in other species including domestic (Brigham & Meyrick 1986) and laboratory animals (Stolk *et al.* 1992; Hudson *et al.* 1977). Results similar to ours were reported by Lopez and Yong (1986) who showed that the number of the bronchoalveolar cell counts in the lavage fluid of rats continued to increase significantly 7 days post-instillation. In the present study, only the numbers of macrophages in the alveolar space were elevated to 168 hours after instillation (Figure 1b).

Inflammatory cells whose migration into the alveolar spaces was induced by endotoxin (Figure 1) may have caused the endotoxin-induced type II cell hyperplasia (Figure 2). Although severe depletion of circulating neutrophils does not completely prevent the response of the lung to endotoxaemia (Brigham & Meyrick 1986), it is difficult to exclude the possibility that neutrophils are involved in initiating the type II cell hyperplasia. Neutrophils release growth-enhancing agents that could act directly as mitogens or cause injury which necessitates cell renewal. Because neutrophils exposed to endotoxin may behave differently, it is difficult to show *in vivo* their role in the pathogenic sequence triggered by endotoxin.

In spite of the massive influx of leucocytes into the bronchoalveolar space after a single dose of lipopolysaccharide, cell injury could not be detected by use of cytosolic enzymes for up to 7 days after the initial PMN exudation (Lopez & Yong 1986). Furthermore, in our studies no damaged type I cells were detected by electron microscopy. Therefore, it is unlikely that the type II cells proliferate to replace damaged alveolar type I cells, a phenomenon reported in studies using oxidant gases such as NO_2 (Evans *et al.* 1975). Shami *et al.* (1986) have observed proliferation of terminal bronchiolar and type II alveolar epithelial cells in the lung following migration of inflammatory cells after instillation of carbon particles in mice. Also in their model system, no evidence of type I cell injury was found. Because the magnitude of the proliferative response of type II cells was correlated with that of the influx of inflammatory cells into the alveolar spaces, Shami *et al.* hypothesized that neutrophils and macrophages could stimulate type II cell proliferation. Macrophages are known to release toxic, reactive oxygen species or proteolytic enzymes that can injure epithelial cells (Brigham & Meyrick 1986).

erbB-2 and EGF receptors belong to the same family of transmembrane receptors, and both have intrinsic tyrosine kinase activity (Peles *et al.* 1992). The EGF receptor and c-erbB-2 genes have been amplified and overexpressed in many different human primary tumours including non-small-cell lung cancer. Overexpression of EGF receptor is believed to amplify normal EGF signal transduction (DiFiori *et al.* 1988), and the dysregulation of these genes may be important in human carcinogenesis (DiFiori *et al.* 1988). Therefore, these proteins are not expected to be induced in transient hyperplastic cells. However, both immunohistochemical and Western blot analysis show induction of EGF receptor and $p180^{erbB\text{-}2}$ in endotoxin-induced epithelial cell proliferation.

This staining was partly membrane-specific, as would be expected for $p180^{erbB\text{-}2}$. The increased levels of this receptor during hypertrophy (Figure 5C) and hyperplasia (Figure 5D) suggest that several ligands, including EGF or TGF-α play a role in type II cell proliferation in response to endotoxin. The EGF receptor is a binding site for several members of the ligand families, EGF and TGF-α (Reynolds *et al.* 1981). The increased expression of EGF

receptor and TGF-α in radiation-induced proliferative foci (Gillett *et al.* 1991, 1992) and other tumour models (Tateishi *et al.* 1991; Derynck 1989) may, therefore, be associated with general cell replication mechanisms, instead of having a unique role in neoplastic progression. Many possible ligands for the erbB-2 receptor were found to be synthesized by different rat and human cell lines (Grooteclaes *et al.* 1994). It is conceivable that endotoxin either directly induces synthesis of erbB-2 in the epithelial cells of the lung or stimulates the release of erbB-2-specific ligands through the inflammatory response.

These receptors may have different functions in neoplastic tissue and in transient hyperplasias. One putative ligand for erbB-2, the neu stimulatory factor, was found to retard cell growth and to induce differentiation of mammary tumour cells (Peles *et al.* 1992). Similarly, erbB-2 may be involved in stopping the endotoxin-induced hyperplasia and inducing differentiation of the newly formed hyperplastic cells after being stimulated by a ligand. It was recently reported that cells expressing both EGF receptor and $p180^{erbB\text{-}2}$ increase the tyrosine phosphorylation of both receptors after EGF treatment, which could result in the recruitment and activation of a different subset of SH2 domain-containing proteins than cells expressing only EGF receptors (Carraway & Cantley 1994). It is therefore possible that both receptors are required to induce the differentiation of hyperplastic cells.

In summary, this study demonstrated that a single intratracheal instillation of rats with endotoxin induces a transient proliferation of type II cells and that the proto-oncogenes, EGF receptor and erbB-2, are induced during this hyperplasia. The ability to isolate normal and hyperplastic type II cells should facilitate mechanistic studies on how endotoxin induces the transient type II cell hyperplasia, and the role of EGF receptor and erbB-2 in transient hyperplasias and in the uncontrolled proliferation of type II cells in pulmonary adenocarcinoma.

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