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Acute effects of 4-ipomeanol on experimental lung tumors with bronchiolar or alveolar cell features in Syrian hamsters or C3H/HeNCr mice

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Abstract. 4-Ipomenaol (IPO) has been shown to induce *P*-450-mediated necrosis of Clara cells in experimental animals, and clinical trials were initiated to treat people with bronchioloalveolar cancers with this novel drug. We therefore performed experiments to examine two different animal lung tumor models for acute IPO cytotoxicity: hamster Clara-cell-derived adenocarcinomas and mouse alveolar type II cell tumors. Clara cells serve as stem cells for airway cell renewal and, therefore, tumors derived from Clara cells may likewise differentiate into various bronchiolar cell types, or undergo squamous cell metaplasia. Bronchiolar cell tumors were induced in Syrian hamsters by a single weekly gavage with 6.8 mg *N*-nitrosomethyl-*n*-heptylamine (NMHA)/animal for 35 weeks. NMHA-induced bronchiolar tumors were classified as well-differentiated lepidic bronchioloalveolar carcinomas, acinar adenocarcinoma, adenosquamous carcinoma, and squamous-cell carcinoma. After 35 and 46 experimental weeks, control and carcinogen-treated hamsters were injected once with doses of 40–110 mg IPO/kg i.p. and necropsied 15–48 h later. Solid and papillary tumors with alveolar cell features were induced transplacentally in C3H/HeNCr mice, by treating pregnant animals on gestation day 16 with 0.5 mmol *N*-nitrosoethylurea/kg, i.p. Offspring of control and carcinogen-treated mice were injected at 2–3 months of age with 35 mg or 50 mg IPO/kg i.p. and necropsied either 24–48 h or 5 and 12 days after injection. Light microscopic studies were carried out to assess cytotoxic effects in various tissues in both hamsters and mice; in hamsters, additional ultrastructural studies were performed. When administered to hamsters, IPO induced moderate to severe cytotoxicity in normal and dysplastic bronchiolar lining cells, in most lepidic bronchioloalveolar carcinomas, and in some glandular areas of adenosquamous cell carcinomas. Susceptible cells included normal, anaplastic, and neoplastic nonciliated and some ciliated bronchiolar cells. Undifferentiated and squamous tumor cells were resistant to IPO, as were resident normal alveolar type II cells. However, some adenocarcinomas composed primarily of ciliated and mucous cells also showed no IPO-induced necrosis, indicating a deficiency in appropriate activating enzymes. In the mice, IPO induced bronchiolar cell necrosis and, at the high dose, also severe pulmonary edema. No cytotoxicity was observed in normal or hyperplastic alveolar epithelium, nor in either solid or papillary growth forms of mouse alveolar cell tumors. In conclusion, these experiments show, in original tumor settings of the lung, that it is possible to achieve cell-specific cytotoxic effects based on cellular composition and functional maturity, i.e., toxicity in carcinomas of predominantly nonciliated bronchiolar cells but not in tumors of alveolar type II cell lineage.

Key words: 4-Ipomeanol – Lung cancer – Alveolar cell – Clara cell – Histogenesis

Introduction

The treatment of solid tumors, in particular lung cancers, remains an enigma. After years of testing cancer drugs in inappropriate models for solid tumors, such as transplantable mouse leukemia, new approaches aim at more tumor-specific therapeutic regimens and will require different models (Griswold and Harrison 1991; Double 1992). One new approach currently under way involves the use of 4-ipomeanol (IPO) in clinical trials on patients with bronchioloalveolar adenocarcinomas (Christian et al. 1989). IPO is a 3-substituted furan produced in moldy sweet potatoes, which is metabolically activated by *P*-450 enzymes to a toxic form (Boyd et al. 1975; Boyd 1976, 1977). Although species differences exist with regard to tolerance and cell types involved, all mammals so far examined (rats, mice, hamsters, rabbits, bovines, and dogs) exhibit Clara cell cytotoxicity (Dutcher and Boyd 1979; Devereux et al. 1982; Doster et al. 1983; Smith et al. 1987; Durham et al. 1985; Li and Castleman 1990). At

Abbreviations: IPO, 4-ipomeanol; NMHA, *N*-nitrosomethyl-*n*-heptylamine; APA-FCRDC, Animal Production Area, Frederick Cancer Research and Development Center

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high doses, ciliated cells and pulmonary endothelium may also become necrotic, causing edema and hydrothorax; in calves and mice, IPO may also cause some alveolar type I cell changes (Durham et al. 1985; Li and Castleman 1990). No cytotoxic effects of IPO have been demonstrated in alveolar type II cells of any species tested, including mice and hamsters (Boyd 1977; Devereux et al. 1982; Gram 1989; Durham et al. 1985), although some covalent binding of IPO was demonstrated in isolated alveolar type II cells of rabbits (Devereux et al. 1982). Metabolism of IPO is also detectable in normal human lung tissue as determined by measurement of [^{14}C]IPO covalent binding (McLemore et al. 1990) or by complementary-DNA-expressed human cytochromes *P*-450 (Czerwinski et al. 1991). However, the cytotoxic potential of IPO in humans is not known. Human lung tumors transplanted into the lungs of nude mice were reduced in size and number following treatment with IPO; in vitro, various small-cell and non-small-cell lung cancer cell lines and tumors showed metabolic activation of IPO and cytotoxicity, as assayed by reduced colony-forming abilities (Falzon et al. 1986; McLemore et al. 1988, 1990). However, there are no histological studies on the acute effects of IPO on tumors of human or animal origin.

For the work reported here, two different experimental lung tumors were chosen to examine the acute effects of IPO. In the first experiment hamsters were exposed to *N*-nitrosomethyl-*n*-heptylamine (NMHA), and developed lung tumors of bronchiolar cell lineage (Rehm et al. 1993). These tumors expressed a specific 10-kDa Clara cell protein and were composed of cells with ultrastructural features of Clara cells, such as an abundance of smooth endoplasmic reticulum and nonosmiophilic secretory granules showing ultrastructural immunolabelling for the Clara cell protein (unpublished data). Clara and other mucous cells have been shown to serve as stem cells for airway cell renewal with terminal differentiation into ciliated or mucous cells. Airway cells may also undergo squamous cell metaplasia (Evans et al. 1978; Becci et al. 1978; McDowell and Trump 1983). Likewise, NMHA-induced tumors were composed of cells showing various types of airway cell differentiation, similar to those of the mouse induced by *N*-nitrosobis-(2-chloroethyl)ureas (Rehm et al. 1991). NMHA-induced bronchiolar hamster tumors were classified as well-differentiated lepidic bronchioloalveolar carcinomas, acinar adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma (unpublished data).

In the second experiment, solid and papillary tumors were induced transplacentally in mice with *N*-nitrosoethylurea. These tumors have been found to arise from alveolar walls and to show features of alveolar type II cells, including features exhibited by fetal and intermediate alveolar epithelium (Evans et al. 1975). No specific markers indicating a Clara cell lineage could be demonstrated (Rehm et al. 1988). The different growth patterns and cellular changes represent modifications associated with dedifferentiation, i.e., solid tumors being adenomas, whereas papillary tumors are adenocarcinomas (Foley et al. 1991; Belinsky et al. 1993). Although, some investigators consider papillary tumors to be derived from Clara cells (Kauffman et al. 1979; Kauffman 1981; Thaete and Malkinson 1991; Gunning et al. 1991), such claims could not be confirmed (Ward et al. 1985; Rehm et al. 1991).

Materials and methods

Hamsters. Male Syrian golden hamsters (Cr:RGH) were received from APA-FCRDC (Fredrick, Md.), and housed individually in polycarbonate cages within a barrier-type facility at a room temperature of 20–22° C, a relative humidity of 50±10%, and 12 changes of room air/h. The animals were given Purina autoclavable laboratory chow in pellets and HCl-acidified tap water (pH 2.5) ad libitum. Animal care was provided in accordance with the procedures outlined in the *Guide for the care and use of laboratory animals* (1985). The hamsters were 9 weeks old at the beginning of treatment with 6.8 mg *N*-nitrosomethyl-*n*-heptylamine (NMHA)/animal in 0.2 ml ethyl acetate/corn oil (1 : 2) by gavage once a week for 35 weeks (Lijinsky and Kovatch 1988). Control hamsters were treated with vehicle only. The present study is based on 12 NMHA-treated and 13 control hamsters submitted to single i.p. injections of IPO in corn oil in doses ranging from 40 mg/ml to 110 mg/kg and sacrificed 15–48 h later. IPO was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI. Initially 5 carcinogen-exposed hamsters were treated with IPO at experimental week 35, but, at this time there were insufficient lesions for study. By experimental week 46, however, the incidence of pulmonary lesions had appreciably increased and the remaining animals (7) were treated. When possible, anesthetized animals were perfused through the heart with saline and the lungs removed and inflated with one of several different fixatives, including 10% neutral buffered formalin, Bouin's solution (4–5 h followed by transfer to 70% ethanol), or 2.5% glutaraldehyde in 0.1 M cacodylate buffer (1–2 h, followed by transfer to cacodylate buffer). Grossly visible tumors were stored and processed on an individual basis to enable tracing of specific lesions at the ultrastructural level after light-microscopic examination. In addition to lung and trachea, nasal passages, liver, kidney, and all tissues with grossly visible changes were also fixed and processed for histological examination.

Mice. Male and female C3H/HeNCr MTV mice were obtained from the APA-FCRDC, Frederick, Md., and housed 5/cage under the same conditions as were the hamsters. The mice were free of serum antibodies to *Mycoplasma* sp. and to all known murine viruses, including pneumotropic agents such as Sendai virus and pneumonia virus of mice. At the age of 8 weeks, the mice were mated and the females checked every morning for vaginal plugs to determine the first day of gestation (day 1). Lung tumors in mice were induced transplacentally with *N*-nitrosoethylurea as reported previously (Rehm et al. 1988). On gestation day 16, the pregnant females were injected i.p. with 0.5 mmol *N*-nitrosoethylurea/kg (Sigma, St. Louis, Mo.) dissolved in trioctanoin (Eastman Kodak, Rochester, N.Y.). Pregnant control mice were treated with trioctanoin only. The present investigation is based on the study groups of 19 control mice and 24 mice exposed transplacentally to *N*-nitrosoethylurea, all of which were subjected to a single IPO injection in the dose range of 35–50 mg/kg at the age of 2–3 months. In addition, 2 controls and 6 mice with *N*-nitrosoethylurea-induced lung tumors were not treated with IPO for comparative purposes. None of the mice died from IPO treatment. Most mice were necropsied 24–48 h after treatment, and 8 were sacrificed at later times 5 or 12 days following IPO exposure. Lungs, nasal passages, kidneys, adrenal glands, liver, pancreas, and salivary glands were placed in formalin or Bouin's fixative and embedded in paraffin.

Microscopic procedures. Light-microscopic studies were carried out on frozen sections stained with oil red O and on paraffin sections subjected to various stains, including hematoxylin and eosin (H&E) and alcian blue/periodic acid Schiff's reagent, with prior diastase digestion, to demonstrate mucopolysaccharides. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex procedure with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.). To detect hamster Clara cell antigen (CCA), rabbit anti-(hamster CCA)antiserum was used at a dilution of 1 : 7000 (Singh et al. 1985; Strum et al. 1990). Trypsinization was necessary on formalin-fixed material to detect Clara cell antigen (30–40 min at 37° C of 0.02% type II crude trypsin from porcine pancreas; Sigma Chemical Co., St. Louis, Mo.).

For electron microscopy, hamster lung tumors were postfixed in 2.5% glutaraldehyde and 1% osmium tetroxide for 1 h, stained with

Table 1. 4-Ipomeanol (IPO) toxicity in control Syrian hamsters [a]

Dose (mg/kg)	*n*	Adrenal glands	Bronchioles	Trachea	Nasal passages	Liver	Glandular stomach
40	2	+++ [b]	+	–	–	–	–
50	1	+++	+	–	–	–	–
60	3	++	+	+	–	–	–
70	1	++	++	+	–	–	–
80	1	+++	++	+	+	–	–
90	1	+++	++	+	++	+	+
100	2	+++	++	+	++/+++	+	++
110	2	+++	++	+	+++	++	+

[a] Hamsters were injected i.p. with IPO and necropsied when found dead, moribund, or no later than 24–48 h after treatment
[b] Degree of toxicity: –, none; +, mild; ++, moderate; +++, severe

Table 2. IPO toxicity in *N*-nitrosomethyl-*n*-heptylamine(NMHA)-treated Syrian hamsters [a]

IPO dose (mg/kg)	*n*	Adrenal glands	Bronchioles	Trachea	Nasal passages	Liver	Glandular stomach
60	1	– [b]	+	–	–	–	–
70	2	–/++	–/+	–/+	–	–	–
80	2	+++	+	–/+	+	–	–
110	7	++/+++	+/++	+	++/+++	+/+++	–/++

[a] Nine-week-old hamsters received 35 weekly doses of 6.8 mg NMHA/animal by gavage. Hamsters were injected i.p. with IPO and necropsied when found dead, or no later than 20–30 h after treatment
[b] Degree of toxicity: –, none; +, mild; ++, moderate; +++; severe

0.5% uranyl acetate and embedded in epoxy resin. Semithin sections were stained with toluidine blue and thin sections with uranyl acetate/lead citrate.

Results

Hamsters

Doses of up to 80 mg IPO/kg were generally well tolerated. The first death of a control hamster occurred after 15 h at 100 mg IPO/kg and two NMHA-treated hamsters died 20 h following exposure to 110 mg IPO/kg. At the dose of 110 mg IPO/kg all animals clearly showed signs of morbidity and were sacrificed 24–30 h after IPO treatment.

Table 3. Toxicity of IPO [a] on pulmonary tumors of hamsters induced by NMHA [b]

Parameter	Bronchiolo-alveolar carcinoma				Adenosquamous carcinoma			Adeno-carcinoma [c]	
Degree of toxicity [d]	–	+	++	+++	–	+	++	–	+
Number of tumors	0	1	1	2	8	6	2	20	4

[a] Ten hamsters with lung tumors received 70–110 mg IPO/kg, i.p.
[b] Nine-week-old hamsters received 35 weekly doses of 6.8 mg NMHA/animal
[c] Includes acinar as well as papillary growth patterns
[d] Degree of toxicity: –, none; +, mild; ++, moderate; +++, severe

Light microscopy of the lung

Dose-dependent cytotoxic effects of IPO occurred in various organs (Tables 1 and 2). Necrosis of pulmonary cells of control animals was restricted to cells lining airways and did not involve alveolar, endothelial, or other cell types present in the lung. Since IPO cytotoxicity in the respiratory tract was found to be dose-related, the highest IPO dose of 110 mg/kg was chosen to treat hamsters with pulmonary tumors for the maximal possible response in tumors.

Tumors responding with mild to severe cytotoxicity were those classified as bronchioloalveolar adenocarcinoma and adenosquamous carcinomas (Table 3; Fig. 1 A, B). Bronchioloalveolar tumors were composed of anaplastic bronchiolar cells that had grown out from terminal airways to populate alveolar walls (Rehm et al. 1993). By light microscopy, severe necrosis of neoplastic bronchiolar cells was characterized by nuclear pyknosis or karyorrhexis, cytoplasmic vacuolization, and sloughing of cells from the basement membranes of alveolar walls (Fig. 1 A, B). Occasionally, mild hemorrhages were also observed. Affected cells included nonciliated and ciliated cells, but not alveolar type II cells. Necrotic debris accumulated in alveolar spaces, leaving the original alveolar structure intact. The basement membranes were denuded or lined either by resident, trapped alveolar type II cells, or cells the exact nature of which could not be identified. In adenosquamous tumors, only glandular portions exhibited IPO-induced cellular necrosis (Fig. 1 B), showing lumens filled with sloughed necrotic cells. However, a basal acinar layer remained and squamous cells were not affected; scattered degenerating squamous cells, as well as other degenerating tumor cells, were present in tumors of hamsters whether or not they had received IPO treatment.

Degenerating Clara cells usually lose immunoreactivity for the Clara cell antigen rapidly within a few hours. For this reason, it was difficult to identify degenerating nonciliated cells as Clara cells by immunomorphological methods. On the other hand, some hamsters treated with IPO exhibited only mild cytotoxic effects, although bronchioles and proliferative lesions were composed of cells strongly immunoreactive

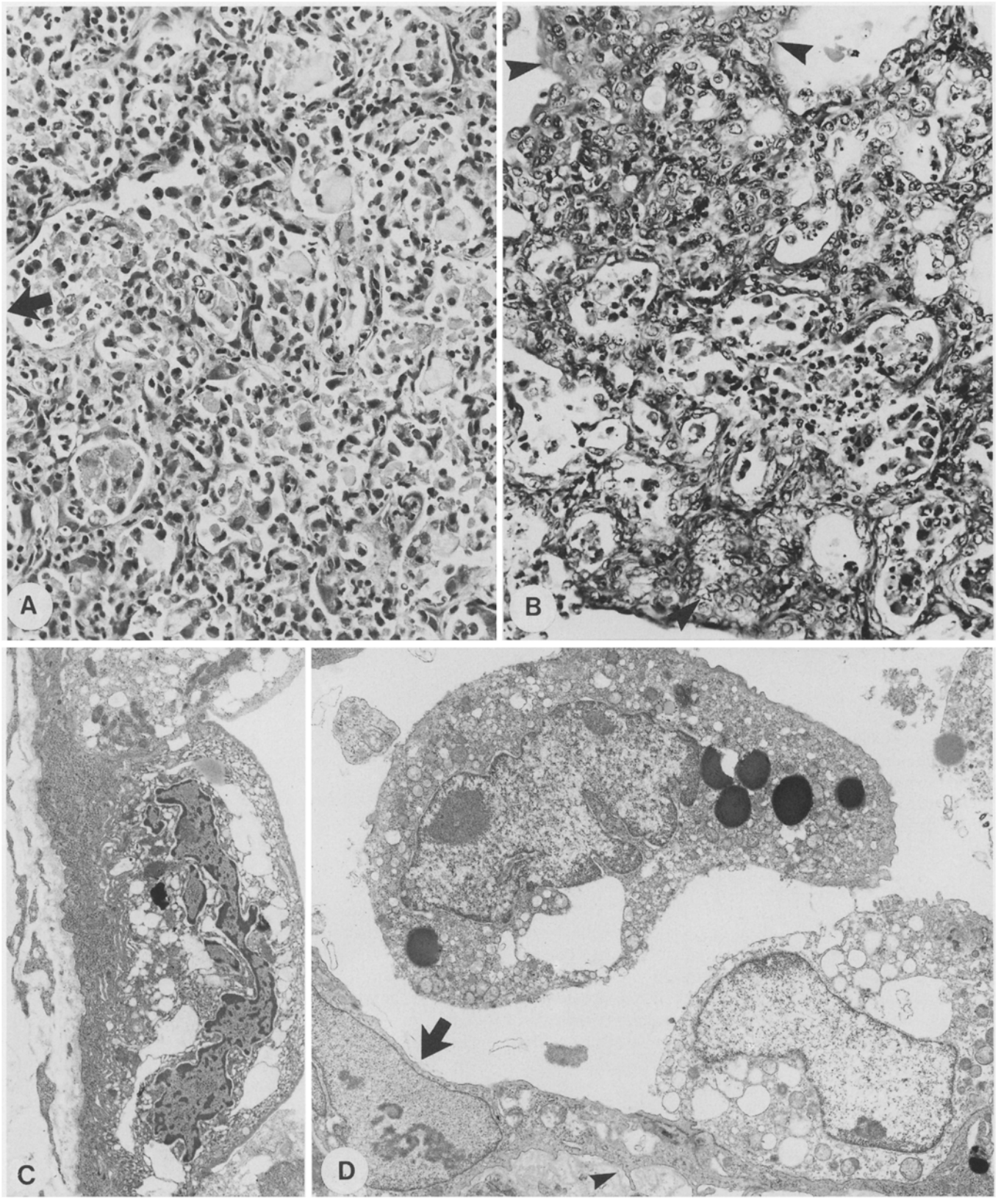

Fig. 1. A Severe necrosis of bronchioloalveolar carcinoma of hamster 24 h after treatment with 110 mg 4-ipomeanol (IPO)/kg. Cells are sloughed from original alveolar walls; *arrow* indicates denuded basement membranes (H&E, × 250). **B** Moderate cellular necrosis of glandular portion in adenosquamous cell carcinoma of hamster 24 h after treatment with 80 mg IPO/kg. Note intact residual cells lining acinar structures and unaffected squamoid cells (*arrowheads*) (H&E, × 250). **C** Ultrastructure of dysplastic nonciliated cell of *N*-nitrosomethyl-*n*-heptylamine-treated hamster lining bronchiole 24 h after treatment with 110 mg IPO/kg. Note highly irregular nuclear shape and vesicular appearance of cytoplasm due to dilatation of endoplasmic reticulum (uranyl acetate/lead citrate, × 12 600). **D** Ultrastructure of bronchioloalveolar carcinoma of hamster 24 h after treatment with 110 mg IPO/kg. Two nonciliated degenerating cells, one sloughed into the lumen, with vesicular endoplasmic reticulum and dense lipid droplets. Specific secretory Clara cell granules are no longer present. *Arrow* indicates cell with scant cytoplasm and no signs of degeneration; *arrowhead,* basement membrane (uranyl acetate/lead citrate, × 11 600)

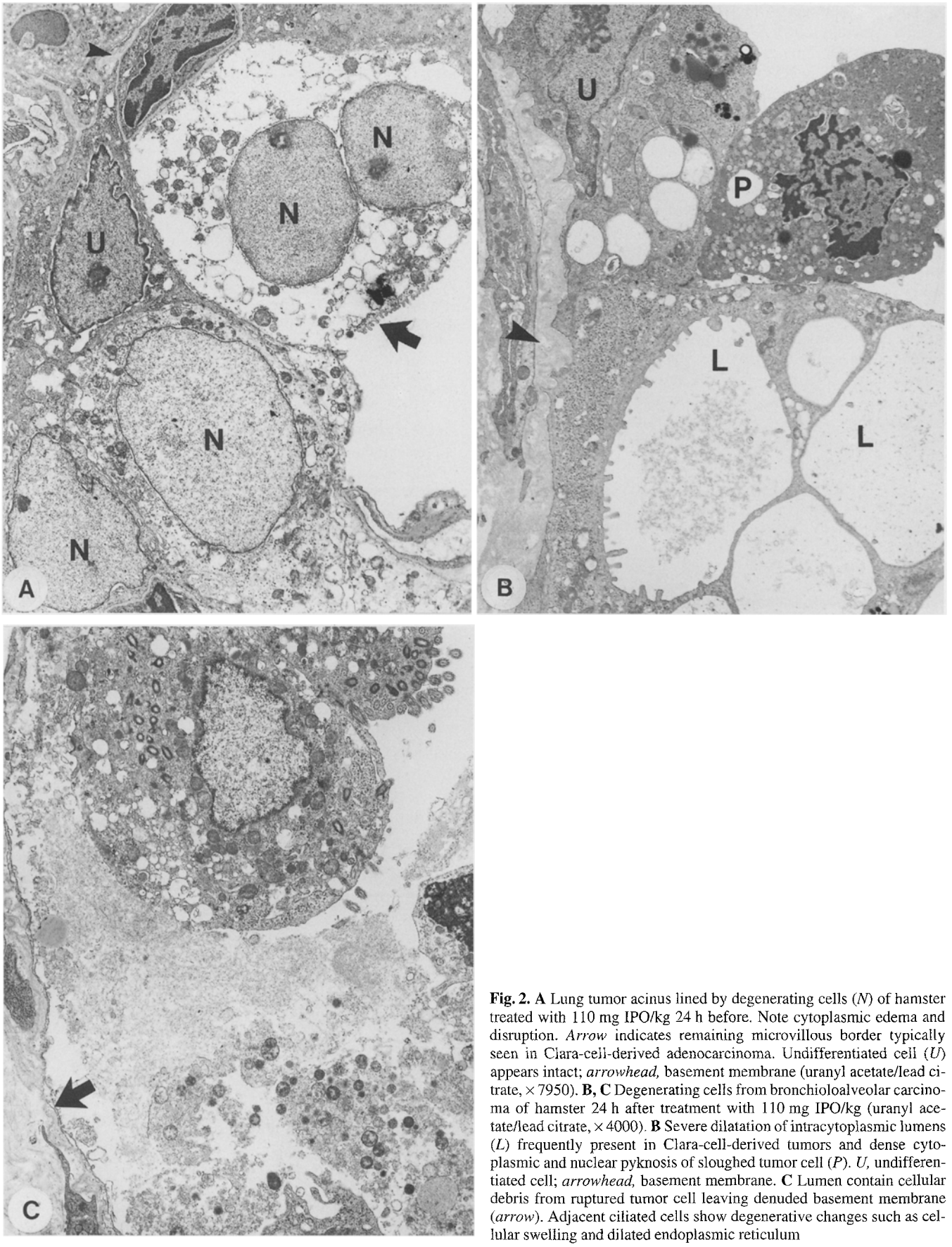

Fig. 2. A Lung tumor acinus lined by degenerating cells (*N*) of hamster treated with 110 mg IPO/kg 24 h before. Note cytoplasmic edema and disruption. *Arrow* indicates remaining microvillous border typically seen in Clara-cell-derived adenocarcinoma. Undifferentiated cell (*U*) appears intact; *arrowhead,* basement membrane (uranyl acetate/lead citrate, × 7950). **B, C** Degenerating cells from bronchioloalveolar carcinoma of hamster 24 h after treatment with 110 mg IPO/kg (uranyl acetate/lead citrate, × 4000). **B** Severe dilatation of intracytoplasmic lumens (*L*) frequently present in Clara-cell-derived tumors and dense cytoplasmic and nuclear pyknosis of sloughed tumor cell (*P*). *U,* undifferentiated cell; *arrowhead,* basement membrane. **C** Lumen contain cellular debris from ruptured tumor cell leaving denuded basement membrane (*arrow*). Adjacent ciliated cells show degenerative changes such as cellular swelling and dilated endoplasmic reticulum

for Clara cell antigen. Other types of adenocarcinomas that usually were not affected by IPO toxicity included ones having acinar or papillary growth patterns.

Electron microscopy of the lung

At the ultrastructural level, dysplatic cells lining bronchioles (Fig. 1 C) and neoplastic nonciliated bronchiolar cells (Fig. 1 D, Fig. 2 A, B) susceptible to IPO toxicity exhibited dilated smooth endoplasmic reticulum, cytoplasmic swelling or increased density, and nuclear pyknosis or loss of heterochromatin. Electron-dense round globules of degenerating cells (Fig. 1 D) represented mostly lipids, as demonstrated by oil red O stain. Intracytoplasmic lumens, which are typical features of tumors of bronchiolar cell lineage, were grossly dilated, compressing the remaining cytoplasm (Fig. 2 C). Most degenerating cells appeared to be nonciliated; however, some ciliated cells were also affected (Fig. 3). Resistant tumor cells, i.e., those showing no obvious degenerative changes (Figs. 1 D, 2 A, B), were cells that possessed little cytoplasm and no specific organelles. The surviving cell in Fig. 2 D is similar to those seen during early squamous differentiation (unpublished data). Normal alveolar type II cells with lamellar bodies and neoplastic squamous cells, which were identified by the formation of tonofilaments, did not develop signs of cytotoxicity.

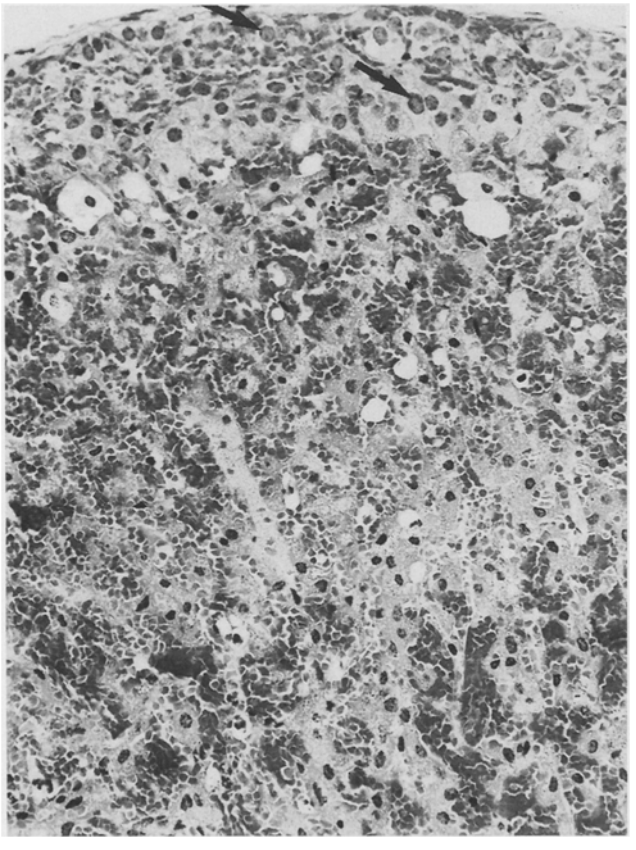

Fig. 3. Adrenal cortical hemorrhage and necrosis in zona fascicularis of hamster treated with 100 mg IPO/kg. Cells of zona glomerulosa are still viable (*arrows*) (H&E, × 250)

Extrapulmonary cytotoxicity

Cells lining the upper respiratory tract (bronchi, trachea, nasal passages) were also affected and, at high doses, the most severe necrosis occurred in the olfactory epithelium and Bowman's gland. Also at higher doses, coagulative necrosis was present in the liver and ulcers were found in the glandular portion of the stomach (Tables 1, 2). Most sensitive were the adrenals, developing severe hemorrhage and necrosis of the cortex even at the lowest dose of 40 mg IPO/kg. Macroscopically, adrenals were increased up to three times their normal size and appeared dark brown to black. Histological evaluation showed diffuse hemorrhage of the cortex with degenerating or necrotic cells of the zona reticularis and fascicularis (Fig. 3). The cells of the zona glomerulosa and the medulla were usually unaffected.

Mice

At the highest IPO dose of 50 mg/kg, mice developed moderate to severe pulmonary edema and hydrothorax. No instance of IPO cytotoxicity was found in normal alveolar type II cells even when associated with severe edema (Fig. 4 A). Likewise, both alveolar type II cell hyperplasia, and solid and papillary tumors were resistant to the cytotoxic effects of IPO (Table 4; Fig. 4 A–C). Bronchiolar cell necrosis (Fig. 4 B, D) was most apparent 24 h after IPO injection and more extensive at 50 mg/kg than at 35 mg/kg. Cells regenerated rapidly; 5 or 12 days after IPO treatment bronchioles were lined by mildly hyperplastic, basophilic epithelium. Other lesions induced by IPO included severe renal cortical necrosis in males, necrosis of glandular duct epithelium in the nasal cavity in females, and occasional cellular degeneration of nonciliated respiratory cells of the trachea and nasal passages in both sexes.

Discussion

The A/J mouse lung tumor has most recently been suggested as a model for the development of chemointervention strategies (Belinsky et al. 1993). On the other hand, several transplantation models of orthotopic human lung cancers have

Table 4. Numbers of C3H mice and alveolar lung tumors [a] tested for cytotoxic response to IPO [b]

Dose (mg/kg)	Number of mice with tumors	Alveolar type II cell tumors		Number of mice without tumors [c]
		Solid	Papillary	
0	6	4	21	2
35	10 [d]	7	46	10 [d]
40	3	3	11	3
50	5	6	22	4

[a] Lung tumors were induced transplacentally on day 16 of gestation by i.p. injection of mothers with 0.5 mmol *N*-nitrosoethylurea/kg

[b] Most mice were examined at 2–3 months, 24–48 h following i.p. injection of IPO

[c] Offspring of mothers treated with vehicle only

[d] Two mice each were examined 5 or 12 days after IPO treatment

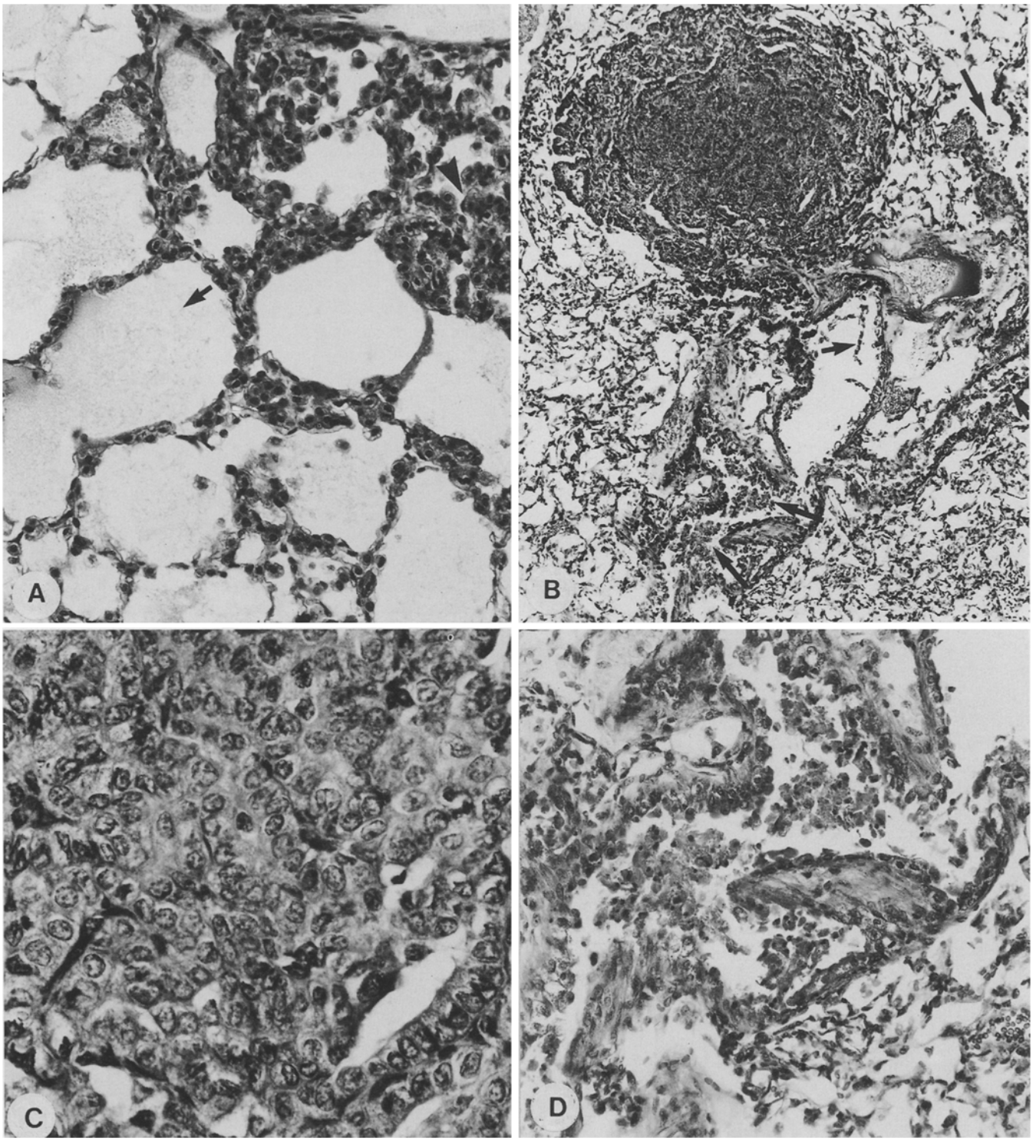

Fig. 4 A–D. Lungs from mice 24 h after treatment with 50 mg IPO/kg. **A** Alveoli distended by proteinaceous edema fluid (*arrow*). Absence of cytotoxicity of normal and hyperplastic, round alveolar type II cells lining alveoli at edge of solid tumor (*arrowhead*). **B** Intact papillary tumor shown at higher magnification in **C.** Only bronchiolar cells are affected by degeneration and necrosis sloughing into lumen of airway (*arrows*) shown at higher magnification in **D** (H&E, **A** × 250; **B** × 100; **C** × 630; **D** × 250)

been described for responses to chemotherapy (Howard et al. 1991; Kal et al. 1991; Zeligman et al. 1992). Advantages of the transplantation models include the use of human tumor tissue and comparatively rapid answers resulting from a reduced latency period. However, in the course of passaging tumors in tissue culture or animals, clonal selection and loss of characteristic features may occur, limiting the usefulness of the model. We therefore decided to examine two different indigenous lung tumor models to test acute cytotoxic responses to IPO, a lung cancer drug currently undergoing clinical trials on patients with bronchioloalveolar cancers (Christian et al. 1989). Such neoplasms may consist of neoplastic

alveolar type II and/or Clara cells, since the human lung tumor classification system does not consider the histogenetic origin of non-small-cell lung cancers (WHO 1981; Singh et al. 1984).

The cytotoxicity of IPO depends on the amount of alkylating intermediates produced by biotransformation, which, in turn, relates to the quantity or efficacy of specific *P*-450 forms in IPO metabolization (Dutcher and Boyd 1979; Larsson and Tjälve 1988; Gram 1989). There are species differences among experimental animals in types of *P*-450, which may account for species differences in response to IPO (Plopper et al. 1992), and similar variations may exist amoung humans in normal and neoplastic tissue (Czerwinski et al. 1991). Pulmonary epithelial cells susceptible to IPO cytotoxicity include Clara and ciliated cells, and, particularly in the bovine, alveolar type I cells. Clara cell toxicity is directly related to the intracellular metabolism of IPO, whereas degenerative changes of alveolar type I and ciliated cells may be attributed secondarily to activated IPO released from necrotic endothelial or Clara cells respectively (Li and Castleman 1990). IPO binding without resultant toxicity has been demonstrated in isolated alveolar type II cells of rabbits (Devereux et al. 1982). Detailed ultrastructural examinations in mice have found no indication of damage of alveolar type I or type II cells (Durham et al. 1985). Autoradiograms of tritium-labelled 4-ipomeanol-treated lungs from rats, Syrian hamsters, and mice showed almost exclusive localization of radioactive granules over Clara cells and little or no labelling of the alveolar parenchyma (Boyd 1977; Gram 1989). Since the extent of IPO-induced cytotoxicity is dose-related (Boyd 1977), the highest possible dose was chosen to treat hamsters and mice in the current experiments. In agreement with others, most mice developed dyspnea at 50 mg/kg, but survived (Boyd 1980). Severe pulmonary edema was evident only in mice indicating the presence of specific *P*-450 enzymes not found in hamster lung endothelium. We could not confirm 140 mg/IPO as the LD_{50} noted in hamsters by others (Dutcher and Boyd 1979). In the current study, 100–110 mg/kg was associated with three early deaths, and at 110 mg IPO/kg all hamsters showed clear signs of morbidity after 20–24 h.

Although the hamsters are resistant to the early fatal effects of IPO on the pulmonary vasculature, they ultimately die from hepatotoxicity (Dutcher and Boyd 1979). Liver necrosis may also be induced in rats by IPO if animals are pretreated with 3-methylcholanthrene, which increases hepatic cytochrome *P*-450 concentrations and the subsequent metabolism of IPO (Statham and Boyd 1982). Hepatocellular toxicity in the hamster, however, is most likely due not to increased metabolism but to the depletion of glutathione. Similarly, an enhanced pulmonary toxicity was seen in rats following experimental depletion of glutathione by pretreatment of rats with diethylmaleate (Boyd et al. 1982).

Upon exposure to IPO, few bronchiolar-cell-derived hamster lung tumors exhibited severe necrosis, but the majority of the hamster lung tumors showed little or no acute cytotoxic response. Primarily affected were well-differentiated lepidic bronchioloalveolar tumors consisting of Clara, mucous, ciliated, and undifferentiated cells growing along basement membranes of alveolar walls. Also affected by IPO were glandular portions of adenosquamous carcinomas. However, even in responsive tumors, undifferentiated cells remained unaffected by IPO. These cells either represented a most recent cell population that had not yet matured or were dedifferentiated tumor cells with little smooth endoplasmic reticulum and *P*-450 enzymes with the capacity to metabolize IPO. For the same reasons, no or very little cytotoxicity was found in individual squamous cells and squamous cell carcinomas, in less well-differentiated adenocarcinomas with an acinar pattern, or in tumor areas composed of large numbers of ciliated cells. In some NMHA-exposed hamsters, cells immunoreactive for the Clara cell antigen did not respond with cellular necrosis following IPO treatment, which may be indicative of loss of fully functional *P*-450 species in dysplastic or neoplastic Clara cells. Increased resistance may also be conferred by increased levels of glutathione in anaplastic of neoplastic cells; however, such a pathway seems unlikely.

As shown by others, IPO treatment in the present study did not affect normal alveolar type II cells of the hamster or mouse, nor did solid alveolar or papillary mouse lung tumors exhibit cytotoxicity at any dose. Mice received IPO at 2–3 months, well before papillary tumors become large, dedifferentiated, and invasive. In the same mice, however, Clara cells clearly demonstrated cellular necrosis in response to IPO treatment. These results are in agreement with our previous observations that both solid tumors, made up of round cells, and papillary tumors, composed of cuboidal to columnar cells, exhibit features specific for alveolar type II cell lineage, rather than expressing markers specific for Clara or other bronchiolar cells. Treatment with Clara-cell-specific toxins may, therefore, be included as a functional marker for bronchiolar cell lineage. As with many other such markers, whether morphological or biochemical, it has to be kept in mind that they may be fully expressed in well-differentiated lesions and frequently lost in the malignant tumor type. However, in most cases, the loss of a marker occurs progressively, rather than abruptly, and it is possible to associate a marker with a certain phenotype if sufficient tumor numbers are studied at different times during tumor progression. None of the 100 papillary mouse lung tumors examined in this investigation exhibited any indication of cellular toxicity due to IPO. The most likely explanation is that *N*-nitrosoethylurea-induced mouse lung tumors show an inability, similar to normal alveolar cells, to produce the toxic metabolite(s) of IPO. This metabolic incompetence is probably due to a deficiency of appropriate *P*-450 enzymes.

In a recent study by Forkert et al. (1992), other compounds that also cause specific Clara cell cytotoxicity were likewise ineffective at inducing necrosis of solid or papillary mouse lung tumors. In this case, however, lack of toxicity was interpreted as complete loss of metabolizing capacity of Clara-cell-derived tumors.

Studies on cell lines from human lung tumors showed that metabolic activation of IPO was greater and colony formation was reduced in a cell line exhibiting Clara cell morphology when compared to a cell line with alveolar type II cell morphology (Falzon et al. 1986). When the Clara cell line was transplanted intrabronchially to nude mice, those mice receiving subsequent IPO treatment survived longer and there was a decrease in the size and number of lung tumors

(McLemore et al. 1988). Although of different cellular origin, both tumors were classified as bronchioloalveolar carcinoma (WHO 1981). This emphasizes the need for a correct histogenetic classification as suggested by McDowell and Trump (1983) if cell-specific treatments are to be effective.

In normal human lung tissue, in human lung cancer cell lines, and in primary human lung cancers of different histological types (bronchioloalveolar carcinoma, adenocarcinoma, squamous-cell carcinoma, small- and large-cell cancers), wide variations in metabolic activation of IPO were demonstrated by [^{14}C]IPO covalent binding (McLemore et al. 1990). Although most of the experimental animal data indicated a correlation between the extent of cellular binding of IPO and its cytotoxicity, tissues may, nevertheless, show no toxicity despite IPO binding (Boyd et al. 1975; Larsson and Tjälve 1988). The lack of toxicity may be attributed to nontoxigenic metabolism of IPO or the presence of adequate detoxification systems (Statham and Boyd 1982; Boyd et al. 1982). Therefore, binding studies alone may not be sufficient to predict IPO cytotoxicity. From the study by McLemore et al. (1990), squamous and small-cell carcinomas indicated in vitro IPO metabolic activity. In vitro studies performed by Fiebig et al. (1990) showed a response of only small-cell cancers to IPO. In vivo experiments of the same tumors, however, showed no growth inhibition by IPO treatment. In the present hamster experiment, squamous cells developed no significant toxicity. Although no small-cell lung cancers were observed, cytotoxicity of such tumors in the hamster would not be expected, since neither normal or hyperplastic neuroepithelial bodies, composed of well-differentiated neuroendocrine cells (Rehm et al. 1993), revealed a cytotoxic effect at any dose of IPO.

Adrenal toxicity has not been previously ascribed to IPO. Adrenal cortical epithelial toxicity, without hemorrhage, has been reported for various other natural and/or synthetic compounds (Hallberg 1990). In cases of hemorrhagic necrosis, including our IPO-treated hamsters, the initial insult probably occurs within the cortical sinusoidal endothelium, which is followed by secondary ischemic epithelial necrosis of the zona fascicularis and reticularis. Cells of the zona glomerulosa were less severely affected, suggesting an intact capsular blood circulation.

Hamsters in the present study also manifested cytotoxicity of the respiratory and olfactory epithelium of the nasal passages and concomitant toxicity in the glandular tumors arising in these areas (data not shown). Not only should patients currently enrolled in trials with IPO, therefore, be monitored for possible pulmonary, hepatic, and renal side-effects (Christian et al. 1989), but clinicians should also be aware of possible effects on the adrenal cortex and nasal passages.

In summary, the current experiments are the first to analyze lung tumors for a cell-specific cytotoxic effect in their original location, unaltered by experimental manipulations such as transplantation and culture conditions. The results suggest (a) the necessity for correct histogenetic lung tumor classification if cell-specific treatments are to be used, (b) that Clara-cell-derived neoplasms may retain *P*-450 cytochromes capable of metabolizing IPO to toxic forms, and (c) that, in the course of tumor progression, desired cell-targeted toxicity may be lost or reduced as a result of changes in cellular metabolic capabilities.

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