Inhibition of Mouse Lung Tumor Development by Hyperoxia

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ABSTRACT

The hypothesis was tested that continuous hyperoxia would enhance the development of lung tumors in mice. In strain A/J mice treated with a single dose of urethan (1000 mg/kg) and exposed to 70% O2 for 16 wk, an average of 5 tumors per lung developed, whereas in animals kept in air, an average of 20 tumors per lung was found. When the animals were returned to air after oxygen exposure, it was found that a difference of 15 tumors per lung between the two groups persisted up to 1 yr later, indicating that O2 was tumoricidal. The shortest duration of O2 exposure to be effective was 4 wk, and delay of O2 exposure up to 12 wk after urethan still was effective in reducing the number of developing tumors. Histopathology showed that continued exposure to 70% O2 produced some hyperplasia of the bronchiolar epithelium and only very discrete changes in the pulmonary parenchyma. Analysis of cell proliferation patterns with a continuous [3H]thymidine labeling technique showed a persistent high cell labeling in the bronchiolar epithelium and a temporary increase in alveolar wall cell labeling. Chronic hyperoxia failed to alter the activities of pulmonary superoxide dismutase or glucose-6-phosphate dehydrogenase. Ornithine decarboxylase, on the other hand, was increased as long as the animals remained exposed to oxygen. It was concluded that hyperoxia kills developing tumor cells in mouse lung.

INTRODUCTION

Reactive molecular species of oxygen may be involved in tumor promotion. The bulk of evidence supporting this hypothesis was obtained in experiments examining the effects of free-radical generating chemicals or of free-radical scavengers on mouse skin or suitable in vitro cell systems (1-3). Additional support for the oxygen free-radical hypothesis was obtained in early experiments which studied the influence of oxygen on lung tumor development in strain A mice (4-6). Exposure of strain A mice for 2 days to elevated concentrations of O2 (70-100%) immediately after injection of urethan or dibenz(a, h)anthracene significantly increased the average number of lung tumors found 3-4 mo later. Hyperoxia also increased the activity of pulmonary ornithine decarboxylase (7), an event commonly associated with tumor promotion.

Some experimental data, however, are not in agreement with the oxygen free-radical hypothesis of tumor promotion. Repeated exposure to 100% oxygen of Swiss-Webster mice treated with urethan is without any apparent effect on lung tumor development (8). And, although the free-radical scavenger BHT inhibits tumor promotion in mouse skin (9), it is a most effective tumor-enhancing agent in mouse lung (10), mouse colon (11), mouse liver (11), rat bladder (12), rat thyroid (13), and rat liver (14) and has effects similar to the classical tumor-promoting agent 12-O-tetradecanoylphorbol-13-acetate in two different in vitro systems (15, 16).

We decided to examine the effect of prolonged hyperoxia on lung tumor development in mice. Available evidence suggests that hyperoxia, i.e., inhalation of oxygen at a higher fraction than 20%, increases the production of oxygen free-radicals in lung slices, isolated perfused lungs or lung nuclei, and mitochondria (17-20). One would therefore expect that hyperoxia might enhance lung tumor formation. We found the opposite to be true: prolonged hyperoxia severely inhibits lung tumor development.

MATERIALS AND METHODS

Chemicals. Urethan (ethyl carbamate), cytochrome c, xanthine, xanthine oxidase, pyridoxal phosphate, dithiothreitol, L-ornithine, and a commercial kit for measuring the activity of glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents used were of the highest available purity. Amershams-Searle Co., Arlington Heights, IL, provided [methyl-3H]thymidine (specific activity, approximately 2 Ci/mmol), and L-[U-14C]ornithine (specific activity, 52.6 mCi/mmol) was obtained from New England Nuclear, Amherst, MA. Alzet Model 2002 osmotic minipumps were purchased from Alza Corporation, Palo Alto, CA.

Animals. Male A/J mice, 4-6 wk old, were ordered from the Jackson Laboratory, Bar Harbor, ME. Upon arrival the animals were kept for 2 wk in quarantine and then assigned at random to the different treatment groups. The animals, when not in exposure chambers, were housed on hardwood bedding in plastic shoe-box cages, 10 to a cage, under a 12-h light-12-h dark cycle at a temperature of 21 ± 1°C and a relative humidity of 40-60%. They had access to a conventional laboratory diet (Rodent Laboratory Chow 5001; Ralston Purina Co.) and hyperchlorinated water ad libitum throughout.

Oxygen Exposure. Animals were placed within their cages into a 450-liter-capacity plastic chamber in which an atmosphere of 70% O2 was maintained by mixing 100% O2 with air in appropriate proportions. Flow rate of the air-oxygen mixture was 18-22 liters/min. Temperature within the chamber was 23-25°C, and relative humidity was 60%. Oxygen concentrations were routinely monitored with an oxygen analyzer and did not vary more than 2% around the nominal concentration of 70%. Industrial hygiene kits (Sensidyne Gasco analyzer tubes) were used to measure chamber concentrations of NH3 and CO2. Ammonia could not be detected in the chamber (sensitivity of the method, 0.2 ppm), and less than 0.3% CO2 was found.

Scoring of Lung Tumors. The detailed protocols for each experiment are given in "Results." To determine the number of tumors per lung, the animals were killed by asphyxiation, and then 1 ml of Tellynsiczky's fixative was instilled into the lung through the trachea (for composition of the fixative, see Ref. 21). Lungs, heart, and mediastinum were excised en bloc and dropped into the fixative. After at least 24 h in fixative, the tumors visible on the lung surface were counted under a magnifying glass; experimental groups were coded. The procedure gives a reliable count of all tumors present in a mouse lung (21). Some tumors were embedded in paraffin, cut, stained with hematoxylin-eosin, and classified under the light microscope into tumors derived from type 2 alveolar cells or derived from bronchiolar nonciliated (Clara) cells. The criteria of Kaufman et al. (22) were used.

To obtain an index of tumor incidence, the percentage of tumor-bearing mice per total number of mice in each treatment group was calculated. Tumor multiplicity is defined as the average number of tumors per lung, obtained by dividing the total number of tumors

Received 9/9/85; revised 11/25/85; accepted 12/16/85.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Research sponsored by the Office of Health and Environmental Research, United States Department of Energy, under Contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.
2 Recipient of support under an appointment to the Laboratory Cooperative Postgraduate Research Training Program administered by the Oak Ridge Institute for Science and Education, under Contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.
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4 The abbreviations used are: BHT, butylated hydroxytoluene; SOD, superoxide dismutase; ODC, ornithine decarboxylase; G6PDH, glucose-6-phosphate dehydrogenase; LI, labeling index.

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counted by the total number of mice per group, including non-tumor-bearing animals. Statistical comparisons were made by using the $\chi^2$ test for tumor incidence and Student's $t$ test for multiplicity (or, where appropriate, analysis of variance). The null-hypothesis was rejected whenever a $P$ value of 0.05 or less was found.

Autoradiography. Details of the procedure have been described (23, 24). Briefly, osmotic minipumps filled with 1 mCi of [methyl-3H] thymidine were implanted s.c. Two wk later the animals were killed by an overdose of pentobarbital, and the lungs were fixed by inflation with neutral buffered formalin. Thin (1-$\mu$m) glycol methacrylate sections were dipped in Kodak NTB2 emulsion, exposed for 10 days, developed, and stained with Lee's methylene blue basic fuchsin. A minimum of 500 cells in 8–10 terminal bronchioles and a minimum of 2000 sessile alveolar wall cells were counted and differentiated as previously described (23, 24). The percentage of cells labeled nuclei per total number of bronchiolar or sessile alveolar wall cells was defined as the LI. Criteria for classification of the cells into type 1 alveolar cells, type 2 alveolar cells, interstitial cells, and capillary endothelial cells have been reported in previous papers (23, 24). The number of labeled type 2 alveolar cells per 10,000 alveolar wall cells was calculated from the LI and the differential cell count as described earlier (24). Student's $t$ test or, where appropriate, analysis of variance was used for statistical analysis of the data.

Enzyme Assays. For biochemical analysis, animals were deeply anesthetized with Metofane and exsanguinated by cutting the abdominal aorta. The lungs were perfused with 0.9% NaCl solution through the right ventricle until uniformly white. Each lung was homogenized in 2 ml of 50 mM phosphate-0.1 mM EDTA buffer (pH 7.8) (SOD) or in 2 ml of 30 mM phosphate-5 mM diethiothreitol-0.1 mM pyridoxal phosphate-0.1 mM EDTA buffer (pH 7.1) (ODC-G6PDH). G6PDH activity was measured by directly incubating an aliquot of lung homogenate in the presence of substrate, whereas SOD and ODC were measured in an aliquot of supernatant following a 15-min spin of the homogenate at 20,000 rpm. SOD activity was expressed as units per mg of protein, a unit being defined as the quantity of enzyme required to produce a 50% inhibition of the rate of reduction of cytochrome c under the conditions of assay (25). ODC activity was measured as production of [14C]ornithine, trapped in hyamine, and activity was calculated as pmol of [14C]ornithine per mg of protein released during a 60-min incubation period (26). The activity of G6PDH was estimated by measuring the conversion of NADP to NADPH in the presence of glucose 6-phosphate and was calculated as the amount of substrate transformed per min per mg of protein (27). All enzyme assays were run under conditions where activity was linear with time and with amount of protein present in the enzyme source.

RESULTS

Inhibition of Tumor Development by Hyperoxia. Strain A/J mice were given injections i.p. of urethan (1000 mg/kg). Control animals received 0.9% NaCl solution i.p. The animals were left 4 days in room air, and then half of them were placed into the chamber ventilated with 70% O$_2$, and the other half were placed in a similar chamber ventilated with air.

While in oxygen, the animals were weighed periodically. Table 1 shows that the animals tolerated continuous exposure to an atmosphere of 70% O$_2$ well. There was no mortality attributable to the hyperoxic environment, and overall weight gain over the 16-wk period was comparable between animals kept in air and animals under hyperoxia. An initial very slight weight loss was less than 10%.

After 16 wk, one group of oxygen-exposed and one group of animals kept in air were killed. All remaining animals were kept in air, and additional groups were killed 24, 36, and 52 wk after the injection of urethan. The number of tumors was determined, and the results are shown in Table 2. Animals given urethan and maintained in air had 16 wk later an average of 20 tumors per lung. Exposure to 70% O$_2$ reduced the average number of tumors to 5 per lung. With time, the number of tumors in both groups increased further. However, at all time points examined, animals originally exposed to O$_2$ during the first 16 wk had approximately 15 tumors less in their lung than the air controls. About 100 tumors were examined by light microscopy and classified into type 2 cell tumors and Clara cell tumors (22). It was found that hyperoxia did not influence the types of tumors present. A similar percentage of Clara cell tumors (15%) and alveolar type 2 cell tumors (85%) was found in both the air and 70% O$_2$ groups. It was concluded that 70% O$_2$ reduced the probability of development of urethan-induced tumors as long as hyperoxic conditions lasted (16 wk) and that the deficit could not be made up by returning the animals to air. In a group of animals not given injections of urethan at the beginning of the experiment and kept in 70% oxygen for 16 wk before returning them to air, the average number of lung tumors found at 1 yr was twice the number of tumors found in animals kept in air throughout.

In the second experiment, A/J mice treated with 1000 mg of urethan per kg or with 0.9% NaCl solution were placed, 4 days after the initial injection, into an atmosphere of 70% oxygen or were kept in a similar chamber ventilated with air. Groups of 28–30 animals were removed from the oxygen 2, 4, 6, and 8 wk later and returned to air. One group remained in 70% O$_2$. Sixteen wk after the urethan injection, all animals were killed.

Again it was found that hyperoxia profoundly affected tumor development (Table 3). Animals which were kept in 70% O$_2$ for the entire 16 wk had only one-fourth as many tumors per lung than had animals kept in air. Exposure to O$_2$ for 4 or even 4 wk was sufficient to significantly reduce the average number of tumors; a 2-wk exposure had no effect. In control animals which had received 0.9% NaCl solution instead of urethan, oxygen did not increase the number of "spontaneous" tumors per lung nor did it increase the percentage of animals with lung tumors. Oxygen at 70% was thus capable of inhibiting lung tumor development in animals treated with urethan and in this case reduced the average number of tumors to 5 per lung. With time, the number of tumors in both groups increased further. However, at all time points examined, animals originally exposed to O$_2$ during the first 16 wk had approximately 15 tumors less in their lung than the air controls. About 100 tumors were examined by light microscopy and classified into type 2 cell tumors and Clara cell tumors (22). It was found that hyperoxia did not influence the types of tumors present. A similar percentage of Clara cell tumors (15%) and alveolar type 2 cell tumors (85%) was found in both the air and 70% O$_2$ groups. It was concluded that 70% O$_2$ reduced the probability of development of urethan-induced tumors as long as hyperoxic conditions lasted (16 wk) and that the deficit could not be made up by returning the animals to air. In a group of animals not given injections of urethan at the beginning of the experiment and kept in 70% oxygen for 16 wk before returning them to air, the average number of lung tumors found at 1 yr was twice the number of tumors found in animals kept in air throughout.

<table>
<thead>
<tr>
<th>Time (wk after injection of urethan)</th>
<th>Body wt (g)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>70% O$_2$</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<tr>
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<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

* Number of animals per group, 30 throughout.
*b Mean ± SD.

<table>
<thead>
<tr>
<th>Time of sacrifice (wk)</th>
<th>No. of tumors/lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% O$_2$</td>
<td>Air</td>
</tr>
<tr>
<td>16</td>
<td>5.0 ± 0.8* (29)</td>
</tr>
<tr>
<td>24</td>
<td>14.7 ± 1.1 (29)</td>
</tr>
<tr>
<td>36</td>
<td>20.5 ± 1.1 (24)</td>
</tr>
<tr>
<td>52</td>
<td>22.6 ± 1.0 (26)</td>
</tr>
<tr>
<td>52 (saline controls)</td>
<td>1.4 ± 0.3 (29)</td>
</tr>
</tbody>
</table>

* All animals given urethan (1000 mg/kg) i.p. and killed at time indicated after urethan.
*b Animals kept in 70% O$_2$ during the first 16 wk after urethan injection.
*c Animals kept in air throughout.
*d Mean ± SE.
*e Numbers in parentheses, number of animals.
$f P < 0.05 compared to animals kept in air throughout (t-test).
received 0.9% NaCl solution.

Delayed Onset of Oxygen Exposure. In this experiment, all mice were given injections of urethan (1000 mg/kg) and one group (Group A) was placed 4 days later into 70% oxygen. Six wk later this group was returned to air, and a second group (Group B) was placed into 70% O2. After another 6 wk (wk 12), Group B was removed into air, and Group C was placed for 6 wk into O2. Group D was kept in air throughout, and all animals were killed the moment Group C was returned to air (wk 18). Table 4 shows that animals kept for 6 wk in O2 had a significantly lower labeling index in sessile lung cells than if exposure was delayed, and animals in Group A developed significantly fewer tumors than did animals in Groups B and C.

Cell Kinetic Studies. Four groups of animals were used: mice treated with urethan or with 0.9% NaCl solution and kept in 70% O2 and mice treated with urethan or with 0.9% NaCl solution and kept in a chamber ventilated with air. At the beginning of the experiment, four mice from each group received a minipump filled with 1 mCi of [methyl-3H]thymidine implanted under the skin of the back. Two wk later the animals were killed, and new animals from each group received a minipump s.c. Model 2002 minipumps release their content at a controlled rate of 0.5 µl/h; a continuous in vivo supply of labeled thymidine was thus available during each 2-wk period, labeling all pulmonary cells synthesizing DNA during this time. The procedure allows the determination of the cumulative labeling index in sessile lung cells for each consecutive 2-wk period (23, 24).

In animals treated with 0.9% NaCl solution, the cumulative overall parenchymal labeling index for any 2-wk period was around 3%; one apparent burst seen at 6 wk was statistically not significant and due to one of four animals having an unexplained high LI (Fig. 1). Exposure to 70% O2 produced an initial burst of cell proliferation during the first 4 wk. The average LI then fell, although between 6 and 16 wk, it remained at all times higher than in the air-exposed controls. After 16 wk, all animals were returned to air. Upon removal from the oxygen, there was a small increase in the overall LI, and then between 16 and 18 wk, it fell to control values.

In animals treated with 1000 mg of urethan per kg and kept in air, there was a substantial initial but transient increase in the overall alveolar LI, a phenomenon observed repeatedly in previous similar studies (23, 24). During this time, oxygen had little additional effect on cell proliferation (Fig. 2). After 6 wk, cell proliferation in animals exposed to 70% O2 was similar to the one seen in animals not given urethan and exposed to 70% O2.

In strain A mice, the main target cell for urethan is the type 2 alveolar cell, and 70–80% of all lung tumors presumably originate from this cell (21). Table 5 shows that, in mice treated with urethan and air, there was during the first 4 wk a high proliferative activity in this particular cell population (Group C). During this time, oxygen exposure of the urethan-treated animals produced an effect which was not quite additive (Group A). After 6 wk, the number of labeled type 2 alveolar cells in urethan-treated animals fell to control levels. In the animals exposed to hyperoxia, the number of labeled type 2 alveolar cells remained high until the animals were removed from the hyperoxia atmosphere; however, no significant differences were found between animals treated with urethan and animals originally given injections of 0.9% NaCl solution. Only upon removal from oxygen, during wk 16–18, was there a transient increase in type 2 cell labeling in animals given the carcinogen in comparison with their controls.

While the labeling index of cells in the alveolar wall initially showed a substantial increase, it eventually reached a plateau at levels about twice those found in air-exposed animals. Continuous hyperoxia had a much more profound effect on the
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Fig. 2. Overall labeling index for sessile cells in the alveolar zone of mice given injections of urethan (1000 mg/kg) and kept in air (I) or exposed to 70% O2 on wk 1—16 (8). Minipumps were implanted in selected animals every 2 wk, and the mice were killed 14 days later. The LI is defined as the percentage of labeled cells in the alveolar walls, and each value gives the mean from four animals. * significantly different (P < 0.05) from air controls. Bars, SE.

Table 5 Labeled type 2 alveolar cells per 10⁶ alveolar wall cells

<table>
<thead>
<tr>
<th>Time (wk)*</th>
<th>Urethan + O₂ (Group A)</th>
<th>0.9% NaCl solution + O₂ (Group B)</th>
<th>Urethan + air (Group C)</th>
<th>0.9% NaCl solution + air (Group D)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>205 ± 28*</td>
<td>188 ± 38</td>
<td>124 ± 20*</td>
<td>36 ± 10</td>
</tr>
<tr>
<td>4</td>
<td>313 ± 20</td>
<td>287 ± 26</td>
<td>227 ± 32*</td>
<td>80 ± 14</td>
</tr>
<tr>
<td>6</td>
<td>244 ± 45</td>
<td>247 ± 24</td>
<td>59 ± 20</td>
<td>84 ± 27</td>
</tr>
<tr>
<td>8</td>
<td>155 ± 34</td>
<td>179 ± 40</td>
<td>43 ± 6</td>
<td>62 ± 14</td>
</tr>
<tr>
<td>10</td>
<td>245 ± 25</td>
<td>173 ± 33</td>
<td>52 ± 11</td>
<td>47 ± 9</td>
</tr>
<tr>
<td>12</td>
<td>244 ± 29</td>
<td>269 ± 19</td>
<td>55 ± 7</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>14</td>
<td>171 ± 43</td>
<td>254 ± 26</td>
<td>34 ± 10</td>
<td>61 ± 26</td>
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<tr>
<td>16</td>
<td>166 ± 43</td>
<td>225 ± 19</td>
<td>96 ± 13*</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>18</td>
<td>145 ± 16*</td>
<td>70 ± 25</td>
<td>54 ± 13</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>20</td>
<td>42 ± 8</td>
<td>38 ± 8</td>
<td>64 ± 21</td>
<td>42 ± 10</td>
</tr>
</tbody>
</table>

* Denotes the time during which cumulative LI was measured, i.e., 2 wk, cumulative LI during the first 2 wk after urethan, or 0.9% NaCl solution.
* Mean ± SE (n = 4 throughout).
* P < 0.05 compared to Group D.
* P < 0.05 compared to Group B.

However, 2 and 4 wk after removal from oxygen, the epithelium regained an appearance indistinguishable from that seen in the control mice (Fig. 4C).

The only changes in the alveolar parenchyma were seen in the animals exposed to oxygen for 2 wk. In these animals there was a mild diffuse increase in interstitial macrophages and type 2 epithelial cells. These changes were no longer visible in the groups exposed to oxygen for longer periods of time. Even after 16 wk of exposure to oxygen, few changes in the parenchyma could be observed (Fig. 5). Specifically, there was no thickening of alveolar walls or extension of cuboidal epithelium on the surfaces of the alveolar ducts. A minimal increase in alveolar macrophages and type 2 epithelial cells was occasionally seen in animals exposed to longer than 2 wk of oxygen.

Enzyme Assays. Exposure of 70% O2 had no effect on the activity of two pulmonary enzymes usually involved in antioxidant defense mechanisms. Both SOD and G6PDH activities calculated per mg of lung protein remained essentially unaltered during the first 2 mo of hyperoxia. Expression of enzyme activities on a total per lung basis or on a per cell basis (e.g., per mg of DNA) gave essentially similar results (data not shown). In view of these negative findings, assays for these two enzymes were discontinued after 2 mo.

Histopathology. At all time points studied, the lungs from the air control mice demonstrated no significant pathological changes. The bronchiolar epithelium was a single layer with few labeled cells (Fig. 4A). The bronchiolar epithelium of the oxygen-exposed mice was distinctly different from the controls, even at the earliest time points studied. In the mice exposed for 2 wk to hyperoxia, the bronchiolar epithelium was hyperplastic with piling up of Clara cells. Numerous nuclei were labeled. After 3–4 wk of oxygen exposure, epithelium which was several layers deep was frequently observed (Fig. 4B). This epithelial hyperplasia was seen at all remaining time points of oxygen exposure. After removal from oxygen, a dramatic drop in cell labeling could be observed, although the epithelial hyperplasia could still be seen 2 wk after removal from the oxygen exposure.
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little oxygen effects are seen. The animals gain weight in a normal fashion and fail to show any dramatic changes in their lung structure as examined by light microscopy. During oxygen exposure, cells in the alveolar walls and particularly in the lining of the small airways show increased DNA synthesis. Biochemically it is possible to find an increase in the activity of pulmonary ODC, whereas two enzymes commonly associated with pulmonary antioxidant defense mechanisms, SOD and G6PDH, show no changes in activity. Thus, the effects of prolonged hyperoxia are minimal except on developing tumors.

A 16-wk exposure to 70% O2, beginning a few days after injection of a carcinogen, reduced tumor multiplicity to 25% of control values. When animals treated in this way were kept in air until 1 yr after the injection of urethan, we found that they continued to grow tumors after being returned to air but would never make up the deficit initially produced by oxygen exposure. Table 4 also shows that hyperoxia reduces tumor multiplicity even if given for the first time 12 wk after urethan. We conclude from these observations that oxygen severely interferes with the development of many growing tumors.

There was only one exception to the generalization that oxygen interfered with tumor development. In animals treated with 0.9% NaCl solution and kept for the initial 16 wk in 70% O2, more tumors were found after 1 yr than in animals kept in air throughout. The data could be interpreted to mean that O2 "promotes" the occurrence of "spontaneously" occurring lung tumors in A/J mice. The experiment as designed would, however, also allow the interpretation that O2 acted as a carcinogen in itself. A more likely explanation is that O2 treatment for 16 wk with its accompanying hyperplasia of alveolar type 2 cells (Table 5) simply increased the number of cells at risk to undergo spontaneous transformation.

It is not clear why hyperoxia should kill tumor cells in the lung. Since 70% O2 was highly effective in preventing tumor development, we did not find enough tumors in the present study to establish labeling indices for tumors. To get this information, we will require a separate and differently designed study. We can only speculate why oxygen would be cytotoxic to tumor cells. At concentrations of 95% or more, oxygen is severely cytotoxic to the pulmonary cells of many animal species, including humans (28). Lower O2 concentrations usually produce milder signs of toxicity in resting lung cells while still affecting dividing lung cells. Under these circumstances, oxygen concentration as low as 50–60% may interfere with DNA synthesis in mouse lung and compromise the recovery phase (29, 30). In primates, dividing cells are susceptible to 60–80% of oxygen, whereas resting cells appear to remain unaffected (31). However, it cannot be stated categorically that all dividing cells in a damaged lung remain sensitive to hyperoxia; otherwise it would not be possible, as we did, to observe a continued doubling of the alveolar labeling index for as long as the animals were kept in the hyperoxic environment (Figs. 1–3). It is also possible that transformation of lung cells occurs at a similar rate in animals exposed to oxygen and in air, but that transformed cells are more susceptible to oxygen, perhaps because of reduced antioxidant defense capacities (32).

The concentration of O2 used, 70%, has actually very little pulmonary toxicity as indicated by a lack of morphological changes in the lung parenchyma. Initially, the 2-wk cumulative LI in 0.9% NaCl solution-treated animals exposed to 70% reached 10% and then remained constant around 5% through the duration of the exposure. In lungs damaged by one single dose of BHT, the 2-wk cumulative LI can reach 50% (33). Pulmonary SOD did not increase; this was not entirely unexpected, since it has been shown before that even 100% O2 produces only small increases in mouse pulmonary SOD (34). Glucose-6-phosphate dehydrogenase, a second enzyme associated commonly with lung antioxidant defense mechanisms (35), also failed to increase. On the other hand, ODC activity showed an initial burst to levels which were up to 10 times higher than in controls and remained significantly elevated throughout the entire exposure period. Since tumor multiplicity decreased, ODC activity under those circumstances may have reflected O2-induced cell proliferation rather than having been associated with tumor promotion. It remains unclear why there was no increase in ODC activity in animals treated with urethan and kept in air during the early phase of alveolar cell proliferation, during wk 2 and 4.

Our data are at some variance with the free oxygen radical theory of tumor promotion. Development of murine lung tumors can be enhanced by such agents as butylated hydroxytoluene, phorbol, or saccharin, even if exposure to a carcinogen and the enhancing agent is separated by several months (10). On the other hand, it has to date not been possible to promote lung tumor development by BHT when mice were treated with

Fig. 4. Terminal bronchioles from mouse lungs. In A, air exposure control demonstrates the regular array of single cells lining the bronchiolar lumen. In B, 70% oxygen exposure for 6 wk results in marked epithelial hyperplasia with piling up of Clara cells, which result in a lining epithelium multiple cells deep. Also note the high frequency of labeled nuclei. In C, 2-wk recovery in air after 16 wk of continuous 70% oxygen exposure results in return to the usual appearance of a single lining epithelial layer. One-μm autoradiograph sections stained with Lee’s methylene blue and basic fuchsin, x 400.
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Fig. 5. Lung from mouse exposed to 70% oxygen for 16 wk. Although the hyperplastic epithelium is observed, the alveolar duct regions and parenchyma are normal in appearance. Lee's methylene blue and basic fuchsin, × 20.

Fig. 6. Activity of pulmonary ODC. Animals treated with urethan (1000 mg/kg) or 0.9% NaCl solution were kept in 70% O2 for 16 wk or in air. ODC activity was measured at times indicated; ■, urethan plus 70% O2; ●, 0.9% NaCl solution plus 70% O2; □, urethan plus air; ○, 0.9% NaCl solution plus air. Points, mean; bars, SE (n = 5 throughout); *, significantly higher (P < 0.05) than air controls; **, significantly higher or lower than animals treated with 0.9% NaCl solution and O2.

subcarcinogenic doses of a carcinogen. This may preclude the application of the terms “initiation-promotion” to lung tumor development in mice (36).

Nevertheless, should oxygen free radicals indeed play a crucial role in the modification of gene expression by initiated cells (2), then it was not unreasonable to expect that lung tumor multiplicity should increase in hyperoxia. Since this was not the case, some older experiments with mouse lung which apparently support the oxygen free radical hypothesis (4–6) need to be reinterpreted. In all these studies, animals were exposed to 70–100% O2 immediately after urethan or dibenz(a,h)anthracene administration. Such a procedure may alter blood flow and drug delivery to the lung or alter carcinogen metabolism and disposition. A 2-day exposure to 100% O2 also will in mouse lung produce extensive cell damage, followed by a round of cell proliferation (37), and such an event may influence potential repair mechanisms. The previous in vivo data on the effect of hyperoxia are thus open to several interpretations, one of them being that hyperoxia administration shortly and for a brief period of time after a carcinogen has cocarcinogenic and not promoting activity.

In conclusion, we have shown that hyperoxia severely interferes with the development of lung tumors in mice without otherwise having much pulmonary or systemic toxicity. We also have found since that hyperoxia at concentrations of 40–70% may reduce development of lung tumors in rats treated with a single-dose intratracheal instillation of 3-methylcholanthrene. It needs to be established to what extent our observations may be exploitable for therapeutic purposes.

ACKNOWLEDGMENTS

We wish to thank Dr. G. Helman for helping with some of the histopathological evaluation of the lung tumors and Jim Wesley for his excellent methacrylate autoradiographs. June Whitaker and T. Stephens for technical help, and Jeanie Shover for help in preparing the manuscript.

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