Dynamics of Neoplastic Development in Carcinogen-exposed Tracheal Mucosa

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ABSTRACT

The qualitative and quantitative cellular changes occurring in tracheal epithelium in vivo during the process of carcinogenesis were investigated by use of an in vitro assay which measures the formation of epithelial foci (EF); this method is called EF assay. The cell or cells giving rise in vitro to an EF which survives and proliferates for at least 1 month after tracheal cells have been seeded into culture dishes was defined as the epithelial focus-forming unit (EFFU). Viable epithelial cells were harvested from heterotopic tracheal grafts immediately and at 2, 4, and 8 months after they had been exposed in vivo for 4 weeks to 165 \( \mu \)g of dimethylbenz(a)anthracene. This carcinogen exposure was known to induce a 9% incidence of invasive tracheal carcinomas. The cell suspensions obtained from individual tracheas (20 tracheas per time point) were seeded into culture dishes, and the number of EF was scored 1 month later. Normal tracheal cells and cells from tracheas exposed to 100 \( \mu \)g of 12-O-tetradecanoylphorbol-13-acetate produced no EF. In contrast, 80 to 90% of the cultures of all tracheas, exposed to dimethylbenz(a)anthracene 8 months prior to the collection of cells, developed one or more EF. The first 4 months after termination of dimethylbenz(a)anthracene exposure, the number of EF per trachea remained constant at 3 to 4. Between 4 and 8 months, the number of EF per trachea increased 3- to 5-fold.

Three different types of EF could be distinguished experimentally: EF that could not be subcultured (EFs); EF that could be subcultured but did not grow in soft agarose (EFs,ag-); and EF that could be subcultured and that also grew in soft agarose (EFs,ag+). During the 8 months after exposure, EF that can be subcultured (EFs) increased from 53 to 84%, and EFs,ag+ increased from 23 to 57%. Simultaneously, the frequency of EF0 dropped from 47 to 16%. The relative frequency of EFs,ag+ remained the same with time. Studies with 'mixed-population' cell cultures derived from the same tracheas with which the EF assays were carried out showed similar trends, namely, an increase in subculturability and tumorigenicity in cultures established at increasingly longer time intervals after carcinogen exposure. Our findings suggest the existence, in the epithelium of carcinogen-exposed tracheal, of EFFU's endowed with different in vitro growth capacities. The data are consistent with a gradual conversion of EFFU's with limited growth capacity to EFFU's with neoplastic growth capacity as a function of time after carcinogen exposure (EFFU0 \( \rightarrow \) EFFUag0 \( \rightarrow \) EFFUag+). A tumor induction study, in which tracheal transplants were exposed during a 4-week period to 165 \( \mu \)g dimethylbenz(a)anthracene, showed an incidence of 9% invasive tracheal carcinomas. In comparison, the EF assay demonstrated that at 8 months 80% of such tracheas contain cells with neoplastic potential (EFFUag+). This suggests that, left in the host animals, only a fraction of these cells actually succeed in establishing a malignant tumor.

INTRODUCTION

The hypothesis that carcinogenesis is a multiphasic process has received wide acceptance among cancer researchers (e.g., Refs. 8, 10, and 26). The most convincing evidence in support of this concept still stems from so-called 2-stage (or multistage) skin carcinogenesis studies which demonstrate that tumor initiation and tumor promotion are 2 seemingly separable effects or events (4, 6). Recently, distinct phases of carcinogenesis have been demonstrated in organs such as the liver (9, 19, 25, 29), mammary gland (7), urinary bladder (15), and respiratory tract (21, 28). However, further characterization of these changes, presumed to constitute neoplastic disease, has generally met with limited success (23). Some major obstacles have been encountered in defining the sequential and progressive events at the cellular and molecular level. One of these is the lack of specific and unequivocal markers for the 'preneoplastic' stage(s). Another is the fact that most, if not all, methods used for identification of phenotypic markers of putative preneoplastic cell populations in vivo, such as cytological and histological or histochemical and biochemical abnormalities, are destructive tests. Thus, it has been impossible to provide direct evidence for the progenitor-progeny relationships between phenotypically distinct cell populations which have evolved following carcinogen exposure. However, the unequivocal demonstration of the developmental and progressive nature of the neoplastic disease process (i.e., all events occurring subsequent to the interaction of the ultimate carcinogen with the crucial cellular targets, leading to the appearance of recognizably malignant cell populations) depends on the demonstration of such relationships between sequentially occurring cell populations. Tissue and cell culture systems seem to offer new possibilities for study of the sequential changes presumed to occur during the in vivo development of neoplasia (for review, see Ref. 23). It has been shown that, shortly after carcinogen exposure, cells which have a markedly increased growth capacity in vitro can be isolated from organs known to be targets for the carcinogen (2, 3, 5, 12-14, 16, 21, 22, 28). These cell populations can be subcultured indefinitely. Thus, subsequent
generations of cells originating from the same cell pool can be examined for the emergence of neoplastic characteristics.

We previously reported (22) investigations of the evolution in vitro of neoplastic cells from tracheal epithelium, isolated immediately after carcinogen exposure. The studies reported here make use of these earlier observations but focus on the in vivo rather than the in vitro development of preneoplastic and neoplastic cell populations during an 8-month period following exposure of tracheal epithelium to the carcinogen DMBA\(^3\) (165 \(\mu\)g delivered in 4 weeks). Tracheas were sampled at different times after carcinogen exposure to be assayed for the presence of EFFU's operationally defined as a cell or group of cells from which expanding foci of epithelial cells originate when tracheal cells are cultured in vitro. Epithelial cells from normal tracheas or TPA-exposed tracheas did not proliferate under the culture conditions used. Thus, the capacity to form EF was the biological indicator by which carcinogen-altered cells were recognized. There are at least 3 different types of EF: EF\(_0\), EF\(_\text{s,ag} \), and EF\(_\text{s,ag}^-\). Correspondingly, there are EFFU\(_0\), EFFU\(_\text{s,ag} \), and EFFU\(_\text{s,ag}^-\) in the tracheal epithelium which give rise to these different EF in vitro. It is clear from the foregoing that the EF assay plays a key role conceptually and operationally in the reported investigations. Only those carcinogen-altered cells which can give rise to an EF will be detected with the assay system described. The experiments described in this communication are an attempt to quantitate the emergence of carcinogen-altered and neoplastic cell populations in tracheas following in vivo exposure to DMBA and to study the dynamics of development of cell compartments endowed with different proliferative and neoplastic potentials.

MATERIALS AND METHODS

In Vivo Exposure of Tracheal Epithelium to TPA or DMBA. Specific-pathogen-free female Fischer 344 rats were used. The methods for transplanting tracheas and exposing them to carcinogen have been previously described (11, 17). Tracheal transplants were exposed over a 4-week period to TPA or DMBA contained in beeswax pellets. Exposure was terminated by removal of the pellets. During the 4 weeks of exposure, 165 \(\mu\)g of DMBA or 100 \(\mu\)g of TPA were delivered to each trachea. Doses delivered were determined by assay of the amount of DMBA contained in beeswax pellets. Exposure was terminated with CMF-HBSS plus 20% FBS and the supernatants were removed. The cells were counted in a hemacytometer, and the viability was determined by means of erythrosin B dye (0.2%) exclusion. The average viability in all groups was 55 to 60%. In addition to the total viable cell count, the number of cell clumps and the number of cells per clump were scored. The number of clumps and the number of cells per clump were similar in all experimental groups. For each 100 cells counted, approximately 70 were present as single cells, 15 were present in clumps consisting of 2 cells, and 15 were present in clumps of 3 to 10 cells.

To evaluate the effectiveness of the trypsinization procedure in the collection of epithelial cells, we fixed all tracheas in Bouin's fixative after they were trypsinized. Each trachea was cut into 2-mm segments, and 4 histological step sections were cut from each (16 to 20 sections per trachea). No sections with remaining epithelial cells were found in the tracheas harvested immediately after exposure and at 2 months. At 4 and 8 months, 1 of 20 and 2 of 20 sections, respectively, showed a small patch of epithelial cells remaining.

Tracheal cells obtained by trypsinization were seeded into two to three 60-mm plastic-grid dishes at a density of 10\(^4\) to 10\(^5\) viable cells per dish. Approximately 0 to 20% of the cells attached at 48 hr after seeding were of a fibroblast-like morphology. The epithelial cell attachment frequency (i.e., the total number of attached cells with epithelial-like morphology divided by the total number of viable cells plated) was determined 48 hr after the cells were seeded from a count of the number of attached epithelial-like cells. All dishes were maintained in Ham's F-12 plus 10% FBS, penicillin (100 IU/ml), and streptomycin (100 \(\mu\)g/ml), insulin (0.1 \(\mu\)g/ml), and hydrocortisone (0.1 \(\mu\)g/ml). The culture medium was changed weekly.

Determination of EFFU in Tracheal Grafts. The cells recovered from tracheal grafts (control and TPA- and DMBA-exposed tracheas) during the 8 months after carcinogen exposure were seeded in 2 to 3 culture dishes, and 1 month later the number of proliferating EF was determined (Chart 1). From that, the number of EFFU's for each trachea was calculated. The EFFU is operationally defined as a cell or group of cells giving rise to an EF. Criteria for epithelial-like tracheal cells have been described previously (21, 22). If a focus of epithelial-like cells, still persisting 1 month after seeding in vitro, appeared to be proliferating, it was considered to be an EF. Such criteria as increase in size of the focus with time or the presence of mitotic cells within the focus were used to identify EF. Proliferating EF appear similar to that illustrated by Marchok et al. (Fig. 4 of Ref. 22). An EF 1 month after seeding consisted of anywhere from 10\(^2\) to 5 \(\times\) 10\(^4\) cells. Senescing epithelial

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\(^3\) The abbreviations used are: DMBA, dimethylbenz(a)anthracene; EFFU, epithelial focus-forming unit; TPA, 12-O-tetradecanoylphorbol-13-acetate; EF, epithelial focus; Subscript zero, EF that cannot be subcultured; Subscript s, EF that can be subcultured and does not grow in soft agarose; Subscript s,ag, EF that can be subcultured and grows in soft agarose; CMF, calcium and magnesium free; HBSS, Hank's balanced salt solution; FBS, fetal bovine serum; Subscript s, EF that can be subcultured.
to 5 weeks of age. Three weeks later, a whole-body dose of (dye-excluding) cells. The rats had been thymectomized at 4
female Fischer 344 rats were inoculated i.m. with 10^6 viable
was tested for tumorigenicity in immunosuppressed rats during
37°. The viability of the thawed cells, as determined by dye
agarose were thawed after being stored at liquid nitrogen
Temperatures for 2 to 6 months. Cells were rapidly thawed at
Isolation and attempt to subculture twice
Testing EF for growth in soft agarose

Chart 1. Diagrammatic representation of sequential steps in the EF assay
procedure. Tracheas were exposed in vivo to 165 µg DMBA for 4 weeks, and
cells were collected for in vitro culture immediately, 2, 4, or 8 months after
exposure. Cells were then followed in vitro as diagrammed in order to score the
number of epithelial-like cells per trachea with altered in vitro growth capacity
(EF), to assess subculturability of isolated EF (EF), and to evaluate the capacity
of EF, to grow in soft agarose (EF,ag).

foci (not scored as EF) were identified by the presence of large
spread-out cells with foamy-appearing cytoplasm.

Isolation and Passage of EF-derived Populations and
Mixed-Cell Populations. Cell populations derived from individual
EF were prepared as follows. Once an individual EF, well
isolated from adjacent EF, attained a diameter of 1 cm (8 x
10^5 to 5 x 10^6 cells), one-half was scraped off the culture dish and
transferred to a 35-mm plastic Petri dish after it had been
dispersed by repeated rapid pipetting (Chart 1). All cells from
each 35-mm dish were then passaged once. So-called “mixed-
population” cell cultures contained representatives of all epi-
thelial-like cells with altered in vitro growth potential obtained
from one trachea. This included the remaining halves of foci
previously removed as well as those foci of epithelial cells left
intact on the primary dish. All fibroblast-like cells were removed from
the primary mixed-population dish by means of partial
tryptsinization (3-min exposure at 37° to 0.2% trypsin-0.25%
EDTA in CMF-HBSS plus 5% chicken serum) in combination
with selective scraping. After all observed fibroblast-like cells
were removed, the entire dish was passaged once. Isolated
foci and mixed cell cultures were passaged in each successive
passage into sequentially larger dishes (i.e., all cells from 35-
mm dishes were passaged into 60-mm dishes and subse-
quently into 100-mm tissue culture Petri dishes). All cell pop-
ulations to be tested for tumorigenicity in vivo or for growth in
agarose were thawed after being stored at liquid nitrogen
temperatures for 2 to 6 months. Cells were rapidly thawed at
37°. The viability of the thawed cells, as determined by dye
exclusion, was between 60 and 90%.

Tumorigenicity Testing. Each mixed-population cell culture
was tested for tumorigenicity in immunosuppressed rats during
their third in vitro passage. Both thigms of immunosuppressed
female Fischer 344 rats were inoculated i.m. with 10^6 viable
(dy-excluding) cells. The rats had been thymectomized at 4
to 5 weeks of age. Three weeks later, a whole-body dose of
400 rads was delivered. Three weeks following the first expos-
ure, a second dose of 600 rads was given; this was immedi-
ately followed by an i.p. injection of 1 to 2 x 10^7 bone marrow
cells to assure survival (21). Rats were inoculated within 24 hr
after the last x-ray exposure.

Testing for Anchorage-independent Growth. Marchok et
al. (22) and Terzaghi and Nettesheim (Table 1) have found that
anchorage-independent growth in vitro of carcinogen-exposed
populations of tracheal epithelium correlates well with onco-
genicity in vivo when the minimum in vivo tumor latency is short
(i.e., <42 days). In this study, we are, therefore, utilizing a test
for anchorage-independent growth to assess the oncogenicity of
all fourth-passage, isolated, EF-derived populations. The
method used for preparation of the agarose plates was similar
to that of MacPherson (20). Cells (5 x 10^4/dish) were seeded
in the top layer containing 0.3% agarose (Sigma Chemical Co.,
St. Louis, Mo.; type II, electrophoretic grade). The entire dish
was scored 3 weeks after plating. Any population yielding at
least 1 colony of 20 cells or more on each of 2 duplicate dishes
(i.e., 0.004% plating efficiency) was considered positive.

RESULTS

Tracheal transplants were exposed to 165 µg of DMBA which
was released from intraluminal beeswax pellets over a period of
4 weeks. At this time, the pellets were removed from all
transplants. Intact normal tracheas or tracheal grafts with pel-
lets containing 100 µg of the phorbol ester TPA served as
controls. Beeswax-TPA-exposed tracheal transplants were in-
cluded in order to control for the possible effects of tracheal
transplantation and exposure to beeswax containing a noncar-
cinogenic agent, which also induces marked hyperplasia, on
the in vitro growth potential of tracheal epithelial cells. Tracheas
were harvested from the host animals, either immediately after
removal of the pellets or 2, 4, and 8 months thereafter. The
epithelial lining was removed from the tracheas by a combined
physical and enzymatic procedure. The cells thus obtained

Table 1
Relationship between anchorage-independent growth in vitro and tumorigenicity
in vivo of mixed-population cell lines

<table>
<thead>
<tr>
<th>Rats</th>
<th>No. of cell lines in agarose</th>
<th>No. of cell lines - agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumorogenic MLP &lt; 42 days</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Tumorogenic MLP &gt; 42 days</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Non-tumorogenic</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

a Two 60-mm dishes per cell line, each with 5 x 10^5 cells in 0.3% agarose.

The entire dish was scored for growth 3 weeks after the cells were seeded. Colo-
nies of 20 or more cells were scored.
b 10^5 cells were inoculated i.m. into immune suppressed syngeneic rats. MLP,
minimum latency period, time (days) from cell inoculation to first palpable tumor.
c The average plating efficiency in agarose was 0.02%; the range was 0.008
to 0.2%.
d The average plating efficiency in agarose was 0.008%; the range was 0.004
to 0.01%.
e Inoculated rats were followed over the life span of the animal.
f All 3 showed plating efficiency in agarose (0.004%) and 2 of the 3 yielded
nodules that regressed 2 to 3 weeks after their appearance.
were dispersed, counted, and plated into 60-mm dishes, as described in "Materials and Methods."

**Histological Evaluation of Carcinogen-exposed Tracheal Epithelium.** Tracheal grafts (6 to 8/group) were excised and fixed in Bouin's fixative for histological evaluation immediately and at 2, 4, and 8 months after exposure. Each trachea was cut into 2-mm segments, and 4 histological step sections were cut from each. Immediately following exposure, tracheas were found to be lined principally by hyperplastic epithelium and normal-appearing mucociliary epithelium. A few areas of keratinizing metaplasias were also seen. At 2 months after exposure, there was a marked increase in the amount of atrophic epithelium interspersed with areas of "normal" epithelium. Again, some areas of squamous metaplasia were visible. By 4 months after exposure, there was a marked increase in the number of squamous metaplasias, some with atypias (e.g., mitotic figures, variability in cell size, and nuclear-cytoplasmic ratios). Atrophic epithelium was also seen. At 8 months, the epithelium appeared similar to that observed at 4 months. No invasive carcinomas were seen in the small sample of tracheas described in this report. However, in a large in vivo experiment carried out in parallel with our experiments, 9% of 86 tracheas were found to develop invasive carcinomas, with the first grossly recognizable tumor appearing 10 to 12 months after exposure.  

**Study of EF.** The principal end point measured during this first phase of the study was the formation and survival of EF in vitro scored at 1 month after the cells were plated in order to establish the number of EFFU's per trachea. The EFFU may be 1 or more cells from which an EF develops. The relevant data are summarized in Table 2. The mean number of viable cells obtained at various times after exposure ranged from 6 to 9 x 10^6/traquea. Thus, the cell yield was fairly constant throughout. The average epithelial-like cell attachment frequency, as determined at 48 hr, was roughly 1% for the first 4 months after exposure but increased markedly at 8 months to 6% of the total viable cells seeded. It should be noted that the epithelial cells obtained from carcinogen-exposed tracheas had attachment frequencies consistently lower than those observed in the control groups. At 1 month after the tracheal cells were plated in vitro, no surviving EF were recognized in any of the culture dishes seeded with cells from the 60 control tracheas. In contrast to the controls, between 80 and 90% of the carcinogen-exposed tracheal cells yielded EF, and the relative frequency of tracheae yielding EF appeared to be constant at this level over the entire 8 months of study. The average number of EF per positive trachea remained relatively constant at 3 to 4 during the first 4 months after exposure (Table 2). However, between 4 and 8 months, a rather marked increase occurred, and the average number of EF per positive trachea at 8 months was 3 to 5 times higher (15 EF per trachea); the frequency of EF increased from approximately 5/10^6 tracheal cells to 20/10^6. It might be significant that the increase in attaching epithelial cells and the increase in EF per trachea occurred simultaneously. For further examination of the change in EF per trachea with time after carcinogen exposure, the tracheas were grouped according to their respective numbers of EF. As can be seen in Chart 2, until 8 months the majority of positive tracheae (73%) had only 1 or 2 EF or less, and from the total of 51 positive tracheae, only 4 yielded 9 to 10 EF per trachea. In contrast, at 8 months only 5 tracheae yielded 4 EF or less, and from a total of 16 positive tracheae, 13 yielded 9 to 10 EF or more. These data suggest that in at least one-half of all the tracheae assayed at 8 months, the EFFU compartment must have expanded during the last 4 months of the study.

After a minimum of 1 month in culture, when the individual EF had reached a diameter of 1 cm (0.8 to 5 x 10^4 cells), the foci were isolated, and attempts were made to subculture them at least 2 times. As shown in Table 2, only 53% of the EF isolated from tracheae harvested immediately after carcinogen exposure could be repeatedly subcultured. This number increased to 84% for EF from tracheae removed 8 months after carcinogen exposure. In addition to the number of EF, we also determined the "growth rate" of EF by measuring the time required for the individual foci to reach a diameter of 1 cm and the time required for the cell populations derived from individual EF to grow to confluency during the third passage (10^6 cells were seeded per 60-mm dish). We were particularly interested in determining whether a more rapid growth rate could be detected in the EF obtained from tracheae assayed 8 months after carcinogen exposure. In a large in vivo study with 165 mg DMBA, the first tumor occurred 10 months after the end of carcinogen exposure; therefore, one might expect cell populations obtained at 8 months to have progressed to a stage closer to malignancy and as a consequence to grow more rapidly in vitro than those isolated earlier. The data did not reveal any such trend. In all groups, the mean time required for EF to reach 1 cm in diameter ranged from 4 to 8 weeks, and the mean duration of the third passage ranged from 3 to 4 weeks (Table 2).

We then studied the development of neoplastic cells in cell populations derived from EF. Fourth-passage cell cultures derived from individual isolated EF were tested for their capacity to grow in soft agarose. Whenever possible, we tested the cultures of 4 EF from each trachea; however, not every trachea yielded 4 subculturable EF (Table 2). The results of the test for anchorage-independent growth in isolated EF (Table 3) indicate that over the 8-month period of observation the percentage of EF that increased from 23 to 57%. The frequency of tracheae yielding EF increased from 15% immediately after carcinogen exposure to 80% at 8 months.

**Study of Mixed-Population Cell Cultures.** The second test for the presence of neoplastic cells was carried out on the mixed-population cell cultures. As described in "Materials and Methods," the mixed-cell populations are made up of representatives of all carcinogen-altered epithelial-like cells from each trachea. Third-passage cell populations were tested for tumorigenicity by inoculation of 10^6 cells into the thighs of immunosuppressed isogenic rats. The results of this study are summarized in Table 4. Only 45% of the tracheae harvested immediately after the end of carcinogen exposure yielded mixed-cell cultures that survived the third passage. This number increased steadily during subsequent months, reaching 85% at 8 months. Of the cell cultures obtained from tracheae immediately and at 2 months after the end of carcinogen exposure, only 20% were tumorigenic. On the other
Table 2

<table>
<thead>
<tr>
<th>Time after exposure</th>
<th>No. of tracheas assayed</th>
<th>Av. no. of viable cells/trachea (×10^9)</th>
<th>Av. cell attachment frequency (%)</th>
<th>% of tracheas with EF</th>
<th>% of tracheas with EF, c</th>
<th>Av. no. of EF/trachea</th>
<th>% of EF, c</th>
<th>Av. time to 1-cm EF (wk)</th>
<th>Av. duration of third passage of EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>20</td>
<td>6 ± 4</td>
<td>1 ± 0.9</td>
<td>80</td>
<td>3</td>
<td>53</td>
<td>4 ± 1</td>
<td>3 ± 2</td>
<td></td>
</tr>
<tr>
<td>2 mos.</td>
<td>18</td>
<td>9 ± 9</td>
<td>1.2 ± 0.8</td>
<td>89</td>
<td>61</td>
<td>46</td>
<td>8 ± 3</td>
<td>3 ± 2</td>
<td></td>
</tr>
<tr>
<td>4 mos.</td>
<td>21</td>
<td>7 ± 8</td>
<td>1.5 ± 0.9</td>
<td>90</td>
<td>71</td>
<td>43</td>
<td>8 ± 4</td>
<td>4 ± 3</td>
<td></td>
</tr>
<tr>
<td>8 mos.</td>
<td>20</td>
<td>7 ± 10</td>
<td>6 ± 5</td>
<td>85</td>
<td>85</td>
<td>84</td>
<td>7 ± 2</td>
<td>3 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

a Tracheas were exposed for 4 weeks to DMBA-containing pellets. The tracheas were removed from the host animals immediately after the end of exposure or 2, 4, or 8 months later for in vitro culture.
b The number of attached epithelial-like cells was scored 48 hr after the cells were plated and is expressed as the percentage of total viable cells (attachment frequency).
c When EF attained 1-cm diameter, cells from one-half of each focus were scraped off and transferred into a separate dish. A focus was considered subculturable if it could then be sequentially subcultured 1 more time (third passage). Number of successful subcultures + total attempted subcultures × 100.
d Mean ± S.D.

Table 3

<table>
<thead>
<tr>
<th>Time after exposure</th>
<th>No. of EF tested</th>
<th>% of EF positive for growth in agarose</th>
<th>% of tracheas with at least 1 agarose of positive EF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>13</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>2 mos.</td>
<td>32</td>
<td>34</td>
<td>27</td>
</tr>
<tr>
<td>4 mos.</td>
<td>26</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>8 mos.</td>
<td>49</td>
<td>57</td>
<td>80</td>
</tr>
</tbody>
</table>

e A bottom layer of 0.5% agarose plus a top layer of 0.3% agarose containing 5 × 10^9 cells in each of 2 dishes per cell line.
f Those which had reached a diameter of 1 cm. One-half of each focus was scraped off the dish and passaged separately. After 1 more passage, these cells were tested for growth in agarose.
g Cells were exposed in vivo to 165 μg DMBA and cultured in vitro immediately and at 2, 4, and 8 months after exposure.
h Up to 4 subculturable isolated EF per trachea were tested. Some tracheas, however, did not yield as many as 4 subculturable EF.
i Cell lines with a plating efficiency ≥ 0.004% (i.e., at least 1 colony ≥20 cells on each of 2 dishes seeded with 5 × 10^9 viable cells) were considered positive. Average plating efficiency, 0.04% with a range of 0.004 to 0.1%.

Table 4

<table>
<thead>
<tr>
<th>Time after exposure</th>
<th>% of tracheas yielding mixed-population cell cultures</th>
<th>% of cell cultures yielding tumors</th>
<th>% of tracheas yielding tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>45</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>2 mos.</td>
<td>55</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>4 mos.</td>
<td>76</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>8 mos.</td>
<td>85</td>
<td>78</td>
<td>85</td>
</tr>
</tbody>
</table>

a A total of 22 tumors were observed; of these 21 were diagnosed as squamous cell carcinomas and 1 as an adenocarcinoma. No sarcomas were observed.
time between inoculation of the cell cultures into isogenic recipients and the first appearance of palpable tumor nodules). Chart 3 shows the average total time of observation of mixed-cell population lines broken down into the phases described in a to c. No systematic differences in mean in vitro times were observed between cell cultures established from tracheas immediately or for up to 8 months after DMBA exposure or between tumorigenic and nontumorigenic cell populations. There were no significant differences in mean tumor latencies (time from inoculation of cell lines to development of tumor). The in vitro times ranged from 15 to 25 weeks, and the mean tumor latency periods ranged from 2 to 22 weeks. The tumor latency data for individual mixed-population cell lines are presented in Chart 4. The in vivo tumor latency was 9 weeks or less for most of the cultures. Thus, neither the in vitro times nor the tumor latencies showed any clear trend toward a reduction in the time required for the expression of malignancy, as one might have expected, considering the fact that the tracheal populations harvested from the tracheas at 8 months had as much as 4- to 8-month "head start" over those harvested immediately and up to 4 months after carcinogen exposure. The minimum total time for full expression of oncogenicity to occur in our system was 17 weeks (13 weeks in vitro plus 4 weeks in vivo). This was observed in one of the cultures initiated immediately after exposure.

**DISCUSSION**

Chemical and physical carcinogens cause changes in tissues and cells which are fully realized only many months or years after the carcinogenic insult has occurred, namely, when a recognizable tumor mass begins to invade the tissues of origin. This late emergence of recognizable neoplastic cell populations, long after some precursor cells enter into the neoplastic process following carcinogenic insult, has been an enigma to cancer researchers for decades. The view that in the course of the development of neoplastic diseases successive generations of qualitatively different cell populations evolve has recently received considerable support from various studies (1, 18, 19, 22, 27, 28). However, the significance of these findings for the elucidation of carcinogenesis in vivo needs further study. The present experiments are the first attempt at quantification of the process of carcinogenesis as it develops in vivo in terms of cell populations recognizably altered by carcinogen. The development and use of the EF assay has allowed us to identify and assess some of the quantitative as well as qualitative changes triggered in vivo by carcinogen exposure.

In these studies, we are principally concerned with the identification and quantification of "carcinogen-altered" cells which have, as a result of carcinogen exposure, acquired different growth properties. These properties are not observed in any control cell populations. The growth properties of interest are the capacity of tracheal epithelial cells to form proliferating epithelial foci in vitro which, when isolated, survive subculture in vitro and grow in soft agarose or form carcinomas in vivo. Other procedures used during the EF assay may also cause certain cell subpopulations to be selected. The survival of cells during tissue dissociation, the efficiency with which epithelial cells attach to the primary culture dish, and the survival of cells during freezing and thawing procedures may inadvertently be selective. These inadvertent selection procedures are hopefully being minimized by recent technical improvements. With improved cell isolation techniques, we have increased 2-fold cell viability in the tissue dissociate and increased 10-fold the efficiency with which epithelial-like cells attach to the primary culture dish. Currently available data from these experiments indicate that while this improved technique allows us to culture a larger number of EF from a larger fraction of similarly exposed tracheas, the relative proportions of EF₀, EFₛ, and EFₛₐₜₜ subpopulations detected remain essentially the same. Thus, it appears that in the experiments described in this paper, we have not markedly selected against any one of the cell subpopulations of interest.

The principal observations made in this study are as follows. As a result of exposure of tracheal epithelium to carcinogen in vivo, cells attain an increased in vitro growth potential and are recognizable as EFFU's. With the type of carcinogen exposure used, the incidence of EFFU-containing tracheas was at a constant level of 80 to 90% throughout the study, and the...
frequency of EFFU was more or less stable for 4 months at an average level of about 5 per 10^6 viable cells or 3 EFFU's per trachea. Marked expansion of the pool of EFFU's occurred between 4 and 8 months, to 20 EFFU's per 10^6 viable cells. The EFFU population moved from what appears to have been a steady state into a growth state. Within the EFFU pool, the EFFU increased linearly with time from 53% immediately after carcinogen exposure to 84% at 8 months (Chart 5). The relative frequency of the neoplastic EFFU-ag as measured by growth in agarose also increased from 7 to 48% at 8 months. In contrast, the EFFU pool dropped from 47 to 16%, and the EFFU-ag pool remained constant. The experiments with mixed-population cell cultures inoculated in vivo showed an increase in tracheas yielding oncogenic cell lines from 10 to 65%.

In principle, the observations made with the EF assay and with the mixed-population assay lead to similar conclusions in spite of the fact that the assays differ in important respects. One important difference is the fact that the measurement of tumorigenicity registers a capacity of cells which may not yet exist at the time at which the cells are inoculated into animals, but may develop only several months later. The measurement of growth in agarose, on the other hand, measures a capacity which exists at the time the cells are inoculated in the semisolid medium. Thus, it was perhaps not surprising that the number of EFFU-ag or the percentage of tracheas containing EFFU-ag was low early after carcinogen exposure; but it was unexpected that 80% of the mixed-population cell lines established immediately after carcinogen exposure remained non-tumorigenic, even though the tumorigenesis assay was carried out over a time span of more than 10 months (Chart 3).

Conceivably, the number of tumorigenic or agarose-positive cells was sufficiently small during the early sampling periods to have more frequently escaped detection. Or perhaps the experimental procedures used in the mixed-population assay interfere in some way with a critical early phase of the development of neoplastic cells.

The data reported are consistent with the hypothesis that while the process of initiation is essentially complete with the end of carcinogen exposure (the percentage of tracheas containing EFFU is constant), further development of the neoplastic disease continues after termination of exposure (changes in EFFU population). We believe that our findings are most compatible with a model of neoplastic development involving gradual conversion of EFFU0 to EFFU-ag. In Chart 5, one finds that as time after carcinogen exposure increases, the EFFU0 population decreases, the EFFU-ag population increases, and the EFFU-ag population remains constant. One might interpret this as suggesting that the conversion rates of EFFU0 to EFFU-ag and EFFU-ag to EFFU-ag are roughly similar. Within this model, one or all EFFU pools may be self-replicating, and some subpopulations may never become oncogenic. We cannot presently rule out the alternative to this model of neoplastic progression, namely, the selective growth of an initially very small EFFU-ag subpopulation.

Finally, our findings must be discussed in the light of a tumor induction study involving 86 tracheal transplants which received the same carcinogen exposure. The incidence of invasive carcinoma was 9%, with the first grossly recognizable tumor appearing at 10 months. The study reported here shows an incidence of tracheas containing neoplastic cells which is as much as 5-fold higher, with the first tumor in the mixed-population assay observed 4 months after exposure. Thus, in exposed cell populations isolated in vitro, the expression time (time to first recognizable tumor) is reduced and the frequency of cells with neoplastic potential increased. These studies suggest that normally only a small proportion of the cells endowed with neoplastic potential are allowed to realize their full potential in the intact tissue. Various tissue (7) and host factors appear to prevent them from expanding into recognizable tumors.

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