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Edited by

GEORGE KLEIN

SIDNEY WEINHOUSE

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CONTENTS

Chromosomes and Cancer in the Mouse: Studies in Tumors, Established Cell Lines, and Cell Hybrids

DOROTHY A. MILLER AND ORLANDO J. MILLER

Polyomavirus: An Overview of Its Unique Properties

BEVERLY E. GRIFFIN AND STEPHEN M. DILWORTH

The Pathogenesis of Oncogenic Avian Retroviruses

PAULA J. ENRIETTO AND JOHN A. WYKE

Adjuvant Chemotherapy for Common Solid Tumors

DAVID A. BERSTOCK AND MICHAEL BAUM

NEOPLASTIC DEVELOPMENT IN AIRWAY EPITHELIUM

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I. Introduction

Bronchogenic carcinoma is the most common fatal neoplastic disease in many parts of the world. It has been estimated that in 1982, 129,000 new lung cancer cases will be diagnosed in the United States alone, and that roughly one-quarter of all cancer deaths are due to lung cancer (Surgeon General, 1982).

The etiology of bronchogenic carcinoma is more firmly established than that of most other cancers. The evidence is overwhelming that most lung cancers are caused by tobacco smoke inhalation, the incidence depending on duration of exposure and dose rate (i.e., number of cigarettes smoked per day) (Doll, 1978; Doll and Hill, 1964; Doll and Peto, 1978; Hammond,

ADVANCES IN CANCER RESEARCH, VOL. 39

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1966; Wynder, 1972; Wynder and Graham, 1950; Wynder and Hoffman, 1972; Wynder and Stellman, 1979). With cessation of smoking, the lung cancer incidence rate ceases to increase within a few years, but according to the analysis of most recent data, the risk to develop lung cancer does not return to that of nonsmokers (Doll and Peto, 1978; Peto, 1977), suggesting irreversibility of the sustained damage. In contrast, morphologically recognizable tobacco smoke-induced abnormalities of the bronchial mucosa (e.g., atypical cells) have been reported to subside in ex-smokers (Auerbach *et al.*, 1962, 1977). These seemingly contradictory findings will be discussed later in this article. In addition to cigarette smoking, several occupational agents have been linked to causation of lung cancer (for review see Doll and Peto, 1981; Frank, 1978) such as coke oven effluent (Lloyd, 1971; Radford, 1976), asbestos (Hammond *et al.*, 1979; Selikoff *et al.*, 1968), radioactive contaminants in uranium mines (Archer *et al.*, 1974; Lundin *et al.*, 1969), and bis(chloromethyl)ether (Figueron *et al.*, 1973). It has been estimated that in the United States 10,000–12,000 cases of lung cancer annually might be attributable to occupational exposures (Doll and Peto, 1981; Peto, 1977). Whether urban air pollution is a significant factor in the rising lung cancer incidence of nonsmokers (Doll and Peto, 1981; Mohr *et al.*, 1977) is uncertain and a matter of continuing debate (Doll and Peto, 1981; Enstrom, 1979).

Bronchogenic carcinoma, then, is one of the few types of cancers with a well-defined etiology. What is not certain, however, is what factors other than carcinogen exposure are important determinants of lung cancer induction. There are some leads which indicate that host and environmental variables may play a role. One of these is interindividual variability in carcinogen metabolism and DNA binding of the bronchial epithelium (Harris *et al.*, 1978). This may provide an important basis for differences in lung cancer susceptibility. Another possible factor suggested by several epidemiologic studies is dietary composition. Low intake of vitamin A or carotenoids may predispose to lung cancer induction (for review and discussion see Doll and Peto, 1981; Peto *et al.*, 1981). The effect of dietary variables on cancer development is supported by studies in laboratory animals (Clayson, 1975; Fink and Kritchevsky, 1981; Mass and Kaufman, 1983; Nettesheim, 1980; Newberne and Rogers, 1981; Rogers and Newberne, 1975; Wattenberg, 1975).

Because the etiology of most bronchogenic carcinomas is related either to tobacco smoke, to certain occupational exposures, or to a combination of the two, high-risk groups for lung cancer are relatively easy to define. This makes it feasible to study the pathologic changes preceding the development of invasive cancer of the airways with the hope of constructing a morphologic sequence of preneoplastic stages. This has been accomplished with

considerable success with the use of exfoliative cytology and fiber optic bronchoscopy. The studies by Saccomanno *et al.* on the uranium miners (Saccomanno, 1978; Saccomanno *et al.*, 1965, 1970, 1974) while exemplary are by no means the only efforts of this type (Fontana *et al.*, 1975; Frost *et al.*, 1973; Johnston and Frable, 1976, 1979; Kato *et al.*, 1980, 1982; Kierzenbaum, 1965; Kinsella, 1959; Koss, 1969; Marsh *et al.*, 1973, 1976; Melamed *et al.*, 1977a,b; Nasiell, 1966; Nasiell *et al.*, 1978, 1982; Papanicolaou, 1954; Schreiber, 1978; Woolner *et al.*, 1981).

In recent years, experimental studies in laboratory animals exposed to known respiratory tract carcinogens have been conducted with similar goals in mind, namely, to define the tissue and cell changes leading to cancer of the tracheobronchial mucosa. Because the mucosal pathology observed in these studies is remarkably similar to that described in humans exposed to carcinogens (for review see McDowell *et al.*, 1978; Nettesheim and Griesemer, 1978; Nettesheim and Klein-Szanto, 1982; Nettesheim and Schreiber, 1975; Schreiber, 1978), these experimental studies provide information which is very useful for developing a better understanding of the morphogenesis of bronchogenic carcinoma. The obvious attraction of animal experimentation in this regard is that it offers the opportunity to expose target tissues to known quantities of chemically well-defined carcinogens over predetermined periods. Thus it is easier to address questions concerning, e.g., progression and reversal of putative preneoplastic lesions.

The scope of this article is purposely limited to the subject of "neoplastic development" (Foulds, 1975) in the mucosa of the conducting airways. We intend to summarize and critically analyze primarily the information relevant to the induction and development of those cell and tissue changes which may progress to invasive cancer. Because of this intentionally narrow focus, we will leave largely unattended many important subjects of respiratory tract carcinogenesis such as studies of lung cancer etiology, cocarcinogenic mechanisms, the morphology and biology of different lung cancer types, carcinogen metabolism by airway epithelium, the topographic peculiarities of various lung tumors, and the diverse experimental lung cancer models and their different uses. Many of these subjects have been reviewed (Hanna *et al.*, 1970; Harris, 1978; Harris *et al.*, 1978; Karbe and Park, 1974; McDowell *et al.*, 1978; Nettesheim and Griesemer, 1978; Nettesheim and Klein-Szanto, 1982; Nettesheim and Schreiber, 1975; Nettesheim *et al.*, 1981a; Reznik-Schüller and Reznik, 1979; Saffiotti, 1969; Severi and Stewart, 1966).

The reader will find that many morphologic features of neoplastic development which will be described for the airways are reminiscent of events and changes known to occur in liver carcinogenesis (Farber and Cameron, 1980; Ogawa *et al.*, 1979; Williams, 1980), mammary carcinogenesis (Me-

TABLE I
NEOPLASTIC DISEASE

Induction phase	Interaction of carcinogen with molecular targets
	Fixation of "heritable" cell damage resulting in recruitment of cells into the neoplastic process
Preneoplastic phase	Self-sustained evolution of cellular disorders
	Abnormal cell replication and differentiation
	Progression — promotion
Neoplastic phase	Loss of growth control
	Tumor formation
	Invasion — metastasis

dina, 1978), or cancer development in the cervix uteri (Reagan, 1964). These similarities which cut across not only different target organs, but also a variety of species, suggest that the tissue changes observed during carcinogenesis are common and probably important phenotypic manifestations of the evolving neoplastic disease process. The study of hepatocarcinogenesis has produced much information regarding histochemical and biochemical changes accompanying the various morphologic alterations, some of which are considered to be preneoplastic (e.g., Farber and Cameron, 1980). Such information is virtually nonexistent in the field of respiratory tract carcinogenesis. However, due to recent progress in tissue and cell culture of respiratory tract epithelium (Heckman *et al.*, 1978; Kato *et al.*, 1980; Lane, 1978; Lechner *et al.*, 1981; Marchok *et al.*, 1975; Mossman and Craighead, 1975; Terzaghi and Nettesheim, 1979; Wu *et al.*, 1982), it has been possible to gain insights into the cellular dynamics of neoplastic development which are beginning to add a new dimension to the studies of carcinogenesis (Nettesheim, 1980, 1982; Nettesheim *et al.*, 1982a,b; Terzaghi and Nettesheim, 1982; Terzaghi *et al.*, 1982). Thus, the investigations of neoplastic disease in various organs are shedding light on different aspects of the same family of cellular disorders, rounding out our understanding of the pathogenesis of cancer.

The concept of "neoplastic disease" which underlies the discussions in this article (Table I) encompasses not only the malignant phase of cancer but also the entire evolution of cellular changes which precede and lead up to the invasive and metastatic endphase of the disease. The knowledge we have gained largely during the last two decades about the evolution of this disease as it occurs in the conducting airways is the subject of this article.

II. Experimental Approaches and Methodologies

A variety of experimental models have been developed over the last 25 years to study respiratory tract carcinogenesis in experimental animals. Their characteristics and utilities have been discussed during a series of

conferences (Hanna *et al.*, 1970; Karbe and Park, 1974; Nettesheim *et al.*, 1970; Severi and Stewart, 1966) and in a number of reviews (Kuschner, 1968; Laskin *et al.*, 1970; Nettesheim and Griesemer, 1978; Nettesheim and Klein-Szanto, 1982; Nettesheim and Schreiber, 1975; Saffiotti, 1969, 1970). For the purpose of this presentation, it may suffice to outline briefly the principal features of those respiratory tract carcinogenesis models which have been used specifically for studies of neoplastic development in the tracheobronchial mucosa.

A. *In Vivo* Exposure Systems and Tumor Induction Models

1. *Intratracheal Instillation Methods*

The most popular tumor induction systems in respiratory tract carcinogenesis involve intratracheal instillation of carcinogens using a variety of experimental animals, most commonly rodents. The carcinogens employed, among others, are polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]pyrene (BaP) (e.g., Saffiotti *et al.*, 1968, 1972a,b), metals (Ho and Furst, 1973), or radionuclides (for review see Kennedy and Little, 1978), which are usually delivered as particulates or adsorbed to so-called carrier particles (e.g., FE_2O_3), suspended in a liquid medium for purposes of administration. The particulates deposit primarily in the small airways and alveoli and are cleared by various routes. The rate of clearance depends on the physicochemical characteristics of the material administered, including particle size and solubility (Creasia *et al.*, 1976; Henry and Kaufman, 1973; Saffiotti, 1970). PAHs have been shown to persist in the tissue for at least several days if not weeks following a single administration. Ten to twenty weekly or biweekly instillations are employed in most tumor induction studies. The site of tumor development as well as the morphologic spectrum of tumors induced in various respiratory carcinogenesis models depend on a number of variables such as the animal species, the physicochemical properties of the carcinogen, and the carcinogen dose (Becci *et al.*, 1978b; Blair, 1974; Mass and Kaufman, 1982; Montesano *et al.*, 1970a,b; Mossman and Craighead, 1978, 1979; Schreiber *et al.*, 1975a; Yarita and Nettesheim, 1979; Yoshimoti *et al.*, 1980). The most extensive studies to characterize the intratracheal instillation system have been carried out by Saffiotti and his collaborators (Montesano *et al.*, 1970a; Saffiotti, 1969, 1970; Saffiotti *et al.*, 1968, 1972a,b). Except for undifferentiated small cell carcinoma, all major types of lung cancers can be readily induced. Because intratracheal instillation is the most commonly used method for carcinogen exposure of respiratory tract tissues, much of the information concerning morphogenesis of airway neoplasia stems from studies employing this method. However, with some noted exceptions, most of this information is anecdotal. The method has some serious limitations if employed in morphogenetic studies: many

airway segments are exposed simultaneously and the investigator has little control over local dose, dose rate, or duration of exposure because of the uneven distribution and persistence of the instilled material in various parts of the airways.

2. *Pellet Implantation Technique*

A method which has to be regarded as a major advancement in studying morphogenesis of bronchogenic carcinoma is the pellet implantation technique, first developed by Kuschner *et al.*, (1957, 1966, Laskin *et al.*, 1970) in rats and subsequently adapted for use in other species. Pellets containing the test materials (e.g., radionuclides, PAHs, chromates) are fabricated to fit the size of the airway which is to be exposed. The pellets are introduced into the bronchus and are fixed in place. The leaching chemical exposes the underlying mucosa. In the case of radionuclides, the pellet serves as a radiation source from which various types of radiation are emitted depending on the nature of the radionuclide used. The advantage of this exposure system is that a limited, preselected area of airway mucosa is exposed to a known concentration of carcinogen, thus the resulting lesions have the same exposure history, when collected at the same time after start of exposure. A possible disadvantage is damage to the airway which can result in obstruction and pneumonia. While the earlier studies were carried out in rodents, more recently the method has been adapted for experimentation in dogs (Matsumura *et al.*, 1978). A further modification of this approach is the injection of carcinogen into the submucosa of large airways with the aid of a fiberoptic bronchoscope (Cohen *et al.*, 1978; Hayata *et al.*, 1977; Kato *et al.*, 1982; Matsumura *et al.*, 1978). Initial studies suggest that this experimental model may provide many new opportunities to study the development of bronchogenic carcinoma with the diagnostic tools commonly used in the clinic. Even though, it might be difficult to avoid severely toxic tissue reactions.

3. *Tracheal Lavage Method*

Another "localized tumor induction system," so-called because exposure occurs in a specific location and limited region of the organ system, which has good potential for studies of neoplastic development, is the tracheal lavage technique (Schreiber *et al.*, 1975b; Yarita and Nettesheim, 1978). A segment of trachea is rinsed in the anesthetized animals with the use of a double cannula (Fig. 1). The washing fluid which contains the water-soluble carcinogen such as methyl- or ethylnitrosourea is delivered through one of the cannulas and collected with the other. Only 5–10 mm of trachea are exposed. This method was originally developed to collect exfoliating cells from rat or hamster airways (Schreiber and Nettesheim, 1972). To induce

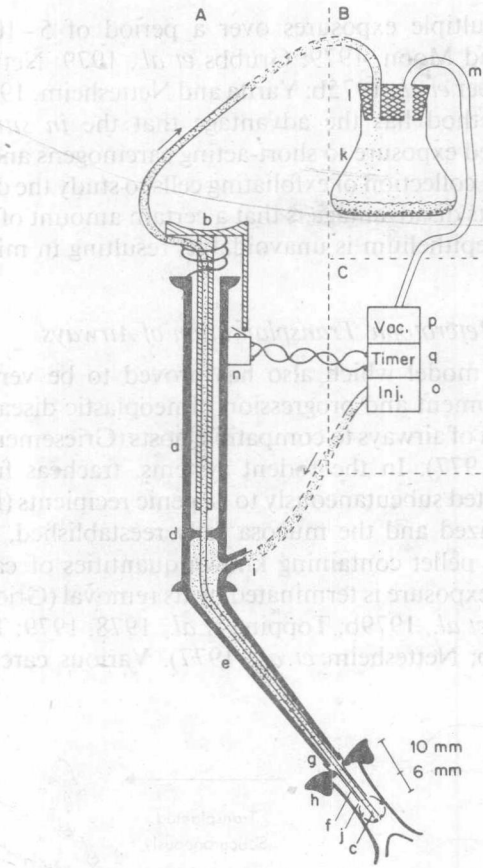

FIG. 1. Intratracheal catheter. (A) Catheter consisting of barrel (a) with plunger (b) to which aspiration tubing (c) is mounted. Slide seal (d) has an opening for the tubing. A shaft (e) is attached to the lower end of the barrel; its distal end is covered by a short, stiff Teflon tube (f) (o.d. 1.6 mm, i.d. 1.2 mm) which is inserted 10 mm deep into the trachea until the joint (g) of the shaft with the tube stops at the vocal cords (h). The carcinogen solution enters the shaft through an opening (i) and leaves through an annular outlet (0.1 mm wide) (j), washes 6 mm of the tracheal wall, and enters the aspiration tubing at its distal end (polyethylene tube—o.d. 1 mm, i.d. 0.6 mm) which has been pushed out through the tip of the outlet (j) after insertion of the catheter so that it projects 6 mm into the trachea. (B) Collection system. The upper end of the collection vessel (k) is sealed by a rubber stopper (l) which holds perforations for the aspiration tube (through which the aspirated carcinogen solution is delivered) and the vacuum line (m). (C) Regulating unit. This unit is turned on by the microswitch (n) when the plunger (b) is pushed down. The automated injector (o) (Sage Model 237-1) injects the 1-ml carcinogen solution. Simultaneously, the vacuum valve (p) opens the vacuum line for recovery of the carcinogen solution at the distal end of the aspiration tube; the timer (q) terminates the injection and the vacuum after 5 seconds. (Modified from Schreiber and Nettesheim, 1972.)

tracheal tumors, multiple exposures over a period of 5–10 weeks are required (Grubbs and Moon, 1979; Grubbs *et al.*, 1979; Nettesheim and Yarita, 1979; Schreiber *et al.*, 1975b; Yarita and Nettesheim, 1978; Yarita *et al.*, 1978). This method has the advantage that the *in situ* trachea is accessible for repeated exposure to short-acting carcinogens and cocarcinogens as well as for the collection of exfoliating cells to study the development of mucosal lesions. Its disadvantage is that a certain amount of traumatization of the tracheal epithelium is unavoidable, resulting in mild epithelial hyperplasia.

4. *Heterotopic Transplantation of Airways*

An experimental model which also has proved to be very useful for studying the development and progression of neoplastic disease is heterotopic transplantation of airways to compatible hosts (Griesemer *et al.*, 1977; Nettesheim *et al.*, 1977). In the rodent systems, tracheas from normal donors are transplanted subcutaneously to isogenic recipients (Fig. 2). After the graft is vascularized and the mucosa fully reestablished, which takes about 2–3 weeks, a pellet containing known quantities of carcinogen is inserted at one end; exposure is terminated by its removal (Griesemer *et al.*, 1977; Klein-Szanto *et al.*, 1979b; Topping *et al.*, 1978, 1979; Topping and Nettesheim, 1980a,b; Nettesheim *et al.*, 1977). Various carcinogen dose

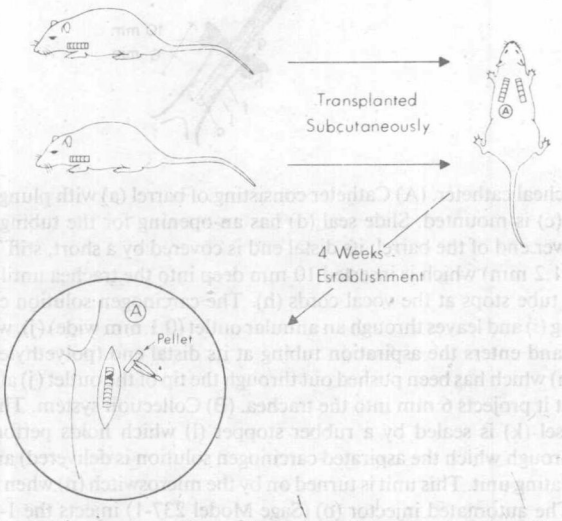

FIG. 2. Diagrammatic representation of the tracheal transplant system. Tracheas are grafted subcutaneously on the backs of syngenetic recipients. Four weeks later when the grafts are fully established they are opened at one end and pellets containing the carcinogen are inserted. (From Nettesheim *et al.*, 1978.)

rates can be achieved by changing the carcinogen concentrations and/or the composition of the pellet matrix (Pal *et al.*, 1978). A number of modifications of this experimental approach have been introduced. These include the development of an open-ended, flow-through tracheal implant system which has several distinct advantages (Shiba and Marchok, 1983). Multiple exposures to low carcinogen doses can be administered which should greatly reduce acute toxicity. The physical form of the carcinogen is not limiting, i.e., it can be solid, liquid, or an aerosol and can be introduced into or passed through the tracheal lumen at a constant flow rate. With this model, exfoliating cells can easily be collected from the lumen for diagnosis of epithelial changes without sacrifice of the tissue. Another new development is the transplantation of human airway mucosa (Harris, 1976; Okamoto *et al.*, 1980; Shimosato *et al.*, 1980; Valerio *et al.*, 1981) or of human airway epithelial cells (Klein-Szanto and Terzaghi, unpublished) to immunodeficient nude mice. Preliminary reports describe the induction of carcinomas by chemical carcinogens in such heterotransplants (Okamoto *et al.*, 1980; Shimosato *et al.*, 1980). We expect that these methods will lead to many exciting studies of carcinogenesis of human airway epithelium. The advantages of the airway transplantation systems for studies of neoplastic development are that the carcinogen exposure is well defined and limited to a small segment of airway.

B. *In Vivo–in Vitro* Systems for the Analysis of Neoplastic Development

The major limitation of the experimental models described above is that the endpoints measured are mostly morphological. Morphologic diagnosis necessitates destruction of the very cells and tissues being studied and precludes tracking of the descendants of a given, carcinogen-exposed cell population through the course of neoplastic development. To overcome this limitation, combined *in vivo–in vitro* models of carcinogenesis were developed which make it possible to study a gamut of cellular and biochemical changes in cell populations retrieved from tracheobronchial mucosa at different stages of tumor induction, without disrupting the continuity of successive cell generations. Very early stages in carcinogenesis can be studied, as "carcinogen-altered" epithelial cells have a selective survival and growth advantage *in vitro* over most other cells, which becomes apparent soon after carcinogen exposure. At predetermined time intervals after administration of carcinogen *in vivo*, epithelial cell populations are obtained from the exposed tracheas to assess the stage of neoplastic development or to study "neoplastic progression" *in vitro*.

1. *The Tracheal Explant-Outgrowth System*

To study early biological changes of the epithelium during the postinitiation phase resulting from *in vivo* exposure of tracheas to carcinogen, small tissue explants are established *in vitro* from preexposed tracheas (Heckman *et al.*, 1978; Marchok *et al.*, 1977, 1978). The explant cultures are maintained for 7–10 days in a culture medium which stimulates the outgrowth of epithelium, resulting in primary epithelial cell cultures (Fig. 3A and B).

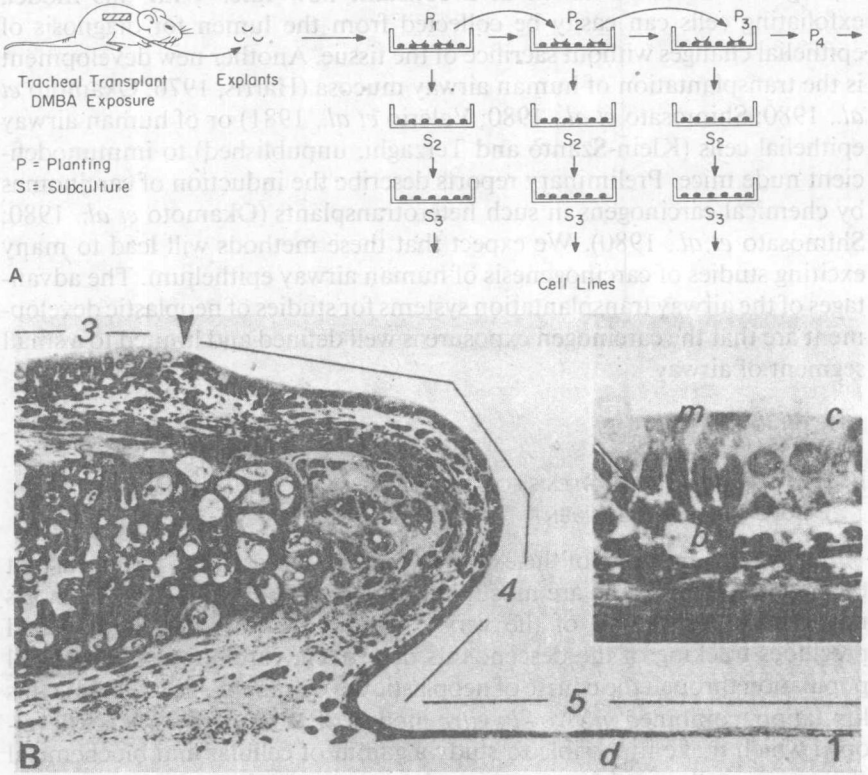

FIG. 3. *In vivo–in vitro* system for the establishment of epithelial cell cultures from tracheal transplants preexposed *in vivo* to carcinogens. (A) Diagrammatic representation of procedure. (B) Cross section of a tracheal explant and outgrowth after 4 days in culture. The epithelium extends from the lumenal surface to the culture dish (d). In labeling index determinations, zone 5 comprises the outgrowth and zone 4 the side of the explant. Zone 3 comprises the 20 cells extending from the end of the elastic lamina (arrow) on to the lumenal surface. Zones 2 and 1 are not shown, but include the next 20 cells and central portion of the lumenal surface, respectively. Inset: Differentiated cells on an explant after 4 days in culture. Normal ciliated (c), mucous (m), and basal (b) cells are present. In some areas of the epithelium, the intercellular spaces appear broadened (arrow). ×1000. (Modified from Nettesheim *et al.*, 1978; Heckman *et al.*, 1978.)