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IN VIVO-IN VITRO STUDIES OF THE EVOLUTION OF EPITHELIAL NEOPLASIA

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DISCLAIMER

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I. Introduction: The Hypothesis of Neoplastic Development

Typically the process of carcinogenesis as observed in vivo is characterized by a long latency period which is, as the term implies, concealed and more or less clinically asymptomatic. Depending on dose, dose rate, and potency of the carcinogenic insult, months, years, or even decades elapse between the first exposure and the appearance of the neoplasm. In the case of chronic, low-level carcinogen exposure, this is in part a function of the accumulation of a "critical" dose, but long latency periods are also observed following single carcinogenic insults. Since it is known that persistance of the carcinogen is not required for the later development of malignancy, genetic or epigenetic changes must have occurred which are fully expressed only when the appearance of a "tumor" signals the presence of a cell population endowed with destructive and unlimited growth capacity. What events occur in the time span between exposure and the onset of recognizable tumor growth is still largely unknown.

From a multitude of clinical and experimental observations (for review and discussion see references 11, 12, 13) the concept has developed that following the "initiating" event(s), progressively changing cell populations emerge which possess an increasing propensity to acquire the biological properties of cancer cells. Morphologically and biochemically abnormal cells appear during the latency period which consistutes what are believed to be preneoplastic lesions. Depending on the animal species and the organ

system, these have been described as hyperplastic areas, early and late hyperplastic nodules, and hyperbasophilic foci (liver); as metaplasias with varying degrees of atypia, dysplasias, and carcinomas *in situ* (uterine cervix, bladder, larynx, and bronchus); and as alveolar hyperplasias, ductal hyperplasias, or atypical lobules (mammary gland) to name just a few (for discussion see 35).

The concept, however, that neoplastic disease is an evolutionary progression in which "initiated" cells give rise to successive generations of preneoplastic cells and these in turn give rise to neoplastic progeny, is largely based on inferences and circumstantial evidence. While this evidence is indeed highly suggestive, the exact relationships between the various presumed precursor lesions on one hand and between them and full fledged neoplasia on the other hand, are not known. A temporal sequence does not necessarily imply a progenitor-progeny relationship. This lack of real insight into the process of neoplastic development provokes many fundamental questions. Are the carcinogen-induced structural cell and tissue abnormalities which can be observed in epithelial tissues phenotypic expressions of the neoplastic process, i.e. are all of these lesions precursors of cancer, or are some perhaps "end-stage" lesions? Are the apparent sequential changes following carcinogen exposure morphological manifestations of an evolutionary progression in the sense of progenitor-progeny relationships? An alternative is that they are independent expressions of qualitatively and quantitatively different abnormalities

having varying "incubation" periods. Are some of the lesions reversible-abortive attempts of neoplastic development? What stages of neoplastic development can be discerned? What stages do various lesions represent? Is neoplastic development perhaps a process of selection rather than progression? Or is it both? These and many other related questions pose great challenges to the students of carcinogenesis. They are of fundamental significance for our understanding of the development of epithelial neoplasias and are also of practical importance if we are to make progress in the early diagnosis and therapy of neoplastic diseases.

One of the most common fatal neoplasias in man is bronchogenic carcinoma. Its etiology is clearly linked to tobacco smoke and certain occupational exposures such as coke oven effluents, asbestos, chromates, uranium ore dust, and others (for a recent review see 14). Time and dose dependent cytological and histological abnormalities have been reported in smokers and in smoking uranium miners and other "high risk" occupations (2,3,4,15,30). A description of a temporal sequence of lesion development, largely based on cytological specimens (29) lists "regular" metaplasia as the earliest and probably most non-specific change, followed by metaplasia with varying degrees of cellular atypia (and histological disorganization) carcinoma <u>in situ</u> and invasive carcinoma. A variety of tumor induction studies (e.g. 18, 31, and for review see 27) as well as several morphogenesis studies with periodic sampling of tissue or cytology specimens (1,20,26,32),

clearly indicate that a morphological sequence, similar to that described in humans, can be produced in laboratory animals by radiation as well as chemical carcinogens. In principal, this provides the basis for experimental studies of the putative precursor lesions observed during the evolution of respiratory tract neoplasia. However, numerous technical and logistic obstacles need to be overcome in order to make such investigations feasible: More often than not the diagnosis of preneoplasia is made by morphological means, which in most cases preclude further study. Even if the visual observations are made on live tissue with the aid of a bronchoscope, the possible heterogeneity within lesions and the usually small tissue mass available for study present serious difficulties. In addition, the most important information concerning the "exposure history" of any given lesion is almost certainly missing: How old is the lesion, to what carcinogen dose was the tissue of origin exposed, what is the duration of exposure, when did exposure cease, etc.? The fact that both in man as well as in most animal models it is impossible to predict from what precise target area the lesion(s) to be studied will arise, introduces another serious complication. In the most widely used experimental lung cancer models, the carcinogen is introduced into the airways by inhalation or by intratracheal injection. In either case it is difficult to determine with any degree of accuracy the carcinogen dose sustained by a given area of tracheal or bronchial mucosa.

To systematically investigate the evolution of neoplasia in respiratory tract epithelium from its inception and the development and fate of preneoplastic lesions, it was necessary to design an experimental system that would minimize these problems. In the following we will describe the development of such an experimental model and discuss our recent findings obtained in studies concerned with the evolution of epithelial neoplasia. Finally we will discuss related investigations of other investigators.

II. An Experimental Model for the Study of Incipient Neoplasia

The essential features of the experimental model are as follows: (a) Carcinogen exposure is carried out in vivo. A preselected area of respiratory tract tissue, namely trachea, is exposed to a known quantity of carcinogen for a predetermined period of time. (b) To establish epithelial cultures in vitro and to expand the exposed cell populations, the pre-exposed tissues or cells are placed in vitro under conditions favoring growth of epithelial cells. (c) Abnormal growth behavior is used as an indicator for "carcinogen altered" (initiated?) cell populations. (d) Phenotypic changes indicative of neoplastic transformation are sought during continued in vitro maintenance of epithelial cultures.

A. In vivo Exposure System

1. Heterotopic tracheal transplants

It was previously shown by Crocker and Nielsen (9) that tracheas from suckling rats can be transplanted successfully into isogenic recipients and that such transplants survive indefinitely. After initial studies in a variety of animal species (19), we chose the Fisher-344 inbred line of rats for our transplantation experiments. Donor and recipient animals are 8-12 weeks old. Tracheas are transplanted subcutaneously on the backs of appropriately prepared recipients near the scapula, one trachea on each side (F-1). During the first few days the transplants live by diffusion, and many of the epithelial cells die and slough off. However between days 3 and 4, vascularization of the graft occurs followed by a marked regenerative response of the remaining epithelium. By day 7 the tracheal graft assumes a near-normal appearance and is lined by tall columnar mucociliary epithelium. By 2 weeks the grafted trachea is practically indistinguishable from the hosts own trachea (16). F-1

2. Carcinogen exposure

Routinely tracheal transplants are exposed to carcinogens 4 weeks after grafting by inserting carcinogen-containing pellets into the tracheal lumen. Tumor induction studies (17,26) showed the tumor response to be carcinogen dose dependent (Table I). The majority of the tumors are squamous cell carcinomas. The appearance of carcinomas is preceded by various epithelial abnormalities and focal lesions, T-I

metaplasias with and without cellular atypias of varying degrees (Fig. 2) F-2
similar to those described in humans. In the *in vivo-in vitro* studies
to be described below, tracheas exposed in this manner to different
doses of DMBA were used as source of "initiated" tissues or cells.

B. *In vitro* Systems for Analysis of Cellular Changes

Two different approaches are being used to study the initiated
cells *in vitro*. The first one is an explant-outgrowth system (23-25)
in which epithelial cell cultures are established from outgrowths
which develop when pieces of trachea are placed on the bottom of
tissue culture dishes (Fig. 3). Multiple outgrowths can be obtained F-3
from the same explant by repeated planting (Fig. 4) In this way cells F-4
are allowed to establish themselves in culture over a period of days
or a few weeks with a minimum amount of disruption and trauma. In the
second approach (37), the entire epithelium is removed from pre-
exposed tracheas by a combination of mechanical and enzymatic procedures
and dispersed cells are seeded into culture dishes. In this case the
in vitro study starts with a known number of cells, which are all at
the same time submitted to a considerable trauma and stress.

III. *In vivo-In vitro* Studies with the Explant-Outgrowth System

Studies were designed to determine whether tracheal epithelium
exposed *in vivo* for a relatively short period of time would acquire
in vitro growth characteristics different from that of normal tracheal
epithelium (22,24). Tracheal grafts were removed from their hosts

after exposure to DMBA for 2 weeks, during which time 150 µg and 640 µg of the carcinogen was delivered from pellets containing 165 µg and 1000 µg of DMBA respectively. Control grafts were either not exposed, received empty pellets, or pellets containing 0.5% croton oil. Histological examination of sections prepared from randomly selected tracheal grafts showed that the entire mucosal surface of tracheas exposed to the two DMBA doses had been converted to a thick squamous epithelium. The various control tracheas showed mostly hyperplastic (croton oil) or normal mucociliary epithelium (Fig. 5). Multiple outgrowths were established from explants obtained from these tracheas by repeated in vitro planting. F-5

The most striking observation made during this part of the experiment is that many outgrowths established from carcinogen-exposed tracheal pieces survived as primary cultures after removal of the explants, while outgrowths from control explants did not survive beyond 3 weeks in Waymouth's medium containing fetal bovine serum with or without insulin and hydrocortisone. Only when the medium was enriched with additional amino acids, fatty acids, putrescine, and sodium pyruvate could primary cultures from control tracheas be maintained (Table II). T-II

The next step in the experiment was to determine whether the primaries established from the various tracheal explants could be subcultured. As can be seen in Table II, cell lines were established from the low as well as the high carcinogen dose groups. In marked contrast,

no cell lines could be established from the control primaries even with use of the enriched medium. Typical examples of the morphology of some of the primary cell cultures as well as the subcultured cell lines are shown in Figure 6a & b. All the primaries as well as the cell lines were epithelial in character. Many of the cell lines stained positive for keratin with the Papanicolaou procedure. Others clearly showed the presence of keratohyalin granules in the top layers of the sloughing cells. The epithelial nature of the cell lines was further ascertained by electron microscopic demonstration of desmosomes and tonofilaments (Fig. 6c). F-6

We then determined whether the cells that have acquired the capacity to grow indefinitely in vitro are neoplastic, or will become so after propagation in vitro. The cell lines obtained from the low and high carcinogenic doses were propagated continuously in vitro and were tested periodically for two characteristics--loss of anchorage dependency of growth and in vivo tumorigenicity. The results of this last phase of the study are summarized in Figure 7. F-7

The epithelial cell lines derived from tracheas exposed to the low carcinogen dose were negative for growth in soft agar and for in vivo tumorigenicity when first tested after about 150 days in culture. Two tested positive for growth in soft agar after 350 days, but so far are negative in the tumorigenicity test.

Some of the cell lines derived from tracheas exposed to the high DMBA dose were already positive in soft agar when first tested between

100 and 150 days in culture. The others tested positive between 200-400 days in culture. Once the cell lines had become positive, the efficiency for colony formation in agar increased with subsequent passages (see 22). Six out of 8 cell lines have become tumorigenic between 175-425 days in culture. The tumorigenicity tests were carried out in immunosuppressed, isogeneic recipients. Two of the cell lines produced tumors that regressed when first tested (1000 CD, 1000 MG). Invasive carcinomas developed upon inoculation of later passages. All tumorigenic lines have produced invasive keratinizing squamous cell carcinomas at one time or other (Fig. 8). F-8

The following conclusions can be drawn from the experiments described above. (1) Carcinogen-exposed tracheal epithelium acquired a markedly enhanced in vitro growth capacity as early as 2 weeks after start of exposure, i.e. many months before tumors develop in the original hosts. This alteration manifested itself in two ways: primary epithelial cultures from carcinogen-exposed tracheas had much simpler nutritional requirements than cultures from control tracheas; and permanent cell lines could be established only from cultures derived from carcinogen-exposed tracheas. (2) Progression of neoplastic development occurs in vitro. This was evident from three types of observations: cell lines that were negative in the test for anchorage independent growth at early passages became positive at later passages; cell lines first negative in the tumorigenicity test became positive later on; two of the cell lines first producing regressing tumors produced invasive carcinomas when inoculated at later passage.

It is clear from this study, that, among the cells harvested from tracheas at the termination of a 2-week exposure to the high carcinogen dose, were the progenitors of later appearing neoplastic cells. It remains to be seen whether the cells that have acquired the capacity to survive in cell culture after exposure to the low dose of DMBA will also eventually exhibit tumorigenicity.

IV. In vivo-In vitro Studies with Dispersed Epithelial Cells

In the experiments reported so far, carcinogen-exposed tracheal mucosa was sampled and studied in vitro at the end of a short carcinogen exposure period. We decided that it would be important to know whether cells sampled at different times during the "post initiation phase," i.e., at different times after termination of the in vivo carcinogen exposure, would similarly show an increased in vitro growth capacity. Would the relative frequency with which such cells occur increase or decrease as a function of time? Would the growth rate of cells obtained early after initiation differ from that of cells obtained late after initiation? Would cells sampled late in the post-initiation phase show signs of neoplastic transformation earlier or at higher frequency than cells sampled early? Would we be able to identify any "markers" indicative of the relative position in the neoplastic evolution in which cell populations obtained at various times after initiation, have arrived?

The studies initiated to approach these questions are currently in progress (37) and are therefore still incomplete. They have nevertheless, even at this early date, yielded important information.

The carcinogen exposure was carried out as detailed above. Tracheal transplants were exposed for 4 weeks (instead of 2 weeks as in the previous study) to beeswax pellets containing 165 µg of DMBA. At this time the pellets were removed. The DMBA dose delivered was 160 µg. Cells were removed from tracheal transplants by trypsinization at 0 time (i.e., time of pellet removal), 8 weeks, 16 weeks, and 32 weeks post-exposure and were seeded in vitro.

Total cell yields, percent viability (dye exclusion), and general cytology of the cell suspension were noted. The cells obtained from each tissue were plated separately at comparable viable cell densities. Forty-eight hours after seeding, each dish was scored to determine the total number of attached epithelial cells (seeding efficiency). Two weeks after seeding, the dishes were scored for proliferating epithelial foci and the size of each focus was recorded. All dishes were monitored as necessary for changes in size of each focus or disintegration of foci previously detected. Foci attaining a size of 1 cm in diameter were isolated and subcultured. After two to three successive passages, such cell populations were frozen down for future studies.

The rationale for particular details of the above described protocol are as follows. (1) Parameters such as total cell yields, viability, seeding efficiency, and cell morphology allow us to make some judgement

concerning state of the tissue under study, e.g. whether the tissue is atrophic, hyperplastic, or metaplastic. (2) The presence of proliferating foci at 2 weeks or more after plating is an early indication that cells with an "altered" in vitro behavior are present (normal cells do not survive for more than a few days). (3) Changes in the size of individual foci with time yield some measure of the relative in vitro growth rate of the cells constituting a particular focus. (4) The capacity of a focus-derived population to proliferate through the third passage completes the initial phase of testing for altered growth potential in vitro. (5) Frozen, focus-derived cell populations will be used in future in vivo and in vitro studies to detect markers of neoplastic transformation in populations obtained either at a fixed time interval after exposure to different carcinogen doses or at various time intervals after exposure to the same carcinogen dose.

Table 3 summarizes preliminary data obtained at various times after a 4-week exposure of tracheal transplants to a dose of 160 µg of DMBA. Several points are of interest. Except for the group tested at 8 weeks post-exposure (this group is presently being retested), the fraction of tracheas with proliferating foci (1 month after plating) is remarkably constant from 0 time to 32 weeks post-exposure. Second, the fraction of tracheas with proliferating foci which yield 2nd and 3rd passage cell strains also remains relatively constant (60-75%). Third, the minimal time necessary for foci to attain a size of 1 cm diameter is very similar (22-25 days) at all time intervals tested. Likewise, the distribution of T-3

growth rates of the most rapidly proliferating foci in positive tracheas remains quite similar from 0 to 32 weeks after exposure (Fig. 9). The latter 3 observations suggest that neither subculturability nor in vitro growth rate reflect the relative degree to which the carcinogen-induced alteration approaches the neoplastic state (or the relative position in the latency period). Because the first tumors can be detected histologically in vivo at 32 weeks (unpublished observation), one would expect systematic changes in these parameters from 0 to 32 weeks if they reflected more than the presence of the initial carcinogen-induced alteration. Fourth, (Table 3) the average number of proliferating foci per positive trachea (tracheas with 1 or more proliferating foci) remains essentially constant until 16 weeks after exposure but is markedly increased at 32 weeks post-exposure. We believe that this suggests the proliferative expansion of already existing lesions in the tracheas rather than development of new ones since neither the number of tracheas with foci change with time (except for the possible reduction at 8 weeks) nor the proportion of foci which can be subcultured. F-9

In order to demonstrate the applicability of this approach to other organ systems and to carcinogens administered systemically, rats were given total doses of 225 and 450 mg/kg of body weight of n-nitroso-heptamethylen-eimine (NHMI) intragastrically over periods of 15 and 30 weeks respectively. Cells were then removed by trypsinization from the lungs, tracheas, and esophagi at 3 weeks and 20 weeks after the last exposure, the exact procedure varying with the particular tissue involved. The results from this pilot study are summarized in Table 4. Following systemic nitrosamine administration, cells with an altered growth capacity can be detected in T-4

the lung, trachea, and esophagus with the in vitro assay. All three organs are known to be targets for the carcinogenic action of NHMI (36). The number of animals with "altered" cell populations in these target organs increases as a function of time after exposure. This is in contrast to the observations made following topical administration of DMBA to the trachea. The reason for this apparent difference is presently not clear. With larger sample sizes and additional dose groups now under study, we hope to elucidate this discrepancy.

These experiments, though incomplete, support in general the findings of the in vivo-in vitro studies using the explant-outgrowth system since they also show the appearance of cells endowed with an increased in vitro growth capacity in carcinogen exposed organs during the tumor latency period. Noteworthy is the finding in the study with DMBA that the number of tracheas yielding in vitro foci and cell lines remains relatively constant throughout the 32 weeks studied, but that the number of foci that can be obtained from these tracheas dramatically increases at this last time point. This suggests that the size of the carcinogen-altered cell populations within these tracheas may remain constant for many weeks until, at 32 weeks, they have started to expand. Final conclusions can be drawn only when we have obtained information on the neoplastic potential of the cell lines derived from the foci. Also crucial for interpretation of the data are the results from the ongoing in vivo studies concerned with the development and fate of carcinogen-induced epithelial lesions.

V. Discussion: Altered *in vivo* Growth Capacity, A Common Feature of Carcinogen-Exposed Cells

The main purpose of the experiments described above is to develop means for the investigation of the "neoplastic process" as it occurs in fully matured and organized epithelial organs or tissues. We therefore exposed respiratory tract mucosa to carcinogens *in vivo* and studied subsequently the altered growth behavior of epithelial cell populations obtained from the pre-exposed mucosa at different times during the "latency" period. This *in vitro* assay, while introducing a certain artificiality into the studies, provides the opportunity to expand the cell populations we hope to investigate, to observe them while alive, and to study them quantitatively under controlled and manipulable conditions. The term "neoplastic process" as used in this context is meant to encompass all events occurring subsequent to initiation. Thus it deals also with those cell and tissue alterations that are not included in the term neoplasia but, if recognized at all, are usually categorized as preneoplastic.

In vitro studies with preneoplastic and early neoplastic lesions, i.e. tissues initiated *in vivo*, are not new. The first investigations seem to have been carried out with human tissues. Wilbanks reviewed in 1969 (38) and in 1975 (39) tissue culture studies concerned with various preneoplastic lesions of the uteri. He and others have found that epithelial cells from such lesions are endowed with an increased survival and growth capacity as measured *in vitro*. Cells from cervical intraepithelial neoplasia could be subcultured 30-50 times, unlike epithelial cells from normal cervix. Other investigators (8) have drawn attention

to altered in vitro growth characteristics of mesenchymal cells underlying such cervical lesions.

Shabad and his collaborators reported in 1966 and in a number of subsequent publications (for reference, see 33) similar findings in initiated animal tissues. These investigators described that explants from embryonic lung tissue of rats and mice, transplacentally exposed to various carcinogens, exhibit a marked enhancement of growth in vitro. They also reported development of adenomas after culture of such tissues for 4-14 days. Subsequently several other workers extended these observations made on tissues transplacentally exposed to carcinogens. DiPaolo (10) developed a host-mediated in vivo-in vitro assay for chemical carcinogenesis using transplacental exposure of hamster embryos to a variety of carcinogens. Laerum and Rajewsky (21) and Roscoe and Claisse (28) found that glial cells from rat fetuses exposed to N-ethyl-N-nitrosourea had acquired increased growth capacities as determined in vitro. The former authors also showed that such cells acquired through a series of stages, neoplastic characteristics, in vitro. Similar observations were reported by Borland and Hard (5,6) who showed that cells isolated from kidneys of rats injected with a single dose of dimethylnitrosamine (DMN) have acquired an increased in vitro survival capacity. While control cells die off after a few passages, cells (probably fibroblasts) from carcinogen-exposed rats isolated from 20 hours to seven days following DMN injection can be continuously cultured. Such cells exhibited various phenotypic characteristics believed to be associated with neoplastic transformation. Observations made by Brand and his collaborators (7) in foreign body tumorigenesis

studies also indicate that during the process of carcinogenesis *in vivo*, cells appear which can be detected *in vitro* by virtue of their growth advantage over normal cells.

All of these studies, including our own, demonstrate that carcinogen-exposed cells of various origins have one feature in common, a markedly increased capacity to survive and grow *in vitro*. This profound change occurs very early, long before neoplastic cells appear. It is tempting to speculate that this acquisition of altered growth control constitutes one of the fundamental changes occurring during the early developmental phase of the neoplastic process. Obviously at this stage, during the tumor latency period, control of growth is not completely lost since there is no indication that the cells, when left *in vivo* or when reinjected into compatible hosts, grow without limit. Thus *in vivo*, regulatory mechanisms, not existing or functioning *in vitro*, keep the carcinogen-altered cell population in check preventing the expression of this newly acquired growth potential.

We do not mean to imply that all cells having acquired this increased growth capacity will necessarily become malignant. Conceivably, however, such cells have an increased propensity to become neoplastic. This increased growth capacity may even be a prerequisite for the ultimate expression of the neoplastic state. It may be one of the changes required, but by itself not sufficient, for the development of neoplasia. In our initial studies (see Fig. 8) several epithelial cell lines derived from tracheas exposed to the low carcinogen dose did *not* become neoplastic even after *in vitro* maintenance for over 450 days. Recent experiments on the

effects of the promoting agent tetradecanoyl phorbol acetate (TPA) on tracheal epithelium in vitro (34) have shown that TPA, which is not (or only weakly) carcinogenic, can also induce an increased growth capacity. Whether these cell lines obtained from TPA-exposed epithelial cells will show signs of transformation remains to be determined. This will be important for the interpretation of the data discussed here and may help to elucidate the significance of the alteration of growth control in the early developmental phase of the neoplastic process.

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Fig. 1. Diagrammatic representation of the *in vivo* carcinogen exposure system using heterotopic tracheal transplants.

Fig. 2. Metaplastic lesions induced in heterotopic transplants by carcinogenic aromatic polycyclic hydrocarbons.

a) nonkeratinizing metaplasia with little or no cellular atypia, orderly stratification.

b) cornifying metaplasia with disorderly arrangement of closely packed small, undifferentiated cells.

c) nonkeratinizing metaplasia with considerable cellular atypia and loss of polarity in some parts of the lesion.

d) nonkeratinizing (left) and keratinizing (right) lesion with almost complete loss of differentiation and stratification in the left half of the lesion, cellular atypia and lymphocyte infiltration.

Fig. 3. Tracheal explant with outgrowth

a) Explant above center of the outgrowth, which is a mixed epithelial cell-fibroblast culture SEM.

b) Cross-section through explant showing outgrowth cells which have migrated onto the culture dish.

Fig. 4. Diagrammatic representation of the *in vivo-in vitro* system for the establishment of epithelial cell cultures from tracheal transplants pre-exposed *in vivo* to carcinogens.

Fig. 5. Morphological appearance of explants and early outgrowth.

a) mucosa from control trachea

b) mucosa from DMBA-exposed trachea showing extensive squamous metaplasia

c) early outgrowth from DMBA-exposed explant, explant margin visible in left upper corner. Marchok, et al., 1977 (24).

Fig. 6. Epithelial cell cultures derived from DMBA-exposed explants.

a) primary culture, 60 days after removal of explant

b) cell line derived from above primary culture. Note similar morphological appearance and piling up of cells in several areas.

c) electron micrograph showing desmosomes and tonofibrils of cultured cells, indicative of their epithelial nature. Marchok, et al., 1977 (24).

Fig. 7. Diagrammatic representation of development of phenotypic changes in cell lines derived from carcinogen-exposed tracheal epithelium. Cross-hatched area indicates when cell line tested positive for growth in 0.3% agar; black area indicates when cell line tested positive for tumorigenicity (0.5-1.0 X 10^6 cells i.m.) and produced invasive squamous cell carcinomas; grey area indicates that cell line produced benign tumors (or keratinic cysts) which regressed after several weeks. Cell line designation on left; circled numbers indicate number of passages at that time. Marchok, 1977 (22).

Fig. 8. Tumors formed in immunosuppressed rats following i.m. inoculation of epithelial cell line (1000 MG, see Fig. 7).

a) highly keratinizing cystic tumor which subsequently regressed induced by inoculation of cells from passage 13.

b) invasive keratinizing squamous cell carcinoma following inoculation of cells from passage 26.

Fig. 9. Growth index of epithelial foci derived from carcinogen-exposed tracheas. Horizontal axis: time required for the first focus of any trachea producing foci to reach a size of 1 cm in diameter; vertical axis: the relative number of tracheas with (first) foci of a given growth rate. Numbers in right corner of each frame indicate duration of in vivo carcinogen exposure and postexposure time in vivo in weeks. Terzaghi, 1977 (37).

Table I*

Twenty-two months tumorigenesis study with low DMBA doses

DMBA (μg) per pellet	Effective no. of tracheas	Time until carcinoma development (months)[a]								Total number of tracheas with histologically confirmed carcinomas
		8	10	12	14	16	18	20	22[b]	
210	18	2[c]	1	4	2	3	1		(2)4	17
115	18	3[d]		5	1	2			(3)4	15
40	18			1	1				(2)1	3
10	18								(3)	0
Beeswax only	16								(2)	0
Untreated	32								(4)	0

[a] The time when progressively enlarging tumors reached 1.0 cm in size. All tumors were confirmed histologically. Two tracheas were grafted per rat.

[b] All tracheas remaining after 22 months were harvested and examined microscopically for evidence of neoplasia.

[c] Number of tracheas with tumors. The numbers in parentheses indicate number of transplants with sarcomas.

[d] One tracheal carcinoma metastasized to the lung.

*Griesemer, et al., 1977 (17).

Table II*

Establishment of primary cell cultures and cell lines from successive plantings of tracheal explants exposed to DMBA

Group / Medium[a]	1st Planting			2nd Planting			3rd Planting			4th Planting			5th Planting			6th Planting			7th Planting		
	E	M/O	S/T[b]	E	M/O	S/T	E	M/O	S/T	E	M/O	S/T	E	M/O	S/T	E	M/O	S/T	E	M/O	S/T
I (150 µg DMBA)																					
W-FBS	11	0/11	—	11	2/9	2/2	9	1/7	—	6	0/2	—	2	0/2	—						
Wihc-FBS	6	0/6	—	6	0/6	—	6	3/6	3/3	6[c]	3/6	3/3	6	1/6	—						
II (640 µg DMBA)																					
W-FBS	10	2/10	—	10	1/2	1/1	2	1/2	—	2	1/2	—	2	1/2	0/1	2	0/2	—	2	0/2	—
Wihc-FBS	5	2/5	2/2	5	2/5	2/2	5	3/5	2/2	5	3/5	2/2	2	1/2	1/1	4	2/4	1/1	4	2/2	1/1
													2[c]	2/2	2/2						
III (0 DMBA)																					
W-FBS	11	0/0	—	11	0/11	—	11	0/11	—	11	0/11	—	11[c]	6/9	0/6	11[c]	2/11	0/2	11[c]	4/11	0/2
Wihc-FBS	11	0/11	—	11	0/11	—	11	0/11	—	11	0/11	—	11[c]	3/10	0/3	10[c]	6/9	0/3	8[c]	7/8	0/4

[a] Medium in which cultures were initiated at the first planting. All explants were moved into Wihc-FBS at the second planting.

[b] E, number of explants; M/O, number of primary cultures maintained/number of outgrowths; S/T, number of primary cultures subcultured for at least five passages/number of primary cultures tested.

[c] Explants moved into WRihc-FBS medium

*Marchok, et al., 1977 (25).

Table III

Focus formation and establishment of cell lines from tracheal epithelium obtained at different times after 4 weeks of exposure to DMBA.

Weeks after pellet removal	Total number of tracheas	Percent tracheas with proliferating foci 1 month after plating *in vitro*=A	Percent tracheas yielding 2nd and 3rd passage cell strains=B	B/A X 100	Average number proliferating foci per trachea	Earliest time (days) to 1 cm diam. focus after plating *in vitro*
0	20	80	60	75	2.7	23
8	18	39	23	60	2.4	24
16	21	81	55	68	4	22
32	20	74	58	73	12	25
Control	15	0	0	0	0	--

*Terzaghi, 1977 (37)

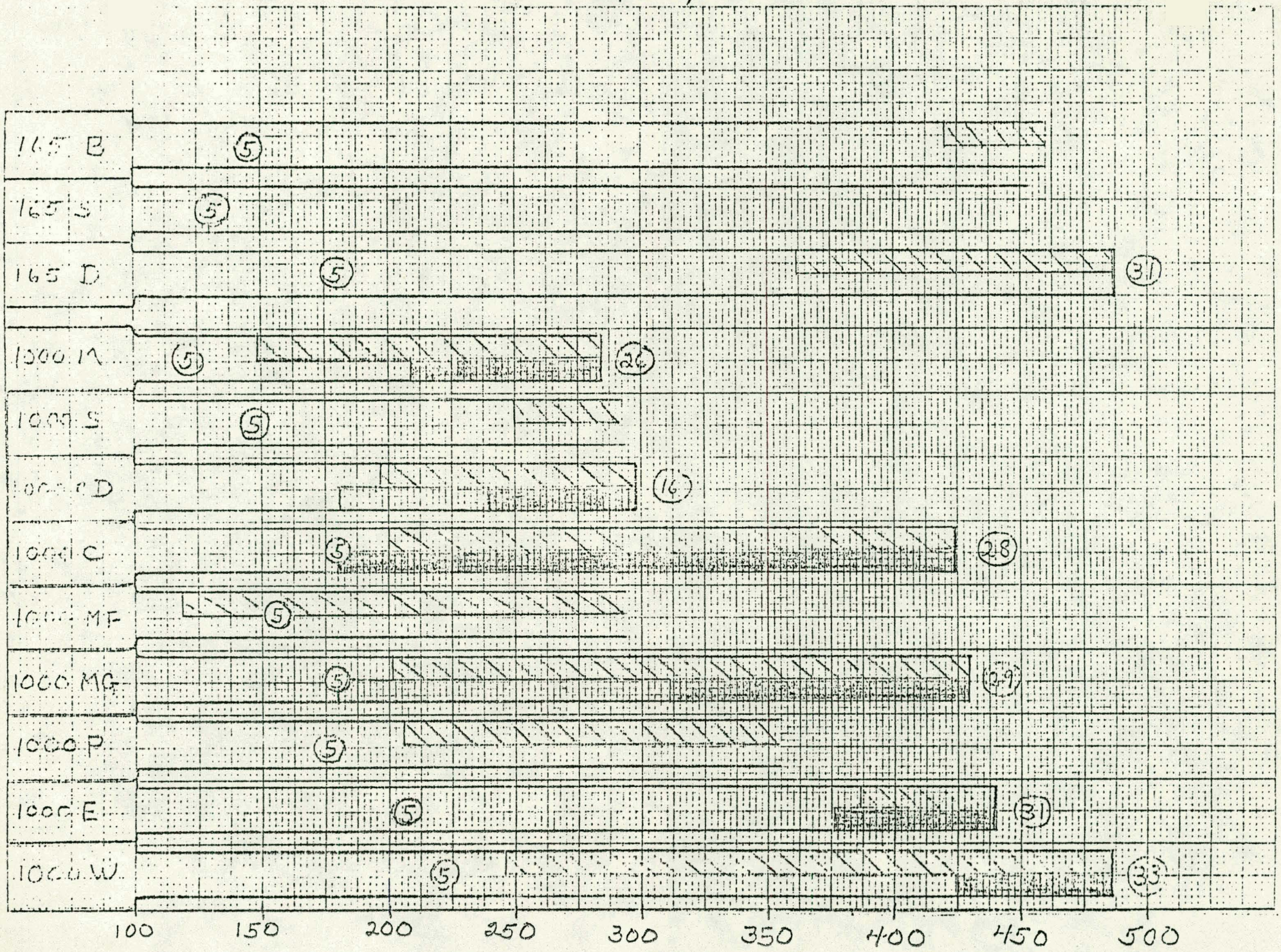
Table IV*

Focus formation with epithelium from different organs obtained after exposure to N-nitroso-heptamethyleneimine.

Total dose mg/kg	Weeks after last exposure	Number of rats with proliferating foci in different organs	
225	3	trachea	0/3
		lung	0/3
	20	trachea	1/3
		lung	1/3
450	3	trachea	0/3
		lung	2/3
	20	trachea	4/4
		lung	4/4
		esophagus	2/2

*Terzaghi, 1977 (37)

Fig. 7


165 B
165 S
165 D
1000 M
1000 S
1000 D
1000 C
1000 MF
1000 MG
1000 P
1000 E
1000 W
5
5
5
26
5
16
3
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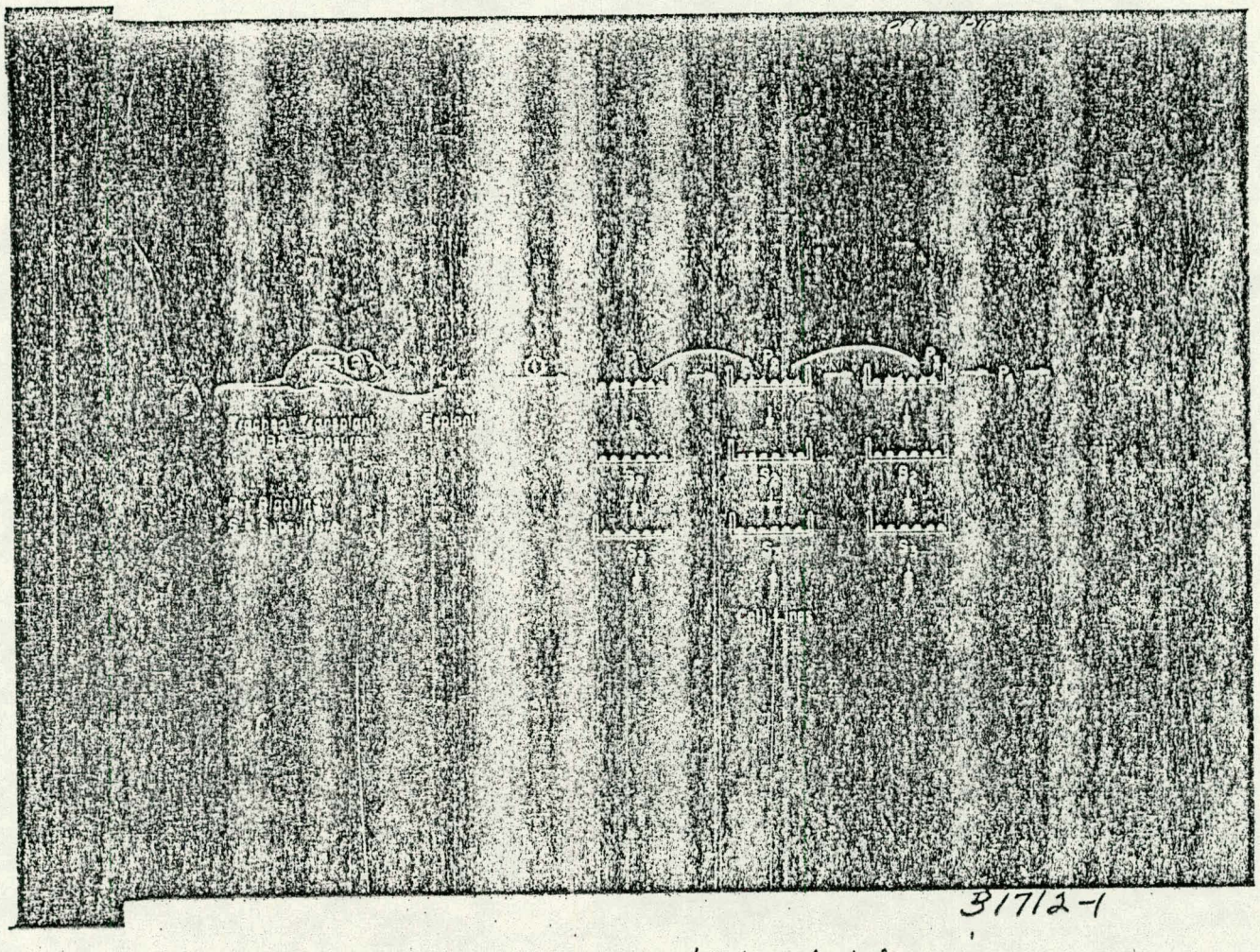
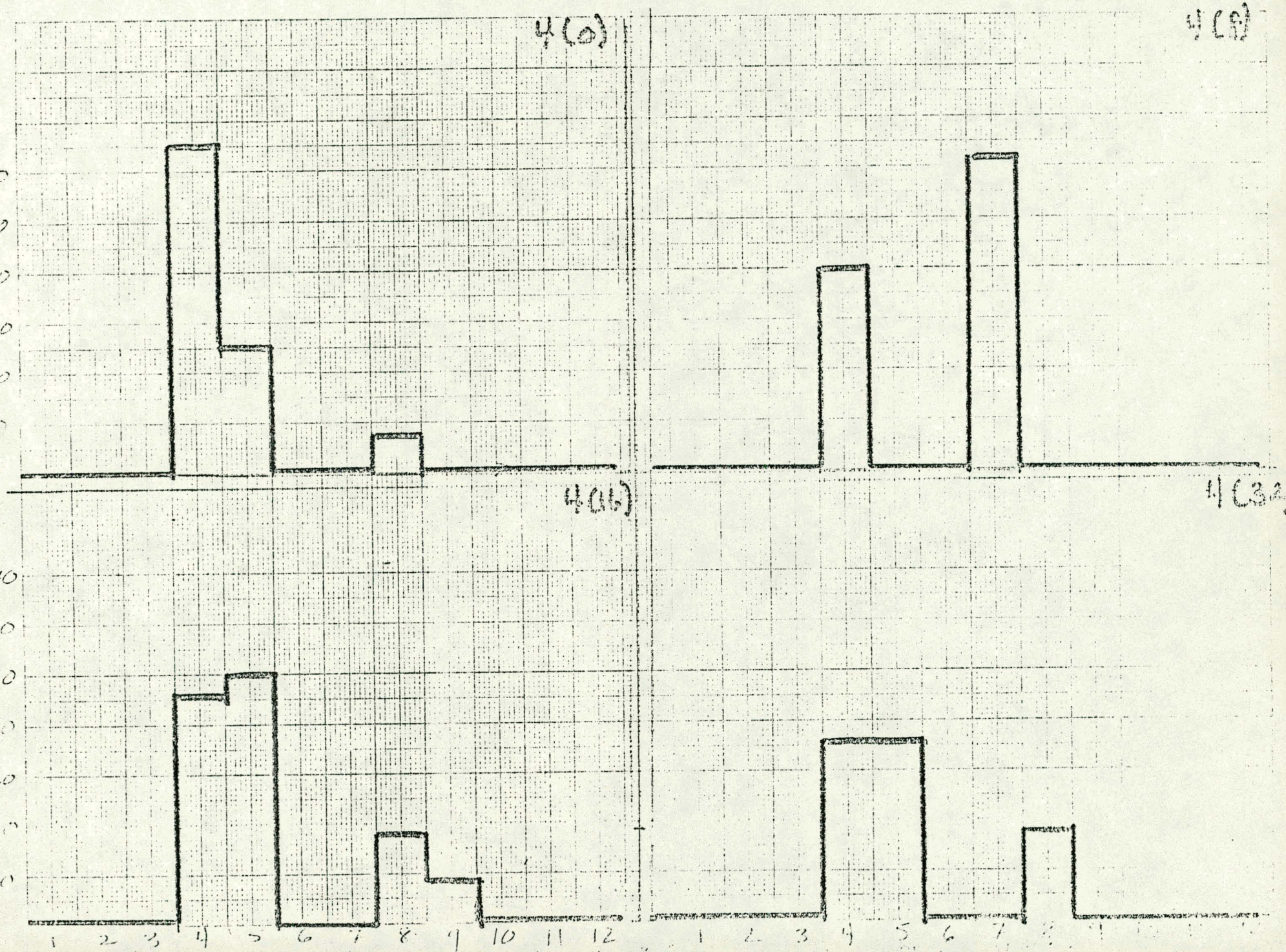
3
31
5
33
100
150
200
250
300
350
400
450
500
Time in vitro (Days)



31712-1

Diagrammatic representation of the in vivo-in vitro system for establishing epithelial cell lines from tracheal transplants exposed in vivo to carcinogen.

F189


4(a)
4(b)
4(c)
4(d)
4(e)
4(f)
10 Millimeters to the Centimeter
% of Total Rainfall
60
50
40
30
20
10
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7

32912

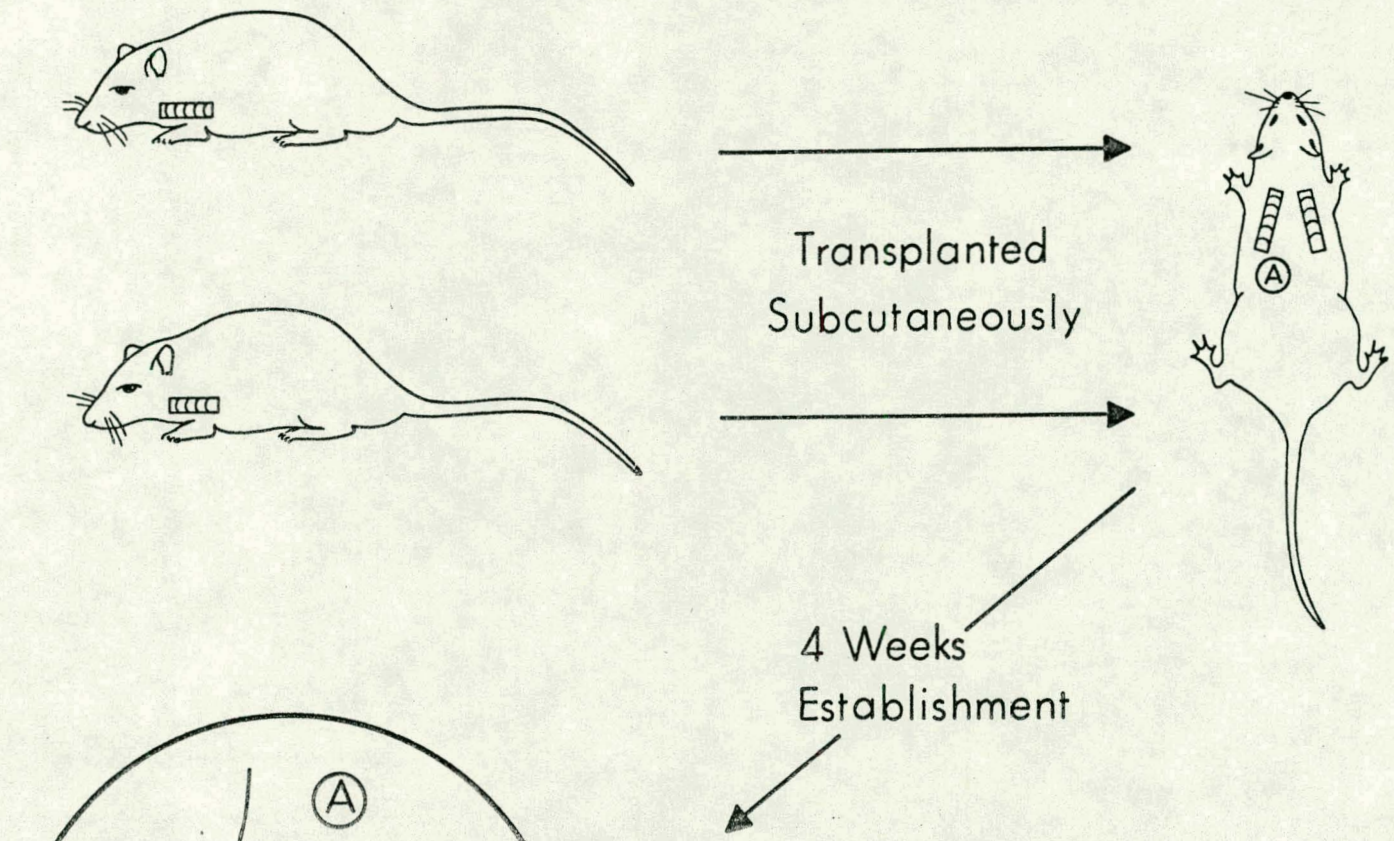

Transplanted
Subcutaneously
A
4 Weeks
Establishment
A
Pellet

Fig. 1

33162

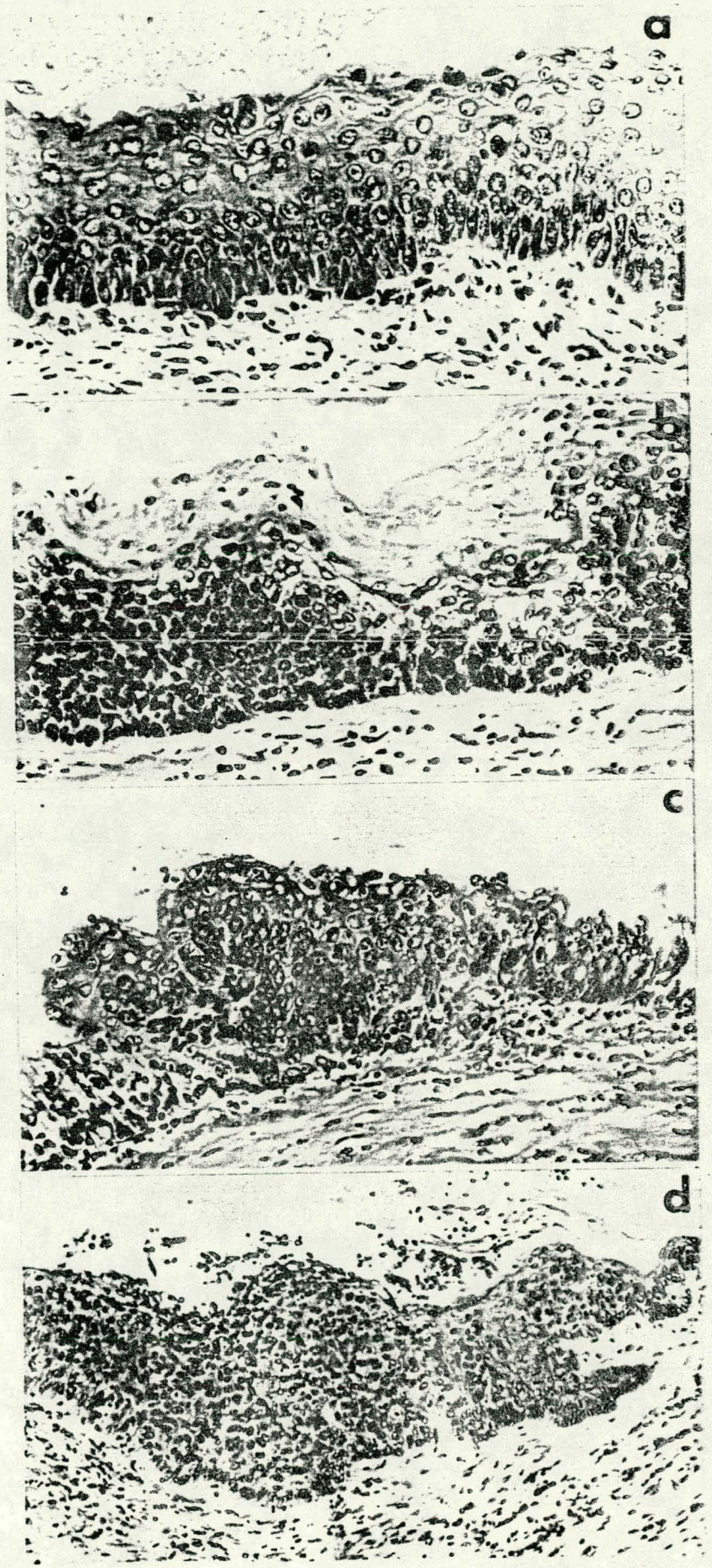


Fig. 2

33164

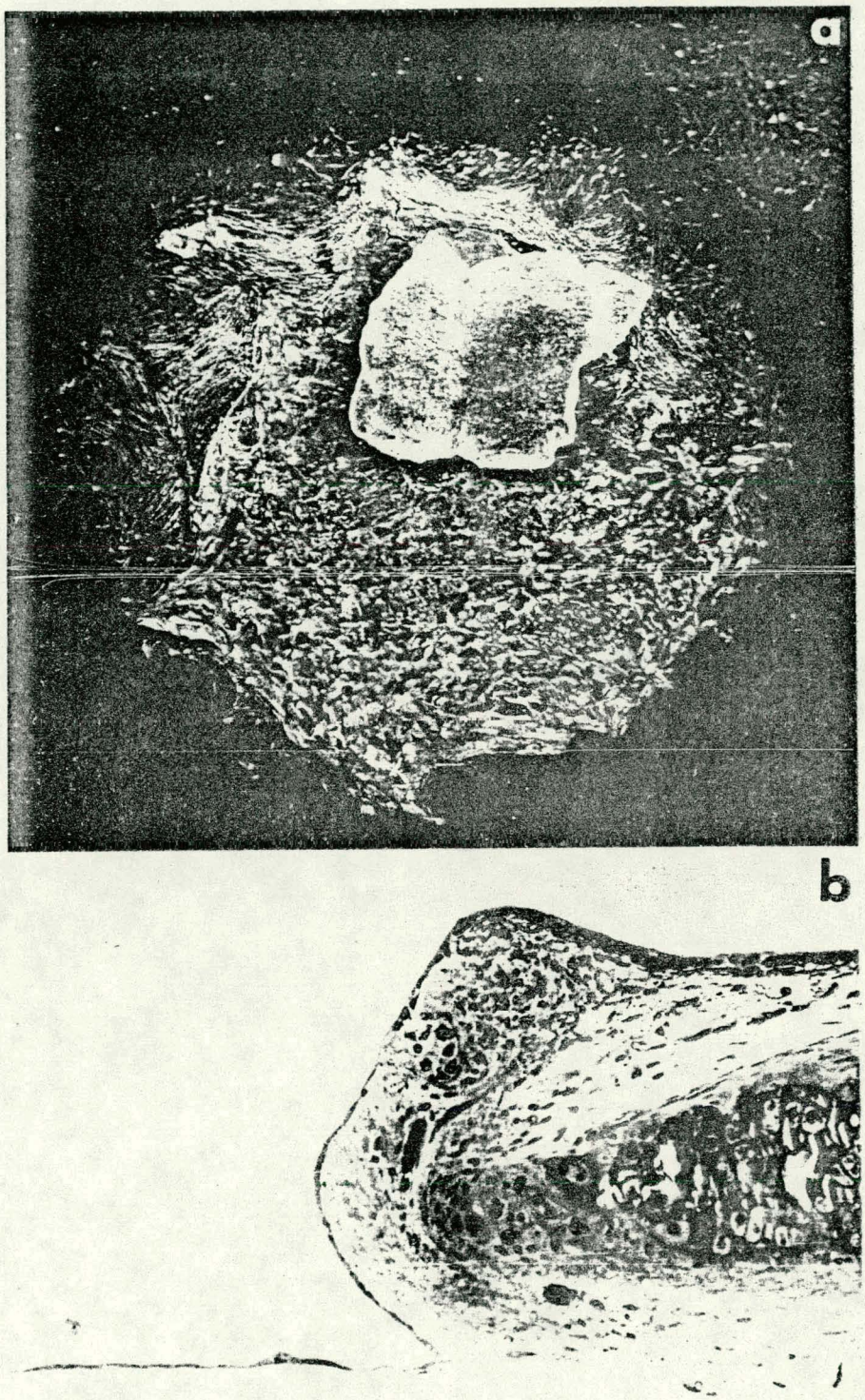


Fig. 3

31712-1

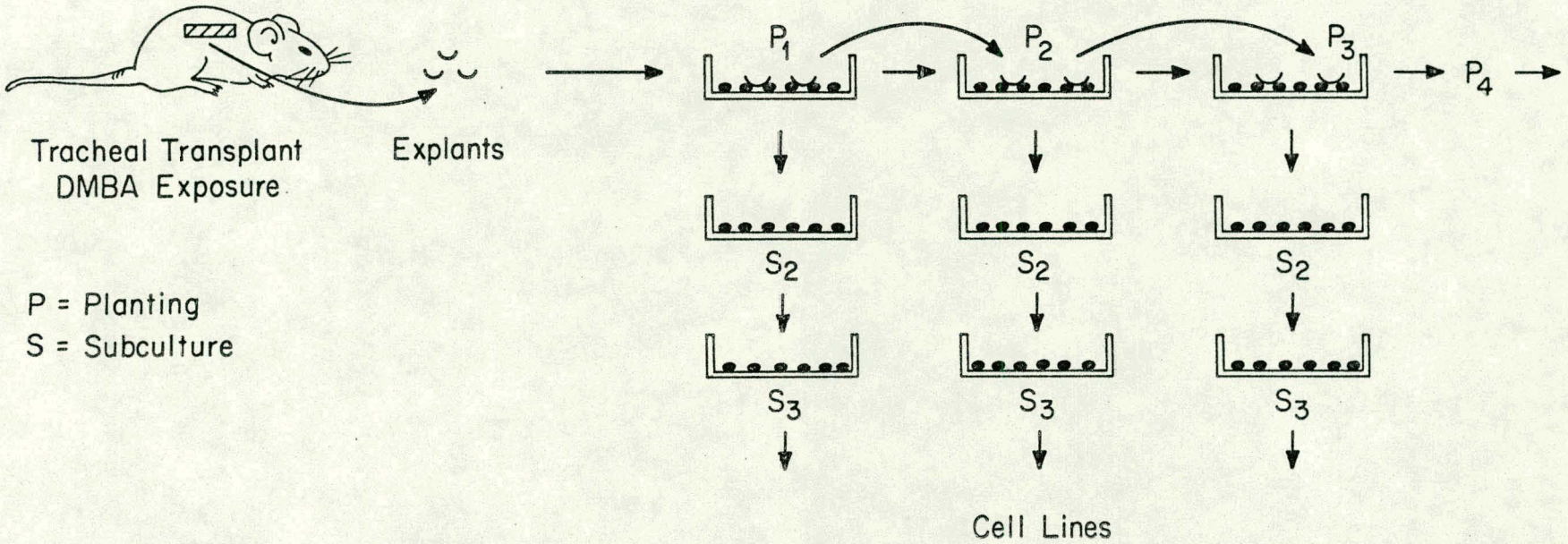

Tracheal Transplant
DMBA Exposure
Explants
P = Planting
S = Subculture
P1
P2
P3
P4
S2
S2
S2
S3
S3
S3
Cell Lines

Fig. 4

33166

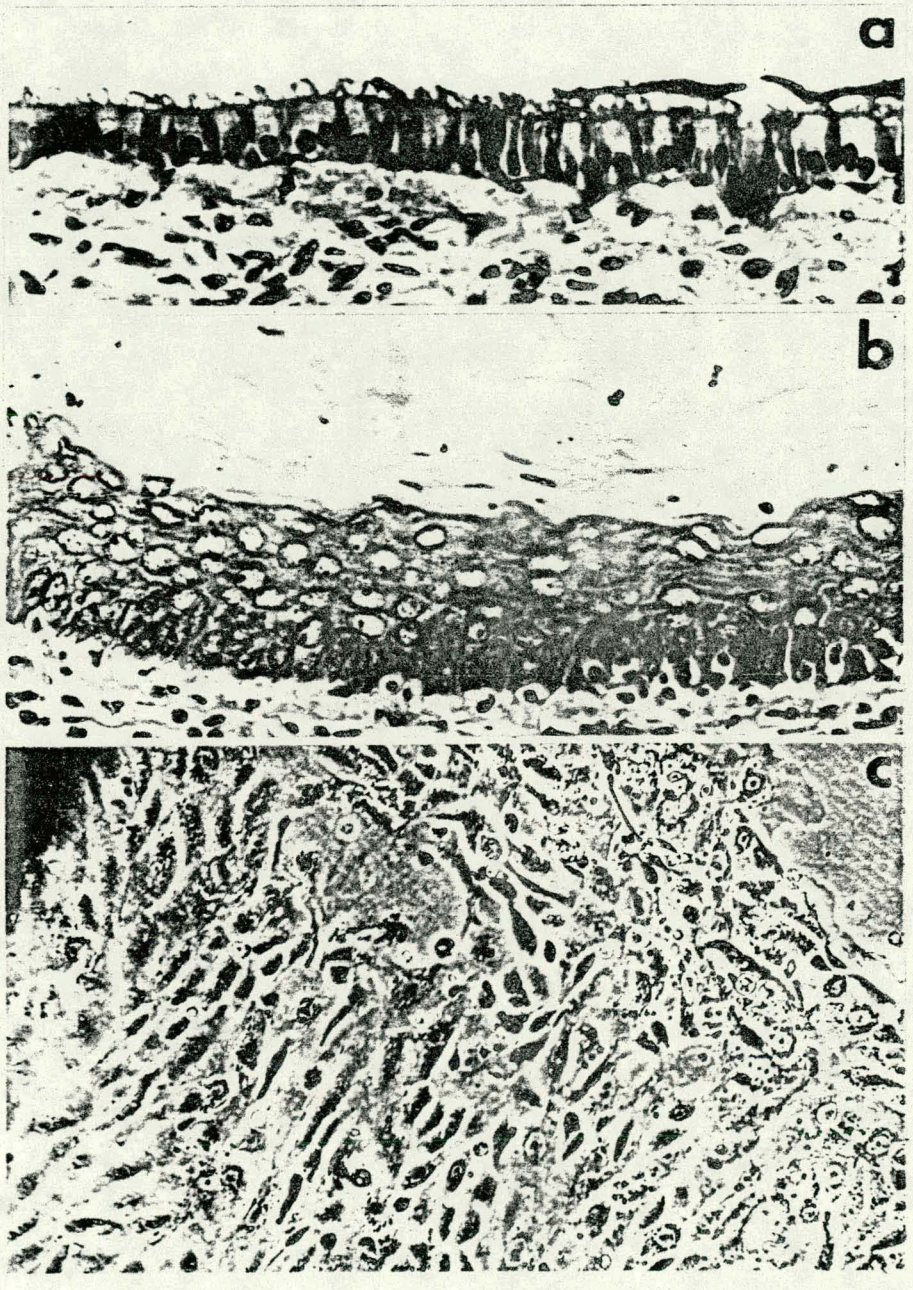


Fig. 5

33167

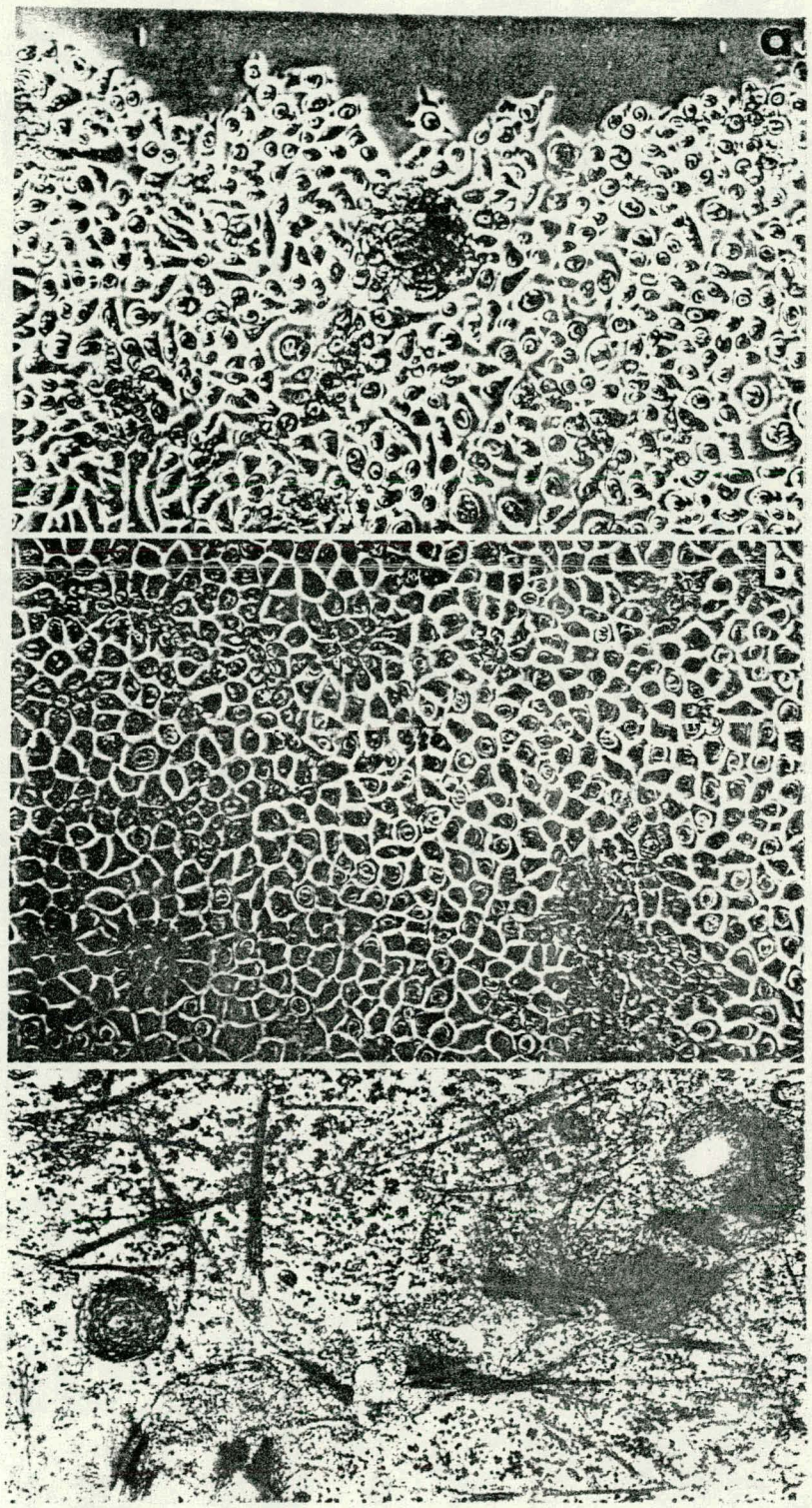


Fig. 6

33168

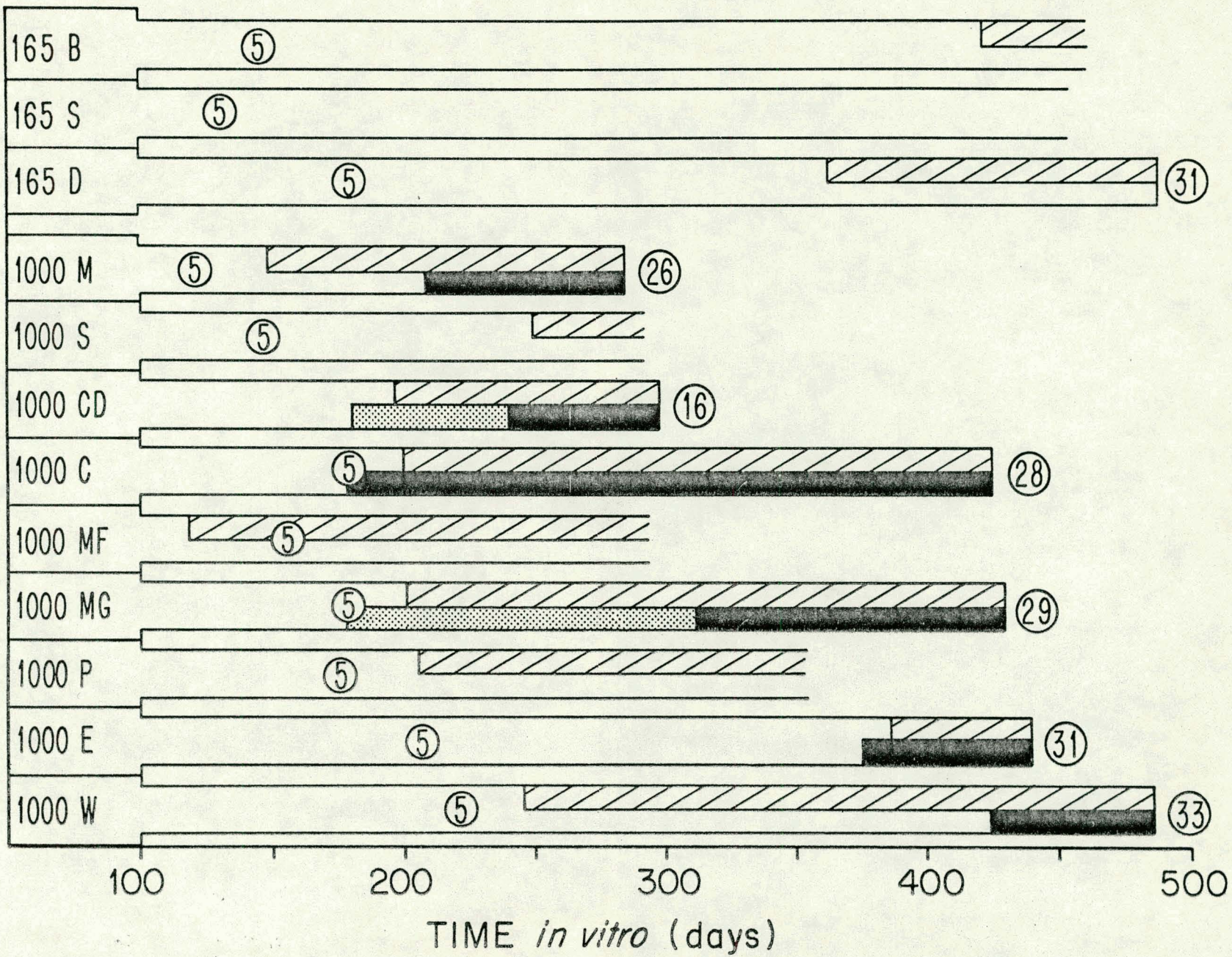

165 B
165 S
165 D
1000 M
1000 S
1000 CD
1000 C
1000 MF
1000 MG
1000 P
1000 E
1000 W
5
31
26
16
28
29
33
100
200
300
400
500
TIME *in vitro* (days)

Fig. 7

33170

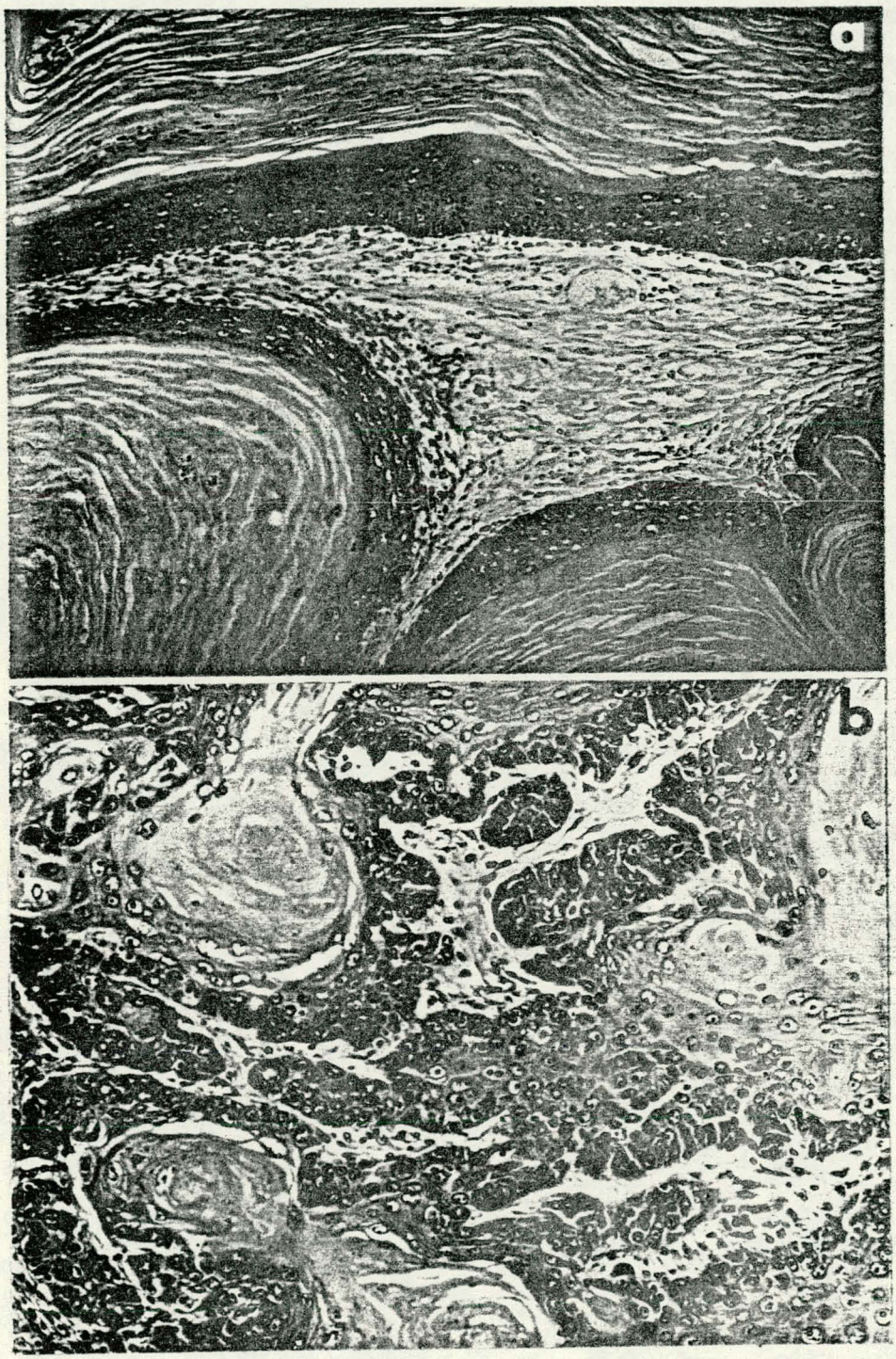


Fig. 8

33165

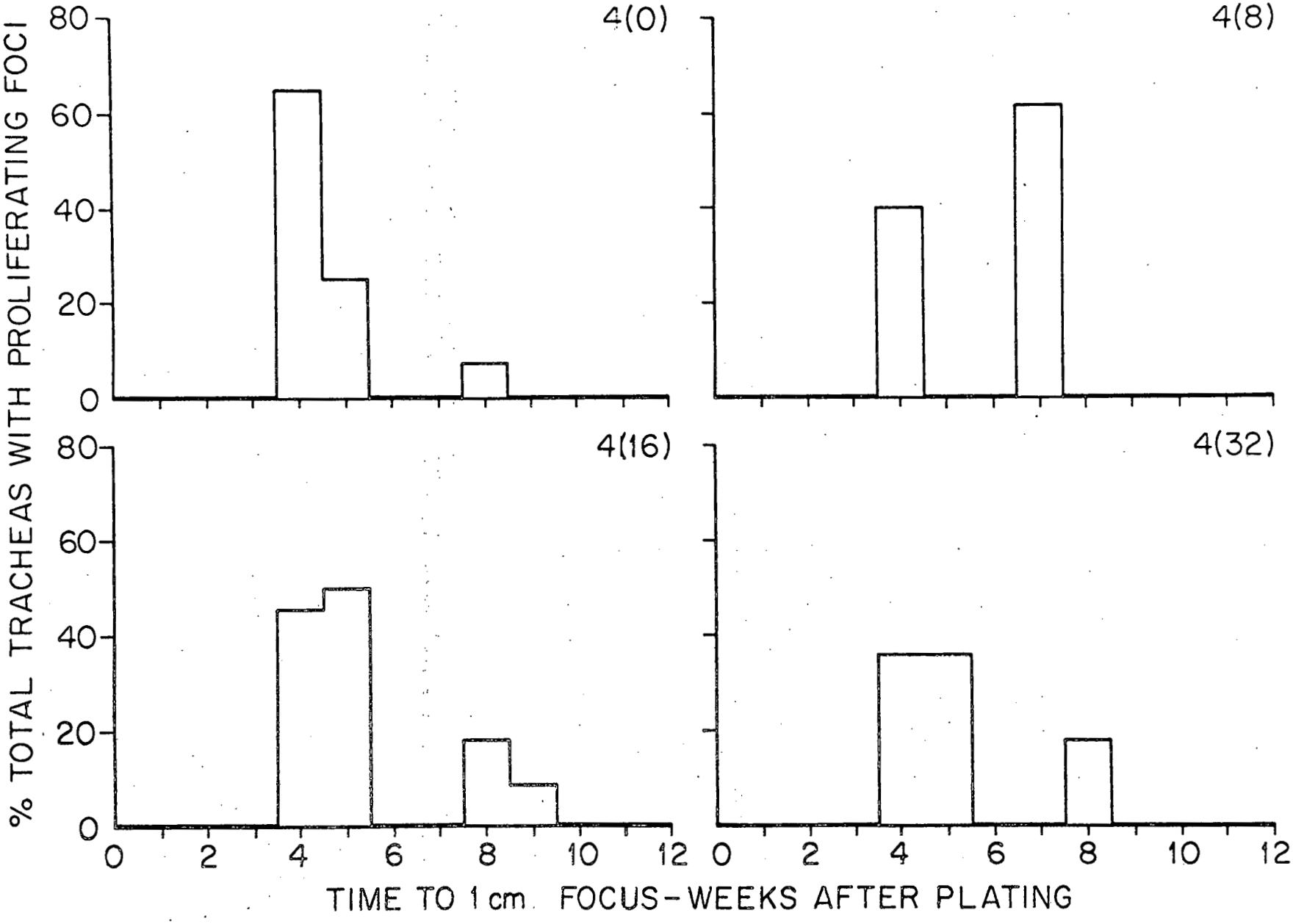

4(0)
4(8)
4(16)
4(32)
% TOTAL TRACHEAS WITH PROLIFERATING FOCI
0
20
40
60
80
0
2
4
6
8
10
12
TIME TO 1 cm FOCUS - WEEKS AFTER PLATING

Fig 9