Studies with a New Experimental Model in Respiratory Carcinogenesis[1]

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Contents

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I. Introduction

The anatomical and physiological complexities of the respiratory tract pose a number of serious problems to the student of respiratory carcinogenesis. To investigate the pathogenesis of "lung cancer" (i.e. neoplasias in various segments of the respiratory tract) in laboratory animals, most investigators have used intratracheal injection on inhalation of carcinogen as means of exposure. The complications inherent in these approaches stem from the fact that the various segments of the respiratory tract contain different cell types with different metabolic activity (1) and susceptibility to different various carcinogens. In addition, the distribution of carcinogen within the respiratory tract and the dose within each segment is beyond the control of the investigator.

We have therefore developed methods in our laboratory to expose a preselected and limited segment of the respiratory system, namely the trachea, to carcinogenic agents. One such experimental model involves the use of heterotopic transplants as target for carcinogenic test substances (2,3,4). Another uses the trachea of hamsters in situ which is exposed to a water soluble carcinogen with the aid of a device originally developed for the collection of exfoliating cells from the trachea of laboratory rodents (6). A first report describing the success of this approach was published from our laboratory several years ago (7). Since then we have made an effort to further develop and characterize the experimental model so it may become a useful tool in experimental carcinogenesis (8). The subsequent report is an attempt to give an overview over these efforts which are still in progress.

II. Materials and Methods

The basic principals of the equipment and procedures have remained the same since we first described the "intratracheal sampling device" for collection of exfoliating cells from the respiratory tract of rats and hamsters (6). Figure 1 explains the essential parts and their function of the intratracheal washing apparatus. A late version of the equipment currently in use in our laboratory is pictured in Figure 2. When the apparatus is used for carcinogen exposure instead of collection of cells, the rinsing fluid is replaced with carcinogen solution of N-nitroso-N-methylurea (NMU) in 0.1 M sodium citrate, pH 5.7. The NMU is stable for at least three hours at room temperature. F-1 F-2

Anesthetised hamsters are fastened to a slanted board in a position similar to that used for intratracheal injection (5). The catheter is inserted under vision through the open mouth of the hamster into its larynx and trachea to a constant depth of catheter insertion (marker on cannula matched with vocal cords). The fluid delivery system and the vacuum pump are activated and the carcinogen fluid is pushed out of the tip of the shorter (and wider) outer tubing, runs down the tracheal wall, and is then collected at the tip of the longer (and thinner) inner tubing (see Fig. 1). In the studies described here, the distance between the ends of the delivery and the collection tubing was 0.5 cm with the aim to rinse the epithelial surface between the 4th and the 10th tracheal ring. The rinsing time was 7 seconds per exposure and the volume of carcinogen solution (which is fully recovered) 0.5 ml.

Hamsters are exposed in this manner either once or repeatedly. They are killed at predetermined times after the last exposure or when moribund.

III. Results and Discussion

A. Early Changes of Tracheal Mucosa Induced by NMU

Several experiments were carried out to determine the "early" changes induced in the tracheal epithelium by NMU using the tracheal exposure device. We wanted to know whether epithelial abnormalities would be limited to the 4th to 10th tracheal rings as intended, what type of mucosal changes would occur, whether these would persist after cessation of exposure, and what the effect of NMU concentration, and the frequency of exposures (1 or 2 exposures per week) would be. It was also essential to determine when during or after a series of exposures, neoplastic lesions would appear.

The early mucosal changes induced by 1% NMU can be briefly summarized as follows. With 5 to 10 exposures, the tracheal epithelium changed to a low cuboidal epithelium with areas of flat and "bizarre" cells (Fig. 3A and B). F-3 Other areas showed hyperplastic and hypertrophic epithelium and small squamous metaplasias. Four to six weeks after the last exposure, most of these changes disappeared with only few metaplastic patches remaining. With 15 to 20 exposures the atrophic and toxic changes were widespread and severe. Metaplasias were commonly seen. Six to 8 weeks after the last exposure, most of the tracheal lining had returned to normal, but in the groups receiving 20 exposures, small early invasive carcinomas were detectable in several animals. In general the toxic changes were considerably more severe in animals receiving exposures twice per week than in animals being exposed once per week. With 0.5% NMU, the mucosal changes were much milder. Hyperplastic reactions rather than atrophic changes were commonly seen. Metaplasias were small and less

frequent. With a concentration of 0.25% NMU, changes other than mild hyperplasia were rare. An occasional metaplastic patch occurred after 20-30 exposures. The morphology was not markedly different from animals exposed to citrate buffer only. The tracheal changes found in such hamsters, killed shortly after 30 exposures (0.5% NMU), are schematically represented in Figure 4 to show type, distribution, and frequency of various epithelial abnormalities. Repeated tracheal washing (up to 30 times) with citrate buffer only caused mild epithelial hyperplasia and an occasional focal squamous metaplasia. These changes regressed rapidly. The various sacrifice studies show that with up to 20 repeated NMU exposures (2 exposures per week), carcinomas are not present at the time of the last exposure. In the 10 animals sacrificed per group at the end of exposure, no neoplasms could be found. Neoplasms did develop 4 or more weeks later, depending on the dose schedule. F-4

B. Induction of Tumors in the Trachea by NMU

The initial studies were all carried out with an NMU concentration of 1%. It became clear, however, that this concentration was toxic, particularly when repeated exposures were performed more than 20 times. In Figure 5 the mortality data during repeated NMU exposures are summarized. With an NMU concentration of 1% and 20 exposures (2 exposures per week), mortality is 10%. With further exposures the incidence increases rapidly. With a lower NMU concentration, mortality is insignificant even with as many as 30 exposures. The mortality occurring in these studies is due to a necrotising tracheitis followed by tracheal obstruction. The washing procedure itself causes no mortality. F-5

Figure 6A and B summarize the mortality rate and the rate of tumor development after cessation of carcinogen exposure as a function of the NMU concentration. With 20 as well as with 30 exposures, the median survival time after the last exposure is 10 weeks with 1% and approximately 40 weeks with 0.25% NMU. In control hamsters, median survival time is over 50 weeks. F-6

The details of the tumor response as a function of the number of exposures as well as carcinogen concentration are summarized in Table 1. It can be seen that 10 exposures (1% NMU, 2 exposures per week) result in a minimal tumor response. Only 2 out of 12 hamsters had tumors and both tumors were benign. With increasing number of exposures (1% NMU), the number of tumor bearing and invasive-tumor bearing animals increases, while tumor induction time decreases. The difference in the two NMU concentrations (1.0% versus 0.5%) amounts to a difference of only 10-20% in terms of tumor incidence. However, the mean tumor induction times are 10-14 weeks longer in the groups receiving 0.5% NMU than in those exposed to 1% NMU. Analysis of the relative frequency of tumor types shows an interesting distribution (Table 2). Benign polyps and papillmoas make up 40% or more of all tumors in the low dose groups (10 and 15 exposures 1% NMU, 20 exposures 0.5% NMU), but only 20% or less in the higher dose groups. Conversely, invasive carcinomas constitute 50-70% of all tumors in the high dose groups. The relative frequency of noninvasive carcinomas (the term is used here to be synonymous with carcinoma in situ) also tended to increase with increasing dose. An exception is the group receiving 15 exposures to 1% NMU which has tumor incidence of about 40%. Among the invasive carcinomas, 5 histological types were distinguishable. (Tumors are classified according to T-1 T-2

predominant cell type.). Of these, the epidermoid carcinoma was most frequent (50% of all invasive carcinomas) followed by adeno carcinoma and large cell carcinoma (about 20% each). Combined epidermoid-adeno carcinomas and small cell carcinomas were rare (about 9% and 4% respectively). Of interest is the observation that the lower concentration of NMU induced the higher incidence of adenocarcinomas, namely, 43% of all invasive carcinomas in both groups receiving 0.5% NMU as compared to only 13% in all groups receiving 1% NMU. The main histological tumor types are depicted in Figures 7 through 9. F-7-9 Several types of preneoplastic and neoplastic lesions deserve special comment. Among the lesions classified as carcinoma in situ, a small cell and a squamous type could be clearly distinguished (Fig. 7A and B). The anaplastic large cell carcinomas (8A) usually showed isolated cells with PAS positive droplets. The anaplastic small cell carcinomas (Fig. 8B, C) were made up of either polygonal or fusiform cells. Whether these neoplasms are related to human oat cell carcinomas remains to be determined. One type of tumor was of special interest since it might be easily overlooked. This neoplasm grows almost exclusively endophytic in narrow sheets without any significant luminal tumor mass (Fig. 9). It seems to be a highly invasive neoplasm. Distant metastases were not observed in any of the animals probably because the hamsters died in most cases early from tracheal obstruction.

The topography of neoplasias and other major epithelial abnormalities is influenced by both NMU concentration and frequency of exposure (Fig. 10). At F-10 the low NMU concentration, tumors occur mostly below the 5th tracheal ring (Fig. 10A and B). The epithelium bordering on the neoplasias is either hyperplastic or normal. With 1.0% NMU, particularly in the 30 exposures group (Fig. 10C),

tumors commonly occur in or extend to the proximal portions of the tracheas. The epithelium next to the neoplastic lesions is frequently metaplastic, showing various degrees of atypia. The tracheal "maps" show that the tracheal region affected by the NMU exposures is larger than anticipated. With 0.5% NMU and 20 exposures, tumor development is largely confined to the region from the 6th to the 13th tracheal ring. However with higher concentrations and particularly with more frequent exposures, the heavily exposed region clearly is larger.

IV. Summary and Conclusion

Tracheal tumor induction in hamsters with NMU shows a clear dose-response relationship. Multiple exposures are required to induce tumors. With 10-20 exposures, most of the neoplasms occur in the region between the 6th to 13th tracheal rings. With low doses, non-invasive neoplasms are most common. With high doses invasive carcinomas are the most frequent type of tumor. Adeno carcinomas appear to be most common with the lower (0.5%) NMU concentration. Saml1 cell carcinomas were induced; whether these are similar to small cell carcinomas in humans remains to be determined. The experimental model described appears to offer many attractive features for the study of neoplastic evolution in respiratory tract epithelium. Every major histological types of neoplasm known to occur in the bronchial tract of humans can be induced in this system.

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Table 1

RESPIRATORY TRACT TUMOR RESPONSE IN HAMSTERS GIVEN MULTIPLE INTRATRACHEAL EXPOSURES TO NMU

	NO. OF EXPOSURES	EFFECTIVE NO. OF HAMSTERS	NO. OF TUMOR-BEARING HAMSTERS	NO. OF HAMSTERS WITH CARCINOMA (ALL TYPES)	NO. OF HAMSTERS WITH CARCINOMA (INVASIVE TYPE)	MEAN TUMOR-INDUCTION TIME (WK)	TIME TO FIRST TUMOR (WK)	
							FROM START OF EXPOSURE	FROM END OF EXPOSURE
1% NMU	10	12	2 (17)[a]	0 (0)	0 (0)	50	33	28
	15	12	9 (75)	5 (42)	2 (17)	51	25	17
	20	15	10 (67)	10 (67)	10 (67)	30	18	8
	20[b]	23	22 (96)	20 (87)	17 (74)	35	19	< 1
	25	17	15 (88)	15 (88)	12 (71)	28	13	< 1
	30	17	16 (94)	16 (94)	16 (94)	28	18	3
0.5% NMU	20	18	14 (78)	9 (50)	7 (39)	44	27	17
	30	17	15 (88)	14 (82)	13 (76)	39	19	4
CITRATE BUFFER	20	13	0	0	0	–	–	–
	30[c]	17	0	0	0	–	–	–

[a] Numbers in parentheses are percentages.

[b] This group received 20 exposures, 1 exposure per week instead of 2, as all other groups. Two animals died after 19 exposures.

[c] This control group has been under study for 65 weeks.

Table 2

NUMBER AND MORPHOLOGIC TYPES OF TUMORS IN HAMSTERS GIVEN MULTIPLE INTRATRACHEAL EXPOSURES TO NMU

	NO. OF EXPOSURES	TOTAL NO. OF TUMORS[a]	NO. OF TUMORS OF VARIOUS TYPES								
							HISTOLOGIC TYPES OF INVASIVE CARCINOMA				
			SARCOMA	PAPILLOMA AND POLYP	NONINVASIVE CARCINOMA	INVASIVE CARCINOMA	EPIDERMOID	ADENO	COMBINED EPIDERMOID AND ADENO	ANAPLASTIC	
										LARGE CELL	SMALL CELL
1% NMU	10	2	0	2	0	0	0	0	0	0	0
	15	12	0	5	5	2	1	0	0	1	0
	20	14	0	2	2	10	7	0	0	2	1
	20[b]	43	0	5	11	27	16	3	1	7	0
	25	27	0	4	8	15	8	3	2	2	0
	30	43	1	1	19	22	9	4	2	5	2
0.5% NMU	20	20	0	9	2	7	1	4	0	1	1
	30	28	0	6	5	16	4	6	3	3	0
CITRATE BUFFER	20	0	0	0	0	0	0	0	0	0	0
	30[c]	0	0	0	0	0	0	0	0	0	0
TOTAL		189	1	34	52	99	46	20	8	21	4

[a] Not included are 11 hemangioma-like growths occurring in the tracheas of hamsters of 5 of the 8 NMU-exposed groups.

[b] This group received 20 exposures, 1 exposure per week instead of 2, as all other groups. Two animals died after 19 exposures.

[c] This group has not yet been terminated. It has been carried so far for 65 weeks.

Figure Legends

Fig. 1. Diagram of the intratracheal sampling device. A, collection system. The lower end of the collection vessel (a) is sealed by a rubber stopper (b) carrying a round coverglass; the upper end is sealed by a rubber stopper (c) that holds (d) the aspiration tube through which the aspirated specimen is delivered and (e) the vacuum line. B, sampling catheter. The main component of the catheter is a barrel (f) with plunger (g) to which the aspiration tubing (d) is mounted; the slide seal (h) has an opening for the tubing. A shaft (i) is attached to the lower end of barrel; its distal end is covered by a short, stiff Teflon tube (j) (outside diameter, 1.6 mm, inside diameter, 1.2 mm) that is inserted into the larynx (up to the dotted line). Washing fluid enters the shaft through an opening (k) and leaves through an annular outlet (l) (0.1 mm wide). The aspiration tubing (d) (polyethylene tube, outside diameter, 1 mm; inside diameter, 0.6 mm) is pushed out through the tip of the outlet by application of pressure to the plunger (g), so that it projects into the trachea. C, regulating unit. The unit is turned on by the microswitch (m) when the plunger (g) is pushed down. The automated injector (n) (Sage Model 237-1) injects the washing fluid and the vacuum valve (o) opens the vacuum line; the timer (p) terminates vacuum and injection of washing fluid.(6).

Fig. 2. Apparatus used to expose hamster tracheas to carcinogen.

Fig. 3. Early toxic changes in hamster tracheas exposed to NMU. This type of change is particularly common with multiple exposures to 1% NMU. A, flat cells. B, "bizarre" cells; these cells are large, irregularly shaped with large nuclei ( ).

Fig. 4. Tracheal "map" showing distribution and type of lesions in hamsters killed 1-8 weeks after 30 exposures to 0.5% NMU.  Hyperplasia,  squamous metaplasia,  tumor. E = epidermoid carcinoma, I = carcinoma in situ, P = papillima or polyp. Each column represents the trachea of one hamster. Tracheal rings were kept in sequence, two-four sections per tracheal ring.

Fig. 5. Mortality during repeated exposures to different concentrations of NMU. Hamsters were exposed two times per week. Data are based on a series of studies involving over 500 animals.  , 1% NMU;  0.5% NMU;  , 0.25% NMU;  citrate buffer.

Fig. 6. Mortality after exposure to various concentrations of NMU (2 exposures per week).  , 1% NMU; , 0.5% NMU,  , 0.25% NMU;  , citrate buffer. Solid symbols = carcinoma, striated symbols = carcinoma in situ, striped symbols = papilloma or polyp, open symbol = no tumor, C = cannibalized, no histology available. A, hamsters received 20 exposures. B, hamsters received 30 exposures.

Fig. 7. Typical carcinomas in situ. A, neoplasm composed of small cells with high nuclear-cytoplasmic ratio. B, neoplasm composed of larger cells; signs of squamous differentiation are evident ( ).

Fig. 8. Typical anaplastic carcinomas. A, anaplastic large cell carcinoma with low nuclear-cytoplasmic ratio. B, invasive small cell carcinoma, with small densly staining cells ( ).

Fig. 9. Neoplasm with predominantly endophytic growth. Notice strands of neoplastic cells in submucosa. Serial sections reveal little evidence of intraluminal growth.

Fig. 10. Tracheal "maps" showing location and type of tumors in hamsters dying after having received multiple NMU exposures.  , hyperplasia;  , squamous metaplasia;  , metaplasia and hyperplasia;  , tumor. E = epidermoid carcinoma, A = adenocarcinoma, C = combined epidermoid and adeno carcinoma, L = large cell carcinoma, S = small cell carcinoma, I = carcinoma in situ, P = papilloma or polyp. Each column represents the trachea of one hamster. The numbers on bottom of the charts indicate time of death after the last exposure of each hamster. A, 0.5% NMU, 20 exposures; B, 0.5% NMU, 30 exposures; C, 1.0% NMU, 20 exposures; D, 1.0% NMU, 30 exposures.

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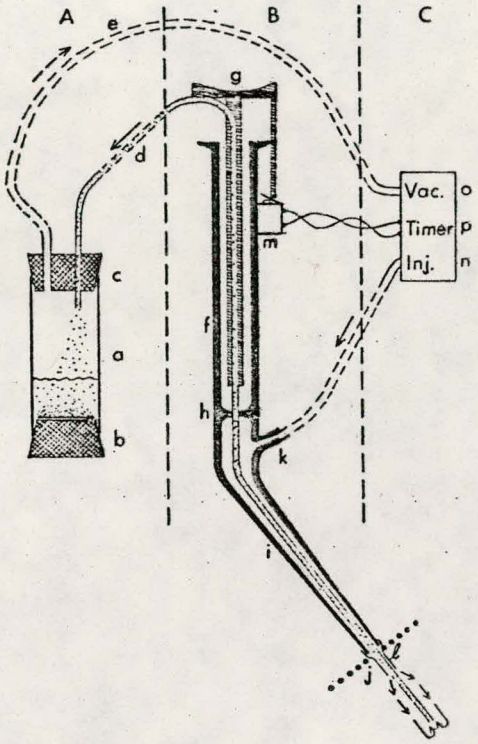

Fig 1

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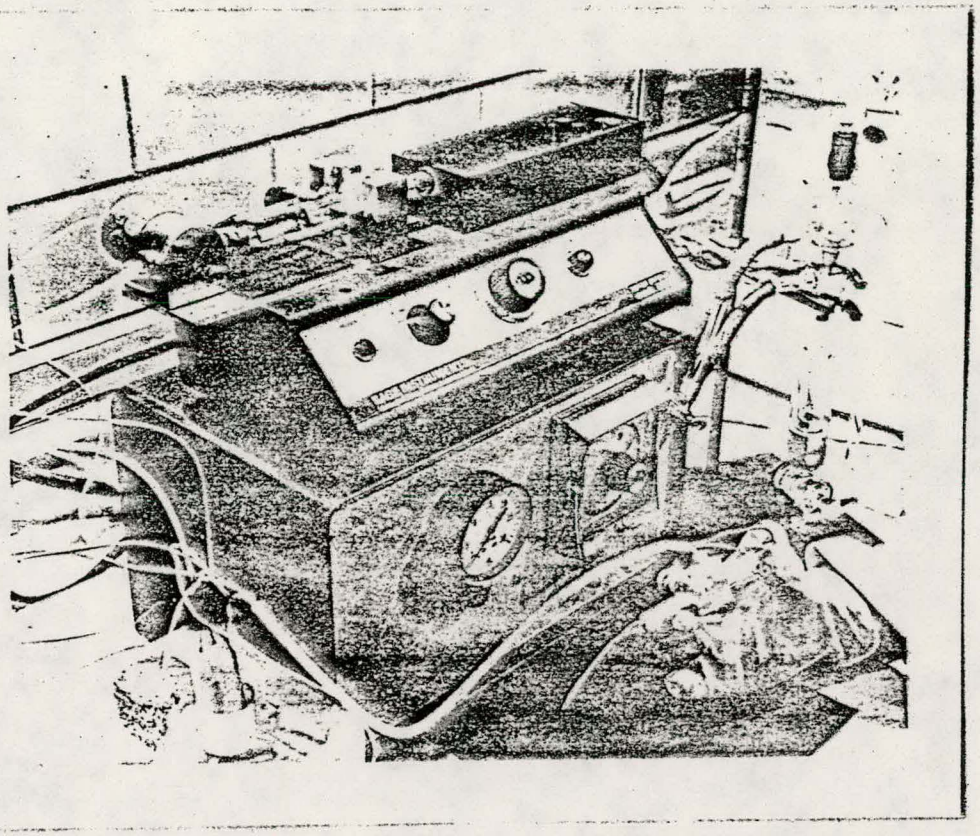
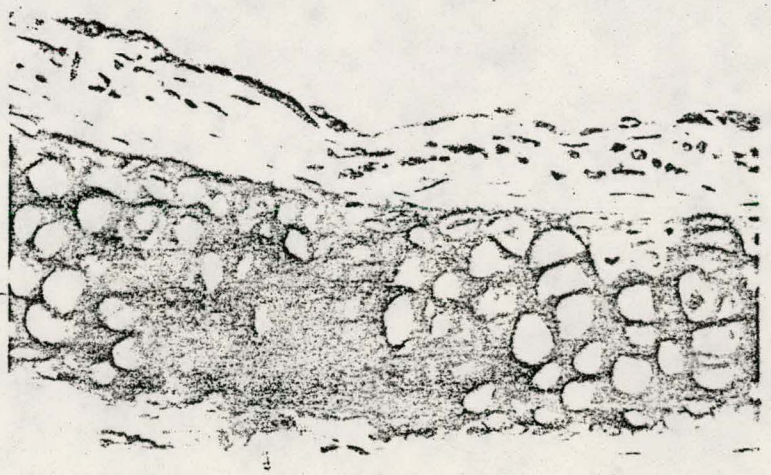


Fig 2

Fig 3 A

Fig 3B



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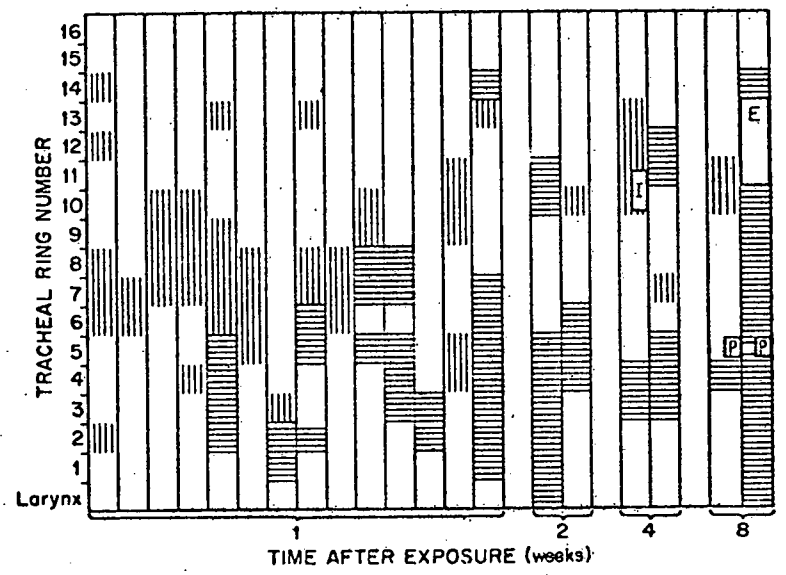
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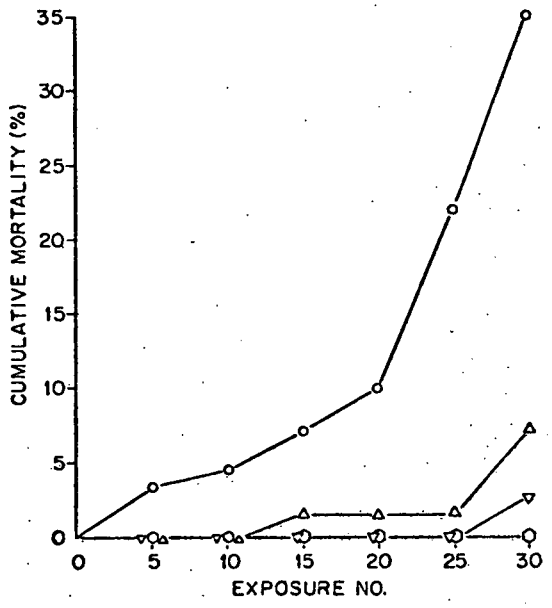
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
Larynx
E
I
P
P
1
2
4
8
TIME AFTER EXPOSURE (weeks)

Fig 4

34064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30
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15
10
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0
5
10
15
20
25
30
EXPOSURE NO.

Fig 5

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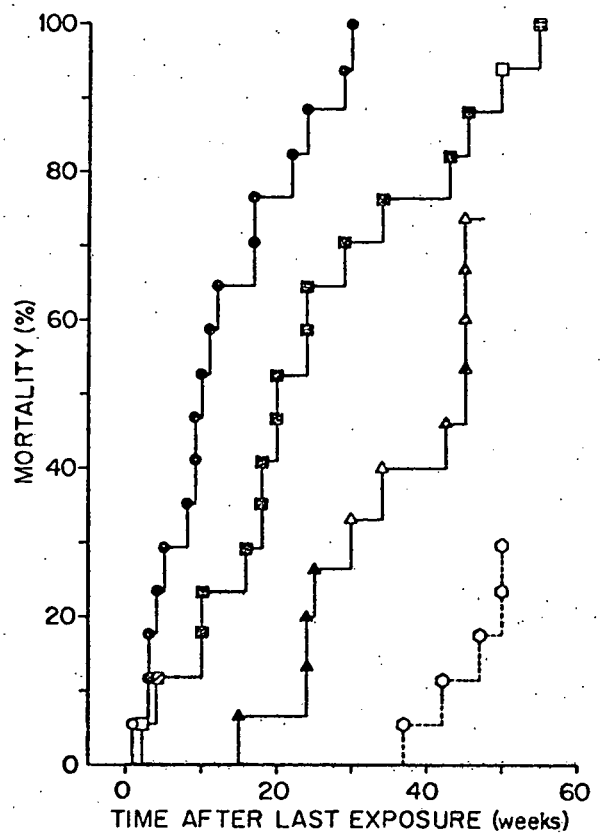
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40
20
0
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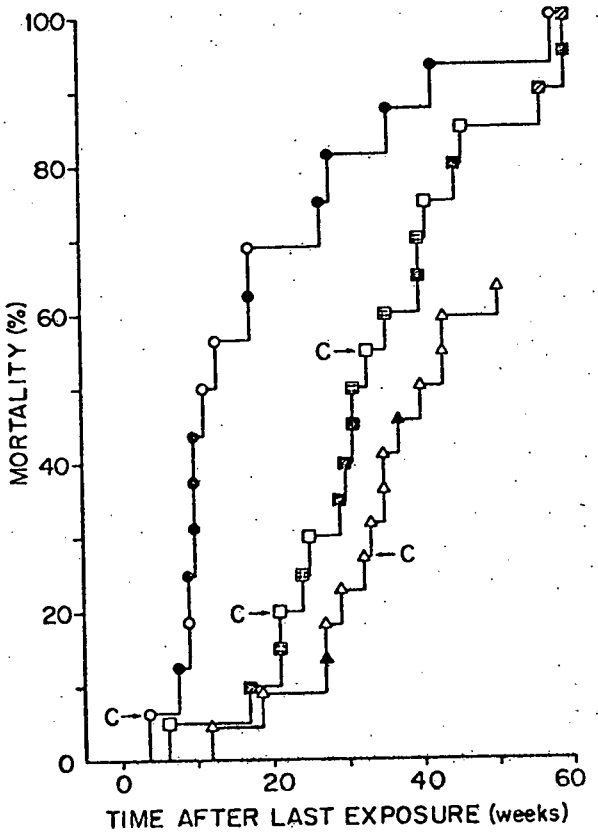
20
40
60
TIME AFTER LAST EXPOSURE (weeks)

Fig 6A

34057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TIME AFTER LAST EXPOSURE (weeks)

Fig 6B

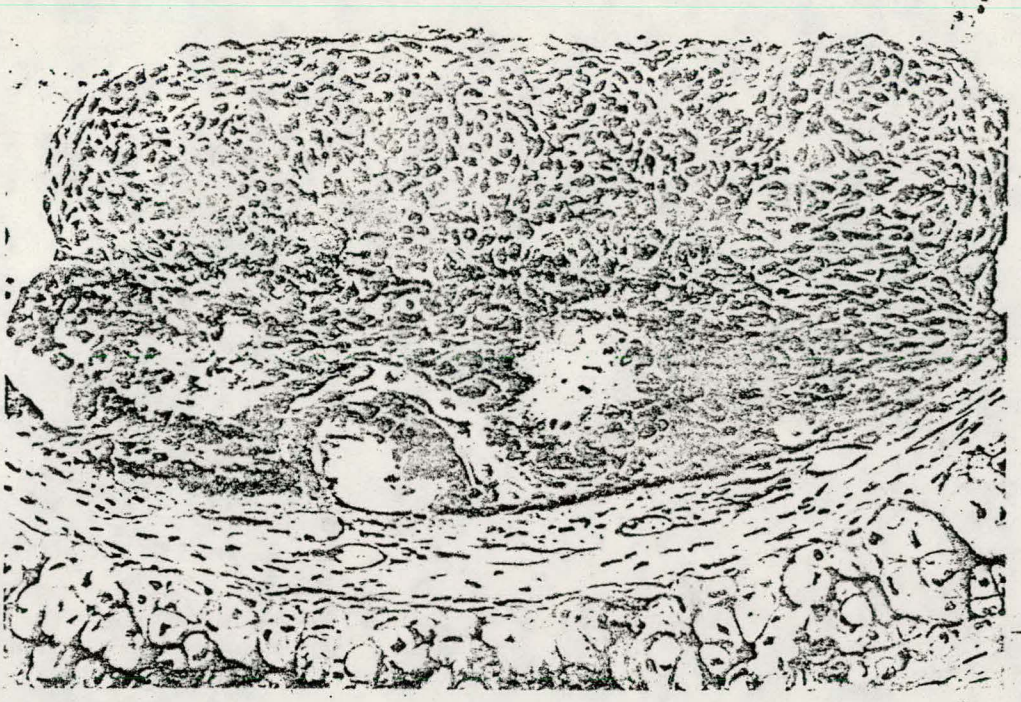
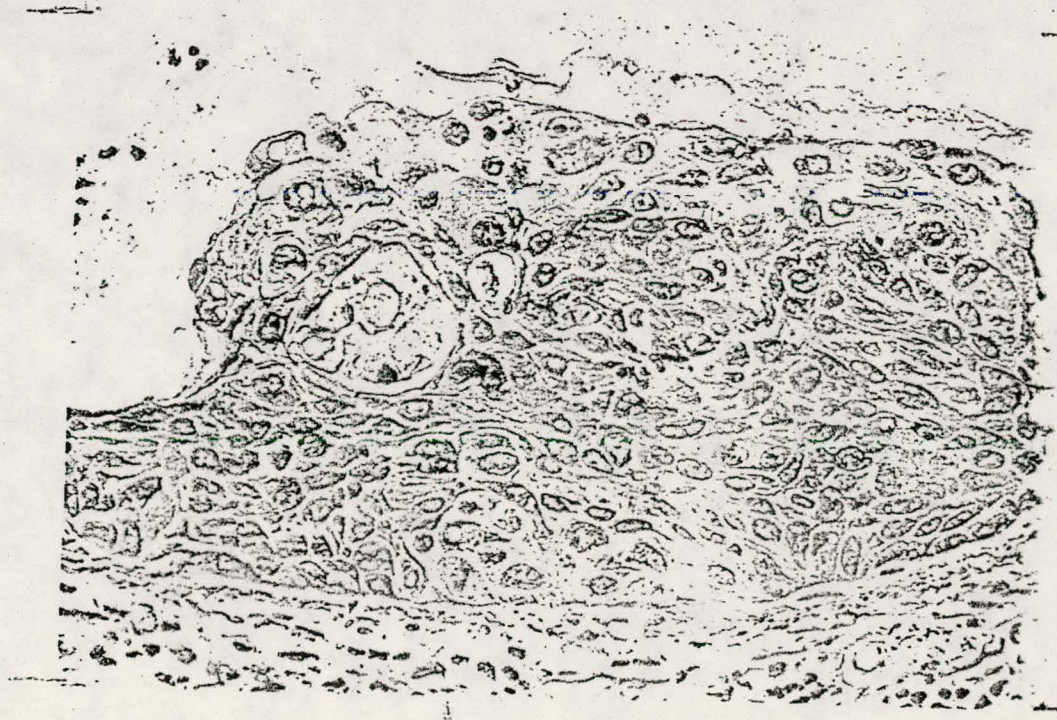


Fig 7A

Fig 7B



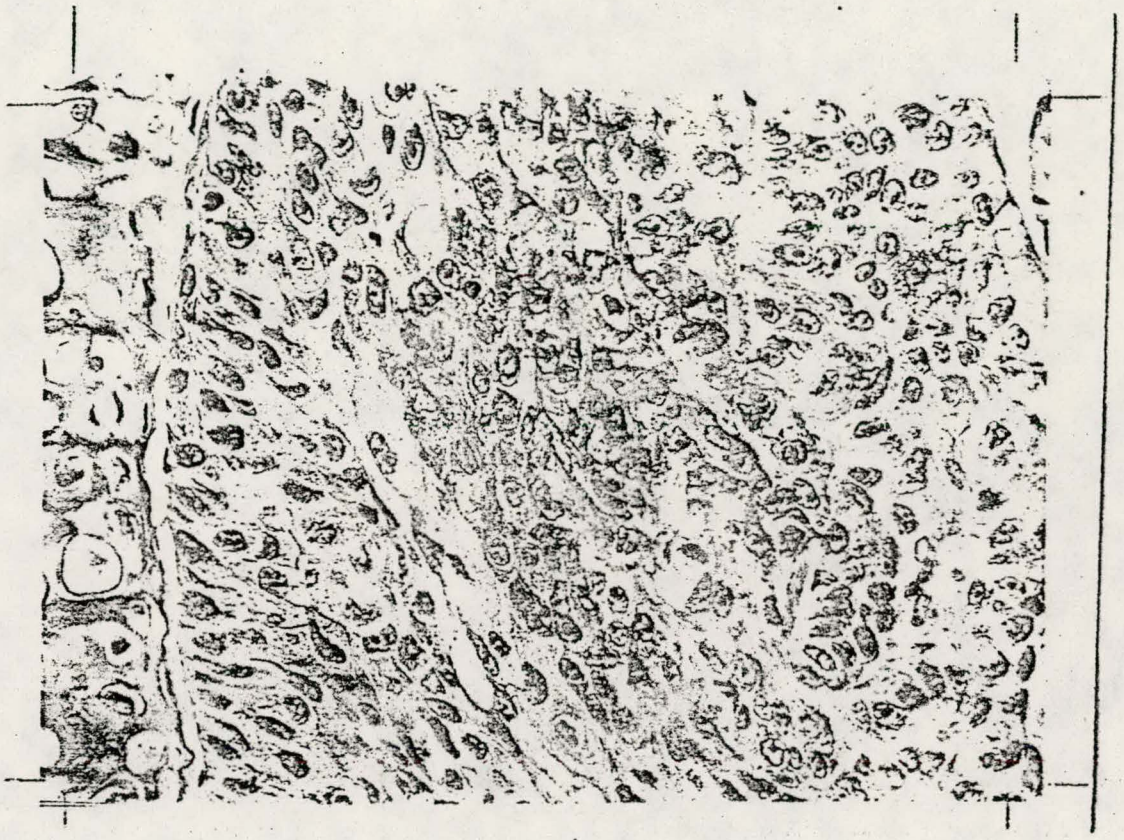
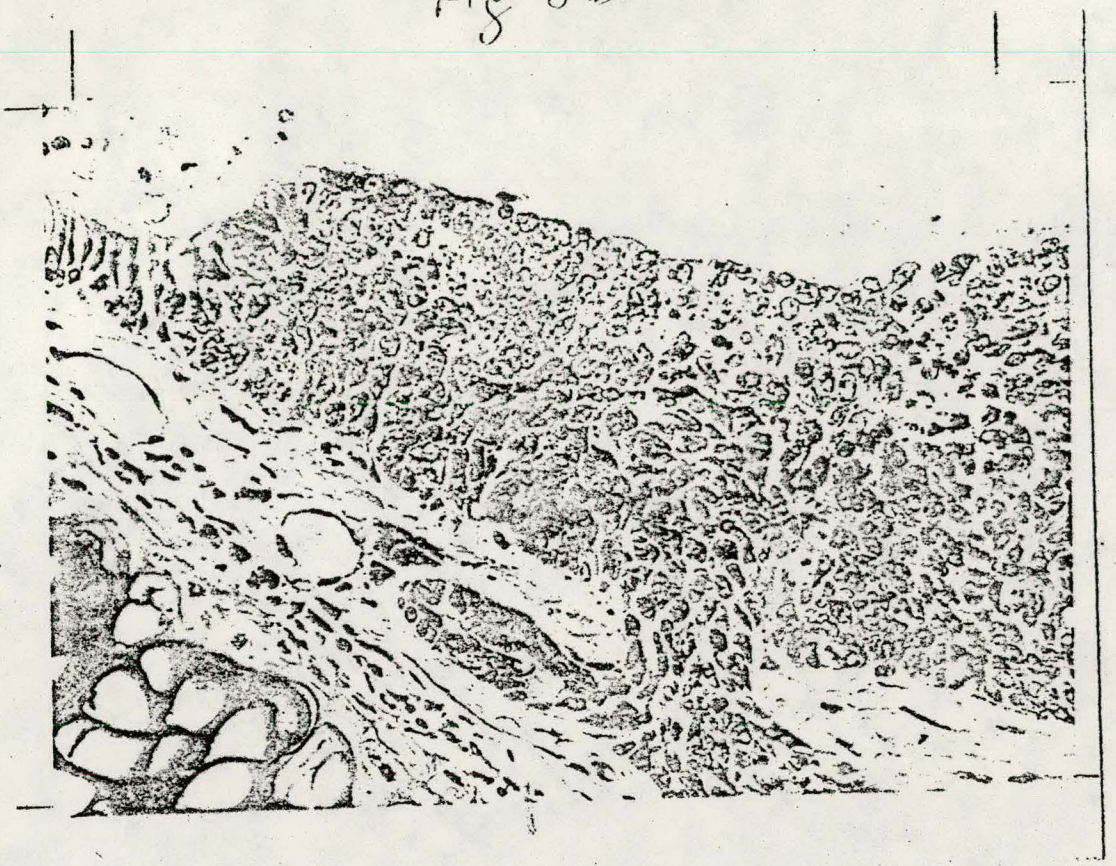


Fig 8A

Fig 8B



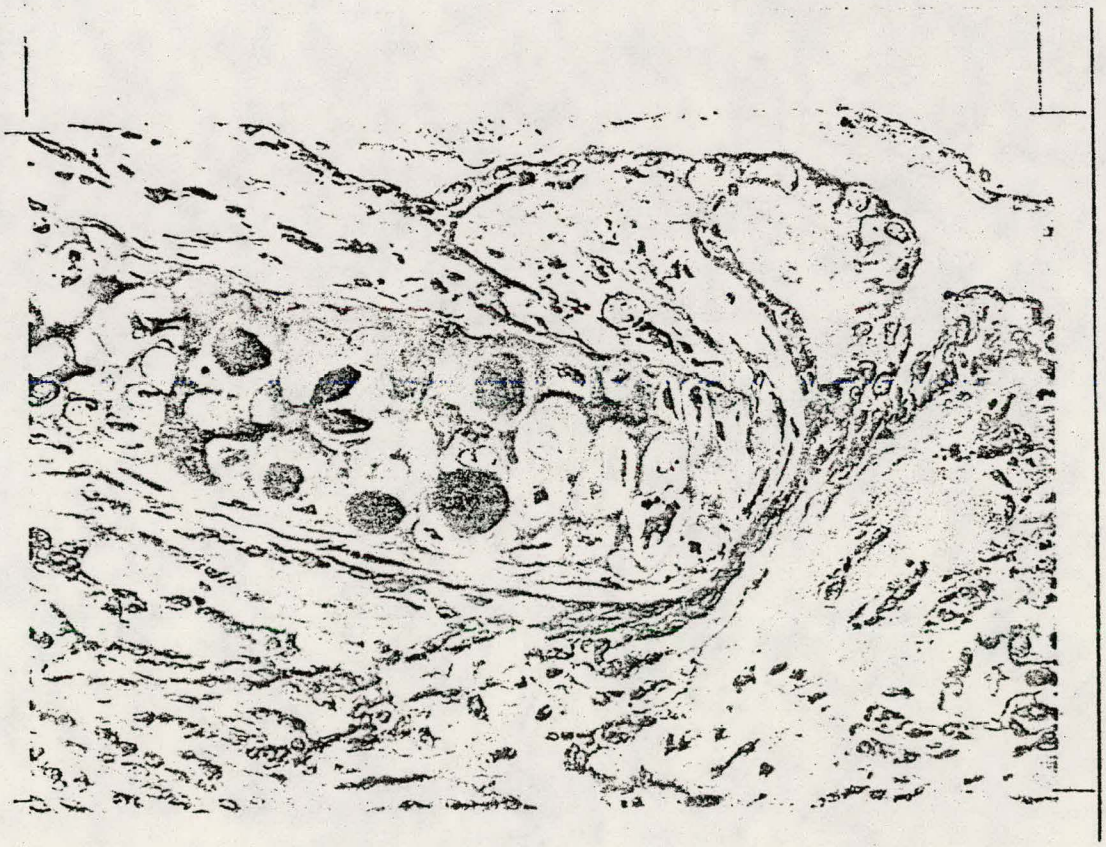
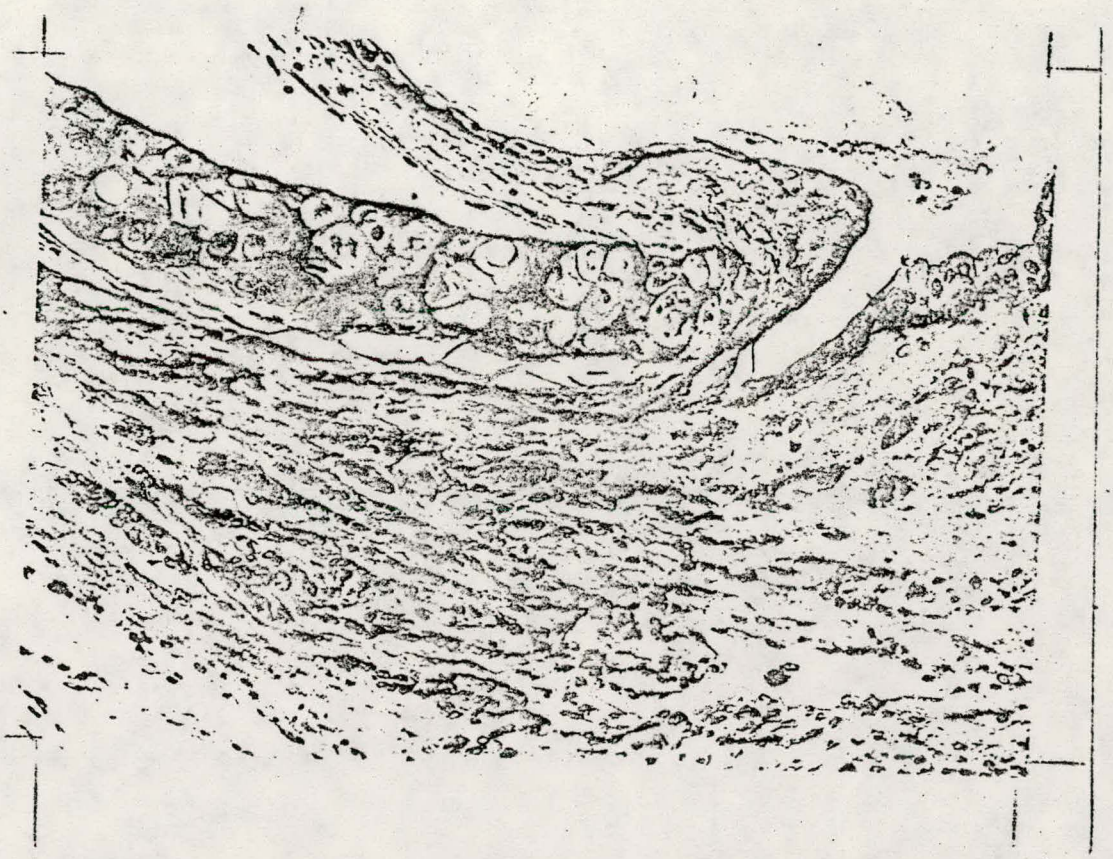


Fig 9



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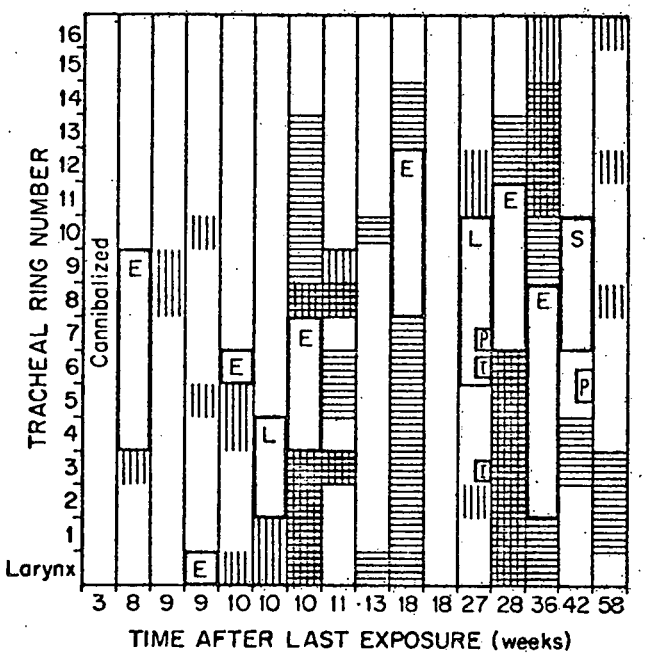

Fig 10 A

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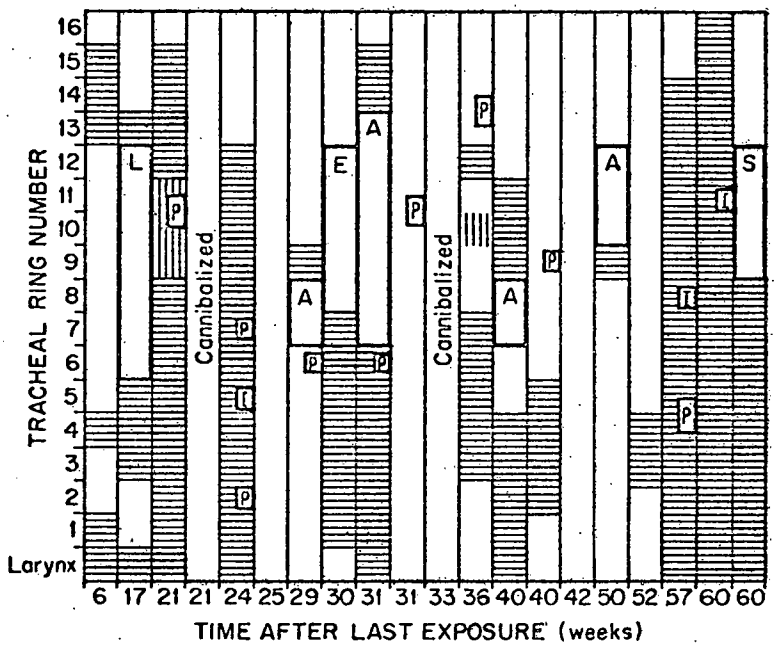

Fig 10B

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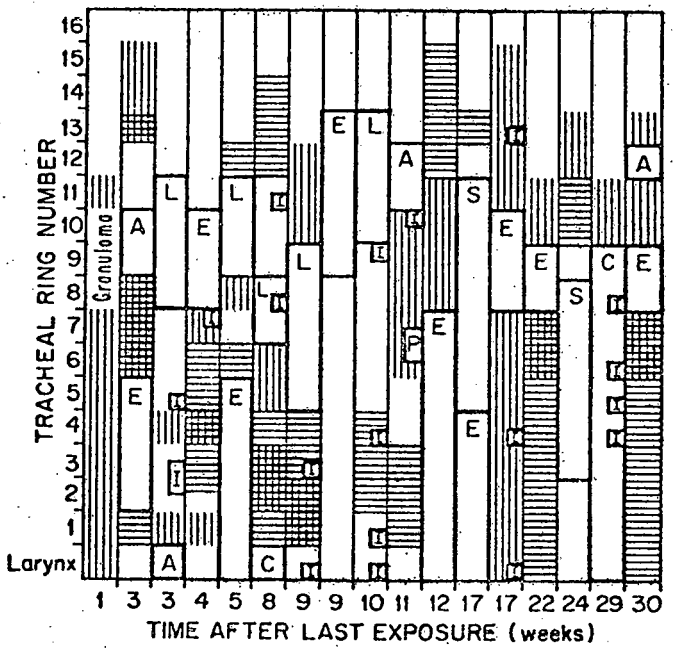

Fig 10C

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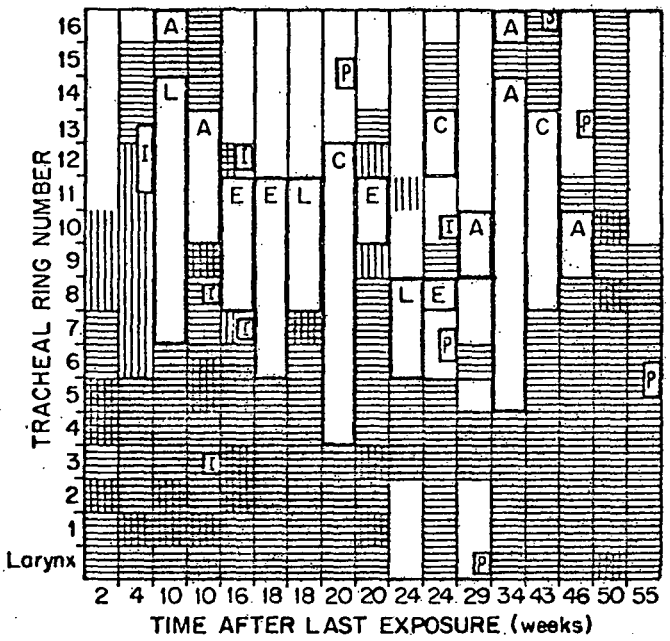

Fig 10 D