A Preliminary Study on the Effect of Cigarette Smoke and Air Pollution upon Cells in Culture[1)2)]

By

Masahiro Mizutani

Zoological Institute, Hokkaido University

(With 14 Text-figures)

Current etiological investigations which make use of statistical methods have presented evidence which suggests a causal relation existing between lung cancer on the one hand and cigarette smoke or the polluted air in industrial towns on the other. It has been shown that tobacco smoke and the air pollution of industrial cities contain small quantities of the polycyclic aromatic hydrocarbons (Cooper *et al.* 1954), some of which are carcinogenic. Further, evidence has been offered that tobacco tar, smoke condensate, and 3,4-benzpyrene, one of the components of smoke from cigarette paper and of air pollution collected from big cities or industrial areas, induce malignancies in experimental animals (Blacklock 1957, Engelbreth-Holm and Ahlman 1957, Graham *et al.* 1957, Passey 1957, Wynder *et al.* 1957, Gellhorn 1958, Orris *et al.* 1958, Reddy *et al.* 1961, Mizutani *et al.* 1961, and some others). Also, histological and cytological studies on the effect of cigarette smoke and smoke condensate upon cells *in vivo* and *in vitro* has pointed out a possible carcinogenic factor in the smoke condensate (Leuchtenberger *et al.* 1958, 1960a, b, Lasnitzki 1958, Nakanishi *et al.* 1959, Awa *et al.* 1961, and some others).

Under the treatment with smoke condensate and benzpyrene alone and in combination, Nakanishi *et al.* (1959) observed in a human lung strain *in vitro* a reduction of the modal chromosome number with the appearance of dicentrics. Working with cultured human lung cells exposed directly to the smoke which was collected from each of whole cigarette, tobacco alone and cigarette paper alone, Awa *et al.* (1961) found that paper smoke was notably toxic to the cells, in contrast to the other materials. However, knowledge in this field is rather restricted.

The present study was undertaken to examine whether any cytological change occurs, particularly change in the chromosomes, in cultured human lung cells after prolonged applications of cigarette smoke or acetone-soluble air pollution.

It is the author's pleasant duty to express his gratitude to Professor Sajiro Makino for invaluable criticism and improvement of this manuscript. Further thanks are offered to Professor Sanshi Abe, Dr. Fumio Tsunoda, and Miss Masako Omote, Department of Public Health, School of Medicine, for supply of the specimen of acetone-dissolved air

1) Contribution No. 477 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) Supported by a grant to S. Makino from the Damon Runyon Memorial Fund for Cancer Research (DRG-563B).

pollution, and to Dr. Yasushi Ohnuki for his constructive advice with friendly assistance.

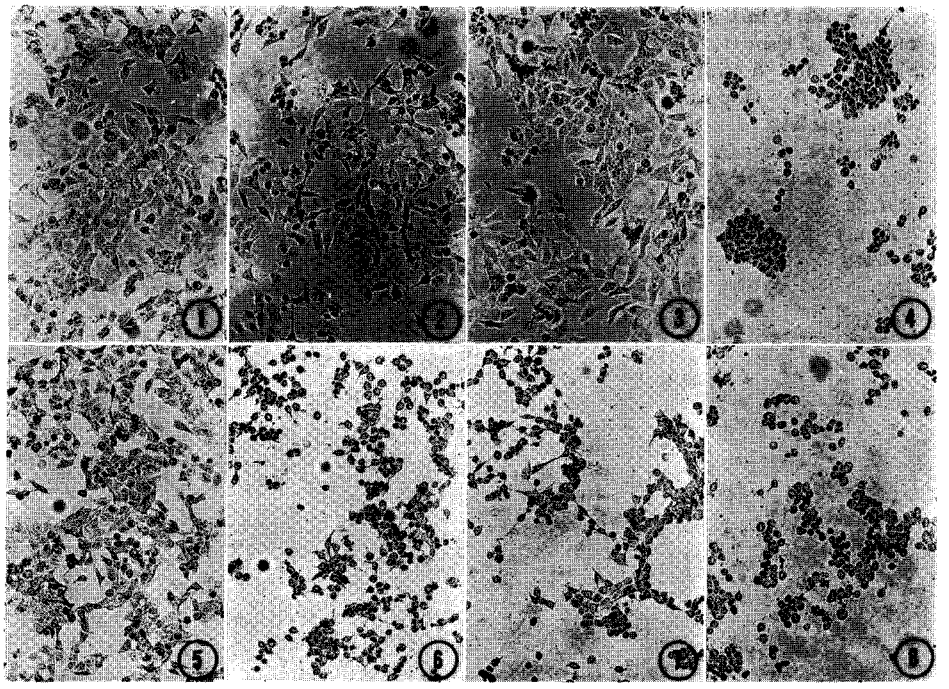
Material and Methods: A human foetal lung strain (HL-C strain) established by Nakanishi *et al.* (1959) was cultivated in T-flasks or square culture bottles. Initial seeding in each experimental culture was adjusted to become monolayer by the 3rd or 4th day. Then, cells were directly exposed to tobacco smoke collected from filter-tip- or non-filter-tip cigarettes, or treated with air pollution dissolved in acetone. Trypsin (0.2%) was used for subculture. Nutrient medium was Eagle's with 10% inactivated horse serum or Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 100 units of penicillin/ml, and 10% inactivated bovine serum.

In the smoke experiments, nutrient medium covering the monolayer of cells was removed, and then smoke from test material was introduced by sucking with a 50 or 100 ml syringe connected with a cigarette directly into the bottom of the container through a sterilized rubber stopper equipped with a long inlet and a short outlet glass tube. After the milky colour of smoke disappeared completely, fresh nutrient medium was added. In air pollution experiments, nutrient medium was replaced by fresh and then acetone-dissolved air pollution was dropped into the culture flask. After treatment of cells with cigarette smoke or with air pollution, nutrient medium was changed at least once in a passage, with the same procedure as in the first one. Observation of chromosomes was made following the water pretreatment acetic dahlia squash method.

Results

Cigarette smoke: HL-C cells were morphologically of epithelial type with well-defined nucleoli. In control cultures on the 3rd or 4th day after setting up culture, many round-shaped cells in process of mitosis were observed in the cell population (Figs. 1, 5). They were directly exposed to 35 ml of smoke collected from three kinds of cigarettes for 5 seconds, and then the smoke was removed from the culture flask with the aid of a suction bottle. As the cells showed little evidence of injury after ten hours, the 2nd treatment was performed. The cells were checked at ten hours after the 2nd exposure. In two culture flasks treated with cigarette #1 (filter-tip) and #2 (filter-tip), a large number of cells were found still healthy, but the number of damaged cells as evidenced by blebbing of the cytoplasm or by pycnotic nuclei was greater in #1 than in #2. Furthermore, mitotic cells were quite few in number in #1 in comparison with #2 (Figs. 2, 3). A small number of cells unusual in shape were observed in a flask after exposure to cigarette #3 (non-filter-tip) (Fig. 4). Old medium was replaced by fresh one at ten hours after the 2nd treatment, and the recovery of cells from injury was checked 24 hours later. Cells exposed to #2 seemed to be in the state of recovery, but elements treated with #1 showed a further decrease in cell number. HL-C cells were exposed to 70 ml of each smoke from the same three kinds of cigarettes; remarkable degeneration of cells occurred in all treated cultures (Figs. 6–8). The degree of cell damage diminished in the order of #3, #1, and #2, as observed in the previous test. On the basis of these preliminary runs, it was decided to use non-filter-tip cigarettes for further experiment, since such cigarettes seemed to exert the severest effect upon cells, in contrast to filter-tip cigarette.

HL-C cells grown well in square culture bottles were directly exposed to 10, 15, 20, 25, 30, 35, 40, and 50 ml of smoke collected from non-filter cigarettes (#4), respectively, in order to seek the most suitable dose for long-term exposure of the cells. The effects observed 48 hours after treatment are not constant in each repeated test, probably due to technical difficulty in getting equal volumes of smoke in each. However, 20 ml of smoke appeared to be the highest dosage for prolonged exposure.



Figs. 1–8. Photomicrographs of HL-C cells directly exposed to smoke collected from three kinds of cigarettes, in contrast to untreated controls. For detail, see text. 1 and 5, untreated control. 2 and 6, cells treated with #1 cigarette-smoke. 3 and 7, cells treated with #2 cigarette-smoke. 4 and 8, cells treated with #3 cigarette-smoke.

The chromosomes of the HL-C strain were studied in the 191st subculture as control. The most frequent chromosome number was 68, in 34 cells out of 50, with a narrow range from 66 to 70 (Figs. 9, a and 10). In the 200th subculture the number of most frequent occurrence was also 68 (18 cells out of 30) with a variation ranging from 65 to 70 (Figs. 9, b). In the 5th and 12th subcultures of the smoke-treated line, the distribution and stemline chromosome-number were found approximately the same as those of the untreated controls (Figs. 9, e-f and 11). After approximately two months, the chromosome-number was again checked in the

211th control culture. The chromosome modality showed 64 in 16 cells out of 30, with a range from 61 to 69 (Fig. 9,c). The chromosomal modality in the 217th control subculture and in the 20th experiment subculture was studied. The modal value in the untreated control was also 64, in 18 cells out of 40, with a variation ranging from 61 to 69 (Fig. 9, d). In the experimental line the modal chromosome

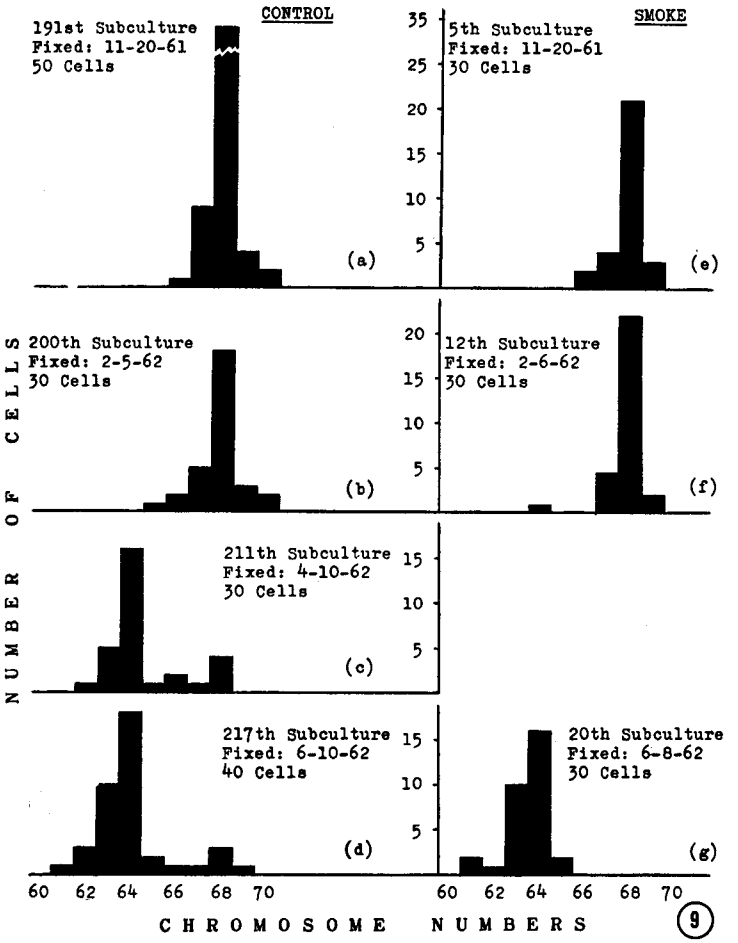
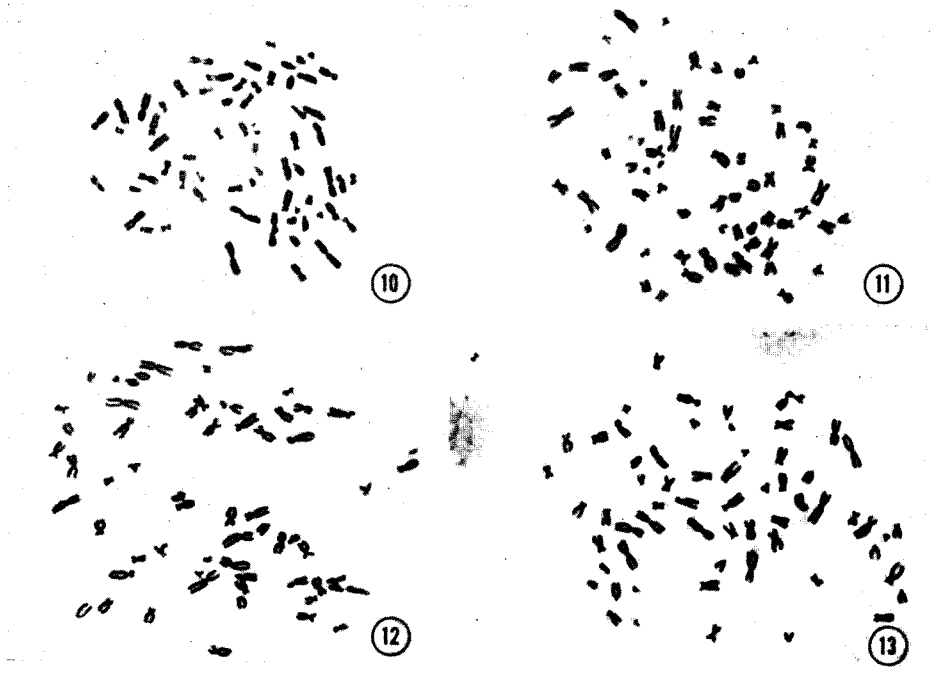


Fig. 9. Distribution of chromosome numbers in cells of HL-C strain treated with #4 cigarette-smoke, in contrast to untreated control.

value was also 64, in 15 cells out of 30, with a range from 61 to 65 (Figs. 9, g and 12). It is then evident from the above data that no noticeable change has occurred in the chromosomal modality of HL-C cells following the cigarette smoke treatment

even to the number of 40 treatments during 248 days.



Figs. 10–13. Photomicrographs of the chromosomes of HL-C strain treated with #4 cigarette-smoke or acetone-dissolved air pollution, in contrast to untreated culture. 10, an untreated control cell, 68 chromosomes. 11, a cigarette-smoke-treated cell in the 3rd subculture, 68 chromosomes. 12, a cigarette-smoke-treated cell in the 6th subculture, 64 chromosomes. 13, an air pollution-treated cell in the 3rd subculture, 64 chromosomes.

Air pollution: HL-C cells in the 196th subculture were seeded in small culture tubes. On the 3rd day after setting up the cultures they were treated with air pollution material dissolved in acetone and with acetone alone, at the concentrations of 0.01, 0.05, and 0.1 mg/ml in nutrient medium. A dose of 0.01 ml caused no visible morphological change in cells after 48 hours. Then the above-mentioned dose was adopted for further experiment.

In the 3rd subculture after acetone treatment the stemline chromosome-number was found to be 64 (13 cells out of 30) with a range from 62 to 68 (Fig. 14, a). The modality of chromosome numbers in the 6th acetone subculture was also 64 (14 cells out of 30) ranging from 61 to 69 (Fig. 14, b). The 3rd subculture after air pollution treatment showed the chromosome modality at 64 (8 cells out of 30): this value approximates the one in controls (Figs. 13 and 14, c). In the 6th subculture the most frequent chromosome-number was 63 (17 cells out of 30) with

a range from 62 to 71 (Fig. 14, d). In addition, chromosome breakages were observed in 4 cells out of 30 in the 6th subculture, but no such cells were found in controls.

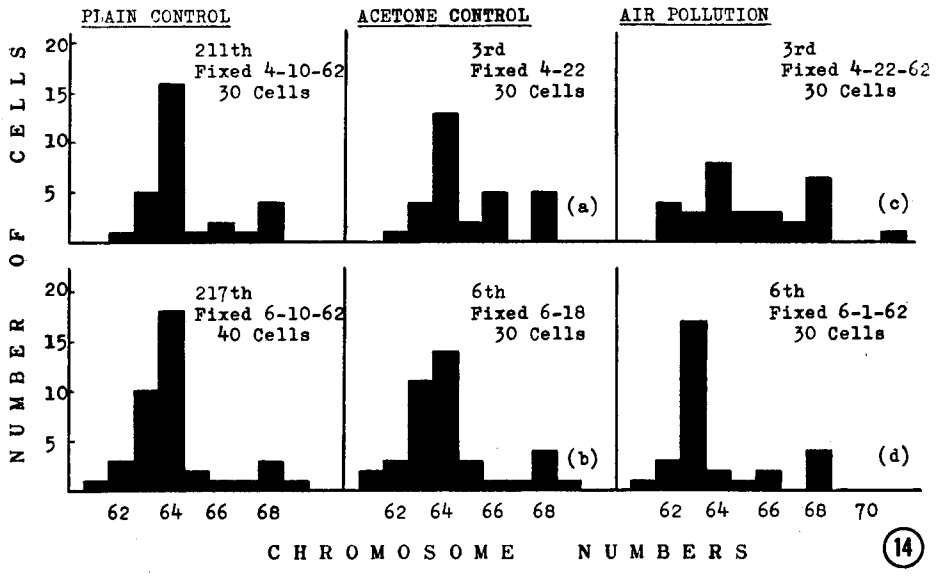


Fig. 14. Distribution of chromosome numbers in cells of HL-C strain treated with acetone-dissolved air pollution, in contrast to untreated control.

Discussion

Leuchtenberger *et al.* (1958, 1960a, b) studied lung tissues of mice exposed to cigarette smoke, and reported that bronchitis, basal-cell hyperplasia and typical basal hyperplasia were found in the majority of animals, while a few developed squamous metaplasia. According to Awa *et al.* (1961) the smoke collected from cigarette paper induced severe vacuolization of the cytoplasm together with pycnotic nuclei, a decrease in the rate of mitotic index, and a considerable increase in the occurrence of abnormal division in a human lung *in vitro* strain, while smoke from whole cigarette and tobacco alone caused little evidence of cellular damage. HL-C cells directly exposed to a large amount of cigarette smoke became rounded, and finally broke down becoming free from the glass surface of culture flasks. As seen in the photomicrographs presented by Nakanishi *et al.* (1959), certain cigarette tar produces injury to mitochondria in culture cells. Therefore, death of the cells due to cigarette smoke might have been caused by the inhibition of respiration in them resulting from the injury of mitochondria.

Nakanishi *et al.* (1959) have observed the shift of chromosome modality from 77 to 76 with the appearance of dicentric chromosomes in a human lung strain as having been caused by treatment with cigarette-smoke condensate. Those findings suggest that long-term exposure of cultured lung cells to cigarette smoke might induce some cytological change, particularly change in chromosomes. No shift in stemline chromosome-number, however, occurred in HL-C cells within the scope of the present experiment. No one has reported the induction of lung cancer in animals by exposing them to the smoke of tobacco (Passey 1962). A possible explanation of such lack of information is that carcinogenic substances in the cigarette smoke as well as other active fractions are too small to produce any positive result during the period which experiments have hitherto used.

Knowledge concerning the effect of air pollution upon cells grown *in vitro* is very meagre. As far as the author is aware, the only cytological study in this field has been done by Rounds *et al.* (1962). They showed that *in vitro* cells exposed to an atmosphere of gaseous exhaust showed a decrease of total mitotic index and an increase of abnormal mitotic figures. Further, chloroform-soluble constituents of auto-exhaust produced a marked stimulation in the growth rate of cells, with a decrease in the mitotic time apparently contributing to the decrease in the number of mitotic figures. Tsunoda (1962) has reported the existence of a fairly large amount of 3, 4-benzpyrene in automobile exhaust. According to his personal information, one of the main constituents of air pollution material used in this study is 3, 4-benzpyrene. Nakanishi *et al.* (1959) treated HL-C cells with benzpyrene and observed the shift of chromosome modality from 77 to 76 during a one-month experiment. Further, they reported that the shift of the modal value of chromosomes was hastened, when benzpyrene was combined with cigarette-smoke condensate.

In the present study, HL-C cells subjected to treat with air pollution material at a concentration of 0.01 ml showed normal growth. The most frequent chromosome-number shifted from 64 to 63 after repeated treatments for 135 days. The reduction of chromosome modality observed in this study may be one of some interest, in reference to the data presented by Nakanishi *et al.* (1959), though it is premature to conclude that the shift of chromosome modality was caused by benzpyrene which existed in air pollution. In many well established cell strains a gradual decrease of chromosome modality during long *in vitro* life seems to be a common feature (Hsu 1961, Awa *et al.* 1961). In addition, the effect of acetone should be considered in this experiment (Pace 1962). In view of the above facts, therefore, it may be safe to conclude that air pollution material serves to enhance the shift of the modal value of chromosomes.

Summary

Cells of the human lung strain (HL-C) were directly exposed to cigarette smoke. Smoke from non-filter-tip cigarette affected the cells rather remarkably, while filter-tip cigarette smoke caused less damage to the cells. Prolonged exposure of the HL-C cells to non-filter-tip cigarette smoke failed to induce any change of chromosome modality even after the 20th subculture.

Shift of modal value of chromosomes from 64 to 63 occurred in the 6th subculture of HL-C cells, after repeated subjection to air pollution. Very probably, benzpyrene, one of the main constituents of polluted air, served to enhance the shift of the stemline chromosome-number.

Literature

Awa, A., Y. Ohnuki and C.M. Pomerat 1961. Some comparative effects of smoked paper, tobacco and cigarettes on chromosomes *in vitro*. Texas Rep. Biol. & Med. **19**: 518–528.

Blackclock, J.W.S. 1957. The production of lung tumours in rats by 3, 4-benzpyrene, methylcholanthrene and the condensate from cigarette smoke. Brit. J. Cancer **11**: 181–191.

Cooper, R.L., A.J. Lindsey, and R.E. Waller 1954. Chemistry and industry, pp. 1418.

Engelbreth-Holm, J., and J. Ahlman 1957. Production of carcinoma in ST/Eh mice with cigarette tar. Acta. path. et microbiol. Scandinav. **41**: 267–272.

Gellhorn, A. 1958. The carcinogenic activity of cigarette tobacco tar. Cancer Research **18**: 510–518.

Graham, E.A., A. B. Croninger, and E.L. Wynder 1957. Experimental production of carcinoma with cigarette tar. IV. Successful experiments with rabbits. Cancer Research **17**: 1058–1066.

Hsu, T.C. 1961. Chromosomal evolution in cell population. Intern. Rev. Cytol. **12**: 69–161.

Lasnitzki, I. 1958. Observations on the effects of condensates from cigarette smoke on human foetal lung *in vitro*. Brit. J. Cancer **12**: 547–552.

Leuchtenberger, C., R. Leuchtenberger, and P.F. Doolin 1958. A correlated histological, cytological, and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke.I. Bronchitis with a typical epitherial changes in mice exposed to cigarette smoke. Cancer **11**: 490–506.

Leuchtenberger, C., R. Leuchtenberger, W. Zebrun, and P. Shaffer 1960a. Ditto. II. Varying responses of major bronchi to cigarette smoke, absence of bronchogenic carcinoma after prolonged exposure, and disappearance of bronchial lesions after cessation of exposure. Cancer **13**: 721–732.

Leuchtenberger, R., C. Leuchtenberger, W. Zebrun, and P. Shaffer 1960b. Ditto. III. Unaltered incidence of grossly visible adenomatous lung tumors in female CF_1 mice after prolonged exposure to cigarette smoke. Cancer **13**: 956–958.

Mizutani, M., Y. Ohnuki, Y.H. Nakanishi, and C.M. Pomerat 1960. The development of a near-diploid *in vitro* strain from a smoke condensate induced mouse tumor. Texas Rep. Biol. & Med. **18**: 455–469.

Nakanishi, Y.H., M. Mizutani, and C.M. Pomerat 1959. Smoke condensates on lung cells in tissue culture with special reference to chromosomal changes. Texas Rep.

Biol. & Med. **17**: 542–590.

Orris, L., B.L. Van Duuren, A.I. Kosak, N. Nelson, and F.L. Scmitt 1958. The carcinogenicity for mouse skin and the aromatic hydrocarbon content of cigarette-smoke condensate. J. Nat. Cancer Inst. **21**: 557–562.

Pace, D.M. and A. Elliott 1962. Effects of acetone and phenol on established cell line cultivated *in vitro*. Cancer Research **22**: 107–112.

Passey, R.D. 1957. Carcinogenicity of cigarette tars. Brit. Empire Cancer Campaign. Thirty-fifth Annual Rept. : 65–66.

———— 1962. Some problems of lung cancer. Lancet II: 107–112.

Rounds, D.E., A. Awa, and C.M. Pomerat 1962. The effect of automobile exhaust on cell growth *in vitro*. A. M.A. Arch. Environ. Health, in press.

Reddy, D.G., D.B. Reddy, and P.R. Rao 1960. Experimental production of cancer with tobacco tar and heat. Cancer **13**: 263–269.

Tsunoda, F. 1962. Studies on the aromatic hydrocarbons, 3, 4–benzpyrene, from automobil exhaust. (in Japanese). Jap. J. Public Health, in press.

Wynder, E.L., P. Kopf, and H. Ziegler 1957. A study of tobacco carcinogenesis. II. Dose-response studies. Cancer **10**: 1193–1200.