Sodium Fluoride and Cell Growth

W. D. ARMSTRONG,* M.D., PH.D.; C. H. BLOMQUIST,† PH.D.; LEON SINGER,* PH.D.; MARY E. POLLOCK,† PH.D.; LEROY C. McLAREN,† PH.D.

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Berry and Trillwood (1963) found that sodium fluoride added to culture media to give an augmented fluoride concentration of 0.045 to 4.5 p.p.m. reduced the growth rate of HeLa cells and of strain L mouse fibroblasts. These concentrations of fluoride were described as "equivalent to those recommended for use in drinking-water"—that is, 1 p.p.m. These results have been used in the public press in Great Britain and the United States to bring into question the health safety of water fluoridation. We have repeated some of the work of Berry and Trillwood and extended it to include more numerous replications of the experiments—analyses for fluoride in the media, determinations of cell protein as an additional index of cell multiplication, the use of another strain of human cells, and observations of effects of fluoride on continued multiplication of established cell cultures. We found results contrary to those of Berry and Trillwood, and we draw conclusions which do not impugn the safety of use of properly fluoridated water.

Materials and Methods

Two established lines of human epithelial cells were used: HeLa cells derived from a uterine cervical carcinoma (Gey *et al.*, 1952) and oesophageal epithelium (Minn. EE) established from an infant with a tracheo-oesophageal fistula (Syverton and McLaren, 1957). The culture medium ($Ca_5Hu_5E_{90}$) was 5% calf serum, 5% human serum, and 90% Eagle's (1959) minimal essential medium containing amino-acids and vitamins. The medium also contained penicillin 100 units, streptomycin 100 μg., neomycin 50 μg., and mycostatin 50 μg./ml. The fluoride content was 0.05 p.p.m. The sodium fluoride solution used to add fluoride to the media was sterilized by filtration through a type HA Millipore membrane.

When cell counts were to be made the medium was removed and the cell monolayer was washed once with Ca-Mg-free saline (GKN) (McLaren *et al.*, 1959). The cells were dispersed from the glass surface with a solution of 0.05% trypsin and 5×10^{-4} M Versene (Na_4EDTA) in GKN. Further dilutions were made to give cell counts of less than 50,000/ml. The number of cells in the final dilution was determined with a Coulter Counter, Model A. Protein was determined by the method of Oyama and Eagle (1956) after the medium was removed and the monolayer of cells washed with GKN solution. A measured volume (5 or 5.5 ml.) of the alkaline tartrate-$CuSO_4$ reagent was added to a tube containing the cells and allowed to stand two days to ensure complete solution of the protein. Fluoride was determined by the method of Singer and Armstrong (1954, 1962). One of us (C. H. B.) made all counts of numbers of cells and protein determinations and summarized these results without knowledge of the amount of fluoride which had been added to the cell cultures.

In Experiment 1, which was essentially a repetition of the first HeLa-cell experiment of Berry and Trillwood, the following were added to 250-ml. flat Kimble Neutraglas bottles: 9.5 ml. of medium, 0.5 ml. of test solution (NaF solution of varied but known concentration or 0.02% NaCl solution), and 0.5 ml. of HeLa-cell suspension containing 1,200,000 cells. Five replicates of each test solution were prepared. The cultures were incubated at 37° C. for three days and four separate cell counts were made from the diluted contents of each bottle.

The cells in Experiments 2 and 3 were allowed to become established in culture in normal medium in 16×125-mm. screw-cap tubes before the test solutions were added. At Day 0 the original medium was removed and 2 ml. of test medium, prepared from 38 ml. of $Ca_5Hu_5E_{90}$ medium plus 2 ml. of test solution (fluoride or saline), was added. Cultures were interrupted at intervals to seven days for cell counts and at seven days for protein determinations. HeLa cells were used in Experiment 2. The tubes were originally inoculated with 1 ml. of cell suspension containing 190,000 cells in normal medium and incubated for two days at 37° C. before changing media. The cell enumeration at each fluoride concentration was obtained from three counts of each of three replicate tubes. The protein determinations were made from three different replicate tubes.

Experiment 3 was carried out in the same way as Experiment 2 except that Minn. EE cells were used and the protein determinations were made on five-day cultures. The original inocula contained 280,000 cells.

The possibility that the high fluoride contents of the media were not sustained throughout the period of exposure of the cells to the media, perhaps by reaction of fluoride with the glass or cells, had to be considered. Separate experiments which duplicated the course of Experiments 1 and 2 were employed to test this possibility, using a medium which contained 4.7 p.p.m. of fluoride by analysis. The media removed from five culture bottles which were prepared from 9.5 ml. of media and 0.5 ml. of HeLa-cell suspension contained, after three days of incubation, 4.4 p.p.m. ± 0.39 (S.D.) of fluoride. The expected fluoride content resulting from dilution of 9.5 ml. of medium by 0.5 ml. of cell suspension is 4.5 p.p.m. of fluoride. Five cultures of HeLa cells were allowed to establish growth in normal medium in tubes for two days. The original media were removed and replaced with the fluoride-containing medium. After five days of incubation these media contained 4.7 p.p.m. ± 0.26 (S.D.) of fluoride. These results indicate that the cells were exposed to media of sustained fluoride content during the periods of cell multiplication.

Results

The results shown in Table I were obtained in experiments with media of fluoride contents which were found by Berry

TABLE I.—*Effect of Added Fluoride on Growth of HeLa Cells in Three-day Cultures*

Cultures	Added Fluoride Conc. of Media (p.p.m.)	No. of Cells per Culture (× 0·002*) (mean ± S.D.)
A	0·45	7,810 ± 810
B	0·045	8,260 ± 1,410
C	Saline control	7,390 ± 460
D	4·4	7,710 ± 610

* Original cultures diluted 500 times for counting.

* Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota.
† Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota.

and Trillwood (1963) to depress the growth of HeLa cells in seven-day cultures. Our results, on the contrary, show no relation of these amounts of fluoride in the media to multiplication of HeLa cells.

Tables II and III present the observations of effects of fluoride in the culture media on cell multiplication and protein synthesis when added to established cell cultures of HeLa and Minn. EE cells. These experiments were carried out to observe the effects of fluoride on cell growth at frequent time intervals over the course of incubation of the cultures. Also, since no deleterious effects were obtained with the amounts of fluoride used in Experiment 1, much higher fluoride concentrations were employed in these experiments.

The results in Table II, with one possible exception, show no statistically significant effects on multiplication of HeLa cells, or on their increase of protein contents, when the results with the saline control (Culture H) are compared with those obtained with cultures whose media contained up to 15 p.p.m. of fluoride. The only possible exception is the result for protein determination at Day 7 with the cultures containing 15 p.p.m. of fluoride. This latter finding may have been a consequence of loss of the cells from the glass surfaces, which is a frequent observation with hypermature HeLa cells grown as a monolayer on glass surfaces. On this account it is probable that the most reliable results of cell counts in this experiment and in Experiment 3 are those obtained before Day 7. Loss of attachment of cells to the glass surfaces in the seven-day HeLa-cell cultures used by Berry and Trillwood may account for their observations.

The determinations of protein in Experiment 3 were made at Day 5 in order to eliminate the effects on these results of loss of cells from the glass surfaces as the cultures aged. A similar pattern of results was obtained in Experiment 3 with Minn. EE cells as with HeLa cells (Experiment 2). At Day 5 the protein contents of Cultures E (15 p.p.m. fluoride) were statistically lower ($P=0.007$) than those of Cultures H (saline control), and the cell counts of the same cultures were at the borderline of significant difference at Day 7 ($P=0.08$). At Day 3 the presence of 15 p.p.m. of fluoride had no effect on multiplication of Minn. EE cells, but from Day 3 to Day 7 the number of cells did not increase in these cultures (Cultures E).

The results obtained with the established cell cultures clearly indicate no effect of up to 10 p.p.m. of fluoride in the media on growth of HeLa and Minn. EE cells. Evidence for an incipient retardant effect on cell growth, particularly of Minn. EE cells, was observed at a fluoride concentration of 15 p.p.m.

Discussion

Our results regarding concentrations of fluoride required to affect the growth of human epithelial cells can be compared with those of two other recent studies of fluoride effects on cultures of other kinds of cells. Proffit and Ackerman (1964) examined the effects of fluoride on D.N.A. and collagen synthesis by organ cultures of rapidly growing rat metacarpal bones. Tritium-labelled thymidine uptake, indicating D.N.A. formation, from the media was not affected by up to 20 p.p.m. of fluoride, but was reduced by higher amounts of fluoride in the media. Collagen synthesis, denoted by uptake of ^{14}C-labelled proline, was reduced only by fluoride concentrations in excess of 10 p.p.m. Albright (1964) worked with cultures of murine leukaemic lymphoblasts and found no inhibition of cell multiplication at a fluoride concentration of 3×10^{-4} M (5.7 p.p.m.). A possible retardant effect on cell growth was noted at 4.4×10^{-4} M (8.4 p.p.m.) of fluoride, and this effect was definite at 5.9×10^{-4} M (11.2 p.p.m.) of fluoride.

The appraisal of the physiological significance of the results of our studies reported in this paper, and those of other workers who have studied the effects of fluoride on cell cultures, should be made in relation to the fluoride concentrations of human body fluids. Singer and Armstrong (1960) found the mean fluoride contents of the plasma of persons using water containing 0.15 to 2.5 p.p.m. of fluoride to lie within the range of 0.14 p.p.m. ± 0.005 (S.E.) to 0.19 p.p.m. ± 0.0085 (S.E.). Persons who used water containing 5.4 p.p.m. of fluoride had a slight, but significantly elevated, mean plasma fluoride content of 0.26 p.p.m. ± 0.0124 (S.E.). Rapid and effective mechanisms due to renal excretion and skeletal sequestration of fluoride (Armstrong, 1960) operate to prevent large rises of plasma fluoride content even when the intake is enormous in comparison to the amount of fluoride which could be derived from fluoridated water. Patients were treated with 50–100 mg. of fluoride a day by mouth for periods up to 34 weeks. The highest plasma fluoride concentration found (Armstrong *et al.*, 1964) was 1.8 p.p.m. after 14 days of treatment, and the plasma fluoride content of the same patient was

TABLE II.—*Effect of Fluoride on Multiplication of Established HeLa-cell Cultures—Experiment 2.*

Culture		Day 0	Day 1	Day 3	Day 6	Day 7	Flouride Added to Media (p.p.m.)
E	Cells*	13,000 ± 1,560	18,900 ± 360	33,800 ± 1,910	37,900 ± 2,740	33,600 ± 2,140	15
	Protein†	0·14 ± 0·039				0·22 ± 0·020	
F	Cells	15,700 ± 540	17,700 ± 1,010	27,300 ± 510	37,000 ± 5,890	32,900 ± 1,130	0·5
	Protein	0·20 ± 0·011				0·34 ± 0·045	
G	Cells	15,100 ± 500	17,800 ± 980	35,200 ± 2,000	38,800 ± 2,220	38,900 ± 2,690	10
	Protein	0·18 ± 0·032				0·32 ± 0·025	
H	Cells	14,700 ± 280	18,600 ± 460	29,100 ± 1,700	35,900 ± 1,670	38,700 ± 820	Saline control
	Protein	0·16 ± 0·018				0·32 ± 0·030	
I	Cells	17,900 ± 1,030	18,800 ± 530	32,300 ± 720	39,900 ± 2,170	35,000 ± 2,190	5·0
	Protein	0·18 ± 0·052				0·35 ± 0·013	

* Number of cells per culture (× 1/15); mean (nearest 100) ± S.D. † mg. protein per culture; mean ± S.D.

TABLE III.—*Effect of Fluoride on Multiplication of Established Minn. EE Cell Cultures—Experiment 3.*

Culture		Day 0	Day 3	Day 5	Day 7	Fluoride Added to Media (p.p.m.)
E	Cells*	22,670 ± 570	65,800 ± 2,990	65,600 ± 3,530	63,400 ± 7,340	15
	Protein†	0·34 ± 0·044		0·55 ± 0·057		
F	Cells	23,300 ± 690	66,500 ± 5,090	74,200 ± 11,500	84,700 ± 4,310	0·5
	Protein	0·32 ± 0·005		0·72 ± 0·035		
G	Cells	23,200 ± 660	47,100 ± 3,800	50,200 ± 8,900	74,500 ± 13,960	10
	Protein	0·27 ± 0·031		0·66 ± 0·102		
H	Cells	23,100 ± 620	57,000 ± 750	79,900 ± 12,170	84,800 ± 11,010	Saline control
	Protein	0·28 ± 0·029		0·78 ± 0·037		
I	Cells	22,000 ± 670	71,400 ± 5,460	77,800 ± 14,300	92,200 ± 5,400	5
	Protein	0·26 ± 0·039		0·74 ± 0·089		

* Number of cells per culture (× 1/15); mean (nearest 100) ± S.D. † Mg. protein per culture; mean ± S.D.

0.45 and 0.48 p.p.m. after 33 and 34 weeks of continued treatment. It seems certain, then, that the fluid environment of cells cannot reach the fluoride concentrations which are required to inhibit cell growth of those cells that have been studied under *in vitro* conditions.

Summary

Sodium fluoride was added in known amounts to cultures of HeLa and human oesophageal cells, and the multiplication of the cells was determined by cell counts carried out by an operator who was unaware of the fluoride contents of the media. Amounts of added fluoride up to 4.5 p.p.m. did not affect the multiplication of HeLa cells when the fluoride was added to the media at the same time as the media were inoculated with the cells. No effect on cell multiplication or on protein synthesis was found by up to 10 p.p.m. added fluoride with HeLa cells or oesophageal cells. At 15 p.p.m. added fluoride an incipient retardant effect on growth of oesophageal epithelial cells was noted.

These results indicate that higher fluoride concentrations than those found in human body fluids are required, under *in vitro* conditions, to alter epithelial cell growth.

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New Observations on Distribution of Neoplasms of Female Breast in Certain European Countries

A. J. LEA,* M.B., CH.B.

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It must first be made clear that we are here dealing with death rates from neoplasms as distinct from their incidences, which unfortunately are not known. However, treatment of neoplasms of the breast does not vary greatly from one country to another, so that errors introduced by such differences as do exist are likely to be small and not greatly to influence the results.

The very low death rate from neoplasms of the breast in Japanese women as compared with the rates for women in western Europe has been known for some years (Segi, 1955). Various explanations, ranging from differences in breast-feeding habits (Stocks, 1958) to racial factors (Steiner, 1954), have been suggested to account for this. If the difference were due mainly to racial factors, then it might show in the distribution of these neoplasms in a country in which part of the population was of Mongoloid origin and part of European, provided that the two sections of the population could be distinguished geographically and that such distinction corresponded with the method of recording the vital statistics. Such a country is Finland, where the Lapp population in the north has a Mongoloid origin, and where the death rate from neoplasms of the breast is known to be low for the whole country (Segi and Kurihara, 1962). Unfortunately, the necessary data for Finland were not readily available, but those for Norway and Sweden were to hand. It was known that the death rates for neoplasms of the breast in these countries, though higher than in Finland, were considerably lower than for Western Europe in general (Segi and Kurihara, 1962). It was also known that part of the population in the north of these countries was of Lapp origin (Coon, 1939).

Examination of crude death rates from neoplasms of the breast for the individual counties of Norway and Sweden showed that wide variations existed in both countries, the rates in the north being much less than those in the south. By pooling the data for the two countries and plotting it against the mean latitude of the various counties it was apparent that the gradient ran fairly smoothly from north to south. Addition of similar data for England and Wales and Scotland showed that the gradient continued south to the Channel Islands. This result was very surprising, but improvement of the data by using all available for a nine-year period, correcting for age distribution and constructing a mortality index for the latitude range of 50° to 70° North, merely had the effect of smoothing out some of the irregularities. It was found that the probability of the correlation between the mortality index and the mean latitude being due to chance was less than 1 in 1,000. It was obvious that these results could not be explained by a Mongoloid origin of part of the population.

Factors which might be associated with latitude were considered, the obvious one being temperature. It was found possible to make an estimate of the mean annual temperature for each degree of latitude for the countries under investigation from data published by the Meteorological Office. A high degree of correlation between mean annual temperature and mortality rate from neoplasms of the breast ($P<0.001$) was found. However, for London, the area providing the largest population and most reliable statistics on death rates, the published mean temperature was much lower than it should have been to fit the hypothesis. A check from the individual monthly values showed that the annual maximum was 10° F. (5.5° C.) too low, and correction of this entirely removed the apparent discrepancy.

If the association between mean annual temperature and death rate from neoplasms of the breast held good for the rest of the world, then it was obvious that populations living in the extreme

* Statistics Unit, Imperial Cancer Research Fund, London.