Radiation induced chromosome aberrations in human foetal cells grown in vitro [1]

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Preface

The senior author is responsible for the initiative, planning and supervision of this investigation which after some preliminaries was started in 1959. Several difficulties, in obtaining the necessary tissue material from suitable human foetuses and limitations of available scientific manpower during a period of rapid expansion of the research programme at the Institute for Medical Genetics, at Uppsala, prevented an earlier conclusion of this work.

However, the intervening years have brought very few publications on the effect of ionizing radiations on human cells grown *in vitro* and, in fact, none duplicating this report. Experiments with human cell cultures have numerous short-comings, many of which we are not as yet in a position to appreciate fully. Only further systematic studies can fill the gaps and eventually make it possible to better understand and more precisely evaluate the use of human cell cultures as a tool in genetics and radiation research.

In the meantime, conclusions must be guarded, and it is necessary to accept the fact that such investigations are likely to raise more questions than they answer. Maintaining this critical attitude, I regard this investigation merely as one of the first unsophisticated approaches to this complex problem. I hope, nevertheless, that it will stimulate a few more workers to undertake experiments with the same or similar techniques.

Dr. M. Fraccaro, with the assistance of J. Lindsten, Med. et Fil. Kand., was in charge of the practical details of the experiments, including the cell culture work and the preparation of slides for microscopy. Furthermore, together with Karl

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Fredga, Fil. Kand., they analysed the majority of the cells scored in the main experimental series, and Dr. Fraccaro assisted in the preparation of this report.

The participation of other members of the staff of this institute and of the staff of this particular project has been indispensable. Acknowledgements are due to the following co-workers for substantial and significant contributions.

Berta Santesson, Ph. D., Karl Fredga, Fil. Kand. and J. Lindsten, Med. et Fil. Kand., held appointments as senior (B. S.) and junior (K. F. and J. L.) research assistants in cytogenetics. Their conscientious and careful chromosome analyses and recording of the primary data is much appreciated.

The statistical calculations were supervised by Mr. E. Lander, chief of the statistical section at the institute.

Indispensable biophysical and radio-biological advice and practical aid was generously given by H. Börje Larsson, civil engineer, Ph. D., associate professor of radiobiology at the University of Uppsala.

Many thanks are due to Professor The Svedberg, Ph. D., Director of the Gustaf Werner Institute for Nuclear Chemistry at Uppsala, for permission to use the Co-60 irradiation equipment.

Finally, the cultivation of the cells *in vitro* was handled by the technical staff of our cell biology laboratory under the skilful direction of Miss Elsa-Britta Larsson, chief technician.

Introduction

The problem of genetical radiation hazards for human populations has two main aspects. One is the extent and type of damage, mainly as gene mutations and chromosomal aberrations, induced by acute or chronic radiation exposure of the gonads. The other is the fate of the zygotes, embryos or individuals who carry such genetical deviations.

Extensive and detailed information is now available, for a great variety of organisms, about the types of changes which can be induced in genes and chromosomes by direct exposure to ionizing radiations. The fundamental similarity of these structures, in the vast majority of living organisms, suggests that, in principle, human chromosomes and genes should react to ionizing radiations according to the same qualitative pattern. Important differences are likely to be quantitative rather than qualitative, *i.e.* to concern radio-sensitivity rather than type of damage.

In these investigations we have chosen to study the effect of acute doses of radiation, varying from 0-150 r, on the chromosomes of human cells, grown *in vitro*. The cell cultures originated from foetal brain and lung tissue. Post-metaphase chromosomal aberrations were used as a quantitative measure of the genetical damage caused by the irradiation.

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Review of relevant literature

Radiation Effects on Human Chromosomes. *In vitro* Experiments

In a large number of investigations the effects of different kinds of ionizing radiation on plant and animal chromosomes have been analysed in great detail (cf. the monographs of Lea, 1956; Read, 1959; Bacq and Alexander, 1961 and Evans, 1962). On the other hand, relatively few experiments with human cells have been reported (Bender, 1957; Puck, 1958; Bender, 1960; Chu et al., 1961).

Essentially the same technique was used by Bender (1957) and Puck (1958). They measured the radiation effect by counting the number of chromosomal aberrations detectable at metaphase. In both investigations the cells were treated with colchicine before fixation and staining.

Bender's (1957) cell cultures were derived from the normal kidney of a one-year-old girl. Morphologically, the cells of these cultures were epithelioid. Cells from the second to the fourth sub-cultures were used in the experiments. These cultures were euploid and contained an average of 8 per cent polyploid cells. They were exposed to acute doses of 25 and 50 r and fixed after 42, 49 and 72 hours. A total of 974 diploid cells in mitotic metaphase were analysed with respect to different types of chromosome breaks and scored according to an operational system of classifiction. The rate of spontaneous aberrations in the control cultures was about 0.01 breaks per cell. Combining the 42 and 49 hour recovery periods, a calculation of the relation between effect and dose gave a value of 0.003 chromosome breaks per cell per roentgen. The doubling dose was estimated at 3.3 r. Bender was well aware of the deficiencies of this set of experiments and interpretations and conclusions were presented with critical reservations. Nevertheless, these observations are of great interest also because they were the first radio-genetical experiments carried out on human euploid cell cultures.

In a subsequent paper Bender (1960) presented an enlarged series of experiments with essentially the same results. Furthermore, he could also show that neither sex nor age of the donor of the cells used for culture influenced the aberration rate.

Puck (1958) used cell cultures derived from the normal skin of four different individuals for his radiation experiments. Some of these cultures had been transferred fifty times while others were used within 2-3 weeks of the initial biopsy. The morphology of the cells was fibroblastlike. The cultures were euploid even after 50 transfers and contained only 3 per cent polyploid cells. The doses used in the experiments varied from 0-150 r with a dose rate of 143 r per minute. In some experiments a dose rate of 50 r per minute was used, without any apparent difference. In most experiments the cells were fixed within 72 hours of irradiation but some recovery periods varying from a few minutes to 7 days were also included.

In all Puck's experimental and control cultures a total of 299 unequivocal metaphases were scored. The spontaneous aberration rate was high, i.e. 25 chromosome breaks in 116 control cells or 0.2 breaks per cell. In the 183 irradiated cells 240 breaks

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were recorded, corresponding to an average of about 0.02 breaks per cell per r. While realising the difficulty in finding metaphases suitable for analysis, the results from such a small series of radiated cells need further confirmation, in particular since there is a considerable discrepancy between the rate of spontaneous as well as induced aberrations between Bender's and Puck's data.

Morkovin and Feldman (1959) pointed out that the corrections of the dosimetric values used by Puck (1958), and for which Morkovin holds himself responsible, mean that numerical conclusions drawn from these data also need correction. Furthermore, they pointed out that the value given by Puck for the number of " hits " per mitosis per r of 0.027 should be corrected to a value of about 0.019. The dose reported by Puck to produce, on the average, one chromosome abnormality in a euploid human cell should be corrected to 57-85 rads absorbed dose instead of 40-50 r. This reduces the differences between Puck's and Bender's figures but there is still a considerable discrepancy.

Another investigation on X-ray induced chromosome aberrations in human euploid cells grown *in vitro* has been published by Chu et al. (1961). Their cell cultures were derived from surgical material from a variety of tissues. The morphology of the cultured cells was in most instances fibroblast-like. Three acute doses were used, i.e. 25, 50 and 100 r, and the chromosome aberrations were scored at intervals after the irradiation, ranging from 6 to 67 hours. A total of 1,293 metaphases were analysed. No differences with respect to radio-sensitivity were observed between tissues or between individuals, nor between tissues within individuals. The aberrations were divided into two major categories, chromatid and chromosome. Shortly after irradiation, only chromatid aberrations were found, and in the later post- irradiation periods only chromosome aberrations. The frequency of spontaneous aberrations was on the average 0.1 per cell in all control cultures. The average aberration frequencies induced by all the different doses added together were 0.022 at 18 hours and 0.015 at 67 hours after exposure (if iso-chromatid aberrations are considered to result from one break) or 0.034 and 0.016, respectively (if iso-chromatid aberrations are considered to result from two breaks).

The recent discovery that phytohemagglutinins induce active multiplication of peripheral blood cells *in vitro* provided a new tool for genetics and radiation research. This technique has already been used in a few investigations on the effect of radiation on human chromosomes.

Ohnuki et al. (1961) irradiated human blood cells *in vitro*, at the fourth day of culture, with a single dose of 400 r from a Co-60 source. They analysed 226 metaphases in such cultures from three individuals and found that 128, or 56.6 per cent, had damaged chromosomes, mainly in the form of acentric fragments and dicentrics. The occurrence of spontaneous aberrations in the control cultures was not mentioned. A considerable increase of tetraploid cells was observed in the irradiated cultures, *i.e.* 5.4 per cent against an average of 0.1 per cent in the non-irradiated control cultures.

Bender and Gooch (1961) irradiated human blood immediately after it was drawn

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and studied the chromosome aberrations induced in the cultured leucocytes. In the control cultures the aberration rate was about 0.02 chromatid breaks per cell and no aberrations of the chromosome type were observed. In the irradiated cultures, aberrations of the chromosome type, only, were found and these aberrations had been induced at a rate of about 0.004 chromosome breaks per cell per r. The following types of aberrations were recorded: ring chromosomes, dicentrics, symmetrical translocations and deletions. Their frequencies were close to those observed by the same authors in other systems. Such investigations are of great importance since this system approaches more than any other type of experiment human cells irradiated *in vivo*.

Quite recently, Bender and Gooch (1962) reported the results of further similar experiments. The dose effect calculated on the basis of these new data was 0.0024 chromosome breaks per cell per r, which is very close to the value of 0.003 calculated by Bender (1960) for human epithelioid cells grown *in vitro*.

Other radiation effects on human cell cultures

In addition to the scoring of induced chromosomal aberrations, radiation damage to cells can be determined in various other ways, *e. g.*, by recording the depression of the mitotic activity or the DNA synthesis, the formation of giant cells or by the construction of survival curves for irradiated cell cultures.

Cell death following irradiation (mitotic or genetic death) has been studied intensively, and adequate quantitative data are available for several different plant and animal systems. The best way to study this phenomenon quantitatively is by determining the ability of the cells to form colonies or clones. A cell may divide after irradiation but if this does not lead to colony formation the cell line is counted as killed. The formation of giant cells is often seen in cell cultures. These giant cells do not divide but continue to synthesize nucleic acids (DNA and RNA) and proteins and grow, often to immense sizes.

Most of these experiments have been made with established cell lines. In spite of their human origin such cell lines may be quite different biologically from euploid human cells. Puck and Marcus (1956) irradiated cell cultures originating from single human HeLa cells (derived from a cervical carcinoma). The capacity of these cells to form macroscopic colonies was studied quantitatively.

Distinct effects were observed after exposure to doses as low as 75 r. After a dose of 500 r, only one per cent of the seeded cells were able to form large colonies (*i.e.* of > 50 cells). Cells incapable of forming large colonies are not necessarily dead but may produce colonies containing giant cells. Such giant cells occurred at 6.7 per cent after a dose of 50 r and about 100 per cent after 600 r. Furthermore, the cells were not killed by doses of 800 r, but their ability to divide was destroyed. Puck and Marcus explained their results in terms of the chromosome target theory, claiming that the major damage is genetical, consisting of lesions in one or several chromosomes. Furthermore, Yamada and Puck (1961) studied the life cycles of hyperploid S 3

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HeLa cells which were given sublethal doses of X-rays. Extensive reversible mitotic lag, irreversible reproductive death and chromosomal aberrations were observed at doses below 250 rads, indicating that the common cause of these phenomena was the chromosomal damage produced by the radiation. Yamada and Puck (*l. c.*) also pointed out the differential effects of the radiation on some of the biophysical and biochemical cellular activities, such as active transport or capacity to synthesize virus particles which remain unaffected by doses of several thousand roentgens.

Essentially the same conclusions were reached by Tolmach (1961) in further experiments with this cell strain. The general metabolic activity, cell growth and possibly the rate of cell division after a radiation lag, were quite radio-resistant.

Using Puck's technique, Marin et al. (1960) analysed the survival curves of irradiated cells from the epithelioid strain EUE, derived from human foetal tissue. The mean lethal dose, LD_{37}, defined as the dose in roentgens needed to reduce the surviving colony-producing inoculates to 37 per cent of their original level, was determined to be 50-60 r. A certain amount of variability was observed which was independent of the dose but influenced by other experimental factors, such as the density of the cell inoculum which was observed to affect the single cell plating efficiency.

The dynamics of cell populations, the formation of giant cells and other cytopathological effects of high doses of radiation (300 to 3000 r) were studied by Fogh *et al.* (1961) in a hypo-tetraploid EL strain derived from human amnion. Surviving cells from cultures exposed once or twice to 1,000 r showed no significant differences in growth behaviour as compared to the parental cultures.

Puck et al. (1957) calculated survival curves for cultured normal human cells derived from a variety of tissues and exposed to different doses of X-rays. The mean lethal dose, LD_{37}, for normal diploid human cells was about 50 r., *i.e.* half that for aneuploid hyperdiploid cells. All epithelioid cells appeared to be more radio-resistant than the fibroblastlike ones.

These workers also observed a high frequency of morphological and biochemical " mutants " among surviving cells after doses of 500 to 900 r. The biochemical " mutants " differed with respect to nutritional requirements.

Elkind and Sutton (1959) investigated X-ray damage and recovery in two cell strains derived from the Chinese hamster (Cricetulus griseus) grown *in vitro*. This is mentioned here as it is of interest in relation to Puck's work on human cell strains. The X-ray doses were given by fractions to test for the repair of accumulated damage, and survival curves were constructed. Elkind and Sutton (*l. c.*), also found that, after irradiation, essentially all the surviving cells were undamaged, in spite of their different radio-sensitivity. Apparently, the accumulated damage was repaired before the first division after irradiation. This is an interesting difference in comparison with the observations of Puck et al. (1957) of a high yield of " mutants " in the progeny of cells surviving 5 to 7 LD_{37} doses.

Comprehensive discussions of the problems pertinent to investigations of radiation-induced damage in cell cultures and subsequent recovery have been published recently by Puck (1960), and by Bender and Wolff (1961).

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Radiation effects on mammalian chromosomes other than human

Bender (1960) was concerned over the report by Puck (1958) that " fibroblasts " grown *in vitro* have much higher chromosome aberration rates than epithelioid cells, in control as well as irradiated cultures, and considered it imperative that chromosome aberration rates be determined for mammalian cells *in vivo*.

Bender (1960) and Bender and Gooch (1961) reported on some such experiments with the Chinese hamster (Cricetulus griseus). Males were given whole-body doses of 25, 50 and 100 r of X-rays. Femoral bone marrow preparations were made at various intervals after irradiation according to the technique of Ford and Hamerton (1956).

No aberrations were found in 281 metaphases scored in the controls of the *in vivo* bone marrow experiments while the spontaneous rate of aberrations in the human *in vitro* kidney cells varied between 0 and 0.013 breaks per cell (Bender 1957). Two hours after the whole-body irradiation of the hamster the *in vivo* bone marrow aberration rate was 0.0051 breaks per cell per r, falling rapidly with time after irradiation.

The absence of " spontaneous " aberrations *in vivo* should be compared with the high rate of " spontaneous " aberrations found by Bender (1960) in the diploid Yerganian line 1290-2 of Chinese hamster embryonic fibroblasts and by Wakonig and Ford (1960) in the diploid V-2 cell line of similar origin. These latter " spontaneous " rates were reported as 0.21 breaks per cell and " 13 per cent aberrant metaphases ", respectively.

Bender (1960) concluded that the control aberration frequencies of zero per cent for hamster bone marrow *in vivo*, about 1 per cent for human epithelioid cells *in vitro* (Bender 1957) and about 25 per cent for human and hamster " fibroblasts " *in vitro*, clearly indicate that epithelioid cells *in vitro* display a radio-sensitivity close to that of rapidly dividing cells *in vivo*, whereas " fibroblasts " are much more sensitive.

An association between ploidy and radio-sensitivity was reported by Stroud *et al.* (1961) who observed that diploid pig kidney cells (37 chromosomes) stopped growing *in vitro* after a second dose of 500 r, while hypo-tetraploid monkey kidney cells (78 chromosomes) continued to grow after three such doses of irradiation.

Effects of therapeutic or diagnostic radiation exposures on human chromosomes

Tough et al. (1960) discovered a large number of chromosomal abnormalities in leucocyte cultures derived from two patients who had been given relatively heavy doses of X-rays over their spines (250 rads single acute dose and 10 fractionated doses of 1,500 rads, respectively).

Before treatment, the incidence of cells with structural chromosome abnormalities in the blood cultures from these two patients was 2 per cent and 1 per cent, respectively. This incidence increased to a maximum of about 40 per cent aberrant cells

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shortly after the treatment and showed a tendency to decrease during the next few days. The observed aberrations included dicentric chromosomes, rings and fragments.

Similar increases of chromosomal aberrations in peripheral blood cultures have been reported by Boyd et al. (1961) for 4 patients with thyrotoxicosis treated with I^{131} and for 2 patients with thyroid adenocarcinoma who subsequent to surgery received I^{131} treatment for widespread metastases. Their doses of I^{131} varied from 7 to 150 mC. In all instances, even though the number of analysed mitoses was not always sufficient to provide statistically significant results, some 4-13 per cent of the cells showed structural chromosome abnormalities. Certainly, these data indicate strongly that the intake of 100 mC of I^{131} may produce changes in the modal number of the chromosomes and an increase of chromosomal aberrations in leucocyte cultures quite similar to those described by Tough et al. (1960) as following a single dose of 250 rads to the spine. Furthermore, Boyd et al. (1961) suggested, but with considerable reservation, that even smaller doses, of the order of 10 mC I^{131}, used in the treatment of thyrotoxicosis, may produce significant changes.

Steward and Sanderson (1961) discovered what was probably a dicentric chromosome in 2 out of 31 cells in a leucocyte culture (prepared by the Hungerford technique) from a patient with Klinefelter's syndrome who had received diagnostic radiation a few hours before the blood sample was taken. The authors were very critical of their observations and cautiously suggested as alternatives that the abnormal chromosomes could have been produced by background irradiation, could be a cultural artifact, or, perhaps, be related to the patient's abnormal karyotype (47/XXY).

Conen (1961) reported the cytogenetical findings from a 7 week old infant who because of multiple congenital anomalies had been subjected to a total of 22 diagnostic X-ray exposures. The dose was not mentioned. The cytogenetical analyses of blood leucocyte and bone-marrow cultures showed an unusually high incidence of non-modal chromosome counts and several different types of structural abnormalities of the chromosomes, including dicentrics and probable chromatid deletions.

Bender and Gooch (1962) examined peripheral blood cell cultures from eight individuals who at a radiation accident had received whole-body mixed γ-ray and neutron fission irradiation. The blood cell cultures were prepared 29 months after the accident. Five of these individuals were estimated to have received a dose of more than 230 rads. Their cultures contained 7-23 per cent of cells with deviant chromosome counts and 2-20 per cent of metaphases with one or more chromosome aberrations, such as rings, dicentrics and minutes.

Material and methods

The cell cultures used in this investigation originated from brain and lung tissue of four human foetuses. All foetuses were morphologically normal with their stages of development corresponding to their ages. Their sexes, total heights, approximate ages and code numbers were as follows:

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Foetus code no.	Sex	Height	Approx. age
VI	Female	20 cm	18 weeks
VIII	Female	13.5 cm	15 weeks
IX	Male	23 cm	19 weeks
X	Male	12 cm	14 weeks

Further reference to this material will be by code number.

Cultures technique

Particular care was taken to irradiate the cells as soon as possible after they had become attached to the glass coverslips and had shown unequivocal signs of sufficient and active multiplication. The primary cultures were irradiated within 4-8 days after their preparation and the transferred cultures within 2-3 days of transplantation (cf. table 1).

The primary cell cultures were prepared as follows. The foetuses were sent from the Division of Obstetrics and Gynecology, Royal Academic Hospital of Uppsala

Tab. 1. Timing of experimental procedures

Origin of culture	Interval between first preparation and first transfer, days	Interval between first preparation and irradiation, days
Foetus VI, female Lung	17	19
Foetus VIII, female Lung	11	14
Foetus X, male Lung	No transfer	4
Foetus VIII, female Brain	No transfer	8
Foetus IX, male Brain	No transfer	8

to the laboratory under sterile conditions immediately after the operation. The brains and lungs were removed under sterile conditions and placed in Hanks' balanced salt solution (Hanks, 1955). Small pieces of tissue were excised and washed repeatedly in a balanced salt solution containing antibiotics. Next, the specimens were transferred

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into Petri dishes containing culture medium and minced into minute fragments with a pair of sharp ophthalmological scissors. Small amounts of medium (for composition see below) containing the minute tissue fragments were distributed to glass coverslips placed in non-wettable plastic Petri dishes (50 mm in diameter).

As nearly as possible, estimated macroscopically, the same amount of cellular material was distributed to the different Petri dishes of each series of experiments and controls. One well-known effect of ionizing radiation on living cells is a dose dependent depression of the mitotic activity, often called mitotic inhibition. In the experiments Lung VI, Lung VIII, Brain VIII and Brain IX all post-metaphases of each individual culture have been analysed. It can be seen in tables 2-5 that in

Tab. 2

Incidence of post-metaphases classified as equivocal with respect to chromosomal aberrations

Origin of culture (foetus)	Corrected dose (rads)	Length of post-irradiation period at fixation of cultures					
		24 hours			48 hours		
		N. of scored post-metaphases	Cells with equivocal aberrations	% equivocals	N. of scored post-metaphases	Cells with equivocal aberrations	% equivocals
Lung VI,	0	230	27	11.7	58	8	13.8
female	23	146	17	11.6	51	4	7.8
(1st transfer)	136	128	11	8.6	2	—	—
Lung VIII,	0	3119	186	6.0	2157	89	4.1
female	22	2011	93	4.6	2118	113	5.3
(1st transfer)	67	1121	61	5.4	1865	117	6.3
	89	793	57	7.2	456	33	7.2
	0	334	34	10.2	515	15	2.9
Lung X,	9	341	41	12.0	522	23	4.4
male	22	322	22	6.8	572	28	4.9
(primary)	45	323	18	5.6	540	16	3.0
	89	319	19	6.0	518	18	3.5
	134	324	16	4.9	260	19	7.3
Brain IX,	0	281	18	6.4	292	16	5.5
male	9	173	9	5.2	283	19	6.7
(primary)	45	118	11	9.3	117	12	10.3
	89	52	3	5.8	97	11	11.3

all of these experiments, increasing doses were associated with a decreasing number of recorded post-metaphases. An analysis of this correlation indicates that the attempt to obtain, for each series of experiments, cultures with originally the same or nearly the same, number of cells have been fairly successful.

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After an interval of 2-5 hours, giving the material time to sediment and adhere to the glass, 2 ml. of medium were added. The medium consisted of 20% pooled human serum, 35% medium 199 (Parker, 1950, Difco Lab. Detroit, USA), 5% bovine embryo extract EE 20 (Difco dessicated EE 100 diluted to EE 20 with Hanks' balanced salt solution) and 40% Hanks' balanced salt solution. Phenol red was added as a p^H indicator (Hanks, 1955). Penicillin (100 I.U./ml. medium) and streptomycin (1 mg/ml. medium) were always added to the medium.

The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. This was achieved by keeping the Petri dishes in communicating plastic boxes stacked upon each other, the bottom box containing sterile distilled water, into which flowed an air-CO_2 stream, controlled by gas flow-meters. This whole construction was kept in a thermostated room with a temperature tolerance of 37 ± 0.5 centigrade. While such a rigidly controlled environment is not required for ordinary karyotype studies, it might be important for quantitative studies of chromosomal aberrations. It remains to be investigated whether or not small temperature differences, *e.g.* of $\pm$1-2 centigrades, have a significant effect on the rate of chromosomal aberrations in human cell cultures. In fact, such temperature effects have been observed in some animal and plant material *in vivo*. It is with this in mind that we have standardized the environmental conditions of the cells and reported them in detail.

For the transplantations the cells were loosened by treatment with 0.08% trypsin (Difco 1:250) in Hanks' solution. The trypsin was permitted to act for about 10 minutes at 37°C. The action of the trypsin was then stopped by adding an equal amount of fresh medium. The obtained cell suspension was centrifuged at 400 rpm for 10 min. (+4°C), washed in Hanks' solution, re-suspended in fresh medium, transferred to new Petri dishes, and handled as with primary culturing.

A series of parallel cultures was prepared to study the behaviour and the morphology, *in vitro*, of the cells derived from foetal brain and lung. For this purpose glass Petri dishes were used. The living cultures were studied with the aid of a phase-contrast inverted microscope. For high power magnification studies fixation and staining was required. The cell aggregates attached to the cover-slip or to the bottom of the Petri dishes, were fixed with absolute alcohol (1 min.), stained with Giemsa solution, diluted 1 : 20, dehydrated with acetone (few seconds), cleared in xylol and mounted in Depex (T. G. Gurr).

Characteristics of cultured cells

After the initial procedures and seeding, described in the preceding section, the fragments and dispersed cells attached rapidly to the glass. Migrations from the fragments usually began after a few hours and a substantial mitotic activity could be observed within the first 24 hours.

The time required for a complete mitotic cycle was estimated at between 20 and 30 hours. The mitotic activity was clearly asynchronous, because cells in all stages of mitosis were observed at any given time.

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The primary brain cultures always contained two types of cells, both having characteristic cytoplasmatic prolongations, but differing significantly with respect to the size of their nuclei. One type had a large round nucleus and the other a very small nucleus. The cytoplasmatic prolongations were also of different sizes, being significantly thinner in the small-nucleated cells. This gave a characteristic morphology to the cell aggregates when fixed and stained *in toto*, as exemplified in Fig. 1. Representative samples of these cell types in living and fixed preparations are shown in Figs. 2, 3, 4 and 5. Both cell types probably originated from glial elements. This cellular dimorphism was still present after the cells had been sub-cultured by trypsi-

Fig. 1. Appearance of a cell culture, derived from human foetal brain, growing on the bottom of a glass Petri dish. Several small and large cellular aggregates. Giemsa stain

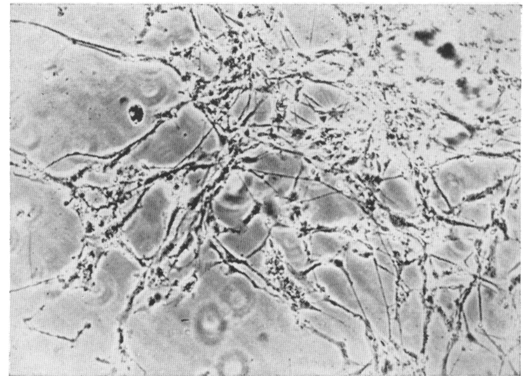

Fig. 2. Part of a live cell culture, derived from human foetal brain, showing a network of cytoplasmatic prolongations. Phase contrast

nization. A tendency for cells of similar type to re-aggregate preferentially after trypsinization was also observed.

Most of the cells derived from the lung tissue were fibroblast-like (Fig. 6) but small round cells were also observed, particularly in the primary cultures. These cells were dispersed in the cultures and no aggregates of epithelial-like cells were observed. In all transplanted cultures the cells were consistently fibroblast-like.

On the whole, the morphology and behaviour of the cells in the primary and in the first transplantation cultures did not differ markedly. Chromosome numbers and morphology were repeatedly investigated in both primary and transplanted

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cultures. Chromosome preparations were made by the aceto-orcein squash technique described by Böök et al. (1959).

The modal chromosome number was consistently 46 and all cells, analysed in detail, had apparently normal karyotypes. Occasional polyploid cells were also observed. Their frequency averaged about 5 per cent of the analysed cells. Structural

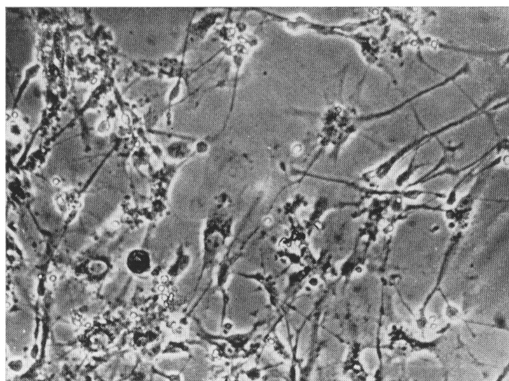

Fig. 3. Part of a live cell culture, derived from human foetal brain, showing two types of cells with large or small nuclei. Phase contrast

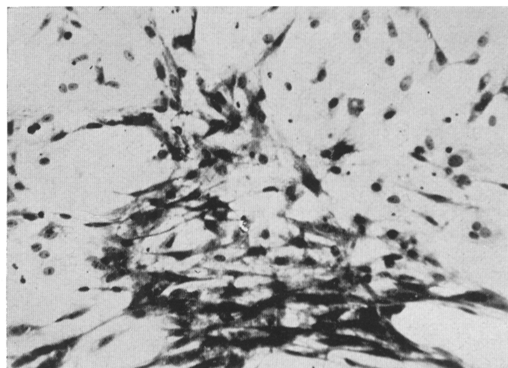

Fig. 4. Part of a cell culture, derived from human foetal brain, showing predominantly cells with large nuclei. Giemsa stain

re-arrangements of various types were occasionally observed at metaphase, with an even lower frequency.

Both primary and first transplantation cultures were used for the irradiation experiments. Although this means that not all cultures subjected to irradiation received identical treatment, experience has shown that the first sub-cultures do not differ significantly from primary cultures with respect to cellular morphology or chromosome number.

Radiation technique and dosimetry

This tissue culturing was initiated at the Institute for Medical Genetics, and the irradiations were carried out at the Gustaf Werner Institute for Nuclear Chemistry, both at Uppsala. The Petri dishes with the cultures were transported in a thermostated carrier, and except for the handling preliminary to and during the actual irradiation, the cells were constantly maintained at 37°C. The irradiation periods were very short (6-90 seconds), and it is not likely that the lower surrounding temperature (15-17°C) during this handling could have had any important effects on the results of the irradiation.

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The control cultures were treated in exactly the same way as the irradiated cultures, except for the actual radiation exposures. They were also placed in position for irradiation in the radiation room for the same time but received no dose.

The radiation was supplied from a 100 Curie Co-60 source with a mean energy of 1.25 MeV. The doses varied between 0 and 136 rads. The exposure times were between 6 and 90 seconds at a room temperature of 15 to 17°C. During irradiation the plastic Petri dishes, lids removed, were placed on a circle, 50 cm from the radiation source.

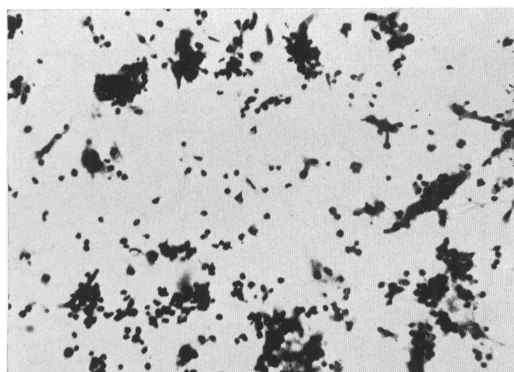

Fig. 5. Part of a cell culture, derived from human foetal brain, showing predominantly cells with small nuclei. Giemsa stain

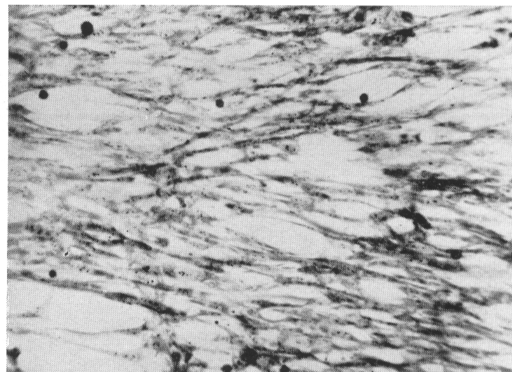

Fig. 6. Part of a cell culture, derived from human foetal lung, showing an aggregate of fibroblast-like cells. Giemsa stain

Dose determinations were made according to Fricke's $FeSO_4$-dosimetry (cf. Fricke and Morse, 1927), using a G-value of 15.6 Fe^{++} ions per 100 eV absorbed energy. Measurements of absorbed dose were made in horizontal and vertical planes through the source. The data were used to construct curves from which the doses at the points of irradiation could be read.

In all experiments, the Petri dishes with their cell cultures were placed in a plane at an angle of about 25° below the horizontal plane through the radiation source. With this arrangement the correction factor for position, including distance, was 0.96.

For the determination of the actually absorbed doses, a further correction is necessary with respect to the amount of radiation lost during the passage through the culture medium. The thickness of this fluid medium above the cells was 1.4 mm. However, as the radiation struck at an angle of about 65°, the build-up distance became 3.3 mm. This " build-up " factor, as a percentage of the maximum dose, was calculated from the depth-dose curves published by Hine and Brownell (1956) at 0.96. With this correction for the absorption in the culture medium, the dose rate was calculated at 105 rads per min. This value was correct at the time (24th De-

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cember, 1957) when the radiation source was calibrated to be 100 Curie. The dose rate at later times was calculated by multiplying with the factor

$$e^{-\frac{0.693\,t}{5.3}}$$

where t is the time (in years) after the 24th December, 1957, and 5.3 is the half-life period for Co-60. Further details about this radiation equipment will be found in a paper by Kinell and Larsson (1960).

Preparation of material for cytological analysis after irradiation

After irradiation the culture medium was removed from all cultures and replaced with pre-warmed fresh medium. Cytological preparations were made at 24 and 48 hours after irradiation. The coverslips were lifted from the Petri dishes, washed in Hanks' solution to remove all traces of serum and immediately fixed in Kahle's modified fixative. The cells were stained with basic fuchsin after hydrolysis with 1 N-HCl for 15 min. at 60°C and counterstained with light-green. This procedure gave a satisfactory differentiation between the chromosomal and nucleolar material.

Principles of analysis and scoring

In the experiments Lung VIII, Brain VIII and Brain IX, all post-metaphases on each coverslip were scored. In the experiment Lung X, a random sample of post-metaphases was analysed.

Visible chromosome aberrations at post-metaphase, *i.e.* anaphases and telophases, were scored. This group of aberrations is composed of a variety of different chromosome irregularities. They include both numerical and structural changes ranging from small acentric fragments at the limit of visibility to lagging of groups of chromosomes.

With presently available techniques, and a chromosome number of 46, it was not possible to analyse the abnormal post-metaphases in such detail as to make sure whether they contained one chromosomal aberration, only, two or even more. However, with respect to observations on human metaphase chromosomes *in vitro*, after acute doses of ionizing radiation within the same ranges as here, very few abnormal post-metaphases in our cell cultures were likely to contain more than one chromosomal aberration.

Therefore, and for brevity, " aberration " has also been used in the meaning of an aberrant cell (in post-metaphase), *i.e.* containing at least one chromosomal aberration. For example, to be strictly correct, " the frequency of post-metaphase aberrations " should have been written: " the frequency of abnormal post-metaphases containing at least one chromosomal aberration ".

In the scoring process each aberration was sketched on paper. This procedure allowed an approximate identification and classification of the various types of abnor-

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malities. However, because of remaining uncertainties of classification, it was decided that the comparative results were better presented in terms of frequencies of all types of post-metaphase aberrations, added together.

The kinds of anaphase and telophase abnormalities which were most frequently recorded have been sketched in Fig. 7, and representative photographs of various

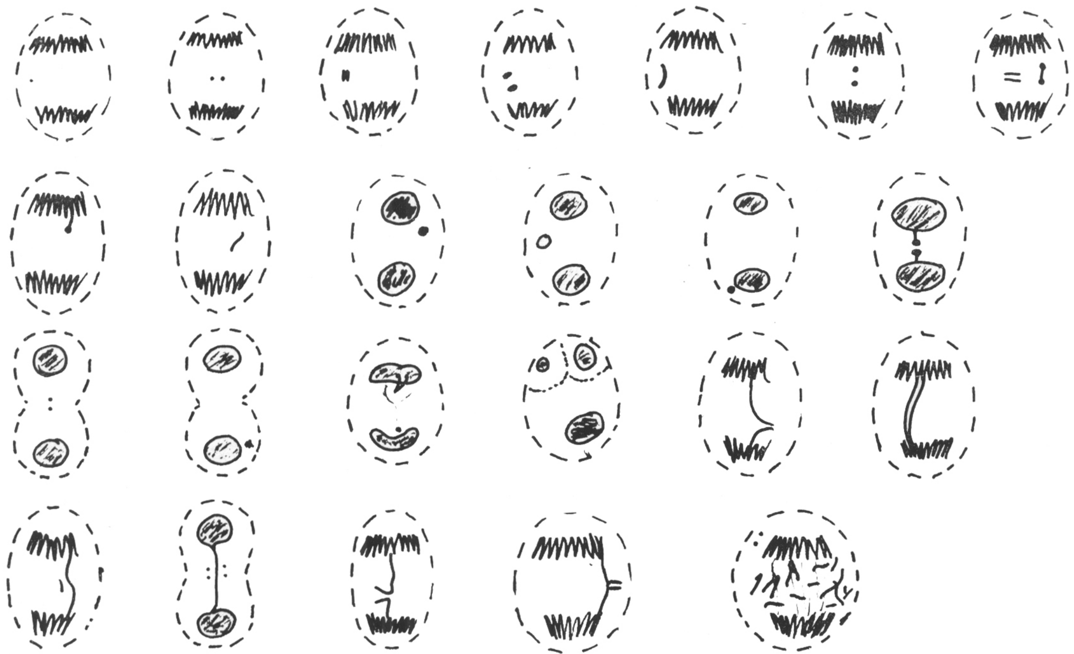

Fig. 7. Schematic drawings of different types of post-metaphase aberrations, observed and scored in the irradiated cell cultures and their controls

aberrations are shown in Fig. 8. Sometimes it was difficult to decide whether an aberration had occurred or not. This was often the case with small questionably euchromatic bodies which might be minute acentric chromosome fragments. However, it was decided to register also these doubtful cells. Their relative frequencies, in the representative experiments, are given in Table 2. Apparently, the frequency of these " equivocal " cells ranging from 3 to 14 per cent, was not dependent on the dose of radiation. A proportion of them, however, are likely to consist of true aberrations and therefore the final frequencies of aberrations given in Tables 3, 4 and 5 are probably underestimated by a factor proportional to the incidence of these equivocal post-metaphases.

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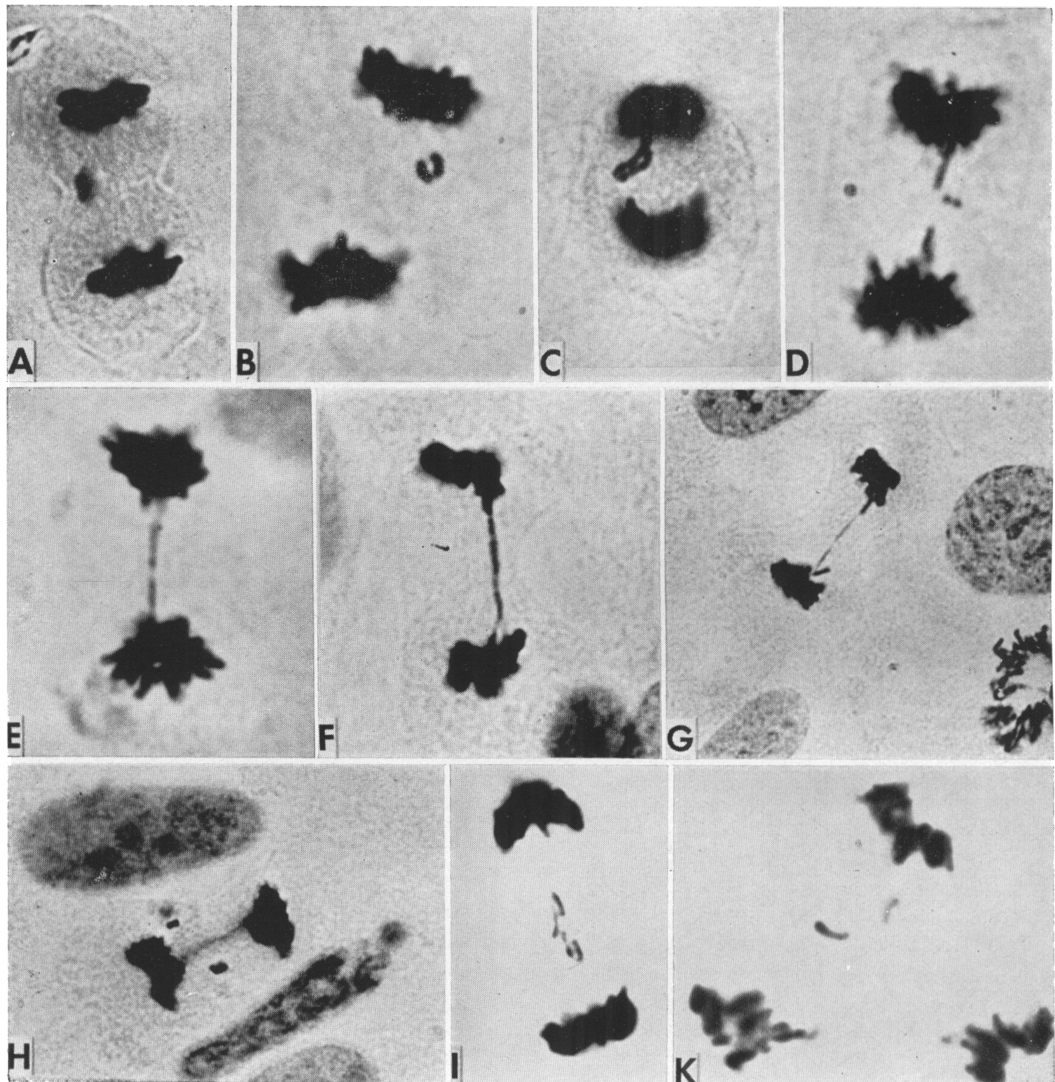

Fig. 8. Photomicrographs of a sample of different types of abnormal post-metaphases, recorded in irradiated cell cultures derived from human foetal brain and lung; a and b, acentric chromosome fragments; c, large acentric or lagging chromosome; d, broken bridge and two (double) acentric fragments; e, f, g and h, different types of bridges with or without visible acentric fragments; i, lagging chromosomes; and k, tripolar anaphase with lagging chromosomes

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Results

On the whole, we have been reluctant to add the results from different sets of experiments. The value of combining data from different foetuses or from experimental irradiations carried out at different times is questionable. Furthermore, the conditions of culture and the subsequent fixation and staining procedures cannot be identical, even if everything has been done to standardize all procedures. Therefore, recognizing the inherent variations characteristic of biological systems in

Tab. 3. Results of the pilot experiment

Origin of culture (foetus)	Dose, rads		Result of scoring 24 hrs after the acute radiation dose		
	Theoretical *	Corrected	N. of post-metaphases	Thereof aberrant cells	
				N.	%
Lung VI,	0	0	203	7	3.4
female	25	23	129	4	3.1
(1st transfer)	150	136	117	21	17.9

* For practical reasons based on distance and dose rate in air as constants during varying exposure times.

response to whatever influence or treatment, the results should be interpreted in terms of sets of limits rather than of average values. The total number of analysed post-metaphases and the absolute and relative frequencies of aberrations are given separately for each individual experiment in tables 3, 4 and 5. Confidence limits at the 95 per cent level are also included in tables 4 and 5.

Chromosomal aberrations in the control cultures

The incidence of chromosomal aberrations in the control cultures of the various experiments is given in tables 3, 4 and 5. These aberrations did not differ qualitatively from those of the irradiated cultures. In the " brain " cultures [4] (cf. table 5) the frequency of " spontaneous " aberrations at 24 hours after exposure was consistently 3 per cent. Approximately the same incidence (3.4 per cent) was observed

[4] For brevity we have used a few expressions which, quite obviously, are inadequate. It should not be difficult, however, to understand such references as " brain " cultures, meaning cell cultures derived from brain tissue, or zero dose and 48 hour recovery period, referring to a control culture which, for technical reasons, has been entered in one of the tables under these headings, etc.

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Tab. 4. Incidence of abnormal post-metaphases (i. e. containing at least one chromosomal aberration) in cell cultures derived from human foetal lung tissue, subjected to acute doses of radiation from a Co-60 source

CL 95% = 95 per cent confidence limits

Origin of culture (foetus)	Dose (rads)		Length of post-irradiation period at fixation of cultures							
			24 hours				48 hours			
	Theoretical	Corrected	N. of post-metaphases	Thereof cells with aberr. N.	%	CL 95 %	N. of post-metaphases	Thereof cells with aberr. N.	%	CL 95 %
Lung VIII, female (1st transfer)	0	0	2933	18	0.6	0.4-1.0	2068	8	0.4	0.2-0.8
	25	22	1918	34	1.8	1.2-2.5	2005	12	0.6	0.3-1.0
	75	67	1060	54	5.1	3.8-6.6	1748	47	2.7	2.0-3.6
	100	89	736	88	12.0	9.6-14.5	423	23	5.4	3.4-8.0
Lung X, male (primary)	0	0	300	7	2.3	0.9-4.7	500	8	1.6	0.7-3.1
	10	9	300	8	2.7	1.2-5.2	499	11	2.2	1.1-3.9
	25	22	300	10	3.3	1.6-6.0	544	22	4.0	2.6-6.0
	50	45	305	16	5.2	3.0-8.4	524	24	4.6	3.0-6.7
	100	89	300	28	9.3	6.2-13.2	500	37	7.4	5.2-10.1
	150	134	308	42	13.6	10.0-18.0	241	32	13.3	9.2-18.3

Tab. 5. Incidence of abnormal post-metaphases (i. e. containing at least one chromosomal aberration) in cell cultures derived from human foetal brain tissue, subjected to acute doses of radiation from a Co-60 source

CL 95% = 95 per cent confidence limits

Origin of culture (foetus)	Dose (rads)		Length of post-irradiation period at fixation of cultures							
			24 hours				48 hours			
	Theoretical	Corrected	N. of post-metaphases	Thereof cells with aberr. N.	%	CL 95 %	N. of post-metaphases	Thereof cells with aberr. N.	%	CL 95 %
Brain VIII, female (primary)	0	0	312	9	2.9	1.3-5.4	—	—	—	—
	25	23	280	14	5.0	2.7-8.2	—	—	—	—
	75	67	135	17	12.6	7.4-19.6	—	—	—	—
	150	135	102	19	18.6	11.6-27.5	—	—	—	—
Brain IX, male (primary)	0	0	263	8	3.0	1.3-5.8	276	4	1.4	0.4-3.6
	10	9	164	12	7.3	3.8-12.4	264	8	3.0	1.3-5.8
	50	45	107	8	7.5	3.3-14.2	105	6	5.7	2.1-12.0
	100	89	49	6	12.2	4.5-24.0	86	10	11.6	5.7-20.3

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in the preliminary experiment Lung VI (table 3). However, between the other two series of "lung" cultures there is a difference (table 4). The incidence in Lung X was 2.3 per cent and in Lung VIII 0.6 per cent. The 95 per cent confidence intervals are 0.9-4.7 and 0.4-1.0 per cent, respectively.

Further comparisons between these two series of experiments revealed some interesting features. The frequencies of unequivocal post-metaphase aberrations at the doses of 22 and 89 rads with 24 or 48 hours' recovery periods were higher for the Lung X cultures with one exception only, *i.e.* at 89 rads and 24 hour recovery periods which gave 9.3 per cent for Lung X and 12.0 per cent for Lung VIII. This exception appears again at 89 rads, counting *equivocal* aberrations only, and consequently remains, if unequivocals and equivocals are added together. Furthermore, at the 48 hour recovery periods, only the frequencies of equivocals were consistently higher for the Lung VIII cultures. Adding equivocals and unequivocals for each culture changes this general picture only in so far as the Lung VIII and Lung X cultures now obtain the same frequency at zero dose and 48 hour recovery period (4.5 per cent).

None of these differences reach statistical significance. They have interest only in showing trends which might be worth while to follow up in further investigations.

It should be of some importance to know whether or not radiation effects vary significantly between foetuses (individuals) and for tissues (*n.b.* here cell cultures derived from different tissues) between and/or within individual foetuses.

Disregarding the preliminary experiment Lung VI, in view of the small number of analysed cells, we could postulate that cell cultures derived from the lungs of the foetuses VIII and X are different with respect to the frequency of "spontaneous" as well as radiation induced chromosomal aberrations. Expressed differently, lung X has a higher radio-sensitivity and a higher frequency of "spontaneous" aberrations.

One might question this tentative conclusion for the following reasons. Although everything was done to standardize the experiments, the influence of systematic errors or unnoticed minor differences in technique cannot be excluded.

Another similar objection should be based on the possibility of a systematic difference in the scoring procedure. This objection should not argue from the fact that in the Lung VIII series, all cells of the cultures were scored and in the Lung X series random samples of 300 and 500 cells each, unless one wants to cast doubt on the actual, randomisation procedure.

Whatever the nature of such systematic errors, their effects should be systematic, too. With very few exceptions, the aberrations or suspected aberrations were checked by two investigators. A few early series were screened independently by two investigators to test the standardization. Their results were in close agreement.

The Lung VIII series were analysed before the Lung X series. A systematic difference due to training and accumulated experience of the investigators should have appeared as an increase of recorded aberrations, in particular minor ones which a less experienced investigator might have missed. This agrees with the fact that an aberration frequency of 0.6 per cent was recorded at zero dose and 24 hours' recovery

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period for the Lung VIII culture series, while the corresponding frequency for the Lung X series was 2.3 per cent. Therefore, the values of the Lung VI series should have been lower than 0.6 per cent. Actually, they were consistently higher but the relatively small number of observations prompts cautious interpretations and conclusions. The same trend was observed with respect to the incidence of equivocal aberrations in the same series. Furthermore, the Brain VIII culture series ought to have had lower frequencies than the Brain IX series but no differences were found.

Another result which rather disagrees with the idea of this kind of systematic scoring differences, is that while the frequencies of equivocal aberrations at the 24 hour recovery periods are higher for the zero and 22 rad doses in the Lung X series as compared to the Lung VIII series, these frequencies were lower after the 89 rad dose. Finally, in the same two series the earlier one (Lung VIII) has consistently higher frequencies of equivocal aberrations at the 48 hour recovery periods.

The absence of significantly consistent trends of systematic errors such as those discussed here, makes it more likely that the observed differences between the Lung VIII and Lung X series have some biological foundation.

Realising that our instruments of measurement are very crude, nevertheless we feel that the results suggest that the frequencies of " spontaneous " chromosomal aberrations as well as those induced by ionizing radiation (γ-rays) depend not only on known (and unknown) environmental factors of which some can be controlled, but also on biological, probably genetical differences between the cells and the individuals to whom these cells belong.

Although with considerable reservation and only for the purpose of obtaining a different type of estimate, including equivocal as well as unequivocal aberrations, all zero dose cell cultures belonging to each of the two series Lung VIII and Lung X have been added together. In this way we obtain, as an estimate of the frequencies of " spontaneous " post-metaphase chromosomal aberrations in cells derived from human foetal lung and grown *in vitro* a figure of 6 per cent for Foetus VIII and 8 per cent for Foetus X.

This difference is fairly modest ($0.02 < P < 0.05$). Nevertheless, it is interesting and invites further explorations. The fact that the cells with the highest rate of spontaneous aberrations (*i.e.* the Lung X series) were of male sex may or may not reflect the generally more pronounced vulnerability of the male as compared to the female. A final point of importance which should be added here is that these two foetuses (VIII and X) were of approximately the same age. With respect to the above comparisons, this excludes one further biological factor which might cause individual differences. It is well known that the stage of development at which the foetus is subjected to ionizing radiation is of crucial importance for the final result. So far, what is known in this field refers to certain malformations, such as microcephaly and cleft palate. It remains to be investigated whether or not such effects occur also at the cellular level.

Although Lung VIII and Brain VIII originated from the same foetus, there is

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a significant difference between their rates of spontaneous aberrations, being 0.6 and 2.9 per cent, respectively (cf. table 4 and 5).

It is, of course, important to realize that all the reservations which must be included in this chapter, and at this juncture limit, in some respects severely, the value of this work. Very likely, there may be important errors, other than those discussed here. However, regarding this work rather as a preliminary to future more sophisticated approaches, we have felt that the present difficulties in manipulating and classifying the human material, whether individuals or cells, should not prohibit venturing the following tentative conclusions.

With respect to the frequency of " spontaneous " chromosomal aberrations occurring in human cell cultures derived from foetal lung and brain our observations suggest:

1. differences between cells derived from different individuals, (*e.g.* Lung VIII versus Lung X, 0.6 per cent-2.3 per cent).

2. differences between cells derived from different tissues in the same individual (*e.g.* Lung VIII versus Brain VIII, 0.6 per cent-2.9 per cent).

3. differences with respect to sex. The existence of such a variation, which might be included under point (1) above, receives further support from the observations of a higher radio-sensitivity as measured by the number of aberrant post-metaphases per 100 cells per rad (*cf.* table 6). This will be further discussed in the following section. On the other hand, the cells derived from Brain VIII, female, and Brain IX, male, had approximately the same frequency of " spontaneous " aberrations (2.9 and 3.0 per cent, respectively). A difference between " Lung " and " Brain ", perhaps worth noticing here, is that the material derived from Lung VIII-Lung X belonged to foetuses of the same age, whereas the material of Brain VIII-Brain IX includes an additional possible cause of variation in as much as foetus VIII, female, was younger and about half the size of foetus IX.

4. with considerable reservation due to the above mentioned objections which can be made against the addition of observations from different series of cell cultures, the following base lines of " spontaneous " aberrations (unequivocals, only):

a) for cultured cells derived from foetal lung 0.7 per cent (41/5801 cells).

b) for cultured cells from foetal brain 2.5 per cent (21/851 cells).

Induced aberrations, dose rates and doubling dose

Before initiating the main project, a pilot experiment comprising a small series of cell cultures, was undertaken to test the function of all practical arrangements and technical details. These cell cultures originated from the lungs of an eighteen week old female foetus with a total length of 20 cm.

The irradiated cultures were given test doses of 23 and 136 rads (corrected doses, actually absorbed by the cells). These preliminary experiments and results have been summarized in table 3. Fixation and staining were undertaken at intervals

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of 24 and 48 hours after irradiation. In this particular series the 48 hour results have been left out because too few post-metaphases were available for analysis.

The insufficient number of cells probably explains the lack of significant differences between the cultures which received an acute dose of 23 rads and their matched controls. However, the acute dose of 136 rads produced a clear enough result although the calculated frequency of 17.9 per cent has a large standard deviation.

The results of the main experimental series are shown in tables 4 and 5. For easy comparison of differences as well as trends in the dose-effect relations, these

Tab. 6. Average frequencies (in per cent) of abnormal post-metaphases (i. e. containing at least one chromosomal aberration) per rad for all doses in the different series of irradiated cell cultures, derived from foetal lung and brain tissue

Foetus N. and sex	Tissue origin of cell culture	Length of post-irradiation period at fixation	
		24 hours	48 hours
VI, female	Lung	0.14	0.10
VIII, female	Lung	0.10	0.04
X, male	Lung	0.15	0.14
VIII, female	Brain	0.19	—
IX, male	Brain	0.37	0.20

results are also presented diagrammatically (fig. 9, 10 and 11). The theoretical as well as the corrected exact doses of radiation have been included in the tables together with the aberration frequencies observed at 24 and 48 hours after exposure. However, for the series Brain VIII the 48 hour values are lacking. This series of cultures was lost due to fungous infections, and it was decided that a repetition of this whole series was not likely to contribute much new or important information.

All cultures which were fixed and stained 24 hours after exposure showed a consistent increase of their aberration frequencies, beginning at a dose of 22-23 rads. The only exception was the Brain IX series with a marked increase at 9 rads which, however, is not statistically significant.

Furthermore, it should be stressed again that our instrument of measurement in this investigation is a very crude one. There are no observations suggesting an interpretation other than a continuous positive correlation between radiation dose and incidence of aberrations.

From a biological point of view one could hardly envisage a curve passing through a series of threshold values. Even the existence of a lower threshold, below which radiation is innocuous, has been much debated and remains very dubious.

The cell cultures fixed 48 hours after irradiation displayed a consistent trend towards lower frequencies of aberrant post-metaphases as compared to those fixed 24 hours after treatment. In the experimental series Lung VIII, comprising the largest

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number of scored post-metaphases, these differences are all statistically significant at the 5 per cent level or less.

As pointed out in the preceding section, our observations suggest a higher frequency of spontaneous aberrations in the cultures derived from foetal brain as compared to those derived from foetal lung. This is most clearly seen in the constellation

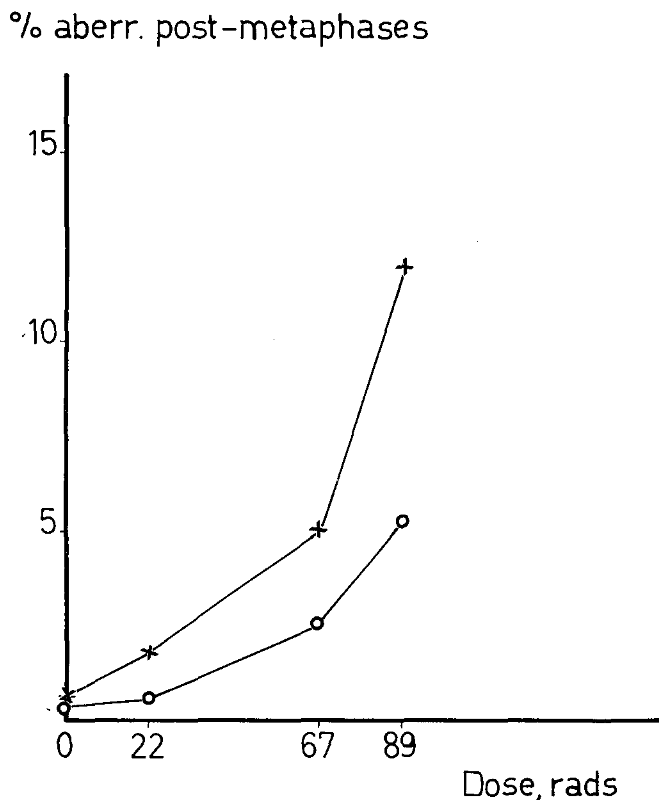

Fig. 9. Relation between frequency (in per cent) of post-metaphase aberrations and corrected absorbed dose in rads. Radiation source: 100 Curie Co-60. Cell cultures of the experimental series Lung VIII, (+) 24, and (o) 48 hours after the acute doses of radiation

Lung VIII *versus* Brain VIII (0.6 and 2.9 per cent, respectively) while in other comparisons age and sex differences enter as possible additional causes of variation.

The results of our radiation experiments also indicate that the cultures derived from foetal brain have a higher degree of radio-sensitivity than those derived from foetal lung and that this difference might be proportional to the " base line " difference of the spontaneous aberration rates. In the series Lung X and Brain IX the

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" base lines " are fairly similar but the Brain IX cultures showed a higher incidence of aberrations at all doses.

The most efficient comparison is, of course, between the series Brain VIII and Lung VIII because they originated from the same foetus. Here, again, the brain series had a consistently higher aberration frequency at all doses, only this time the

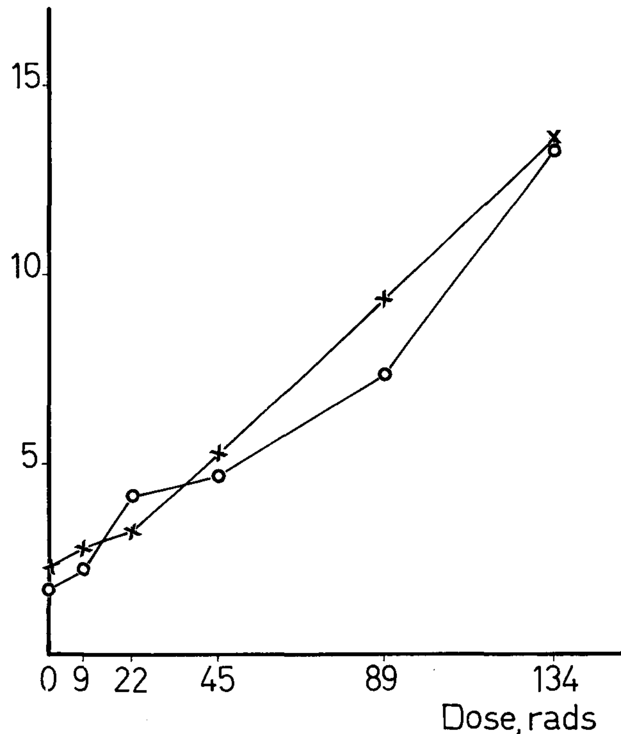

Fig. 10. The same relation as in fig. 9, but for the experimental series Lung X, (+) 24 and (o) 48 hours after the acute doses of radiation

differences appeared as resulting from a combination of a higher " spontaneous " as well as higher induced rate.

As mentioned in the previous section, part of the differences observed between cell cultures derived from different individuals could be due to a sexual dimorphism. With respect to the main series of experiments (*i.e.* Lung VIII and X, Brain VIII and IX) and the rate of "spontaneous" aberrations there is nothing but a slight suggestion. It is only when comparing the effects of irradiation on XY and XX cell cultures that this possibility appears worthy of close examination.

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As compared by aberrations per 100 cells per rad (cf. table 6), the male cultures had higher values than the female ones (derived from foetal lung as well as brain). However, it should be observed that the value of 0.37 for the male Brain IX series is distorted by the comparatively high number of aberrations recorded after the dose

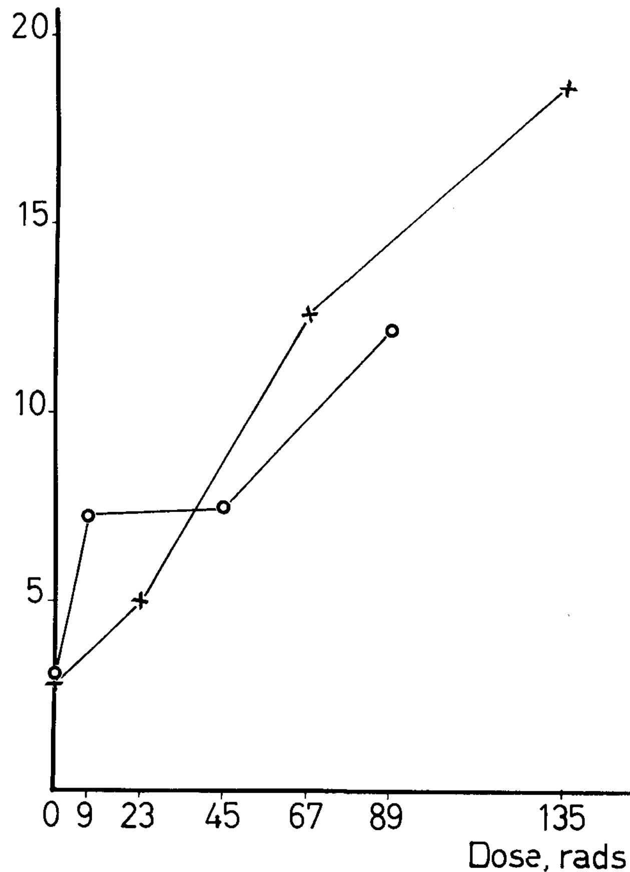

Fig. 11. The same relation as in figs. 9 and 10, but for the experimental series Brain VIII (+) and Brain IX (o) and at 24 hours after acute exposure, only

of 9 rads. This single entry contributes as much as 0.81 against 0.17 and 0.14 for the other two dose levels. This value of 0.81 could, of course, be a chance deviation of no significance whatsoever. However, the fact that the 48 hour value of aberrations per 100 cells per rad, based on the scoring of 455 post-metaphases, was also rather

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on the high side (*i.e.*, 0.20) in relation to all the other values in table 6, makes it likely that we are dealing with trends of biological significance.

Even assuming that the figure of 0.81 is wrong by a factor of two leaves a minimum estimate of 0.24 aberration per 100 cells per rad as an average for this series of experiments.

Another, probably quite important, characteristic of this experimental series should be brought in at this point. As can be inferred from tables 4 and 5 and the diagrams of figs. 9, 10 and 11, there is a tendency of the rate of aberrations to decrease with increasing dose, or in other words, the inclination of the tangent of the curve to the X-axis is decreasing. A further characteristic feature, very likely of the same origin, is the general trend of a decreasing frequency of aberrations with an increasing period of time elapsing between radiation exposure and fixation, more pronounced at high doses and high radio-sensitivity of the irradiated cells.

The simple explanation of these phenomena, at least the essentials of it, is that the radiation will kill or seriously damage some cells so as to make it impossible for them to appear for scoring at subsequent mitoses. Theoretically, one could envisage any number of transitions from the heavy acute doses of perhaps several thousand rads which likely kill every single cell, thus inducing a frequency of chromosomal aberrations of zero per cent, to infinitesimally small doses inducing sub-microscopical or chemical changes in the chromosomes followed by processes of competition and selection over periods of many cell generations.

With respect to the increase of post-metaphase chromosomal aberrations induced by γ rays from a 100 Curie Cobalt 60 source in cell cultures derived from human foetal brain and lung, our main results may be summarized as follows.

1. The number of aberrations induced per 100 cells per rad varied from 0.10 to 0.15 for cultures derived from foetal lung and from 0.19 to 0.37 for cultures derived from foetal brain. These figures refer to observations made 24 hours after acute doses varying from 9 to 136 rads of actually absorbed radiation. At 48 hours after radiation the values were somewhat lower but showed the same general trends (*cf.* table 6).

2. Comparisons between the frequencies of aberrations induced in the different types of cell cultures, referred to as the Lung and Brain series, suggest a higher radiosensitivity of the Brain series. Furthermore, a sexual dimorphism is suggested by the fact that within the Brain as well as the Lung series, XY (male) cultures had a higher aberration rate than XX (female) cultures.

3. The doubling dose, defined as the dose of radiation required to double the base line aberration frequencies of the control cultures, has been estimated from the data of tables 4 and 5. The limited number of observations at some of the doses made it necessary to use a graphical method instead of a regression analysis. The estimates of the doubling dose in these experiments range from 15-40 rads (*cf.* table 7).

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General discussion

In this work we have tried to measure the effect of ionizing radiation on human cells *in vitro* by scoring chromosome aberrations detectable at post-metaphase, while in similar experiments previously carried out by other workers, chromosome aberrations detectable at colchicine-blocked metaphases were scored. In the latter case the aberrations can be classified according to the different chromatid or chromosome types, which was not possible in our material.

There were two main reasons to select post-metaphase aberrations for a yardstick. Firstly, not being used earlier for radiation experiments with human cells *in vitro*, such data might contribute foundations for new aspects on the problem of

Tab. 7. Estimates of the doubling dose for post-metaphase chromosomal aberrations in irradiated human cell cultures derived from foetal lung and brain tissue

Length of post-irradiation period at fixation of cultures	Estimated doubling doses (rads) in cell cultures derived from:			
	Lung VIII female	Lung X male	Brain VIII female	Brain IX male
24 hours	15	35-40	30	30-40
48 hours	35	25	—	15

radiation damage to the human genetical material. Secondly, when these experiments were planned, it was decided that the scoring at post-metaphase with the aid of a series of schematical drawings (*cf.* fig. 7) could be made with a higher degree of accuracy and reproducibility. This was particularly important because, even allowing a period of several years for this project, it would have been unrealistic to rely on one and the same individual to analyse in detail several thousands of cells. An additional reason which, however, turned out to be of little or no importance (*cf.*, *e.g.* Bender 1960) was that we wanted to avoid bringing in an additional factor (colchicine) which might affect the aberration rate.

It seems likely that the recorded number of chromosomal aberrations in our irradiated cell cultures is on the low side. Some cells might have been damaged in such a way that they were either killed or otherwise made incapable of further mitotic division.

For the above reasons we have abstained from detailed comparisons with the results of similar investigations which were reviewed in the first part of this report.

" Spontaneous " aberrations were observed in all control cultures. The tendency of the " brain " cultures to display a higher rate of spontaneous aberrations is still clear, but not as definite as it appeared from the analysis of the first series of experi-

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ments, *cf.* Lindsten (1959) and Fraccaro (1960). It should be observed that the two series of cultures (Lung and Brain) had a different cell morphology. The cells derived from foetal lung were consistently fibroblast-like while the cells derived from foetal brain were of two different types, characterized by a considerable difference in nuclear size.

The observed overall higher mitotic activity of the " lung " cultures associated with a trend of lower aberration rates suggests that the frequency of " spontaneous " aberrations is independent of the rate of mitotic multiplication.

Puck (1958) and Bender (1960) found a higher rate of " spontaneous " aberrations (and higher radio-sensitivity) for fibroblast-like cells as compared to epithelial-like cells *in vitro*. The rate of " spontaneous " aberrations in our " lung " cultures was low, and we have no suitable material for comparison, both types of cells in our " brain " cultures being probably of glial origin. The occurrence of apparently spontaneous chromosomal aberrations in cells grown *in vitro* is a well known phenomenon but systematic investigations of the incidence and type of such " spontaneous " aberrations with reference to tissue and individual origin are still largely lacking. Recently, Sax and Passano (1961) reported a frequency of 3.6 per cent " spontaneous " chromosomal aberrations detectable at post-metaphase in human fibroblasts cultured for two months. Aging of three months produced an increase of this frequency to 10 per cent.

During the extensive investigation of associations between karyotype and various diseases or defects at this institute, a large number of cell cultures derived from skin, bone marrow, connective tissue, blood leucocytes and other tissues of several hundred individuals, including healthy relatives, have been examined. In this material several different types of structural chromosome changes, apparently unrelated to the karyotype or other phenotypical characteristics of the individual have been observed occasionally. A sample of such structural changes observed in cell cultures from various individuals are shown in fig. 12.

These " spontaneous " aberrations are of the same type as those observed after exposure to ionizing radiations (*cf., e.g.* our fig. 12 with figs. 9-11 in the paper by Wakonig and Ford, 1960).

In our experimental system, doses of about 20 rads consistently produced an increase of chromosomal aberrations. In one of the " brain " cultures a marked increase was produced by a dose of 9 rads, only. A consistent increase in the frequency of aberrations with increased dose occurred 24 as well as 48 hours after exposure. The incidence of abnormal post-metaphases is, however, almost consistently lower at 48 hours. This indicates a differential survival or prolonged mitotic inhibition in the cell population. This is in agreement with other results, *e.g.* those by Chu *et al.* (1961).

Irradiation of cells at any stage of the mitotic cycle will induce chromosomal aberrations, generally detectable at the first metaphase or anaphase following the exposure. The aberrations of the chromatid type are produced with high frequency in cells which have already duplicated their chromosomes and therefore are the

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first ones to enter metaphase after irradiation. Cells scored later, 48 hours in our experiments, are likely to have been irradiated during their interphase period prior to chromosome duplication. In such cells, aberrations of the chromosome type only will be induced.

A trend of differential radio-sensitivity between cells derived from foetal lung and from foetal brain was recorded. As mentioned previously in this paper, this sensitivity is apparently in part proportional to a difference in the rate of "spontaneous" aberrations between the two types of cultures. In consequence, the number of aberrations per 100 cells per rad (table 6) was higher in the "brain" cultures than in the "lung" cultures.

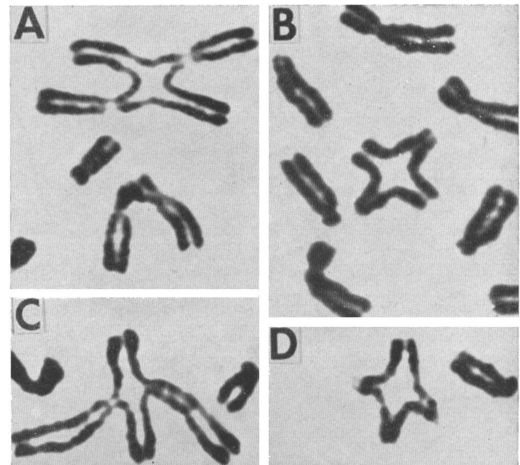

Fig. 12. Four examples of chromosome configurations at metaphase, indicating somatic pairing and segmental interchange. Such irregularities are occasionally observed in single cells of cultures derived from individuals with abnormal as well as normal karyotypes. Acetic orcein stain. Phase contrast

The estimated doubling dose, however, was lower for the Lung series (15 rads) as compared to the Brain series (30 rads) in the comparable experiments with cell cultures derived from foetus VIII. These are no more than suggestions and only extensive new experiments can clarify these trends.

To our knowledge, these are so far the only irradiation experiments with human cell cultures in which chromosomal aberrations have been analysed and scored at post-metaphase (anaphase and telophase) and not at metaphase. On the whole, our results are in agreement with those of previous investigations of the effects of ionizing radiations on human, and other mammalian cells grown *in vitro*. The difference in cell morphology and incidence of "spontaneous" aberrations observed between the two types of cultures used for the experiments may in part explain the observed differences in radio-sensitivity.

Summary

The effect of ionizing radiation, from a 100 Curie Cobalt-60 source, on diploid human cells grown *in vitro* has been investigated.

The analysed cell populations originated from foetal brain and lung tissue. Other known variations of this material were age and sex of the foetuses, primary and first transfer cultures.

The effect was measured by recording post-metaphase chromosomal aberrations

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in cell cultures fixed and stained 24 and 48 hours after acute irradiation. " Spontaneous " aberration frequencies were determined in matched control cultures.

Although conclusions must be guarded, in view of the insufficient knowledge of factors influencing human cell populations *in vitro*, our observations can be summarized, tentatively, in the following main points.

1. The frequency of " spontaneous " aberrations appears to vary with respect to (a) differences between the individuals from whom the biopsies were taken, (b) tissue of origin whether within or between individuals and (c) the sex of the cultured cells.

The qualified estimates of the overall averages of " spontaneous " aberrations were, (a) for the cell cultures derived from foetal lung 0.7 per cent (41/5,891 scored cells) and (b) for the cell cultures derived from foetal brain 2.5 per cent (21/851 scored cells).

2. In the irradiated cell cultures which received doses varying from 9-136 rads of absorbed dose the number of aberrant post-metaphases per 100 cells per rad varied from 0.10-0.15 for cultures derived from lung tissue and from 0.19-0.37 for cultures derived from brain tissue, all at 24 hours after the acute dose. At 48 hours after irradiation the frequencies were somewhat lower but the same trends remained.

3. The cell cultures derived from brain tissue appear to have a higher radiosensitivity than those derived from lung tissue. Furthermore, a sexual dimorphism is suggested because, in all series of cultures, those composed of XY cells had a higher aberration frequency as compared to those with XX cells.

4. The irradiation doubling dose (for definition, see p. 27) was estimated at 15-40 rads (*cf.* table 7).

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RIASSUNTO

Si è studiato l'effetto di radiazioni ionizzanti, provenienti da una fonte di Cobalto-60 a 100 Curie, su cellule umane coltivate *in vitro*.

Le popolazioni cellulari analizzate provenivano da tessuto fetale cerebrale e fetale polmonare. Altre variazioni conosciute di questo materiale riguardavano l'età ed il sesso dei feti, nonchè le possibili differenze fra culture primarie e secondarie.

L'effetto fu misurato registrando le aberrazioni cromosomiche di post-metafase in culture cellulari fissate e colorate 24 e 48 ore dopo forte irradiazione. Le frequenze delle aberrazioni « spontanee » venivano determinate in culture analoghe di controllo.

Pur non potendo trarre conclusioni, data l'insufficienza delle nostre conoscenze sui fattori che influenzano le popolazioni di cellule umane *in vitro,* possiamo, comunque, tentare di riassumere le nostre osservazioni come segue:

1. La frequenza delle aberrazioni « spontanee » risulta variare rispetto (a) alle differenze fra gli individui da cui sono state prelevate le biopsie, (b) al tessuto di origine, proveniente sia da uno che da più individui, e (c) al sesso delle cellule coltivate.

Stime ragionevoli delle medie generali delle aberrazioni « spontanee » sono state (a) 0,7% per le culture di cellule provenienti da tessuto fetale polmonare (41/5.891 cellule contate) e (b) 2,5% per le culture di cellule provenienti da tessuto fetale cerebrale (21/851 cellule contate).

2. Nelle culture cellulari irradiate, con dosi da 9 a 136 rads della dose assorbita, il numero di post-metafasi aberranti per 100 cellule per rad variava da 0,10 a 0,15, per culture derivate da tessuto polmonare, e da 0,19 a 0,37 per culture derivate da tessuto cerebrale, entro 24 ore dopo l'applicazione della dose acuta. A 48 ore dopo l'irradiazione, le frequenze erano alquanto inferiori, ma la tendenza generale era la stessa.

3. Le culture di cellule provenienti da tessuto cerebrale sembrano avere una maggiore radiosensibilità di quelle provenienti da tessuto polmonare. È anche possibile un dimorfismo sessuale, in quanto, in tutte le serie di culture, quelle composte da cellule XY presentavano una maggiore frequenza di aberrazioni, rispetto a quelle composte da cellule XX.

4. La dose raddoppiante l'effetto dell'irradiazione (v. definizione a p. 27) è stata valutata in 15-40 rads (cfr. tabella 7).

RÉSUMÉ

L'effet de la radiation ionisante, venant d'une source de 100 Curie de Cobalt-60, exercé sur des cellules diploïdes humaines cultivées *in vitro,* a été étudié.

Les populations de cellules analysées provenaient du tissu foetal, cérébral et pulmonaire. D'autres variations connues dans cette matière se rapportaient à l'âge et au sexe des foetus ainsi qu'aux différences qui pouvaient exister entre les cultures primaires et celles qui avaient été une fois transférées.

Nous avons mesuré l'effet en enregistrant les aberrations chromosomiques de post-métaphase dans des cultures de cellules fixées et colorées 24 et 48 heures après l'irradiation. La fréquence des aberrations « spontanées » était déterminée dans des cultures analogues de contrôle.

Bien qu'on doive se garder de tirer des conclusions, étant donné l'insuffisance de nos connaissances des facteurs influençant des populations de cellules humaines cultivées *in vitro,* nous essayerons de résumer nos observations dans les points principaux suivants:

1. La fréquence des aberrations spontanées semble varier suivant (a) les différences entre les individus desquels provenaient les biopsies, (b) les différences entre des tissus provenant d'un seul individu ou bien de plusieurs, (c) le sexe des cellules cultivées.

Une estimation raisonnable de l'ensemble des aberrations « spontanées » en moyenne était

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(a) pour les cultures de cellules pulmonaires fœtaux 0,7 per cent (41/5.891 cellules comptées) et (b) pour les cultures de cellules cérébrales foetaux 2,5 per cent (21/851 cellules comptées).

2. Dans les cultures de cellules irradiées, ayant reçu des doses de 9 à 136 rads de dose absorbée, le nombre de post-métaphases aberrantes sur 100 cellules sur rad variait, 24 heures après l'application de la dose aiguë, de 0,10 à 0,15 pour les cultures provenant du tissu pulmonaire et de 0,19 à 0,37 pour les cultures provenant du tissu cérébral. 48 heures après l'irradiation les fréquences étaient un peu plus basses, mais la tendance générale restait la même.

3. Les cultures de cellules provenant du tissu cérébral semblent être plus sensibles à la radiation que celles provenant du tissu pulmonaire. En outre, un dimorphisme sexuel est possible, parce que, dans toutes les séries de cultures, celles qui étaient composées de cellules XY avaient une fréquence plus haute d'aberrations que celles composées de cellules XX.

4. La dose qui double l'effet de l'irradiation (pour définition, voir p. 27) était estimée à 15-40 rads (tableau 7).

ZUSAMMENFASSUNG

Die Wirkung ionisierender Strahlung von einer Quelle von 100 Curie Colbat-60 auf *in vitro* gewachsene diploide menschliche Zellen ist untersucht worden.

Die analysierten Zellenpopulationen entstammten embryonalen Gehirnund Lungengeweben. Weiterhin bekannte Variationen dieses Materials waren Alter und Geschlecht der Embryos, primäre und erstmalig transplantierte Kulturen.

Die Wirkung wurde durch Registrierung der postmetaphasen Chromosomenabweichungen bei Zellenkulturen, die 24 und 48 Stunden nach akuter Bestrahlung fixiert und gefärbt worden waren, gemessen. « Spontane » Frequenzabweichungen wurden in angepassten Kontrollkulturen bestimmt.

Obgleich in Anbetract der unzureichenden Kenntnis von beeinflussenden Faktoren bei menschlichen Zellenpopulationen *in vitro* Schlüsse nur mit Vorsicht geäussert werden dürfen, können unsere Beobachtungen doch versuchsweise zu folgenden Hauptergebnissen zusammengefasst werden:

1. Die Frequenz von « spontanen » Abweichungen scheint zu differieren mit Rücksicht auf a) Unterschiede bei den Individuen von denen Biopsien genommen wurden, b) den Ursprung des Gewebes im Individuum selbst oder unter den Individuen, und c) das Geschlecht der kultivierten Zellen.

Die angemessenen Schätzungen des allgemeinen Durchschnitts von « spontanen » Abweichungen waren a) für Zellenkulturen aus embryonaler Lunge 0,7 Prozent (41/5,891 gezählte Zellen) und b) für Zellenkulturen aus embryonalem Gehirn 2.5 Prozent (21/851 gezählte Zellen).

2. Bei den bestrahlten Zellenkulturen, die eine Dosis von 9-136 rad absorbierter Dosen erhielten, variierte die Anzahl der abweichenden Postmetaphasen pro 100 Zellen pro rad von 0.10 - 0.15 für Kulturen, die Lungengeweben entnommen waren und von 0.19 - 0.37 für Kulturen, die Gehirngeweben entnommen waren, jeweils bei 24 Stunden nach der akuten Dosis. Bei 48 Stunden nach der Bestrahlung waren die Frequenzen etwas niedriger, aber es verblieben dieselben Tendenzen.

3. Die dem Gehirngewebe entnommenen Zellenkulturen scheinen eine höbere Strahlenempfindlichkeit zu besitzen als diejenigen aus dem Lungengewebe. Des weiteren gibt sich ein sexueller Dimorphismum zu verstehen, da bei allen Serien von Kulturen, diejenigen mit XY-Zellen eine höbere Abweichungsfrequenz verglichen mit denjenigen mit XX-Zellen aufwiesen.

4. Die Doppeldosis der Bestrahlung (Definition s. S. 27) wurde auf 15-40 rad geschätzt (vgl. Tabelle 7).

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