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Effects of in utero exposure to ionising radiation during the early phases of pregnancy

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CONTENTS

FOREWORD

Under the terms of the Treaty establishing the European Atomic Energy Community, the Community, amongst other things, establishes uniform safety standards to protect the health of workers and of the general public against the dangers arising from ionizing radiation. The standards are approved by the Council, on a proposal from the Commission, established taking into account the opinion of the Group of experts referred to in Article 31 of the Treaty. The most recent version of such standards is contained in Council Directive 96/29/Euratom of 13 May 1996 laying down basic safety standards for the protection of the health of workers and the general public against the dangers arising from ionizing radiation.

The European Commission organises every year, in cooperation with the Group of experts referred to in Article 31 of the Euratom, a scientific seminar to discuss in depth a particular topic of radiation protection suggested by the Group.

Despite more than one hundred years of research on the biological effects of ionising radiation, the exact consequences of radiation exposure in the early stages of human pregnancy still remain to be better understood. The major reason is, of course, the problem of obtaining direct information for humans. Frequently, pregnancy will go undetected until early organogenesis and, even after detection, it is difficult t identify the exact time of each developmental step retrospectively. Thus, the information obtained from animal experiments has to be extrapolated to the human situation.

The aim of the present seminar was to summarise the recent information available for radiation risk during the early stages of gestation (i.e. the first trimester) and to look whether the above-mentioned Directive continues to ensure an adequate level of protection to the citizens of the European Union.

Leading scientists in this area presented the latest information.

The seminar also dealt with mental effects of radiation exposure during early childhood.

Genetic Predisposition and Genomic Instability in Preimplantation Mouse Embryos

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1. INTRODUCTION

No observations in humans are available for the analysis of radiation risk during the preimplantation period, as conception is generally not noticed at that early period of pregnancy. Therefore the risk analysis can only be achieved on the basis of animal experiments which have mainly been performed with mice and rats. With respect to the preimplantation period there exsists the advantage that the duration and also the general biological processes (cf. cell proliferation and differentiation) during this developmental period are very similar for most mammalian species. Thus the duration of the preimplantation period is 5 days for mice, 7 days for rats and 8 days for humans (SSK 1984; Streffer and Molls 1987), although the time periods of the total prenatal development vary to a much larger degree between mammalian species. On the first sight the preimplantation period is determined by cell proliferation processes from the zygote (1-cell) to the hatched blastocyst with about 100 cells (mice) to about 250 cells (humans). The hatched blastocyst is then implanted into the uterus for further development (Carlson 1994). However, cell differentiation starts early during this period. After entrance of the sperm into the oocyte and the fusion of the male and female pronuclei the whole genetic information of the new individual is determined.

For decades until recent years it was postulated that malformations cannot be induced by a radiation exposure during the preimplantation period. For many years lethality of the embryo was thought to be the only health effect of an exposure by ionising radiation or other toxic agents during the preimplantation period (Friedberg et al. 1973; Schlesinger and Brent 1978) although reports about developmental defects had already been published in the sixties. The lethal effects are always connected with chromosomal aberrations and cell death of blasomeres (Weissenborn and Streffer 1988). The radiosensitivity can vary during the preimplantation period especially during the zygote stage dramatically (Yamada and Yukawa 1984). Thus the LD_{50} changes from 1.5 Gy to 0.3 Gy gamma-rays (Cs-137) within hours during the development of the zygote of mice. Significant lethal effects are usually observed in dose ranges of 0.2 Gy low LET radiation.

The data on developmental abnormalities were apparently not so convincing that they were accepted by the broad scientific community as no dose effect relationship was observed (Rugh et al. 1969). Only in recent years it could be shown by several groups that with certain mouse strains malformations can be induced by a radiation exposure during the preimplantation period. These effects which occur with the highest frequency after an irradiation of the zygote are apparently related to a genetic predispositon which will be discussed later.

In 1935, Job concluded from his experiments, in which preimplantation stages of rats were exposed to ionising radiation, that these early embryos either died or survived without any detectable malformation (Job et al., 1935). In 1950, Russell and Russell found similar results for mice and L. B. Russell coined the well-known “all-or-none-rule” in 1956 for the radiation damage in mammalian preimplantation embryos, “killing or normality” (Russell, 1956).

Since that time, quite a number of publications have confirmed the results reported by Job and Russell for rats (Hicks, 1953; Brent and Bolden, 1967, Roux et al., 1983) and mice (Russell et al., 1959; Friedberg et al., 1973; Schlesinger and Brent, 1978; Mazur, 1984). It was generally accepted that during the preimplantation period the pluripotency of the blastomeres or the low degree of differentiation of the later stages could compensate for cell loss to a certain extent and the further radiation damage was repaired. Therefore, no malformations were expected to be induced during this developmental stage (Streffer and Müller 1996).

2. INDUCTION OF MALFORMATIONS

However, starting in 1959 first results were published that have cast some doubt on the general validity of the rule mentioned above (Rugh and Grupp, 1959, 1960): exencephalies were observed after single or fractionated exposures of murine preimplantation stages. These results provoked a lot of criticism (for a review see Mole 1992). The major points of this criticism were the lack of a clear dose-response relationship and of sound control data. During the subsequent years, however, further information was obtained. Thus, Ohzu (1965) observed a marked increase in the frequency of polydactyly of the forefeet of mice after radiation exposure on days 0.5 or 1.5 after conception; this author however, attributed these malformations to indirect effects.

In 1988, an increased frequency of malformed fetuses on day 19 of gestation was observed after exposure of zygotes 1 - 3 h post conception with either X-rays or neutrons (Pampfer and Streffer, 1988). Almost exclusively gastroschisis (a hernia, open bowl, with externalisation of the gut and other organs) were found in these studies with the mouse strain “Heiligenberger Stamm” (HLG). Gastroschisis can also be found as a malformation in humans, therefore this malformation is of special interest. This type of malformation occurs with a comparatively high frequency in this mouse strain already in the controls (around 1% in the control group at the time of the first study and up to 3% in some later studies with completely inbred mice). The observed increase was very pronounced (about 20% malformed fetuses of all surviving fetuses after 2Gy of X-rays or 0.75 Gy of fast cylclotron neutrons, average energy around 6 MeV). The effect was clearly dose-dependent with a dose effect relation without a threshold and the frequency of malformed fetuses significantly different from the concurrent controls (as mentioned, about 1%) after radiation doses of 0.25 Gy X-rays and 0.125 Gy neutrons and higher doses. Basically similar results were reported by the group of Generoso (Rutledge et al., 1992), although the types of malformations were different and the frequency of malformed fetuses less pronounced after radiation exposure of (C3HxC57Bl) F_1 mice. This working group reported the induction of malformations also after exposures to various chemical agents especially alkylating agents at the zygote stage (Rutledge 1997).

Thus the possibility of the induction of malformations during the preimplantation period in some mouse strains is not restricted to ionizing radiation. Quite a number of chemicals (e.g. N-methyl-N-nitrosourea, ethylene oxide, ethyl methanesulfonate, diethyl sulfate, dimethyl sulfate) are able to induce malformations after application during early embryonic stages (Bossert and Iannaccone, 1985; Generoso et al., 1988; Rutledge et al., 1992). Whether the mechanisms of induction are comparable for all agents under study is not clear in the moment.

There are two important aspects associated with radiation exposure of Heiligenberger embryos on day 1 of gestation: Firstly, there is a statistically significant dose-dependent increase in the number of malformed fetuses of the HLG-mice after exposure to X-rays or fast neutrons (Pampfer and Streffer 1988), and secondly, the extent of this effects, this means the radiosensitivity, changes within a few hours and days of prenatal development (Müller and

Streffer 1990). The latter aspect will play some role in the discussion of indirect effects. Successive experiments showed that an increase in the number of malformations after radiation exposure is not restricted to the zygote stage. All preimplantation stages of the radiosensitive mouse strain HLG do show a certain probability to react with the induction of malformed fetuses after radiation exposures (Müller und Streffer, 1990). The sensitivity, however, is reduced when one compares the data with the results obtained after irradiation during the zygote stage (1 – 3 hours p.c.).

The results obtained after radiation exposure of zygotes and of later preimplantation stages offer the unique possibility to test the assumption that radiation dose-response curves of processes that require damage to only one cell do not show a threshold dose, whereas in the case that damage to several cells is necessary a threshold has to be expected (Hulse and Mole 1982; Müller et al. 1994; Streffer and Müller 1996). The data presented reveal, that indeed after exposure of 1-cell embryos there is no indication of a threshold dose, whereas exposure of 32- to 64-cell embryos clearly goes with a threshold dose. The latter result indicates that in the multicellular situation it is not sufficient to damage only one cell in order to induce the malformation. Obviously, other cells can compensate for such a type of damage; only after damage to several cells and therefore exceeding a certain threshold dose, this compensation is no longer working, because too many cells have been damaged (Müller et al. 1994). This requirement also explains, a least partly, the lower sensitivity of stages beyond day 1 of murine development.

Very early during these experiments it was assumed that the effects observed were strain dependent. In order to test this assumption, comparable experiments using C57Bl and HLG mice in parallel were performed (Müller et al., 1996). Indeed it turned out that C57Bl mice did not respond with an increase in malformations after radiation exposure of preimplantation stages (1.7%, 2 malformations in 121 fetuses in the controls, 0%, no malformation in 54 fetuses in the 1.0 Gy group), whereas exposure during organogenesis resulted in C57Bl in the expected augmentation of the number of malformed fetuses (almost 60%). Actually, the radiation response of C57Bl mice during organogenesis was even more pronounced in the C57Bl mice than in HLG mice with respect to radiation-induced malformations. Thus for C57Bl mice a radiation effect for induction of malformations is only found after exposures during the major organogenesis and not during the preimplantation period and for HLG mice the induced rate is about the same in both developmental periods.

The analysis of the spectrum of the types of malformations in HLG mice clearly demonstrated that the maechanism of the development of malformations is different for induction during the preimplantation period and the organogenesis. After radiation exposure with 1.0 Gy X-rays during the preimplantation almost 90% of the malformations were of the type of gastroschisis while this was only in about 50% the case after exposure during organogenesis (Streffer and Müller, 1996).

Cross-breeding of HLG and of C57Bl mice resulted in no or only a very small, insignificant increased risks of malformations after radiation exposures to the zygote stage (Müller et al. 1995). This was the case independently whether the father came from the HLG- or from the C57Bl-mice (Table 1).

Thus, the pronounced sensitivity of the zygote to respond to radiation exposures with an increased number of malformed fetuses (gastroschisis) is specific for the HLG mouse genome. The analysis of protein expression in the fetal liver showed that a number of changes occurred in the fetuses which manifested a gastrochisis (Hillebrandt and Streffer 1994). In further experiments it could be shown that the genetic predisposition is inherited through a recessive

trait in which 2 or 3 genes are involved and through gene linkage analysis it was shown that one of these genes is located on chromosome 7 of the HLG mice in the position around 28 cM where a number of genes for imprinting are located (Hillebrandt et al. 1998).

The involvement of a genetic predisposition could be underlined further by the observation that a preconceptional X-irradiation of HLG mice also caused an increase of gastrochisis in the next generation (Müller and Schotten 1995; Müller et al.1999). When HLG-females were X-irradiated 1-4 weeks before ovulation radiation doses of 2 and 3 Gy (dose rate 60 Gy/h) significantly increased the frequency of gastroschisis to 9% (37 malformation in 410 fetuses) and 17% (34 malformations in 196 fetuses). Besides gastroschisis quite a number of dwarfs (fetuses with reduced body weight) developped. This effect was already significant with radiation doses of 1 Gy X-rays. Oocytes irradiated more than 4 weeks before conception were so radiosensitive that they did not survive even after 0.5 Gy at this high dose rate. However, when the radiation exposure was performed with gamma-rays (Cs-137) at a dose rate of 11 mGy per h a number of oocytes survived,were fertilized and fetuses with gastroschisis could be observed (8 malformed fetuses in 46 fetuses) after a radiation dose of 1.4 Gy. In the controls 4 malformed fetuses were found in 276 fetuses.

An increase of gastroschisis was also observed after preconceptional exposures of male mice with 2.8 Gy Gamma-radiation (Cs-137) and a dose rate of 0.28 Gy/h (Müller et al. 1999). The highest effect was observed when the males were irradiated 4-5 weeks before conception showing that the meiotic stages of sperm development are most radiosensitive. No increase of malformations was seen after irradiation of spermatogonia (X-ray exposure 8 weeks before mating). The meiotic are generally most sensitive. This was also aboserved with the preconceptional irradiation of female mice. The preconceptional irradiation of male mice 1-8 weeks before mating also significantly increased the rates of preimplantation death and early resorptions (Müller et al 1999).

These data generally confirm earlier reports of Nomura (1982;1988) as well as Kirk and Lyon (1984) although the increased rate of malformations was less and the spectrum of malformations not so much limited to certain specific types in these earlier publication. In all experiments a high rate of fetuses with a reduced birth weight (dwarfism) was also observed. These data with preconceptional irradiation of males demonstrate that indirect effects can be completely excluded for the induction of these malformations. It has been argued after irradiation of females that the increase of malformations is due to malnutrition caused by radiation sickness of the mother. It is clearly shown by the data with males that the observed effects are of genetic nature, more or less only genetic material is transferred with the sperm and the mothers or oocytes were not exposed at all in these experiments.

Similar observations as with the HLG- and C57Bl-mouse strain have been made by Jacquet et al. (1995) who found an increase of malformations after irradiation of zygotes of CF1 mice 7 hours after conception with a dose range of 100 to 1000 mGy X-rays, but the effects were variable with respect to dwarfism. The CF1 mouse strain was also used by Rugh and coworkers in their experiments mentioned above. But no effects at all were oberved by Jacquet et al. (1995) for BALB/c mice. This finding may be correlated with a differences of DNA repair in both mouse strains used by Jacquet et al (1995). Gu et al. (1997) studied the induction of malformations in ICR mice after gamma-irradiation (Cs-137) during the preimplantation period. The authors also found a significant increase of malformations after radiation doses of 0.25 – 2.5 Gy. The time of highest radiosensitivity was 2 hours post conception, but a radiation effect was also observed 72 and 96 hours after conception.

In general these studies show that developmental defects can be caused by exposures to ionizing radiations during the preimplantation period. The first three hours during which meiosis is completed for the female genome the radiosensitvity is apparently higher but also exposures at later times of this developmental period can induce developmental changes. In all these experiments it was observed that the effects are mouse strain dependent and that generally a genetic predisposition is very much in favour of this effect. Therefore no malformations have been observed if this phenomenon of genetic predisposition has not been considered in earlier studies or mammals without such a predisposition were used for such radiation experiments. Therefore the data have led to the described controversies in the literature. In the HLG mice the radiatiosensitivity with respect to the induction of malformations can be the same during the preimplantation period as well as during the major organogenesis.

The induction of malformations after exposure during the preimplantation period is certainly a very rare event if one considers the risk of a total human population. Despite these very interesting findings the general rule is still that the most important radiation risk during the preimplantation period is the death of the embryo. However, in rare cases the induction of malformation is possible if a genetic predisposition exists for such malformations. Then the risk can be appreciable. In this case family histories give some indication for such a risk. Further under these circumstances the time window for such a risk is small. Nevertheless such biological models are very important in order to study the risk of mutagens and developmental toxicity (Rutledge 1997) and conclusions can also be made with respect to genetic effects. The investigation of these effects after irradiation especially of zygotes further has the advantage that the rate of developmental defects is higher than after preconceptional irradiation and therefore less animals are needed for the evaluation of such risks.

3. INDUCTION OF GENOMIC INSTABILITY

The induction of chromosomal aberrations has been measured during the first three mitotic cell divisions after irradiation of HLG-zygotes with X-rays as well as with neutrons. It has been found that new aberrations originate in the second and third mitotic division, however, later apparently the rate of chromosomal aberrations decreases (Weissenborn and Streffer 1988). In further experiments skin biopsies were taken from the fetuses 19 days post conception just before the time of birth and these skin biopsies were brought into culture for fibroblast outgrowth. The studies of chromosomal aberrations in these fibroblasts showed that the frequency was increased in the cells from those fetuses where the irradiation had taken place in the zygote stage and which did not show a developmental defect. This radiation effect was even higher in the fibroblasts from those fetuses which had developped a gastroschisis after zygote irradiation (Pampfer and Streffer 1989).

These chromosomal aberrations are apparently not caused directly by irradiation. If this would have been the case a new organism could not have developped from the irradiated zygote. It is evident that the genome has been affected by the earlier radiation exposure in such a way that new chromosomal breaks developped in much later cell generations without any further radiation exposure. The quality of the aberrations also differ. Thus chromosome breaks are dominating over chromatid breaks directly after irradiation (ratio about 2.7), whereas there are less chromosome breaks than chromatid breaks in the delayed chromosomal damage with increased genomic instability (ratio about 0.7). All chromosomes are apparently involved in this phenomenon of genomic instability. A dose response relation was found in the dose range of 0.5 to 2.0 Gy X-rays. From the investigations of gene linkage described above it looked like that the increase of chromosomal aberrations was connected with the genetic predisposition

for gastroschisis. However, studies with C57Bl - mice revealed that the genomic instability was also found in this mouse strain after zygote irradiation. The effect was even higher than in the C57Bl – mice (Table 2).

4. TRANSGENERATIONAL RADIATION EFFECTS

In further studies the normal appearing fetuses after zygote irradiation were grown up to sexual maturity. Females from these mice were mated in the age of 8 - 12 weeks with healthy non-exposed males and the fetuses from these matings (generation 2 after radiation exposure with 1 Gy X-rays) were isolated on day 19 post conception by Caesarian section. The fetuses and uterine content were studied with respect to resorptions and fetal deaths. A large number of the females irradiated in the zygote stage were sterile or had no implantations at all. However, what might be more important in the second mouse generation which never was exposed to ionizing radiation significant developmental disturbances were observed (Pils et al. 1999). The number of surviving fetuses was decreased, early and late resorptions were significantly increased. For gastroschisis the rate in the unexposed fetuses (second generation) was slightly increased to 6.5% in comparison to the controls with 3.5%. This difference was just not statistically significant ($p>0.05$) (Table 3). The genomic instability measured by chromosomal aberrations in fetal liver cells of unexposed fetuses of the second generation was also increased.

It is very interesting that the X-irradiation of HLG zygotes caused an increased rate of gastroschisis not only in the mice which developped from these zygotes but also a slight increase was also found in the next generation. But even more interestingly there occurred an accumulation of fetuses with gastroschisis in certain mothers which may be due to a radiation-induced genomic instability in these mice (Pils et al. 1999). Thus in 9 mothers out of 21 of this group from the second generation 2 fetuses with gastroschisis were found per mother (Table 4). This accumulation of malformed fetuses is significantly increased in comparison to the controls. Apparently some of the female mice have been damaged through the zygote irradiation in their genome in such a way that they give birth to their descendants with increased rates of the malformation.

5. SUMMARY AND CONCLUSION

1. Death of the conceptus is the main radiation effect after exposures during the preimplantation period. These effects are accompanied or even caused by chromosomal aberrations. The radiosensitivity can change dramatically during this period. Significant effects are usually not observed after radiation doses below 0.1 Gy low LET radiation.

2. In mouse strains with a genetic predisposition for certain developmental defects the rate of such defects can be increased by exposures to ionizing radiations. The radiosensitivity is highest during the first hours after conception (development of the zygote) and is under these circumstances about the same as during major organogenesis.

3. Significant increases of malformations have been observed after radiation doses above 0.25 Gy X-rays and 0.12 Gy fast neutrons for such situations. Exposures during the zygote stage lead to dose effect relations without a threshold. It appears possible that such effects can also occur in humans when a genetic predisposition exists in individuals. These are rare cases.

4. Radiation exposures during the preimplantation period can increase genomic instability measured through chromosomal aberration which is manifested in the fetus and in the new born mouse. These effects have been found after radiation doses above 0.5 Gy X-rays. This phenomenon is of a more general nature, it apparently occurs independent of a genetic predisposition.

5. The development of genomic instability is transmitted to the next mouse generation which was measured by the increase of chromosomal aberrations. But also the rate of developmental defects and disturbancies of pregnancy was transmitted to the next mouse generation after radiation exposure to the zygote.

6. In the mouse strain with a genetic predisposition the developmental defects can also be induced by preconceptional radiation exposures to males as well as to female mice. This situation including radiation exposures to zygotes may be a good tool in order to study genetic effects.

6. LITERATURE

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Table 1: Radiation – induced (1Gy, 3 h p.c.) Malformations in HLG-, C57 BI- and in Cross-bred (H x C) or (C x H)Mice

		No of Fetuses	Malf. Fetuses	Perc. Malf.	P-value
HLG	Co	270	2	0.74	<0.01
	Irr	110	15	13.64	
C57BI	Co	121	2	1.65	>0.05
	Irr	54	0	0	
H x C	Co	162	0	0	<0.05
	Irr	128	4	3.13	
C x H	Co	222	1	0.45	>0.05
	Irr	190	5	2.63	

Table 2: Chromosome Aberrat. in Fibroblasts from Fetuses (19 d p.c.) of Mice after X-Irradiation of Zygotes (1 h p.c.)

(Number of Aberrations/Number of Metaph. and %)

Mouse Strain	Contr.	%	1 Gy	%	2 Gy	%
C 57 BL	22/795	2,8	136/626	21,7	109/400	27,5
HLG	29/400	7,3	48/400	12,0	56/322	17,4

Table 3: Teratogenic effects in the second generation after X-irradiation of HLG/Zte mice with 1 Gy of the first generation in the zygote stage

	Irradiated group	Controls
Gastroschisis	6,5%	3,5%
Early resorptions	17,2 % *	9,28%
Late resorptions	2,4% *	0%
Sterile individuals	62%*	34%
Surviv. fetuses	76%*	90%

* P<0.05 (Comparison controls against irradiated groups)

Table 4: Distribution of malformations among mated females of the first and second generation

Mated females of	Females with at least one malformation	Females with 1 malf. fetus	2 malt. fet.	3 malf. fet.
1st generation				
Control	**18**	**17**	**1**	
Exposed	**46**	**42**	**3**	**1**
2nd generation				
Control	**7**	**6**	**1**	
Exposed	**21**	**12**	**9***	

*** Significantly different at P<0.05 when compared to the controls and at P<0.01 when compared to the first generation**

GENETIC SUSCEPTIBILITY TO RADIATION-INDUCED EFFECTS IN EMBRYOS

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1. INTRODUCTION

Embryonic development can be grossly divided into three periods, corresponding each to a peculiar sensitivity to the various effects of radiation.

- The first period is the so-called “preimplantation period”, and extends from the fertilization up to the implantation of the embryo into the uterine walls. During this, the fertilized egg, or zygote, will travel the oviduct (or Fallopian tube) to arrive after a few days into the maternal uterus. During its journey through the oviduct, the embryo undergoes a series of divisions, reaching the 2-cell, 4-cell, 8-cell and 16-cell stages. The 16-cell embryo is called the “morula”, and the 32-cell embryo, ready to implant, is called the “blastocyst”. The duration of the preimplantation period does not greatly differ between the various mammalian species.

- Implantation consists into the attachment of the blastocyst to the uterus, its penetration through the epithelium and the beginning of the complex interactions of the embryo with its mother. In humans, this process occurs essentially during the second week of pregnancy. Substantial morphological changes occur at that time, with the differentiation of the embryonic cells into the three layers : ectoderm, endoderm and mesoderm. This very important step is known as the gastrulation. A relatively long period follows then, during which organs are formed according to a well defined sequence for each species. This period, called “organogenesis”, extends up to day 56-60 in humans. Near its end, the human embryo measures about 30 millimeters and weighs 2-2.7 grammes.

- The third period is the foetal period. Like for the organogenesis, the duration of this period shows great variations among species : nearly 70 % of the total pregnancy in humans, but only 30 % in the small rodents. This is the period of general growth and functional maturation of the newly formed organs. Important developments occur during this period, for example neurogenesis and synaptogenesis, and formation of the external genital organs.

This seminar is essentially devoted to the risks of an irradiation during early pregnancy. In the first part of my talk, I will, therefore, try to give an overview of recent data on the radiation-sensitivity of the embryo during the preimplantation period, with special emphasis on the susceptibility of the newly fertlized egg, or "zygote", to radiation- and chemical-induction of

malformations. Results obtained with a number of chemicals may shed new insights on this important question.

With the advent of refined molecular biological techniques, mice deficient for a number of genes have been available, and the influence of such genes on embryonic development and radiation-sensitivity has been the object of many publications. It would be very difficult to give even a brief overview of those, and I will essentially limit the second part of my talk to the results obtained with mice deficient in some genes whose influence on radiation-sensitivity of adult tissues has been well established, if not yet fully understood. I hope to show that the use of animals deficient for specific genes offers new and exciting perspectives for the study of the mechanisms of ionizing radiation on the highly sensitive embryonic cells.

2. PREIMPLANTATION EMBRYO AND TERATOGENIC EFFECTS : A PECULIAR SENSITIVITY OF THE ZYGOTE?

2.1. Positive results following irradiation

In man, it is virtually impossible to investigate events occurring in the embryo before its implantation into the uterus, because there is no way to know whether fertilization took place before the most sensitive radioimmunoassay tests have detected an increased concentration of human chorionic gonadotrophin (hCG) in the urines, indicative of a trophoblastic activity. However, it is largely admitted that many pregnancies come to an end before having been diagnosed clinically, and even before the first missed menstruation. Direct observations of human preimplantation stages are thus extremely rare and, in order to have a precise idea of the effects of an exposure to toxic agents like radiation, one is obliged to rely on results obtained from experiments on laboratory animals.

Lethality has been recognized as the main effect of irradiation during the preimplantation period. It has also long been admitted that embryos escaping killing by radiation would develop without anomaly, due to the fact that the cells are still undifferentiated at these stages and that loss of one or a few cells can eventually be compensated by other cells. The fact that definite positive results were obtained by the laboratory of Prof. Streffer (Pampfer and Streffer, 1988; Müller and Streffer, 1990) obliged the scientific community to reconsider this problem, leading to some controversy. The results obtained in Essen showed that there was a strong mouse strain-specificity in the sensitivity to the teratogenic effects of radiation during the pre-implantation period and pointed to the peculiar sensitivity of the one-cell embryo, or zygote. The main malformation induced by irradiation of preimplantation embryos of the Heiligenberger strain, 1 hour after fertilization, was gastroschisis. This malformation was also encountered in unirradiated embryos, but its frequency was very clearly increased by irradiation with X-rays or neutrons. This suggested that the frequency of malformations could be increased by irradiation in those mouse strains already showing a specific predisposition for these malformations. There were marked differences in the sensitivity of the various preimplantation stages, the one-cell stage being the most sensitive, but there was no time during the preimplantation development that did not show an increased teratogenic risk. The data were also compatible with an absence of threshold dose for the induction of malformations during the one-cell stage.

Since that time, Gu and colleagues, in Japan, also reported about malformations in ICR embryos which had been gamma-irradiated at various preimplantation stages (Gu et al., 1997).

Like in Essen, the highest effects were obtained after irradiation of one-cell embryos, in this case 2 hours after fertilization, but other preimplantation stages were also sensitive. In contrast to the results of Essen, the malformations observed were very variable and included exencephaly, cleft palate, chest hernia, abdominal hernia, open eye, anophtalmia, abnormal tail and polydactyly. No external malformations other than open eye were observed in unirradiated fetuses. The average incidence of all types of external malformations among mice irradiated at 2 h postconception with 0, 0.1, 0.25, 0.5 and 1.0 Gy were 0.19, 0.49, 3.1, 6.1 and 4.2 %, respectively. The increase was significant from the dose of 0.25 Gy. The decrease observed at 1.0 Gy was attributed to the fact that, at that dose, the lethal effects were dominant, resulting into a probable elimination of a number of abnormal embryos. Another interesting point of that study was that the susceptibility to external malformations appeared even higher in pre-implantation stages than during organogenesis : a dose of 0.5 Gy given on day 8 induced only exencephaly and exophtalmia, and the incidence of external malformations induced by that dose did not differ from that observed in unirradiated mice.

In our laboratory too, we X-irradiated zygotes 7 hours after fertilization and obtained some evidence of teratogenic effects in the CF1 strain, but not in the BALB/c strain (Jacquet et al., 1995). The abnormalities observed in the CF1 strain included exencephaly, polydactyly, hypodactyly and gastroschisis. In unirradiated animals, only 1 of 862 fetuses showed exencephaly, and there were no other malformations. Although the incidence of malformed fetuses was always low, it showed a tendency to increase with the dose of radiation, from 0.12 % in the unirradiated group to 2.27 % in the group irradiated with 1.0 Gy, the difference with controls being significant for the doses of 0.1, 0.5 and 1.0 Gy. In addition to external malformations, we also observed a number of underdeveloped or "dwarf" fetuses. The incidence of dwarfs increased significantly following irradiation with 0.5 and 1.0 Gy. When dwarfs were added to the malformed fetuses to calculate the incidence of abnormal fetuses, the proportion of those increased from 1.8 % in the unirradiated group to 5.2 % and 5.7 % in the groups irradiated with 0.5 and 1.0 Gy, respectively. Like in the study of Gu et al. (1997), the number of surviving fetuses was very low in females given 1 Gy, and it is possible that a number of abnormal embryos of this group had been eliminated soon after implantation.

The group of Rutledge performed similar experiments in hybrid mice (SEC X C57BL), which were given 1.5 or 2 Gy of X-rays 2.5 h after fertilization (Rutledge et al., 1992). The anomalies found in the fetuses were mainly hydrops or generalized fetal edema, wall defects such as gastroschisis, eye defects, exencephaly and cleft palate. Except for the latter, these anomalies were also found in the control fetuses, but their proportions increased from 1.2 % to 3.6 % and 6.2 %, repectively, in groups irradiated with 1.5 Gy and 2.0 Gy. The increase was marginally significant for the dose of 2.0 Gy.

An important feature of all the above considered studies is that, in contrast to most previous studies, a very narrow and accurate timing for the determination of conception was performed (mating possibility restricted to 2 hours instead of during all the night), allowing a better synchronization of the embryonic development. This resulted in irradiation of homogeneous developmental stages and, logically, in more defined radiation responses.

To summarize, various results obtained during recent years and using the same experimental protocols have suggested that ionizing radiation could be potentially teratogenic in a few mouse strains, when administered as early as at the one-cell (or "zygotic") stage. The Japanese and German results also suggested that irradiation of later pre-implantation stages could induce similar effects, though to a lesser degree. While the German studies have clearly shown

that, in the Heiligenberger strain, a genetic predisposition exists for induction of gastroschisis, this is not necessarily so for the various malformations induced in other mouse strains.

2.2. Positive results following the administration of chemicals

In view of the controversy caused by the few positive results obtained after irradiation of pre-implantation embryos, an examination of some recent results obtained by teratologists using chemical compounds will be instructive.

The laboratories of Generoso at Oak Ridge and Rutledge in Seattle have been involved in a decade of study of mutagen effects on zygotes of hybrid mice (for review, see Rutledge, 1997).

Treatment of the female mice 1 to 9 h post-mating with a variety of short-lived agents revealed to result into various unusual effects. Thus, a number of mutagens induced embryonic lethality at different stages of gestation, including mid and late gestation and even stillbirths or death before weaning. Furthermore, many chemicals revealed also able to induce malformations after zygotic treatment. The malformations observed in 17-18 day fetuses after treatment of the zygotes form a special class that differs from the set induced during organogenesis, in that the defects are more restricted in nature : hydrops (generalized fetal edema), bent limbs and tail, abdominal wall defects and eye defects represent the majority of anomalies induced by such treatments. Interestingly, exencephaly and digital defects have not been observed after treatment of the zygotes by chemicals, although those have been reported in various papers following irradiation. The list of chemicals able to induce malformations after exposure at the one-cell stage includes : ethylene oxide, ethyl methanesulfonate, diethyl sulfate, dimethyl sulfate, acrylamide, ethylnitrosourea and triethylenemelamine.

A set of chemicals have also been shown to produce changes in the post-zygotic pre-implantation embryo, resulting in embryonic death and malformations such as limb defects, cleft palate, exencephaly, open eyelids, skeletal defects and digital numerical aberrations (poly- or hypodactyly). Exencephaly, not induced in the zygotic stage, is a consistent finding and the incidence of cleft palate is high only when the blastocyst is treated. Dwarfism, the most common defect after germ cell treatment, is not frequent. The list of such agents includes : methylnitrosourea, cyclophosphamide, ethylnitrosourea, retinoic acid (vitamin A) and 5-azacytidine (for review, see Dwivedi and Iannaccone, 1997). Importantly, not all of the effects were manifest in utero. Eighty four to 90 h old blastocysts were exposed in vitro to methylnitrosourea, transplanted to surrogate mothers and allowed to go to term (Iannaccone, 1984). While no malformations, radiographic, morphometric, karyotypic or histologic defects were detected, the pups from the treatment group had excess perinatal mortality and 58 vs. 22% mortality at one year.

2.3. Mechanisms responsible for the teratogenic effects observed after exposure of pre-implantation stages

Even if the mechanisms of induction of malformations could strongly differ between chemicals and irradiation, the teratogenic effects observed after treatment of mouse preimplantation embryos by a number of chemicals undoubtly add further relevance to the few positive results reported after irradiation of the same embryonic stages.

Some of the mechanisms involved in the teratogenic effects observed after irradiation of Heiligenberger zygotes have been evoked by Prof. Streffer. Similar studies have not been performed in other radiation sensitive strains, like the ICR and CF1 strains.

The mechanisms for zygotic damage after exposure of zygotes to chemical mutagens have not yet been established. Even though maximally tolerated doses of mutagens have been used, embryo transfer experiments have shown that the effects are generally zygotic rather than maternal. Lack of an increase in the incidence of background anomalies indicates that, in contrast to the situation observed in the Heiligenberger mouse strain after irradiation, chemical mutagens do not act by increasing the rates of spontaneous anomalies. Like for irradiation, it is suggested that genetic modifiers can play a role, but broader studies involving the influence of genetic background or even species effects are still lacking.

The finding that some effects induced by an exposure of preimplantation embryos to chemicals were expressed long after birth is of importance and underlines the usefulness of performing long-term studies, to have a precise idea of the full potential impact of such exposure. In this context, it may be interesting to note that Rugh et al. (1964) reported that 1 Gy of X-rays to the newly fertilized egg of the CF1 strain caused as much as 98% of the males and 97% of the females surviving to 18 months of age to develop cataracts. Only 13% and 17% of the control males and females, respectively, developed this defect at the same age. A comparable study was made at the 0.1 Gy X-ray exposure level but, with this dose, no significant effect was obtained.

3. THE EARLY POSTIMPLANTATION STAGES ARE HYPERSENSITIVE TO DNA DAMAGE

3.1. The gastrula responds to low doses of irradiation by a p53- and Atm-dependent apoptosis

Early postimplantation development in mammals is associated with a dramatic increase in the proliferation rate of undifferentiated stem cells that form the primary embryonic layers, ectoderm, endoderm and mesoderm, and with the start of differentiation of the embryo. Gastrulation occurs during the second week of pregnancy in humans. The potential cost to the embryo of a very rapid proliferation rate is a high production of damaged cells. Although embryos do show some DNA repair capacity, the extent of damage that the embryo can tolerate during early gastrulation are not very clear, as are the mechanisms for the elimination of unrepaired cells. This question was addressed last year by Heyer and colleagues (Heyer et al., 2000).

Heyer et al. (2000) showed that during gastrulation, the mouse embryo becomes hypersensitive to DNA damage induced by low doses of irradiation. In general, two major pathways can be activated in response to DNA damage : cell cycle arrest (which allows the cell to repair DNA damage before it becomes fixed as a mutation) or apoptosis (also defined as "programmed cell death"). At early gastrulation (6.5 to 7.5 days after fertilization), the cell cycle was not perturbed in response to low irradiation. Instead, damaged cells were eliminated by apoptosis, with a threshold as low as 0.05 Gy. Apoptosis was also restricted to the time of gastrulation : it was not observed in preimplantation embryos irradiated with doses up to 0.5 Gy, or in embryos irradiated 8.5 days after fertilization. Heyer et al. (2000) also investigated the molecular mechanisms of apoptosis in the gastrula, using embryos

deficient in various genes∗, under others *p53* which is one of the key regulators in the DNA damage pathway to cell cycle arrest and apoptosis : the apoptotic response appeared to be dependent on *p53* but not on $p19^{ARF}$ or *DNA-PK*, two other genes involved in cell cycle regulation and DNA damage response. Heyer and colleagues also found that *Atm*, which encodes the protein deficient in the human neurodegenerative and cancer predisposition condition ataxia telangiectasia, functions upstream of *p53* in the embryonic apoptotic pathway operating during early gastrulation.

Interestingly, the expression of *Atm* and *p53* was upregulated in both the embryonic and extraembryonic region within 1 h post-irradiation in wild-type embryos, but only in the embryonic region did this upregulation lead to the induction of apoptosis. The extraembryonic cells contribute to tissues that enable the embryo to survive within the maternal uterus. But these extraembryonic cells are transient and do not contribute to the embryo proper. What are the differences between the embryonic and extraembryonic lineages that lead to a difference in the ability to undergo apoptosis in response to DNA damage is not known. There are a number of genes that have distinct expression patterns in the embryonic versus extraembryonic regions during the pre-gastrula period, however, whether any of these genes are responsible remains to be determined.

3.2. What are the consequences of an inhibition of the apoptotic process for the embryo, in normal conditions and following irradiation?

Apoptosis is an essential physiological process in the normal development of embryos, either for eliminating abnormal embryonic cells or for controlling the number of developing embryos in various species (Schwartzman and Cidlowski, 1993).

It has been reported that, in the absence of irradiation, homozygous mice from mouse strains carrying null mutations of the tumour suppressor gene *p53* can survive normally to birth, but then succomb rapidly to tumours, predominantly of the thymic lymphoid lineages (Jacks et al., 1994). Results of Armstrong et al. (1995) showed, however, that a significant proportion of female $p53^{-/-}$ mice also die during embryogenesis (duration of gestation in the mouse : 18-21 days) or in the period between birth and weaning, being subject to a spectrum of abnormalities. In a significant proportion of $p53^{-/-}$ female embryos, the normal process of neural tube closure failed, leading to exencephaly (day 11.5) and subsequently anencephaly (at birth). This also occurred, but at a much lesser extent, in males. There were also other defects: craniofacial malformations, including ocular abnormalities and defects in upper incisor tooth formation, and polydactyly (i.e. a higher number of digits) of the hindlimbs. Similar sex distortion associated with exencephaly has been reported for both humans and mice. The transition from exen- to anencephaly by the time of birth was necessarily associated with massive cell loss. Electron microscopic and histological analysis of the exencephalic embryos confirmed the presence of apoptosis in neural tissue from *p53*-deficient mice. These results

∗ Most genes are present in two copies, each on a separate "homologous" chromosome, one inherited from the mother and one from the father. Individuals carrying two normal copies (or "alleles") of one particular gene are homozygous (+/+) for that gene, while those carrying one normal copy and one abnormal (mutated) copy of the gene are heterozygous (+/-). Individuals homozygous (-/-) for the mutation, also called homozygous null, have two mutated copies of the gene considered and, as a consequence, cannot synthesize the protein encoded by this gene. Animal models of human disorders can be created by replacing the normal pair of genes by two defective copies (such -/- animals are called "knockout" animals).

demonstrated a clear link between p53 deficiency and developmental abnormalities. But they also indicated the existence of other, p53-independent mechanisms of apoptosis, operating at least in the developing central nervous system between late organogenesis and birth.

Heyer et al. (2000) were interested by the biological consequences of the lack of Atm- and p53-dependent apoptosis in response to low dose irradiation during gastrulation. Embryos from heterozygous matings (only 3 litters each) were irradiated with 0.5 Gy 6.5 days after fertilization∗. None of the *Atm* or *p53* homozygous null embryos survived to birth, and very few heterozygous or wild-type youngs were obtained. Heyer did not say whether the few heterozygous youngs obtained from these experiments were normal.

In our laboratory, we also mated *p53* heterozygous mice and X-irradiated the embryos with 0.5 Gy 7 days after fertilization, corresponding to the gastrula stage (Baatout et al., 2001a-b). Almost normal proportions of *p53* heterozygous and homozygous null embryos were obtained at the end of gestation. Moreover, in both irradiated and control groups, developmental abnormalities were found, affecting mainly the homozygous null embryos and, at a lesser degree, the heterozygous ones. The proportion of abnormal embryos was, however, significantly increased in the irradiated group (23.4 % vs. 12.9 % in controls). In the control group, the abnormalities consisted in exencephaly and dwarfism. In the irradiated group, gastroschisis, polydactyly, cleft palate and cephalic edema were also found.

These results point to the importance of the p53 tumour-suppressor protein for normal development. They also clearly show that homozygous $p53^{-/-}$ (or heterozygous $p53^{+/-}$ at a lesser extent) embryos may be more at risk for radiation-induction of external malformations during the early postimplantation stages, and that such embryos are able to survive to birth.

4. THE INFLUENCE OF P53 ON THE RADIATION SENSITIVITY OF EMBRYOS DURING ORGANOGENESIS

4.1. P53-dependent apoptosis suppresses radiation-induced teratogenesis : the studies of Norimura and coworkers

In a first paper by Norimura and colleagues, $p53^{+/+}$ and $p53^{-/-}$ mouse embryos were implanted into recipient wild-type (i.e. normal $p53^{+/+}$) females. Pregnant mice were X-irradiated on either days 3.5 (preimplantation period, approximately equivalent to day 5 in humans) or 9.5 (organogenesis period, roughly equivalent to day 27 in humans) of gestation, killed on day 18 (one day before delivery) and examined for fetal anomalies and prenatal deaths (Norimura et al., 1996). X-irradiation of $p53^{+/+}$ mouse embryos with 2 Gy on day 3.5 induced a very high preimplantation mortality and no anomalies, as could be foreseen. As much as 22% of the $p53^{-/-}$ fetuses which had been irradiated at the same time showed malformations, but this proportion did not differ from that obtained for unirradiated $p53^{-/-}$ fetuses (23 % malformations), confirming once again the role of p53 for normal development. After X-irradiation during the organogenesis period (day 9.5), $p53^{-/-}$ mice had only a 7 % incidence of prenatal deaths and a 70 % incidence of anomalies, whereas $p53^{+/+}$ mice had a 60 % incidence

∗ According to Mendel's law, mating of a $p53^{+/-}$ female with a $p53^{+/-}$ male will theoretically give 50 % $p53^{+/-}$ embryos, 25 % $p53^{+/+}$ and 25 % $p53^{-/-}$ embryos.

of deaths and only a 20 % incidence of anomalies. The results obtained following irradiation during the organogenesis period were confirmed in a following work (Nomoto et al., 1998).

Altogether, these results supported the idea that, in principle, embryos at preimplantation and early organogenesis stages are resistant to radiation-induced malformations but sensitive to killing because they have a tissue repair capacity, in which cells with radiation-induced teratogenic injury commit "altruistic suicide" for the health of the whole body. Analysis of apoptotic cells in various tissues of embryos irradiated with 2 Gy on day 9.5 supported this idea : they led to the conclusion that p53-dependent apoptosis suppresses radiation-induced malformation by removing teratogenically injured cells. It is obvious that dead cells must be replaced by healthy ones during the repair of teratogenic injury. It was tempting, for Norimura and colleagues, to assume that mouse fetuses in the major organogenesis period are susceptible to malformations after irradiation with doses above a threshold dose because the rate of differentiation of embryonic organs at this stage is too rapid to allow replacement of an above-threshold number of injured cells by healthy ones after proliferation; that is, suppression of radiation-induced teratogenesis by p53-dependent apoptosis is limited to cases of below-threshold doses of radiation. X-irradiation with doses below 0.5 Gy does generally not produce many anomalies, whereas a dose of 2 Gy produces a high frequency of anomalies. Competent removal by apoptosis of damaged cells from irradiated tissues is dramatically impaired if one of two wild-type *p53* alleles is lost, resulting in even more malformations in the p53-deficient mice.

It is generally admitted that the threshold dose for malformations after embryonic irradiation is much larger after protracted exposures than after single brief exposures.

In the most recent paper of the Norimura group, Kato et al. (2001) report that protracted exposure of mice over two days from days 9.5 to 10.5 of gestation to a γ-ray dose of 2 Gy at 1.2 mGy/min was not teratogenic for $p53^{+/+}$ mice whereas the same protracted exposure was teratogenic for $p53^{-/-}$ mice. Their results support the hypothesis that disappearance of radiation-induced teratogenic damage in embryonic tissues after irradiation at a low dose-rate depends on two independent cellular functions : proficient DNA repair by virtue of low dose-rate irradiation, and vigorous p53-dependent apoptosis. DNA repair is not perfect and residual cells with unrepaired radiation-induced teratogenic damage are deleted from the tissue only when there is vigorous p53-dependent apoptosis, which is the case in $p53^{+/+}$ mice but not in $p53^{-/-}$ mice.

Kato et al. (2001) proposed that mouse embryonic tissues have a p53-dependent "guardian" that abort cells with radiation-induced damage. In a review of the preceding findings of Norimura and colleagues, Brash (1996) already proposed the concept of cellular proof-reading : cells, like DNA polymerases, can erase their mistakes, protecting so both the tissue and the organism by committing apoptosis.

4.2. P53-dependent apoptosis can induce radiation-induced teratogenesis : studies by others

There are indications that the *p53* gene can also have negative effects on the development of the embryo. In a first study, Wang et al. (1999) reported that digital defects (= hypodactyly or missing metacarpals or metatarsals) induced by irradiation 10 days after fertilization (late organogenesis) were due to excessive cell death by apoptosis (Wang et al., 1999). In a second study, Wang et al. (2000) X-irradiated pregnant mice with different *p53* status 11 days after

fertilization (doses: 1, 3 or 4 Gy), in order to examine the possible involvement of p53 in this effect. The authors found that wild-type $p53^{+/+}$ mice were the most sensitive to radiation-induction of digital loss, $p53^{+/-}$ were intermediate and $p53^{-/-}$ were the most resistant. According to Wang et al. (2000), the *p53* gene would have a preventive effect against malformations in the preimplantation period or in the early period of organogenesis. Indeed, at that time, apoptotic death of an appropriate number of cells would facilitate normal development by deleting radiation-damaged cells as the embryo has sufficient plasticity to compensate for the lost cells. However, the gene would have a causative effect for malformations in the late period of organogenesis. During that period, apoptosis plays a critical role in shaping the organs and structuring their architecture. Excessive cell death in particular regions of some organs where p53 is highly expressed would lead to malformations.

It must be stressed that excessive cell death is probably responsible for many malformations caused by an irradiation during early organogenesis, depending on the dose (see the studies of Norimura and colleagues), the organ and the embryonic distribution of p53 and other proteins involved in the apoptotic pathway(s), which varies greatly among different tissues as well as between different stages of development (Gottlieb et al., 1997). Studies performed with chemicals also suggested that the pattern of craniofacial malformations induced at different times during the organogenesis period, beginning from the gastrula, was related to the particular vulnerability of cells in the vicinity of normal programmed cell death. Excessive cell death in these regions, for which the embryo may be unable to compensate, could represent an important mechanism of teratogenesis (Sulik et al., 1988). In another study, eye defects (lens agenesis) were significantly reduced in $p53^{-/-}$ mouse fetuses compared to $p53^{+/+}$ fetuses, after exposure as early as on day 8 to 2CdA, a potent inducer of double strand breaks, and this fact was also related to excessive apoptosis, which disturbed the integrity of optic organ primordia in the $p53^{+/+}$ fetuses (Wubah et al., 1996).

4.3. Conclusion

Studies performed in mice irradiated at different times of organogenesis generally suggest that fetal malformations could indirectly result from a lack of elimination of teratogenically damaged cells. Such case is supposed to occur predominantly in fetuses suffering deficiencies in genes involved in the induction of the apoptotic process. It is tempting to assume that there is a link between the accumulation of cells carrying unrepaired or misrepaired DNA damage and the expression of malformations. In other cases, on the contrary, malformations could directly result from excessive cell death in the forming organ or structure. Support for a role of excessive cell death in the expression of malformations comes also from various studies performed with chemicals. The pattern of spontaneous or radiation-induced malformations observed in wild-type fetuses seems to differ at least partly from that observed in p53-deficient fetuses. Thus, exencephaly is not rare in $p53^{-/-}$ or $p53^{+/-}$ fetuses and is increased after irradiation at different times of organogenesis, whereas this malformation is much less frequent in $p53^{+/+}$ fetuses irradiated at the same times with the same doses. Similarly, polydactyly but not hypodactyly is frequently observed in unirradiated $p53^{-/-}$ or $p53^{+/-}$ fetuses and is increased after irradiation during organogenesis (at least up to 10 days after fertilization), while hypodactyly is characteristically induced in $p53^{+/+}$ fetuses irradiated during late organogenesis (Armstrong et al., 1995; Norimura et al., 1996; Nomoto et al., 1998; Kato et al., 2001; Baatout et al., 2001a-b; Wang et al., 2000).

5. GENES INVOLVED IN THE SUSCEPTIBILITY TO CANCER AND/OR IN DNA REPAIR : THEIR INFLUENCE ON DEVELOPMENT

The number of genes required to control the normal functions of mammalian cells and organisms has been estimated to 60,000-70,000 (Fields et al., 1994). To monitor damage and to maintain the genes without significant alteration is a major concern for the cell, and repair processes have evolved in all organisms to correct errors made in replicating the genes and to restore damaged DNA. In view of the multiplicity of types of damage requiring repair, it would not be surprising if the overall number in humans is a few hundred genes. The consequences of loss of repair capacity can be seen in a number of human syndromes showing hypersensitivity to environmental agents. These syndromes generally show multiple symptoms, including cancer-proneness, neurological disorders and immune dysfunctions. An important question is whether individuals affected from such mutations are also more susceptible to spontaneous or radiation-induced developmental effects. The question is even more crucial for the potentially more frequent heterozygotic disorders. In view of the high number of genes concerned with this complex problem, we will limit this short discussion to some of them, for which there exists some suspicion in favour of such sensitivity.

In addition to the frequent somatic mutation of *p53* in sporadic cancer, germline mutation of one allele of this gene in humans causes an inborn predisposition to cancer known as Li-Fraumeni syndrome. Individuals with Li-Fraumeni syndrome are highly prone to the development of sarcomas and a variety of other tumor types, including carcinomas of the breast and brain (Malkin, 1993). Similarly, $p53^{+/-}$ mice exhibit decreased longevity and increased tumor incidence. The tumor spectrum observed in *p53* heterozygous mice is similar (although not identical) to that seen in humans with Li-Fraumeni syndrome (Jacks et al., 1994). As developed earlier in this paper, the studies performed in Mol and elsewhere have shown that p53 heterozygous mouse embryos had a greater risk to undergo spontaneous or radiation-induced malformations.

ATM has been shown to be a key regulator in response to DNA damage by activating different responses such as cell cycle checkpoints, DNA repair and apoptosis. Individuals suffering from ataxia-telangiectasia (about 1 per 100,000) show mutations in the two copies of the *ATM* gene. They show a complex phenotype : cerebellar ataxia, neuromuscular degeneration, dilated ocular vessels (telangiectasia), immunodeficiency, chromosomal instability and an increased incidence of some cancers and neoplasms, under others lymphocytic leukaemia and non-Hodgkin's lymphoma. They are also highly radiosensitive, as an apparent result of an inability to recover from DNA breakage, leading to a higher level of residual chromosomal damage. A number of studies have suggested that individuals heterozygous for the ataxia-telangiectasia defect, who are much more frequent than ataxia-telangiectasia homozygotes (they represent about 1 % of the population), could be at increased risk of cancer, especially breast cancer. $Atm^{-/-}$ mice are viable but growth retarded and infertile (due to meiotic failure), and the majority of them die before four months from thymic lymphomas (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). $Atm^{+/-}$ mice survive similarly to wild-type mice, showing no particular increase in the incidence of tumors (Barlow et al., 1996). The results of Heyer et al. (2000) showed that $Atm^{-/-}$ embryos irradiated with 0.5 Gy during gastrulation were unable to develop to term whereas $Atm^{+/-}$ and wild-type littermates did. However, as underlined earlier in this text, these experiments were performed on a very small number of animals (3 litters of heterozygous matings $Atm^{+/-}$ x $Atm^{+/-}$) and only 5 embryos were obtained at birth (2

heterozygous and 3 wild-type). Clearly, the radiation-sensitivity of heterozygous *Atm* during early organogenesis deserves further investigations.

ATR, like *ATM*, is a protein kinase that is activated by DNA damage and initiates signaling important in cell cycle checkpoints. Recent studies also suggest that *ATR*, like *ATM*, could regulate p53 in response to DNA damage-induced phosphorylation of p53 (Tibbets et al., 1999). It is currently not known whether *ATR* defects exist in humans; however, the region to which *ATR* maps in humans is a site of alteration in lung cell carcinomas (Cimprich et al., 1996). It has been found that disruption of the *ATR* gene leads to a decrease in survival and increase in tumorigenesis in heterozygous $ATR^{+/-}$ mice and to very early embryonic lethality in homozygotes $ATR^{-/-}$. Embryonic lethality observed in *ATR* homozygotes occurred soon after implantation of the blastocyst and before 7.5 days p.c. The study of Brown and Baltimore (2000) also showed that in $ATR^{-/-}$ embryos, chromosomal fragmentation preceded apoptosis of the embryos, suggesting that the early death of $ATR^{-/-}$ embryos was caused by a widespread loss of genomic integrity. The fact that, in contrast to $ATM^{-/-}$ and $p53^{-/-}$ mice, $ATR^{-/-}$ mice are not viable, indicates that *ATR* must function in some manner that is not redundant with *ATM* and is independent of *p53* regulation. Studies on the influence of ATR heterozygosity on radiation-induction of embryonic malformations are still lacking.

Bloom's syndrome is a rare autosomal recessive disorder characterized by proportional dwarfism, telangiectatic erythema, immune deficiency and an increased risk for all cancers. One of the defining features of the disease is the presence of chromosome aberrations in cultured Bloom's cells. Other genome instability syndromes like Fanconi anemia, ataxia telangiectasia and Werner syndrome, also have increased levels of chromosome gaps, breaks and rearrangements. However, unique to Bloom's cells, are increased exchanges between homologous chromosomes ("sister chromatid exchanges"). Mouse embryos homozygous for a targeted mutation in the murine Bloom's syndrome gene (*Blm*) were found to be developmentally delayed (like their human equivalents) and to die by embryonic day 13.5 (some evidence also exists for intrauterine lethality in humans : the proportion of Bloom's individuals is less than expected for transmission of an autosomal recessive trait). Interestingly, there was apparently no cell cycle delay in the early embryos : the growth retardation observed in mutant embryos resulted exclusively from a wave of increased apoptosis occurring specifically in embryonic cells during gastrulation (Chester et al., 1998). Increased apoptosis has not been described in human Bloom's syndrome. However, the possibility that small size exhibited in Bloom's syndrome newborn is also caused by a similar mechanism during embryonic development has clearly to be considered. Heterozygous mice appeared to be normal and were phenotypically indistinguishable from wild-type littermates until at least 1 year of age (Chester et al., 1998). The effects of irradiation on embryonic development of such mice remains to be investigated.

Fanconi's anaemia is also an autosomal recessive, cancer-prone disorder, most commonly presenting with acute myeloid leukaemia (the risk is increased by a 15,000 factor), although solid tumours are also found. The symptoms of this disorder are variable and may include bone marrow failure and a number of congenital abnormalities such as growth retardation, skin hyperpigmentation, hypogonadism, renal malformations, microcephaly, variable degrees of mental retardation and microphtalmia (Glanz and Fraser, 1982). Fanconi's anaemia cells show high levels of chromosome aberrations and are hypersensitive to DNA cross-linking agents (like mitomycin C). The extent to which Fanconia's anaemia cells are sensitive to ionizing radiation has been an object of discussion (like for the Bloom's syndrome), although

they have been recognized as more sensitive than normal cells (Deschavanne et al., 1986). Cells from Fanconi's anaemia patients have been classified into eight genetic groups, consistent with the heterogeneity of symptoms found. Mice defective for one of these genes have been generated and showed some of the features of the human disease like impaired fertility (Chen et al., 1996), but investigations on the effects of prenatal irradiation in such mice have not yet been undertaken.

A number of genes involved in DNA damage repair have been cloned, by their structural homology to genes involved in specific repair pathways in lower organisms. In those, the main pathway for the repair of DNA double-strand breaks involves homologous recombination. The genes responsible in the budding yeast are *RAD50, RAD51, RAD52, RAD53, RAD54, RAD55, RAD57, MRE11* and *XRS2* (the so-called "*RAD52* gene group"). Several human or mouse homologues of the *RAD52* gene group have been cloned. In the mouse, *mRAD50*$^{-/-}$ and *mRAD51*$^{-/-}$ embryos have been shown to die around the gastrula stage. Apparently, death did arise from a failure of cell proliferation rather than from high levels of apoptosis (Lim and Hasty, 1996; Luo et al., 1999). Radiation sensitivity assays performed on early blastocysts showed that the mutated embryonic cells were hypersensitive to gamma irradiation, implying that the cell-proliferation defect may be secondary to a defect in DNA repair. As underlined earlier, gastrula is a period of embryonic development during which proliferation increases dramatically, with estimated mean cell cycle times of 2-4.4 h in the ectoderm (Hogan, 1986). The bulk of spontaneously occurring double strand-breaks is likely to arise during DNA replication, and available evidence suggests that such breaks are repaired primarily through homologous recombination with the sister chromatid. The death of *mRAD50*$^{-/-}$ and *mRAD51*$^{-/-}$ embryos at the gastrula stage, when rapid proliferation normally occurs, may reflect that sister chromatid-based repair of such spontaneously arising DSBs is abrogated in the absence of mRAD50 or mRAD51, leading to the accumulation of unrepaired or misrepaired spontaneous DSBs over several cell cycles (Luo et al., 1999). These data argue that homologous recombination is linked to cellular proliferation, and that this link is responsible for the failure of *mRAD50*$^{-/-}$ and *mRAD51*$^{-/-}$ cells to meet the intense replicative demand of early embryogenesis.

The RAD51 protein has been shown to interact directly with p53, as well as with BRCA1 and BRCA2 proteins, the products of two genes involved in the susceptibility to cancer (Scully et al., 1997; Sharan et al., 1997). Inherited mutations in *BRCA1* may cause breast cancer and ovarian cancer, while inherited mutations in *BRCA2* will cause mostly breast cancer with a lower risk of ovarian cancer compared to *BRCA1* (Easton et al., 1993). Neoplasia appears to be associated with loss of heterozygosity of the non-mutated alleles in tumours that arise in these patients. Up to now, and despite their importance in cancer predisposition, the molecular function of the BRCA genes was unknown, and the finding of their interaction with RAD51 provided the first evidence that their role may be in DNA repair. Like *mRAD50*$^{-/-}$ and *mRAD51*$^{-/-}$ embryos, *BRCA1*$^{-/-}$ and *BRCA2*$^{-/-}$ embryos are not viable and arrest during development at similar times (Hakem et al., 1996; Sharan et al., 1997). Why does *BRCA1*$^{-/-}$ or *BRCA2*$^{-/-}$ deficiency cause cell lethality in early embryos, whereas *BRCA1*- or *BRCA2*-deficient mammary or ovarian cells proliferate and form tumours is not known. One explanation is that the proliferation arrest is cell-type specific. The early embryo is very sensitive to unrepaired breaks and so the cells are not viable. In contrast, mammary or ovarian cells deficient in one of these two genes can survive with an unstable genome, but presumably they accumulated genomic alterations that contribute to the malignancy. Taken together, these data suggest that BRCA1, RAD51 and BRCA2 may be involved in detecting and repairing

double-strand breaks, thereby controlling cell cycle progression. Loss of this repair/monitor system has a direct effect on cell proliferation in the early embryo and tumorigenesis in the adult (Sharan et al., 1997). The fact that the absence of some repair genes leads to embryonic lethality shows that these genes have important roles in basic cellular processes influencing tissue development. Embryos heterozygous for the *RAD50*, *RAD51, BRCA1* or *BRCA2* genes have not yet been investigated for their suceptibility to developmental defects induced by irradiation during organogenesis.

While in lower organisms, double-strand breaks are mostly repaired by the homologous recombination pathway, non-homologous end joining seems to be the main mechanism in mammalian cells for such repair. DNA-dependent protein kinase (DNA-PK) plays a key role in non-homologous end joining pathway. DNA-PK is related to the ataxia telangiectasia gene product and consists of a catalytic subunit (DNA-PKcs) and a DNA-targeting component Ku, which itself is a heterodimer of two polypeptides, Ku70 and Ku80. This dimer binds to broken ends of DNA, recruiting the catalytic subunit and confering kinase activity on the complex. When activated, DNA-PK phosphorylates many transcription factors, including p53 (Lees-Miller et al., 1992). The *scid* (severe combined immune-deficient) mouse strain, which is radiosensitive, shows a single DNA base alteration in the gene encoding the catalytic subunit of DNA-PK. This mutation gives a protein truncated by 83 amino acids in *scid* cells, leading to partial abolition of the kinase activity. Mice homozygous for the *scid* mutation lack functional T- and B-lymphocytes, and about 15 % of them develop lymphomas (Custer et al., 1985). They are three to four times more radiosensitive than normal cells and have a reduced ability to repair double-strand breaks. Mice heterozygous for the mutation also show an increased sensitivity to gamma rays. Additionally, it was recently shown that mouse embryos deficient in both DNA-PKcs and ATM are not viable and die at the gastrula stage (Gurley and Kemp, 2001).

Transgenic mice were also be obtained, which were "knockout" for the gene encoding the catalytic subunit of DNA-PK, resulting in the total absence of DNA-PKcs (Jhappan et al., 1997). All these so-called *slip* mice died within 5-6 months from thymic lymphoblastic lymphomas. Similarly, knockout mice for the genes encoding Ku86 or Ku70 could be obtained. The mice derived from such mutations showed important growth reduction and were very radiosensitive. Additionally, Ku86 defective mice developed a high frequency of spontaneous thymic and disseminated T-cell lymphomas (Nussenzweig et al., 1997; Gu et al., 1997; Li et al., 1998).

To our knowledge, only one publication was dealing with the radiation-sensitivity of embryos defective in the non-homologous end joining pathway. Thus, it was reported that gamma-irradiation of *scid* mice on day 8 of gestation, with 0.75 Gy, led to embryonic mortality as well as to various developmental effects (Shoji et al., 1998).

Damage to individual bases in DNA may be corrected simply by removing the base, cleaning up the site, and resynthesis. This process, called the base-excision repair pathway (BER), requires several different enzymes. The latter part of the process may also be used to repair single-strand breaks in DNA. Attempts to generate mice defective in the BER have generally led to embryonic lethality, as seen with the major apurinic/apyrimidinic (AP) endonuclease (APE) (Xanthoudakis et al., 1996) and polymerase β (Gu et al., 1994), two factors that are recognized as central players in this pathway. Three additional genes having a less-well defined relationship to BER, *XRCC1*, *LIG1* and *PARP*, also have been knocked out (Wilson and Thompson, 1997; Bentley et al., 1996; Ménissier-de Murcia et al., 1997). Whereas

XRCC1 and LIG1 disruptions lead to lethality during embryogenesis, the poly(ADP-ribose) polymerase (PARP)-deficient embryos survive, but they are smaller than average and their litter sizes are reduced. They are also abnormally sensitive to killing by γ-rays. Another gene, *Aag*, has been successfully knocked out (Engelward et al., 1997). This gene encodes a DNA-glycosylase that initiates BER by excising damaged bases from DNA. It represents, therefore, the first fully viable model that is deficient in a recognized BER component. It will be interesting to see how these animals respond to DNA-damaging agents in terms of viability, development and carcinogenesis. Like for other above-mentioned genes, it will be particularly interesting to look for developmental effects induced by an irradiation of heterozygous embryos.

6. CONCLUSIONS

During development, as during an animal's entire lifespan, cells are constantly subjected to environmental and metabolic conditions that may cause damage to genomic DNA. If left unrepaired, these modifications can cause mutations that could result in loss of viability. Mutations incurred in early embryogenesis could be transmitted to large populations of cells as embryogenesis proceeds. These mutated cells might contribute to teratogenesis and might be incorporated into the germ line. Therefore, to ensure survival, cells are equipped with mechanisms to repair these DNA modifications. In addition, proliferating cells can also delay cell cycle progression to avoid replication or segregation of damaged DNA. Finally, as a safeguard, cells of multicellular organisms have the option of undergoing apoptosis in response to DNA damage. The survival strategies used by the embryo may vary according to the stage of development or the tissue concerned. However, data in this field are still very scarse, as are the mechanisms involved.

Experimental studies with mice have generally shown that irradiation during the pre-implantation period of the embryo induces a high incidence of prenatal deaths but virtually no malformations; irradiation after the implantation period makes fetuses progressively resistant to radiation-induced prenatal death; and the incidence of gross anomalies at term is high only in mice irradiated during the period of major organogenesis.

Even if embryonic mortality will remain by far the principal risk associated with an irradiation during the preimplantation period, I think that it should be hazardous to exclude completely the possibility of teratogenic effects after such exposure. Even if the mechanisms involved may be different, studies performed with a number of chemicals have definitively shown that malformations can be induced during this period, and that zygotes are generally, but not always, more at risk for such effects than the following preimplantation stages. The studies performed in the laboratory of Prof. Streffer have shown that the zygotes of the Heiligenberger mouse strain are also sensitive to radiation-induced teratogenic effects, and suspicion exists for the existence of a similar sensitivity, although at a lesser degree, in some other strains.

Overall, these results are important, since they contradict the long-standing dogma in teratology that induction of fetal abnormalities requires the exposure of the embryo during the period of organogenesis. Furthermore, this means that chemicals and ionizing radiation can induce anomalies in the embryo by various mechanisms. Teratogenic effects induced during teratogenesis are classically described as deterministic. Their occurrence and severity would depend on the number of cells killed and they do not occur below a certain threshold dose. Fetal anomalies induced by treatment of a 1-cell embryo should necessarily be the result of

some defect compatible with cell survival. Such effects should then possibly be of a genetic (stochastic) nature and be transmitted to all cells of the organism, as it should be after treatment of the germ cells. In line with this, it should be underlined that the zygote stage could in some manner be considered as the final stage of the female germ cell maturation.

With the advances of molecular biology, mice deficient for various genes involved in DNA damage response have become more and more available, and studies performed with such animals will undoubtly greatly help to understand the mechanisms of peculiar radiation-sensitivity of the embryo. A number of studies performed very recently in this field used mouse strains deficient for the *p53* gene, which is known to play a key role in apoptosis and cell cycle regulation in adult tissues. Apoptosis plays a capital role in embryonic survival as well as in the occurrence of malformations, but the studies published todate have shown that its pathways are complex and can vary between stages of development or even organs. Thus, some studies have shown that, like in oocytes (Perez et al., 1997), p53 is not always involved in the apoptosis occurring in the various embryonic tissues. Moreover, apoptosis can protect the embryos from developing malformations or, alternatively, increase their frequency after irradiation, depending again on the stage at which irradiation occurs. The fact that high levels of malformations can arise in the supposed absence of apoptosis point to the important possibility of malformations mechanisms different from cellular death leading to a cellular deficit in the developing structures.

With the recognition of *p53* homologues and their functions (*p63* and *p73*) (Mills et al., 1999; Yang et al., 1999; Yuan et al., 1999), it will be important to examine the complexity of the *p53* gene and its role in radiation teratogenesis further. The fact that an interference with the apoptotic processes can lead to teratogenic effects after protracted exposure at very low dose-rates also deserves further attention.

Investigations performed todate with mice deficient in various other genes involved in DNA repair and cellular responses supported the idea that defects in DNA repair or response pathways are particularly critical just prior to or during gastrulation. Mice homozygous for such mutations were generally found to die around that critical stage, and it will be important to investigate more deeply the influence of an heterozygous state on embryonic survival and normality after exposure to low doses of irradiation during this period. Moreover, it should not be surprising that genes whose mutations lead to an increased probability to develop cancer, due to their important role in cell cycle control and DNA repair processes, also determine to a large extent the sensibility of the embryo to various developmental effects.

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Health consequences after irradiation in utero – human data.

Risk of leukemia and mental retardation

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ABSTRACT

The relation between childhood leukemia, mental retardation and pre- and postnatal exposure to low-dose ionizing radiation remains debatable. Ionizing radiation is considered a risk factor for leukemia for exposure at all ages (1, 2), although no radiation-related excess of leukemia has been identified among atomic bomb survivors exposed in utero and prospectively followed (3-7).

Ionizing radiation to the developing brain has been shown to result in decreased cognitive function (8), although the lowest dose that affects cognitive function yet has to be defined. The underlying mechanisms by which ionizing radiation causes brain damage are still largely unknown, although reduced neuronal migration, cell death, and decreased perfusion have been suggested (9).

In the present paper, current knowledge on the carcinogenic and cognitive effect of pre- and postnatal exposure to ionizing radiation is reviewed. Two recent Swedish studies are taken as examples of possible ways to address the epidemiological problems involved when approaching these problems.

LEUKEMIA RISK AFTER EXPOSURE TO IONIZING IRRADIATION

Introduction

Acute lymphatic leukemia is the most common malignancy among children, accounting for about one-third of all malignancies found in individuals younger than 15 years (10-13). The incidence peaks at two to four years of age and suggesting events early life, including the intrauterine period, being causally related to childhood leukemia (10-13). Perinatal risk factors such as maternal renal disease, use of supplementary oxygen at delivery, postpartum asphyxia, birth weight of >4500 g, and hypertensive disease during pregnancy, are factors, besides ionizing radiation, that has been linked to an increased risk of childhood leukemia (14).

In 1958, Stewart et al. (15) reported that prenatal radiation from diagnostic x-rays was associated with an increased risk of leukemia during childhood. A relationship was found between cancers in children less than 10 years of age and diagnostic x-ray exposure to their mother's abdomen during pregnancy. In subsequent reports, children up to 15 years of age were included and a relative risk of 1.5 was found for all childhood cancers (15, 16).

Other reports with similar findings followed and contributed to major changes in medical practice (1, 15-30). There became a consensus that low-dose fetal exposure was harmful and

x-ray examinations decreased over time, largely replaced by ultrasound. However, the causality was debated (26, 29, 30).

Most case-control studies and meta-analyses have shown a small increased risk for childhood leukemia following a history of prenatal radiation (1, 15, 16, 18, 19, 22, 27-29), which is in contrast to most cohort studies that have not supported this association (1, 17, 20). Results from twin studies are contradictory (31-34).

The largest and most comprehensive study is the Oxford Survey of Childhood Cancer with more than 15,000 case-control pairs. This cohort is important since there are few studies that have the statistical power to study the carcinogenic effects of an exposure of 10-100 mGy (29).

Evidence of causality

Since the reports in the late 1950s that in utero exposure to ionizing radiation is associated with cancer and leukemia in childhood, the issue has been debated. Evidence for a causal association derives mainly from case-control studies while cohort studies, most notably the A-bomb survivor data, report no association.

The evidence of causal association between in utero exposure and a subsequent childhood cancer are several (Table 1). Meta-analyses are consistent with a relative risk close to 1.40, and the variation between study results is not large and could be due to chance alone. Few studies have addressed the dose-response relationship but one study showed an increasing risk with increasing number of x-ray films (16). There also seems to be a reduction in malignancies over time that parallels a reduction in exposure based on estimates of dose per film (29).

The largest study to date, the Oxford Survey of Childhood Cancer, reported a 50% increase in risk following prenatal x-ray for both myeloid and lymphatic leukemia (29). A possible explanation for these findings was recall bias since exposure data were based on maternal interviews and not medical records. It is likely that a mother of a child that has recently developed leukemia has a tendency to remember any exposure better than women with a perfectly healthy child. These concerns where minimized with the publications of MacMahon et al. (18, 23, 26). The large series relied on medical records and not mothers recall and the initial study (18) confirmed the results by Stewart et al. (15, 16) (Table 2). In an extended series published in 1984, no longer an excess of solid tumors was found (23).

The underlying reason for examination could confound the results if related to leukemia. Early case-control studies were criticized for not adjusting for concomitant disease in the mother and/or the fetus. However, in case-controls studies of twins, where mothers a presumably x-rayed to determine fetal position, no difference in risk estimates has been shown when compared to singletons (21).

Evidence against causality

Despite the wealth of knowledge described above, uncertainty of the causal relationship remains (Table 3).

It has been argued that the reason for, rather than the x-ray exposure itself, explains the elevated risks. The sharpest argument for this argument is the lack of increased risks among

the risk A-bomb survivors (3). Using the risk estimates derived from the initial studies by Stewart et al. (35), 14-15 cases of childhood cancers deaths are anticipated. Only two cancers and no leukemia have been identified among the 753 survivors exposed to >10 mGy (mean dose 310 mGy).

Already many years ago it was argued that it was peculiar that diagnostic x-ray in utero would increase the risk of all solid tumors by 40-50%. Especially since tissue varies in susceptibility to ionizing radiation and childhood tumors have dissimilar origins. Similar risk coefficients for leukemia, lymphoma, Wilms' tumor, CNS tumors, and neuroblastoma were reported by Bithell and Stewart (16). Besides, the extended study by Monson and MacMahon (23), failed to show an increased risk of solid tumors (Table 2).

Excess relative risk per Gy (ERR/Gy) based on case-control studies has been found to be 40% for both leukemia and solid tumors after in utero exposure (1). The corresponding figures for children exposed under the age of 10 years to the atomic bombs were 17% and 2%, respectively (5). It has to be remembered that the latter increase, refer to risks seen for the whole life span and not only childhood. The identification of incident cases of cancer among the survivors did not start until 1958, which influences the risks. It could also be argued that the cells that gives rise to some of the childhood cancers are capable of dividing only a short time after birth. This is however not true for the red bone marrow and there is no obvious reason why the same cells should react differently to exposure just before compared to after birth.

Animal experiments indicate a variety of tumors being induced by fetal irradiation but no difference in risk estimates between leukemia and solid tumors (36). Increased risk of myeloid leukemia was seen after postnatal exposure but not after in utero exposure. With few exceptions, previous studies analyzed childhood leukemia as one entity, lumping acute lymphatic and myeloid leukemia together (16). In some studies (16, 23), potential confounding factors such as children with Down's syndrome, an accepted risk factor for leukemia (37, 38), were included. In addition, most studies lacked statistical power, due to small sample size.

Twin studies are somewhat puzzling in that no increased risks of childhood leukemia's have been seen in any cohort studies despite substantial x-ray exposure (33, 34).

Swedish experience – leukemia risk

A recent Swedish case-control study by Naumburg et al (Naumburg E, Bellocco R, Cnattingius S, Hall P, Boice JD Jr , Ekbom A. Intrauterine exposure to diagnostic x-ray and risk of childhood leukemia subtypes. Radiat Res, January 2002, in press) examined the association between prenatal exposure to diagnostic x-ray examinations (for different types of examinations and stages of pregnancy) and the risk of childhood lymphatic and myeloid leukemia.

All children, born and diagnosed with leukemia (excluding children also diagnosed with Down's syndrome) between 1973 and 1989 in Sweden (578 lymphatic and 74 myeloid), were selected as cases and matched (by sex and year of birth) to a healthy control child. Exposure data were blindly abstracted from all available medical records. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by conditional logistic regression.

Prenatal x-ray examinations resulting in direct fetal exposure were not associated with a significant increased risk for childhood leukemia, OR=1.11 (95% CI 0.83-1.47) nor for lymphatic leukemia, OR=1.04 (95% CI 0.77-1.40; Table 4) or myeloid leukemia, OR=1.49 (95% CI 0.48-4.72; Table 5). For lymphatic leukemia no effect of age at diagnosis, trimester of exposure, number of x-ray examinations, type of examinations or time of investigation (Table 4). Routines for pelvimetri were changed in Sweden in the late 1970s.

A slight, but non-significantly increased risk after abdominal x-ray examinations compared to no and other examinations (Table 5) was seen for myeloid leukemia. The conclusion was that x-rays performed during pregnancy in the 1970s and 1980s in Sweden did not discernibly affect the risk of childhood leukemia and lymphatic leukemia.

Conclusion - leukemia risk

Exposure to low doses of ionizing radiation in early childhood is associated with an increased risk of cancer and leukemia and it is, therefore, likely that exposure to ionizing radiation in utero presents a leukemogenic risk to the fetus. It is, however, somewhat peculiar that cohort studies have failed to identify an increased risk of childhood cancer or leukemia related to in utero exposure to x-ray examinations and previous case-control studies find a consistent risk of approximately 40-50% for all different types of tumors. Most studies had methodological weaknesses. The largest case-control study to date, presented in this paper, failed to identify an increased risk after adjustments had been made for subtype of leukemia and maternal health problems. Taken together, these findings argue against a profound carcinogenic effect of in utero exposure to low doses of ionizing radiation.

RISK OF MENTAL RETARDATION AFTER EXPOSURE TO IONIZING IRRADIATION

Introduction

Most studies of potential cognitive effects after exposure to ionizing radiation in early childhood are based on children treated for cancer or leukemia where the underlying disorder and other therapeutic modalities, such as chemotherapy, influence neurocognitive function (39). Cranial doses of 18 Gy or more have been shown to cause severe mental retardation (40-45). Psychological disturbances have also been noted after surgery for brain tumors prior to radiotherapy (42). Endocrine abnormalities caused by radiotherapy probably also affect mental capacity (41, 46).

Children exposed in utero as a consequence of the atomic bombs in Hiroshima and Nagasaki experienced an increased prevalence of mental retardation and reduced school performance (8, 47-51). The highest risk of damage to the frontal lobes was seen at a gestational age of 8-15 weeks, the time of rapid proliferation of neurons and neuroblast migration from the cerebral ventricles to the cerebral cortex, although no significant effect on cognitive ability was seen at a fetal dose-range below 100-200 mGy (8, 50). Severe mental retardation was seen in surviving children exposed during the period 8-15 weeks of gestational age. For those exposed to more than 500 mSv increased risk were seen (Table 6).

Ron et al. (52) compared cognitive abilities in approximately 11,000 Israeli children receiving a mean brain dose of 1.3 Gy for ringworm of the scalp (tinea capitis). The irradiated children had lower school examination scores, intelligence test scores, psychological test scores, and

completed fewer school grades than the non-exposed group, although the difference was not statistically significant. A 40% excess risk of being treated for a psychiatric disorder was found in 2,215 American children treated with x-ray for ringworm of the scalp at seven years of age compared to controls (53).

Swedish experience – risk of mental retardation

Ionizing radiation influences the developing brain but he lowest dose that affects cognitive ability yet has to be defined and few studies have the possibility to address this problem. No studies have evaluated the possible adverse cognitive effects of low doses of ionizing radiation, defined as <100 mGy, to the developing brain. Computed tomography (CT) scans are becoming increasingly popular and absorbed brain doses are not negligible and we therefore evaluated the potential effects of ionizing radiation, very early in childhood, on adult cognitive functioning in an unique Swedish cohort (Hall P, Pedersen NL, Ekbom A, Ingvar M, Lundell M, Granath F. Low doses of ionizing radiation affects the cognitive development of the human brain. Submitted for publication).

Material and methods

Results from military service enlistment tests were matched to a cohort of boys receiving radiotherapy for skin hemangioma (n=3,094) within the first 18 months of life. Cognitive test results and the likelihood of attending higher education were related to absorbed brain dose.

The mean age of the children at first treatment was 7 months (median 6 months, range 0-17 months). Mean absorbed brain dose was 52 mGy (Table 7) and the hemangiomas were located anywhere on the body, but were concentrated to the head and face (36%). All children were treated with radium applicators (β and γ rays) and/or x-rays as described in detail previously (54, 55). The dose contribution from treatments with radium-226 needles/tubes in glass capsules was measured on a child phantom with the original applicators. For all other kinds of treatments, original depth-dose curves and tables were used. For each treatment the distance between the treatment location and the brain was adjusted according to the age and size of the child at the time of treatment.

Information on year of enlistment examination, education, number of siblings, birth order, and father's occupation and education was available from the military registry. Father's occupation was used as a proxy for socio-economic status and was stratified in four groups: unknown and farmers, blue-collar workers, low level white-collar workers, and high level white-collar workers.

During the period of the study a pupil with a sufficient grade point average could attend the equivalent to high school/A-levels at the age of 16 years. The subjects were categorized as those attending high school and those that did not.

The Swedish psychological tests for inscription have been used for more than a half a century, with some minor changes over the years. The enlistment battery used for all individuals in our cohort included tests General Instructions, Concept Discrimination, Technical Comprehension and Spatial recognition. General Instructions and Concept Discrimination are believed to measure general verbal ability and are somewhat education sensitive. These two tests were analyzed as one entity (using the average score) as they co-varied considerably. In Spatial Recognition and Technical Comprehension, which reflect suitability for technical and

mechanical training, logic reasoning is rewarded. The tests are not sensitive to education to the same extent as the other tests. At the time of testing all test scores for a given year of testing were standardized to a mean of 5 and a range from 1to 9.

The influence of radiation on cognitive abilities was initially evaluated for educational attainment recorded at the time of the induction to the military service. The probability of having entered the equivalent of high school was modeled by logistic regression with skull dose, age at treatment (0-3, 4-6, 7-9, 10-12, 12+ months), number of siblings (0-2, 3, >3), father's occupation and year of the military testing as categorical explanatory variables. Doses were divided into four dose categories, 0-19 mGy, 20-99 mGy, 100-249 mGy, ≥250 mGy. Results were presented as odd ratios and 95% confidence intervals (CI).

Results

The proportion of boys attending higher education after the age of 16 years decreased with increasing brain dose (Table 8). The influence was most pronounced for those who could be described as sons of middle class fathers. A positive dose-response relationship was seen and was most pronounced for cognitive tests that reflected processes in the frontal part of the brain. Tests reflecting scholastic skills, rather than spatial recognition, were most influenced by the ionizing radiation. As can be seen in Table 9 it seems as those boys attending high school had the most pronounced effect on the tests reflecting scholastic skills. When contrasting test reflecting scholastic skills to spatial recognition the effect of ionizing radiation seemed even more pronounced.

Conclusion – risk of mental retardation

Previous studies have not addressed risk at this dose level. The average brain dose for a child treated for tinea capitis is in the range of 1-1.5 Gy and the adverse cognitive effects of doses below 100 mSv has not previously been evaluated.

The Swedish study suggests that low doses of ionizing radiation very early in childhood influence cognitive abilities 15-20 years after exposure. The results are worrisome in the light of increasing use of CT scans in infants after minor head trauma.

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Table 1 Arguments supporting the theory that in utero exposure to ionizing radiation increases the childhood cancer risk.

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1. *Consistency*. Nearly all studies are statistically consistent with a relative risk close to 1.40 for leukemia.

2. *Dose response*. Risk of childhood cancer has been found to increase with number of x-ray films.

3. *Coherence*. Apparent lower risks in more recent age cohorts where the dose per examination is supposedly lower.

4. *Confounding*. Confounding variables has been sought but none has been found.

5. *Selection bias*. The underlying reason for examination does not seem to influence risk as supported by results from twin studies.

6. *Risk estimates*. Estimates of leukemia risk after in utero exposure are generally comparable to risk after childhood exposure.

Table 2. Number of pregnancies, proportion of children exposed to diagnostic x-ray examinations, and relative risk, RR. Adopted from (18, 23).

	Control sample		Leukemia			Solid tumors		
	No. of pregn.	% of x-ray	No. of children	% of x-ray	RR	No. of children	% of x-ray	RR
Initial study	7,230	10.6	292	16.1	1.48	246	15.4	1.45
Extension	7,046	8.2	305	12.8	1.58	261	8.4	1.06
Both	14,276	9.4	597	14.4	1.52*	507	11.8	1.27

*Statistically increased risk, 95% confidence interval 1.18–1.95

Table 3 Grounds for uncertainty regarding the causal nature of the association between exposure to in utero radiation and childhood cancer.

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1. A-bomb survivors studies finds no increased risks of solid tumors or leukemia.
2. All major cohort studies are negative.
3. The biological implausibility of equally relative risks of all solid tumors given the variability in tissue susceptibility.
4. Risks are higher for in utero compared to newborn exposures.
5. Twin studies have lower risks of childhood cancer despite more x-ray examinations.
6. Supporting animal evidence is weak.

Table 4 Risk of *lymphatic leukemia* following prenatal x-ray exposure, age at diagnosis, trimester of exposure, by number of x rays, type of prenatal x-ray exposure and abdominal x-ray examinations performed before 1980 and after

		Cases	Controls	Adjusted OR[1]	95% CI
All	unexposed	449	450	1.00	
	exposed	103	102	1.04	0.77-1.40
Age at diagnosis, years					
<2	unexposed	84	79	1.00	
	exposed	17	22	0.69	0.32-1.47
2-4	unexposed	174	179	1.00	
	exposed	33	28	1.23	0.70-2.16
5-16	unexposed	191	192	1.00	
	exposed	53	52	1.17	0.71-1.63
Trimester of	unexposed	449	450	1.00	
exposure	first	3	4	0.73	0.16-3.28
	second	18	9	2.00	0.84-4.59
	third	80	89	0.93	0.67-1.30
	unknown[2]	2	0	-	
No. of x rays	unexposed	449	450	1.00	
	one	96	91	1.08	0.79-1.48
	two or more	7	11	0.70	0.27-1.91
Type of x ray	unexposed	449	450	1.00	
	abdominal	55	54	1.01	0.68-1.51
	other x ray	31	28	1.07	0.62-2.83
	unknown[3]	17	20	-	

continuation

Table 4 to be continued

		Cases	Controls	Adjusted OR[1]	95% CI
Time period					
<1980	unexposed	260	276	1.00	
	abdominal	43	37	1.16	0.73-1.86
	other x ray	26	22	1.14	0.63-2.09
	unknown[3]	14	14	-	
≥1980	unexposed	183	174	1.00	
	abdominal	12	17	0.67	0.31-1.46
	other x ray	5	6	0.81	0.24-2.73
	unknown[2]	3	6	-	

[1]OR was calculated by means of conditional logistic regression. The adjusted model was based on mother's age at birth, gestational age, parity, smoking, cesarean section, and birth weight.

[2]Unknown information of trimester of exposure.

[3]Unknown type of x-ray exposure.

Kolla fotnötterna i Tab 4 och Tab 5 så att de stämmer med dina intentioner. Det ska inte vara bindestreck mellan x och ray här. Men det ska vara bindestreck när det står x-ray exposure.

Table 5 Risk of *myeloid leukemia* by number of x-rays, type of prenatal x-ray exposure, and abdominal x-ray examination performed before 1980 and after.

		Cases	Controls	Adjusted OR[1]	95% CI
All	unexposed	54	61	1.00	
	exposed	18	11	1.49	0.48-4.72
No. of x-ray	unexposed	54	61	1.00	
examinations	one	17	10	2.25	0.82-6.12
	two or more	1	1	0.61	0.03-11.6
Type of x ray	unexposed	54	61	1.00	
	abdominal	13	7	1.74	0.53-5.74
	other x ray	3	4	1.04	0.20-5.33
	unknown[2]	2	0	-	
Time period					
<1980	unexposed	35	38	1.00	
	abdominal	6	4	2.73	0.31-9.66
	other x ray	3	4	1.01	0.19-5.30
	unknown[2]	2	0	-	
≥1980	unexposed	19	23	1.00	
	abdominal	7	3	2.61	0.40-16.9
	other x ray	0	0	-	0.24-2.73
	unknown[2]	3	6	-	

[1]OR was calculated by means of conditional logistic regression. The adjusted model was based on mother's age at birth, gestational age, parity, smoking, cesarean section, and birth weight.

[2]Unknown information on type of x-ray exposure.

Table 6 Total number of A-bomb survivors exposed at 8-15 weeks of gestational age, number with severe mental retardation and proportion of mentally retarded in relation to dose (Sv)

Dose	Total no. at risk	Mentally retarded	Proportion of mentally retarded, %
+1.00	26	12	46
0.50-1.00	43	4	9
0.10-0.49	215	2	1
0.005-0.09	212	3	1
<0.005	1,069	9	1

Table 7 Average absorbed doses to the frontal and posterior parts of the brain in relation to the localization of the skin hemangioma (closest to the skull).

Hemangioma localization	No. of individuals	Mean dose to parts of the brain, mGy (maximum dose, Gy)		
		Frontal	Posterior	Average dose, mGy
Skull	552	172 (2.8)	121 (1.8)	147
Face	642	79 (2.4)	50 (0.4)	64
Other parts	1,675	15 (0.3)	16 (0.3)	16
Average dose, mGy	-	60	44	52

Table 8 Number of individuals and the proportion within fathers' occupational category attending the equivalent of high school in relation to frontal and posterior brain dose among 2,816 boys[1] irradiated for skin hemangioma

	Fathers' occupation							
	Blue collar		Low level white collar		High level white collar		No information	
Brain dose, mGy	No.	%	No.	%	No.	%	No.	%
Frontal dose								
0-19	458	12.2	429	37.8	327	60.1	84	10.7
20-99	345	11.3	272	33.1	183	61.2	57	15.8
100-249	226	8.4	160	25.6	97	52.6	62	11.3
≥250	43	7.0	36	16.7	22	50.0	15	-
Posterior dose								
0-19	459	12.0	435	36.8	329	59.3	84	11.9
20-99	459	11.1	339	33.9	241	60.6	90	11.1
100-249	142	7.0	116	20.7	54	51.9	38	13.2
≥250	12	8.3	7	-	5	40.0	6	

[1]53 subjects were excluded due to missing information on education.

Table 9 Mean test results adjusted for number of siblings, treatment age, and year of military testing in relation to frontal brain dose in 2,210 boys[1] irradiated for skin hemangioma, and 1,524 boys[2] not attending high school.

		Military test			
		CD-GI[3]	TI	SR	(GI+CD+TI)-SR
Brain dose, mGy	No.	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)
All irradiated for skin hemangioma					
0-19	1038	5.60 (0.06)	5.44 (0.07)	5.36 (0.07)	0.18 (0.06)
20-99	677	5.64 (0.07)	5.38 (0.08)	5.49 (0.08)	0.07 (0.08)
100-249	410	5.42 (0.09)	5.31 (0.10)	5.48 (0.10)	-0.09 (0.09)
≥250	86	5.32 (0.18)	4.77 (0.21)	5.44 (0.21)	-0.30 (0.19)
p-value[4]		0.03	0.003	0.50	0.0012
IRRADIATED FOR SKIN HEMANGIOMA NOT ATTENDING HIGH SCHOOL					
0-19	682	5.05 (0.07)	5.01 (0.08)	5.00 (0.09)	0.03 (0.08)
20-99	463	5.05 (0.08)	4.95 (0.10)	5.11 (0.10)	-0.09 (0.09)
100-249	312	5.00 (0.09)	5.06 (0.11)	5.23 (0.12)	-0.21 (0.11)
≥250	67	4.80 (0.19)	4.68 (0.22)	5.19 (0.24)	-0.44 (0.22)
p-value[4]		0.16	0.25	0.19	0.008

[1]417 boys were excluded since they were tested 1954-1958 and test results were not comparable. Additionally 241 boys were excluded due to missing information on education and number of siblings.

[2]417 boys were excluded since tested 1954-1958, 193 boys because of missing information on education and number of siblings, and 735 boys since they did not attend high school.

[3]CD-GI= mean of concept discrimination and general instruction, SR=spatial recognition, TI=technical instruction.

[4]Test for trend, based on the mean in each strata and dose treated as a continuous variable.

Conclusions and Potential Implications

RIHSS Working Group of the Article 31 Group of experts1

INTRODUCTION

This document presents the main conclusions and potential implications of the Scientific Seminar on the Effects of in Utero Exposure to Ionising Radiation during the Early Phases of Pregnancy, held in Luxembourg on 6 November 2001. While it is not intended to report in an exhaustive manner all of the opinions that were expressed by the speakers or by the audience, it takes into account the discussions that took place during the subsequent meeting of the "Article 31" Group of experts on 7 November 2001. The content of the document has been prepared with the assistance of a rapporteur2, then discussed within the RIHSS (Research Implications on Health Safety Standards) Working Party of the Article 31 Group of experts. The final text is the responsibility of the RIHSS Working Party.

1. RIHSS Seminars: rationale

The RIHSS Working Party of the Article 31 Group of experts was set up with the task of helping to identify the potential implications of recent research results or new data analysis on the European Basic Safety Standards (BSS) Directive and on the related Recommendations and guidance.

The approach adopted is the following: on the basis of input from the Directorate General Research of the European Commission and of information provided by individual members of the Article 31 Group of experts, each year the Working Party proposes relevant themes to the Article 31 Group that could be discussed during a subsequent seminar. After selection of the theme and approval of a draft programme by the Article 31 Group, the Working Party deals with the practical organization. The seminars involve invited speakers -- mainly leading experts -- who are asked to synthesize clearly the state-of-the-art in the field, with special attention paid to new information. Additional experts, identified by members of the Article 31 Group from their own country, take part in the seminars and act as peer reviewers. The Commission convenes the seminars on the day before a meeting of the Article 31 Group, in order that members of the Group can discuss the potential implications of the combined scientific results.

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1 The members of the RIHSS Working Party who contributed to the preparation of this document were the following members of the Article 31 Group: R. Clarke , J. Piechowski, P. Smeesters (Chairman of the WP), A. Susanna. They were assisted by the following officials of the European Commission: V. Ciani, M. Sarro-Vaquero, C. Desaintes, E. Morere-Molinero.

2 Rapporteur: Prof. Dr Wolfgang-Ulrich Müller, Institut für Medizinische Strahlenbiologie, Universitätsklinikum Essen, D45122 Essen, Germany.

2. Background and purpose of the seminar

Despite more than one hundred years of research on the biological effects of ionising radiation, the exact consequences of radiation exposure on the early stages of human pregnancy still remain to be better understood. The major reason is, of course, the problem of obtaining direct information for humans. Frequently, pregnancy will go undetected until early organogenesis and, even after detection, it is difficult to identify the exact time of each developmental step retrospectively. Thus, the information obtained from animal experiments has to be extrapolated to the human situation.

Most of the animal experiments which are relevant for conclusions on radiation risk at very early gestational stages have been done in mice and rats. It is reasonable to extrapolate to humans, at least for the period up to implantation, because timing and developmental processes are rather similar in most mammals.

The penetration of the sperm head into the oocyte results in the formation of the zygote and then the gestation starts (see Fig. 1 for an overview). After several cell divisions, compaction results in the formation of the morula, which consists of between 16 to 32 cells. The development of a cavity within this cell ball leads to a blastocyst, which hatches from the zona pellucida and is ready for implantation. This process takes place around day 5 to 6 in the mouse and slightly later in humans (although the exact timing is not known and may vary from woman to woman; in general, it is assumed to occur around day 9 to 12). Gastrulation and implantation run partly parallel and, after the three fundamental cell layers devoted to the formation of the various tissues have appeared, the embryo is ready for organogenesis. This stage lasts until day 12 to 13 in the mouse (until about day 50 in humans) and is followed by the foetogenesis until term (day 19-20 in mice and about day 270 in humans).

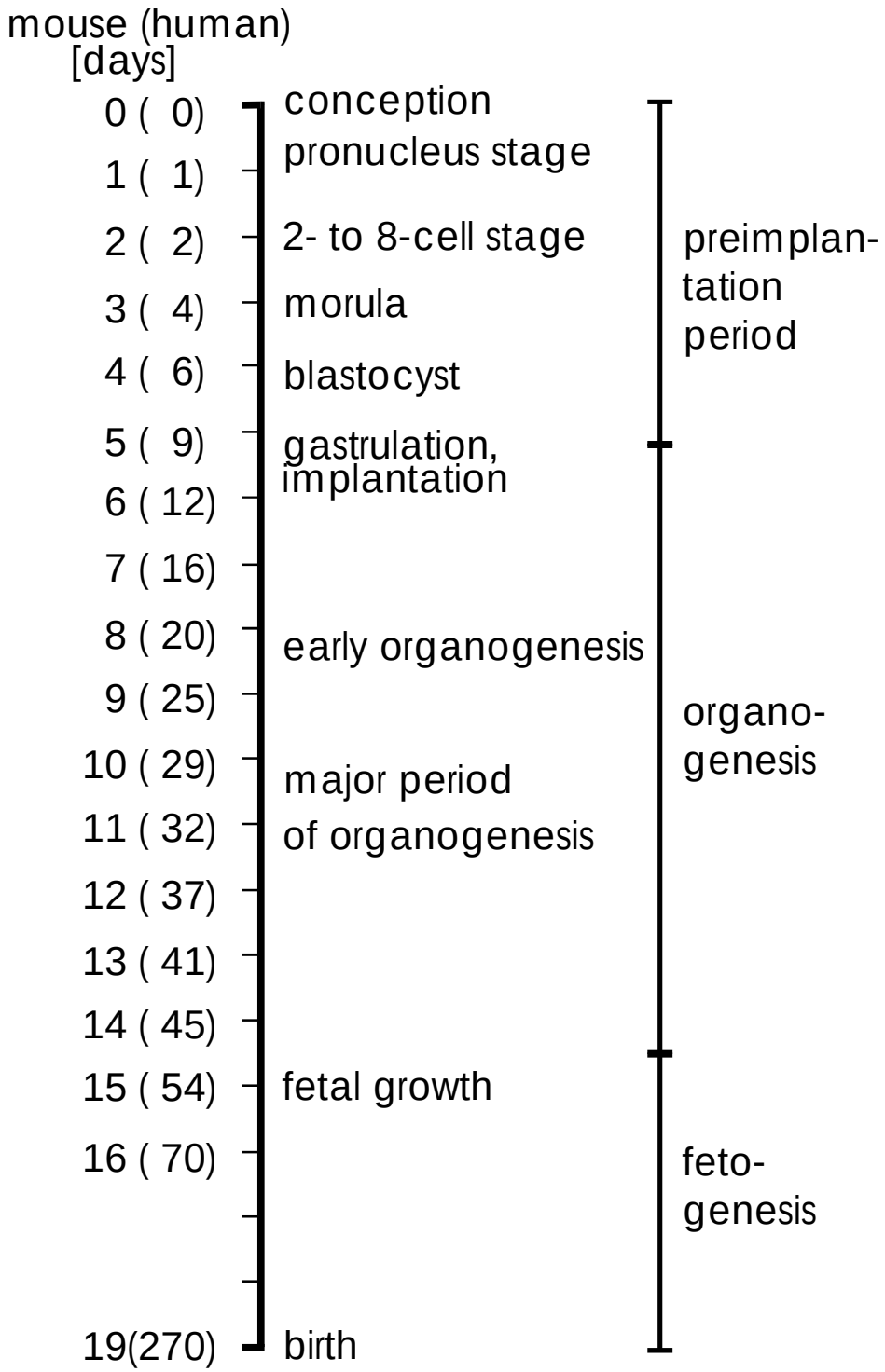

Fig. 1: Major developmental stages of murine and human gestation

(Please note: 1. There is considerable variability from individual to individual so that no exact timing of the specific developmental events is possible. 2. The human time-table is non-linear; thus, the lines of the three major stages shown on the right apply to the mouse, but are out of proportion for humans).

Detailed summaries on the known effects of ionizing radiation on the various stages of gestation are available.3

The classic view of radiation risk during the first trimester of human gestation has been summarized in textbooks and by important national and international radiation protection organisations in the following way:

- Above a certain level of dose (frequently quoted value is 100 mSv), exposure to ionizing radiation during the preimplantation stage results in embryonic death or in healthy survivors ("all-or-none-rule").

- Above a certain level of dose, exposure to ionizing radiation during organogenesis bears a significant risk of induction of visible congenital malformations. But many scientists refrain from stating above which dose this occurs. Most frequently, a value of 100 mSv is quoted, a figure recommended by ICRP.

- Since the studies of Stewart et al., which more or less have been confirmed by others (for an overview see reference [1] in the footnote 3), it is standard knowledge that ionizing radiation applied during pregnancy can induce childhood leukaemia. There are indications that all stages of gestation are affected, and it is assumed that the risk factor is roughly equal to the risk factor for children.

- The analyses of mental disorders of those children exposed *in utero* in Hiroshima and Nagasaki have shown that severe mental retardation can be induced, primarily during weeks 8 to 15, and, to a lesser extent, during weeks 16 to 25. During the weeks 8 to 15, the epidemiological data are compatible with a threshold at about 0.1 to 0.2 Gy, but do not rule out a dose effect relationship without threshold. The assumed mechanisms are cell death, impairment of cell proliferation and impairment of migration. In addition, a dose dependent decrease in the intelligence quotient (IQ) has been observed for those children exposed *in utero* in Hiroshima and Nagasaki.

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3 1. Doll, R.; Wakeford, R.: Risk of childhood cancer from fetal irradiation. Br.J.Radiol. 70 (1997) 130-139

2. European Commission, Radioprotection Publication 100 : Guidance for protection of unborn children and infants irradiated due to parental medical exposures (1998)

3. ICRP Publication 49: Developmental Effects of Irradiation on the Brain of the Embryo and Fetus (1986)

4. ICRP Publication 60: 1990 Recommendations of the International Commission on Radiological Protection (1991)

5. ICRP Publication 84: Pregnancy and medical radiation (2000)

6. NRPB Volume 4 No 4: Diagnostic medical exposures: Advice on exposure to ionising radiation during pregnancy (1993) p. 5-14

7. SSK Volume 2: Effects of pre-natal irradiation; G. Fischer Verlag, Stuttgart (1989) 2nd edition.

8. UNSCEAR Annex J: Developmental effects of irradiation in utero; in: *Sources and Effects of Ionizing Radiation*, New York: United Nations, Sales No. E.77.IX.1 (1977) p. 655-725

9. UNSCEAR Annex C: Biological effects of pre-natal irradiation; in: *Sources and Effects of Ionizing Radiation,* New York: United Nations, Sales No. E.86.IX.9 (1986) p. 263-366

10. UNSCEAR Annex H: Radiation effects of the developing human brain; in: *Sources and Effects of Ionizing Radiation*, New York: United Nations Publication, Sales No. E.94.IX.2 (1993) p. 805-868.

The purpose of the seminar was to summarize the recent information available for radiation risk during the early stages of gestation (i.e. the first trimester) and to look for possible implications of these results for radiation protection. In addition, very recent information was given on mental effects of radiation exposure during early childhood.

3. Main Points Arising from the Presentations and Discussion of their Potential Implications

Some new insights have been gained during the last years with regard to risk after radiation exposure in early gestational stages. These new insights have been presented by the three speakers during the seminar. In the following summary, only those aspects that were judged most important as regards their potential implications will be addressed. As regards the full texts and the literature evaluated by the authors, the reader should refer to the specific articles published in this document.

3.1. Genetic Predisposition and Genomic Instability in Preimplantation Mouse Embryos

C. Streffer informed the audience that in contrast to the understanding in the past ("all-or-none-rule", i.e. the preimplantation embryo either dies or survives without visible damage after radiation exposure), there are mouse strains that respond with a dose-related increase of the frequency of *malformations* when irradiation occurs during the preimplantation stage. Such observations have been made not only in Germany, but also in the United States, in Belgium and in Japan. In addition to ionising radiation, some chemicals (in particular alkylating ones) were able to induce malformations when applied during the preimplantation stage.

In these observations, the sensitivities of the various preimplantation *stages* to the induction of malformations varied markedly, with the (one-cell) zygote stage (first day) being particularly sensitive. In Streffer's experiments with HLG mouse strain, the observations on the *zygote stage* were compatible with *the absence of a threshold dose.*

C. Streffer mentioned several facts showing that "*genetic predisposition*" is an important factor in many of the above-mentioned observations.

C. Streffer stressed in his talk that one should keep in mind that, even in those strains which respond with an increased malformation rate to radiation exposure during preimplantation stages, *embryonic death still predominates*.

Another interesting observation mentioned by Streffer was the induction of *genomic instability*, after irradiation (at a dose above 0.5 Gy) during the preimplantation period of various mouse strains, including those not carrying a genetic predisposition to congenital malformations. This genomic instability appears later in the foetus 'life" and persists in the non-exposed second generation.

3.2. Genetic Susceptibility to Radiation-Induced Effects in Embryos

- In the first part of his talk, **P. Jacquet** presented the experiments performed in Mol and summarized the literature on radiation and chemically induced malformation risk in the **preimplantation stage**.

He drew the attention on the fact that strain peculiarities play an important role. Whereas in the HLG strain (Streffer's experiments) a high *spontaneous* frequency of one *specific* type of malformation was observed (i.e. gastroschisis), which was increased by radiation exposure, this was different in P. Jacquet's (CF1 strain) and in the Japanese (ICR strain) experiments. In both cases, spontaneous malformations were very rare and the types of induced malformations were various and

not necessarily seen spontaneously. This means that *the existence of a specific genetic predisposition, while important, is not a condition* for the radiation induction of malformations after exposure during the preimplantation stage.

Otherwise, while in the HLG strain the threshold dose is at about 1 Gy for the 32- to 64-cell stage (blastocysts), in other experiments, with other mouse strains, external gross malformations were observed even in the mice irradiated with 0.1 Gy at 72 h (morula) and 96 h (blastocyst) postconception. This means that the level of the *threshold dose* for the radiation induction of malformations after exposure during the preimplantation stage could be highly *variable (from stage to stage and from species to species) and relatively low.* Remember that, according to Streffer's talk, a threshold dose may *not* exist during the short zygote stage, at least when a genetic predisposition exists.

- **A lot of potential implications** can be identified from **Streffer's and Jacquet's** talks concerning **preimplantation stages**.

  If there exist mouse strains *with a genetic predisposition* to the radiation induction of specific congenital malformations, *the same is probably true for human beings*: there are indeed families showing *clusters* of spontaneous congenital malformation. Irradiation, during the preimplantation stage (when the pregnancy is not yet recognized), of individuals bearing such predisposition could then induce malformations after very low doses during the short zygote stage (possibility of absence of threshold), or after larger doses (variable threshold doses), during later stages of the preimplantation period.

  As there are besides some similar observations with mouse strains showing *no predisposition to specific malformations*, here again the same could be true for human beings. Irradiation, during the preimplantation stage, could then induce malformations even in the absence of visible family clusters of malformations. It is interesting to note here that, according to UNSCEAR 2001, 50% of the human congenital abnormalities have some genetic *component* (even if not evidenced by the existence of a visible cluster).

  Two questions are raised by the above-mentioned issues. First, knowing this, how can we be sure that radiation-induced congenital malformations are " very rare" (and as such "negligible") after irradiation during the preimplantation stage? Secondly, is the giving up of the ten-day rule in radiology still justified with these new observations?

  Last but not least, the observations concerning induction of genomic instability, *not linked with* genetic predispositions, have potential implications for the high dose medical examinations performed during the preimplantation stage in women not aware of being pregnant.

- **P. Jacquet** reported also about new results published recently on the mechanism of radiation induced malformations after exposure during the **early post-implantation stage (including gastrulation)** and during **organogenesis**. In these studies, the role of genes regulating apoptosis and DNA-repair was analysed.

During gastrulation (day 9-12 by women), apoptosis is induced by relatively low doses (vigorous apoptosis already at 0.05 Gy). This may be considered as a welcome protection against malformations (damaged cells are removed: "altruistic suicide"). The genes *ATM* and *p53* are involved in this process. At least for *p53* there are strong indications that mice with defects in both copies of this tumour-suppressor gene ("homozygous" mice) are particularly sensitive with regard to *malformation* induction during the gastrulation stage; the same applies to those mice with one defective copy ("heterozygous"), but to a lesser degree. The induced malformations are not always

lethal. Apparently, cells with *unrepaired or misrepaired* damage are no longer or only insufficiently removed by apoptosis resulting in malformed fetuses. Note that this is not the classic deterministic mechanism according to which the effect only depends on cellular death leading to a cellular deficit in the developing structures.

Research on genetic susceptible mice (Norimura, Nomoto) defective in p53 (homozygotes and heterozygotes) also showed a particular sensitivity with regard to *malformation* induction, when irradiation occur in early organogenesis. *Protraction of the exposures offers no protection*. Here again the cause of the congenital malformations is not an increased loss of cells (classically considered deterministic effect) but the persistence of unrepaired or misrepaired DNA-damaged cells *(*"*teratogenically damaged cells*").

P. Jacquet reviewed also the role of other *genes* (besides *p53*) *involved in DNA-damage response or in cancer development* like *ATM, ATR, BLM, BRCA1, BRCA2, LIG1, PARP, XRCC1,* and the mammalian homologues of the *RAD52* gene group. In most cases, those mice with defects in both copies (homozygous mice) die early in gestation. Data for mice with only one defective copy are still lacking and it will be important to investigate the influence of such heterozygous state on embryonic survival and normality after exposure to low doses of irradiation during early development. As underlined by P. Jacquet, it would not be surprising if genes whose mutations lead to an increased probability to develop cancer, due to their important role in cell cycle control and DNA repair processes, also determine to a large extent the sensitivity of the embryo to various developmental effects.

- **Potential implications for the early post-implantation stage (gastrulation)** : what about human beings with genetic disorders in the pathway of apoptosis, in particular in the case of the potentially more frequent heterozygotic disorders? They could also show radiation-induced congenital abnormalities after irradiation during this *so-called "safe moment"* in the pregnancy. Again: Is the giving up of the ten-day rule in radiology compatible with these observations?

- **Potential implications for the organogenesis**: there are many genes implicated in the DNA-damage response and involved in the genetic susceptibility to cancer induction by irradiation ; if the mechanisms are similar (misrepair), it is plausible that a genetic susceptibility to the radiation-induction of congenital abnormalities is associated with the human genotypes leading to cancer-proneness . For such human beings with genetic disorders in the pathway of apoptosis or DNA-repair, there could then be a *higher risk* of congenital malformation after irradiation during organogenesis (even when irradiation is protracted), or a *lower threshold* for the induction of malformations. The use of a generally applicable threshold dose (like the 100 mSv figure) for the radiation induction of malformations after exposure during organogenesis could be an unjustified simplification.

3.3. Health Consequences after Irradiation in Utero or in early Childhood – Human Data. Risk of Leukaemia and Mental Retardation

P. Hall summarised the studies available for risk of childhood leukaemia and mental problems after *in utero* or early childhood radiation exposure and reported about new Swedish results in both fields.

One of the intriguing aspects of childhood leukaemia after radiation exposure during pregnancy is the observation that most of the case-control studies report an increased risk, whereas the cohort studies (in particular, the Hiroshima/Nagasaki data) do not.

There are arguments that support the assumption that radiation exposure during pregnancy increases the number of childhood leukaemia cases:

- virtually all case-control studies are consistent with a relative risk of about 1.4;
- there is a dose-response relation;
- the data are coherent in the sense that more recent age cohorts (with presumably lower doses per examination) show lower risks;
- no confounders could be identified up to now;
- selection bias could be ruled out;
- leukaemia risk after *in utero* exposure is similar to the risk after childhood exposure.

There are, however, questions on the validity of the causal relation between *in utero* exposure and childhood leukaemia:

- all major cohort studies are negative;
- the originally reported increase in relative risk for all solid tumours to the same extent is not plausible when taking into consideration the differences in tissue susceptibility;
- twin studies show lower risks of childhood cancer despite more X-ray examinations;
- animal experiments do not support a causal relationship substantially.

P. Hall reported about a recent Swedish study with diagnostic X-ray exposures between 1973 and 1989, in which no statistically significant increase in risk was observed for childhood leukaemia (this result was obtained not only for all leukaemia's, but also for the subgroups lymphatic and myeloid leukaemia).

In his conclusion he mentioned that it is likely that radiation exposure during pregnancy presents some leukaemogenic risk, the magnitude of which being still uncertain but probably low.

Finally, P. Hall talked about recent results on mental effects of radiation exposure during early childhood because of skin hemangiomas. This Swedish study (unpublished) suggests that low dose of ionizing radiation (mean absorbed brain dose was 52 mGy) very early in childhood influences cognitive abilities 15-20 years after exposure. The lowest dose that would be effective is not known right now, but the mean dose of about 50 mGy points to a rather low dose, well in the range of computer tomography.

- As regards potential implications, it is obvious that these results are worrisome in the light of the increasing use of *CT scans* in infants after *minor* head trauma.

4. Discussion

A basic question is how much scientific evidence is needed before the scientific community feels it is necessary to apply the precautionary principle. Are these new observations concerning the risks of irradiation during pregnancy and early childhood and their potential implications sufficient to warrant action. In other words, can they be considered as "early warnings" asking for precautionary

measures. A related question is to know if the various stakeholders (besides the experts) would need the same amount of evidence before recommending action. Here are the main conclusions of the discussions on this matter, including those within the article 31 group of experts the day after the seminar:

4.1. Concerning irradiation of the developing brain :

- The general opinion was that, given the validity of the results, these new observations *have* potential implications for current ***use of CT-scans in infants***. Information on the new studies presented has to be disseminated among the relevant physicians

- The biological mechanisms of radiation-induced effects on cognitive abilities when irradiation occurs after birth are unknown.

- These observations raise the question as to whether irradiation of the CNS *in utero* during the most active periods of brain development (particularly during the weeks 8-15) can also involve a risk of *detectable* late effects on *cognitive abilities* after relatively *low* doses.

4.2. Concerning irradiation in utero during the first trimester:

- On the basis of the new observations presented, the majority opinion is that there is ***no need to change current regulations (BSS directive) and current dose limits in the framework of practices***. There are three arguments in favour of this opinion:

  - There is no emerging evidence that, during this period of the pregnancy, stochastic risks coefficients (cancer, hereditary effects) are higher than it is currently assumed;

  - The current observations are still compatible with *threshold doses,* for the radiation-induction of congenital malformations after irradiation during the first trimester, including the *major part* of the preimplantation period;

  - Although there could be *no* threshold dose for the radiation-induction of congenital malformations in genetically susceptible animal strains when irradiation occurs during the short zygote-stage (< 1 day), and although the same could be true in humans, the *probability* of induction of congenital malformations is *assumed to be very low*.

- ***Nevertheless,*** many experts have expressed the view that the new observations presented have ***various practical implications, particularly in the medical field:***

  - As regards *genetic susceptibility* to radiation induced effects in embryos:

    - During the zygote-stage (about 1 day), no threshold dose for the radiation-induction of congenital malformations has been observed in genetically susceptible animal strains. After irradiation during other stages of the pre-implantation period and during the early post-implantation period, both periods being generally considered safe as regards risks to live births from irradiation, non lethal congenital malformations *have* been induced in animals

with a genetic predisposition to specific congenital malformations or with *genetic disorders* in the pathways of apoptosis or DNA-repair; after irradiation during the organogenesis, *more* congenital malformations have been induced in these animals with genetic disorders. The same susceptibilities *could exist* in humans. It was largely agreed that *there could be for some inviduals a* ***higher risk*** *of radiation-induced malformations or* ***lower thresholds*** *and that* ***the risk*** *could also exist* ***during the "safe" periods of pre- and early post-implantation.***

- As a consequence, some experts expressed *doubts about the "definite" and generalized character of the 100 mSv threshold dose* for developmental effects after irradiation during the first trimester of pregnancy, currently applied by many as a practical criterion.

- While many experts have expressed the view that genetic predisposition (to congenital malformations) most probably exists also in humans, the *frequency* of these predisposed individuals is *not known* ; many experts assume this frequency being low, while other have doubts

- Based on the above-mentioned considerations, several experts have expressed the opinion that *it is worth thinking about on how to detect* a possible predisposition to the radiation-induction of congenital abnormalities (e.g. familial history).

- Several experts have expressed the view that possible predispositions to the radiation-induction of congenital abnormalities *have to be taken into account, due to the higher potential exposures, in the medical field and in intervention situations* (emergency teams).

♦ As regards general risks of irradiation during the *preimplantation* period (including in *non*-predisposed individuals):

- As the radiation-induction of congenital malformations has *also* been observed after irradiation during the preimplantation period of mouse strains showing no predisposition to specific malformations, here again the same could be true for human beings. Irradiation, *above some threshold dose*, during the preimplantation stage, could then induce malformations even in the absence of visible family clusters of malformations.

- Similarly, *genomic instability* has been induced after irradiation above 0.5 Sv during the preimplantation period and this was *not linked with* genetic predisposition.

- These observations have clear potential implications for the ***high dose medical examinations performed during the preimplantation stage in women not aware of being pregnant.***

- Several experts considered that the new observations presented are at least a ***confirmation of the prudent approach*** followed in the BSS and in the guidance4 of the article 31 experts concerning protection of unborn children irradiated due to parental medical exposures; it was mentioned that other documents addressing the same audience are less cautious on this field.

- There was general agreement on the fact that ***more research is needed*** in this field, particularly as regards

    - heterozygotes for the genes implicated in DNA check-point integrity and DNA-repair,
    - induction of genomic instability,
    - and sensitivity of the Central Nervous System during its developmental stages

5. Conclusions and recommendations

- On the basis of the new observations suggesting late effects on cognitive abilities after brain irradiation during early childhood, the ***use of CT-scans in infants should be carefully justified and optimized.***

- Current regulations (BSS directive) are characterized by a prudent approach and ought not to be changed on the basis of the new observations concerning the effects of irradiation during the first trimester of pregnancy. Nevertheless, these new observations have various practical implications, particularly ***in the medical field*** and in intervention situations:

    - ***Due to genetic factors***, after irradiation during the *first trimester* of pregnancy**,** including the pre- and post-implantation periods, there could be for some inviduals a ***higher risk*** of radiation-induced malformations or ***lower thresholds***;

    - Even in the absence of genetic factors, irradiation during the *preimplantation* period (when women are not aware of being pregnant) could induce ***congenital malformations*** or ***genomic instability*** (above some threshold dose);

    - This requires cautiousness in the medical field; the application of the ***ten-day rule*** (plan the non-urgent examination within the ten days following the beginning of the menstruation), whenever the abdominal dose could be significant, would largely reduce these problems.

- ***More research is needed*** in this field, particularly as regards:

    - heterozygotes for the genes implicated in DNA check-point integrity and DNA-repair

4 Radiation Protection 100, see particularly paragraph 31, asking the planned exposure being postponed if there is any uncertainty concerning pregnancy, and paragraph 33, concerning the further use of the ten-day- rule in particular cases.

- induction of genomic instability,
- and sensitivity of the Central Nervous System during its developmental stages

ABSTRACT

The Treaty establishing the European Atomic Energy Community requires the Community to establish basic standards for the protection of the health of workers and the general public against the dangers arising from ionising radiation.

The purpose of the seminar was to summarise the recent information available for radiation risk during the early stages of gestation (i.e. the first trimester) and to look for possible implications of these results for radiation protection.

This recent information is dealt with in three complementary papers focussed on:

- Genetic Predisposition and Genomic Instability in Pre-implantation Mouse Embryos
- Genetic Susceptibility to Radiation-Induced Effects in Embryos
- Health Consequences after Irradiation in Utero or in early Childhood - Human Data. Risk of Leukaemia and Mental Retardation.

The publication is completed by considerations on the conclusions that can be drawn from the seminar and on the potential implications of the informations presented on the developments of the European Union radiation protection legislation.

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14

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