DNA SYNTHESIS IN ATAXIA TELANGIECTASIA

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De foto op de omslag toont een gefuseerde cel met twee kernen, afkomstig van verschillende patienten met ataxia telangiectasia. Dit laatste is herkenbaar aan de twee verschillende typen plastic bolletjes die zich in het cytoplasma bevinden. De kernen zijn beide gemarkeerd met radioactief thymidine, hier zichtbaar als zwarte korrels.

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1. GENERAL INTRODUCTION

1.1 Inherited diseases with hypersensitivity to carcinogens

The principle that genetic changes are the basis of most human cancers, was first postulated by Boveri (1914). Identification and characterization of these changes is the main goal for geneticists studying cancer today. A strategy to identify genes involved in tumorigenesis can be the study of germinal mutations that are responsible for hereditary cancer. At least 200 human single gene traits are known that predispose to some form of neoplasia (Mulvihill, 1977). Most of these inherited conditions are rare and generally the neoplasia is only one of many other clinical manifestations. Apparently, environmental factors contribute to the appearance of the tumors in these disorders. Of those factors, physical and chemical agents that are known to cause cancer in normal individuals are of special interest. The majority of these carcinogenic agents are able to interact with the DNA. This results in DNA damage, that may ultimately give rise to genetic changes which initiate malignant transformation of the cell.

A number of human inherited disorders exist that combine cancer-proneness with an abnormal response to DNA damaging agents. The best characterized are xeroderma pigmentosum (XP), ataxia telangiectasia (AT) Fanconi's anemia (FA) and Bloom's syndrome (BS). The study of these syndromes can be expected to provide insight in the processes that are responsible for the production of genetic changes that are the basis of human cancer.

Patients having XP are very sensitive to sunlight exposure and have an increased risk of skin carcinoma. The cells from most XP patients have a reduced ability to repair a specific type of DNA damage induced by ultraviolet light. Because of this defect these cells become hypersensitive to UV light. Cell death occurs already at low doses, and the surviving cells become mutated or transformed with relatively high frequency. These observations indicate that genes governing DNA repair processes are an example of inherited factors that can play a role in tumorigenesis.

Fanconi's anemia, ataxia telangiectasia and Bloom's syndrome are characterized by a propensity to many types of tumours, predominantly of the lymphoreticular system. Cells from these patients show enhanced frequencies of chromosomal damage and are hypersensitive to mitomycin C (FA,

BS), ionizing radiation (FA, AT), UV light (BS) or DNA-alkylating agents (BS,AT?). In none of these diseases the primary genetic defect could be identified yet.

This thesis will deal with ataxia telangiectasia (AT), a disorder with clinical hypersensitivity to therapeutic X-rays and many other abnormalities in various tissues, including the nervous and endocrine systems, the immunological apparatus and the skin. Because the clinical radiosensitivity in the patients is also expressed at the cellular level, the study of cultured AT cells provides an experimental system that will help to identify the genes and gene products that are involved in the response to ionizing radiation.

1.2 Cellular responses to ionizing radiation

1.2.1 Types of DNA damage

Unlike most chemical mutagenic agents, that directly interact with molecular structures in the DNA, the action of ionizing radiation is largely indirect. Since water is the most abundant cellular constituent, the ionizing action of radiation primarily produces reactive intermediates derived from H_2O. These intermediates, mostly radicals, are unstable and react with other molecules in the cells. The result is a broad spectrum of damage in various cell components. This contrasts with the much more defined types of lesions produced by most chemical mutagens. With respect to DNA, almost every chemical bond may be affected by ionizing radiation exposure and the relative frequencies of the different types of DNA lesions are influenced by other factors, such as the partial oxygen pressure, or the presence of radical scavengers.

The DNA lesions can be classified according to the overall effect they have on the DNA molecule: (i) lesions that interrupt the continuity of the sugar-phosphate backbone: DNA single-strand and double-strand breaks of various types. Those that can be sealed directly by the action of ligase are called 'clean' breaks. The sealing of most breaks ('dirty breaks') requires additional complex enzymatic reactions. (ii) Base damage. Examples are ring-opened structures or glycols, such as the dihydrodihydroxythymine adducts. Generally the basepairing is disrupted at the site of these lesions. (iii) Apurinic/apyrimidinic sites can be produced either directly or after enzymatic processing of base damage. (iv) Chemical cross-

links may produce topological anchorage with other DNA components or with proteins.

1.2.2 DNA repair processes

In living cells enzymatic mechanisms exist, that can repair many of the DNA lesions produced by the action of physical or chemical agents. The result of these enzymatic reactions is the disappearance of DNA damage after some time. The molecular nature of DNA repair processes in mammalian cells is largely unknown. Most of the models are adopted from microbial genetics, where more details of DNA repair mechanisms have been elucidated (for reviews see Lehmann and Karran, 1981; Friedberg, 1984). Using such models two types of repair pathways can be discriminated:

A. The DNA lesion is enzymatically reversed without any reactions involving the rest of the DNA molecule. Examples are photoenzymatic monomerization of pyrimidine dimers, lesions that are caused by short-wave UV light, or the methyltransferase reaction, where the methyl group of a methylated base is transferred to an acceptor protein.

B. The other pathway can be followed for the removal of many types of DNA damage. It is called excision repair because it involves the sequential cutting of the DNA-backbone near the damaged site by lesion-specific endonucleases, and exonucleolytic removal of a number of nucleotides from the damaged strand. The size of the excised patch depends on the type of damage: for most lesions produced by ionizing radiation it appears to be less than 10 nucleotides. The resulting single-strand gap is subsequently filled in and resealed by polymerase and ligase reactions, respectively.

In the case of excision repair this description is oversimplified. Genetic studies of cells from patients with xeroderma pigmentosum indicate, that in repair of UV-damage complex enzymatic reactions precede the endonucleolytic step. Also, evidence has been obtained for preferential repair of some regions in the genome of mammalian cells.

1.2.3 Biological consequences of DNA damage

Cells exposed to radiation display an abnormal behaviour in many respects. Only some of the biological effects of ionizing radiation will be mentioned here.

An early effect of radiation exposure is a delay in cell cycle progression. This results in a reduced growth rate and appears to occur during all phases of the cell cycle. The duration of the delay is dependent on the radiation dose and is not the same in all phases. The G2-phase appears to be the most sensitive in this respect.

When after some delay irradiated cells start to divide again, the chromosomes that are formed during mitosis are frequently damaged. These aberrations can affect one or both chromatids, dependent on the phase of the cell cycle in which the radiation was administered. Many types of chromosomal damage can be observed: there are the simple ones, such as gaps and breaks; acentric, dicentric and ring chromosomes are examples of complex rearrangements.

The ultimate radiation effect is cell killing. Cellular death can be defined in many ways, but for cells that proliferate in culture it has become common practice to measure cellular survival as the ability to reproduce and form a colony. In this way also effects on the following cell cycles are taken into account. However, the parameter cell death (described as inability to survive) is not clearly defined with respect to the 'killing event'. This may be a complete desintegration of the cell, a detachment from the substratum or a long-lasting block in cell cycle progression with continued metabolic activity. Because the ability to proliferate is influenced by many factors the cellular survival will be dependent on the conditions of culture and of the type of cell studied.

Finally, the cells that survive after a radiation treatment may have undergone persistent genetic changes, that can become apparent as chromosomal abnormalities (numerical changes or rearrangements), gene mutations, morphological changes or altered growth characteristics.

One approach to elucidate the molecular mechanisms of the radiation response is to investigate the effects of DNA damage on DNA structure and function. In this approach the replication of DNA is an important parameter.

1.2.4 DNA synthesis in irradiated cells

The slowing down of progression through the S-phase of the cell cycle after exposure to ionizing radiation is reflected by a decreased overall rate of DNA synthesis (for a review, see Walters and Enger, 1976). A typical dose-response curve for the inhibition of DNA replication in cultured human fibroblasts is shown in Fig.1. It follows a biphasic pattern. The first component is believed to be caused by the inhibition of replicon initiation. Since this is a coordinated event in a number of adjacent replicons, the target-size for radiation-induced inhibition of replicon-initiation is relatively large (Watanabe, 1974; Makino and Okada, 1974; Painter and Young, 1975, 1976; Povirk, 1977). The second component of the inhibition curve is thought to represent interference in replication fork movement ('chain elongation') directly caused by damaged sites in the DNA, since the target-size roughly corresponds with that of a single average replicon (Painter and Young, 1975, 1976).

The control of replicon initiation is determined by the higher order structure of chromatin (Mattern and Painter, 1979). The nuclear matrix, a threedimensional networklike structure made from fibrous proteins is an important part of this. The nuclear DNA is attached to the matrix at distances of about 75 µm (Mc Cready et al., 1980) resulting in supercoiled loops of the size of several replicons (Cook and Brazell, 1975; Benyajati and Worcel, 1976; Paulson and Laemmli, 1976; Comings and Okada, 1976). Because the replication of DNA occurs physically linked to the nuclear matrix (Mc Cready

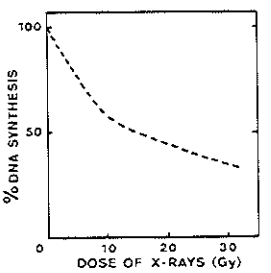

Fig. 1. The rate of DNA synthesis after exposure to X-rays. Normal human fibroblasts were exposed to different doses of radiation and incubated for two hours in the presence of tritiated thymidine. Cells were harvested and radioactivity was counted by liquid scintillation spectrometry.

et al., 1980; Vogelstein et al., 1980) it has been hypothesized that the loop structures correspond to the coordinately initiated replicon domains. The fact that ionizing radiation inhibits replicon initiation suggests that an alteration of the chromatin affecting the loop structures is involved. The exact nature of this structural change and the type of DNA lesion that may be responsible for it are unknown.

The subject of this thesis is the study of the replication of DNA in cells from patients with ataxia telangiectasia. In the experimental work described in the appendix it is shown that in AT cells exposed to ionizing radiation DNA replication proceeds with a rate that differs from that in irradiated cells from normal individuals.

2. ATAXIA TELANGIECTASIA

2.1 Introduction

Ataxia telangiectasia (AT, McKusick number 20890) is a human genetic disease with an autosomal recessive inheritance pattern (Taljoedin and Fraser, 1965). According to the latest estimates the overall incidence is around 1 in 100.000 (see Harnden and Bridges, 1982). The clinical symptoms are expressed in a wide variety of tissues, including the skin, the nervous and endocrine systems and the immunological apparatus. Some of the manifestations of the disease can be traced back to early abnormalities in fetal development. The syndrome is also characterized by a strong predisposition to cancer combined with an excessive clinical sensitivity to therapeutic X-ray doses (Gotoff et al., 1967; Morgan et al., 1968; Cunliffe et al., 1975; Roberts and Ward, 1975; Pritchard et al., 1982).
In 1975 it was shown by two collaborating British groups that cultured fibroblasts from patients with AT are hypersensitive to ionizing radiation (Taylor et al., 1975). In the last decade extensive molecular and cell biological studies have aimed at a better characterization of the response of cultured AT cells to ionizing radiation and to other mutagenic agents. These efforts have not resulted yet in the identification of the primary genetic defect, but merely to the notion that the manner in which cells respond to ionizing radiation is extremely complex.

The purpose of this chapter is to provide a summary of the clinical characteristics of the AT syndrome and a critical update of the cellular and molecular studies. This overview does not pretend to be exhaustive and for additional information the reader is referred to a number of reviews (Sedgwick and Boder, 1972; Paterson et al., 1979, 1980; Friedberg et al., 1979; Lehmann, 1977, 1982a; Bridges, 1981; Huang and Sheridan, 1981; Weichselbaum and Little, 1980; Gatti and Hall, 1983) and two excellent monographs (Bridges and Harnden, 1982; Gatti and Swift, 1985).

2.2 Clinical characteristics

The first clinical description of AT patients was made by Syllaba and Henner in 1926. Later the disease has been 'rediscovered' by Louis-Bar (1941). The first extensive clinical studies were from Boder and associates (1957). The following sections summarize the most important clinical symp-

toms of the AT syndrome; detailed information is available from a number of elaborate treatises (Boder and Sedgwick, 1958, 1963, 1977; Kraemer, 1977; McFarlin et al., 1972; Thieffy et al., 1961; Sedgwick and Boder, 1972).

2.2.1 Neurological complications

The most common neurological abnormalities that occur in AT are cerebellar ataxia and, to a lesser extent, extrapyramidal disorder. The abnormalities become apparent usually at the age of 2 to 8 years and are slowly progressive. There are difficulties with gait, posture, speech and eye movement. When the disease progresses the patient will very often become confined to a wheel chair. Mental abilities of the patient appear normal, but an arrest in cognitive development at the age-level of 10-11 years is common (Sedgwick, 1982; Boder and Sedgwick, 1972).

The ataxic features are caused by cerebellar athrophy. The Purkinje cells are much reduced in number and those still present are often atrophic or displaced into the granular layer (Paula-Barbosa et al., 1983; Vinters, 1985). It is believed that the degeneration of the Purkinje-cells sets in already early in fetal development (Rakic, 1985). Other large cells of the nervous system, such as the anterior horn cells, also may degenerate. Abnormalities in peripheral nerves occur as well.

2.2.2 Immunological abnormalities

The majority of the AT patients suffer from recurrent respiratory tract infections, mainly of bacterial origin. Viral challenges (eg. vaccinations) are tolerated normally. It can be expected that this immunologic incompetence is related to some profound defects in both humoral and cellular immunity that are seen in most (but not all) patients. (Mc Farlin et al., 1972; Dutau et al., 1975; Keller et al., 1978; Jason and Gelfand, 1979; Gatti et al., 1982; Fiorilli et al., 1983; Waldmann et al., 1983).

With respect to the humoral immunity, there are marked deficiencies in IgA, IgE and IgG2, caused by a failure to synthesize these species. (Mc Farlin et al., 1972; Strober et al., 1968; Yount, 1982; Oxelius et al. 1982). The antibody response to bacterial or viral antigens is usually poor (Sedgwick and Boder, 1972; Waldmann, 1982), which is believed to be caused by defective T-helper cell functions (Levis et al., 1978; Waldmann

et al., 1983). However, intrinsic B-cell defects were found as well in some patients (Weisbart et al., 1980; Mitsuya et al., 1981; Waldmann et al., 1983b). Molecular genetic analysis of the Cα and Cμ gene regions in IgA deficient AT patients revealed no apparent abnormalities (Waldmann, 1982; Waldmann et al., 1983b).

Defective T-cell function is also believed to be involved in cellular immunodeficiency. Skin graft rejection (Kuan et al., 1974) and delayed type hypersensitivity reactions may be depressed (Rosenthal et al., 1965; Schuler et al., 1972), and there are poor in vitro responses to mitogens (like phytohaemagglutinin) or antigens. The latter appears to be caused not by a failure of recognition or receptor binding but rather an inability to respond to the membrane signal (McFarlin and Oppenheim, 1969; O'Connor and Linthicum, 1980). Finally, a reduced ability to generate virus-specific MHC-restricted cytotoxic lymphocytes was reported (Nelson, 1980).

The exact mechanism of the immune dysfunction in AT is still unclear. Most probably many of the features will be related to an early abnormality in AT, that is the fact that the patients show an underdeveloped, rudimentary or even absent thymus and gut-associated lymphoid tissue (Petersen et al., 1966; McFarlin et al., 1972). This finding has been the basis for the hypothesis that the immunological problems in AT are caused by defects in embryonic organ maturation (Waldmann et al., 1983a).

2.2.3 Cancer susceptibility

Another major clinical hallmark of AT is the increased risk of the patients to develop malignancies. The overall incidence of cancer in AT is about 10%, which is high since practically all of these cancers arise during the first two decades of life (Levin and Perlov, 1971; McFarlin et al., 1972; Kersey et al., 1973; Berkel and Ersoy, 1974; Aiuti et al., 1978; Spector et al., 1978, 1982). The majority of the tumors are of lymphoreticular origin (Hodgkin and non-Hodgkin lymphomas, leukemias) but carcinomas are also frequent, mainly in the stomach, the ovaries and the liver (Spector et al., 1982). The survival of AT patients with cancer is relatively short (4-7 months), and the therapy of the patients is complicated by their excessive responses to radiotherapy or chemotherapy regimens (Pritchard et al., 1982; Spector et al., 1982; Abadir and Hakami, 1983), and serious pneumonia.

The types of tumours that occur in AT patients and their relative frequencies are different from those observed in children that were exposed to radiation. This suggests that environmental exposure to radiation or radiomimetic chemical agents is not the only factor that plays a role in the tumorigenesis in AT.

There are also differences in malignancy patterns between AT patients and children with immunodeficiency. Moreover, the tumour spectrum in AT patients with and without immunological incompetence is similar, suggesting that cancer-proneness and immunodeficiency are also not simply causally related in AT.

2.2.4 Cutaneous manifestations

A characteristic feature of AT is the development of telangiectasia (small dilated bloodvessels). Such skin changes are common in healthy aged individuals, but in AT patients they already appear at an age of 2 to 8 years. They are most prominent on the conjunctivae of the eyes and across the butterfly area of the face. With increasing age telangiectasies may also occur on the rest of the face, the ears and dorsa of hands and feet (Reed et al., 1966). This skin pattern is suggestive for an involvement of sunexposure and has also been regarded as a manifestation of premature aging in AT. Other skin abnormalities are disturbances in pigmentation (e.g. café-au-lait spots), warts and eczema.

It is possible that the abnormal response of some cultured AT fibroblast strains to longwave UV-light (Paterson and Smith, 1979) is related to some of these clinical findings.

2.2.5 Developmental abnormalities

An apparent impairment of tissue differentiation is another characteristic of AT. Defective organ maturation has become evident from a number of observations. Besides the underdevelopment of thymus tissue mentioned above, there is also an almost consistent elevation in the plasma levels of alphafetoprotein, suggesting an abnormal maturation of the liver (Waldman and McIntyre, 1972; Richkind et al., 1982; Keller et al., 1978; Berkel et al., 1985; Simons and Hosking, 1974; Ohama and Ikuta, 1982). The level of carcinoembryonic antigen, another oncofetal protein produced by fetal gut, liver and pancreas, is also frequently elevated (Sugimoto

et al., 1978). In addition, there is ovarian agenesis (Bowden et al., 1963; Boder and Sedgwick, 1958; Miller and Chatten, 1967) with abnormally frequent dysgerminomata, a tumor of undifferentiated germ cells (Dunn et al., 1964; Spector et al., 1982). Finally, AT patients may produce a fetal type of collagen (McReynolds et al., 1976).

All these observations have led to the hypothesis that there is a defect in the interaction between two major germ lines during embryogenesis, i.e. the entoderm and the mesoderm (McFarlin et al., 1972; Waldmann and McIntyre, 1972; Sugimoto et al., 1982). It is believed that this interaction is a step in the development of thymic (Auerbach, 1960) and germinal tissues (Miller and Chatten, 1967) and other organs including gut and liver. This hypothesis may provide a framework to describe some of the clinical symptoms of the AT patients. However, the cellular characteristics of AT (radiosensitivity, chromosomal instability) are difficult to fit in this description.

2.3 Cytogenetics of AT

One of the characteristics of AT is chromosomal instability. Abnormal chromosomes in cells of AT patients were first reported by Hecht et al. (1966), and numerous studies confirming this observation have followed since then (reviewed by Harnden, 1973 and by Taylor, 1982). Spontaneous chromosomal aberrations are not evident in cells from every patient to the same extent. Consistently found is an elevated level of chromosome aberrations after exposure to ionizing radiation, in comparison to irradiated cells from normal individuals.

2.3.1 Spontaneous chromosomal aberrations

The occurrence of abnormal chromosomes in cells of AT patients can be due to an increased frequency of virtually all types of aberrations, including gaps, breaks, dicentrics, fragments, rearrangements and interchanges (e.g. Gropp and Flatz, 1967; German, 1972; Hecht et al., 1973; Bochkov et al., 1974; Pfeiffer, 1970; Cohen et al., 1973, 1975; Taylor et al., 1981). Many patients show high numbers of cells with stable chromosomal rearrangements (translocations and inversions)(Harnden, 1974; Oxford et al., 1975; McCaw et al., 1975; Aurias et al., 1980 and Taylor et al., 1981), and very often identical patterns are seen in many of these cells within

an individual patient ('clones'). The breakpoints of the translocations are highly non-random: translocations and inversions involving chromosomes 7 and 14 are the most frequent, with a specific involvement of the chromosome bands 14q12 and 14q32, 7p14 and 7q35 (Hecht et al., 1973; Bochkov et al., 1974; Lisker and Cobo, 1970; Oxford et al., 1975; Rary et al., 1975; McCaw et al., 1975; Nelson et al., 1975; Hayashi and Schmid 1975; Cohen et al., 1975; Hook et al., 1975; Al Saadi et al., 1980; Webb et al., 1977; Jean et al., 1979; Scheres et al., 1980; Aurias et al., 1980,1983; Aurias, 1981; O'Connor et al., 1982 and Taylor, 1982). It is interesting that the breakpoints involved in translocations that are occasionally observed in blood cells from normal individuals occur also often in these two chromosomes and the same bands appear to be the usual sites of rearrangement (Hecht et al., 1975; Beatty-De Sana, 1975; Welch and Lee, 1975; Aurias et al., 1980, 1982; Zech et al., 1978; Hecht and Kaiser-McCaw, 1982). This parallellism may be explained in two ways. Either the abnormality in AT reflects an enhanced rate of normal cytogenetic processes, also occurring in the absence of a defective AT gene (Hecht and Kaiser-McCaw, 1982), or, alternatively, the apparently normal individuals with these translocations are in fact AT heterozygotes (O'Connor et al., 1982). The frequency of AT heterozygotes may be high enough to account for this phenomenon (see section 2.7.2). Moreover, chromosomal instability and rearrangements involving chromosome 14 have also been observed in cells from a few heterozygotes that were investigated in this respect (Cohen et al., 1975; Oxford et al., 1975; Aurias et al., 1980; Kohn et al., 1982).

The specific involvement of chromosomes 7 and 14 may implicate the existence of hotspots for breakage. In this respect it is remarkable, that three of the four specific breakpoints may occur in the vicinity of genes involved in the immune response. The immunoglobulin heavy-chain gene is located on band 14q32 (Kirsch et al., 1982; Cox et al., 1982) and the α and β chains of the human T cell receptor were assigned to 14q11-q12 and 7q13-q36, respectively (Collins et al., 1985; Croce et al., 1985; Caccia et al., 1985). These genes can become rearranged in the course of cell maturation. The possibility exists, that the double strand breaks generated during the recombination events are processed improperly in AT (Fiorilli et al., 1985). If this is so, there must be a reason why preferential breakage is not observed at the site of the λ and κ light chain genes on chromosomes 22 and 2, though instances of such events have been described in

B cells (Kirsch et al., 1984). Alternatively, it is possible that rearrangements in these stretches of the genome confer some growth advantage to the cells, allowing them to accumulate. The altered immunological status of AT patients may promote selective outgrowth of cells with specific types of chromosomal changes. On the other hand, the preferential involvement of chromosomes 7 and 14 is also evident in cytogenetically abnormal AT fibroblasts (Cohen et al., 1973; Aurias et al., 1980) and it is hard to explain this on the basis of peculiarities of the heavy chain and T-cell receptor genes.

In contrast with cultured fibroblasts and stimulated lymphocytes of both T and B cell origin (O'Connor et al., 1982) increased spontaneous chromosome breakage was not observed in cells derived from bone marrow (Hecht et al., 1973; Cohen et al., 1975; Al Saadi et al., 1980) or in Epstein Barr virus-transformed B cells (Cohen et al., 1979; Kidson et al., 1982), even when these cells were obtained from patients having abnormal peripheral lymphocytes. While the information on the bone marrow cells comes from a few single cases only, the variability with the cell types may still indicate that the growth properties and/or requirements can differ between cells with and without aberrations. Selection may play a significant role in culture as well. For instance, in the fibroblast strain AT5BI, reported to contain cytologically abnormal clones in one study (Webb et al., 1977), no abnormalities were observed in our own laboratory (C.R. Bartram, pers. communication).

2.3.2 Radiation-induced chromosomal aberrations

Hypersensitivity of AT cells to ionizing radiation in terms of chromosome breakage was first reported by Higurashi and Conen (1973). Many reports have confirmed and extended this finding. Upon exposure to gamma-rays, X-rays, fast neutrons or tritium β-emissions there is an increased level of chromosomal aberrations in all cell types that were studied (Rary et al., 1974; Taylor et al., 1976; Taylor, 1978; Natarajan and Meijers, 1979; Littlefield et al., 1981; Natarajan et al., 1982; Gianelli et al., 1982; Taalman et al., 1982; Taylor, 1982; Bender, 1980; Zampetti-Bosseler and Scott, 1981; Nagasawa and Little, 1983).

The types of induced chromosomal damage are not the same in AT and normal cells. In the latter, exposure of cells in the G0 or G1 phase re-

sults in chromosome type aberrations (both chromatids involved) whereas irradiation in G2 produces chromatid type aberrations only. In AT cells chromatid type damage is observed after exposure in all three cell cycle phases (Taylor et al., 1976; Taylor, 1978; Natarajan and Meijers, 1979; Bender et al., 1985). This finding has been interpreted by these and other authors (Lehmann, 1977) as evidence for a defect in the repair of single strand DNA breaks. Breaks formed in G0/G1 would be sufficiently longlived in AT to be converted into double-strand breaks during DNA replication, which would cause chromosome breakage. This would fit in the classical hypothesis that chromosomal aberrations are essentially the result of double-strand DNA breaks (Evans and Scott, 1969; Kihlman, 1971; Bender et al., 1974). On the other hand it does not fit easily with biochemical studies, indicating normal rejoining of DNA breaks in AT (see section 2.5.2).

Recent studies of prematurely condensed chromosomes (PCC) in non-proliferating fibroblasts after radiation exposure indicate a time-dependent decrease in PCC-fragments that is more pronounced in normal cells than in AT cells (Cornforth and Bedford, 1985). These results suggest a defect in the processing (repair?) of potentially clastogenic damage in AT, and indicate that entry into the S-phase is not required for the production of chromosomal breaks after irradiation in the G1-phase.

Elevated levels of chromosome breakage were also found in AT cells after exposure to clastogenic agents such as bleomycin (Taylor et al., 1979; Kohn et al., 1982; Cohen et al., 1981; Shaham et al., 1983), streptonigrin (Taylor et al., 1983), neocarzinostatin and tallysomycin (Cohen and Simpson, 1983). Reactive radicals were shown to be involved in the action of all of these radiomimetic agents. Combined treatment with furocoumarins and long-wavelength UV light also caused increased chromosome breakage in one AT cell strain (Natarajan et al., 1981). The significance of this finding remains to be determined. The responses to an other cross-linking agent (Mitomycin C) and to an alkylating compound (MNNG) were normal in AT lymphoblastoid lines (Cohen and Simpson, 1983).

The frequencies of spontaneous sister chromatid exchanges (SCE), as well as those induced by X-rays, bleomycin, EMS, adriamycin or mitomycin C were the same in AT and normal cells (Chaganti et al., 1974; Galloway and Evans, 1975; Hayashi and Schmidt, 1975; Bartram et al., 1976; Hatcher et al., 1976; Galloway, 1977; Kohn et al., 1980; Kohn et al., 1982; Cohen and Simpson, 1982; Nagasawa and Little, 1983; Hook and Heddle, 1983). SCEs are

preferentially produced by agents that inhibit DNA replication by disturbing replication fork movement (Painter, 1980b). The fact that normal levels of SCEs are induced in AT by radiation and radiomimetic chemicals is compatible with the finding that DNA synthesis is not inhibited as strongly in AT as in normal cells (discussed in section 2.6.2).

2.3.3 The AT clastogenic factor

In the sera obtained from AT patients a factor was demonstrated causing chromosomal aberrations in cultured cells from normal individuals (Shaham et al., 1980). This 'clastogenic factor' appeared also to be present in culture medium conditioned by AT fibroblasts, but not by AT lymphoblastoid cell lines (Shaham et al., 1980; Cohen and Simpson, 1980b, 1982a). The factor proved to be a pronase and RNAse resistant heat labile molecule of a MW between 500 and 1000 Daltons (Shaham and Becker, 1981).

Low MW clastogenic factors were also detected in the sera of patients suffering from a variety of human disease, including systemic lupus erythematosus, rheumatoid arthritis, Crohn's iliocolitis and Bloom's syndrome (reviewed by Emerit, 1982). The in vitro action of these factors is suppressed in the presence of anti-oxidants such as CuZn-superoxide dismutase, suggesting that active oxygen species (like O_2^-) play a role in their formation. Active oxygen species are not only involved in the action of ionizing radiation, but also in that of chemicals like bleomycin, neocarzinostatin, adriamycin and tumor promoting phorbol esters (Nagasawa and Little, 1981; Emerit et al., 1983). They are also produced by normal cellular oxygen metabolism (Fridovitch, 1978). It has been suggested that the clastogenic factors may all be secondary phenomena in these disorders, but still contribute to some of the clinical characteristics (Emerit, 1982).

It is not known whether the clastogenic factor in AT resembles those found in other human disoders; data on its sensitivity to anti-oxidants were not reported. The cellular hypersensitivity in AT to radiation delivered under anoxic conditions (see section 2.4.1) argues against a primary defect in the metabolism of active oxygen species.

2.3.4 Chromosomes and cancer

In many types of human tumours the involvement of specific karyotypic abnormalities is evident (Sandberg, 1980). Well-known are chronic myeloid leukemia (CML) with the typical Philadelphia translocation t(9;22)(q34;q11) and Burkitt's lymphoma (BL) most often exhibiting a translocation between chromosomes 8 and 14 (q24;q32)(for reviews see de Klein and Hagemeijer, 1984; Nowell et al., 1984). Molecular analysis of these specific translocations at the DNA level has resulted in the isolation of the DNA sequences from the region of the chromosomal breakpoints. In CML it was found that a piece of chromosome 9 that is translocated to chromosome 22, contains a part of the proto-oncogene *c-abl*, the cellular homologue of the gene that confers oncogenic potential to abelson murine sarcoma virus. By this rearrangement, *c-abl* oncogene sequences are combined with the DNA of a gene located in the breakpoint region of chromosome 22. Transcription of this fusion gene results in a modified gene product that may play a role in the initiation or maintenance of the transformed state of the myeloid cell. In Burkitt's lymphoma cells another oncogene *c-myc* (homologous to a gene in avian myelocytomatosis virus), that is normally located on chromosome 8, is moved to a position on chromosome 14 in the immediate vicinity of the immunoglobulin heavy chain gene. This results in a truncated and activated version of the *c-myc* gene, that appears to become regulated by DNA sequences controlling immunoglobulin gene expression.

As mentioned above, the band 14q32 is often involved in translocations that occur in AT cells, but it is not known whether the AT-breakpoint is also in the immunoglobulin chain gene. IgA-deficient AT patients were reported to have normally rearranged heavy chain genes (Waldmann et al., 1983). However, these patients have not been studied cytogenetically. Another recently discovered oncogene called *tcl-1* (Croce et al., 1985) has also been localized in chromosome band 14q32 and thus may be involved as well.

Extensive studies of the chromosomes in the neoplastic cells of AT patients have not been carried out yet. The few available reports deal with tumors of lymphoreticular origin and indicate that the specific rearrangements involving chromosome 14, seen in the non-transformed blood cell clones, are very common in the malignant cells of leukemic AT patients as well (Kaiser-McCaw et al., 1975, 1978; Lampert, 1969; Harnden, 1977;

Levitt et al., 1978; Sparkes et al., 1980; Saxon et al., 1979, 1980; Bernstein et al., 1981). Kaiser-McCaw and Hecht (1982) reviewed six AT patients, two with chronic T-lymphocytic leukemia (CLL), two with acute T-cell leukemia (ALL), one with Burkitt's lymphoma and one with Hodgkin's disease (HD). In the CLL-patients the leukemic cells appeared to have arisen from a lymphocytic clone with a t(14;14)(q12;q32) already present before any clinical manifestations of the leukemia. In the ALL patients the leukemic cells did not contain a chromosome 14 abnormality, although the characteristic t(14;14) was already present in the PHA-stimulated lymphocytes before clinical onset of the cancer. A similar phenomenon was reported in a third AT patient with T-ALL (Wake et al., 1982). As expected, a characteristic t(8;14) was seen in the Burkitt case. Finally, the HD-cells were monosomic for chromosome 14 but otherwise normal. Such a 14^- variant clone was detected in peripheral blood 18 months before the clinical appearance of HD.

It has been speculated (Kaiser-McCaw et al., 1975) that a clone with an abnormality involving band 14q12 in AT patients may be considered as the precursor for neoplastic disease. This may be valid for certain types of leukemia (CLL), but cannot be generalized to explain the exhanced frequency of cancer in AT. Studies on more AT cancer patients and on other types of tumours will be necessary to identify specific abnormalities in the neoplastic cells. It will also be important to characterize the breakpoints involved in the rearrangements more extensively using cytogenetic and molecular techniques. Recently, Aurias et al. (1983) have described an AT patient, with a lymphocytic clone exhibiting a t(14;14) where one of the breakpoints was at q11.1-q11.2, clearly different from the site usually seen in AT patients.

Irrespective of whether or not in AT the most frequently observed chromosomal anomalies play a role in cancer susceptibility, the hypothesis that AT chromosomal instability may lead to cells with rearranged genes governing growth control (e.g. oncogenes) remains attractive. Whether such cells will develop into a full-blown malignancy may also be influenced by other factors, including the immunological status of the patient, the degree of maturation of the cell, or the presence of reactive radicals in the various organs (Hayashi and Asada, 1977; Pryor, 1982; Fahl et al., 1984).

2.4. Cellular hypersensitivity to mutagens

The cytotoxicity of DNA damaging agents is measured as the effect on the proliferative activity of cultured cells. The dose-dependent inactivation curves of colony-forming ability after exposure to radiation are negatively exponential and grossly in agreement with the target theory of radiation action (Lea, 1956; Chadwick and Leenhouts, 1973; Gilbert et al., 1980). Often, an initial shoulder is observed, but this can vary with the experimental conditions. In most cases survival curves obtained after treatment with chemical mutagens are negatively exponential as well, and the data are therefore analysed in a similar way.

2.4.1 Radiosensitivity

The clinical radiohypersensitivity of AT patients is also expressed at the cellular level. Hypersensitivity to ionizing radiation in cultured AT fibroblasts was discovered by Taylor et al. (1975). In subsequent studies this observation was confirmed with fibroblast strains and lymphoblastoid cell lines from at least 50 different AT patients (Friedberg et al., 1979; Lehmann, 1980) with no exception. Measurement of colony-forming ability of cultured AT cells was therefore recommended as a diagnostic aid in AT (Cox et al., 1978). The Do values (the dose equivalent to one lethal hit) were in the range of 0.40-0.75 Gy, implying that AT cells are 2-3 times more radiosensitive than cells from normal individuals.

The difference between normal and AT cells is dependent on the type of radiation. On the one hand, X-rays, γ-rays and cumulated exposure to ^{3}H- or ^{125}I-decays all yielded the same sensitivity ratios (Ritter, 1981). On the other hand, Cox et al. (1982) using accelerated ^{238}Pu α-particles found evidence for a dependence on the linear energy transfer (LET) of the radiation type. Upon increasing the LET the difference between AT and normal cells became less pronounced, a finding confirmed by Paterson et al. (1982) using high LET-neutrons and by Tobias et al. (1984) with accelerated neon and argon particles. Since these results were primarily due to a greater biological effectiveness of high-LET rays in the normal cells, it was suggested that high-LET radiation induces a higher amount of irreparable damage than low-LET radiation, which tends to mask the difference between AT and normal cells (Cox et al., 1982).

Irradiation with γ- or X-rays at atmospheric oxygen pressure results

in a damage spectrum that differs from that obtained under hypoxia. The amount of base damage is relatively little affected, but strand breaks are less frequent than after an aerobic exposure (Paterson and Setlow, 1972). In normal cells, aerobic irradiation is about 2.2 times more cytotoxic than anaerobic exposure. In AT cells this ratio is the same (Ritter et al., 1979) but at a lower survival level (Paterson et al., 1979; Paterson and Smith, 1979; Kinsella et al., 1982). This result was interpreted as evidence in favour of primary involvement of base damage in the hypersensitivity of AT cells (Paterson, 1978).

The response of AT cells to non-ionizing radiation is generally normal. This was clearly shown for UV-C light (254 nm)(Lehmann et al, 1977; Arlett, 1977; Weichselbaum et al., 1978; Paterson and Smith, 1979; Scudiero, 1980; Ikenaga et al., 1983) and heat (Raaphorst and Azzam, 1983). UV-B light of 313 nm was more cytotoxic to 2 out of 4 AT cell strains (Paterson and Smith, 1979; Smith and Paterson, 1980), but this observation could not be reproduced using the same cell strains (Arlett et al., 1982).

Taken together it is clear that AT cells show a consistent hypersensitivity to ionizing radiation, but a normal sensitivity to the non-ionizing part of the radiation spectrum.

2.4.2 Chemical mutagen sensitivity

After the first observation by Hoar and Sargent (1976) that AT cells are also hypersensitive to radiomimetic chemical agents, a large amount of data has accumulated on this subject. The available information is summarized in Table 1, and contains many conflicting results. An illustrative example is presented by the cell strain AT4BI. Arlett et al. (1982) found a normal sensitivity after exposure to the two alkylating agents methylmethanesulfonate (MMS) and methylnitronitrosoguanidine (MNNG). Scudiero (1980) reported hypersensitivity to MNNG only, whereas Paterson and Smith (1979) observed the reverse. Probably, different experimental conditions are the basis of these discrepancies, but the exact nature of the relevant factors is not clear. One of these is the use of different culture media, that vary in the concentration of components like nicotinamide, which is known to influence the cytotoxicity of some DNA damaging agents (Paterson et al., 1982; Jaspers et al., 1982a paper II)). Subtile differences in culture conditions may influence the DNA damage spectrum as well, which

Table 1. HYPERSENSITIVITY TO CHEMICAL MUTAGENS

Chemical Agent[a)]	Defective Strains vs Strains Tested	Sensitivity Ratios[b)]	Literature References
MMS	5/7	c)	Hoar and Sargent, 1976
	0/5	-	Scudiero, 1980
	3/4	1.5	Paterson and Smith, 1979
	0/5	-	Arlett and Harcourt, 1978
			Arlett et al., 1982
	2/2	1.3	Ikenaga et al., 1983
	2/3	1.3-1.6	Barfknecht and Little, 1982
MNNG	2/3	2	Paterson and Smith, 1979
	5/5	1.8	Scudiero, 1980
	0/3	-	Jaspers et al.,1982a (Paper II)
	0/1	-	Arlett et al., 1982
	0/2	-	Ikenaga et al., 1983
	0/3	-	Barfknecht and Little, 1982
MNU	2/2	1.5	Arlett et al, 1982
			Teo and Arlett, 1982
ENU	3/3	1.2-3	Paterson and Smith, 1979
	0/2	-	Arlett et al., 1982
EMS	0/1	-	Arlett et al., 1982
	3/3	1.8	Barfknecht and Little, 1982
MMC	4/6	c)	Hoar and Sargent, 1976
	0/3	-	Arlett and Harcourt, 1978
	0/3	-	Jaspers et al., 1982a (Paper II)
	0/2	-	Ikenaga et al., 1983
NCS	8/8	2.5	Shiloh et al., 1982a,b; 1983a
	3/3	c)	Tatsumi et al., 1981
	2/2	2-3	Babilon et al., 1985
Bleomycin	2/2	1.5	Lehmann and Stevens, 1979
	3/3	3	Taylor et al., 1979
	4/4	c)	Cohen et al., 1981
	3/3	2-4	Paterson et al., 1982
	3/3	2.5-3	Jaspers, unpublished
	2/2	4	Ikenaga et al., 1983
4NQO	2/3	2	Paterson and Smith, 1980
	0/2	d)	Arlett et al., 1982
	1/2	3	Ikenaga et al., 1983
	2/3	2.3	Barfknecht and Little, 1982
ActD	8/11	c)	Hoar and Sargent, 1976

a) Agents: MMS = methymethanesulphonate; MNNG = N-methyl-N'-nitro-N-nitrosoguanidine; MNU = methylnitrosourea; MMC = mitomycin C; 4NQO = 4-nitroquinoline-1-oxide; NCS = neocarzinostatin; ActD = Actinomycin D.

b) Sensitivity ratios AT over normal, based on Do or D10 values given by the authors or derived from their data.

c) Ratios could not be determined from the data.

d) Large variability between normal cell strains.

could play a role after exposure to 4NQO (Jaspers et al., 1982a (paper II)). Finally, since the cloning efficiency may influence the response to mutagens (Beverstock and Simons, 1982), it may be of importance that AT cells were reported to require special serum batches for optimal growth (Shiloh et al., 1982c).

Discrepancies have arisen in the results obtained after exposure to a crosslinking agent (MMC), alkylating agents (MMS, MNNG, EMS, ENU) and 4NQO. The response of different AT cell strains to these chemicals varied and in most cases the observed hypersensitivity ratios did not exceed the value of 2. This contrasts with the response to ionizing radiation, being consistent in AT, with a sensitivity ratio of 2-3.

Hypersensitivity of AT cells to bleomycin, neocarzinostatin and streptonigrin is a consistent finding in a number of studies. The action of these three agents is mediated by reactive radicals. In addition, Shiloh et al. (1983c, 1985) have found hypersensitivity to a number of other agents of this type, including adriamycine, H_2O_2, carminomycine and tumor promoting phorbolester TPA. This suggests that the pertinent DNA lesion is a special type of strand break, that needs some enzymatic processing in order to be closed. Some agents that produce relatively low levels of active radicals, such as near UV, Mitomycin C and actinomycin D (Lown et al., 1976; Parshad et al., 1980) can cause a slight hypersensitivity in AT cells, which may be difficult to detect because of other chemical reactions with the DNA. Other reagents of this type, e.g. cadmium chloride (Ochi et al., 1983) remain to be tested in AT.

2.4.3 Potential lethal damage recovery

Potential lethal damage (PLD) is defined as DNA damage that becomes lethal to the cell, unless it is processed (repaired or modified) into a non-lethal entity. The concept of PLD thus implicates the existence of two processes competing for the DNA lesion: one of these leads to cell recovery and the other is an event that fixes the DNA damage into a structure that causes cell death. DNA replication is generally considered to be a process of the second type, since it may convert DNA lesions into stable genetic changes. On the assumption that DNA replication is the earliest critical event in converting PLD into lethal damage, experiments can be designed to selectively measure PLD recovery under conditions of non-proliferation.

With cultured fibroblasts these conditions are achieved by serum-deprivation or high cell densitiy, resulting in an arrest of the cells in the G1 phase of the cycle.

In case of irradiation with UV light, temporary G1-arrest can allow cultured cells to recover completely, if its duration is sufficiently long (see Fig.2). Since PLD-recovery was absent in excision-repair defective xeroderma pigmentosum cells (Maher et al., 1979; Yang et al., 1980; Simons 1979) it was concluded that here PLD recovery was caused by excision repair.

The same experimental design was applied to the study of PLD recovery after exposure to ionizing radiation and neocarzinostatin. In this system AT cells did not show recovery and by analogy with XP this was interpreted as evidence in favour of a DNA repair defect (Weichselbaum et al., 1980; Cox et al., 1982; Shiloh et al., 1983; Pritchard et al., 1982; Arlett and Priestly, 1983, 1984).

However, the recovery pattern in the normal cells is very different from that observed after UV exposure: the increase in cellular survival is limited and lasts only about six hours, irrespective of the damaging dose (Cox et al., 1982; Shiloh et al., 1983)(see Fig. 2). This implies that the assumption of DNA replication being the critical 'fixing' event is incorrect. Apparently G1-arrest does not fully protect against killing. Data showing that inhibition of DNA replication for the first 24 hrs after γ-ray exposure does not influence cell survival (Smith and Paterson, 1983) support this idea (see also O'Neill and Flint, 1985).

Therefore, the results of the PLD-experiments are in line with at least two alternative interpretations: firstly, AT cells may be defective in DNA repair and secondly, there is some abnormality in the critical event in AT. The recovery data suggest that in normal cells there is a radiation-induced response that postpones the critical fixing event for some hours, allowing partial repair of the DNA damage. In AT cells this response appears to be absent. This last interpretation seems more in line with other findings on cell-cycle progression in AT.

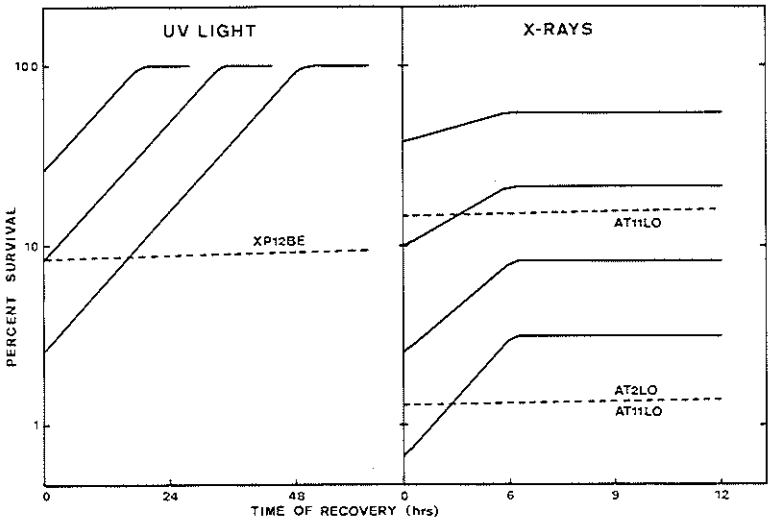

Fig. 2. PLD-recovery in human fibroblasts after exposure to UV-light and ionizing radiation. Continuous lines represent normal cells exposed to different radiation doses, broken lines represent radiation-sensitive cell strains, either XP or AT. These schematic representations are constructed on the basis of data obtained by Cox et al. (1982), Maher et al. (1979) and Yang et al. (1980).

2.5 DNA Repair Characteristics and Mutagenesis

The most direct measurement of DNA repair is monitoring the time-dependent disappearance of defined DNA lesions. Since only a limited number of DNA lesions has been characterized one has often to rely on less specific procedures, such as the incorporation of labelled precursors due to repair DNA synthesis. Finally, there is the possibility of assaying the activity of enzymes known to be involved in DNA repair processes. All three options have been chosen in the various studies of repair in AT.

2.5.1 Radiation-induced DNA damage

The removal of several types of DNA lesions induced by ionizing radiation was investigated in AT cells. Quantitatively the most prominent lesions that are produced, the single and double strand DNA breaks, were as-

sayed using techniques with varying sensitivity, allowing the use of a wide dose range between 1 and 500 Gy. Normal rates of repair of DNA breaks were found using alkaline sucrose gradient centrifugation (Vincent et al., 1975; Taylor et al., 1975; Sheridan and Huang, 1977; Lehmann and Stevens, 1977; Kantor et al., 1980), S1 nuclease digestion (Sheridan and Huang, 1977, 1979) or nucleoid sedimentation (Lavin and Davidson, 1980). Measured with the sensitive DNA elution techniques (Bradley and Kohn, 1979; Kohn, 1981; the repair of single-strand DNA breaks turned out to be either normal (Fornace and Little, 1980; Hariharan et al., 1981) or very slightly affected in only some AT fibroblast strains (Van der Schans et al., 1980; Jaspers et al., 1982a(Paper II)). Similar results were obtained with the double-strand breaks (Van der Schans et al., 1981; Jaspers et al., 1982a (paper II); Cockerelle et al., 1981).

Other defined types of DNA lesions that were repaired with normal rates in AT cells include 5,6-dihydroxydihydrothymine (Remsen and Cerutti, 1977) and apurinic/apyrimidinic sites (Sheridan and Huang, 1978). Finally, there exists an uncharacterized type of DNA lesion, that can be recognized by a crude endonucleolytic enzyme preparation from Micrococcus luteus (Paterson et al., 1978). In some AT cell strains these socalled 'endonuclease-sensitive sites' were removed less efficiently than in normal cells, after exposure to very high doses of ionizing radiation (>200 Gy)(Paterson et al., 1976, 1977, 1979). However, in well-controlled experiments these observations could not be reproduced in another laboratory (Van der Schans et al., 1982).

Ionizing radiation-induced repair DNA synthesis has been studied by many investigators. Since small patches of DNA are displaced in excision of ionizing radiation-induced DNA damage, high radiation doses must be administered to obtain significant signal-to-noise ratios. While a number of authors reported normal responses (Ford et al., 1981; Henderson and Basilio, 1983; Shiloh et al., 1980), decreased levels of repair synthesis were observed in some AT cell strains in several other studies (Paterson et al., 1976, 1977, 1979; Lavin and Kidson, 1978; Vincent et al., 1980; Van der Schans et al., 1980; Smith and Paterson, 1981; Chen et al., 1978; Taalman et al., 1982). These observations have resulted in the postulation of two distinct classes of AT cell strains, called excision-repair proficient (exr^+) and deficient (exr^-). The exr^- phenotype was not observed in lymphoblastoid cells.

In the case of irradiation with UV light, all repair responses appeared to be the same as in normal cells. For instance, thymine dimers were removed at normal rates (Paterson et al., 1976, 1977; Kantor et al., 1980) and the levels of repair DNA synthesis were normal (Kraemer, 1977; Scudiero, 1978; Jaspers and Bootsma, 1982b (paper V); Ahmed and Setlow, 1978; Lehmann and Stevens, 1980).

2.5.2 Chemically induced DNA damage

The early finding of Hoar and Sargent (1976) that AT cells were sensitive to some chemical mutagens has prompted several investigators to study the repair of DNA damage in AT after exposure to such compounds. The literature data, summarized in Table 2, indicate normal repair rates of 'UV-type' DNA adducts (such as those induced by AAF or 4NQO), DNA interstrand crosslinks caused by mitomycin C, and DNA strand breaks caused by bleomycin or neocarzinostatin. With respect to the DNA alkylating agents some controversy has arisen in the literature. Whereas all reports agree on a normal capacity to repair damage induced by MMS, conflicting data were obtained with methylnitronitrosoguanidine (MNNG). Scudiero (1980) observed defective repair replication after exposure with MNNG in the exr^- AT cell strains and a normal response in the exr^+ cells. These results could not be reproduced by others using the same cell strains (Lehmann, 1982; Lehmann et al., 1982) or other strains (Shiloh et al., 1980). Moreover, AT cells appeared to be proficient in the removal of the six most prominent DNA lesions caused by MNNG (Shiloh and Becker, 1982, 1983) or methylnitrosourea (Medcalf and Lawley, 1981).

A similar confusing situation exists with respect to the agent 4NQO. In two out of four AT strains the alkali-stable ('X-ray-like') DNA lesion was repaired less efficiently than in normal cells (Smith and Paterson, 1980), but this could not be reproduced by others using the same AT cells (Lehmann et al., 1982).

What causes these discrepancies is not clear. It seems probable that they have the same basis as the inconsistencies in cellular survival after exposure to these agents.

Table 2. REPAIR OF CHEMICALLY INDUCED DNA DAMAGE IN AT CELLS

Chem. Agent[a]	Parameter Investigated	Techniques Used[b]	Defective Strains /Strains Tested	Literature References
AAF	Adduct removal	CI	0/1	Amacher and Lieberman,1977
	Repair synth.	AUT,BP	0/1	Ahmed and Setlow, 1978 Ahmed, 1980
BLM	SingStr.Breaks	ASG	0/1	Hurt et al., 1983
	SingStr.Breaks	ASG		Lehmann and Stevens, 1979
	SingStr.Breaks	AlkEl	0/2[c]	Fornace and Little, 1980
	SingStr Breaks	AlkEl	2/3[c]	Jaspers and Regulski, unp.
	DoubStr.Breaks	NeuEl	0/4	Van der Schans et al., 1982
	SingStr.Breaks	Nucl.Sed.	0/2	Morris et al., 1983
MMC	Repair Synth.	BNDC	0/6	Shiloh et al., 1980
	Repair Synth.	LSC	0/1	Lehmann and Stevens, 1980
MMS	Repair Synth.	LSC	0/2	Lehmann and Stevens, 1980
	Repair Synth.	BNDC	0/6	Scudiero, 1980
	Repair Synth.	BDG	0/6	Shiloh et al., 1980
	Repair Synth.	BP	0/1	Ahmed, 1980
EMS	Repair Synth.	BP	0/1	Ahmed, 1980
MNU	Adduct Removal	CI	0/1	Medcalf and Lawley, 1981
MNNG	Repair Synth.	BDG	0/1	Henderson and Ribecky, 1980
	Repair Synth.	BNDC	4/6	Scudiero, 1980
	Repair Synth.	LSC, BDG, BNDC	0/2[d]	Lehmann, 1982
	Repair Synth.	BNDC	0/6	Shiloh et al., 1980 Shiloh and Becker, 1981
	Adduct Removal	CI	0/6	Shiloh and Becker, 1982 Shiloh et al., 1983
4NQO	Adduct Removal	ASG	2/4[e]	Smith and Paterson, 1980
	Repair Synth.	AUT	0/1[e]	Ahmed, 1980
	Repair Synth.	AUT	0/1[e]	Jaspers, unp.
	Adduct Removal	ASG	0/1[e]	Lehmann et al., 1982
NCS	Repair Synth.	BNDC	0/4	Shiloh et al., 1982

a) AAF, N-acetoxy-2-acetylaminofluorene; BLM, bleomycine; MMC, mitomycin C; MMS, methylmethanesulphonate; EMS, ethylmethaneulphonate; MNU, N-methyl-N-nitrosourea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; 4NQO, 4-nitroquinoline-1-oxide; NCS, neocarzinostatin.

b) CI, chromatografic identification; AUT, autoradiography; BP, BUdR-photolysis; ASG, alkaline sucrose gradient sedimentation; AlkEl, alkaline elution; NeuEl, neutral elution; BNDC, BND-cellulose chromatography; LSC, liquid scintillation counting of non-proliferating cell cultures; BDG, buoyant density centrifugation: nucl.sed., nucleoid sedimentation.

c) Difference only very slight.

d) Cell strains found defective by Scudiero.

e) Cell strain found defective by Smith and Paterson.

2.5.3 Enzymology of DNA repair

All enzyme activities believed to play a role in DNA excision repair that were tested were found to be normal in AT cells. These include uracil-DNA-glycosylase (Kuhnlein et al., 1978), apurinic endonuclease (Inoue et al., 1979, 1981; Moses and Beaudet, 1978), the three DNA polymerases α, β and γ (Bertazzoni et al., 1978). Also superoxide dismutase and catalase activity, two enzymes that protect the cell against damage were normal (Sheridan and Huang, 1979; Abeliovitch and Cohen, 1978; Brown and Harnden, 1978). Glutathion-levels were also normal (Kinsella et al., 1982).

In bacteria evidence was found for a 'cleaning exonuclease' that modifies radiation-induced strand breaks in such a way, that they can be used as starting points for the action of DNA polymerase I (Inoue and Kada, 1977). The assay for this 'primer activating activity' was also applied to human cell extracts and it was found to be considerably reduced in AT fibroblasts (Inoue et al., 1977, 1982). From these data it was postulated that AT cells may be defective in the repair of some class of 'dirty' DNA breaks. A comparable enzyme activity, but at a much lower level, was demonstrated in human extracts by Edwards et al. (1980). Also here a reduced activity was seen in AT cells, but other have failed to reproduce these results (Lehmann, 1982). The enzyme activity has been demonstrated in extracts of human cells using exogenous purified DNA as a substrate. The exact biological role of it in the living cell is still unclear.

2.5.4 Mutagenesis

In xeroderma pigmentosum cells a reduced ability to remove thymine dimers is correlated with an enhanced frequency of mutations induced by UV light (Maher and McCormick, 1976). Data on the mutagenicity of ionizing radiation in AT cells were reported by three laboratories. In contrast to XP, AT cells were found to be either hypomutable by γ-rays (Arlett and Harcourt, 1978) or equally mutable as normal cells (Simons, 1982; Tatsumi and Takebe, 1985). Spontaneous mutation frequencies in AT cells were normal in these studies.

2.5.5 *DNA repair defect in AT?*

Summarizing the data from the previous sections it appears that unequivocal evidence for a DNA repair defect in AT cells is lacking. In those cases where apparent abnormalities were observed, they were not consistent in AT. This was especially clear with the repair DNA synthesis induced by ionizing radiation. The abnormalities were confined to a minor group of AT patients and were only detectable in fibroblasts.

All studies involving radiation-induced repair DNA synthesis were performed with high doses of ionizing radiation. Since the dose ranges are generally two orders of magnitude higher that those used to study chromosome breakage, cell survival, mutagenesis or cell cycle progression, the biological relevance of these repair data remains questionable. In this respect it may be of interest that Shiloh et al. (1980) observed defective repair synthesis in cells from an apparently normal individual, which was correlated with an altered induction of ornithine decarboxylase (another high-dose phenomenon, BenHur et al., 1981), whereas cell survival was normal.

In another study (Jaspers and Bootsma, 1982b (paper V)) it was shown, that high doses of X-rays affect the rate of UV-induced repair synthesis in AT cells, but not in normal cells. After exposure to 200 Gy UV-UDS was slightly stimulated in two exr^+ cell strains but inhibited to 65% in two exr^- AT cells strains. First of all these data provide strong evidence for different molecular defects in the two classes of AT, but at the same time suggest that high radiation-doses can selectively affect repair processes in AT that are not directly related to the X-ray response. It may be that decreased levels of repair DNA synthesis induced by ionizing radiation in exr^- AT cells are rather a consequence of the AT radiosensitivity than the cause of it.

Taken together, all these data indicate, that DNA repair functions in a normal way in AT cells. This idea is supported by results from studies on the effects of radiation on cell-cycle progression, discussed in the following sections.

2.6 Cell Cycle Progression

2.6.1 Growth of AT cells in culture

Cultured AT cells have a reputation of sluggish growth and low cloning efficiency (Harnden, 1974; Hoar, 1975; Elmore and Swift, 1976; Ros, 1975), but these parameters vary with the cell strains and culture conditions used. AT cells share this property with cells from patients with Bloom's syndrome or Fanconi's anemia, so this may well be secondary to their intrinsic chromosomal instability.

In one laboratory AT lymphoblastoid cell lines had significantly longer doubling times than normal cells, which was mainly accounted for by a lengthening of the S-phase (Cohen and Simpson, 1980a). In contrast, such an abnormality was not observed with AT lymphoblasts in two other studies (Kohn et al., 1982; Imray and Kidson, 1983). Murnane and Painter (1982) found evidence for an increased duration of the S-phase in AT fibroblasts using cell fusion techniques (discussed in section 2.7.1), but in experiments carried out in our laboratory normal doubling times and S-phase periods were observed in two of the same fibroblast strains (results see Table 3).

An explanation for these different observations may be offered by the results from a more systematic study of this subject by Shiloh et al. (1982c). They, as well as others (Thompson and Holliday, 1983) noted a slightly decreased lifespan, which could be related to the progeric skin changes found in AT patients. In addition, AT fibroblasts had a greater requirement for epidermal growth factor (EGF) and fibroblast growth factor than normal cells. This made AT cells more susceptible to poor serum-batches. The authors postulated that there is an abnormality in the processing of these factors by AT cells in vitro. Since the abnormal response to ionizing radiation is consistently observed in AT, independent of cell culture conditions, it appears that slow growth in culture is probably nor directly related to AT radiosensitivity. On the other hand, the growth factor requirements of AT cells may play a role in other features of the syndrome. In this respect it is interesting that the gene coding for the EGF receptor (the *erbB* proto-oncogene) is located on chromosome 7p14-7p11 (Shimizu et al., 1980; Meera Khan and Smith, 1984), a region with preferential breakage in AT (Aurias et al., 1980). One could speculate, that, because of the special growth factor requirements changes in the expression of this gene (e.g.

caused by chromosomal rearrangement) provide selective advantage for AT cells, which could explain the frequent involvement of this chromosomal region in the cytogenetically abnormal clones of AT patients.

Table 3. CELL CYCLE PARAMETERS OF NORMAL AND AT FIBROBLASTS

Cell Strain	Passage Number	Apparent Doubling Time (h)	Labelling Index (%)	S-Phase Duration
AT4BI	16	31.8	29.5	9.4
AT5BI	20	34.7	29.9	10.4
C5RO	17	38.0	24.0	9.1
C7RO	18	28.2	33.6	9.5

Legends: Cells from exponentially growing cultures were trypsinized and seeded at a density of 1250 cells/cm^2 onto plastic petridishes for culture in Ham's F10 medium supplemented with 15% fetal bovine serum and 20 mM Hepes pH 7.4. At every 24 h interval 50% of the medium was replaced. For four days cells were harvested at different times and their number was estimated by the DNA content of the dishes using Hoechst-33258 fluorescence spectrometry (Labarca and Paigen, 1980). Apparent doubling times were calculated from the slopes of the semilogaritmic plots of cell number against culture time, obtained by least-square fitting. Parallel cultures were pulse-labelled with ^{3}H-thymidine at 40 hrs after seeding and processed for autoradiography. Labelling indices were determined by counting 1000 cells in duplicate cultures. The duration of the S-phase was approximated by the product of the apparent doubling time and the labelling index. C5RO and C7RO are normal cell strains, AT4BI and AT5BI are exr$^+$ AT cell strains.

2.6.2. DNA synthesis in damaged AT cells

The ionizing radiation-induced delay in cell cycle progression occurs during all phases of the cell cycle, but the G2 phase is the most sensitive. In this phase the block has the longest duration, whereas that in G1 or S is more transient. A block or slowdown in the progression through the S-phase is simply measured by the overall rate of DNA replication. The effect of DNA damage on the rate of DNA synthesis is the subject of this section.

Effects on DNA synthesis are observed after radiation doses that are in the range of doses that produce moderate clastogenic and cytotoxic effects. On the hypothesis that AT cells are defective in DNA repair, one would predict that unrepaired damage will persist in the DNA, causing a

greater or more persistent inhibition of DNA replication. This expectation is based on the behaviour of UV-irradiated xeroderma pigmentosum cells (Rudé and Friedberg, 1977). In contrast to this prediction, the rate of DNA synthesis in AT cells is inhibited to a lesser extent than in normal cells (see Fig. 3A). This discovery was made independently in four different laboratories (Houldsworth and Lavin, 1980; Edwards and Taylor, 1980; de Wit et al., 1981 (paper I), Painter and Young, 1980) and confirmed by many groups later on (for references see Table 4). The diminished inhibition was not caused by a premature entry into the S-phase (Jaspers and Bootsma, 1982a (paper IV), Imray and Kidson, 1983; Ford et al., 1983). The phenomenon was observed in all AT cell types studied, including fibroblasts, lymphoblastoid cell lines (B-lymphocytes) and peripheral blood lymphocytes stimulated by phytohaemaglutinin (T-cells) or pokeweed mitogen (B-cells). The socalled 'exr$^-$' and 'exr$^+$' AT cell strains showed the same response (de Wit et al., 1981 (paper I); Jaspers et al., 1982a (paper II)). After high doses of X-rays, those used to measure the excision repair phenotype, the difference between AT and normal cells was not detectable (data see Fig. 3B). Thus the abnormality in DNA replication correlates well

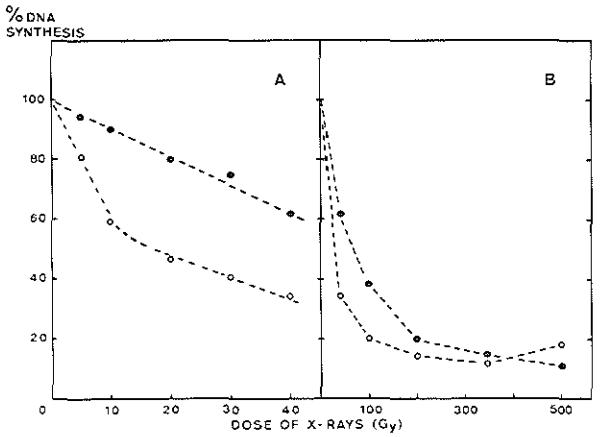

Fig. 3. Inhibition of DNA synthesis by X-rays.
Fibroblasts prelabelled with ^{14}C-thymidine were irradiated with different doses of X-rays, cultured for 60 min in unlabelled medium and for 60 min. in presence of ^{3}H-thymidine. The rate of DNA synthesis was estimated by the $^{3}H/^{14}C$ ratios in acid-precipitable fractions of the harvested cells. C7RO is a normal cell strain, AT3BI is an exr$^-$ AT cell strain. panel A: low doses; panel B: high doses of X-rays. Open circles, C7RO; closed circles, AT3BI.

Table 4. INHIBITION OF DNA SYNTHESIS IN AT BY IONIZING RADIATION

Type of Radiation	Doses (Gy)	Cell Type[a]	Number of Strains	Response Found[a]	Literature References
Gamma-rays	<10	LCL	4	D	Houldsworth and Lavin, 1980
(aerobic)					Ford and Lavin, 1981
	<10	LCL	2	D,N[b]	Edwards and Taylor, 1980
	<15	FIBR	2	D,N	Smith and Paterson, 1980
	<40	FIBR	7	D	Lehmann et al.,1982; p.c. Jaspers et al., 1982c
	400	FIBR	3	N	Smith and Paterson; p.c.
X-rays	<40	FIBR	>20	D	De Wit et al., 1981 (paper I)
(aerobic)					Jaspers et al.,1982a,b,c, (Paper II); unp.
	<50	FIBR	6	D	Painter and Young, 1980; 1982; Painter, 1981
	500	FIBR	2	N	Jaspers, this thesis
	<40	PHAL	6	D	Jaspers et al., 1981b (Paper III); unp.
	<40	PWML	1	D	Jaspers, unp.
	<25	FIBR	2	D	Shiloh et al., 1982a,b
	<8	FIBR	1	D	Edwards and Kaufmann, 1982
	<8	LCL	6	D	Henderson and Basilio, 1983
(anaerobic)	<40	FIBR	4	D	Jaspers et al., 1982a (Paper II)

a) Abbreviations and symbols: LCL, lymphoblastoid cell lines; FIBR, fibroblasts; PHAL, phytohaemagglutinin-stimulated lymphocytes; PWML, pokeweed mitogen-stimulated lymphocytes; D, diminished inhibition of DNA synthesis; N = Normal inhibition; p.c., personal communication; unp., unpublished data.

b) Normal response of AT cells could not be reproduced by several other investigators.

with the other consistent cullular features of AT i.e., chromosome breakage and lethality induced by X-rays. This correlation is further substantiated by studies with a variety of other DNA damaging agents (for reference see Table 5). The relative rate of DNA synthesis was normal after exposure to UV-C and UV-B light, alkylating agents, mitomycin C, actinomycin D and 4-Nitroquinoline-1-oxides, all agents resulting in normal levels of cell killing in AT. Bleomycin, neocarzinostatin, tallysomycin and streptonigrin cause both increased lethality and dimished inhibition of DNA synthesis in

Table 5. INHIBITION OF DNA SYNTHESIS IN AT BY MUTAGENIC AGENTS

Agent[a]	Max. Dose	Cell Type	No. of Strains	Response Found	Literature References
UV-C (J/m^2)	23	FIBR	4	N	De Wit et al., 1981 (paper I) Jaspers er al., 1982d
UV-B (KJ/m^2)	1.2	FIBR	2	N	Jaspers, unp.
UV-A (KJ/m^2)	500	FIBR	2	D[d]	Jaspers, unp.
Bleomycin (µg/ml)	5	LCL	3	D	Cohen and Simpson, 1983
	200	FIBR	1	D	Cramer and Painter, 1981
	40	FIBR	4	D	Jaspers et al, 1982a (paper II)
	40	PHAL	1	D	Jaspers, unp.
	400	LCL	3	D	Edwards et al., 1982
	500	FIBR	3	D	Lehmann et al., 1982
	200	FIBR	2	D	Shiloh et al., 1982a,b
	50	LCL	2	D	Morris et al., 1983
	50	LCL	3	D	Cohen and Simpson, 1982b
NCS (µg/ml)	10	FIBR	4	D	Jaspers et al., 1982c; unp.
	1	FIBR	4	D	Shiloh et al., 1982a,b
	0.15	FIBR	1	D	Povirk and Goldberg, 1982
	10	LCL	3	D	Cohen and Simpson, 1983
	0.08	LCL	1	D	Babilon et al., 1985
MMC (µM)	40	FIBR	3	N	Jaspers et al., 1982a,c (Paper II)
	3	LCL	3	N	Cohen and Simpson, 1982a, 1983
MNNG (µM)	6500	FIBR	6	N	Scudiero, 1980
	17	LCL	1	N	Henderson and Ribecki, 1980
	25	FIBR	4	N	Jaspers et al., 1982a (Paper II)
	100	FIBR	1	N	Cramer and Painter, 1981
	7	LCL	3	N	Cohen and Simpson, 1983
MMS (mM)	1	FIBR	4	N	Jaspers et al., 1982a (Paper II)
DMS (mM)	0.4	FIBR	2	N	Jaspers et al., 1982c
4NQO (µM)	4	FIBR	4	N	Jaspers et al., 1982a (Paper II)
	10	FIBR	2	N	Smith and Paterson, 1980
3Me4NQO[b] (µM)	100	FIBR	2	N	Jaspers et al., 1982c
BUV	c)	FIBR	2	N	Jaspers et al., 1982c
ActD (µM)	5	FIBR	1	N	Cramer and Painter, 1981
Streptonigrin		FIBR	2	D	Taylor et al., 1983
Tallysomycin (µg/ml)	50	LCL	3	D	Cohen and Simpson, 1983

a) Abbreviations and symbols the same as in Tables 1 and 4.
b) 3-Methyl-4NQO induces an alkali-labile type of DNA damage only.
c) Irradiation of DNA substituted with 5-bromo-2'-deoxyuridine by ultraviolet light of 313 nm produces DNA strand breakage.
d) Slight, but significant difference with normal cells.

AT. UV-A light (>340 nm) also had a slight differential effect on AT fibroblasts, which is consistent with its radical-inducing properties.

The steep component is absent in the inhibition curve of AT cells (see Fig.3A) which suggests that the initiation of new replicons is not affected by X-rays. Molecular studies have indicated a failure in the suppression of both replicon initiation and chain elongation (Painter and Young, 1980; Painter, 1981; Ford and Lavin, 1981; Edwards and Taylor, 1982). These data, all obtained with alkaline gradient centrifugation, were confirmed with DNA fiber autoradiography (Ockey, 1982). The rate of chain elongation in AT cells proved to be the most resistant to radiation, being almost unaffected by doses up to 50 Gy. This implies, that the residual inhibition still present in AT cells is caused by failure to initiate replicons, with a dose-response that is similar to the shallow component of normal inhibition curves. Therefore, it was suggested by Painter (1985) that in irradiated AT cells the coordinate replicon initiation must be disturbed, as all replicons seem to behave as separate targets. If this is so, it is likely that an alteration of chromatin structure is involved in the abnormal inhibition of DNA replication of AT cells.

The fact, that radioresistant DNA synthesis is a consistent property of AT cells, may suggest that the primary defect of AT cells is in the regulation of DNA replication. However, there are strong arguments against this. Firstly, recovery from radiation-induced damage is abnormally slow in AT cells even in the absence of S-phase dependent DNA synthesis, with respect to cell killing as well as chromosome breakage (potential lethal and clastogenic damage recovery, see secs. 2.3.2 and 2.4.3). Secondly, the frequency of chromosomal aberrations in AT is enhanced after exposure of the cells in the G2 phase of the cell cycle (Taylor, 1978; Natarajan and Meyers, 1979). It follows, that an abnormal rate of DNA synthesis after radiation exposure is one of the secondary effects of the genetic defect in AT.

2.6.3 Mitotic delay

Exposure to ionizing radiation in any phase of the cell cycle results in G2-arrest. The duration of this arrest, called mitotic delay or division delay is dose-dependent. In the G2-phase there appears to exist a 'point of no return', after which irradiation does not result in mitotic delay. The

timing of this point is also dependent on the radiation dose (Dewey and Highfield, 1976; Tomasovic and Dewey, 1978). The relationship between division delay and other biological endpoints such as cell death, mutagenesis and chromosome aberrations is not clear (for a discussion, see eg. Scott and Zampetti-Bosseler, 1980; Lucke-Huhle, 1982).

In the previous section it was mentioned that irradiated AT cells proceed faster through the S-phase than normal cells. This is compatible with the observation that AT fibroblasts also suffer less mitotic delay after radiation exposure (Zampetti-Bosseler and Scott, 1981). It is not completely certain whether this can be attributed to an effect on the S-phase only or on both G2 and S. The curves describing the fraction of labelled mitoses in AT fibroblasts were interpreted as evidence for a less pronounced G2-arrest but this interpretation is only correct if the G2-block is reversible. Results obtained with fluorescence activated cell sorting (Ford et al., 1984) suggest that in a fraction of the irradiated AT cells the G2-block may be permanent; however this seems not enough to exclude a diminished G2-arrest in AT cells.

With cultured human fibroblasts there is also a radiation-dose dependent delay in progression from G1 to S. In some cells this block may be irreversible (Little and Nagasawa, 1983). In AT fibroblasts neither a G1 block nor a G1-delay was induced by 1 to 4 Gy of X-rays. Virtually all of these cells entered into the S-phase without delay despite high lethality of these radiation doses for AT cells (Little and Nagasawa, 1985). Similar observations were made on bleomycin-treated AT lymphoblasts (Imray and Kidson, 1983).

It appears that the radiation-induced delay in cell-cycle progression that occurs in all cell cycle phases of normal cells, is generally less pronounced in AT cells. This suggests, that some common enzymatic functions control the delay in the different cell-cycle phases. In this respect there is a need of data on the expression of genes that are involved in cell cycle control in irradiated and unirradiated AT cells. A recent study of the gene c-myc that may have such a function, has shown that this is normally expressed in AT lymphoblastoid cells (Lavin et al., 1984).

2.6.4 Chromatin anomalies in AT?

Kinetic studies showed that the diminished inhibition of DNA replication remains evident for at least four hours after radiation exposure, de-

pendent on the dose (Painter, 1981; Jaspers et al., 1982d; Jaspers and Bootsma, 1982a (paper IV), Ford and Lavin, 1981; Edwards and Taylor, 1982). This means that the abnormal rate of DNA synthesis continues to exist after enzymatic rejoining of the DNA strand breaks, which is almost completed within two hours in AT cells (Jaspers et al., 1982a (paper II). Thus, DNA strand breaks cannot be directly involved. This conclusion is supported by studies of Chinese hamster ovary cell mutants, hypersensitive to X-rays (Kemp et al., 1984). These mutants have a defect in the rejoining of radiation-induced double-strand breaks (Kemp et al., 1984; Weibezahn et al., 1985). However, the inhibition of DNA synthesis in the mutants is more pronounced than in the wild type controls (Jeggo et al., 1985), contrary to AT cells.

It has been suggested that abnormalities in chromatin structure may be responsible (de Wit et al., 1981 (paper I); Painter, 1982). Attempts to obtain more direct supportive evidence for this hypothesis were not successful so far. A number of observations are relevant in this respect.

Kraemer et al. (1983) reported that the radiosensitivity in AT was not reflected in the metabolism of the histones and major non-histone proteins. Both their rates of synthesis and their amounts were normal in AT cells.

Burgoyne and Jaspers (unpublished observations) failed to observe a consistent difference in DNAse hypersensitivity of the chromatin of irradiated or unirradiated AT and normal fibroblasts.

Incubation in hypertonic medium is known to inhibit DNA replication and sensitize mammalian cells to the cytotoxic and clastogenic action of X-rays (Dettor et al., 1972). However, X-irradiated AT and normal cells responded similarly to high salt concentration (Painter, 1982).

Sodium-n-butyrate is an agent that inhibits chromatin acetylation and phosphorylation and thus influences DNA-protein interaction in the chromatin. The compound has been shown to arrest cultured cells in the G1 phase (Darzynkiewicz et al., 1980). We have investigated the effect of n-butyrate on the rate of DNA synthesis in AT cells. We found evidence, that is consistent with an arrest of cell cycle progression in G1 of both AT and normal fibroblasts (data not shown). Fig. 4 shows the effects of butyrate in X-irradiated cells. When present at a concentration of 5 mM the X-ray-induced inhibition of DNA replication was abolished. However, the responses of AT and normal cells were similar in this respect.

Finally, AT and normal lymphoblastoid cell lines recovered equally

well from an X-ray-induced reduction in superhelix density, as determined by nucleoid sedimentation (Lavin and Davidson, 1981).

In conclusion, the exact reason for the abnormal response of DNA replication to X-rays in AT cells remains unclear. However, in the experiments mentioned above the occurrence of generalized chromatin anomalies has been tested rather crudely. More fundamental knowledge of DNA-chromatin interactions and their relevance for DNA metabolism will be required to answer the question which chromatin alterations may be important for the radiation response in AT.

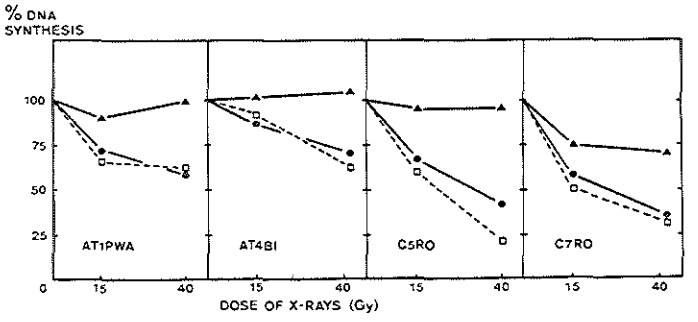

Fig. 4. Effect of X-rays on DNA synthesis after treatment with sodium-n-butyrate.
Fibroblasts from patients with AT (AT1PWA, AT4BI) and from normal individuals (C5RO, C7RO) were prelabeled with ^{14}C-thymidine, incubated for 16 h in the presence of sodium-n-butyrate, exposed to X-rays and cultured for another 4 hrs with butyrate and ^{3}H-thymidine. Cells were harvested and the rate of DNA replication was estimated by the ^{3}H/^{14}C-ratios in each sample. Values of unirradiated cells were normalized to 100%. Concentrations of n-butyrate: 0 MM (□——□), 0.5 mM (●—●) and 5 mM (▲—▲). In the absence of butyrate the inhibition of DNA synthesis is less pronounced in AT cells than in normal cells. In the presence of 5 mM butyrate the DNA synthesis becomes more radiation-resistant in all cell strains.

2.7 Genetics of AT

2.7.1 Complementation analysis of AT [1)]

The suggestion that AT may be genetically heterogenous was made by Hecht and McCaw in 1977 and is based on the variability of the clinical symptoms. Using the varying susceptibility to sinopulmonary infections as a criterion they proposed the existence of at least four different subtypes (Hecht and Kaiser-McCaw, 1982). Whether these differences have a genetic background is not completely clear, since considerable variation in the degree of immunodeficiency has been observed even within AT sibships (Jason and Gelfand, 1979; Saint-Remy et al., 1981). In contrast to the variable clinical picture of AT, cultured cells from AT patients show a rather homogeneous behaviour in many respects. This is especially true with their response to ionizing radiation.

The availability of consistent cellular abnormalities and the fact that the inheritance pattern of the disorder is recessive allow a genetic study of AT by performing complementation analysis. The feasibility of this approach using cultured human cells was shown by De Weerd-Kastelein et al. (1972) with the disease xeroderma pigmentosum. Since then, genetic complementation studies have been carried out on a variety of human inherited disorders, such as gangliosidosis, methylmalonic acidemia, sialidosis, Fanconi's anemia and Cockayne's syndrome. In some instances the molecular basis of the genetic heterogeneity was identified.

Complementation analyses of AT have been undertaken using four different assays. All of these were based on the abnormal response of cultured AT cells to ionizing radiation.

1. Defective gamma-ray induced repair DNA synthesis (Paterson et al., 1977).
2. Defective primer activating activity on irradiated DNA (Inoue et al., 1981).
3. Diminished inhibition of DNA replication after radiation exposure (Jaspers and Bootsma, 1982a (Paper IV); Murnane and Painter, 1982).
4. Enhanced frequency of chromosomal breaks after gamma-irradiation (Chen et al., 1984)

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1) *The text of this section if slightly modified from a publication by N.G.J. Jaspers, R.B. Painter, M.C. Paterson, C. Kidson and T. Inoue, titled: 'Complementation Analysis of AT', in press with Alan Liss Cy., New York.*

With one exception (Inoue et al., 1981) complementation was studied by somatic cell hybridization. Cultured cells from different AT patients were fused using either Sendai virus or polyethylene glycol and the response of the resulting heterokaryons to ionizing radiation was measured.

Paterson et al. (1977) were the first to report genetic heterogeneity in AT. They scored complementation by measuring unscheduled incorporation of tritiated thymidine in cells after exposure to high doses of radiation. Two of the AT cell strains studied failed to complement each other, whereas both complemented a third AT fibroblast strain. The abnormality in repair DNA synthesis is not a consistent phenomenon in AT. Only the class of the exr^- AT fibroblasts is affected and the defect has not been found in AT lymphoblastoid cells.

The lowered activity of a primer activating enzyme, that converts X-ray induced DNA damage into starting points for bacterial DNA polymerase proved to be a characteristic of both the exr^- and exr^+ classes (Inoue et al., 1977; Edwards et al., 1980). Complete restoration of normal activity was demonstrated in a mixture of cell-free extracts obtained from one pair of AT fibroblasts, but not in that from another pair. Thus, the three AT cell strains could be assigned to two complementation groups using the assay.

Another consistent abnormality in AT cell strains is the radiation-resistant DNA replication. In two laboratories this parameter was used for complementation analysis simultaneously and independently (Jaspers and Bootsma, 1982a (Paper IV); Murnane and Painter, 1982). The rate of DNA synthesis was determined by autoradiography on single cells in S-phase after incorporation of tritiated thymidine. Heterokaryons and homokaryons present in the population of fused cells were distinguished by loading the cytoplasm of the cells before fusion with different types of plastic beads. In some cases heterokaryons showed an X-ray induced inhibition of DNA replication that was comparable with that in normal fibroblasts. In homokaryons, or heterokaryons from other combinations of AT patients (sibs for instance) this inhibition was less pronounced (Table 6). The results of the two studies are in agreement with respect to the assignment of cell strains to different complementation groups. In both cases the three cell strains AT3BI, AT4BI and AT5BI were found to complement each other in all combinations.

The combined data from the four studies of AT fibroblasts are summar-

Table 6. COMPLEMENTATION IN AT FIBROBLAST CELLS

Fused Cell Strains[a]	Residual rate of DNA replication[b] after exposure to X-rays (40 Gy)	
	Homodikaryons	Heterodikaryons
C7RO x C7RO	44%	43%
AT4BI x AT5BI	65%;60%	45%
AT4BI x AT6BI	64%;58%	47%
AT5BI x AT6BI	60%;63%	62%

a) C7RO cells are from a normal individual; AT4BI and AT5BI are from unrelated patients, and AT5BI and AT6BI from sibs.
b) The rate is expressed as the percentage of that measured in the corresponding unirradiated cell types, after counting the grains over at least 50 S-phase nuclei.

ized in Tables 7 and 8. Based on these fusions the cells from 11 unrelated cases can be assigned to a least four different complementation groups coded A,B,C and D. Two of these contain the exr$^-$ fibroblast strains (A,B) and C and D harbour cells that were all shown to perform normal levels of repair replication. Therefore, the exr phenotype appears to be reflected by genetic differences. Nothing is known about the capacity for repair synthesis in the strains AT8BI and AT14BI, that are both excluded from groups A, C and D.

Measurement of radiation-induced chromosomal aberrations in fused primary fibroblasts is complicated since these cells proceed to mitosis with very low frequency (Hoehn et al., 1978; Bryantet al., 1979). In large multikaryons the entry into the S-phase is impaired as well (Jaspers et al., 1981a). However, transformed cells proliferate readily after fusion and thus lymphoblastoid cell lines were used by Chen et al. (1984) for studying complementation of the radiosensitivity in terms of chromosome breakage. Since chromosomal aberrations are scored on single cells, discrimination between homokaryons and heterokaryons is also required in this approach. This was accomplished by prelabelling the DNA of one of the fusion partners with 5-bromodeoxyuridine, causing differential staining of the

Table 7. COMPLEMENTATION ANALYSIS OF AT USING CULTURED FIBROBLASTS

	AT3BI	AT4BI	AT5BI	AT2BE	AT10S
AT1BE	- a			+ a	
AT2BE	+ a				
AT1PWA	+ e	- c	+ e	+ e	+ c
AT4JTO	+ d		- d		
AT6BI	+ e	+ e	- e		
AT10S	+ e	+ e	- c		
AT14BI	+ b	+ b	+ b		
AT8BI	+ b	+ b	+ b		
AT17BI	+ b	+ b	- b		
AT5BI	+ bcd	+ bc			
AT4BI	+ bc				

a) Paterson et al., 1977
b) Murnane and Painter, 1982
c) Jaspers and Bootsma, 1982a (paper IV)
d) Inoue et al., 1981
e) Jaspers, unpublished
-, No complementation
+, Complementation

Table 8. COMPLEMENTATION GROUPS AMONG AT-FIBROBLASTS

Group A:	AT1BE, AT3BI
Group B:	AT2BE
Group C:	AT1PWA, AT4BI
Group D:	AT5BI[a)], AT6BI[a)], AT17BI, AT10S, AT4JTO
Unassigned:	AT8BI, AT14BI: both not A, C or D.

a) AT5BI and AT6BI are from sibs

chromosomes with Giemsa. Chromosome breakage was restored to normal levels in some irradiated heterokaryons but not in others. Thus, four different complementation groups were identified in seven unrelated AT patients (Table 9). It is not clear yet, how these groups relate to those found with cultured fibroblasts since the two sets of patients are not overlapping (except cell strain GM 1526, that was obtained from the same individual as AT8BI). Fusions with cells from patients belonging to the Birmingham (BI)-series are in progress (Kidson, pers.comm.).

The combined data from these complementation studies indicate the existence of at least four and possibly nine different complementation groups. As these data are obtained with cells from 17 unrelated patients only, this suggests that extensive genetic heterogeneity exists in AT. A genetic basis of the complementation is suggested by the findings with two AT siblings, although this was established in one case only with one of the four tests. In addition, the complementation pattern was internally consistent, and anomalous behaviour of any cell strain, precluding a clear group-assignment was not yet observed.

Table 9. COMPLEMENTATION GROUPS AMONG AT LYMPHOBLASTOID CELL LINES[a)]

Group 1:	AT1ABR, GM1526 (=AT8BI)
Group 2:	AT3ABR
Group 3:	AT4ABR, AT6ABR, GM717
Group 4:	AT5ABR

a) From Chen et al., 1984

The very frequent occurrence of complementation suggests that the genetic control of the cellular response to ionizing radiation is extemely complex. These genetic data can contribute to the understanding of these processes, since they allow the use of genetically characterized AT cell strains for research. The importance of this is especially clear in studying linkage with polymorphic genetic markers, such as the histocompatibility antigens (Hodge et al., 1980), when these studies involve more than one family.

The genetic complementation analyses are very limited still, and much

further experimentation is needed to provide a large set of characterized AT cell strains. Until then, some reservations concerning the interpretation of the present genetic data remain. For instance, it is not known whether the different complementation assays are equivalent. Only in one case (AT3BI and AT5BI) two independent procedures were followed: the inhibition of DNA replication and the primer activating activity. The finding that with this pair of AT cell strains complementation could be observed even in cell-free extracts suggests that diffusable proteins are directly involved here.

In all studies the response of AT cells to ionizing radiation was considered. Murnane and Painter (1982) reported complementation in unirradiated AT fibroblasts as well. Complementing heterokaryons exhibited a faster rate of tritiated thymidine incorporation than unfused cells or homokaryons. This result was interpreted as evidence for an abnormally slow progression of AT cells through the S-phase. This notion was supported by cell cycle analysis of AT lymphoid cells showing an extended duration of the S-phase (Cohen and Simpson, 1980). However, complementation in unirradiated AT cells was not observed in the other study using the rate of DNA replication as an indicator (Jaspers and Bootsma, 1982a (Paper IV)). The reason for this discrepancy remains unclear sofar, but the inconsistency is reflected by the results from cell cycle studies performed by others (see section 2.6.1).

In conclusion, the results from five independent studies using different parameters measuring radiosensitivity in AT indicate that complementation can be frequently observed in AT cell strains. Whether this extensive heterogeneity is based on genetic changes in different loci in all cases remains to be established.

2.7.2 AT Heterozygotes

The incidence of AT has been estimated to be about 1 in 40,000 to 100,000 (Boder and Sedgwick, 1963; Swift et al., 1976; Harnden and Bridges, 1982). Using the Hardy-Weinberg principle these figures would lead to an incidence of heterozygotes of 1 in 100 to 1 in 150. If there exist n about equally frequent and randomly distributed complementation groups, this estimate becomes still a factor of $\sqrt{n}$ higher, based on population genetic principles.

Definite clinical characteristics reminiscent of AT are not observed

in the heterozygotes, but they do appear to have an increased risk of developing malignancies (Swift et al., 1976). These cancers arise in the breast, ovarium and lymphoid tissues. The cancer mortality rate in young heterozygotes is increased by a factor of 2 at least (Swift, 1982). Susceptibility to nonmalignant disorders such as mild diabetes (Swift, 1984) and ischaemic heart disease (Swift and Chase, 1983) may also be relatively high. The conclusions from these risk analyses have been criticized (German, 1980) since they were based on studies of close blood relatives of AT patients without the use of a clinical laboratory test to identify the carriers individually. In fact, the actual risk of cancer in AT heterozygotes is expected to be still higher.

In the search for such a test many studies were carried out on in vitro cultured cells. It is agreed that lymphoblastoid cell lines and fibroblasts from the heterozygotes as a group are on the average more sensitive to the killing effects of ionizing radiation (Chen et al., 1978; Arlett and Harcourt, 1978; Paterson et al., 1979; Moshell et al., 1980) or neocarzinostatin (Shiloh et al., 1982). However, many AT heterozygotes fall into the normal range in this respect (Kinsella et al., 1982), so the cellular survival test cannot properly function for the identification of heterozygotes. A similar situation exists with respect to other biological parameters such as radiation-induced repair DNA synthesis (Paterson et al., 1979) and chromosomal aberrations (Kidson et al., 1982), or spontaneous chromosome breakage (Oxford et al., 1975; Cohen et al., 1975; Nelson et al., 1975; Taylor, 1982). Arlett and Priestley (1983, 1984) have found defective recovery from potential lethal damage in heterozygote fibroblasts. Whether or not this abnormality is valuable for detection remains to be determined. Cytogenetically abnormal lymphocyte clones have been observed in AT heterozygotes (Kohn et al., 1980; Aurias et al., 1981). The rearrangements most often involve the chromosome 7 and 14 and may be 9 times more frequent in heterozygotes than in normal individuals (Aurias et al., 1980). In many other respects, including the diminished inhibition of DNA synthesis (Jaspers et al., 1981b (paper III)) AT heterozygotes cannot be distinguished from normal controls. It is possible that the expression of the heterozygous state in AT varies with the underlying genetic defect. In some families, but not in others, heterozygotes could be identified using the killing effect of neocarzinostatin as a parameter (Shiloh et al., 1982). Genetic complementation analysis may resolve this.

2.7.3 Genetic counselling

Since AT is a serious inherited disorder for which no adequate medical treatment is available, there is the need of genetic counselling of families with AT.

One factor determining the efficiency of genetic counselling is the availability of a diagnostic test, allowing identification of patients as early as possible, preferably even before the onset of clinical symptoms. To facilitate screening of individuals a clinical laboratory test with relative simplicity is desirable. Of the in vitro cell culturing systems available for this purpose the use of peripheral blood cells seems most appropriate, since this eliminates the time-consuming establishment of long-term cell cultures (lymphoblastoid cell lines or skin fibroblasts). Peripheral white blood cells of AT respond abnormally in various respects; elevated levels of radiation-induced chromosomal aberrations are consistently found in AT lymphocytes. A technically more simple test is the study of the inhibition of DNA replication by X-rays, that has been shown to be diminished in AT lymphocytes (Jaspers et al., 1981b (paper III)). Another test involved the effect of X-rays on PHA-stimulation of lymphocytes. Blood cultures of AT patients incorporate much less tritiated thymidine upon irradiation than cells from normal individuals (Agarwal et al., 1977). In addition, a DNA synthesis-measurement can easily be combined with the assay of alphafetoprotein, the level of which is abnormally high in the blood of almost all AT patients.

A second aid in genetic counselling is the detection of heterozygotes. As discussed in the previous section, at the present time there is no discriminating procedure for routine-screening available yet.

Prenatal diagnoses of AT have been reported by two laboratories. Gianelli et al. (1982) studied the frequency of X-ray-induced chromosomal aberrations in amniotic fluid cells of a fetus at risk and found a normal pattern. Shaham et al. (1981) identified a fetus with AT by the estimation of spontaneous chromosome breakage rate in amniotic fluid cells, and the presence of a clastogenetic activity in the amniotic fluid. This diagnosis was supported by the presence of a cell clone with a 5/14 translocation. A similar procedure has recently been published by Schwartz et al. (1985). In our laboratory the possibility to use the dimished inhibition of DNA synthesis as a marker in prenatal diagnosis was studied. Preliminary data

on normal control amniotic fluid cells and chorion villi showed an X-ray response that is comparable to cultured skin fibroblasts, but with more variability. Whether this variability is related to the heterogeneity of amniotic fluid cells (Van der Veer et al., 19878; Halley et al., 1979) is a subject of further study.

Recently, the use of restriction fragment length polymorphisms (RFLP, Botstein et al., 1980) in genetic counselling has attracted considerable attention. For instance, in Huntington's chorea (an autosomal dominant disease) and Duchenne's muscular dystrophy (an X-linked disorder) RFLPs linked to the (unknown) defective genes have been identified (Gusella et al., 1983; Bakker et al., 1985). Progress in this field with respect to AT has been slow due to a number of difficulties. In the absence of a reliable method to detect heterozygote carriers of the recessive AT gene large, multigeneration families have to be identified and screened to obtain significant linkage data. When more families are to be included in linkage analysis, the existence of genetic heterogeneity must be taken into account. First complementation analysis will be needed to assign each family to a particular complementation group.

Furthermore, the chromosomal localization of genes defective in AT is not known. Therefore a high number of candidate RFLPs must be screened for in the AT families. All these difficulties may be overcome by molecular cloning of AT genes. Investigations to this aim are currently in progress in a number of laboratories (eg. Green et al., 1985).

2.8 Concluding Remarks

The primary defect in AT

The molecular basis for the radiosensitivity in AT has not yet been elucidated. In analogy with xeroderma pigmentosum it was initially hypothesized, that AT cells are defective in DNA repair. However, direct evidence for deficient DNA repair is not available; for instance, the unrepaired DNA lesion could not be identified. The investigation of the rate of DNA replication has resulted in alternative hypotheses, in which DNA repair in AT functions in a normal manner (Painter and Young, 1980; de Wit et al., 1981 (paper I)).

The diminished inhibition of DNA synthesis after ionizing radiation exposure is consistent in AT, but does not appear to be the primary reason for radiosensitivity. As one of the secondary effects of the genetic defect, it is a manifestation of a general abnormality in cell cycle-progression: AT cells show a decreased expression of cell-cycle delay after radiation exposure in both G1, S and G2 phases, This suggests, that cell-cycle delay is an active process that is under control of gene products, of which one or more are defective in AT.

The relation between inhibition of cell cycle progression and other biological endpoints such as cell death and chromosome breakage is not clear. It is possible that the delay functions as a transient 'emergency state', that allows the repair of DNA damage before it can become harmful. Evidence for this comes from experiments with methylated xanthines such as caffeine. These compounds can abolish the G2-arrest after ionizing radiation exposure and synergistically increase chromosome damage and cell lethality (Boynton et al., 1974; Tolmach et al., 1977; Snyder et al., 1977; Oleinick et al., 1978; Griffiths et al., 1978; Tomasovic and Dewey, 1978; Natarajan et al., 1980; Tolmach and Busse, 1980). Painter and Young (1980) have suggested that AT cells may resemble caffeine-treated normal cells. With respect to the inhibition of DNA synthesis and the induction of chromosomal breakage, this resemblance could not be supported (Painter, 1982; Jaspers, unp.; Hansson et al., 1984; Furcinitti, 1983). Moreover, it was shown that caffeine reduces G2-delay in both AT and normal cells (Zampetti-Bosseler and Scott, 1985).

An alternative hypothesis is that X-ray induced deviations of the normal chromatin structure are processed in a abnormal way in AT (de Wit et

al., 1981 (paper I); Jaspers et al., 1982a (paper II)). Proteins recognizing chromatin alterations may be defective in AT, which could prevent cell-cycle delay and promote chromosomal breakage. Such chromatin alterations appear to remain after rejoining of most of the DNA strand breaks. In this hypothesis, it is expected that other processes dependent on chromatin structure can also be disturbed in AT. Abnormalities in the repair of UV damage that occur in X-irradiated AT cells are an example.

The question remains, which may be the primary DNA lesion responsible for the pertinent structural chromatin changes. For several reasons DNA strand breaks can be candidates. The first is, that some AT cells fail to synthesize polyADP-ribose after X-ray exposure (Edwards and Taylor, 1980; Altmann and Dolejs, 1982; Zwelling et al., 1983; Oleinick et al., 1983, 1985). The enzyme katalyzing polyADP ribosylation is located in the chromatin and is stimulated by DNA strand breakage (Benjamin and Gill, 1980; Berger, 1985). A second argument comes from recent experiments by Cox et al. (1984). They found that unirradiated AT cells are unable to functionally restore linearized plasmid that has been introduced into the cells by a calciumphosphate precipitate. This result was interpreted as a defect in the processing of the open ends of double-strand DNA breaks. The possibility exists that a primer activating function is a part of this process. However, as these results are obtained with essentially 'naked' and extrachromosomal DNA, some caution in their interpretation should be taken.

Whatever the mechanism of chromatin processing may be, the genetic complementation data obtained in AT indicate that it is probably highly complex. In AT at least four and possibly nine complementing functions (different gene products?) appear to be involved. Such an extreme complexity in the recognition of a specific type of damage is not unprecedented in mammalian cells: at least nine different gene products may function in the recognition of UV-induced DNA damage, as indicated by complementation analysis of xeroderma pigmentosum and UV-sensitive Chinese hamster cell mutants. In the latter case, genetic and molecular analyses have now resulted in the cloning and characterization of the first gene responsible for one of these functions (Westerveld et al., 1984), which opens the possibility to obtain the relevant protein. Similar studies of AT cells will hopefully lead to the characterization of proteins involved in the response to ionizing radiation.

Chapter III

SUMMARY AND INTRODUCTION TO THE PAPERS

After the discovery that cultured cells from AT patients are hypersensitive to ionizing radiation the suggestion was made that AT could be the 'X-ray-analogue' of xeroderma pigmentosum. The latter syndrome (XP) is characterized by hypersensitivity to short-wave UV-radiation, caused by a reduced ability to properly remove UV-induced DNA damage. The evidence for a DNA repair defect in AT cells is not as strong as in the case of XP (see section 2.2.5 of this thesis). Different XP patients vary in their clinical and cellular UV-sensitivity, and this variability roughly correlates with the capacity to repair the UV damage in the DNA. In AT apparent differences in gamma-ray induced repair DNA synthesis contrast with a rather uniform pattern of radiohypersensitivity.

The rate of DNA replication is affected by low doses of ionizing or non-ionizing radiation. In 1977 it was shown that UV-induced inhibition of DNA synthesis is persistent in highly UV-sensitive XP cell strains; whereas less UV-sensitive cells showed recovery from this inhibition. In the hope to find a consistent biochemical defect in AT cells, it was decided to study the effect of ionizing radiation on the rate of DNA synthesis in AT cells. The first of these experiments are reported in paper I. In contrast to what was expected (assuming analogy with XP) the rate of DNA synthesis in AT cells is more resistant to ionizing radiation than in cells from normal individuals. This behaviour is not seen in x-irradiated cells from XP-patients or in UV-irradiated AT cells. In the same period independent studies from three other laboratories have given identical results. It was hypothesized that AT cells were defective in the processing of chromatin after X-ray exposure rather than defective in DNA repair.

In Paper II the studies of the rate of DNA replication are extended to cultured cells from a larger number of AT patients. The abnormality appear to be a consistent feature of AT. By studying the effect of a variety of DNA damaging agents a correlation between diminished inhibition of DNA synthesis and increased lethality in AT cells is established. In addition, experimental evidence is obtained suggesting that neither DNA strand-breaks nor poly-ADP ribosylation are directly involved in the mechanism of radioresistant DNA synthesis.

The finding in paper II that diminished inhibition of DNA synthesis

is consistent in AT opened the possibility to use this parameter as a diagnostic aid. It is shown in paper III, that a short-term culture of peripheral blood lymphocytes can provide biochemical support for a clinical diagnosis of AT, using the simple test of DNA synthetic rate. This reduces the need of taking skin biopsies or performing more complicated cytogenetic studies.

The availability of a consistent biochemical marker in AT and the fact that the inheritance pattern of the disorder is recessive allow a genetic study of AT by performing complementation analysis. This type of genetic analysis using the radioresistant DNA replication as a marker is described in paper IV. The rate of DNA synthesis is measured in heterokaryons and homokaryons obtained after fusion of cells from several different AT patients. The use of an autoradiographic procedure combined with a cell-labelling technique using cytoplasmic plastic beads allows single-cell analysis. The results indicate the existence of four complementation groups among seven patients studied, suggesting extensive genetic heterogeneity in the AT syndrome.

In Paper V the effect of X-rays is studied on a metabolic pathway believed to function normally in AT cells, i.e. repair DNA synthesis after UV-exposure (UV-UDS). It is demonstrated that the rate of unscheduled DNA synthesis in AT (induced by UV) is changed by X-ray exposure, just like the rate of scheduled (replicative) DNA synthesis. This phenomenon is not seen in normal control cells. The data are in agreement with the hypothesis posed in paper I and II, that the abnormality in AT is in the processing of chromatin after X-ray exposure. The response of UV-UDS to X-rays is different in cells from different AT complementation groups, providing a biochemical reflection of genetic heterogeneity. Only the exr^- AT cells strains experience an inhibition by X-rays of UV-induced repair synthesis. Thus, a possible explanation for the reduced rates of gamma-ray induced repair DNA synthesis in exr^- AT strains can be presented.

SAMENVATTING EN INLEIDING TOT DE PUBLIKATIES

Na de ontdekking, dat gekweekte cellen van patienten die lijden aan AT overgevoelig zijn voor ionizerende stralen, werd geopperd dat AT het 'röntgen-analoog' van xeroderma pigmentosum zou kunnen zijn. Een kenmerk van deze laatste aandoening (XP) is immers een overgevoeligheid voor kortgolvig ultraviolet licht, die wordt veroorzaakt door een te beperkt vermogen om DNA schades te herstellen die optreden na bestraling met dit licht. In tegenstelling tot de situatie bij XP, zijn de aanwijzingen voor onvolkomenheden in het DNA herstel bij AT veel minder duidelijk. Zo kunnen XP-patienten, en ook hun gekweekte cellen, sterk verschillen in hun gevoeligheid voor ultraviolet licht. Deze verschillen zijn in grote lijnen terug te voeren op variaties in het vermogen de DNA schades te herstellen. Bij AT daarentegen, lijken er weliswaar ook verschillen voor te komen in herstelreplicatie van DNA, maar hier vindt men juist een gevoeligheid voor röntgenstraling die vrijwel uniform is.

De snelheid waarmee DNA wordt gesynthetiseerd kan beïnvloed worden door straling in lage doses. In 1977 werd aangetoond, dat in zeer UV-gevoelige XP-cellen de remming van de DNA synthese na UV-belichting lang voortduurt. In XP-cellen die wat minder UV-gevoelig zijn komt de DNA synthese al weer spoedig op zijn oude niveau. We hebben daarom besloten om de effecten van ionizerende straling op de DNA synthese in AT-cellen te bestuderen, in de hoop een consistent biochemisch defect op het spoor te komen. De eerste resultaten van deze experimenten staan in publicatie I. In tegenstelling tot wat de verwachting was (i.g.v. een analogie met XP) blijkt bij AT de DNA synthese snelheid veel minder sterk geremd te worden door straling dan in cellen van gezonde personen. Dit verschijnsel treedt niet op in XP-cellen behandeld met röntgenstraling of in AT-cellen na UV-belichting. In dezelfde periode hebben onderzoekers van drie andere laboratoria onafhankelijk dezelfde bevindingen gedaan. We stelden de hypothese op dat er in AT-cellen een onvermogen bestaat om het chromatine in een volledig functionele staat terug te brengen na blootstelling aan straling i.p.v. een defect in het herstel van schade in het DNA.

In publikatie II worden de studies van de snelheid van DNA synthese verder uitgebreid. Een groter aantal patienten met AT wordt in het onderzoek betrokken en er blijkt dat de eerder gevonden afwijking consistent optreedt. Tevens worden de effecten van allerlei chemische verbindingen die

met DNA kunnen reageren onderzocht, en uit deze experimenten kan geconcludeerd worden dat een afwijkende DNA synthese-remming steeds samengaat met een overgevoeligheid van AT-cellen na blootstelling aan dergelijke stoffen. Resultaten van verdere experimenten suggeren dat breuken in het DNA of poly-ADP-ribosylering geen directe rol spelen in de manier waarop de stralingsresistentie van de DNA synthese tot stand komt.

Nu in publikatie II was aangetoond, dat verminderde remming van de DNA synthese consistent voorkomt in AT, deed zich de mogelijkheid voor deze parameter te gebruiken bij de klinische diagnostiek. Dit punt komt aan de orde in publikatie III. Door de DNA synthese als kenmerk te gebruiken, kan op eenvoudige wijze in kortlopende kweken van lymfocyten uit perifeer bloed de klinische diagnose van AT ondersteund worden. Voor deze laboratoriumtest is men niet langer strict aangewezen op gekweekte cellen uit huidbiopsieën of op veel gecompliceerdere cytogenetische technieken.

Het feit, dat het overervingspatroon van AT recessief is en de beschikbaarheid van een consistent biochemisch kenmerk maken het mogelijk AT genetisch te onderzoeken met behulp van complementatie-analyse. Dit type van genetisch onderzoek aan AT staat beschreven in publikatie IV. De snelheid van de DNA synthese wordt gemeten in heterokaryons en homokaryons die ontstaan na fusie van cellen afkomstig van verschillende AT patienten. De resultaten worden verkregen via analyse op het niveau van een enkele cel door gebruik te maken van een autoradiografische procedure en een celmarkeringstechniek met microscopisch-kleine plastic bolletjes. Er worden aanwijzingen gevonden voor het bestaan van vier complementatie groepen bij zeven patienten die onderzocht zijn, hetgeen aangeeft dat de genetische heterogeniteit in AT wel zeer uitgebreid moet zijn.

Het effect van röntgenstraling op een metabool proces dat overigens normaal functioneert in AT, n.l. door UV geïnduceerde herstel DNA synthese, wordt bestudeerd in publikatie V. Er wordt aangetoond, dat in AT cellen de snelheid van dit type herstelsynthese veranderingen ondergaat onder invloed van röntgenstraling, precies als bij de replicatieve, S-fase afhankelijke DNA synthese. De gegevens stemmen overeen met de hypothese gesteld in publikaties I en II, dat de afwijking in AT betrekking heeft op het metabolisme van chromatine na blootstelling aan röntgenstraling. Het UV-herstel gedraagt zich verschillend in AT cellen die tot verschillende complementatie groepen behoren; blijkbaar is de genetische heterogeneiteit terug te vinden in biochemische verschillen. Alleen in de zg. 'excisie-deficiënte'

AT cellen wordt een sterke vermindering van UV-herstelsynthese waargenomen na röntgenexpositie in hoge doses. De lage snelheid van de DNA-herstel-synthese die door röntgenstraling zelf in deze cellen wordt veroozaakt kan hierdoor verklaard worden.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 30 oktober 1950 in Waarland (Noord-Holland). In 1969 behaalde hij het diploma gymnasium β op het R.K. Lyceum 'Petrus Canisius' te Alkmaar. In 1972 volgde het kandidaatsexamen Scheikunde (S2) aan de universiteit van Amsterdam. De doctoraalstudie, die cum laude werd afgesloten in oktober 1975, omvatte het hoofdvak Biochemie (Prof. J.M. Tager, BCP Janssen Instituut), bijvak Virologie (Prof. J. Van der Noordaa, Lab. voor de Gezondheidsleer) en speciale richting Mathematische Statistiek. In november 1975 kwam hij in dienst als wetenschappelijk medewerker op de afdeling Celbiologie en Genetica van de Erasmus Universiteit, o.l.v. Prof.Dr. D. Bootsma. Aanvankelijk was hij betrokken bij een onderzoek van xeroderma pigmentosum varianten. Dit projekt werd afgesloten met een drietal publikaties. In het kader van een contract met de Commissie van de Europese Gemeenschappen werd gedurende 1980 en 1981 onderzoek verricht aan ataxia telangiectasia. Van de publikaties voortgekomen uit dit werk zijn er vijf opgenomen in de appendix bij dit proefschrift.

Momenteel is ondergetekende in samenwerking met Dr. A.W.M. van der Kamp betrokken bij een onderzoek naar de rol van ultraviolet licht bij het ontstaan van melanomen, dat financieel gesteund wordt door het Koningin Wilhelmina Fonds.

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Velen hebben op hun eigen wijze en in verschillende mate bijgedragen aan de totstandkoming van dit proefschrift. Aan al deze mensen ben ik veel dank verschuldigd. Enkelen wil ik hier met name noemen.

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Bijzondere dank ben ik verschuldigd aan jou, Arthur. Je steun en begrip waren van doorslaggevend belang.

PAPER I

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THE RATE OF DNA SYNTHESIS IN NORMAL HUMAN AND ATAXIA TELANGIECTASIA CELLS AFTER EXPOSURE TO X-IRRADIATION

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Summary

The rate of DNA synthesis was studied in normal cell strains and in strains from patients suffering from the inherited disorder ataxia telangiectasia (AT). After exposure to relatively low doses of oxic X-rays (0—4 krad) DNA synthesis was depressed in AT cell strains to a significantly lesser extent than in normal cells. This response was observed in both an "excision-deficient" and an "excision-proficient" strain. In contrast, there was no difference in DNA-synthesis inhibition between AT and normal cells after UV exposure. After X-irradiation of cells from patients with xeroderma pigmentosum, both complementation group A and XP variants, the observed rate of DNA synthesis was equal to that in normal cells. An exception was the strain XP3BR which has been shown to be X-ray-sensitive. This strain exhibited diminished DNA synthesis inhibition after X-ray doses below 1 krad.

These data suggest a relationship between hypersensitivity to X-rays and diminished depression of DNA synthesis.

Ataxia telangiectasia (AT) is an autosomal recessive multisystem degenerative disorder with an incidence of about 25 per million live births. Its most prominent clinical characteristics comprise both cutaneous (telangiectasia) and neurological abnormalities (progressive cerebellar ataxia), combined with a marked IgA deficiency, an increased incidence of neoplasms and extreme hypersensitivity to X-rays (for a review, see Kraemer, 1977).

At the cellular level some outstanding features are increased chromosomal

Abbreviations: AT, ataxia telangiectasia; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PBS, phosphate-buffered saline (Dulbecco A); TCA, trichloroacetic acid; TdR, thymidine; XP, xeroderma pigmentosum.

instability, both spontaneous and after exposure to ionizing radiation and some clastogenic chemicals, and decreased survival after exposure to ionizing radiations, some DNA-alkylating agents and bleomycin (for a review, see Paterson and Smith, 1979). In cultured fibroblasts from some AT patients a decreased efficiency of repair of DNA damage caused by these agents has been shown (Paterson et al., 1976; Scudiero, 1980). However, cultured cells from a large category of AT individuals show normal levels of DNA repair synthesis after exposure to ionizing radiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and a normal ability to remove γ-endonuclease-sensitive sites from the DNA, while still being hypersensitive to these agents. So far a DNA-repair deficiency has not been clearly demonstrated in the cells from these patients. In an attempt to correlate a possible molecular defect with the increased radiosensitivity of the 2 categories of AT cells, we have investigated DNA synthesis in various cell strains after exposure to X-irradiation under oxic conditions. Here we report the first results from these studies, that show that in AT cultured fibroblasts, after relatively low doses of X-rays, DNA synthesis is inhibited to a significantly lesser extent than in normal cells.

Materials and methods

Cells, routinely cultured in Ham's F10 medium supplemented with 15% calf serum and antibiotics, were seeded into 3 cm petri dishes ($5—10 \times 10^4$ cells per dish). The next day they were prelabelled with [2-^{14}C]thymidine (0.02—0.05 μCi/ml, 60 mCi/mmole) for 6—8 h, in order to have an internal standard proportional to the amount of cells, as has been described by others as well (Painter, 1977; Doniger, 1978). Afterwards the cells were cultured overnight in unlabelled medium and treated with X-rays or UV. We have used relatively low doses of X-rays, an order of magnitude lower than needed to measure repair replication or removal of γ-endonuclease sensitive sites (Paterson et al., 1976, 1977) and more near to the doses used for a study of colony-forming ability after exposure to ionizing radiation (Taylor et al., 1975). Immediately after radiation warm medium containing [^{3}H-*Me*]thymidine (0.5 μCi/ml, 2 Ci/mmole) was added. After 4 h of incubation, the medium was quickly removed, cells were rinsed once with phosphate-buffered saline (PBS) and collected by scraping in 1 ml of cold PBS. The cell suspension was applied to a Whatman GF/C filter wetted with 10% trichloroacetic acid (TCA) and after rinsing with TCA (once) and ethanol (twice) the filters were dried. Then soluene (Packard) and scintillation cocktail were added and the ratio of the ^{3}H to ^{14}C radioactivities was determined. Care was taken to correct for spillover. Within one experiment, the mean result obtained from 4 separate dishes containing sham-irradiated cells was normalized to 100% DNA synthesis. For the other experimental points 2 separate dishes were used.

300 kV X-rays were delivered from a Philips X-ray machine at room temperature under oxic conditions (dose rate 175 rad/min). UV light of predominantly 254 nm came from a Philips TUV mercury lamp at an exposure rate of 0.94 W/m^2.

Results

The rate of DNA synthesis was assessed by the total amount of incorporation of ^{3}H-TdR during the first 4 h after exposure and the ratio of ^{3}H to ^{14}C radioactivity was used as a measure of DNA synthetic activity. After correction for spillover a ratio of at least 2, but generally between 3 and 8 was obtained in unirradiated cells.

Fig. 1 shows that under these conditions DNA synthesis dropped to 24—38% of the control rate in normal cells after exposure to X-ray doses up to 4 krad, but to only 56 and 62% in 2 AT strains. One of these AT strains (AT3BI) has been reported to be deficient and the other (AT5BI) proficient in its ability to carry out excision of radiation-induced DNA damage (Paterson et al., 1976, 1977). The differences in the rate of DNA synthesis between AT and normal cells were statistically significant. A comparison of the results obtained with AT5BI cells with those of 77RD218 cells using a non-parametric significance test (Mann and Whitney, 1947) yielded 2-tailed p-values of maximally 0.016, 0.006, 0.002 and 0.004 for the doses 0.6, 1, 2 and 4 krad respectively. After 0.3 krad the difference was not significant ($p < 0.17$).

In order to test whether the diminished DNA synthesis inhibition after X-ray exposure of AT cells was related to their cellular radiosensitivity, we also investigated the effect of UV irradiation. Fibroblasts from AT patients have a normal colony-forming ability after UV exposure (Lehmann et al., 1977; Arlett

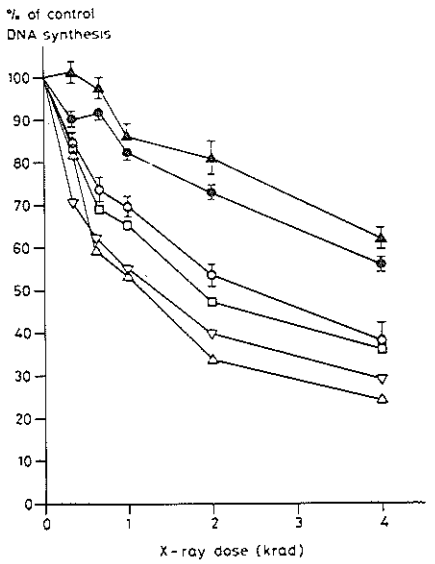

Fig. 1. Rate of DNA synthesis in normal and AT strains after X-rays. Fibroblasts, prelabelled with ^{14}C-TdR and irradiated with indicated doses of X-rays were cultured for 4 h in the presence of ^{3}H-TdR, harvested and processed for scintillation counting. Open symbols indicate normal human cells: ○, 77RD218 (3 Expts.); △, C4RO (1 Expt.); □, C5RO (1 Expt.); ▽, C8RO (1 Expt.). Closed symbols are AT fibroblasts: ▲, AT3BI (exr^{-}, 4 Expts.) and ●, AT5BI (exr^{+}, 3 Expts.). Bars indicate one standard error of the mean.

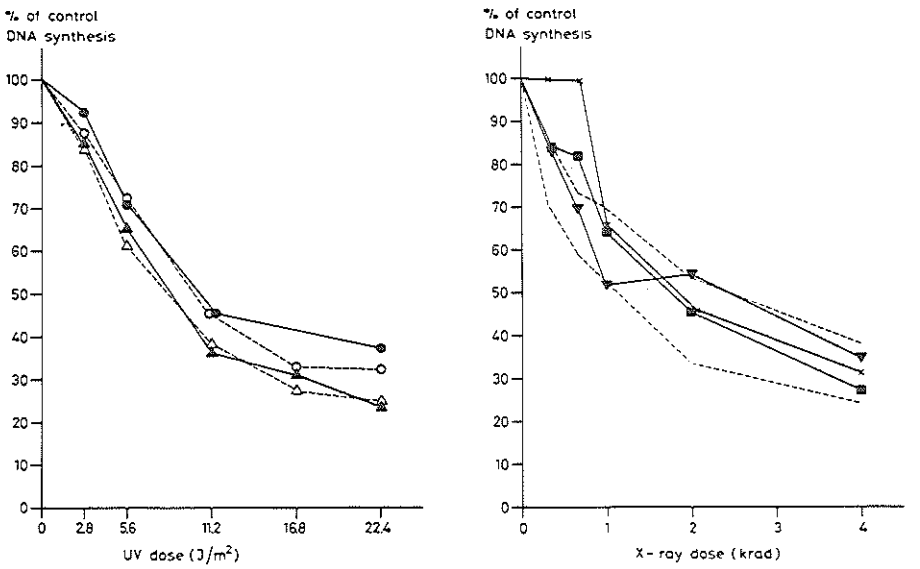

Fig. 2. Rate of DNA synthesis in normal and AT strains after UV. Treatment of cells as in Fig. 1, but UV substituted for X-rays. Open symbols are normal cells: ○, C3RO (4 Expts.) and △, C5RO (4 Expts.). Closed symbols are from AT cells: ●, AT3BI (3 Expts.) and ▲, AT5BI (5 Expts.). In all cases standard errors are less than 3.5%.

Fig. 3. Rate of DNA synthesis in XP strains after X-rays. Treatment protocol same as in Fig. 1. Symbols: ▼, XP4BE (XP variant, 1 Expt.); ■, XP25RO (group A, 2 Expts.) and X, XP3BR (group G, 2 Expts.). Dotted lines indicate the experimental range obtained from normal cells described in Fig. 1.

and Harcourt, 1978). In contrast to the results obtained with X-irradiation, DNA synthesis was depressed in AT and normal cells to an equal extent after exposure to UV light (Fig. 2). The UV dose was chosen in such a way that the inhibition in normal cells was comparable to that found after the X-ray doses used in the experiments of Fig. 1.

In another series of experiments also some xeroderma pigmentosum (XP) cell strains were investigated. Various studies have indicated a normal sensitivity to ionizing radiation in XP (Sasaki et al., 1977; Arlett et al., 1978; Arlett and Harcourt, 1980). Fig. 3 shows that in cells from complementation group A (XP25RO) and in XP variant cells (XP4BE) the DNA synthesis rate after X-irradiation was similar to the rate in normal cells. The strain XP3BR (group G) appeared to be insensitive to very low doses of X-rays (Fig. 3). Recently, it has been shown that these cells are exceptional to the other XP cell strains in exhibiting a moderately decreased colony-forming ability after exposure to ionizing radiation (Arlett, 1979).

Discussion

The analysis of the rate of DNA replication clearly shows, that AT and normal cells differ in their response to X-irradiation. DNA synthesis in AT cells is

depressed to a significantly lesser extent than in normal cells. At least in the case of the strain AT3BI this finding is rather surprising in view of the postulated deficiency in repair of X-ray-induced damage in these cells. It is expected, that, because of the lack of repair, DNA damage would persist, leading to a more extensive blocking of DNA synthetic activity, as has been observed in XP cells after UV exposure (Rudé and Friedberg, 1977). However, a similar behaviour of DNA synthesis was also seen in the strain AT5BI, in which a repair deficiency has not been clearly demonstrated so far. The similar response of "excision-deficient" and "excision-proficient" AT cell strains suggests that the level of DNA replication measured in our experiments is not influenced by the excision repair capacity of the cells.

A relationship between decreased survival and diminished DNA synthesis inhibition after X-ray exposure of AT cells is supported by the results obtained after UV irradiation. Both cell survival and DNA synthesis inhibition were the same as in normal cells. Also, the rate of DNA synthesis after X-irradiation of XP cells, both complementation group A and XP variant, was equal to that in normal cells, being in line with their normal survival after ionizing radiation. An exception could be the strain XP3BR (group G), which exhibits a moderate radiosensitivity in terms of cellular survival (Arlett, 1979). We observed a decrease in DNA-synthesis inhibition after X-ray exposure of these cells as well, although only in the lowest dose range (0—1000 rad), the range that was also used to measure their colony-forming ability. In contrast with our observations on the effect of X-irradiation, Scudiero (1980) has shown that AT cell strains are hypersensitive to the killing action of MNNG, whereas the rate of DNA synthesis in the AT cells (measured during the first hour after treatment with MNNG) did not differ from that in normal cells. Scudiero's data may indicate that different types of DNA damage are involved in increased killing and the deviation from normal DNA-synthesis rate in AT cells.

At the present time, an explanation of the abnormal DNA-replication behaviour in AT cells will be rather speculative, due to our limited understanding of the regulation of DNA replication in mammalian cells. One possibility would be, that the primary defect in AT is a disturbance of the regulation of DNA replication on damaged templates. In those instances where DNA replication escapes its normal regulation, "illegitimate" daughter strands might occur, possibly more extensively damaged (e.g., ss-breaks could be copied into ds-breaks). This may result in enhanced chromosomal instability and consequently in increased cell killing. Experiments to analyse the structure of newly synthesized DNA in irradiated AT cells are in progress. However, against this interpretation are the observations of Taylor (1978) and Natarajan and Meijers (1979) of an increased production of chromosomal aberrations in AT cells after irradiating cells in the G2-phase.

Another possibility may be that the factor which is defective in AT plays a role in maintaining or restoring chromosome structure after induction of DNA damage. In AT this may result in an increase of chromosome aberrations and also in a change in the regulation of DNA synthesis on damaged templates. Similarly, the factor might indirectly be involved in moderating the action of DNA-repair enzymes as well. The genetic heterogeneity in AT, as observed by Paterson et al. (1977) suggests that more than one factor are involved in this con-

trol function. These factors may differ in their specificity to the type of alteration in chromosome structure. This could have different consequences for the DNA-repair activity measured as repair replication in different AT cell strains. Current investigations aim at an identification of the type of lesion responsible for abnormal rates of DNA synthesis in AT.

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PAPER II

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Abnormal Regulation of DNA Replication and Increased Lethality in Ataxia Telangiectasia Cells Exposed to Carcinogenic Agents[1]

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ABSTRACT

The effect of different carcinogenic agents on the rate of semiconservative DNA replication in normal human and ataxia telangiectasia (AT) cells was investigated. The rate of DNA synthesis in all AT cell strains tested was depressed to a significantly lesser extent than in normal cells after exposure to X-rays under oxia or hypoxia or to bleomycin, agents to which AT cells are hypersensitive. In contrast, inhibition of DNA replication in normal human and AT cells was similar after treatment with some DNA-methylating agents or mitomycin C. Colony-forming ability of AT cells treated with these agents was not different from normal cells. Treatment with 4-nitroquinoline 1-oxide elicited a variable response in both AT and normal cell strains. In some strains, including those shown to be hypersensitive to the drug by other workers, the inhibition of DNA synthesis was more pronounced than in other cell strains, but no significant difference between AT and normal cells could be detected.

The rejoining of DNA strand breaks induced by X-rays, measured by DNA elution techniques, occurred within 2 hr after treatment and could not be correlated with the difference in DNA synthesis inhibition in AT and normal cells. After low doses of X-rays, AT cells rejoined single-strand breaks slightly more slowly than did normal cells.

The rate of DNA replication in X-irradiated AT and normal cells was not affected by nicotinamide, an inhibitor of poly(adenosine diphosphate ribose) synthesis.

These data indicate that the diminished inhibition of DNA replication in carcinogen-treated AT cells (*a*) is a general characteristic of all AT cell strains, (*b*) correlates with AT cellular hypersensitivity, (*c*) is not directly caused by the bulk of the DNA strand breaks produced by carcinogenic agents, and (*d*) is not based on differences in the induction of poly(adenosine diphosphate ribose) synthesis between X-irradiated AT and normal cells.

INTRODUCTION

AT[2] is an inherited disorder characterized by a broad spectrum of clinical symptoms (for a review, see Ref. 20). The abnormalities involve the skin (telangiectasia), the immunological apparatus (underdeveloped thymus, decreased levels of IgA and IgE), and the nervous system (progressive cerebellar ataxia). In the patients, there is also a greatly increased risk for various types of cancer, among which the lymphoproliferative disorders predominate. Finally, the patients are extremely hypersensitive to therapeutic treatment with X-rays.

Studies with cultured cells obtained from AT patients have revealed hypersensitivity in terms of cellular survival to various types of ionizing radiation (4, 9, 21, 30, 35, 41, 47) and possibly to some carcinogenic chemical agents (15, 24, 31, 36, 38, 43). AT cells also show chromosomal instability, both spontaneously (8, 13, 42) and after exposure to ionizing radiation (14, 34, 42). In cells from some patients, but not from all, there is a reduction in radiation-induced repair DNA synthesis (33, 46). Recently, it was established in various laboratories that the rate of DNA replication in AT cells after exposure to ionizing radiation is inhibited to a lesser extent than in normal cells (10, 11, 16, 28), independent of the excision repair capacity (10). However, after irradiation with UV at 254 nm, to which AT cells are not abnormally sensitive (3, 22), the rate of DNA synthesis is not different from that in normal cells (10). These observations suggest a relationship between a diminished inhibition of DNA replication and a decreased colony-forming ability in irradiated AT cells. In order to further investigate the possibility of such a relationship, we have undertaken a study of the rate of DNA synthesis in AT and normal fibroblasts after exposure to a variety of carcinogenic agents, with the emphasis on those to which hypersensitivity in AT cells has been observed. In addition, we have tried to obtain information on the type of damage that may be responsible for the depression of the inhibition of DNA replication and on its mechanism.

MATERIALS AND METHODS

Cell Strains and Culture Conditions. The skin fibroblasts were routinely cultured in Ham's F-10 medium, supplemented with 7.5% fetal and 7.5% newborn bovine serum and with penicillin and streptomycin (100 units/ml each). The cells were microscopically screened for *Mycoplasma* contamination at least monthly, using propidium iodide fluorescence. The AT fibroblast cell strains were obtained from Drs. C. F. Arlett (Brighton, England; AT3BI, AT4BI, AT5BI), M. C. Paterson [Chalk River, Ontario, Canada; AT2BE (formerly CRL 1343)], M. Ikenaga (Osaka, Japan; AT10S), J. M. J. T. Scheres (Nijmegen, The Netherlands; AT1NM, AT2NM), and J. Zaremba (Warsaw, Poland; AT1PWA).

Inhibition of DNA Synthesis. The overall rate of semiconservative DNA replication was measured essentially as described before (10). In short, cultures on dishes were prelabeled with [^{14}C]thymidine (8 to 16 hr, 0.02 to 0.04 μCi/ml, 50 mCi/mmol), treated with a particular carcinogenic agent, and incubated for 4 hr in culture medium buffered with 20 mM HEPES (pH 7.4) and containing [^{3}H]thymidine (0.5 to 1.0 μCi/ml, 2 Ci/mmol). Then they were harvested by scraping, and trichloroacetic acid-precipitable radioactivity was determined. The ratio of ^{3}H dpm to ^{14}C dpm was taken as a measure of the overall rate of DNA synthesis. Within a single experiment, each point was a mean of at least 2 separate dishes and was expressed as a percentage of the

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[2] The abbreviations used are: AT, ataxia telangiectasia; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PBS, phosphate-buffered saline (2.7 mM KCl-137 mM NaCl-2.2 mM KH_2PO_4-8.1 mM Na_2HPO_4); 4NQO, 4-nitroquinoline 1-oxide; MMS, methyl methanesulfonate; poly(ADP-ribose), polyadenosine diphosphate ribose.

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mean value obtained from at least 4 untreated control dishes. Statistical analyses were performed with a nonparametric significance test, described by Mann and Whitney (25).

Carcinogen Treatments. X-rays (300 kV) were delivered from a Philips X-ray machine at room temperature with a dose rate of 175 rads/min. Hypoxic conditions were created by placing the cells in 1 ml of cold HEPES-buffered medium and keeping them at 4° for 2 hr under nitrogen in thin-walled plastic boxes. Before irradiation at room temperature, the boxes were sealed. All chemical carcinogen treatments, except MNNG (30 min), lasted for 60 min. Proper concentrations of the agents were prepared in complete culture medium at 37°, supplemented with 20 mM HEPES (pH 7.4), immediately before use. After treatment, the cells were rinsed twice with warm PBS. Stock solutions of 4NQO (100 mM in 10% dimethyl sulfoxide), bleomycin (Lundbeck; 2 mg/ml), and mitomycin C (Christiaens; 0.8 mM) were stored in aliquots at −20°, which were used only once. MMS and MNNG solutions were prepared freshly.

Repair of DNA Breaks. Cell cultures in glass bottles were prelabeled with [^{14}C]thymidine (0.008 μCi/ml) for 2 to 3 days, trypsinized, and seeded in 6-cm Petri dishes (about 2 × 10^5 cells/dish). After 24 hr, they were X-irradiated in ice-cold PBS and then cultured at 37° for different times in prewarmed HEPES-buffered culture medium (pH 7.4) to allow repair. The reaction was stopped in ice-cold PBS, and the cells were harvested by scraping and kept in PBS for less than 5 min before analysis. Single-strand breaks were assayed using the alkaline elution technique originally described by Kohn *et al.* (19) and modified by Cerutti *et al.* (7). Cells were lysed for 1 hr at pH 10 in a solution with 0.2% Sarkosyl, 2 M NaCl, 0.02 M disodium EDTA, and 0.05% proteinase K. The lysate was slowly eluted through a Millipore filter for 15 hr with a solution of tetrapropylammonium hydroxide and EDTA (0.04 M) buffered at pH 12.0. Ten consecutive 4.5-ml fractions were assayed for radioactivity. The fraction of the total activity remaining on the filter was plotted semilogarithmically against the elution time. The initial slopes of the essentially linear plots were determined by least-squares regression analysis of the first 6 time points. This value (S) was inversely proportional to the mean DNA molecular weight of the tested sample (7). In a number of experiments, L1210 mouse lymphoma cells, labeled with [^{3}H]thymidine and irradiated with 150 rads, were included in each elution channel as an internal standard. Double-strand DNA breaks were assayed by neutral elution in the same way as described by Bradley and Kohn (6). Semilogarithmic elution profiles were generally biphasic. The initial slope of the plots (S, calculated as above) was proportional to the X-ray dose and was taken as a measure for the number of DNA double-strand breaks. In the repair tests, all points in a single experiment are averages from duplicate dishes. The percentage of breaks remaining was defined as

$$100 \times (S_t - S_{unirr})/(S_0 - S_{unirr})$$

where S_t is the slope obtained after a repair time of t min and S_{unirr} is the slope before irradiation.

Cell Survival Experiments. Survival was determined as described by Keijzer *et al.* (18). Cells (10^5) were seeded onto 6-cm Petri dishes and exposed to a carcinogenic agent 1 day later. After treatment, they were trypsinized and seeded on feeder layers prepared the day before. Two to 3 weeks later, the cells were fixed with 70% ethanol and stained with Coomassie blue. Only colonies of at least 50 cells were scored.

RESULTS

Effects of X-irradiation. In a previous paper (10), we have reported that, after exposure to X-irradiation, the overall rate of DNA replication in 2 AT cell strains was inhibited to a lesser extent than in 4 normal human cell strains. One of the AT strains (AT3BI) has a reduced level of repair DNA synthesis induced by high doses of γ-rays, while the other (AT5BI) is normal in this respect (30, 33). In order to establish whether

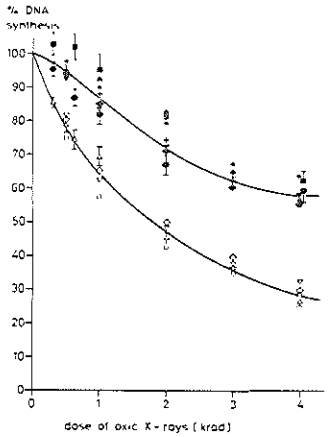

Chart 1. Inhibition of DNA synthesis by X-rays at atmospheric oxygen pressure. *Closed symbols*, AT cell strains. ▲, AT10S (1 experiment); ▼, AT1PWA (2 experiments); ■, AT4BI (5 experiments); ●, AT2BE (3 experiments); ★, AT180CTO (1 experiment); +, AT1NM (1 experiment); ◆, AT2NM (2 experiments). *Open symbols*, normal cell strains. ◇, C1RO (1 experiment); △, C5RO (7 experiments); ▽, C8RO (1 experiment); ○, 77RD222 (1 experiment). In the case of more than 2 experiments, S.E. (*bars*) are given. *krad*, kilorads.

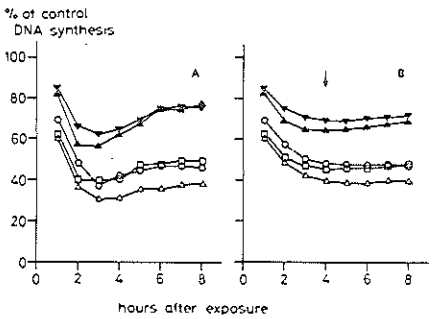

Chart 2. Kinetics of DNA synthesis after exposure to 2 kilorads of X-rays. Cells were either pulse-labeled for 1 hr starting at different times after irradiation (*A*) or labeled for different periods starting immediately after irradiation (*B*) and harvested at the times indicated by the *abscissa*. *Ordinate*, ratio of activities in irradiated and unirradiated control cells. *Closed symbols*, AT cell strains. ▲, AT3BI; ▼, AT5BI. *Open symbols*, normal cell strains. ○, C3RO; □, C4RO; △, C5RO. *Points*, means; *bars*, S.E. C3RO and C4RO, 1 experiment each; other cell strains, 2 experiments.

this phenomenon is a general characteristic of all AT strains, we have extended our studies to an additional 6 AT and 3 normal fibroblast strains. Chart 1 shows that the inhibition of DNA replication after exposure to different doses of X-rays, delivered at atmospheric oxygen pressure, was diminished in all AT cell strains tested compared to the normal cell strains. This panel of strains included AT cells that were shown to be "excision deficient" (AT2BE) (33) and "excision proficient" [AT4BI (33) and AT1PWA, AT10S[3]]. The reduction in inhibition of DNA replication was statistically significant in every arbitrary

[3] Unpublished data.

pair of AT and normal cell strains that was compared. The maximal 2-tailed *p* values were less than 0.02 at all X-ray doses used.

The kinetics of DNA synthesis was studied in AT and normal cells after exposure to 2 kilorads of X-rays. As is shown by Chart 2*A*, a rapid decrease is found in the relative rate of DNA replication in both AT and normal cells immediately after exposure. A minimum was reached at about 3 hr, followed by a gradual recovery of the rate of DNA synthesis at later times. At all times after irradiation, the inhibition of DNA synthesis in AT cells was depressed in comparison to normal cells. This suggests that the type of lesion that is responsible for the abnormal rate of DNA synthesis in AT persists for at least 8 hr.

Chart 2*B* shows the kinetic data obtained after labeling the cells for different periods, starting immediately after exposure to 2 kilorads of X-rays. The curves indicate that a labeling period of 4 hr is optimal to reveal the difference in inhibition of DNA synthesis between AT and normal cells.

We have also studied the effect of X-ray exposure under hypoxic conditions. This type of treatment produces a spectrum of DNA lesions that is different from that found after irradiation at atmospheric oxygen pressure. The number of DNA strand breaks after hypoxic γ-rays is relatively decreased, while there is an increase in the number of base lesions, that can be recognized by an endonuclease from *Micrococcus luteus* (29, 30). AT cells are hypersensitive to hypoxic γ-ray exposure as well (29, 30). The treatment also reduces the rate of DNA synthesis in normal cells (Chart 3), but to a slightly lesser extent than "oxic" X-irradiation (Ref. 10; Chart 1). In AT cells, the inhibition of DNA replication was diminished, the difference between AT and normal cells being similar to that found after exposure under oxic conditions.

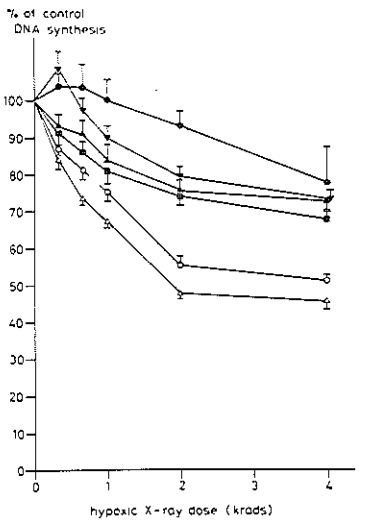

Chart 3. Inhibition of DNA synthesis by X-rays delivered under hypoxia. *Closed symbols*, AT cell strains. ●, AT2BE; ▲, AT3BI; ■, AT4BI; ▼, AT5BI. *Open symbols*, normal cell strains. ○, C3RO; △, C5RO. AT2BE, 2 experiments; all other cell strains, 3 experiments. *Points*, means; *bars*, S.E.

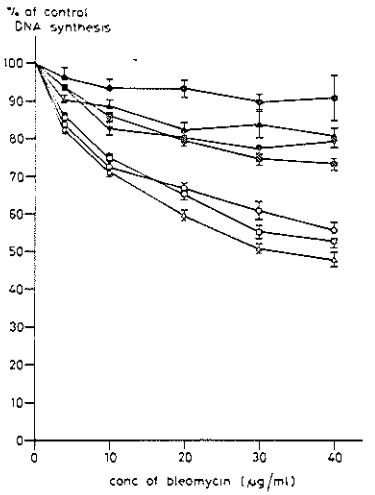

Chart 4. Inhibition of DNA synthesis by bleomycin. *Closed symbols*, AT cell strains. ●, AT2BE (2 experiments); ▲, AT3BI (3 experiments); ■, AT4BI (4 experiments); ▼, AT5BI (3 experiments). *Open symbols*, normal cell strains. ○, C3RO; □, C4RO; △, C5RO (3 experiments each). *Points*, means; *bars*, S.E.

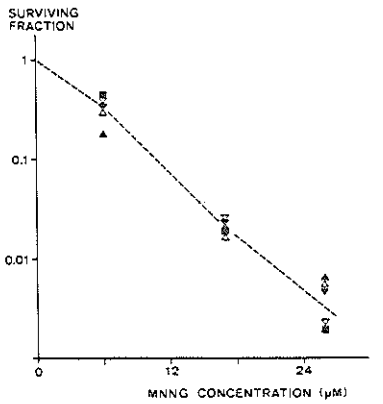

Chart 5. Cellular survival after exposure to MNNG. *Symbols* and cloning efficiencies (in parentheses) in untreated controls: ▲, AT3BI (1%); ■, AT4BI (11%); ▼, AT5BI (19%); △, C3RO (25%); ▽, C5RO (9%). All *points* are means of triplicate dishes.

Effects of Bleomycin. Bleomycin is a radiomimetic agent that causes mainly DNA breaks by a reaction involving the formation of radicals. AT cells are hypersensitive to it in terms of cellular survival (24, 43) as well as induction of chromosomal aberrations (43). In both excision-proficient and excision-deficient AT cells, the inhibition of DNA replication by bleomycin was significantly depressed (Chart 4). After statistical comparison of AT4BI and C3RO, maximal 2-tailed *p* values of less than 0.001 were obtained at all the doses tested.

Effects of DNA-alkylating Agents. In an early report by Hoar

and Sargent (15), evidence was obtained for hypersensitivity of AT cells to the killing action of MMS and MNNG. With respect to the latter agent, Scudiero (36) reached a similar conclusion but found AT cells to be normally sensitive to MMS. Paterson *et al.* (31) reported a slightly increased killing by MNNG and a variable response to MMS. Finally, Arlett *et al.* (2, 3) observed a normal survival in AT cells treated with either of the 2 agents. Also, with our experimental conditions, AT and normal cells were equally sensitive to MNNG (Chart 5). The overall rate of DNA replication after exposure to different doses of MMS or MNNG was equal in normal and AT cells (Charts 6 and 7).

Effects of Mitomycin C. Work by Hoar and Sargent (15) indicated that AT cells are abnormally sensitive to mitomycin C, an agent that induces DNA interstrand cross-links. Their result could not be reproduced by Arlett *et al.* (1–3). In our experiments also, no indication could be obtained for an increased cytotoxicity of the drug in AT (Chart 8). The inhibition of DNA synthesis by doses of mitomycin C up to 40 μM was similar in AT and normal cells (Chart 9).

Exposure to 4NQO. Recently, it was found that 2 out of 3 AT cell strains were more sensitive to 4NQO than were normal 2 strains. In the same report (31), it was claimed that the 4NQO-sensitive AT cell strains (AT2BE, AT4BI) exhibited a reduced ability to repair an alkali-labile type of DNA lesion caused by 4NQO treatment. The results in Chart 10 describe the inhibition of DNA synthesis by 4NQO. The response in the different AT cell strains diverged. In 2 of them (AT2BE and AT4BI), the rate of DNA synthesis was significantly lower than that in the other 2 (AT3BI and AT5BI). However, a similar variability was evident in a panel of 6 tested normal cell strains. Thus, there is no difference in the extent of inhibition of DNA synthesis by 4NQO between AT and normal cells, although

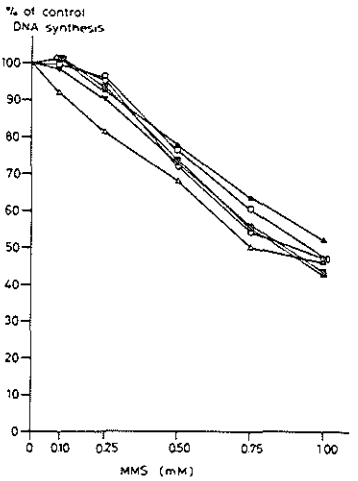

Chart 6. Inhibition of DNA synthesis by MMS. *Closed symbols*, AT cell strains. ▲, AT3BI; ■, AT4BI; ▼, AT5BI. *Open symbols*, normal cell strains. ○, C3RO; □, C4RO; △, C5RO. AT4BI and AT5BI, 4 experiments; all other strains, 2 experiments. Standard errors were always less than 5%.

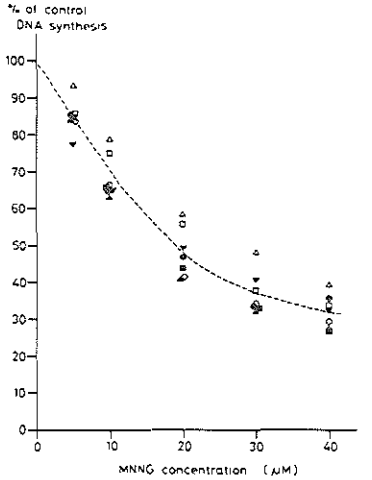

Chart 7. Inhibition of DNA synthesis by MNNG. *Closed symbols*, AT cell strains. ●, AT2BE; ▲, AT3BI; ■, AT4BI; ▼, AT5BI. *Open symbols*, normal cell strains. ○, C3RO; □, C4RO; △, C5RO. AT4BI, 1 experiment; AT5BI and C5RO, 3 experiments; all other cell strains, 2 experiments. Standard errors were always less than 5%.

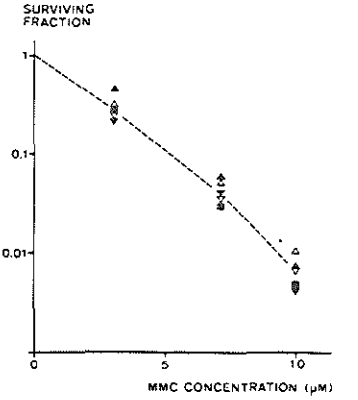

Chart 8. Cellular survival after exposure to mitomycin C: ▲, AT3BI; ■, AT4BI; ▼, AT5BI; △, C3RO; ▽, C5RO. Control cloning efficiencies: AT3BI, 12%; AT4BI, 20%; AT5BI, 14%; C3RO, 32%; C5RO, 17%. All *points* are means of at least 3 dishes.

considerable differences between individual cell strains are observed.

Repair of DNA Strand Breaks. Based on cytogenetic findings, it was postulated (40) that the radiosensitivity in AT could be the result of an inability to repair some class of DNA breaks. However, in work by others, no abnormality in the rejoining of single-strand (12, 30, 45) or double-strand (23, 45) DNA breaks by AT cells could be detected. We have studied the repair of DNA breaks after exposure to low doses of X-rays, those used in this and other work (10, 11, 16, 28) to investigate

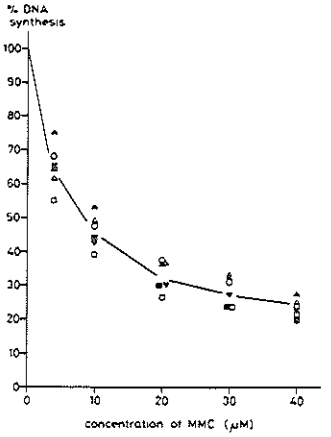

Chart 9. Inhibition of DNA synthesis by mitomycin C. *Closed symbols*, AT cell strains. ▲, AT3BI; ■, AT4BI; ▼, AT5BI. *Open symbols*, normal cell strains. ○, C3RO; □, C4RO; △, C5RO. All *points* are means of 2 independent experiments.

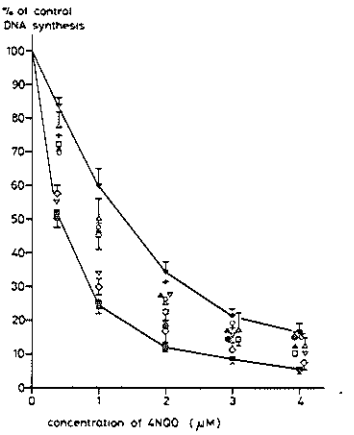

Chart 10. Inhibition of DNA synthesis by 4NQO. *Closed symbols*, AT cell strains. ●, AT2BE (2 experiments); ▲, AT3BI (4 experiments); ■, AT4BI (4 experiments); ▼, AT5BI (4 experiments). *Open symbols*, normal cell strains. ○, C3RO (3 experiments); □, C4RO (2 experiments); △, C5RO (4 experiments); ▽, C1RO (1 experiment); ◇, C8RO (2 experiments); +, 77RD222 (1 experiment). *Error bars* are given where space allows it and in case of more than 2 experiments. Standard errors were always less than 8%.

the rate of DNA synthesis in AT. The sensitive DNA elution techniques for detection of single- and double-strand breaks that were designed by Kohn *et al.* (6, 19) were used for this purpose.

The rejoining of single-strand breaks induced by 500 rads of X-rays showed biphasic kinetics (Chart 11*A*), with normal half-lives of 3 to 6 and 10 to 20 min, respectively. After this low dose of X-rays, AT cells rejoined the single-strand breaks at a slightly reduced rate ($p < 0.05$). This slight reduction in the repair rate in AT appeared to involve mainly the rapid phase, while the rate of the slow phase was hardly affected. After a repair time of 90 min, a significant difference between the elution profiles of irradiated and unirradiated AT and normal cells was no longer detectable (data not shown).

Chart 1*B* shows that the kinetics of rejoining of double-strand breaks produced by 4 kilorads of X-rays was also biphasic. During a rapid phase lasting about 20 min, 80 to 90% of the breaks were rejoined, followed by a slower phase. After 60 to 80 min, the amount of breaks no longer exceeded the limit of detection, which is equivalent to the number of breaks induced by a dose of 400 rads in this assay. No differences between AT and normal cells were evident.

Effect of Nicotinamide. In a recent report, Edwards and Taylor (11) found γ-irradiated AT cells to be defective in the induction of poly(ADP-ribose) synthesis. They postulated that this polymer has a function in the regulation of DNA synthesis after exposure to a DNA-damaging agent. In this hypothesis, the diminished inhibition of DNA synthesis in irradiated AT cells was regarded as a direct result of the low levels of poly(ADP-ribose). On the basis of this hypothesis, we predicted that complete depression of poly(ADP-ribose) synthesis by an externally added inhibitor will affect the rate of DNA synthesis in irradiated normal cells in such a way that it is no longer different from that in irradiated AT cells. This prediction was tested on AT5BI and normal cells treated with X-rays and with nicotinamide as the inhibitor of poly(ADP-ribose) synthetase. At doses of nicotinamide up to 10 mM, concentrations sufficient to completely block the action of the enzyme (5), the difference in inhibition of DNA synthesis between X-irradiated AT and normal cells was similar to that found in the absence of the inhibitor (Chart 12). It appears, therefore, that poly(ADP-ribose) probably does not play a causative role in the depression of DNA replication after exposure to ionizing radiation.

DISCUSSION

The data in this report show that exposure of AT cells to X-rays causes an inhibition of DNA synthesis, which is less pronounced than that found in irradiated normal cells. These results confirm and extend earlier observations by us (10) and other investigators (11, 16, 28) and indicate that the diminished inhibition of DNA replication after exposure to ionizing radiation is a general characteristic of AT cell strains, independent of the capacity of the cells to perform radiation-induced repair

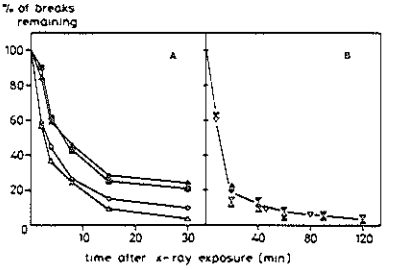

Chart 11. Rejoining of DNA strand breaks induced by X-rays. *A*, single-strand breaks as monitored by alkaline elution; induction dose, 500 rads. *B*, double-strand breaks as monitored by neutral elution; induction dose, 4 kilorads. ▲, AT3BI; ■, AT4BI; ▼, AT5BI; ○, C1RO; △, C5RO; ▽, C3RO. All *points* are means of at least 2 independent experiments. The elution patterns obtained immediately after irradiation (no repair) were similar in AT and normal cells.

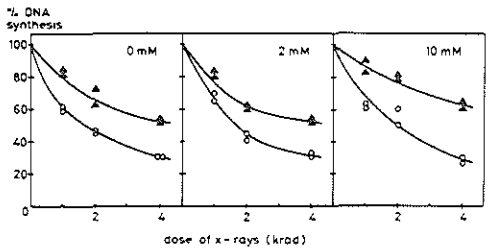

Chart 12. Effect of nicotinamide on the inhibition of DNA synthesis by X-rays. ▲, AT5BI; O, CSRO. Nicotinamide was present in the indicated concentrations during both irradiation and [^{3}H]thymidine labeling. *Points*, single determinations.

DNA synthesis. A relationship is suggested between abnormal regulation of DNA replication and increased cell killing in irradiated AT cells. This assumption is supported by the results from the experiments in which the effects of various other DNA-damaging agents were investigated. A diminished inhibition of DNA synthesis was caused by agents, to which AT cells were unequivocally shown to be hypersensitive, such as different types of ionizing radiation or bleomycin, while on the other hand both a normal inhibition of DNA synthesis and a normal cell killing are observed in AT by UV at 254 nm (3, 10, 22), or DNA-alkylating or cross-linking agents. The interpretation of the experimental results obtained after exposure to MNNG, MMS, and mitomycin C is complicated by the fact that the data in the literature concerning the cytotoxicity of these chemicals in AT are discordant. A reinvestigation of this subject under our experimental conditions revealed no apparent difference in lethality between normal and AT cells after exposure to MNNG or mitomycin C, which agrees with the data of Arlett *et al.* (1–3). A reason for the discrepancies could be that the different authors have used different experimental protocols and culture conditions. For instance, Ham's F-12 culture medium, used in various reports (31, 32, 36, 38), contains 15 times less nicotinamide than does Ham's Medium F-10 and 100 times less nicotinamide than does Dulbecco minimal essential medium. Culturing cells in medium deficient in this vitamin results in low intracellular NAD levels which were shown to affect cellular survival and DNA repair after exposure to DNA-damaging agents (17, 26).

After exposure to 4NQO, a great variability in the response of DNA synthesis in the different cell strains was seen. The carcinogen 4NQO, unlike the other agents tested in this report, is extensively metabolized inside the cells. The drug is consecutively activated and inactivated by enzymes; also, the actual DNA adduct-forming reaction appears to be enzymatically controlled (for a review, see Ref. 39). One could imagine that subtle variations in the enzyme activities involved may lead to considerable differences in the spectrum of lesions produced in the various cell strains. As a result, there should also be a variability in survival of normal cell strains after treatment with 4NQO. Work from other authors has indicated that this may indeed be the case.[4] Further experimentation on the variation of 4NQO cytotoxicity in normal cells will probably clarify this point.

Under our experimental circumstances, there is a good correlation between diminished DNA synthesis inhibition and enhanced cell killing in carcinogen-treated AT cells, with the possible exception of 4NQO treatment. This correlation indicates that the 2 phenomena are a consequence of the same genetic defect in AT. For cytotoxicity screening of chemical agents on AT cells, analysis of the rate of DNA synthesis may suffice. This simple approach will facilitate the handling of large panels of compounds.

If there exists a specific type of DNA lesion that is responsible for the abnormal regulation of DNA replication in AT, our data indicate that this lesion is not produced in biologically important quantities by far UV, mitomycin C, MNNG, MMS, or 4NQO. Relevant DNA damage is produced only by ionizing radiation and bleomycin in our tests. Radiogenic base lesions recognizable by *M. luteus* extracts are probably not involved because of a lack of an effect of oxygen on the diminished inhibition of DNA synthesis in irradiated AT cells. Candidates may be the DNA strand scissions produced by both bleomycin and X-rays. However, the kinetics of repair of both single- and double-strand DNA breaks is very different from that observed for the inhibition of DNA synthesis.

After repair of the bulk of the DNA breaks, there is still a very clear difference in the relative rates of DNA replication between AT and normal cells. With respect to the rejoining of single-strand DNA breaks, a slightly reduced rate was observed in the 2 AT cell strains tested. These data agree with earlier observations made by Van der Schans *et al.* (44), who have used another AT cell strain exposed to γ-rays. Since the differences between AT and normal cells were detectable only at short times after radiation and were relatively small, it is hard to imagine how they could be the cause of the strong effects of radiation on cell killing and chromosomal aberration production. Although the significance of this apparent abnormality in single-strand break repair remains to be established, it seems probable that this is a secondary consequence of the primary genetic defect.

With respect to the type of damage involved, the possibility cannot be ruled out completely that a very small class of DNA breaks may be relatively persistent in AT cells and thereby affect the rate of DNA replication. The limit of detection of the elution assays in our hands was roughly 1 to 2 breaks/10^{10} daltons. This would correspond to a target size of about 50 to 100 replicons, and the size of the replicon clusters characterized by coordinated initiation of DNA synthesis may well be within this range (27).

Due to our limited understanding of the regulation of DNA replication in mammalian cells, it is hard to formulate a molecular mechanism that explains our data. Our results do indicate that a difference in the activity of poly(ADP-ribose) synthetase after exposure to ionizing radiation is very probably not causally involved, as was suggested by Edwards and Taylor (11). It was proposed by others (10, 28) that the primary genetic defect in AT is not located in the repair of DNA damage *per se*. An explanation could be that the defect involves the ability of AT cells to restore a disrupted chromosomal protein structure arising from the helix distortions produced either by the damage itself or by its repair. It is well established that during UV-induced repair processes transient structural alterations in the chromatin occur (37). If conformational abnormalities in the chromosomes after introduction of certain types of DNA damage would be relatively persistent in AT cells, this could have

[4] A. R. Lehmann, personal communication.

an influence on the regulation of various processes, *e.g.*, DNA replication, DNA repair, poly(ADP-ribose) synthesis, and the fixation of these structural changes into the ultimate aberrations seen in mitotic chromosomes.

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PAPER III

THE LANCET, AUGUST 29, 1981

RAPID DIAGNOSTIC TEST FOR ATAXIA TELANGIECTASIA

SIR,—Cells from patients with ataxia telangiectasia (AT) exhibit abnormally high levels of chromosomal aberrations and increased killing after exposure to ionising radiation.[1] In cultured AT lymphoblastoid cell lines[2,3] and skin fibroblasts[4,5] the rate of DNA synthesis is inhibited by radiation to a significantly lesser extent than it is in cells from normal individuals. Our experiments (to be published elsewhere) indicate that this abnormality is a general characteristic of all AT fibroblast cell strains and correlates with cellular hypersensitivity in AT to chemical carcinogenic agents. We report here that abnormal rates of DNA replication are seen after irradiation of phytohaemagglutinin (PHA) stimulated AT lymphocytes as well, and we suggest that this fact can be used in a rapid, simple biochemical test to support the clinical diagnosis of AT.

Lymphocytes were obtained from 8–15 ml of heparinised blood by 1 *g* sedimentation for 2 h, cultured for 2 days in the presence of PHA, and labelled overnight with ^{14}C-thymidine. The cells were then exposed to 300 kV X-rays and labelled for 4 h with ^{3}H-thymidine. The ratio of ^{3}H to ^{14}C radioactivity in trichloroacetic acid precipitates of the cells was taken as a measure of the rate of DNA synthesis. For different doses of X-rays inhibition curves were obtained, as shown in the figure.

There was only a small variability in the profiles obtained from serial tests performed on lymphocytes of the same individual (A and B) and the variation between different normal individuals was small as well (D). In the lymphocytes from three patients, clinically diagnosed as having AT and in two of whom this diagnosis was confirmed cytogenetically, the inhibition of DNA synthesis was less pronounced. D shows that the test is informative with the use of only one X-ray dose (e.g., 3 krad). A clear separation was seen between the ranges obtained in patients and controls, but obligate heterozygotes could not be distinguished from the normal individuals. Since the results are expressed as percentages, they were not influenced by the response of the cells to PHA, which was abnormally weak in two of the three patients, in accordance with observations from others.[6,7]

The procedure described here is more rapid and simple than other laboratory tests for AT, which use cultured fibroblasts or Epstein-Barr virus transformed lymphoblasts. It takes several weeks to establish these cultures and cytogenetic analysis of PHA-stimulated lymphocytes requires highly trained laboratory personnel and is time-consuming. Since the present test is rapid and can easily be scaled down to very small samples it should in principle be suitable for prenatal diagnosis on amniotic fluid cells.

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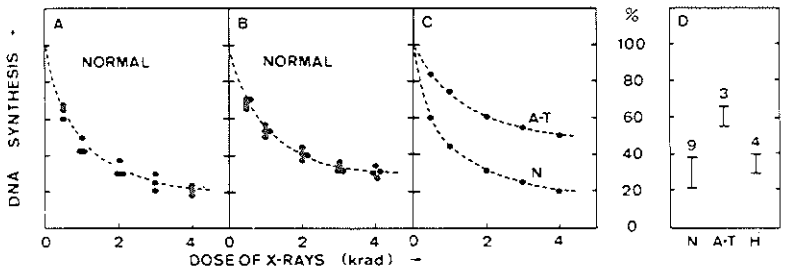

Inhibition of DNA synthesis in PHA-stimulated lymphocytes.
Serial tests on normal individuals were spread over a period of one month (A, three tests) or eight months (B, four tests). Panel C shows an example of a test on an AT patient. Bars and numerals in panel D indicate the range of the values after 3 krad of X rays and the number of different individuals tested. A-T: ataxia telangiectasia; N: normal controls; H: obligate heterozygotes for the AT mutation.

PAPER IV

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Genetics

Genetic heterogeneity in ataxia-telangiectasia studied by cell fusion

(DNA synthesis/x-rays/complementation analysis)

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ABSTRACT The effect of x-rays on the rate of semiconservative DNA replication was investigated by autoradiography in single cells obtained from normal individuals and from patients having ataxia-telangiectasia (AT). In the five AT cell strains studied, the rate of DNA synthesis was inhibited to a lesser extent than in two normal cell strains. By using this abnormal regulation of DNA replication in AT cells as a marker, an experimental procedure was developed that allowed genetic complementation analysis of AT. After Sendai virus-induced fusion of AT cells, the grains were counted over binucleate cells with both nuclei in S phase. In some cases, the inhibition of DNA synthesis caused by x-rays in the heterodikaryons was more pronounced than that in the parental homodikaryons and was comparable to that in normal binucleate cells, indicating complementation. By using this approach, the five AT cell strains that were investigated could be assigned to three complementation groups. The data suggest that extensive genetic heterogeneity exists in AT.

Ataxia-telangiectasia (AT) is a rare, inherited, cancer-prone syndrome clinically characterized by progressive neurological degeneration and immunological incompetence. The patients show a greatly exaggerated response to therapeutic treatment with x-rays (for a review, see ref. 1).

Cultured cells obtained from individuals suffering from the disorder consistently show an increased frequency of spontaneous and radiogenic chromosomal aberrations and hypersensitivity, in terms of cellular survival, to various types of ionizing radiation and to bleomycin (for recent reviews, see refs. 2 and 3). Another consistent characteristic of AT cells that was recently discovered in various laboratories (4–9) is that, in AT cells exposed to ionizing radiation, the rate of semiconservative DNA replication is inhibited to a lesser extent than in normal cells. Still very little is known about the molecular defect underlying these cellular abnormalities.

Besides the consistently observed characteristics of AT cells, varying responses to ionizing radiation in different AT cell strains are shown with respect to some other parameters. Most relevant of these is the capacity to perform repair DNA synthesis after exposure to γ-rays, which is impaired in some AT cell strains, whereas others behave normally (10–12). The existence of these two categories ("excision-deficient" and "excision-proficient") suggests the possibility of genetic heterogeneity in AT. The first genetic complementation studies of AT were carried out by Paterson *et al.* (12). In this analysis, the recovery of a normal level of γ-ray-induced repair DNA synthesis after fusion of AT cells was used as a criterion for complementation. Therefore, these investigations that show the existence of two complementation groups were necessarily restricted to the category of "excision-deficient" AT cell strains. Because the diminished inhibition of DNA replication after radiation exposure is a common characteristic of all AT cell strains, the use of this phenomenon as an indicator in complementation analysis should allow a genetic survey of "excision-proficient" AT cells as well.

This report describes the development of an experimental procedure for genetic analysis of AT by using such an approach. The results of these studies indicate that an extensive genetic heterogeneity exists in AT.

MATERIALS AND METHODS

Cell Strains and Culture Conditions. Fibroblast cell strains from AT patients were obtained from A. M. R. Taylor (Birmingham, England; AT262 and AT3BI, two different biopsies from the same patient), C. F. Arlett (Brighton, England; AT4BI and AT5BI), M. Ikenaga (Osaka, Japan; AT1OS), and J. Zaremba (Warsaw, Poland; AT1PWA). Cell strain AT3BI is defective in γ-ray-induced repair DNA synthesis, and the others are normal in this respect (refs. 10–12; unpublished data). Cells were cultured in Ham's F10 medium supplemented with 2 mM glutamine, 7.5% fetal bovine serum, 7.5% newborn bovine serum, and with penicillin and streptomycin (each, 100 units/ml). The strains were screened for mycoplasma contamination at least monthly with propidium iodide fluorescence.

Cell Fusion Procedure. Confluent cultures were trypsinized and split into two subcultures in medium containing carboxylated polystyrene microspheres ("beads") of 0.78- or 1.83-μm diameter (Polysciences, Philadelphia, nos. 7766 and 7769; final concentration, 1.8×10^5 and 0.8×10^5 beads per ml, respectively). The cells were grown at 37°C for 3 days to allow efficient uptake of the beads. Cells preloaded with different types of beads were fused by using inactivated Sendai virus (13) and were seeded on coverslips in 3-cm Petri dishes (2×10^5 nuclei per dish). When attached, they were rinsed twice with warm phosphate-buffered saline (Dulbecco variant) and cultured further at 37°C in complete medium additionally buffered with 20 mM Hepes (pH 7.4).

Inhibition of DNA Synthesis. Twenty-four hours after fusion, the cells were exposed to 300-kV x-rays in Hepes-buffered medium at room temperature (175 rad min^{-1}) and were incubated further with fresh medium at 37°C. They were pulse-labeled at indicated times for 60 min in Hepes-buffered medium containing [*methyl*-^{3}H]thymidine (46 Ci/mmol, 0.2 μCi/ml; 1 Ci = 3.7×10^{10} becquerels), rinsed twice with ice-cold phosphate-buffered saline, and fixed with Bouin's fixative. Autoradiography was performed with Ilford K-2 dipping fluid. The exposure time was 3–5 days. The grains over at least 50 S-phase nuclei were counted. Only nuclei with more than three grains were considered labeled. The relative rate of DNA replication was defined as the ratio of the mean grain numbers in irradiated and sham-irradiated cells.

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Abbreviation: AT, ataxia-telangiectasia.

RESULTS

DNA Replication in Unfused Cells. In order to investigate the rate of DNA synthesis, the cells were labeled with [^{3}H]thymidine for 60 min after exposure to x-irradiation. The amount of radioactivity incorporated by cells in S phase was determined autoradiographically. Fig. 1 shows the results from an experiment in which the kinetics of DNA replication were investigated in normal and AT cells exposed to x-rays. For this purpose, the cells were pulse-labeled at different times after irradiation with a dose of 2 krad. In both AT and normal cells, an initial decrease in the relative rate of DNA synthesis was observed, followed by a gradual recovery starting at about 4 hr after x-ray exposure. At all times after irradiation, the inhibition of DNA replication in two AT cell strains was less pronounced than in normal cells. One of the AT strains was defective in γ-ray-induced repair DNA synthesis (AT3BI), whereas the other (AT4BI) was normal in that respect. When the grain numbers were arranged in histograms, unimodal profiles were observed in all cases, which indicates that the diminished inhibition of DNA synthesis in AT is not caused by an abnormal subpopulation of cells.

Because the extent of the inhibition of DNA synthesis and its difference between AT and normal cells were maximal between 2 and 3 hr after irradiation, this time-point of labeling (see arrow in Fig. 1) was chosen for a study of the effect of x-rays on other AT cell strains. The results of this experiment (Fig. 2) indicate that the rate of DNA replication was depressed in the AT cells to a significantly lesser extent than in normal cells after exposure to doses of x-rays up to at least 4 krads. All five AT cell strains tested behaved similarly in this respect. These data indicate that autoradiographic analysis of single cells is a suitable method for the detection of differences in the relative rates of DNA replication as observed in x-irradiated AT and normal cells.

DNA Replication in Fused Cells. For genetic complementation analysis, the inhibition of DNA synthesis after x-irradiation was studied in fused AT cells. One day after fusion, the cells were irradiated and pulse-labeled 2 hr later. In the autoradiograms, the grains were counted over unfused cells in S phase and over binucleate cells that contained two S-phase nuclei.

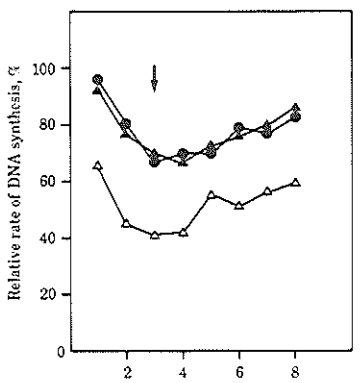

FIG. 1. Kinetics of DNA replication after exposure to 2 krad of x-rays. After irradiation, the cells were cultured for different periods in nonradiative medium, followed by 60 min in medium with [^{3}H]-thymidine, and were fixed at the times indicated in the abscissa. △, Normal cells (C5RO); ▲, AT3BI; ●, AT4BI.

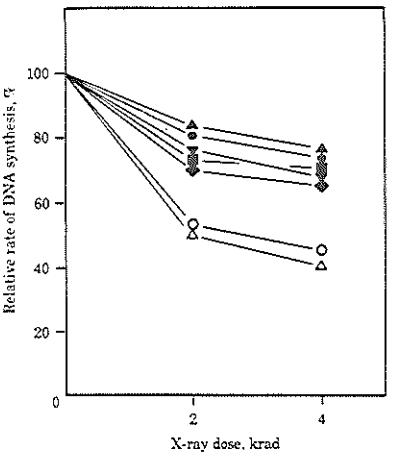

FIG. 2. Inhibition of DNA replication in various cell strains. Labeling with [^{3}H]thymidine started at 2 hr after x-ray exposure. Normal cells: C3RO (○) and C5RO (△). AT cells: AT3BI (▲), AT4BI (■), AT5BI (▼), AT1OS (●), and AT1PWA (◆).

Before fusion, the parental cells were preloaded with polystyrene beads of different sizes, which allowed an easy microscopical identification of the various types of mononucleate and binucleate cells that were present on the same slide. Fig. 3 shows the results of such an experiment, in which the "excision-deficient" AT cell strain AT3BI containing 1.8-μm beads was fused with "excision-proficient" AT4BI cells containing 0.8-μm beads. In a parallel incubation, normal C5RO cells not preloaded with beads were fused. Similar relative rates of DNA synthesis were seen in mononuclear cells and homodikaryons, but the inhibition in the normal cells was more pronounced than in the AT cells.

It appears that the relative rate of DNA synthesis after exposure to x-rays is not affected by the presence of the beads and is also not influenced by cell fusion. The inhibition of DNA synthesis in the AT heterodikaryons (AT3BI/AT4BI) was much more pronounced than in the parental homodikaryons and was comparable to that in normal homodikaryons. These results demonstrate that AT3BI and AT4BI cells complement each other with respect to their defects in the regulation of DNA replication after exposure to ionizing radiation.

Similar studies were carried out by fusion of other pairs of AT cell strains (Table 1). In the control cultures that had not been exposed to x-rays, the mean grain number over AT heterodikaryons was very near to the average of the grain numbers over the two parental homodikaryons. But after x-ray exposure, a difference in some cases between these two values ("observed" and "expected") was evident, resulting in a relative rate of DNA synthesis in heterodikaryons that was comparable to that in normal binucleate cells. In other cases, the radioactivity in the nuclei of the irradiated AT heterodikaryons was not statistically different from the mean of the two parental homodikaryons, indicating the absence of complementation.

No complementation occurred after fusion of AT3BI with AT262. This fusion served as a control, because these two strains were from different biopsies from the same patient. AT262 cells proliferated more slowly in culture than did AT3BI cells, and

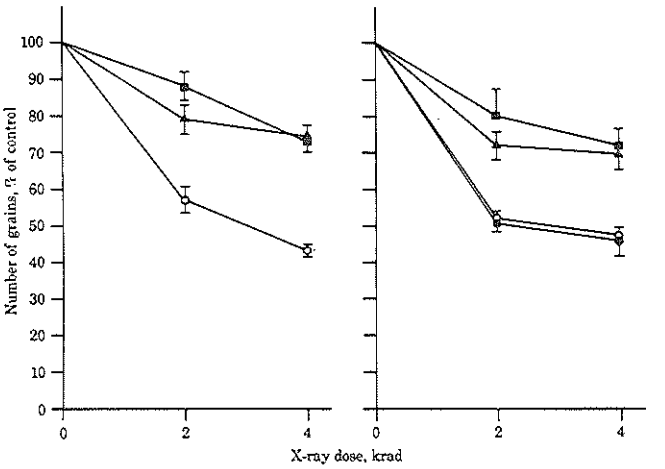

FIG. 3. Inhibition of DNA synthesis in different types of cells obtained by fusion. Conditions were as described in Fig. 2. (*Left*) Mononucleate cells. (*Right*) Binucleate cells. ○, C5RO (normal cells); ▲, AT3BI; ■, AT4BI; and ●, heterodikaryons AT3BI/AT4BI.

they were morphologically distinct—e.g., the nuclear diameter in AT262 was larger.

AT3BI cells complemented the "excision-proficient" AT cells AT4BI (the experiment in Table 1 is an independent repeat of that in Fig. 3) and AT5BI. Because AT4BI also complemented AT5BI, it follows that AT3BI, AT4BI, and AT5BI are in three different complementation groups. The absence of complementation between AT5BI and AT1OS and between AT4BI and

Table 1. Number of grains over binucleate cells after exposure to x-rays

Exp.	X-ray dose, krad	Ataxia-telangiectasia: Homodikaryons, Large beads	Ataxia-telangiectasia: Homodikaryons, Small beads	Ataxia-telangiectasia: Heterodikaryons, Observed	Ataxia-telangiectasia: Heterodikaryons, Expected*	Normal cells	Complementation
1		AT3BI	AT4BI			C5RO	Yes
	0	61.0 ± 3.7	45.6 ± 3.2	56.6 ± 2.7	53.3	55.2 ± 3.2	
	4	26.4 ± 1.4 (44%)	21.4 ± 1.1 (47%)	16.9 ± 1.4 (29%)	23.9 (45%)	13.8 ± 1.1 (25%)	
2		AT3BI	AT262				No
	4	67.5 ± 4.4	106.1 ± 5.6	89.3 ± 4.6	86.8	—	
3		AT3BI	AT5BI			C5RO	Yes
	0	111.9 ± 10.0	108.8 ± 8.6	111.1 ± 5.3	109.8	77.0 ± 3.7	
	2	83.3 ± 5.2 (74%)	81.5 ± 5.0 (75%)	65.9 ± 3.3 (59%)	82.4 (75%)	44.1 ± 2.2 (57%)	
	4	72.2 ± 2.8 (65%)	62.7 ± 2.9 (56%)	46.5 ± 2.2 (42%)	67.5 (62%)	35.2 ± 1.5 (46%)	
		AT1OS	AT5BI				No
	4	75.9 ± 2.8	60.6 ± 2.5	68.8 ± 1.7	68.3	—	
4		AT5BI	AT4BI			C5RO	Yes
	0	84.0 ± 6.8	92.1 ± 7.0	89.4 ± 5.9	88.1	110.0 ± 10.1	
	2	66.1 ± 5.0 (79%)	69.1 ± 5.2 (75%)	55.5 ± 3.9 (62%)	67.6 (77%)	65.8 ± 4.9 (60%)	
	4	58.8 ± 4.2 (70%)	62.6 ± 5.1 (68%)	47.3 ± 3.0 (51%)	60.7 (69%)	54.1 ± 3.8 (49%)	
5		AT1OS	AT4BI			C5RO	Yes
	0	—	—	—	—	62.5 ± 3.8	
	4	60.7 ± 3.9	49.8 ± 3.1	37.2 ± 2.0	55.2	31.8 ± 1.2 (51%)	
		AT4BI	AT1PWA				No
	4	45.1 ± 2.7	55.2 ± 3.4	50.4 ± 2.9	50.1	—	
		AT1OS	AT1PWA				Yes
	4	60.3 ± 2.3	64.1 ± 3.0	44.6 ± 2.3	62.2	—	

The average grain number per nucleus was determined in each binucleate cell. The given data represent the mean ± SEM of at least 25 binucleate cells. Numbers in parentheses indicate the relative rate of DNA replication.

* The "expected" value represents the calculated mean of the grain numbers obtained in the two parental homodikaryons.

AT1PWA indicates that AT1PWA is in the same group as AT4BI and that AT1OS belongs to the same group as AT5BI. Complementation occurred after fusion of AT1PWA with AT1OS, which is consistent with this group assignment.

We conclude from these results that the five AT cell strains tested fall into three separate complementation groups: two of these contain "excision-proficient" AT cell strains and another contains "excision-deficient" cells.

DISCUSSION

Our group (6, 9) and several other laboratories (4, 5, 7, 8) have shown that the inhibition of DNA synthesis in AT cells exposed to ionizing radiation is significantly less pronounced than in normal cells. In these studies, the rate of DNA synthesis was estimated by the amount of tritiated thymidine that had been incorporated by whole cell cultures as measured by liquid scintillation counting. Complementation analysis with this technique is unreliable because of the presence of a significant portion of unfused cells in the cultures and the inhibition of entry into the S phase in multinucleate cells (13). For these reasons, we have chosen to measure the rate of DNA replication at the single-cell level by performing autoradiography and counting the grains above the nuclei in S phase. This procedure is similar to that followed by Rudé and Friedberg, who studied the inhibition of DNA synthesis by UV light in xeroderma pigmentosum (14).

The data obtained by autoradiographic analysis of unfused cells confirm the results from the previous studies of AT (4–9) and show that, after x-ray exposure, the rate of DNA synthesis in single AT cells is inhibited to a lesser extent than in normal cells. The kinetics of DNA replication in x-irradiated cells were very similar to those we found with liquid scintillation assay (9). This similarity suggests that the abnormal inhibition of DNA synthesis in AT found by liquid scintillation measurements is caused by an effect only on DNA-synthesizing cells and cannot be explained by an increased efficiency of entry into the S phase by AT cells upon radiation exposure.

The study of the inhibition of DNA replication in x-irradiated fused cells shows that complementation occurred after fusion of some pairs of AT strains, whereas this was not the case in other pairs. The absence of complementation after fusion of AT262 with AT3BI cells demonstrates that the experimental results were not influenced by differences in morphological characteristics or growth potential of the cell strains used. Therefore, we consider the inhibition of DNA synthesis after x-ray exposure a suitable parameter in genetic complementation analysis of AT. This procedure can be applied for a broad genetic survey of AT cell strains because the abnormality in the inhibition of DNA synthesis is a consistent feature of cultured AT cells. The five AT cell strains investigated in our study could be assigned to three different complementation groups.

To combine our genetic data with those obtained by Paterson *et al.* (12), we also carried out fusions with AT2BE cells, which were shown by these authors to be genetically different from AT3BI. Unfortunately, the proliferation of this cell strain under our experimental conditions was very poor, resulting in an insufficient amount of S-phase nuclei in the preparations. But because our results with AT3BI indicate that differences in the level of repair DNA synthesis may have a genetic basis, it seems probable that also AT2BE cells are genetically different from the "excision-proficient" cells. Therefore, our data and those of Paterson *et al.* (12) indicate that four different complementation groups exist in AT (Table 2). We suggest a provisional nomenclature using the letters A–D. The fact that, in seven different AT cell strains, four complementation groups were identified suggests that an extensive genetic heterogeneity may exist in AT, comparable to that observed in xeroderma pigmentosum (15).

Table 2. Complementation groups in ataxia-telangiectasia

Group	Our data	Paterson *et al.**
A	AT3BI	AT3BI, AT1BE
B	—	AT2BE
C	AT4BI, AT1PWA	—
D	AT5BI, AT1OS	—

* Ref. 12.

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PAPER V

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Abnormal levels of UV-induced unscheduled DNA synthesis in ataxia telangiectasia cells after exposure to ionizing radiation

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Summary

In cultured cells from normal individuals and from patients having ataxia telangiectasia (AT) the rate of unscheduled DNA synthesis (UDS) induced by UV light was investigated by autoradiography. The number of grains in 6 different AT cell strains was similar to that observed in normal cells. Exposure of normal cells to doses of X-rays up to 20 krad had no influence on the rate of UV-induced UDS. In contrast, the UV-induced UDS was significantly modified in AT cells by treatment with X-rays. In AT cell strains that were reported to have reduced levels of γ-ray-induced repair DNA synthesis ('excision-deficient' AT cells) the effect of X-rays on UV-induced UDS was inhibitory, whereas UV-induced UDS was stimulated by X-ray exposure in 'excision-proficient' AT cell strains. Different UV and X-ray dose–response relationships were seen in the two categories of AT cell strains.

These results strongly suggest that different molecular defects are present in excision-deficient and excision-proficient AT cells. They also indicate that the altered levels of repair DNA synthesis after exposure to UV in AT cells may be a secondary consequence of the way such cells handle DNA damage caused by ionizing radiation.

Ataxia telangiectasia (AT) is a recessively inherited disorder, clinically characterized by progressive cerebellar ataxia, conjunctival telangiectasia, immunological dysfunction, abnormally high sensitivity to therapeutic radiation doses and a predis-

Abbreviations: AT, ataxia telangiectasia; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HBM, complete culture medium additionally buffered with 20 mM HEPES (pH 7.4); PBS, phosphate-buffered saline (Dulbecco variant); UDS, unscheduled DNA synthesis.

position to lymphoreticular malignancy (Boder and Sedgwick, 1957; Kraemer, 1977).

Studies, in vitro, on cultured cells from AT patients have mainly concentrated on the effects of ionizing radiation or related DNA-damaging chemical agents. After exposure to radiation, the cellular abnormalities comprise enhanced chromosome breakage and rearrangement (Taylor et al., 1976), increased killing (Taylor et al., 1975; Paterson et al., 1977) and a diminished inhibition of DNA replication (Houldsworth and Lavin, 1980; Edwards and Taylor, 1980; de Wit et al., 1981; Painter and Young, 1980; Lehmann and James, 1982; Jaspers et al., 1982). Some AT cell strains are reported to exhibit a reduced level of radiation-induced repair DNA synthesis (Paterson et al., 1976, 1977). These findings have been taken as evidence that a defect in DNA repair may be the cause of all these cellular abnormalities. Because, in a large category of AT cell strains, DNA repair appears to be normal, some authors have suggested that the defective repair observed in AT cells may be a secondary consequence of the primary genetic defect (de Wit et al., 1981; Painter and Young, 1980; Jaspers et al., 1982). These authors have postulated that an altered chromatin conformation may be involved in the abnormal response of AT cells to ionizing radiation. Based on this hypothesis it could be expected that other molecular mechanisms involving DNA, such as DNA repair and recombination, may be affected in AT cells as well. Here we report a study of the effect of exposure to X-rays on DNA repair synthesis induced by UV-irradiation. AT cells have been shown to function normally with respect to the repair of UV damage (Jaspers et al., 1982; Scudiero, 1978; Kraemer, 1977). Our experiments indicate that, in AT cells but not in normal cells, the rate of UV-induced unscheduled DNA synthesis is modified by exposure to X-rays.

Materials and methods

The human skin fibroblasts were cultured in Ham's F10 medium, supplemented with 7.5% foetal and 7.5% neonatal bovine serum, 2 mM glutamine and antibiotics (penicillin and streptomycin, each 100 U/ml). Ataxia telangiectasia cell strains were obtained from Drs. A.M.R. Taylor (Birmingham, England, AT3BI), C.F. Arlett (Brighton, England, AT4BI and AT5BI), M.C. Paterson (Chalk River, Canada, AT2BE), M. Ikenaga (Osaka, Japan, AT10S) and J. Zaremba (Warsaw, Poland, AT1PWA). AT3BI and AT2BE cells have a reduced capacity for γ-ray-induced repair DNA synthesis, while the other cells are normal in that respect (Paterson et al., 1976, 1977; and our own unpublished observations).

For repair experiments the cells were trypsinized and seeded in 3-cm petri dishes each containing a glass coverslip (10^5 cells per dish). After 1 or 2 days, the cells were rinsed with phosphate-buffered saline (PBS), exposed to UV-radiation and kept at room temperature in complete medium, additionally buffered with 20 mM HEPES (HBM, pH 7.4), for the time needed to deliver the appropriate dose of X-rays. Then this medium was removed and replaced with HBM containing [^{3}H-*methyl*]thymidine (25 Ci/mmole, 10 μCi/ml). After an incubation of 120 min at 37°C the cultures were rinsed twice with ice-cold PBS and fixed with Bouin's fixative. Autoradiogra-

phy was performed with Ilford K-2 liquid emulsion. The exposure time was 4–5 days. Grains were counted above the nuclei of 50 cells in G_1 or G_2 phase, stained with Giemsa.

UV-radiation of predominantly 254 nm was emitted from a Philips 15-W low-pressure mercury tube, providing a fluence rate of 0.94 Wm^{-2}. X-rays (300 kV) from a Philips X-ray machine were delivered at room temperature with a dose rate of 175 rad min^{-1}.

Results

To study the effects of X-rays on the level of UDS induced by UV-radiation, AT and normal cells were kept at room temperature for 30 min, exposed to UV, kept at room temperature for another 30 min and then labelled for 2 h with tritiated thymidine, followed by autoradiography.

During 1 of the 2 periods at room temperature the cells were exposed to X-rays under conditions of normal oxygen pressure. The results of this experiment are presented in Table I. The levels of UDS found after treatment with UV alone were

TABLE I

Cell strain	X-ray dose [a]	Exposure time	Mean number of grains per nucleus		
			0 Jm^{-2}	28.2 Jm^{-2}	X-ray effect [b]
C3RO	0	after UV	1.7±0.2	27.2±1.4	
	4	after UV	1.3±0.2	25.2±1.2	N.S. [c]
C5RO	0	after UV	1.3±0.4	20.0±0.9	
	4	after UV	0.7±0.3	20.3±1.0	N.S.
	4	before UV	–	19.1±0.8	N.S.
AT5BI	0	after UV	1.3±0.4	29.6±1.2	
	4	after UV	0.7±0.3	43.5±1.7	+51% ($p<0.001$) [d]
	4	before UV	–	41.4±0.5	+41% ($p<0.001$)
AT4BI	0	after UV	1.9±0.4	23.7±0.9	
	4	after UV	2.6±0.3	31.9±1.1	+34% ($p<0.001$)
AT1PWA	0	after UV	2.7±0.3	23.2±0.8	
	4	after UV	4.4±0.5	32.1±1.5	+35% ($p<0.001$)
AT3BI [e]	0	after UV	0.6±0.2	29.8±1.1	
	4	after UV	0.8±0.3	23.8±0.8	−21% ($p<0.002$)
	4	before UV	0.6±0.3	24.2±0.8	−20% ($p<0.002$)

[a] Dose in krad. Sham irradiation is represented by 0 krad.

[b] Expressed according to the formula: $100(G_{+uv}-G_{-uv})_{+X\text{-rays}}/(G_{+uv}-G_{-uv})_{-X\text{-rays}}-100$.

[c] N.S. means no significant difference between the UV-induced levels of UDS obtained with and without exposure to X-rays.

[d] p value of significance in the difference of UDS between 0 and 4 krad.

[e] AT3BI was tested in a separate experiment.

similar in normal and AT cell strains. The amount of grains after X-irradiation alone was very small and insignificant. This is in agreement with the concept of a short-patch type of excision repair that is induced by ionizing radiation. In normal cells the combined treatment with both UV and X-rays resulted in levels of UDS that did not differ from those observed after exposure to UV alone. In contrast, X-rays influenced the rate of UV-induced UDS in AT cells significantly. In the cell strains AT4BI, AT5BI and AT1PWA (all excision-proficient cells) UDS was stimulated, whereas in AT3BI (excision-deficient) cells an inhibitory effect of X-rays on UDS was seen. Reversing the order of administration of UV and X-rays did not change these differential effects on UV-induced UDS in AT and normal cell strains.

In another series of experiments the influence of various doses of X-rays on the level of UDS induced by 28.2 Jm^{-2} of UV-radiation was investigated. Fig. 1 shows that X-ray doses up to 20 krad did not exert a significant effect on UDS in normal cells, but, in the excision-proficient AT cell strains, UDS was stimulated. The X-ray effect increased with the dose until a maximal stimulation of 20–30% was achieved at about 3 krad. In the 2 excision-deficient cell strains AT2BE and AT3BI a dose-dependent reduction in UDS was found. Within the X-ray dose range studied there was no evidence for a saturation of the inhibitory effect on UDS in these cell strains.

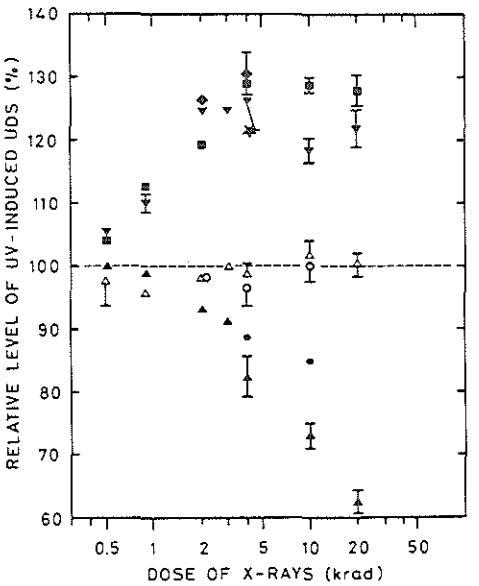

Fig. 1. Effect of various doses of X-rays on the level of UDS, induced by 28.2 Jm^{-2} of UV-radiation. After exposure to UV, cells were X-irradiated and labelled with tritiated thymidine. The relative rate of UDS is defined as $100(G_{+uv}-G_{-uv})_{+X\text{-rays}}/(G_{+uv}-G_{-uv})_{-X\text{-rays}}$ where G stands for the mean number of grains per nucleus. In case of more than 1 experiment, bars indicate the standard error of the mean. Open symbols, normal cells: C3RO (○) and C5RO (△). Closed symbols, AT cells: AT2BE (●), AT3BI (▲), AT4BI (■), AT5BI (▼), AT1PWA (◆) and AT10S (★).

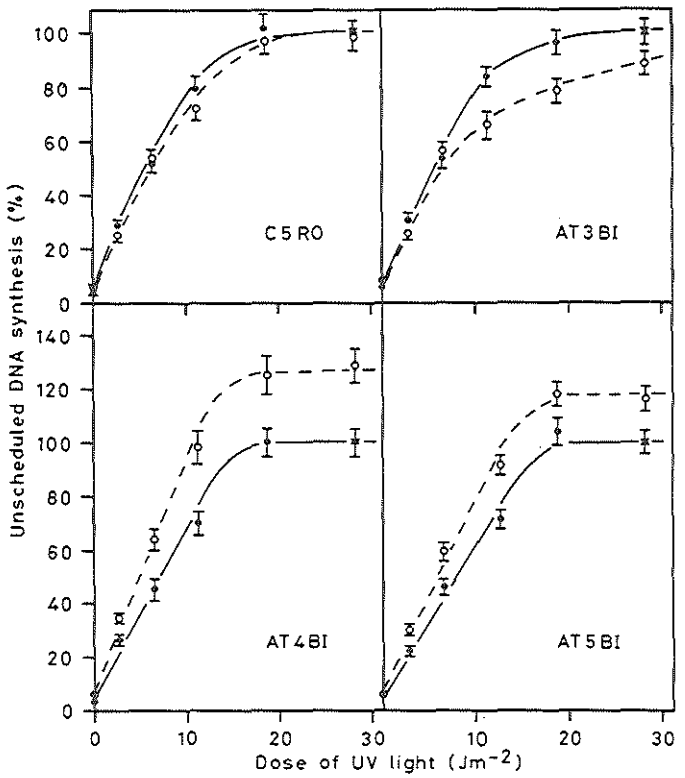

Fig. 2. Effect of X-rays on the level of UDS induced by various doses of UV-radiation. Experimental conditions were the same as for Fig. 1. Symbols and bars indicate the mean and standard error of the number of grains over 50 cells in G_1 or G_2 phase. Closed symbols, sham-irradiated cells; open symbols, 4 krad of X-rays. For ease of comparison, the grain numbers were normalized to 100% by using the value after 28.2 Jm^{-2} and no X-rays, as indicated by the asterisk (★).

The data in Fig. 2 illustrate the effect of a single dose of X-rays on cells irradiated with various fluences of UV-radiation. Without exposure to X-rays a characteristic saturation of UDS was observed at doses above 20 Jm^{-2} in both normal and AT cells. Exposure to X-rays did not significantly change the pattern in normal cells. In excision-proficient cell strains the UDS was stimulated by X-rays at all doses of UV, the amount of stimulation being proportional to the level of UDS. Maximal levels of UDS occurred at the same UV doses as in cells not exposed to X-irradiation. In excision-deficient AT3BI cells, the UV-induced UDS found after exposure to X-rays was less than in control cells and did not reach a saturating level at doses up to about 30 Jm^{-2}. In a separate experiment, AT3BI cells were exposed to 47 Jm^{-2} and a significant effect of 4 krad of X-rays was not observed (data not shown). In excision-deficient AT cells the maximal level of UV-induced UDS was apparently independent of exposure to X-rays, but it was reached at a higher dose of UV-radiation.

Discussion

In the present study, we demonstrated that the rate of UV-induced UDS is modified by exposure to ionizing radiation in AT cells, but not in normal cells. With respect to the normal cells, these data are in agreement with findings recently reported by Gruenert and Cleaver (1981). The response of the AT cells was related to their capacity to perform repair DNA synthesis after introduction of DNA damage by ionizing radiation alone. In the category of excision-deficient AT cells, exposure to X-rays inhibited the rate of UV-induced UDS, whereas in excision-proficient cells the X-rays acted in a stimulatory manner. In the two types of AT cell strains the relationship between the effect of X-rays on UV-induced UDS and the dose of UV or X-rays was also different. These results suggest that the molecular defects in the two categories are distinct, which is in agreement with the results from our recent genetic study of AT, which show complementation between excision-proficient and excision-deficient AT cells (Jaspers and Bootsma, manuscript submitted).

Based on the finding that the relative rate of the 'scheduled' (S-phase-dependent) DNA synthesis is abnormally high in X-irradiated AT cells, a hypothesis was put forward that suggests that an altered chromatin conformation may be responsible for the abnormal behaviour of AT cells after exposure to X-rays (de Wit et al., 1981; Painter and Young, 1980; Jaspers et al., 1982). The modified rates of UV-induced unscheduled DNA synthesis described here fit this hypothesis. If such conformational abnormalities exist, our data indicate that they are induced by DNA damage of the type caused by ionizing radiation, since AT cells not exposed to X-rays appear to function normally. At present, it is not clear which type(s) of chromatin alteration could be responsible for the cellular abnormalities, including the effects of X-rays on UV-induced UDS.

The level of UV-induced UDS is expected to be dependent on two factors. First, the number of UV lesions that is repaired during the period of labelling with tritiated thymidine. This factor is determined by: (1a) the number of damaged sites that is induced; (1b) the concentration of active enzyme molecules and cofactors; (1c) the 'accessibility' of the UV lesions to the repair enzymes; and (1d) the time that is needed to complete a single repair event. Second (2), the level of UDS is dependent on the amount of thymidine that is incorporated per repaired lesion, which is equivalent to the length of the repair-replicated patch of DNA.

Regarding factor 1a, it is possible that DNA, packed in an altered chromatin structure, is more susceptible to UV-radiation, resulting in a different yield of photo-induced DNA lesions. This mechanism is probably not responsible for the X-ray effects on UV-induced UDS in AT, because the order in which the 2 treatments were administered had no influence. In view of the normal levels of UV-induced UDS in AT cells not exposed to X-rays, it appears that a normal amount of UV-specific repair enzymes (factor 1b) is present in AT. A third possibility (1c) is that UV lesions situated in an abnormal chromatin structure are less accessible to the repair enzymes. In that case, it is expected that the maximal level of UV-induced UDS will be similar in X-irradiated and unirradiated cells, but

this level will be reached at a different dose of UV. The UV dose–response relationship that was observed in excision-deficient AT cells is consistent with this assumption. In excision-proficient AT cells the UV dose at which a maximal rate of UDS was reached was not changed by exposure to X-rays. The higher saturation levels of UDS seen after X-irradiation of these cell strains are compatible both with a reduced repair time per lesion (factor 1d) and with an increased size of the repaired patch (factor 2). Our data do not distinguish between these two possibilities.

The rate of repair DNA synthesis after exposure of excision-defective AT cells to ionizing radiation alone may be reduced by a mechanism similar to the one that operates in UV-induced repair. Repair synthesis of DNA after ionizing radiation is usually measured after high doses of radiation. The inhibitory action of the highest dose of X-rays that we used (20 krad) resulted in a residual level of UV-induced UDS of about 65% in AT3BI cells. This value is comparable to the residual level of γ-ray-induced repair DNA synthesis in AT3BI of about 50% as observed by Paterson et al. (1976), who used radiation doses of 30–100 krad. Therefore, the DNA repair deficiency in AT would be a secondary consequence of the primary genetic defect, which could provide an explanation for the absence of a correlation between reduced DNA repair levels and increased lethality in AT after exposure to radiation.

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