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Effect of DNA-damaging Agents on Isolated Spleen Cells and Lung Fibroblasts from the Mouse Mutant "Wasted," a Putative Animal Model for Ataxia-Telangiectasia[1]

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ABSTRACT

Spleen cells from control and wasted (*wst*) mice, a putative animal model for the human genetic disease ataxia-telangiectasia, were tested for inhibition of replicative (semiconservative) DNA synthesis after treatments with bleomycin, γ-irradiation, 4-nitroquinoline 1-oxide, and ultraviolet irradiation. The wasted cells were found to be more resistant than control cells to the first three treatments, but equally sensitive to ultraviolet light. Bleomycin-stimulated repair synthesis in spleen cells was also studied by the CsCl/bromodeoxyuridine method and found to be similar in cells from wasted and control animals. Similarly, no differences in sensitivity to killing by γ-rays, as manifested by relative cloning efficiencies, were demonstrated between primary lung fibroblasts from mutant and control mice. We concluded that observed defects in DNA repair in wasted cells are not identical to those reported in human cells from ataxia-telangiectasia patients.

INTRODUCTION

A-T[5] is a human autosomal recessive disease characterized by a progressive neurological disorder, a marked IgA deficiency, an increased predisposition to cancer, and a spontaneous chromosomal instability. Hypersensitivity to ionizing radiation of cells from A-T patients, expressed as reduced viability and increased cytogenetic damage in cultured fibroblasts, has suggested defective DNA repair in this disease (1, 2). Inoue *et al.* (3-5) and Edwards *et al.* (6) demonstrated a reduced level of the primer activating enzyme in cellular extracts from A-T cells which enhances the priming activity of γ-irradiated DNA for purified DNA polymerase. These findings support the hypothesis that one of the causal factors of A-T is a DNA repair defect.

Recently, Shultz *et al.* (7) described a mouse mutant, wasted (*wst*), which shows pathological changes in central nervous and lymphoid systems and exhibits increases in the frequency of spontaneous, as well as γ-ray-induced, chromosomal aberrations. These findings prompted us to evaluate this mouse mutant as an animal model for A-T. In a previous paper, we reported that both cytogenetic and biochemical defects developed age dependently in specific tissues from wasted mice (8). In this paper, we describe the effects of some DNA-damaging agents on replicative DNA synthesis as well as on repair DNA synthesis in isolated spleen cells of wasted mice. We also describe the results of colony-forming assays performed to examine the sensitivity of wasted mouse lung fibroblasts to the killing effect of ionizing radiation.

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[5] The abbreviations used are: A-T, ataxia-telangiectasia; MEM, minimal essential medium; 4NQO, 4-nitroquinoline 1-oxide; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; MEM-SP, minimal essential medium supplemented with 0.2 mM of serine and 1 mM of sodium pyruvate.

MATERIALS AND METHODS

Chemicals. Bleomycin hydrochloride, purchased from Nihon Kayaku Co., Tokyo, Japan, was dissolved in Eagle's MEM immediately before use. 4NQO, obtained from Wako Pure Chemicals Co., Tokyo, Japan, was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml and stored at −20°C. The stock solution was diluted with MEM just prior to use. Collagenase (type I) was obtained from Sigma, St. Louis, MO. [*methyl*-^{3}H]Thymidine (25 Ci/mmol) was a product of Amersham, Buckinghamshire, United Kingdom.

Animals. Mice were maintained in a heterozygous state in a closed colony system in our laboratory. Affected homozygotes (*wst/wst*) were first recognized at 20 to 22 days of age by low body weights and neurological abnormalities including tremor and uncoordinated body movements. Diagnosis was finally confirmed by low spleen weight (7, 8). On average, two of every three unaffected littermates with a normal appearance are heterozygous for the *wst* gene. However, since any differences among the littermates were undetectable, they were pooled and served as controls.

Isolation of Spleen Cells. The spleen was quickly removed from the animal at 24 days of age following decapitation and then chilled in ice-cold MEM. Spleens from two to four animals of each experimental group were pooled and processed together. The following procedures were carried out at 4°C except where noted. The spleens were disrupted by passage through a fine stainless steel mesh with a rubber policeman with the aid of a few drops of MEM, and the cells were gently suspended in MEM with a Pasteur pipet. The suspensions were allowed to stand for 5 min to eliminate debris and centrifuged at 120 × g for 5 min. The sedimented cells were washed twice with MEM by centrifugation in the same manner and finally suspended in MEM. After staining with Nigrosin, viable cell densities were determined with a microscope.

Measurement of DNA Synthesis Inhibition. Spleen cells at a density of 4×10^6 cells/ml were treated with DNA-damaging agents in one of three ways: (*a*) exposure for 2 h at 37°C to various concentrations of bleomycin or 4NQO in MEM supplemented with 2% FBS, dialyzed against MEM; (*b*) exposure to γ-rays from a ^{137}Cs source at various doses for an isoexposure time of 36 min; (*c*) exposure to UV-light of various doses from a germicidal lamp at a rate of 2.0 J/m^2/s. For UV irradiation experiments, cells were dispensed into microwells in tissue culture trays at a density of 1×10^6 cells/50 μl/well. The irradiations were done at ambient temperature. Following the treatment, the cells were incubated with 25 μCi of [^{3}H]thymidine per ml in the presence of 2% FBS (dialyzed against MEM) for 20 min and chilled in an ice-water bath. Duplicate samples of 40 μl from the reaction mixture were withdrawn and spotted on glass filters (Whatman GF/F) which had been soaked with 100 μl of 1% SDS. After standing for 5 min at room temperature, filters were extensively washed with 5% trichloroacetic acid containing 10 mM sodium pyrophosphate, dried, and counted with a liquid scintillation spectrometer. Nontreated spleen cells incorporated 10,900 to 29,700 dpm of radioactivity per assay which corresponded to 1.24 to 3.37 pmol of [^{3}H]thymidine per 10^6 cells. The ratio of radioisotope incorporation in treated and corresponding control samples was taken as a measure of the inhibition of DNA synthesis. Since the level of DNA synthesis observed in each preparation of cells differed significantly from experiment to experiment, we performed two to three independent determinations for each DNA-damaging agent using animals from different litters. The absolute values of DNA synthesis are given in the legend to each figure.

Measurement of DNA Repair Synthesis after Bleomycin Treatment. Spleen cells in MEM at a density of 8×10^6 cells/ml were preincubated

with 10 μM bromodeoxyuridine in the presence of 2% FBS (dialyzed against MEM) for 10 min at 37°C. After the preincubation, bleomycin and [^{3}H]thymidine were added to the mixture at final concentrations of 1 mg/ml and 50 μCi/ml, respectively. The total volume of the incubation mixture was 4 ml. After incubation at 37°C for 2 h, the cells were collected by centrifugation at 1000 × *g* for 10 min and dissolved in 4 ml of 8 M urea/1% SDS/10 mM EDTA/160 mM sodium phosphate, pH 6.8. The resulting viscous solution was passed 10 times through a hypodermic needle (22 gauge) in order to reduce the molecular weight of DNA. The DNA was purified by extraction with chloroform/phenol followed by column chromatography on hydroxyapatite, as described by Adriaenssens *et al.* (9), and finally dialyzed against 1 mM EDTA/10 mM Tris-HCl, pH 7.4. To the purified solution of DNA (3.6 ml), 0.36 ml of 1.0 N NaOH was added together with 3.83 g of CsCl and 0.88 g of Cs_2SO_4. The solution was then centrifuged at 45,000 rpm for 15 h at 15°C in a Hitachi RP67VF vertical rotor. After the centrifugation, the gradient was divided into 22 fractions, and both absorbance at 260 nm and radioactivity were determined. Fractions containing DNA with normal density were pooled, dialyzed against 1 mM EDTA/10 mM Tris-HCl, pH 7.4, and subjected to a second centrifugation in alkaline CsCl gradients under the conditions described above. DNA with normal density was again collected and subjected to a third centrifugation under identical conditions. Three centrifugations were needed to obtain a clear radioactivity peak corresponding to repair synthesis. The radioactivity of the samples was determined with the aid of toluene/Triton scintillation fluid after neutralization with HCl.

Lung Fibroblasts and Colony-forming Assay. Primary cultures of fibroblasts were initiated from lungs of mice at various ages. Minced lung tissue was digested with 0.05% collagenase in MEM-SP containing 2% FBS for 4 h at 37°C. Cells were suspended by pipeting, washed by centrifugation (80 × *g*, 4 min) with MEM-SP, and incubated for several days in MEM-SP supplemented with 20% FBS. Propagated cells were collected by trypsinization and exposed to γ-irradiation in 0.15 M NaCl/67 mM sodium potassium phosphate, pH 7.0, at 0°C at a dose rate of 1.2 Gy/min from a ^{137}Cs source. Immediately after the irradiation, cells were plated at densities of 1000 to 4000 cells per 6-cm dish in MEM-SP with 20% FBS. Five dishes were used for each experimental point. After 4 wk of incubation, colonies were fixed, stained with Giemsa, and counted. A colony containing 50 cells was counted as a survivor.

RESULTS

DNA Synthesis Inhibition. The extent of DNA synthesis in spleen cells from wasted and littermate control mice after exposure to increasing concentrations of bleomycin for 2 h is shown in Fig. 1. Under these conditions, DNA synthesis in normal cells was inhibited to 20 to 23% of the control value after exposure to the drug at 200 μg/ml, whereas in cells from wasted mice, synthesis was inhibited only to 65 to 70% of the control value. 4NQO was also less effective in inhibiting DNA synthesis in cells from wasted mouse than in normal cells (Fig. 2). In Fig. 3, the effect of γ-irradiation on DNA synthesis is shown, and although the difference in the sensitivity between the two types of cells was not as large as in the case of bleomycin or 4NQO, a significantly reduced DNA synthesis inhibition in wasted mouse cells was reproducibly observed.

In order to test whether the diminished DNA synthesis inhibition in spleen cells of wasted mice occurs when the cells are exposed to other types of DNA-damaging agents, we examined the effects of UV-light on DNA synthesis. In contrast to the results obtained with γ-irradiation or with the above-noted chemicals, an equal extent of DNA synthesis inhibition was observed in cells from wasted mice and from controls after exposure to UV light (Fig. 4), suggesting that any anomaly in DNA metabolism of the spleen cells of wasted mice was specific for certain types of DNA damage.

DNA Repair Synthesis after Bleomycin Treatment. DNA repair synthesis in isolated spleen cells was measured by the bromodeoxyuridine density label method which enables the separation of the DNA synthesis due to repair from that due to extensive chain elongation by semiconservative replication. Under conditions used in the present study, it was necessary to repeat the density equilibrium centrifugation 3 times for clear

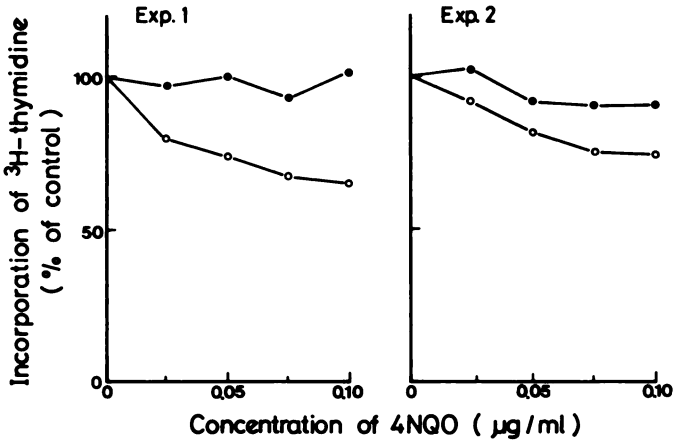

Fig. 2. Effect of various concentrations of 4NQO on DNA synthesis in isolated spleen cells of wasted (●) and control mice (○). The absolute levels of DNA synthesis in nontreated cells were: 10,900 (wasted) and 24,400 (control) dpm/assay in Experiment 1; and 18,800 (wasted) and 18,900 (control) dpm/assay in Experiment 2.

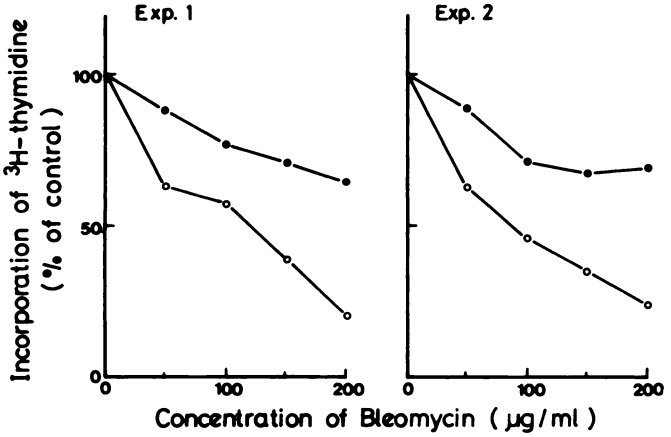

Fig. 1. Effect of various concentrations of bleomycin on DNA synthesis in isolated spleen cells of washed (●) and control mice (○). The absolute levels of DNA synthesis in nontreated cells were: 23,300 (wasted) and 19,500 (control) dpm/assay in Experiment 1; and 13,000 (wasted) and 19,900 (control) dpm/assay in Experiment 2.

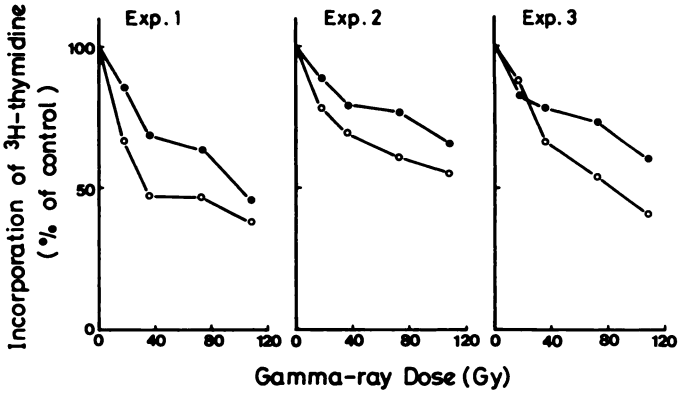

Fig. 3. Effect of various doses of γ-rays on DNA synthesis in isolated spleen cells of wasted (●) and control mice (○). The absolute levels of DNA synthesis in nonirradiated cells were: 20,700 (wasted) and 11,500 (control) dpm/assay in Experiment 1; 14,100 (wasted) and 16,700 (control) dpm/assay in Experiment 2; and 21,000 (wasted) and 29,700 (control) dpm/assay in Experiment 3.

separation of repair synthesis from replicative synthesis in the banding pattern. Measurements of repair synthesis, which is determined as a specific activity of DNA of normal density, are shown in Table 1. This table clearly demonstrates that the cells from wasted mice are quite similar to those from normal mice in respect to their ability to repair damages produced by bleomycin. The trace amount of incorporation in the cells without bleomycin treatment may be ascribed to DNA damage produced during the isolation and/or incubation of the spleen cells *in vitro*.

Colony-forming Assay Using Lung Fibroblasts. Hypersensitivity of fibroblasts to the lethal effect of γ-rays is one of the most characteristic phenotypes of A-T. Evaluation of the sensitivity of cells from wasted mice to γ-rays, however, has not given conclusive results partly because of poor cloning efficiency of cells derived from the mice (10). We therefore carefully examined the conditions for the initiation and maintenance of primary lung fibroblasts from wasted mice. The following points are important for obtaining optimal plating efficiency: (*a*) selection of a good lot of collagenase; (*b*) appropriate digestion time in the presence of FBS; and (*c*) use of cells in the rapidly growing phase of the first passage. Under these conditions, we obtained a cloning efficiency of around 1%, which was the minimum needed for the colony-forming assay. Representative experiments performed to compare the sensitivity of fibroblasts of wasted mice with that of normal mice are shown in Table 2. As shown in this table, a difference in the sensitivity to the killing effect of γ-rays was not detectable between the fibroblasts from wasted *versus* control mice at 24 days of age, at which time degeneration of spleen cells as well as elevated frequency of chromosomal aberrations in bone marrow cells became apparent in wasted mice (8).

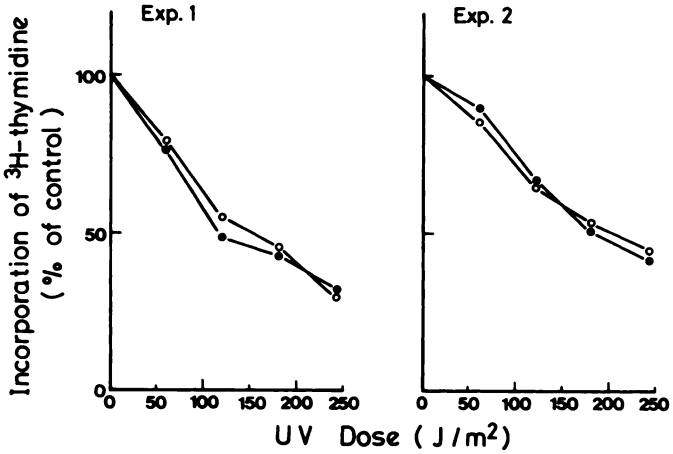

Fig. 4. Effect of various doses of UV-light on DNA synthesis in isolated spleen cells of wasted (●) and control mice (○). The absolute levels of DNA synthesis in nonirradiated cells were: 26,500 (wasted) and 19,500 (control) dpm/assay in Experiment 1; and 26,600 (wasted) and 28,100 (control) dpm/assay in Experiment 2.

Table 1 *DNA repair synthesis in spleen cells from wasted and normal mice after treatment with bleomycin*

Repair synthesis was measured by the bromodeoxyuridine density label method as described in "Materials and Methods." The amount of repair synthesis was determined from patterns of alkaline CsCl density equilibrium centrifugation by quantitating the amounts of [³H]thymidine radioactivity and DNA under the normal density DNA peak (peak fraction only).

Bleomycin treatment (μg/ml)	Animal	Repair synthesis (dpm/μg DNA)
0	Control	8.6
1000	Control	71.1
0	Wasted	5.7
1000	Wasted	72.6

DISCUSSION

The absence of the normal inhibition of replicative DNA synthesis following γ-irradiation (11–13) or treatment with radiomimetic drugs including bleomycin and neocarzinostatin (10, 14–17) is an inherent characteristic of A-T cells. This may have some relationship with the cytological observation by Zampetti-Bosseler and Scott (18) that A-T cells exhibited less mitotic delay after X-irradiation than did normal cells. A possibility has been suggested that the primary defect in A-T is a disturbance in the regulation of DNA replication on damaged templates (13).

Recently, we found the absence of a γ-ray-induced decrease in the mitotic index in bone marrow cells of wasted mice.[6] This observation is confirmed by the results of the present investigation which show that DNA synthesis is more resistant to γ-rays in wasted mouse spleen cells than in control cells. Bleomycin was also less effective in inhibiting DNA synthesis in the wasted mouse cells than in control cells. On the other hand, UV-light, to which A-T cells exhibit normal responses, did not distinguish wasted mouse cells, from those of normal animals as to their sensitivity to DNA synthesis. These results indicate that any defect in DNA metabolism of spleen cells of wasted mice may be specific for damage induced by γ-rays or by an agent which mimics γ-rays. Our results with 4NQO, which is primarily a UV-mimetic DNA-damaging agent, are different from those of Smith and Paterson (12) who found that this agent inhibited DNA synthesis in A-T and normal cells to a similar extent. This discrepancy may occur because 4NQO does not necessarily produce a unique lesion, but rather multiple damage to DNA, some of which may resemble the damage produced by γ-irradiation.

We have previously reported that the primer activating enzyme, which enhances the priming activity of γ-irradiated DNA for purified DNA polymerase, is deficient in extracts of both A-T fibroblasts (3–5) and spleen cells from wasted mice (8). Edwards *et al.* (6) also reported that the primer activating enzyme activity in cellular extract from A-T cells, but not from xeroderma pigmentosum cells, was significantly lower than that from normal human cells. Recently, Cornforth and Bedford reported that the fraction of X-ray-induced breaks in prematurely condensed chromosomes of A-T cells was several times greater than that of normal cells (19). These data indicated that DNA metabolism, possibly DNA repair, was defective in cells from A-T patients as well as from wasted mice. Another line of evidence which supports the idea of abnormal DNA metabolism in A-T cells is that the cells exhibit reduced repair replication stimulated by treatments with DNA-damaging agents including γ-rays (20–22). Scudiero (23) reported that nitrosoguanidine-stimulated repair synthesis was also reduced in some, but not all, A-T strains as compared with controls. In the present report, the data from experiments designed to determine the level of repair replication after treatment with bleomycin showed no difference between spleen cells from wasted and control mice (Table 1). As described in our previous paper (8), the primer activating enzyme activity decreased age dependently in wasted mouse spleen. A decrease in repair replication, therefore, may be detected in cells isolated from older animals. It was, however, difficult to obtain a sufficient number of viable cells from mice older than 25 days of age.

Another important approach to evaluating the wasted mouse as a model of A-T is to compare the relative colony-forming ability of fibroblasts from wasted and control mice after γ-

[6] Unpublished data.

Table 2 *Survival of γ-irradiated primary lung fibroblasts from wasted and control mice at 24 days of age*

Dose (Gy)	Animal	No. of inoculated cells/dish	Colonies/dish	Mean ± SE	Cloning efficiency (%)	Relative survival
Experiment 1						
0	Control	1000	7, 4, 10, 9, 18	9.6 ± 2.3	0.96 ± 0.23[a]	1.00
2	Control	2000	9, 8, 18, 12, 15	12.4 ± 1.9	0.62 ± 0.09	0.65 ± 0.18
4	Control	3000	7, 7, 5, 8, 14	8.2 ± 1.5	0.27 ± 0.05	0.28 ± 0.09
6	Control	4000	1, 4, 3, 6, 3	3.4 ± 0.8	0.09 ± 0.02	0.09 ± 0.03
0	Wasted	1000	2, 4, 1, 10, 2	3.8 ± 1.6	0.38 ± 0.16	1.00
2	Wasted	2000	4, 5, 5, 4, 5	4.6 ± 0.2	0.23 ± 0.01	0.61 ± 0.26
4	Wasted	3000	3, 2, 3, 3, 3	2.8 ± 0.2	0.09 ± 0.01	0.25 ± 0.01
6	Wasted	4000	3, 1, 3, 1, 1	1.8 ± 0.5	0.05 ± 0.01	0.12 ± 0.06
Experiment 2						
0	Control	1000	8, 7, 7, 13, 11	9.2 ± 1.2	0.92 ± 0.12	1.00
2	Control	2000	9, 17, 7, 8, 12	10.6 ± 1.8	0.53 ± 0.09	0.58 ± 0.12
4	Control	3000	4, 2, 7, 4, 5	4.4 ± 0.8	0.15 ± 0.03	0.16 ± 0.04
6	Control	4000	3, 2, 5, 8, 3	4.2 ± 1.1	0.11 ± 0.03	0.11 ± 0.03
0	Wasted	1000	5, 6, 7, 4, 8	6.0 ± 0.7	0.60 ± 0.07	1.00
2	Wasted	2000	4, 6, 10, 7, 10	7.4 ± 1.2	0.37 ± 0.06	0.62 ± 0.12
4	Wasted	3000	4, 4, 7, 5, 4	4.8 ± 0.6	0.16 ± 0.02	0.27 ± 0.05
6	Wasted	4000	3, 5, 2, 1, 2	2.6 ± 0.7	0.07 ± 0.02	0.11 ± 0.03

[a] Mean ± SE.

irradiation. Conclusive results had not been obtained previously because of extremely poor cloning efficiency of primary mouse fibroblasts (10). By using improved conditions for initiation and maintenance of primary lung fibroblasts, we could not detect any significant difference in the sensitivity to the killing effect of γ-rays of lung fibroblasts from wasted mice at 24 days of age.

The results described in the present and previous paper (8) show that certain abnormalities seen in cells from A-T patients are not observed in wasted mice, although the wasted mutation shares many characteristics with A-T. Among the dissimilarities, the most important may be that the lung fibroblasts of wasted mice were not hypersensitive to the killing effect of γ-rays although bone marrow cells in wasted mice exhibited many more radiation-induced chromosomal aberrations (8). It is noteworthy here that anomalies in wasted mice developed in a tissue-specific and age-dependent manner (8). It is thus reasonable to speculate that certain characteristics which are observed only in A-T but not in wasted mouse cells may become evident in wasted mice if other types of cells from mice of different ages are examined. Since A-T comprises a heterogeneous group of diseases with similar but not identical features, it is not surprising that a single gene mutation in the mouse produces some but not all the features attributed to human A-T. The wasted mouse may provide a useful model for investigating the relationships among neuropathology, immune deficiency, and defective DNA metabolism, although it may not be an exact animal homologue for A-T.

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