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Processing of DNA Damage after Exposure to a Single Dose of Fission Spectrum Neutrons takes 40 Hours to Complete

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DISCLAIMER

We have examined the long-term (days) fate of breaks induced in the DNA of human P3 epithelial teratocarcinoma cells by a single dose of JANUS fission-spectrum neutrons (mean energy 0.85 MeV). We used alkaline-filter elution methods that assay totality of single- and double-strand breaks, generally referred to as single-strand breaks (SSBs). When the cells are allowed a period of repair incubation, these breaks are totally sealed by 7 hours after the original exposure, but following the initial repair the DNA is dismantled, as revealed by the reappearance of SSBs. This secondary breakage is almost as extensive as that caused by the original neutron exposure, with a maximum at 16-18 hours after irradiation. Finally, the DNA is once again rejoined, regaining its original size by 40 hours after irradiation. The secondary repair phenomenon may have an editing function, or it may represent the processing of residual damage left unrepaired during the initial rejoining of the backbone breaks.

1. Introduction

Ionizing radiation, such as neutrons, of higher linear energy transfer (LET) than gamma- or x-radiation, have long been known to be more efficient per unit absorbed dose in the killing of cultured cells (Hall *et al.* 1975; Field, 1976; Ngo *et al.* 1977). We confirmed this in our laboratory for human P3 cells exposed to fission-spectrum neutrons from the JANUS reactor (Hill *et al.* 1988, who discussed the use of these cells in radiobiological research). Neutrons have also been shown to be more efficient than low-LET radiations in cellular mutagenesis (Hei *et al.* 1988; Grdina *et al.* 1988, 1989), transformation (Han and Elkind 1979; Hill *et al.* 1985; Miller *et al.* 1988), the production of chromosome aberrations (Schwartz *et al.* 1988), and tumorigenesis (Thomson *et al.* 1981). The reason for this greater relative biological effectiveness (RBE) of neutrons is not known.

DNA damage, which is clearly implicated in mutagenesis as well as transformation (Reddy *et al.* 1982; Weinberg 1989), is also considered to be the critical target for radiation-induced cell death, and the possible importance of unrepaired DNA damage in lethality has been discussed (Dugle *et al.* 1976; Ritter *et al.* 1977; Painter *et al.* 1974; Painter 1980). However, studies of DNA damage and repair have revealed an RBE of less than one for the induction of DNA damage by neutrons. Our measurements of DNA single-strand breaks (SSBs) using the technique of alkaline elution (which measures the sum of all frank strand breaks, including double-strand breaks and alkali-labile sites) confirm the recent observations that these lesions are induced less

efficiently by neutrons than by gamma- or x-rays (Furuno *et al.* 1979; van der Schans *et al.* 1983; McWilliams *et al.* 1983; Peak *et al.* 1989). Further, measurements of DNA damage using the techniques of neutral sedimentation and neutral elution, assays that are considered to measure primarily double-strand breaks (DSBs), have shown that neutrons having RBE values between 1 and 2 are not significantly more efficient than gamma rays at inducing these lesions (Furuno *et al.* 1979; Maki *et al.* 1986; Prise *et al.* 1987; Fox and McNally 1988; Grdina *et al.* 1989). We have confirmed an RBE of 1 for the induction of DSBs by JANUS fission-spectrum neutrons (Peak and Peak, unpublished information).

These results suggest that a higher proportion of the DNA damage caused by neutrons, compared with that caused by radiation of lower LET, must lead to cell death, which implies that the lesions induced by neutrons might be more severe than those induced by low-LET radiation, since their repair might be more difficult. We recently produced evidence of this in studies of the repair of SSBs induced by gamma-rays and fission-spectrum neutrons. In both a rodent (Chinese hamster lung V79) and a human (P3 epithelioid) cell line, neutrons induced SSBs (as assayed by alkaline elution) that were refractory to repair. A statistically significant fraction of SSBs induced by 6 Gy of neutrons remained unsealed after 150 min, whereas the larger number of breaks caused by exposure to 3 Gy of gamma-rays were all rapidly rejoined, with the process complete after 90 min (Peak *et al.* 1989).

The possibility exists that these unrepaired DNA breaks after a 6 Gy exposure may be significant in neutron-induced cell killing; thus it is important to elucidate their long-term fate and establish whether they are eventually repaired. The experiments described here investigate the long-term processing of neutron-induced SSBs.

2. Materials and methods

2.1. Cell culture

Methods for culture of P3 cells were as described previously (Peak *et al.* 1989). Culture medium was RPMI 1640 (GIBCO), supplemented with 10% fetal calf serum, glutamine (20 mmol dm^{-3}), and antibiotics (penicillin, 100 units ml^{-1}, and streptomycin, 100 $\mu g\ ml^{-1}$). The experiments described here used asynchronously growing cells.

2.2. Radiolabeling of cellular DNA

Cells were seeded into 25-ml T-flasks at an initial density of 10^6 cells per flask and allowed to grow for 48 h at 37°C in medium containing [^{14}C]thymidine (ICN, Irvine, CA, 2.3 x 10^6 kBq $mmol^{-1}$) at a concentration of 0.37 kBq ml^{-1}. The cells did not reach confluency during this period. Prior to radiation exposure, cells were incubated for 1 h in medium free of label and were not supplied with any additional labeled thymidine for the remainder of the experiment.

2.3. Irradiation

Immediately prior to neutron irradiation, the T-flasks containing the cells were filled with ice-cold medium and placed in a neutron-compatible incubator specially designed so as not to perturb the radiation field. The cells were kept meticulously cold (0.5°C) during the irradiation. Neutron irradiations were carried out in the high-flux room of the JANUS reactor at Argonne National Laboratory at a dose rate of 35.8 cGy min^{-1}. The characteristics of this room were described previously (Peak *et al.* 1989). All cells were exposed to the same total dose (6 Gy) except for control cells, which were not irradiated. At the end of the irradiation period, cells were immediately transferred to wet ice prior to cold trypsinization or repair incubation. Irradiation of cells with ^{60}Co gamma-rays has been described (Hill *et al.* 1988; Peak *et al.* 1989).

2.4. Post-irradiation treatments

Following neutron exposure, the cold growth medium was removed and cells were rinsed with ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS-A). For control cells and those not permitted a repair period, this was immediately followed by trypsinization on ice. For DNA repair measurements, 5 ml of warm medium was pipetted into each flask and the cells were incubated at 37°C for from 0 to 47 h before trypsinization. In some experiments duplicate T-flasks of cells were treated in parallel. Following irradiation and repair incubation, one of the pair was exposed to trypan blue to measure the viability of the cells.

2.5. Alkaline elution

The alkaline elution assay used in our laboratory for the measurement of DNA damage in mammalian cells has been described previously (Hill *et al.* 1988; Peak *et al.* 1989). Elution was at pH 12.1, so the damage assayed represents total frank scissions plus alkali-labile sites. These DNA lesions are here collectively referred to as single-strand breaks (SSBs). The initial yield of SSBs produced by the neutron exposure was calculated as described previously (Hill *et al.* 1988) by comparison of the elution of DNA from standard cells irradiated with 3 Gy of ^{60}Co gamma-rays. To estimate repair of the DNA damage, we calculated the percentage of initial breaks remaining after repair incubation.

3. Results

We previously showed that repair of SSBs produced by neutron exposure is slower than that following irradiation with gamma rays, and that after repair times as long as 3 h, a statistically significant percentage (10%) of the breaks induced by neutrons remained unsealed (Peak *et al.* 1989).

In the current study, we allowed the cells longer repair times to determine whether this unrepaired fraction of breaks would eventually be sealed, and we demonstrated that all the breaks, as measured by alkaline elution, were sealed within 5-7 h. Figure 1 shows one such experiment that compares elution profiles of DNA from unirradiated cells, cells that had received 6 Gy of neutrons, and those that were incubated for 6 hours following neutron irradiation. Cells exposed to 6 Gy of neutrons

incurred many SSBs in their DNA, as shown by the increased rate of elution compared with DNA from unirradiated control cells. After a 6-h repair incubation, the elution profile was indistinguishable from that of unirradiated DNA, indicating complete rejoining of SSBs.

We continued to monitor DNA size following even longer repair incubation times. The effect of 18 h of repair incubation (also shown in Fig. 1) was that the DNA that had regained its original size after 6 h of repair had, after 18 h, become rebroken and eluted almost as fast as the DNA from cells that had no repair incubation.

The integrity of the DNA at various times throughout the 47-h period following exposure to 6 Gy of neutrons is illustrated in Fig. 2, which summarizes the results of five separate repair experiments. After each repair time the DNA was eluted, and we calculated the breaks as a percentage of those induced by the original radiation insult. Where three or more measurements were made after the same incubation time, the errors are shown. Other points represent the mean of two measurements. Fig. 2 clearly illustrates two distinct phases of DNA repair following neutron irradiation. The primary repair phase was completed between 5 and 7 h after the initial insult, to the extent that SSBs as measured by alkaline elution are all rejoined. Following this initial sealing of the breaks, the DNA was once again broken and this secondary breakage was almost as extensive as that caused by the original neutron exposure. The amount of breakage seen after 16 h of repair incubation was 80% of the initial yield (Fig. 2). The secondary round of breakage was in turn followed by break

rejoining, with the DNA regaining its original size (no measureable breaks remaining) within 40-47 h after neutron exposure.

It is clear from Fig. 1 that the elution profiles of irradiated DNA are virtually identical in shape to the 18-h rebroken DNA. This is evidence that the rebreakage phenomenon is not due to a random degradation of the DNA, which would have caused an initial, very rapid elution. In order to further determine that the secondary breakage of DNA was not just the manifestation of DNA degradation in cells that had not survived the neutron insult, the state of the cells following exposure to 6 Gy of neutrons was monitored by microscopic examination. Cells that had been exposed to this dose of neutrons remained attached to the surface of the T-flask throughout the repair incubation (up to 47 h post irradiation, the longest time measured). During this time there was no decrease in trypan blue exclusion, demonstrating that the cells retained integrity of the membrane. As further evidence that the cells were still viable at the time of the repair measurements, the cells completed a division cycle during the first 24 h repair incubation and reached confluence. However, because cells were only allowed to incorporate labeled thymidine into their DNA during the pre-irradiation period, our alkaline elution measurements did not include newly synthesized DNA.

4. Discussion

It has been suggested that the high biological effectiveness of neutrons for killing and mutating cells may be the result of

an inability of the cells to repair neutron-induced damage (Peak *et al.* 1989), but relatively little is known about the repair of DNA damage after exposure to neutrons. Results from some studies have indicated that strand breaks induced by fast neutrons are rapidly repaired, although in some cases a fraction of the breaks appeared to be refractory to repair (van der Schans *et al.* 1983; Sakai *et al.* 1987; Hesslewood 1978). A few studies have compared the kinetics of break repair following exposure to neutrons and gamma rays and reported that kinetics were similar after radiation doses in the 2-5 Gy range. (McWilliams *et al.* 1983; Maki *et al.* 1986). However, by contrast, another comparison using doses two orders of magnitude larger did show that more neutron- than gamma-induced breaks remained unrepaired (Furuno *et al.* 1979). Our own previously published work showed that a statistically significant fraction of the SSBs induced by 6 Gy of fission-spectrum neutrons remain unrejoined following repair times as long as 3 h (Peak *et al.* 1989). None of these studies has examined repair of breaks after incubation times longer than 3-4 h, so the eventual fate of the unrejoined breaks was unknown.

The experiments reported here have confirmed that DNA breaks induced by neutrons are much more difficult to repair than those induced by gamma rays. It required a full 5-7 h of post-irradiation incubation before all measurable breaks were rejoined. A possible reason for the recalcitrance of neutron-induced DNA damage is suggested by models for DNA damage based on the energy deposition by radiations of different LET. High-LET radiation may give rise to clusters of damage involving both base

and sugar damage at the sites of strand breakage, and these damage sites could be more difficult to repair than the isolated breaks or damaged bases caused by low-LET radiation (Holley and Chatterjee 1990). The importance of such clusters of damage in the repair of double strand breaks has been discussed by Ward (1988). Further, neutrons could give rise to chemical changes in DNA different than those produced by low-LET radiations. This might lead to differences in their repair. Bedford and Goodhead (1989) suggested that the different RBEs shown by radiation of different qualities might depend on a different chemical spectrum of damage being induced in individual nucleotides.

By continuing to incubate the cells for periods of up to 47 h, we have demonstrated for the first time the complex nature of the cellular processing of DNA following neutron exposure, involving two distinct phases of repair. Two possible hypotheses to explain these repair kinetics are illustrated in Fig. 3. The first phase may simply be a ligation process that seals the radiation-induced SSBs to regain the integrity of the DNA, with damage to individual nucleotides left unrepaired. This damage, which could be produced during the first repair process itself (upper panel) or formed as a result of the original radiation insult (lower panel), is then repaired during an excision repair process that takes many hours to complete. The secondary breakage that we see is the result of the excision step in this repair. In the case of repair of photoproducts (pyrimidine dimers) caused by exposure to short-wavelength ultraviolet light, the excision step has been shown to result in measureable DNA breakage in both mammalian cells and yeast (Bradley and Taylor

1981, 1983; Wang and Smith 1986; Frankenberg-Schwager *et al.* 1987). In the first hypothesis, the second phase of repair serves an editing function following an initial misrepair. The second hypothesis, which seems the more likely, is consistent with our knowledge of repair processes. For example, in the situation in which a damaged base occurs on one strand and a SSB on the other, the SSB would be repaired first because base-damage glycosylase is not able to act on single stranded regions of DNA.

Partial DNA rebreakage and subsequent rejoining has been observed by Lett and coworkers (Goldin *et al.* 1980; Okayasu 1987) following low-dose x-irradiation to an ultrasensitive murine cell line L5178Y S/S. We plan measurements of the long-term processing of the DNA of P3 cells following exposure to gamma rays in order to determine whether the complex repair kinetics that we have observed in repair-proficient human cells after exposure to neutrons result from gamma irradiation also.

This work demonstrates that cells with normal repair ability exposed to high-LET radiation may be active in metabolic nucleic acid repair processes for days, rather than hours, after exposure.

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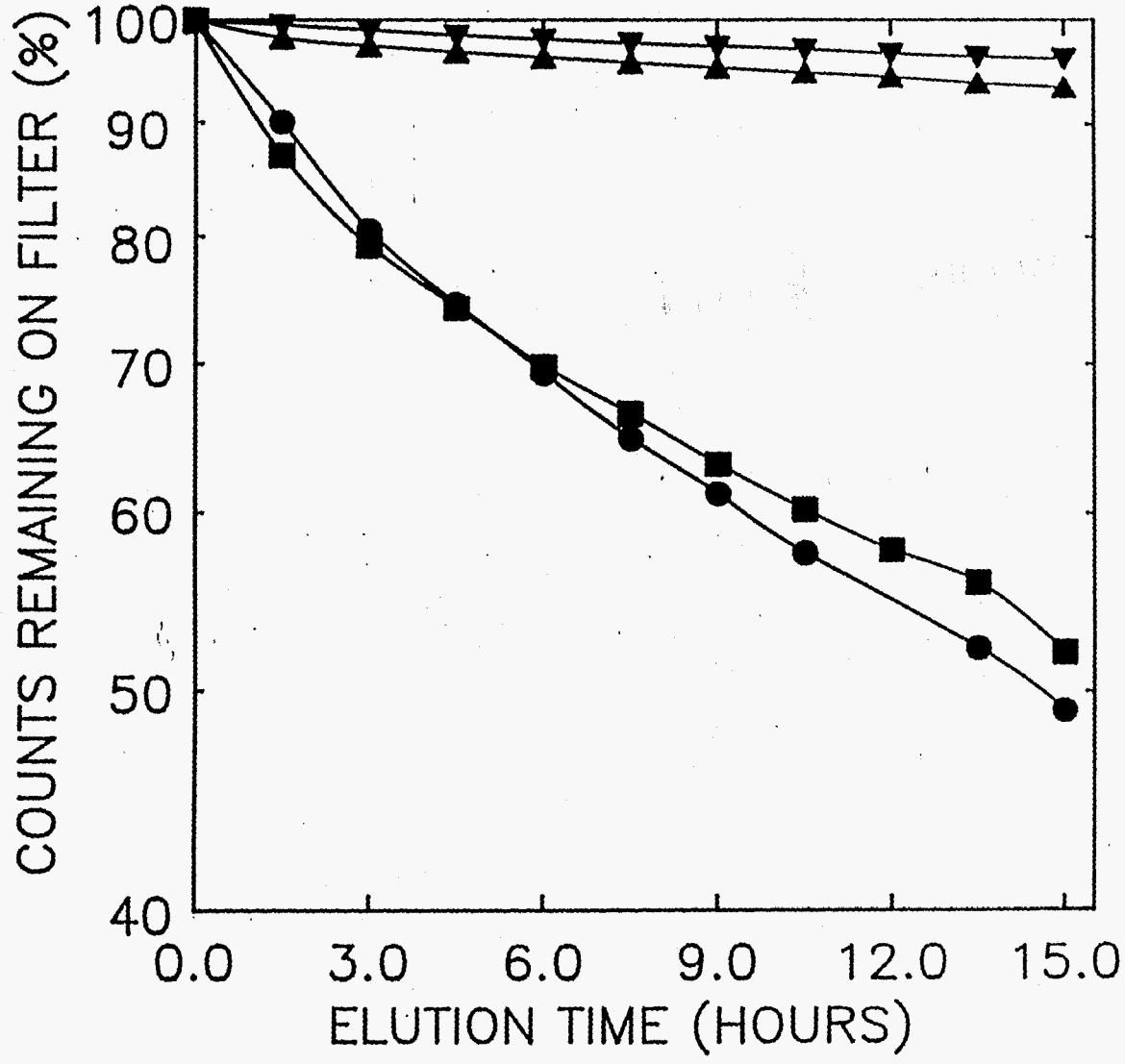
LEGENDS TO FIGURES

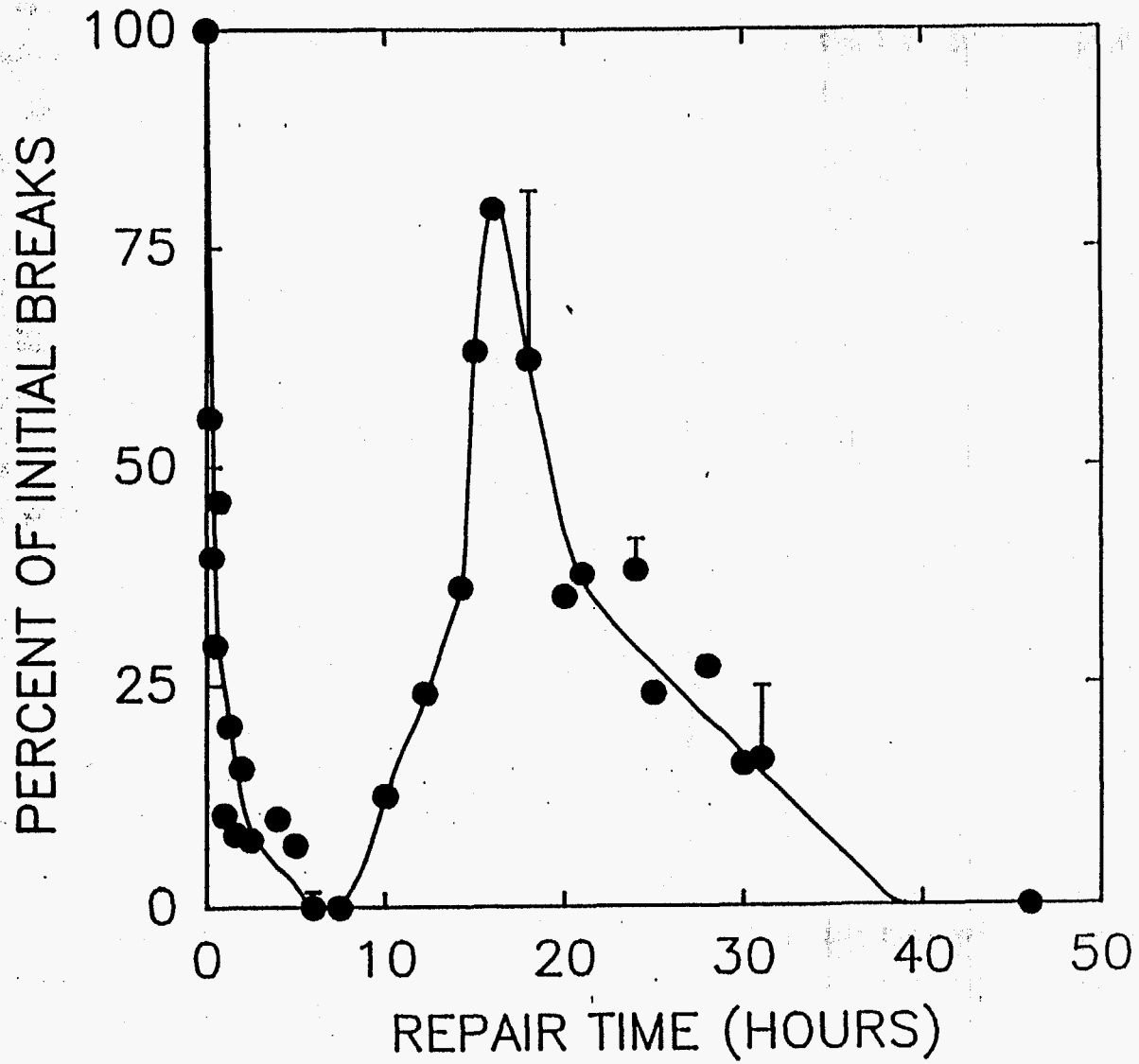
FIG. 1. DNA alkaline elution profiles for P3 cells after exposure to 6 Gy of neutrons followed by repair incubation. Inverted triangles, control unirradiated cells; circles, 6 Gy neutrons; triangles, 6 Gy neutrons plus 6 h repair; squares, 6 Gy neutrons plus 18 h repair.

FIG. 2. Repair of SSBs by P3 cells following exposure to 6 Gy of neutrons as a function of incubation time. Error bars represent one standard error of the mean. Points without error bars are the mean of two measurements.

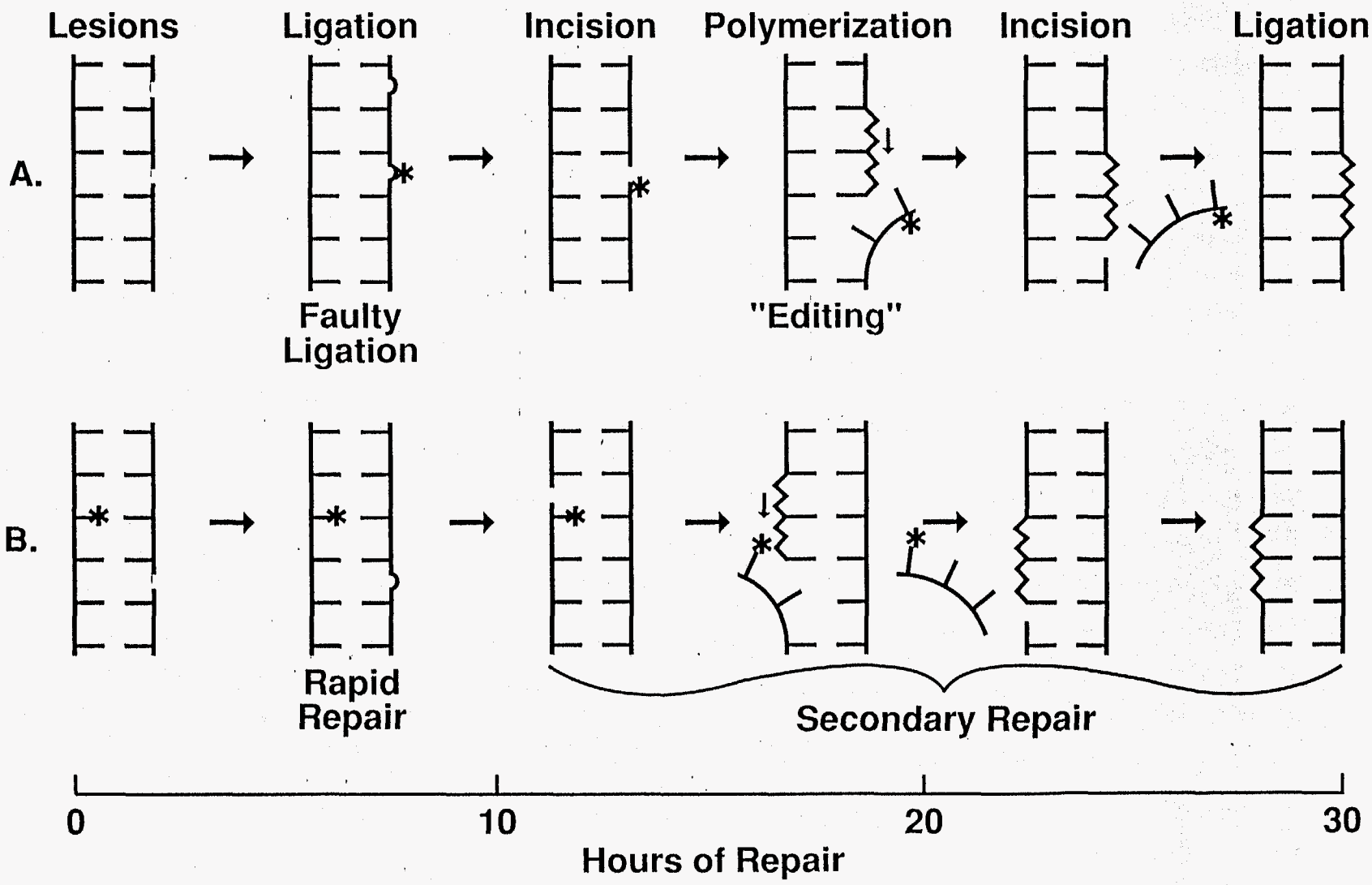
FIG. 3. Hypotheses to illustrate the possible significance of the secondary breakage and rejoining of DNA following exposure to neutrons. A, Removal of damage incurred during the initial rapid sealing of DNA breaks; B, Removal of damage left unrepaired during the initial rapid sealing of DNA breaks.



Peak & Peak Fig. 1



Peak & Peak Fig. 2



Peak & Peak Fig. 3