A cell line deficient in DNA ligase I and sensitive to poly(ADP-ribose) inhibitors, 46BR, was used to examine the relationship between DNA ligation and a large stimulation of repair replication that is seen in cells grown in a poly(ADP-ribose) polymerase inhibitor, 3-aminobenzamide (3AB), after exposure to alkylating agents. Repair replication was stimulated at least 10-fold by 3AB in both normal and ligase-deficient cells. Despite increased 3AB toxicity, repair replication in ligase I-deficient cells was unchanged from that in normal cells. This evidence is consistent with previous observations that the enhancement of repair replication by 3AB is not a direct function of DNA break frequencies. The stimulation may instead result from alkylation damage to other cellular organelles that release nucleases that cause additional damage to DNA, which cells attempt to repair.

Introduction

Poly(ADP-ribose) is a nuclear polymer synthesized from NAD in response to strand breaks in DNA (1–3). The polymer modifies a large number of cellular target proteins, including the poly(ADP-ribose) polymerase, histones and numerous enzymes and other proteins (4–6). Most enzymes that are covalently modified by poly(ADP-ribose) are inhibited, including DNA ligase II (7), topoisomerases I and II (8–10), DNA polymerase α and β and terminal transferase (7,11), RNA polymerase II (12), Ca<sup>2+</sup>, Mg<sup>2+</sup> endonuclease (13), ribonuclease (14) and poly(ADP-ribose) polymerase itself (15).

In view of the large number of substrates for polymerization in cells exposed to DNA breaking agents it is not surprising that the role of poly(ADP-ribose) in cellular recovery is complex and not yet clearly resolved (1–3,5). 3-Aminobenzamide (3AB*) is one of the stronger inhibitors of poly(ADP-ribose) synthesis and also modulates several aspects of DNA repair (16,17). In cells damaged by alkylating agents 3AB at low concentrations produces large (5- to 10-fold) increases in repair replication (18–22) and accelerates the rate of ligation of single-strand breaks associated with repair (23–27). The net single-strand-break frequencies, which represent the balance between rates of break formation and rates of ligation, can increase (1,2,28) or decrease (25–29) under various conditions according to the relative rates of the two opposing processes, which respond differently to different concentrations of 3AB. These processes lead to a variety of logical puzzles that result from trying to generate a self-consistent model for the changes in DNA repair produced by 3AB. Satoh et al. (17) have shown that most long chain polymers are ineffective in inhibiting repair, but may prevent accidental or erroneous non-homologous exchange at sites of damage.

One of the more enigmatic effects of 3AB is the large stimulation of repair replication that can be produced in cells damaged by alkylating agents (18–22). The increase is not due to changes in patch size or acceleration in excision rates and so must be due to a large number of extra patches (18,19,30). One interpretation of the increase in repair replication is that the combination of cellular alkylation damage and 3AB releases endogenous nucleases that create additional damaged sites, which are then the sites of increased repair replication (3). The role of poly(ADP-ribose) polymerase as a target for protease action during apoptosis would be consistent with this view (32).

A unique mutant human cell line (46BR) that has inactivating mutations in both alleles for DNA ligase I and is highly sensitive to the toxic effects of alkylating agents and 3AB has been characterized (31,33,34). Ligase I appears to be important in both DNA replication and repair (31), unlike ligases II and III, which appear to have more specialized functions. This cell line afforded me an opportunity to determine possible relationships between repair replication, DNA ligation and 3AB sensitivity.

Materials and methods

Cell cultures

Normal (GM637) and ligase I-deficient (46BR) (46BR cells were a gift of D. Barnes, ICRF, London, UK) SV40-transformed cells were grown in minimal essential medium plus 10% fetal calf serum and antibodies. For comparative studies of the toxicity of 3AB, 10<sup>6</sup> cells were plated into each well of 4 × 6-well trays, supplemented with 1–14 mM 3AB and grown for 6 days. After this length of time cells were washed free of 3AB and relative cell density was estimated by labeling total cellular RNA. Previous studies using normal and repair-deficient cells, both primary and transformed, have validated the method for estimating relative cell survival by comparison with colony formation (35). Transient effects associated with unique responses in cells, such as Cockayne’s syndrome, that show slow recovery of RNA synthesis are over by 6 days and do not seem to invalidate the method. Cultures were incubated for 4 h with 0.5 μCi/ml acetic acid ethanol (3:1) and then extracted with 4% perchloric acid (16 h, 37°C). The soluble extract was quantified in a water-miscible liquid scintillation fluid and relative cell survival was expressed as 3<sup>H</sup> counts/well at each 3AB concentration relative to the 3<sup>H</sup> in the wells that contained no 3AB. This allowed the relative cell growth to be determined from the relative 3<sup>H</sup> activities.

Cesium chloride–cesium sulfate isopycnic gradients

Replication was determined using cesium chloride–cesium sulfate alkaline isopycnic gradients (19). Cultures were first grown for 2 h in 10 μM bromodeoxyuridine (BrdUrd) and 1 μM fluoro-deoxyuridine (FdUrd) to begin synthesis of BrdUrd-substituted DNA. N-Methyl-N’-nitro-N-nitrosoguanidine (MNNG) was then added from a freshly prepared solution in dimethyl sulfoxide to produce final concentrations of up to 0.6 mM. After 30 min exposure cultures were rinsed and incubated in radioactive medium containing 10 μM BrdUrd, 1 μM FdUrd, 2 mM hydroxyurea, 10 μCi/ml (80 Ci/mmol) [3<sup>H</sup>]thymidine (dThd) and 2 mM 3AB. The same preparation of [3<sup>H</sup>]dThd was used for both cell types to reduce variability. This concentration of 3AB was chosen to be just above the concentration that provides maximal stimulation of repair replication (19) and is above inhibitory concentrations for poly(ADP-ribose) polymerase (16). Larger concentrations produce no further changes in repair.
Nuclei were isolated (23) and lysed in 1 ml 10 mM Tris-HCl, pH 8.0, 10 mM EDTA and 0.5% SDS and diluted to 4.5 ml with 0.15 M NaCl and 0.015 M sodium citrate; the pH was adjusted to 13.0 with NaOH. CuCl and CaSO₄ were added and gradients were run at 42 000 r.p.m. for 36 h and analyzed as described previously (19). For quantification the normal density (repaired) DNA was pooled from gradients, dialyzed and centrifuged a second time to improve purification. Repair replication was quantified on the basis of [³H]dThd incorporated by repair replication per microgram of normal density DNA in isopycnic gradients.

Results
The cell line 46BR was first evaluated to ensure that it retained its 3AB sensitivity, because this characteristic was originally reported for the primary culture (34). The SV40-transformed 46BR cells showed greater 3AB sensitivity than did GM637 (Figure 1); growth was reduced by 50% at 4.8 and 8.0 mM respectively.

Repair replication, as assayed in isopycnic gradients, was at very low levels after MNNG exposure in both cell types, with a flat dose-response over the range 0.2-0.6 mM (Figures 2 and 3). Both cell types, however, showed a very large stimulation of repair replication when grown in 3AB at each exposure concentration of MNNG. The precise shape of the response curves differed and the absolute level of repair replication was greater in 46BR cells than in GM637, both with and without 3AB. However, large stimulations clearly occurred in both cell types. The quantitative differences are most likely due to cell type-specific differences in nucleotide pool sizes and the actual amount of DNA damage caused in each cell type by MNNG. Another factor may be that 46BR was transformed with an SV40 ori+ plasmid, also expressing the gpt gene, which may alter nucleotide metabolism (D.Barnes, personal communication).

Similar experiments with [³H]BrdUrd also showed that both cell types showed a large stimulation of repair replication after exposure to 3AB (data not shown), indicating that the stimulation was not due to differences in cellular preference for dThd over BrdUrd.

Discussion
The increase in repair replication associated with growth in 3AB is a particularly difficult phenomenon to explain, even though the increase is very large (5- to 10-fold or greater) and has been recognized for over 15 years. At first it was considered to be associated with the effect of 3AB on ligation rates, but that would necessitate changes in patch size (1,2). Patch sizes were, however, found to remain at ~30 bases/patch, despite the increased amount of repair (18,19).

One feature of the increased repair replication with 3AB is that it is very large after alkylation damage, but much smaller or negligible after damage from UV light and X-rays (30) and after restriction enzyme breakage in vivo (unpublished observations). Increasing DNA breakage in UV-damaged cells by growth in cytosine arabinoside does not cause 3AB to stimulate repair replication (26). Rufer and Morgan (36) attempted to identify new sites of damage using restriction enzyme-induced mutagenesis, but found that no new sites were induced by 3AB. Like UV- and cytosine arabinoside-induced damage, restriction enzyme damage is uniquely concentrated in DNA. From these observations we can infer that when the cellular damage is concentrated almost exclusively in DNA the activating stimulus for repair replication induced by 3AB is weak or absent. Therefore, previous studies (25,26,30,36) and the results of the present study suggest that for 3AB to stimulate large increases in repair replication there must be prior exposure to a DNA damaging agent that also affects other cellular organelles or systems, rather than DNA alone.

It is likely that an attempt to devise a unitary hypothesis for all the effects associated with 3AB is futile. The wide variety of substrates for poly(ADP-ribose) polymerization means that many independent events could be occurring within
cells exposed to DNA damaging agents and 3AB. In the present study the deficiency in ligase I makes cells more sensitive to the toxic effects of 3AB (Figure 1) but does not eliminate the stimulus to increased repair replication, suggesting that the toxicity of 3AB (34) and its effects on repair replication are independent processes.

Previous work from my laboratory has suggested that the increased repair replication with 3AB could be caused by activation of Ca\(^{2+}\), Mg\(^{2+}\)-dependent nucleases as a result of damage to cytoplasmic structures (13), especially mitochondria, which are stores for intracellular Ca\(^{2+}\). In preliminary experiments I have found that alkylation damage, but not UV light, stimulates large, rapid increases in available cellular Ca\(^{2+}\) and that the stimulation of repair replication by 3AB after alkylation damage can be prevented by calcium chelation (unpublished observation). This observation is consistent with the idea of a nucleolar origin for the increased repair replication. Further studies to identify the nucleosome(s), their mechanism of activation and sites of action and the role of Ca\(^{2+}\) mobilization in mediating cellular damage after exposure to alkylation agents (37) are therefore warranted.

Acknowledgement
This work was supported by the Office of Health and Environmental Research, US Department of Energy, contract no. DE-AC03-76SF01012.

References