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Binding of a Nitroxyl to Radiation-induced DNA Transients in Repair and Repair Deficient of *E. coli* K-12

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Binding of tritiated 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (^{3}H-TAN) to radiation-induced DNA-transients in *E. coli* K-12 strains AB 1157 and JO 307 rec A uvr A has been studied under *in vivo* conditions. After irradiation the cells were washed and resuspended in growth medium and left overnight at 37 °C. Within an uncertainty of about 10 %, no effect of repair could be detected on the yield of TAN bound to DNA for any of the strains. During the period after resuspension. TAN or fragments of TAN leaked out of the irradiated cell samples. This leakage may be attributed to semi-permanent association between TAN and radiation-induced radicals within the cell.

The relevance of different interactions between TAN and transients in DNA is discussed.

I. INTRODUCTION

Nitroxyl free radicals, like 2,2,6,6-tetramethyl-piperidone-*N*-oxyl (TAN), 2,2,6,6-tetramethyl-4-piperidinol-*N*-oxyl (TMPN), and norpseudopelletierine-*N*-oxyl (NPPN) are of considerable interest because of their ability to sensitize anoxic cells to ionizing radiation. Despite intense research, however, the mechanism of their sensitizing effect is still mainly unknown.

It is suggested that these compounds act by interaction with radiation induced damages in vital cellular molecules, of which DNA is assumed to be the most important.[1] In our laboratory we have therefore studied interactions between nitroxyls and radiation induced radicals in DNA and DNA-components. By pulse radiolysis we have demonstrated a correlation between the reactivity of the three nitroxyls mentioned above towards OH-induced radicals in DNA-bases and nucleosides, on one hand, and the sensitizing ability of the nitroxyls on the other.[2,3] Furthermore, *in vitro* experiments have revealed that TAN is able to react with OH-induced DNA-transients in two ways, (1) by an oxydation-reduction reaction yielding reduced TAN (TAN-H), and (2) by binding.[4] Both these reactions can occur even when TAN is added several minutes after DNA is exposed to radiation, illustrating that the DNA radicals responsible for the interaction have a considerable lifetime, at least under *in vitro* conditions.

Similar experiments were performed *in vivo* where TAN was added to cell suspensions at different times prior to or after irradiation.[5] DNA was thereafter extracted and the relative yields of TAN bound to DNA determined. The investigation showed that TAN rapidly penetrated the cellular membrane. Even when TAN was added 2 s after irradiation we found a significant degree of binding. In the latter case, however, no sensitization was observed. Thus, there is no simple correlation between total TAN-binding to DNA and anoxic sensitization.

In order to elucidate further the relevance for sensitization of TAN-binding to DNA, we have in this work studied the influence of recombination and excision repair on the degree of binding. Since post-replication excision of the damage is suggested to be the last step in recombinational repair,[6] both repair mecha-

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nisms result in elimination of damaged fragments from the DNA helix. The release of DNA fragments complexed with TAN may therefore provide some information as to whether the cell considers TAN bound to DNA as a serious damage or not.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions. *E. coli* K-12, strains AB 1157 and JO 307 were used for the experiments. The wild-type strain AB 1157 has both excisional and recombination repair capability, whilst the strain JO 307 is deficient in these repair mechanisms, due to mutations in the rec (A-13) and uvr (A-6) loci.

A single colony of the actual strain was transferred to YET broth, containing 5 g yeast extract, 10 g bacto-tryptone (both from Difco Laboratories, Detroit, Michigan), and 10 g NaCl per liter (pH 7) and incubated overnight. Next morning the suspension was mixed with the double volume of a YET glucose medium, which in addition to the components mentioned above, contained 2 g D(+)-glucose per liter. The culture was incubated under aeration until mid log phase in the mixed medium. Finally, the cells were washed and resuspended in phosphate buffer, pH 7, containing 0.13 M NaCl. The concentration was then about 10^{10} cells per ml.

2.2. Chemicals. TAN was synthesized by catalytic oxydation of the corresponding amine according to the method of Rozantsev,[7] with some minor modifications. The amine (HCl salt) was obtained from Fluka AG, Basel. The tritium labeling was performed by proton exchange from tritiated water of high specific activity (Amersham, Buckinghamshire, U.K.). The stability of the label was checked by measuring the activity in recrystallized TAN after 24 h in water solution, pH 7, at room temperature. No loss of activity was observed.

All other chemicals were of the highest purity commercially obtainable, and were used without purification.

2.3. Experimental procedure. The mixing and flushing setup has been presented earlier (Wold and Brustad [5]), 1 ml samples of bacterial suspension in test tubes, and 1 ml aliquots of 2×10^{-3} M TAN (~ 150 μCi tritium activity) in glass syringes were continuously flushed with highly purified nitrogen gas (less than 6 ppm O_2, Norsk Hydro A/S, Oslo) for at least 30 min before exposure. The TAN solution was transferred to the bacterial suspension under maintenance of anoxia 2 min before exposure. 1 min after irradiation, air was admitted to the exposed mixture. All solutions were kept at ice temperature during the experiment.

The test mixture was irradiated with 4 MeV electrons from a modified AEI-linear accelerator. The dosimetry was performed with the modified Fricke dosimeter system.[8] The oxygen saturated dosimeter solution, contained in a glass tube, was irradiated simultaneously with the test solution.

After irradiation, the test samples were washed 4 – 5 times in phosphate buffer in order to reduce the activity caused by unbound TAN. Then, the cells were resuspended in YET glucose medium.

After centrifugation, the cells were resuspended in cold 5 % trichloroacetic acid (TCA), washed several times with nearly boiling ethanol to remove lipids, and again resuspended in cold TCA. The ^{3}H-activity in this final supernatant was checked and used as a measure for the "background activity". The nucleic acid fraction (mainly DNA [5]) was extracted from the residue by 5 % TCA at 95 °C. After centrifugation, 1 ml samples of the supernatant were counted in a scintillation counter.

3. RESULTS

3a. Radiation induced binding of TAN in E. coli *K-12, strains AB 1157 and JO 307.* When a test solution of TAN and *E. coli* K-12, strain AB 1157 is exposed to 40 krad and treated as described in the previous paragraph, the DNA extract has an activity corresponding to about one TAN molecule per 20 000 nucleotides. In comparison, the frequency of single strand breaks under anoxic conditions is of the same magnitude for this dose.[9]

In order to find whether the extent of binding of TAN to DNA is changed by repair processes, we compared (1) the binding in the repair proficient strain AB 1157 with that in the repair deficient strain JO 307, and (2) the binding in normal and heat-treated cells (10 min at 52 °C) for the repair proficient strain. This treatment is assumed to inactivate some enzymes necessary for repair.[10] Since (a) the radiation affects the survival ratios of the two strains differently because of different sensitivity, (b) usually, different DNA concentrations resulted in extraction from the two irradiated strains, and (c) the heat treatment seems to result in leakage of proteins from the cells (leading to pronounced foaming when flushing), a small difference in TAN binding may be caused by effects other than repair. Therefore, we also compared the two strains with regard to binding of TAN to DNA in intact cells after repair relative to that in heat-treated cells.

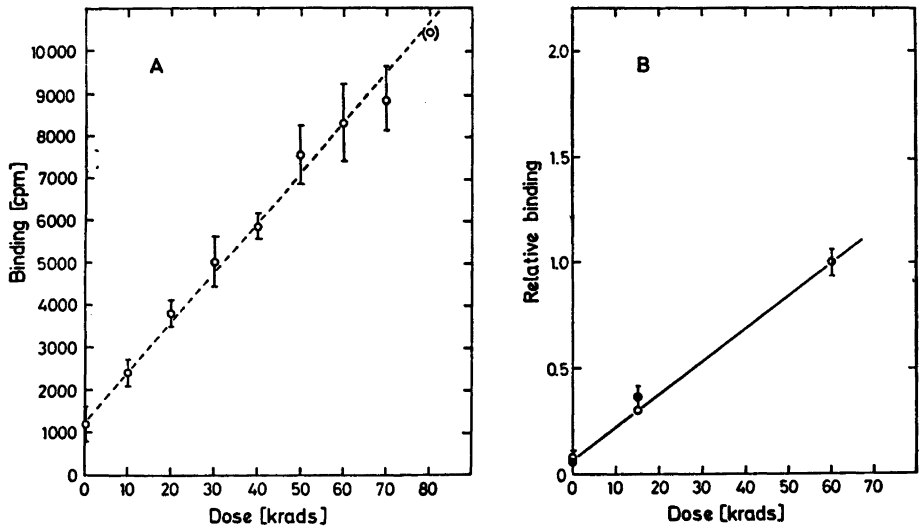

Fig. 1. Binding of ^{3}H-TAN to DNA in *E. coli* K-12 strain AB 1157 as a function of dose. The cells were washed, resuspended in growth medium and left overnight at 37 °C. Next morning, DNA was extracted and measured for ^{3}H-TAN activity.
A. Cells incubated under conditions permitting minimal cell division (see text). No air flushing. Standard error indicated as vertical lines.
B. ○, Same as A. ●, Cells incubated in sufficient medium for 2 – 3 divisions. Air flushing. Standard error for the most uncertain value at each dose indicated as vertical lines. Because of diverging growth conditions during the repair period, resulting in different quantities of DNA, the extraction efficiency is not the same for the cases on Fig. 1B. The values for 60 krad are therefore normalized to 1.0.

Due to the features mentioned above, the results of the different comparisons suffer from a considerable uncertainty. The present conclusion, however, must be that there is no significant reduction in binding level in the repair proficient strain as compared to that in the repair deficient strain.

3.2. Dose-binding curves. As a further check for the ability of repair mechanisms to remove DNA-bound TAN, dose-binding curves were determined for the repair proficient strain. Cell death, or lacking ability for cell division is assumed to be caused by insufficient repair of demages in DNA. Although some repair may occur even at the highest doses at which most cells are "killed", the repair failure ought to be more pronounced at high than at low doses. For recombination repair, post-replication excision is assumed to be the last step in the repair process.[6] This post-replication excision may be blocked if replication and recombination are not completed because of substantial radiation-induced DNA damage. One would therefore expect an even more pronounced repair failure at high doses for recombination than for excision repair. Thus, for both mechanisms a deviation from the linear dose-binding relationship is expected if bound TAN is removed as a result of repair.

Fig. 1A shows the curves for strain AB 1157. The curve is for repair under conditions permitting very little cell division. (The cells were resuspended to a concentration of about 10^9 ml^{-1} without air flushing). In such a highly concentrated suspension about half of the cells in an unirradiated control will die during the 18 h incubation period. Since repair seems to be completed within about 70 min,[11] most cells should have a survival period long enough to excise the damage.

The highest dose in Fig. 1A corresponds to about 1 % survival, while about 80 % of the cells survive the lowest dose applied (unpublished data for lower concentrated cell suspensions).[12]

Fig. 1B shows the importance of sufficient nutrition for cell division during repair. Binding is measured for a high and a low survival ratio

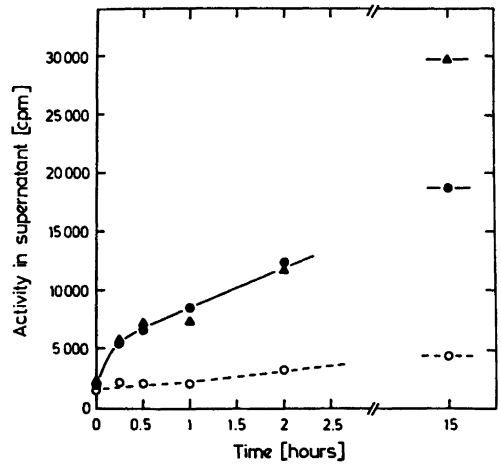

Fig. 2. ^{3}H-TAN activity in growth medium as function of time since suspension. The cells (strain AB 1157) were washed free from unbound ^{3}H-TAN before suspension. O, Unirradiated cells. ●, Cells irradiated with 25 krad in anoxia. ▲, Cells heat-treated (see text) before irradiation (25 krad).

under two different nutritious conditions during the repair period, (a) for medium sufficient for 2–3 cell divisions, and (b) for the growth medium mentioned above. None of the curves show significant deviations from linearity. We have, therefore, no evidence for excision of TAN-DNA fragments. The experiments presented in this section are, however, not sensitive enough to detect low yields of TAN excision.

3.3. TAN leakage from irradiated cells. When irradiated mixtures of TAN and bacteria have been washed several times with buffer, TAN or TAN fragments containing the tritium label continues to leak out, even at room temperature. After 15 h the total activity of the leakage is of the same magnitude as the TAN level in the DNA extracts. TAN also leaks out of unirradiated cells, but not to the same extent.

The feature is demonstrated in Fig. 2, where the leakage is measured from normal and heat treated cells (15 min at 60 °C) of strain AB 1157. All samples were washed 4 times with buffer to remove unbound TAN. At different times after resuspension in growth medium, the samples were centrifuged, and aliquots of the supernatant measured for ^{3}H activity. The cells were immediately afterwards resuspended in the same solvent and again stored. During the first hour of storing the samples were kept at room temperature. Thereafter, the samples were incubated at 37 °C. Thus, much of the leakage occurred at a temperature resulting in reduced biological activity of the cell. During the two first hours, normal cells and heat treated cells (denaturated repair enzymes [10]) yield nearly identical leakage. The leakage is therefore not likely caused by enzymatic repair mechanisms inside the cell.

A possible reason for the leakage is that some TAN may be semipermanently associated with radiation-induced radicals in the cell. With time and warming (to 37 °C) part of this association may break, and free TAN or TAN bound to small molecular debris separates. The proposed association must be much weaker than the covalent binding between TAN and DNA transients reported by Brustad *et al.*[4] since the covalent binding was not affected by heating to 80 °C for 15 min. An alternative explanation is that some of the bound TAN molecules are destroyed, leading to the liberation of the tritium activity. In both cases, the leakage of activity is a consequence of an interaction between TAN and transients within the cell.

The high final level for the inactivated cell sample on Fig. 2 may be caused by cell destruction, permitting rather big molecules and cell debris with bound TAN to separate.

3.4. Investigation of TAN bound to DNA in cells of strain JO 307 after low doses. Strain JO 307 is more sensitive to ionizing radiation than strain AB 1157. We therefore investigated TAN binding and leakage at 800 rad, a dose yielding about the same survival (approximately 10 %, unpublished for low concentrated suspensions [12]) as that caused by the medium dose used in the work on AB 1157. A very low, but probably significant yield of TAN bound to DNA was detected. The leakage of TAN from irradiated cells was only slightly higher than that from unirradiated cells.

Despite this small yield of binding and leakage, TAN has a marked sensitizing effect as regards cell survival of strain JO 307 cells at the applied dose.

4. DISCUSSION

A variety of investigations have led to the conclusion that DNA is a vital target for radiation killing. As a consequence, sensitization is assumed to be connected with some kind of interaction between the sensitizer and radiation-induced damages in DNA. Emmerson [13] has demonstrated clearly that the presence of TAN during irradiation modifies DNA in a way that leads to reduced survival for the cell. He irradiated a donor strain of *E. coli* K-12 in presence and absence of TAN. After conjugation, he found that the sensitizer reduced the 1/e survival dose by a factor of 7.5. Since DNA was the only part of the irradiated donor that was transferred to the recipient, the work establishes that TAN exerts its effect, at least partly, on DNA. Further, Rupp, Zipser, von Essen, Reno, Prosnitz and Howard-Flanders [11] have shown by alkaline sedimentation that DNA synthesized in cells newly irradiated in presence of TAN sediments more slowly that in cells irradiated without sensitizer. In repair proficient strains, DNA synthesized one hour later had nearly recovered its normal sedimentation pattern, while the DNA from repair defective cells maintained the slow sedimentation pattern.

Thus, it seems clear that TAN in some way interacts with irradiated DNA. Two different reactions between TAN and DNA-damages are known, a reduction-oxydation reaction and covalent binding.[4] The binding has gathered most interest, partly because of the assumed resemblance to fixation of DNA damages by oxygen.[14,15] The enzymatic excision of the damage is then suggested to work in a similar way as that for UV-induced thymine dimers.

Some other features, however, can hardly be explained from such a model. TAN sensitized Chinese hamster cells even when washed out of the sample before exposure.[16] In contrast, no pre-irradiation effect of TAN was discovered for *E. coli* K-12 strain AB 2463.[12] In an earlier work on *E. coli* cells we have demonstrated that TAN can be bound to DNA to a nearly full extent on admixture 0.2 s before irradiation, and that a significant degree of binding occurred on post-exposure admixture.[5] Cell sensitization, however, requires the presence of TAN during irradiation, and is very small when the interval between admixture and irradiation is as short as 0.2 s.

The present work does not prove that TAN-DNA fragments are excised from DNA as a result of enzymatic repair processes. As mentioned above, the experimental techniques employed are rather insensitive, and 10 % TAN excision can escape detection. It seems, however, clear that the bulk of TAN-DNA complexes is not excised. This means, alternatively, that (a) total TAN binding does not represent a serious damage, (b) total TAN binding is harmful, but the enzymatic repair mechanisms are not able to handle this kind of damage, (c) part of the binding, say 10 %, is lethal, but can be excised, while the rest is harmless and ignored by the cell.

Experiments that will make detection of small amounts of excised TAN possible are now in progress.

Leakage of TAN or TAN fragments from irradiated cells indicates breaking of some kind of radiation-induced association between the nitroxyl and cell components. The present work does not reveal where TAN is associated, but because of the high reactivity of the compound, it seems reasonable to assume association to a lot of different structures and molecules within the cells, including DNA. Even if such an association is not permanent, it may be strong enough to represent a lethal damage.

It may seem strange to explain sensitization by covalent binding or by some weaker association, since sensitization occurs even when the interaction with the sensitizer is negligible, as is the case for strain JO 307 at low doses. On the other hand, this particular strain is very sensitive to radiation even in the absence of a sensitizer. This general sensitivity may well cause a susceptibility even to weak interactions with the sensitizer. This might for instance be the case if most primary hits in DNA were sublethal. The effect of the interaction with the sensitizer could then be to transfer the sublethal damages to lethal ones. Such a transfer is expected to be more serious for strain JO 307 than for strain AB 1157, due to the deficiency in the enzymatic repair mechanisms of the former strain.

The red-ox process between TAN and DNA transients as reported by Brustad, Jones and

Wold [4] may result in fixation of radiation damage, for instance by the mechanism proposed by Adams and Cooke.[17] In *in vitro* experiments we observed a reaction with irradiated DNA even when TAN was added several minutes after irradiation. Such a long-lived post-irradiation effect on survival is not known for any cell strain. Furthermore, van Hemmen, Mauling and Bleichrodt [18] were unable to detect any inactivation of biologically active DNA by TAN admixture 1.5 ms after irradiation of DNA.

At present, none of the simple models for radiation sensitization seem to yield a satisfactory explanation of all observations. It may be necessary to consider models including other cell components than DNA in order to arrive at a complete understanding of sensitization. One such hypothesis has been presented, where sensitization is ascribed to influence of the sensitizer upon radiation-induced reactions between cell components and DNA.[19]

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