Partial inhibition of postreplication repair and enhanced frequency of chemical transformation in rat cells infected with leukemia virus

(Rauscher leukemia virus/4-nitroquinoline-1-oxide)

RAYMOND WATERS*, NIRMAL MISHRA†, NOEL BOUCK†, GIAMPIERO DIMAYORCA†, AND JAMES D. REGAN*

* Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830; and † University of Illinois School of Medicine, Department of Microbiology, Chicago, Ill. 60680

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ABSTRACT Postreplication repair of DNA and chemical transformation with the carcinogen 4-nitroquinoline-1-oxide were studied in rat cell lines either uninfected or infected with Rauscher leukemia virus. The results indicate that equivalent amounts of carcinogen are bound to the DNA initially and removed during excision repair. However, the lines differ in that the infected line alone exhibits both sensitivity to nitroquinoline-oxide-induced transformation and a partial inhibition of the postreplication repair process after nitroquinoline oxide or UV treatment.

Chronic infection with a leukemia virus can cause low passage rat cell lines to become sensitive to *in vitro* transformation by chemical carcinogens, although the uninfected parent lines are resistant to transformation by chemicals (1). To cause this effect the leukemia virus infection must be underway at the time of the carcinogen treatment (2, 3); thus the viruses appear to serve in some way as co-carcinogens although they by themselves lack any *in vitro* transforming functions.

4-Nitroquinoline-1-oxide (4NQO) is a potent carcinogen both *in vitro* (4) and *in vivo* (5), and is strongly mutagenic in bacteria (6). It is known to cause DNA damage in bacterial (7) and mammalian cells (8). Many of the lesions are repaired by the excision-resynthesis mechanism that operates on UV-induced pyrimidine dimers (7). Hence, UV-sensitive mutants of *Escherichia coli* (7) and cells from patients with xeroderma pigmentosum, which are deficient in UV repair (9, 10), are especially sensitive to treatment with this chemical (11, 12). In an attempt to elucidate events correlated to the leukemia virus-induced sensitization of rat cells, the amount of 4NQO bound to the DNA of such cells and the frequency of 4NQO-induced malignant transformation were measured indirectly. The susceptibility of such lesions to the excision (9, 10) or postreplication repair processes known to operate in human and other mammalian cells (13-15) was then estimated by monitoring, respectively, the removal of photosensitive 4NQO-DNA adducts (16), and the increase in molecular weight of DNA synthesized as a function of time after 4NQO treatment.

MATERIALS AND METHODS

Cell Lines.

F111	Low passage (less than 60 doublings) diploid Fisher rat fibroblast line (1).
R111	Diploid F111 chronically infected with Rauscher leukemia virus (RLV) by G. DiMayorca.
NRK	An established quasidiploid normal rat kidney line.
NRK/RLV	Normal rat kidney chronically infected with RLV by G. DiMayorca.

Abbreviations: 4NQO, 4-nitroquinoline-1-oxide; RLV, Rauscher leukemia virus; BrdUrd, bromodeoxyuridine; M_w , weight-average molecular weight.

HSBP Normal diploid human fibroblasts derived from fetal skin (16).

All cell lines were free of mycoplasma.

Cell Plating; Pre- and Pulse-Labeling; 4NQO and UV Treatment. Cells were grown in Dulbecco's modified medium plus 10% donor calf serum (Flow Laboratories) in an atmosphere of 10% CO₂ at 37°. Cells were plated at 7×10^4 per 5 cm petri dish and incubated for 2 days prior to DNA labeling, treatment, and analysis. DNA was labeled prior to treatment by incubating cells overnight in medium containing either [³H]thymidine (2 Ci/mmol, Amersham/Searle) at 4 μ Ci/ml or [¹⁴C]thymidine (551 Ci/mol, Amersham/Searle) at 0.5 μ Ci/ml. Cells were pulse-labeled with [³H]thymidine (17 Ci/mmol, Amersham/Searle) at 50 μ Ci/ml. Untreated cells were labeled for 30 min. After insult with 4NQO (kindly provided by J. Epler) or UV and incubation in warm growth medium for 1 hr, treated cells were labeled for 60 min. These times allowed approximately equal amounts of DNA to be synthesized in treated and untreated cells, as determined by label uptake. After pulse labeling dishes were rinsed and chased in warm medium.

For the estimation of lesions and postreplication repair, cells attached to the dish were treated with 4NQO in Hanks' salt solution for 90 min. Cells were exposed to UV irradiation after rinsing in phosphate-buffered saline (17).

Sedimentation of DNA in Alkaline Sucrose. Dishes were rinsed with, and cells scraped into, ice-cold 0.02% ethylenediaminetetraacetic acid, 0.8% NaCl. Cells at 7 to 9×10^3 in 50 μ l were layered and lysed on top of a 4 ml alkaline 5-20% sucrose gradient containing 2 M NaCl and centrifuged at 30,000 rpm for 155 min in an SW 56 rotor of a Beckman model L centrifuge at 20°. Pulsed, or pulse-chased untreated and treated, uninfected or infected cells were always run under the same conditions. Fractions were collected by puncturing the tube and dripping onto strips of filter paper (18). Acid-insoluble radioactivity was measured with a toluene/2,5-bis[5'-*tert*-butylbenzoxazolyl-(2')]thiophene (BBOT) scintillator in a Packard scintillation counter. The distribution of radioactivity was converted to weight-average molecular weights by means of a computer program, the details of which have been previously described (16, 19).

4NQO Binding and DNA Repair. Prelabeled cells treated with 4NQO were exposed to 75 kJ·m⁻² of 313 nm irradiation. The 4NQO bound to DNA, upon illumination, results in the photolysis of DNA (16). Hence the relative amount of 4NQO initially bound can be estimated by measuring the number of breaks resulting from 4NQO and 313 nm irradiation. Because a larger 313 nm dose gave no more breaks it was assumed that 75 kJ·m⁻² saturated the number of sites liable to photolysis. Excision repair was estimated both by measuring the proportion of 4NQO remaining bound to the DNA at various times after

FIG. 1. The reciprocal weight average molecular weights (M_w) of rat fibroblasts uninfected (circles) or infected (triangles) with RLV and treated with $0.25 \mu\text{M}$ 4NQO for 90 min. Prior to DNA sedimentation in alkaline sucrose gradients, cells were either subjected to $75 \text{ kJ}\cdot\text{m}^{-2}$ of 313 nm irradiation (solid symbols) or left unirradiated (open symbols).

treatment, and, after UV irradiation, by the bromodeoxyuridine (BrdUrd) photolysis technique. Essentially, the photolysis technique uses BrdUrd as a thymidine substitute after UV irradiation. Hence BrdUrd is inserted into DNA instead of thymidine during the resynthesis step following the excision of UV-induced pyrimidine dimers. Irradiation (313 nm), then causes the inserted BrdUrd to become alkali labile, whereas no such effect occurs in a thymidine control. These alkali-labile sites are then estimated by alkaline sucrose sedimentation, and their numbers are proportional to the amount of repair that has occurred (19).

Transformation. Cells at 10^6 per ml were treated in suspension in TD buffer (isotonic Tris-HCl/saline, pH 7.1) for 1 hr at 37° with various concentrations of 4NQO, washed, and

FIG. 2. Excision repair following $20 \text{ J}\cdot\text{m}^{-2}$ of UV irradiation in uninfected or RLV-infected rat cells and human fibroblasts. Repair was measured by BrdUrd incorporation and 313 nm photolysis (27). Human fibroblasts HSBP, ○; uninfected F111, △; and RLV-infected R111, ▲.

FIG. 3. Typical profiles of newly synthesized DNA as measured by alkaline sucrose sedimentation at 30,000 rpm for 155 min. Uninfected (○) or RLV-infected (●) rat fibroblasts following (A) no treatment, (B) 90 min of $0.25 \mu\text{M}$ 4NQO, or (C) $10 \text{ J}\cdot\text{m}^{-2}$ of UV irradiation. Samples were taken immediately after pulse (0 hr), after a 3 hr or a 6 hr chase.

plated for single colonies in growth medium containing 20% fetal calf serum. The fraction of dense growing morphologically transformed colonies was counted. A small number of these were picked, grown, and tested for ability to plate in soft agar (20). Ten percent of the colonies with transformed liquid morphology were judged to be truly transformed by their ability to form colonies in soft agar. The morphological colony counts were corrected by this factor. The stability of these transformed clones was measured by re-testing after several rounds of passing, freezing in liquid nitrogen, and passing again. The four clones tested retained their ability to plate in soft agar with a plating efficiency of 35–55%.

RESULTS

The relative amounts of 4NQO bound to the DNA of F111 (uninfected) and R111 (infected) cells immediately after treatment, and at various later times, are given in Fig. 1. Slightly more of both alkali-labile bonds without 313 nm irradiation, and breaks induced by 313 nm irradiation were found in the F111 cells following the same treatments. Of the detectable 4NQO–DNA adducts around 60% were removed from both the F111 and R111 DNA by 16 hr after treatment, and around 80% by 30 hr. The removal of lesions after $20 \text{ J}\cdot\text{m}^{-2}$ of UV irradiation and 18 hr incubation in medium as measured by BrdUrd photolysis indicated that the RLV-infected and uninfected rat cells used repaired only 30% as much as normal human cells, which have excised about 50% of the pyrimidine dimers after similar treatment (Fig. 2). An estimate of pyrimidine dimers induced and removed via a UV endonuclease assay (21) gave a similar result (data not shown).

The sizes of newly synthesized DNA as represented by the profiles in alkaline sucrose gradients are shown in Fig. 3 for untreated, UV-irradiated, and 4NQO-treated uninfected or RLV-infected rat fibroblasts. Cells were taken immediately after pulsing or following 3 and 6 hr chases. Fig. 4 represents

FIG. 4. Typical profiles of newly synthesized DNA as measured by alkaline sucrose sedimentation at 30,000 rpm for 155 min. Uninfected (O) or RLV-infected (●) rat kidney cells following (A) no treatment, (B) 90 min of $0.25 \mu\text{M}$ 4NQO or (C) $10 \text{ J}\cdot\text{m}^{-2}$ of UV irradiation. Sampling was immediately after pulse (0 hr), or after a 3 hr or 6 hr chase.

similarly treated uninfected or RLV-infected rat kidney cells. Infection of both fibroblasts and rat kidney cells with RLV only slightly modified the sedimentation of DNA from untreated cells, regardless of whether or not they were chased for 3 and 6 hr. UV irradiation or 4NQO treatments after RLV infection retarded the rate of increase in molecular weight as compared to that seen in uninfected cells. Differences between infected and uninfected cells were difficult to determine in treated unchased samples because their DNA was near the top of the gradients. However, differences became somewhat more apparent after 3 hr chases and considerably more so after 6 hr chases. The gradient profiles presented were the ones most commonly observed in this series of experiments, excepting those for rat fibroblasts following UV irradiation, which show the smallest differences obtained with early passage cells. Fig. 5 compares the reciprocal weight average molecular weights for untreated and insulted, uninfected or RLV-infected rat cells. These results are the averages of at least three experiments. For unchased or chased untreated samples the molecular weights of uninfected cells were an average of 1.02 to 1.1 times those of RLV-infected cells. In one experiment alone a factor of 1.2 was seen for unchased samples but this diminished to 1.1 upon chasing. Both UV irradiation and 4NQO treatment modified this response in that the molecular weights of DNA obtained from uninfected cells were an average of 1.35 times those of RLV-infected cells for unchased samples, increasing to an average of 1.8 after a 6 hr chase. Similar differences were observed following RLV infection of both rat fibroblasts and rat kidney cells. The effect seen was somewhat greater following the 4NQO treatment than after UV irradiation and diminished in magnitude as the cells attained late passages. Regardless of RLV infection, both UV- and 4NQO-treated cells were eventually able to convert their lower molecular weight newly synthesized DNA to the highest molecular weight measurable in our system, that is, 2.8×10^8 .

The *in vitro* induction of transformation by 4NQO in the

FIG. 5. The reciprocal weight average molecular weights (M_w) of uninfected (open symbols) and RLV-infected (solid symbols) rat fibroblast and kidney cells following no treatment (O, ●); $10 \text{ J}\cdot\text{m}^{-2}$ UV irradiation (Δ , $\blacktriangle$), or 90 min of $0.25 \mu\text{M}$ 4NQO ($\square$, $\blacksquare$). Samples were pulsed and chased as in *Materials and Methods*. The results are the average of at least three experiments on each cell line.

infected rat fibroblast line R111 is shown in Fig. 6. The frequency of transformants increased linearly with increasing dose to a maximum of 2.5×10^{-4} . The subsequent fall in frequency as dose continued to rise has been observed before during transformation of infected rat lines (3, 23), but remains unexplained. The peak number of transformants is usually observed at a dose of 4NQO resulting in survival in the culture as a whole

FIG. 6. Transformation of RLV-infected rat fibroblast (R111) per cell plated, as a function of 4NQO dose.

of about 10%. In similar experiments performed with F111, no transformed colonies were observed at any dose; their frequency is therefore calculated to be below 2×10^{-5} .

DISCUSSION

A postreplication mechanism of DNA repair after treatment with UV or chemical mutagens has been reported to function in human and other mammalian cells (13–15). The DNA synthesized in the first hour after UV treatment is generally smaller than that synthesized by unirradiated cells. Pyrimidine dimers are the UV lesions responsible for this reduction in DNA size, as photoreactivation specifically splits these lesions and reverses the effect (13). However, despite the continued presence of these lesions, the ability to synthesize DNA segments of normal size at later times after irradiation returns in human, Chinese hamster ovary (CHO), and L5178Y mouse cells but not in Chinese hamster B14 cells (13). One model suggests that gaps of about 10^3 nucleotides occur between the smaller segments; these are filled, and segments are joined by a postreplication repair mechanism to give high-molecular-weight DNA (13). Alternatively it has been proposed that gaps do not occur and pyrimidine dimers are “bypassed” by a branch migration mechanism that results in high-molecular-weight DNA (14). Regardless of the exact mechanism, it is the step by which high-molecular-weight DNA is synthesized in 4NQO-damaged cells that appears to be partially defective in leukemia-virus-infected rat cells compared to their uninfected counterparts. This defect is seen as a slower rate of increase in molecular weight of DNA synthesized after 4NQO treatment in infected R111 as compared to uninfected F111 cells. The difference in repair between the two lines cannot be attributed to the occurrence of more 4NQO lesions in the R111 line, because the indirect data on binding of 4NQO to the DNA of F111 and R111 cells show, contrarily, that 16% more lesions occur in the uninfected strain, F111. Moreover, it appears not to be due to a more effective removal of these lesions by excision repair in the uninfected line, since rates of excision repair are very similar in the two lines.

It could be argued that the 4NQO lesions responsible for the postreplication repair effect are not measured by photolysis. However, only two classes of 4NQO damage are known (7). One consists of 4NQO–purine adducts that are stable during acid hydrolysis, and are not repaired in excision-deficient bacteria or in xeroderma pigmentosum cells defective in the same process (7, 22). The second class consists of a 4NQO–guanine adduct that is unstable during acid hydrolysis. This is repaired in excision-deficient bacteria and in xeroderma pigmentosum human cells and they are induced in a ratio of about 4:1 acid stable: unstable (7, 22). By using the 313 nm photolysis technique it was shown that 4NQO-induced photo-labile sites are not repaired in xeroderma pigmentosum cells, but 4NQO-induced alkali-labile sites are repaired (16). Assuming a control value of $1/M_w$ of about 4×10^{-9} , our data show these lesions were also induced at a ratio of about 4:1. These facts strongly suggest that the photo-labile adducts correspond to 4NQO–purine adducts that are acid stable and the alkali-labile sites correspond to those that are unstable, and that there is not a separate class of hitherto-undetected lesions.

The slower rate of postreplication repair seen in R111 appears to be a general effect due to leukemia virus infection rather than a specific peculiarity of the interaction of the R111 line with 4NQO, for it is observed following infection of a rat kidney line as well as a rat fibroblast line and in both cases is seen after UV as well as after 4NQO insult. The effect is more pronounced following 4NQO treatment than after UV, which may be a

reflection of the different types of lesions involved. 4NQO produces 4NQO–purine adducts in DNA (7, 8). Unlike UV damage, which is repaired only by long patch excisions, the response of xeroderma pigmentosum cells to 4NQO indicates that there are at least two types of 4NQO lesions in mammalian cells, one subject to short-patch excision repair, the other to long-patch excision repair (16, 22).

Concomitantly with reducing the rate of increase in the molecular weight of DNA synthesized after 4NQO or UV treatment, which suggests a defect in postreplication repair, leukemia virus infection of these cell lines also makes them sensitive to *in vitro* transformation by chemical carcinogens. This sensitization of rat cells to chemical carcinogenesis by leukemia virus infection has been observed previously for many other carcinogens (1, 3, 23, 24), and is reproduced here for 4NQO. The nontransformability of the sister uninfected lines is well established at low passage, although at higher passages uninfected cells also become susceptible to transformation (1). We have noticed a diminution of the differences in postreplication repair between the infected and uninfected lines as the cells increase in age. This may well be related to the induction of an endogenous leukemia virus in the aging uninfected cells, as has been observed by others in similar lines and suggested as an explanation for their sensitivity to carcinogenesis at later passages (1).

The results presented here lend support to a genetic rather than epigenetic hypothesis for the action of chemical carcinogens in that inability to repair carcinogen-induced DNA lesions is correlated with sensitivity to chemical transformation. Although the role of postreplication repair in mammalian cell mutation is still unclear, its delay may well enhance the conversion of 4NQO lesions to stable transforming mutations.

Our *in vitro* results can be correlated with *in vivo* chemical carcinogenesis in that 4NQO is known to interact with DNA *in vivo*, inducing unscheduled DNA synthesis in the specific organs that are targets for its tumorigenic action (5). More importantly, a postreplication repair defect similar to that seen by us in infected rat lines was observed by Lehmann *et al.* (15) in cells derived from one class of xeroderma pigmentosum patients, “XP variants.” These cells are able to excise UV lesions normally, but show a delay of several hours in postreplication repair. However, the patients from whom the lines are derived show the full carcinogenic symptoms of the xeroderma pigmentosum disease. Thus, a defect in postreplication repair similar to that which correlates with carcinogenesis *in vivo* in Lehmann’s human cells correlates with 4NQO-induced transformation *in vitro* in our rat lines.

It remains to be determined whether the response reported here represents a general effect of leukemia virus infections or is limited to RLV interaction with rat cells. If it can be shown to be a general result of leukemia virus infection, then one can suggest that the role of leukemia viruses in the etiology of leukemia is to sensitize the infected cell to spontaneous transforming mutations as it sensitizes the rat cells to transformation by carcinogens like 4NQO.

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