Inhibition of DNA Repair Replication by DNA Binding Drugs Which Sensitize Cells to Alkylating Agents and X-Rays[1] (36818)

DAVID GAUDIN, ROBERT S. GREGG, AND K. LEMONE YIELDING[2]
Laboratory of Molecular Biology, University of Alabama Medical Center, Birmingham, Alabama

In recent years, there has been increasing attention given to the enzymic mechanisms by which a cell can repair a variety of potentially mutagenic damage to its DNA. Of particular interest has been the demonstration that mammalian cells can carry out excision repair (1–3). This is a process in which a chemically altered segment from one strand of a DNA double helix is excised. The excised bases are then replaced using the complementary strand as a template and the ends of the chains closed by a ligase. The net result of this process is to convert a chemically altered DNA back to a biologically active form which we presume to be identical to that existing prior to the damage. The recombination dependent type of recovery, first discovered by Rupp and Howard-Flanders in bacteria (4), also plays a role in mammalian cell recovery (5).

Accompanying the discovery of these repair processes have been numerous observations that inhibitors of repair decrease the survival of cells treated with agents such as uv-light, alkylating agents, or X-rays (6–8). These mechanisms are of particular relevance to tumor therapy with alkylating agents and X-rays because these agents cause chemical alterations to cellular DNA of precisely the kinds which can be repaired by mammalian cells. Furthermore, we have shown that chloroquine or caffeine which inhibit DNA repair in bacteria can also enhance the effect of X-rays and alkylating agents on certain transplantable tumors in hamsters (9, 10). The experiments reported below demonstrate that chloroquine and a variety of other DNA binding drugs inhibit the uv-stimulated uptake of thymidine by normal human lymphocytes subjected to inhibition of DNA synthesis by hydroxyurea. These results are taken as evidence for inhibition of repair replication in accordance with earlier results with bacteria (11).

Because of the general lack of specificity of the alkylating agents and X-rays for tumor cells, it is of obvious importance to determine the effects of repair inhibitors on normal tissues when these inhibitors are used in conjunction with the therapeutic agents. Since the bone marrow is highly sensitive to alkylating agents and X-rays, the white counts of mice have been followed as an indicator of toxicity following treatment with cyclophosphamide either alone or in combination with chloroquine. The effect of chloroquine on the survival of mice following treatment with large doses of X-rays has also been determined.

Methods. These experiments are based on the assay procedure of Evans and Norman (12) who demonstrated the presence of a "DNA repair" capability in human lymphocytes. Blood was collected from normal human donors in 50 ml heparinized "Vacutainers" then passed through a column packed with nylon fibers (Fenwal Laboratories). The blood was then mixed with 0.5 vol of 5% high molecular weight dextran (Sigma type 200 C), allowed to stand for 30 min at 37°, and the supernatant containing lymphocytes removed by aspiration. The white cells were washed by centrifugation, resuspended in phosphate buffered saline (PBS), and irradiated with 200 erg/mm^2 using an ultraviolet light emitting primarily at 2537Å. The cells were then centrifuged from the PBS and

[1] Supported by grants from the American Cancer Society (NP 55F) and the National Cancer Institute (CA-12763) and (CA-12538-01).

[2] RCD Awardee, U.S. Public Health Service (No. GM 22,698).

resuspended in 2 ml of Spinner modified Eagle's minimal essential medium (Baltimore Biological Laboratories) containing 2×10^{-3} *M* hydroxyurea. After standing for 10 min at 37°, 5 μCi/ml of tritiated thymidine (6.7 μCi/mmole, New England Nuclear) were added and the cells incubated for two more hours. At the end of the incubation period, an aliquot of the cells was transferred to the counting solution of Stewart and Ingram (13). Lymphocytic nuclei were then collected on 0.8 μm "Nucleopore" filters and washed successively with PBS, 5% TCA containing 1% sodium pyrophosphate, and 95% ethanol. The air dried filters were then transferred to scintillation vials containing a toluene based scintillation solution and counted. The activity of incorporated tritiated thymidine per 10^4 lymphocytes was determined for each sample. The cell counts were determined with a hemocytometer and were not performed until the end of the incubation period to ensure that any decrease in tritiated thymidine incorporation was not the result of cell lysis produced by the added drugs. Only data from experiments in which at least 85–90% of the cells remained intact at the end of the incubation period were considered suitable for publication. A small amount of incorporation into unirradiated controls was subtracted from each of the uv-irradiated samples. Then the incorporation into irradiated cells incubated in the presence of repair inhibiting drugs was calculated as a percentage of the incorporation into irradiated cells incubated without such drugs.

Results. The results of several experiments using blood drawn from normal healthy donors are shown in Fig. 1. DNA repair replication in the lymhocyte preparation is inhibited by low doses of chloroquine and reaches 50% inhibition at $6–7 \times 10^{-5}$ *M*. Other DNA binding drugs are seen to be even more potent inhibitors of repair replication: 4-(3-diethylaminopropylamino)-7-iodoquinoline for example, is a slightly more effective inhibitor than chloroquine. Quinacrine is also a more potent inhibitor as shown in Fig. 2. This latter drug produces a 50% inhibition of repair replication at slightly less than 1×10^{-5} *M*. By way of contrast, caffeine has no effect

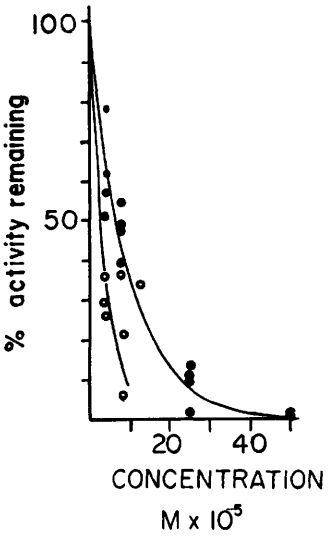

FIG. 1. The effect of varying concentrations of chloroquine (●) and of 4-(3-diethylaminopropylamino)-7-iodoquinoline (○) on the uv-stimulated incorporation of tritiated thymidine into normal human lymphocytes in the presence of hydroxyurea. The different points at each concentration represent blood samples drawn from different individuals. Calculation of the percentage activity remaining was performed as described in Methods.

on the incorporation of tritiated thymidine at concentrations as high as 2×10^{-3} *M*.

Although chloroquine, its iodinated analog, and quinacrine all share the property of binding to DNA not all drugs which bind to DNA inhibit the repair process. For example, streptomycin and D- and L-LSD at concentrations as high as 1×10^{-4} *M* produced no inhibition.

There was a possibility that some of the drugs used here might be inhibiting repair indirectly by inhibiting cellular respiration rather than through their DNA binding properties. In order to distinguish between these two possibilities, experiments were performed in the presence of KCN and dinitrophenol. The data presented in Table I show that these agents have little or no effect on the incorporation of tritiated thymidine which results from uv damage.

As part of our studies on the possible harmful effects of DNA repair inhibitors when used in conjunction with alkylating

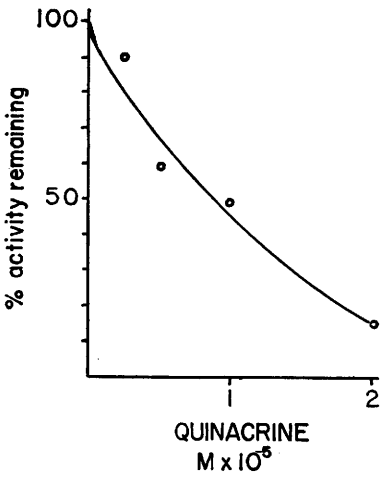

FIG. 2. Inhibition of DNA repair replication by quinacrine. The percentage activity remaining is defined in Methods.

agents or X-rays, we have examined the effects of cyclophosphamide (Cytoxan) either alone or in combination with chloroquine on the white blood counts of mice. In this series of experiments, we have extended previous observations (9) to higher dose levels of cyclophosphamide (80 mg/kg) and found that at these higher doses, the addition of chloroquine to the cyclophosphamide treatment still did not result in a greater lowering of the white count than did the cyclophosphamide alone (Fig. 3). However, the use of repair inhibitors in combination with X-rays is not without possible harmful side effects. As shown in Fig. 4, the administration of chloroquine to mice treated with large doses of whole body X-ray irradiation results in a greatly decreased survival.

TABLE I. The Effect of Dinitrophenol and Potassium Cyanide on DNA Repair Replication in Lymphocytes.[a]

	Conen	
	$1 \times 10^{-3} M$	$1 \times 10^{-4} M$
DNP	75	77
KCN	70	120

[a] The percentage activity of DNA repair replication remaining when the lymphocytes were incubated with the indicated concentrations of dinitrophenol or potassium cyanide. Determination of the percentage activity remaining was as described in Methods.

Discussion. The use of sensitizers to enhance the effects of X-rays and of alkylating agents on experimental tumors *in vivo* (9, 10) led to the present experiments in which it has been demonstrated that these sensitizing agents are potent inhibitors of DNA repair in lymphocytes. In the case of chloroquine, iodoquine and quinacrine, the inhibition occurs over a concentration range which may reasonably be reached *in vivo*. These drugs are relatively nontoxic when administered alone. Furthermore, the experiments reported here demonstrate that chloroquine does not enhance the cytotoxic effect of cyclophosphamide on hematopoietic tissue of mice.

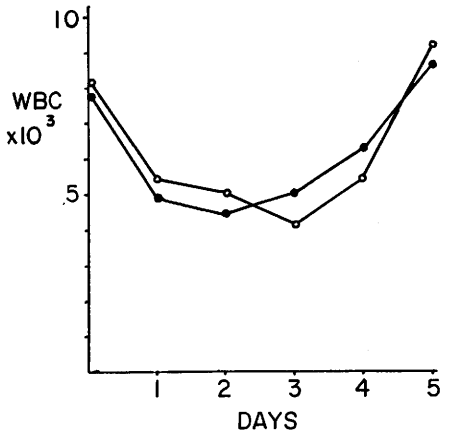

FIG. 3. The effect of Cytoxan (80 mg/kg) either alone (●) or in combination with 30 mg/kg of chloroquine (○) on the white blood counts of mice is shown for different time periods following the intraperitoneal injection of the drugs.

Caffeine did not dcrease the repair replication in lymphocytes following uv-irradiation. This result is consistent with earlier observation by Cleaver and Thomas (5) who also showed that caffeine is an inhibitor of the bypass or recombination dependent recovery mechanism. In this system, caffeine prevents the joining of newly formed short strands of DNA in uv-irradiated cells. It is not known whether caffeine also inhibits the ligase step of repair, a type of reaction which is essential for both excision repair and the bypass type

of recovery. Caffeine does, however, sensitize tumors *in vivo* to the effects of both cyclophosphamide and X-rays (9, 10). Where both types of recovery mechanism are present in the same cells, inhibition of both may be necessary to obtain the optimum amount of sensitization. In earlier experiments the combined use of both caffeine and chloroquine in conjunction with cyclophosphamide or X-rays appeared to result in a greater sensitization of the tumors (9).

The repair replication observed in the presence of KCN or dinitrophenol is convincing evidence that the inhibition of repair caused by other compounds reported here is not due to the indirect effect of inhibiting electron transport or of uncoupling oxidative phosphorylation. Dalrymple, Sanders and Baker (15) similarly found that the presence of dinitrophenol did not inhibit the survival of cells treated with X-rays.

From our earlier experiments, it would appear that inhibitors of the DNA repair mechanism may find some use in the field of tumor therapy when used in combination with either alkylating agents or X-rays (9, 10). However, inhibition of the DNA repair process is not without possible harmful effects. This is seen in the case of X-ray treated mice where the additional administration of chloroquine resulted in a marked decrease in survival (Fig. 4). The shape of these curves shows a remarkable similarity to those of irradiated cells in culture, where the shoulder region is eliminated by inhibitors of the repair process. A chloroquine induced sensitization of the oral mucosa to X-rays has been observed during the treatment of tumors of the head and neck.[3] Furthermore, others have found that diethylstilbestrol increases the tumorigenic effects of X-rays (16). We now have evidence indicating that co-carcinogenesis including the diethylstilbestrol induced sensitization to X-ray tumorigenesis may be the result of DNA repair inhibition (17). Because of these observations, any treatment of humans with repair inhibiting drugs should proceed with caution. Also, the inhibition of repair should be considered as a possible harmful side effect of DNA binding drugs, particularly of those which may be used during X-ray or alkylating agent therapy of tumors or other disease processes.

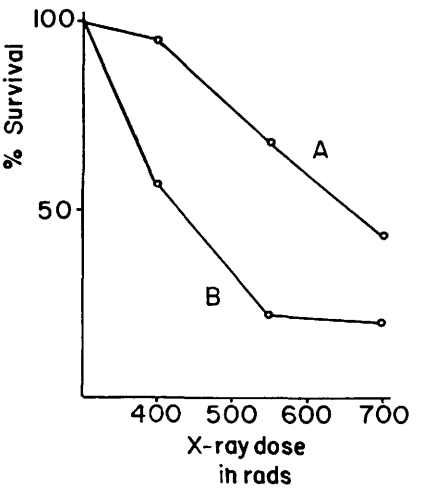

FIG. 4. The effect of chloroquine on the survival of mice treated with X-rays (A) or X-rays in combination with chloroquine (B). Each point indicates the percentage survival in a group of 20 mice.

Neither the D- nor the L-forms of LSD were found to inhibit repair replication although this drug has been shown to bind to DNA (18). Therefore, any possible chromosome abnormalities, if they are in fact caused by this drug, must arise in some other manner.

In order for sensitizing drugs to have any use in tumor therapy, it is not sufficient merely that they sensitize tissues to the effects of X-rays or alkylating agents. If the balance of sensitivities between normal and neoplastic tissues is not altered, the net effect of therapy will not be improved. However, there are several experiments which suggest that such sensitization can be achieved with some degree of selectivity. One such example, the iodinated analog of chloroquine, binds to melanin and selectively concentrates in melanoma (14). Other workers (19) have found that medroxyprogesterone will sensitize endometrial carcinoma to the effects of ionizing radiation. We have demonstrated that this steroid analog and a variety of steroids are effective inhibitors of the DNA repair process

[3] Dr. Carlo Nervi, personal communication.

when used at higher than physiological levels. The tissue selectivity of these drugs may make them useful in sensitizing preferentially certain tissues to the effects of alkylating agents and X-rays. We have also found that prednisone is an inhibitor of the repair process at concentrations which approximate those used therapeutically in treatment of lymphocytic leukemia. The results of our experiments with steroids will be published in a separate paper.

Our investigations with drugs which sensitize tumor cells to the action of alkylating agents or ionizing radiation indicate that this sensitization is the result of DNA repair inhibition. Furthermore, the examples cited above warrant a continued search for drugs which sensitize tissue to the action of X-rays and alkylating agents, and do so with a relative degree of selectivity.

Summary. A number of DNA binding drugs which sensitize living cells to the action of alkylating agents and X-rays have been shown to inhibit the uv-stimulated incorporation of tritiated thymidine by normal human lymphocytes incubated in the presence of hydroxyurea.

It is proposed that such DNA repair inhibiting drugs may have a role in tumor therapy when used in conjunction with alkylating agents or X-rays.

1. Crathorn, A. R., and Roberts, J. J., Nature (London) **211**, 150 (1966).

2. Rasmussen, R. E., and Painter, R. B., J. Cell Biol. **29**, 11 (1966).

3. Regan, J. D., Trosko, J. E., and Carrier, W. L., Biophys. J. **8**, 319 (1968).

4. Rupp, W. D., and Howard-Flanders, P., J. Mol. Biol. **31**, 291 (1968).

5. Cleaver, J. E., and Thomas, G. H., Biochem. Biophys. Res. Commun. **36**, 203 (1969).

6. Elkind, M. M., Whitmore, G. F., and Alescio, T., Science **143**, 1454 (1964).

7. Rauth, A. M., Radiat. Res. **31**, 121 (1967).

8. Fox, M., Int. J. Cancer **3**, 382 (1968).

9. Gaudin, D., and Yielding, K. L., Proc. Soc. Exp. Biol. Med. **137**, 202 (1971).

10. Gaudin, D., and Yielding, K. L., Proc. Soc. Exp. Biol. Med. **131**, 1413 (1969).

11. Yielding, K. L., Yielding, L., and Gaudin, D., Proc. Soc. Exp. Biol. Med. **133**, 999 (1970).

12. Evans, R. G., and Norman, A., Radiat. Res. **36**, 287 (1968).

13. Stewart, C. C., and Ingram, M., Blood **29**, 628 (1967).

14. Lieberman, L. M., Boyd, C. M., Varman, V. M., Bergstrom, T. J., and Beierwaltes, W. H., J. Nucl. Med. **12**, 153 (1971).

15. Dalrymple, G. V., Sanders, J. L., and Baker, M. L., Nature (London) **216**, 708 (1967).

16. Segaloff, A., and Maxfield, W. S., Cancer Res. **31**, 166 (1971).

17. Gaudin, D., Gregg, R. S., and Yielding, K. L., Biochem. Biophys. Res. Commun. **45**, 630 (1971).

18. Yielding, K. L., and Sternglanz, H., Proc. Soc. Exp. Biol. Med. **128**, 1096 (1968).

19. Bonte, J., Decoster, J. M., and Ide, P., Cancer **25**, 907 (1970).

Received May 24, 1972. P.S.E.B.M., 1972, Vol. 141.