Studies on DNA Repair in Human Lymphocytes Treated with Proximate Carcinogens and Alkylating Agents

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SUMMARY

The incorporation of thymidine into DNA by human peripheral blood lymphocytes after exposure to alkylating agents or proximate carcinogens was used to investigate presumed DNA repair in a nondoning human cell system. In the presence of hydroxyurea, which was used to suppress the "background" DNA synthesis in the rare cell in S phase, lymphocyte cultures treated with nitrogen mustard, methyl methanesulfonate, or ethyl methanesulfonate, or with two proximate carcinogens, β-propiolactone or N-acetoxy-2-acetylaminofluorene, incorporated from 4 to 9 times as much thymidine as did controls. Autoradiographs indicated that about 90% of the lymphocytes participated in this response in contrast to the control preparations in which no more than 0.1 to 0.2% of the cells showed labeling with thymidine. Preparation of DNA by isopycnic centrifugation in CsCl and digestion with snake venom phosphodiesterase was used to show that thymidine was actually incorporated into DNA and that less than 20% of this incorporation could be accounted for by terminal addition. No stimulation was noted with the precarcinogens dimethylnitrosamine, 3'-methyl-4-dimethylaminoazobenzene, and 2-acetylaminofluorene or with iodoacetamide which alkylates protein but not DNA. Electron micrographs of cells 15 hr after treatment with nitrogen mustard or methyl methanesulfonate showed considerable evidence of cellular damage, marked especially by separation of the inner and outer nuclear membranes. Very little if any damage was seen with N-acetoxy-2-acetylaminofluorene.

INTRODUCTION

Important advances in our understanding of molecular events preceding chemically induced malignant transformation have occurred during the past decade (14, 23, 24, 27). A large number of carcinogens owe their activity to the generation of unstable electrophilic species which react in specific ways with DNA, RNA, and proteins both in vivo and in vitro (27). Because of the central role of the genome in regulating cellular processes and the large number of agents which react with DNA, it would appear that an understanding of DNA repair processes in chemical and enzymatic terms is important in the metabolic and cellular analysis of carcinogenesis.

The repair process has generally been studied in rapidly dividing bacteria (4, 9, 18, 22, 29, 31, 37) or in continuously dividing mammalian cells (6, 17, 30, 32–34, 38, 39) following exposure to various types of irradiation or to chemicals. While such an approach has already proven to be very useful and rewarding, it would appear that the eventual elucidation of mechanisms of DNA repair might be facilitated by studying the process in nondoning cell populations where damage-induced and normal DNA replication can be separated and thereby studied independently and compared.

Human peripheral lymphocytes, although not an absolute resting cell population, do provide a close approximation to such a system, since the vast majority of the cells are not actively participating in the cell cycle unless exposed to mitogens (13). This cell system has been studied from the point of view of radiation repair (13) but apparently has not been used for carcinogens or other alkylating agents.

As a prelude to subsequent studies of the effects of carcinogens on target organs, we have examined the possible effects of a few selected carcinogens on DNA metabolism in human lymphocytes. This was done on a comparative basis in association with some cytotoxic alkylating agents already known to induce apparent DNA repair in a few mammalian cell systems (17, 34). Some potent proximate carcinogens (27) as well as other alkylating agents were found to stimulate thymidine incorporation markedly into DNA in nondoning human lymphocytes. In contrast, some precarcinogens (27) and IAA, a compound which alkylates primarily protein sulphydryl groups, were without effect on thymidine incorporation. The response patterns of lymphocytes to these agents, with some biochemical, radioautographic, and electron microscopic approaches, are the subject of this communication.

The abbreviations used are: IAA, iodoacetamide; HU, hydroxyurea; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; DMNA, dimethylnitrosamine; BPL, β-propiolactone; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; AAF, 2-acetylaminofluorene; DMSO, dimethyl sulfoxide; PHA, phytohemagglutinin; TdR-3H, tritiated thymidine.

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MATERIALS AND METHODS

Lymphocytes. Human peripheral blood lymphocytes from 20- to 30-year-old men and women were prepared by gelatin sedimentation after defibrination of whole blood (10). The white blood cells in such preparations were approximately 80 to 90% lymphocytes, with an 8- to 15-fold excess of red cells. Lymphocytes (2 to 3 x 10^6) were cultured in 2 to 2.6 ml of modified Eagle's medium (Microbiological Associates, Bethesda, Md.) to which glutamine (2 mM, Microbiological Associates), penicillin and streptomycin (10 units/ml of each, Microbiological Associates), and 20% of the autologous serum: gelatin mixture was added. In one 7-day experiment, the medium was supplemented with tryptose powder, 5 mg/ml (Difco Laboratories, Inc., Detroit, Mich.). No mitogens were added unless specifically indicated. Cells were maintained at 37° and gassed with 95% air:5% CO₂.

Induction of Cell Damage and Use of HU. MMS, EMS, and AAF (both from Eastman Organic Chemicals, Rochester, N. Y.), HN2, (Mustargen, Merck Sharp & Dohme, West Point, Pa.), DNMA (Eastman), IAA (Nutritional Biochemicals, Cleveland, Ohio), HU (NSC 32065, Cancer Chemotherapy Service Center of the National Cancer Institute, Bethesda, Md.), and BPL (Eastman) were made up in 0.9% NaCl solution. The 3'-Me-DAB (K & K Laboratories, Plainview, N. Y.), N-acetoxy-AAF, N-hydroxy-AAF, and AAF (Eastman) were dissolved in ether, acetone, or DMSO (Matheson, Coleman and Bell, Norwood, Ohio) and subsequently diluted with 0.9% NaCl solution. All agents were added to cultures immediately thereafter. Final DMSO concentrations did not exceed 1%, and each agent was tested in both DMSO (36) and one other solvent. After the addition of acetone or ether solvents to 0.9% NaCl solution, the volatile solvent was blown off under nitrogen before the final solution was added to cultures. No differences in the response patterns were noted when HU was added up to 15 min before the damaging agents or immediately afterward. Cultures were exposed to damaging agents for 1 hr after which they were centrifuged (1000 rpm for 10 min in an International PR2 centrifuge at 22°), and the alkylating medium was replaced with fresh medium. Control cultures received 0.9% NaCl solution, DMSO, acetone, or ether. When PHA was used, 1 vial of Difco PHA M was diluted with 5 ml of medium, and 0.03 ml was added to each culture.

Liquid Scintillation Counting. After alkylation, TdR-3H (20 Ci/mmmole, labeled on carbon 2, Amersham-Searle Co., Arlington Heights, III.) was added to a final concentration of 0.75 to 5.0 μCi/ml. In a few experiments, thymidine-14C (59 mCi/mmmole, labeled on carbon 2, Amersham-Searle Co., Arlington Heights, III.) was added to a final concentration of 0.125 μCi/ml. The reaction was stopped by cooling the culture tubes in ice, followed by centrifuging at 1000 rpm for 10 min at 4° and aspirating off the radioactive medium. The cells were washed once with 3 ml of ice-cold 0.9% NaCl solution, precipitated with 3 ml of ice-cold 5% trichloroacetic acid, and spun at 2000 rpm for 15 min at 4°. The precipitates were washed with 5% trichloroacetic acid and extracted with methanol. The pellets were dried at 50–60°, solubilized in Soluene (Packard Instrument Company, Inc., Downers Grove, Ill.), and then a toluene:POPOP scintillation mixture was added. The radioactivity present was measured in a Packard Tri-carb liquid scintillation spectrometer, and the results were expressed as cpm/culture. All recorded values are the average of the counts of duplicate cultures. In most cases, duplicate counts did not vary by more than 10%. Each experiment was performed at least twice.

Autoradiography. Cells in culture were damaged with various agents as described above, washed, and cultured for an additional 3 hr in the presence of TdR-3H (20 Ci/mmmole, 5 μCi/ml). The cells were washed 3 times in culture medium, spread on glass slides, air dried, fixed and washed in methanol, and coated with Kodak NTB emulsion. The coated slices were held in light-free boxes for 2 to 3 weeks at 4°, developed, and stained with hematoxylin and eosin (12).

Isolation and Characterization of DNA. The methods of Flamm et al. (16) and Anet and Strayer (1) were used to prepare DNA. Approximately 60 x 10^6 cells were washed with 0.9% NaCl solution and homogenized in 0.01 M Tris:HC1 buffer (pH 8) with 1% sodium dodecyl sulfate; solid CsCl (K & K Laboratories) was added to give a density of approximately 1.71 g/ml. Aliquots (4.5 ml) were centrifuged at 13,000 rpm for 30 min (fixed angle, Ti 50 rotor, Spinco L2-65B ultracentrifuge, 25°). After removal of the protein, the gradient was overlaid with mineral oil and centrifuged for 18 hr at 45,000 rpm, followed by centrifugation at 33,000 rpm for 40 hr. A 7-drop (ca. 0.1 ml) fraction was collected, diluted with 0.2 ml of buffer, and read in a Zeiss PMQ II spectrophotometer. A 0.05-ml sample from each fraction was removed, acidified with a drop of 2.5 N HCl, and diluted to 1 ml. Samples were counted after the addition of 10 ml of a mixture of 1 part Triton X-100 and 2 parts toluene:POPOP mixture.

DNA was prepared for digestion with snake venom phosphodiesterase as follows. Peak fractions were collected, pooled, and dialyzed against 2 changes of 0.05 M Tris:HC1 buffer (pH 8) with 0.01 M MgCl₂ (300 volumes of buffer per volume of sample). The sample was then treated for 2 hr at 37° with 50 μg/ml of pancreatic RNase (Sigma Chemical Co., St. Louis, Mo.) in which DNase was inactivated by heat treatment at 90° for 15 min. For our studies, we felt that the small amount of DNA-RNA hybrids possibly present in unstimulated lymphocytes could be ignored. Oligoribonucleotides were removed by dialysis against 0.05 M Tris:HC1 (pH 8.5) with 0.01 M MgCl₂ as described above. RNase digestion resulted in no loss of absorbance or counts.

Digestion of DNA and examination of terminal addition were carried out with snake venom phosphodiesterase (Worthington Biochemicals Corp., Freehold, N. J.) in 0.037 M Tris (pH 8.5), 0.075 M MgCl₂, and bovine serum albumin, 0.5 mg/ml. Twenty μg/ml of snake venom phosphodiesterase were added to a DNA sample (A₂₆₀ = 1.032, excluding bovine serum albumin: 17,000 cpm/ml). The reaction was run at 37°, and at several time intervals 0.25-ml aliquots were removed, cooled to 0°, mixed with 0.15 ml of cold bovine serum albumin, and precipitated with 0.15 ml of cold 2 N perchloric acid. After centrifugation (8000 rpm, Sorvall RC2-B), acid-soluble cpm and absorbance at 260 nm were determined.

Cell Viability Studies by Dye Exclusion. Lymphocyte cultures were examined 12 hr after damage for the ability of...
the cultured cells to exclude trypan blue. Cells were resuspended at a concentration of $3 \times 10^6$ cells/ml, and 2 drops of culture were mixed with 1 drop of 2% trypan blue. Cells excluding trypan blue and those taking up the dye were counted in a hemocytometer chamber, and the results were expressed as percentage of cells excluding dye. Cell counts on cultures at Time 0 and 24 hr were done to exclude loss of cells after alkylation.

**Electron Microscopy.** Cells were fixed in Karnovsky's fixative (19) and 4% osmium (3:1) at 4°C for 1 to 2 hr, embedded and sectioned as described by Locker et al. (25), and examined with a Phillips 200 electron microscope.

**RESULTS.**

**Effects of HU in Untreated and Alkylated Lymphocyte Cultures.** Untreated lymphocyte cultures incorporate thymidine during the first 12 hr, presumably due to the small numbers of lymphocytes present that are in S phase (Chart 1). This thymidine incorporation by untreated cultures is suppressed by the addition of alkylating agents or HU. However, in the presence of HU, both alkylating agents cause marked increases in thymidine incorporation when compared to controls treated with HU only (Chart 1). Increasing the HU concentration from 1 to 10 mM enhances the differences between alkylated and control cultures. The stimulation of thymidine incorporated by the alkylating agents is linear for about 6 hr and levels off between 6 and 12 hr. This time sequence is independent of HU concentration for the doses used. Thus, the suppression of thymidine incorporation by HU treatment permits observation of the effects of alkylating agents as revealed by subsequent thymidine incorporation.

**Autoradiography.** The cellular distribution of thymidine incorporation was investigated by autoradiography of unalkylated cells incubated with 0, 1, and 10 mM HU and cells alkylated with 0.0015 and 0.015 mM HN2; 0.01, 0.1, and 1.0 mM MMS; and 1 mM N-acetoxy-AAF both with and without HU (Fig. 1 and 2). In unalkylated cultures, rare lymphocytes (1 to 2 per 1000) were heavily labeled and probably represent cells in S phase. Occasional other cells contained 1 or 2 grains, but the vast majority showed no labeling. With the addition of HU, a few heavily labeled cells were still present, but the labeling of these cells was much less intense. Most likely this is a morphological demonstration of the suppression of DNA synthesis by HU seen in control cultures (Chart 1). Because of the low frequency of heavily labeled cells and the decreasing intensity of the label with increasing concentrations of HU, it was not feasible to determine whether or not HU also decreased the number of labeled cells.

In cultures damaged with HN2, MMS, or N-acetoxy-AAF, the great majority (roughly 90%) of lymphocytes (Fig. 2) were lightly labeled; for example, for the higher dose of HN2 and the doses of MMS each cell was covered by 5 to 9 grains. At the lowest dose of HN2, 1 to 2 grains per cell were noted. None of the red cells or the occasional neutrophils present were labeled. Because HN2, MMS, and N-acetoxy-AAF interfere with replicative synthesis (see below), it was not possible in alkylated cultures to determine the fate of the occasional dividing cell since such cells are already rendered obscure by treatment with HU. Thus, the normal, background incorporation of thymidine occurs in only an isolated cell, while the incorporation following treatment with HN2, MMS, or N-acetoxy-AAF occurs in the vast majority of lymphocytes. This light labeling of most of the lymphocytes in response to alkylation occurs regardless of whether or not HU is added to the medium. Thus, HU itself is not necessary for the phenomenon but is only essential to make it apparent for scintillation counting.

**Response to Alkylating Agents and Carcinogens.** The dose-response curves were determined for 3 alkylating agents (HN2, MMS, and EMS) in the presence of HU (Chart 2). The response to the 3 agents is similar. In each case, with increasing concentration there is increasing stimulation of thymidine incorporation to a maximum of 7 or 8 times control values followed by a decline at higher concentrations. The maximum response was seen with lower concentrations of HN2 than of MMS and in turn required considerably higher concentrations of EMS.

The results of an experiment in which BPL, a potent skin carcinogen, was used to alkylate lymphocytes are presented in Chart 3. In the presence of HU, a 3- to 4-fold stimulation of incorporation of thymidine at a 1 mM concentration of BPL is noted, followed by a drop at 10 mM. These results are similar to those for the 3 noncarcinogenic alkylating agents (Chart 2), although the amount of stimulation is smaller. When the reaction is run without HU, there is a marked fall in incorporation of thymidine at a dose of 1 mM BPL (compare with Charts 1, 4, and 5). This probably represents selective inhibition of DNA synthesis in the occasional dividing cells. These results point out the difficulty in determining which dose represents maximal stimulation in the damaged resting lymphocytes, since the baseline (represented partially by cells in S phase) may change; presumably, however, this effect

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*This value may not be uniform from donor to donor. Although autoradiography was done on only two different donors, a large number of liquid scintillation counts of control cultures reveal a wide range among donors.*
effect. The response to N-hydroxy-AAF in the presence of HU is substantial (3 to 4 times controls) but less marked than that of N-acetoxy-AAF; furthermore, it occurs at a 10-fold higher concentration. These intermediate results might be explained by the reduced uptake and/or a low rate of in vivo esterification of the N-hydroxy compound (27). In the absence of HU, there is depression of DNA synthesis at higher concentrations resembling the response to all the other active agents. However, unlike the other agents examined, N-acetoxy-AAF and N-hydroxy-AAF, in the presence of HU, did not produce a reduction in thymidine incorporation at the highest concentration tested. These compounds were not tested at concentrations higher than 1 mM because, even at that concentration, a marked turbidity developed.

Demonstration of Thymidine Incorporation into DNA. Cells were damaged with 0.1 mM N-acetoxy-AAF in the presence of 10 mM HU as previously described except that the

Chart 2. Effect of alkylation agents on thymidine incorporation by lymphocyte cultures. Cultures (3 \times 10^6 lymphocytes) were alkylated with the indicated doses of HN2, MMS, and EMS for 1 hr and then exposed to a 12-hr pulse of thymidine (0.83 \muCi/ml, 20 Ci/mmmole) in the presence of 10 mM HU. In this and subsequent experiments, the concentration is expressed on a log scale.

Chart 3. Effect of BPL (\#PL) on thymidine incorporation by lymphocyte cultures. Conditions were similar to those presented in Chart 2 except that slightly less Tdr-\(^3\)H (0.77 \muCi/ml) was used. Top curve, cells exposed to BPL alone; bottom curve, results from exposure to BPL and 10 mM HU.

should not be very great since the HU has eliminated most of the DNA synthesis in dividing cells.

The results of a similar study with HN2, 2 precarcinogens (DMNA and 3'Me-DAB), and IAA are presented in Chart 4. There is a peak of thymidine incorporation between 0.01 and 0.1 mM HN2 concentration in the presence of HU and a fall in the amount of incorporation as the HN2 concentration is increased in the absence of HU. Neither of the 2 precarcinogens produces any stimulation of thymidine incorporation by lymphocytes. Similarly, IAA, which alkylates protein sulfhydryl groups (5) and is without apparent effect on DNA, produced no increase in thymidine incorporation.

The results presented in Chart 5 demonstrate that, in the presence of HU, N-acetoxy-AAF produces about a 9-fold increase in thymidine incorporation at 0.1 mM concentration, while AAF, the parent precarcinogen, is entirely without effect. The response to N-hydroxy-AAF in the presence of HU is substantial (3 to 4 times controls) but less marked than that of N-acetoxy-AAF; furthermore, it occurs at a 10-fold higher concentration. These intermediate results might be explained by the reduced uptake and/or a low rate of in vivo esterification of the N-hydroxy compound (27). In the absence of HU, there is depression of DNA synthesis at higher concentrations resembling the response to all the other active agents. However, unlike the other agents examined, N-acetoxy-AAF and N-hydroxy-AAF, in the presence of HU, did not produce a reduction in thymidine incorporation at the highest concentration tested. These compounds were not tested at concentrations higher than 1 mM because, even at that concentration, a marked turbidity developed.

Demonstration of Thymidine Incorporation into DNA. Cells were damaged with 0.1 mM N-acetoxy-AAF in the presence of 10 mM HU as previously described except that the

Chart 4. Effect of HN2, DMNA, 3'-Me-DAB, and IAA on thymidine incorporation by lymphocyte cultures. The conditions are the same as those in Chart 3. The 3'-Me-DAB was prepared in ether as described in the text.

Chart 5. Effect of the precarcinogen AAF, its active carcinogenic derivative N-acetoxy-AAF, and a chemical intermediate N-hydroxy-AAF on thymidine incorporation by lymphocyte cultures. All agents were added in DMSO (final concentration, 0.83%). HN2 (in 0.15 M NaCl solution) is included for comparison. Conditions are as in Chart 3.
DNA Repair in Human Lymphocytes

The study was determined. Because the phenomenon studied appears to be completed in 12 hr, cultures were examined by dye exclusion, cell counts, electron microscopy, and PHA stimulation at various times up to 24 hr after in vitro incubation.

Hemocytometer counts of control untreated cultures at Time 0 and 24 hr revealed no change in cell number. Trypan blue exclusion studies at 0 and 12 hr showed only rare (1%) dye uptake. Electron microscopy of cells prepared at 0 and at 24 hr both with and without 10 mM HU showed no differences (e.g., Fig. 3); the morphology was essentially the same as that previously reported [11] and will not be covered in detail here. Finally, stimulation of cultures on Day 0 or on Day 1 with PHA and assessment of blast transformation by following thymidine-\textsuperscript{14}C incorporation revealed identical responses (Chart 8). These results suggest that, over the period studied, freshly cultured lymphocytes represent a stable cell population. In addition, they suggest that the leveling off of the incorporation curves in alkylated cultures (Chart 1) is not related to extraneous events such as aging, utilization of culture medium, or a shifting cell population. Unstimulated cultures show increased incorporation on each successive day; this incorporation probably represents increasing numbers of dividing cells resulting from multiplication of the few dividing cells originally present.

Retention of Response to Alkylation by 24-Hr Cultures.

Although the data presented in Chart 8 suggest that there is no loss in the ability of control or treated cells to replicate DNA after 24 hr in culture, they do not allow one to assess the effect of culturing on the ability to respond to alkylation. Thus, an experiment was run in which some cultures were alkylated soon after the cells were obtained from the donor

Chart 7. Snake venom phosphodiesterase digestion of DNA from cells damaged with N-acetoxy-AAF and allowed to repair for 12 hr in the presence of TdR-\textsuperscript{3}H (see text for details). Ordinate, percentage of total cpn ($) or total absorbance at 260 nm (o) rendered acid soluble. Insert, comparison of the rate of release of cpn to the rate of release of 260 nm-absorbing material as a function of incubation time. Ordinate, ([\textDelta cpn/min]/[\textDelta A_{260} /min]) \times 10^6; abscissa, reaction time.
while others were held for 24 hr in vitro before alkylation (Table 1). After 24 hr in culture, cells retain the ability to respond to alkylation.

**Dye Exclusion Test.** Trypan blue exclusion studies were performed on control cells and those damaged 12 hr previously with concentrations of HN2 from 0.001 to 0.4 mM, of N-acetoxy-AAF from 0.001 to 1 mM, and of MMS at 0.1 mM. The number of cells from control cultures taking up the dye was about 1%. For the cells damaged with HN2, the results were 0.0001 mM, 0 to 1%; 0.001 mM, 0 to 1%; 0.01 mM, 3.8%; 0.4 mM, 4.9%. For cells damaged with N-acetoxy-AAF the results were: 0.001 mM, 0 to 1%; 0.01 mM, 0 to 1%; 0.1 mM, 1.5%; 1.0 mM, 6.4%. At a concentration of 0.1 mM MMS, 12% of the cells failed to exclude dye.

**Electron Microscopy.** Cells were examined by electron microscopy 15 hr after damage with 0.001 and 0.01 mM HN2, 0.2 mM MMS, and 0.01 and 0.1 mM N-acetoxy-AAF. With the higher dose of HN2 and MMS, apparently dead cells with pycnotic nuclei, loss of cytoplasmic organelles, and large vacuoles were noted. A much more frequent occurrence in both populations (Figs. 4 and 5) was marked cellular damage characterized by loss of cytoplasmic processes, extensive cystic dilatation between the inner and outer nuclear membranes, and peripheral nuclear clumping of chromatin. Such changes involved the majority of cells in these two preparations. At the lower dose of HN2, such changes occurred only infrequently. In contrast to the results with HN2 and MMS, the majority of cells appeared normal with the higher dose of N-acetoxy-AAF (Fig. 6). While a few cells were obviously affected and showed changes similar to those seen with the higher dose of HN2 and MMS, nevertheless, on the whole the population much more closely resembled control cultures. At the 0.01 mM dose of N-acetoxy-AAF, almost no cells were affected.

**DISCUSSION**

Lymphocytes respond in general to several alkylating agents including carcinogenic ones as they do to UV light (10) and to X-irradiation and electrons (38). The need for HU to suppress the background incorporation of thymidine, the time course, the involvement of the majority of cells, and the degree of stimulation in the presence of HU are all comparable. Also, lymphocytes resemble other mammalian cells (6, 17, 30—34, 38, 39) as well as prokaryotes (4, 9, 18, 22, 29, 31, 37) in their response patterns.

Not only is there a qualitative resemblance but there is also a quantitative one in the responses of lymphocytes and other cells to alkylating agents. The relative order of sensitivity of lymphocytes, HN2 > MMS > EMS, agrees in general with the findings in bacteria (5) and in HeLa cells (J. J. Roberts, personal communication). The increased reactivity of HN2 is probably not the result of its difunctional nature since only 25 to 30% (6, 21) of the molecules react with 2 guanines. It is
more likely related to the ease with which electrophiles can be generated from the parent compounds (21). Three precarcinogens (AAF, DMNA, and 3'-Me-DAB), each of which must be metabolized to an active intermediate before it can react with DNA (27, 28), produce no stimulation of thymidine incorporation.

With HN2, BPL, N-acetoxy-AAF, and N-hydroxy-AAF, there is suppression of DNA synthesis in the absence of HU. This probably represents suppression of the occasional dividing cells which, on a cell-for-cell basis, synthesize many times more DNA than the repair cells. Many in vitro studies (e.g., Refs. 35 and 41) have demonstrated similar inhibition of DNA synthesis by alkylated templates.

Purification of DNA by isopycnic centrifugation in CsCl and enzymatic digestion demonstrates that the thymidine incorporated by damaged cells is present predominantly, if not exclusively, in DNA. We have used N-acetoxy-AAF as the active agent because less cellular damage is seen by electron microscopy with this agent than with the alkylating agents, and the guanine C-8 substitution product with the AAF derivative is considered to be much more stable than is the N-7 guaninyl derivative of HN2, MMS, or EMS (20). Thus there are fewer artifacts with which to contend in interpretation of digestion studies.

In our hands, 80 to 85% of this label in the DNA appears to be internal. The small amount of terminal addition (15 to 20%) is not unexpected and is best interpreted as representing areas in which repair is occurring but in which ligation has not yet been completed. Using MMS, Ayad et al. (2) have interpreted their data as representing primarily terminal addition. However, the use of dividing cells, bromodeoxyuridine, MMS, and an indirect assay for terminal addition make their data hard to compare with ours.

An interesting question that becomes important in the eventual understanding of the DNA repair phenomenon in eukaryotes is the intracellular site of damage and repair. Wunderlich et al. (43) have shown that some carcinogens react more rapidly with mitochondrial DNA than with nuclear DNA. Whether the target in the case of human lymphocytes in our study is the nuclear or the mitochondrial DNA has not been established. The small number of mitochondria in unstimulated human lymphocytes make this cell an unsuitable one for such studies. However, Epstein et al. (12) show that, at least in the case of UV damage to skin, thymidine incorporation by N-acetoxy-AAF compared to HN2 (Chart 5) may be a manifestation of this retained viability, although conceivably the peak of the HN2 incorporation may have been missed because of the 10-fold dilutions used.

One disquieting feature of our results is the uniformity of the response to agents with vastly different cellular potentials. Why, for instance, should cells treated with a very lethal agent like HN2, a powerful mutagen like EMS, or a proximate carcinogen like BPL or N-acetoxy-AAF all respond in a qualitatively identical way? The distinction between a carcinogenic and a noncarcinogenic alkylating agent is a relative one since even agents like MMS and EMS are carcinogenic under proper conditions (26). If one postulates that increased thymidine incorporation really does represent repair, then one must conclude either that insignificant damage (damage not essential for the major action of these compounds) is repaired in preference to critical types of damage or that successful repair represents a rate phenomenon in which repair and mutagenesis, malignant transformation, or irreversible cellular injury compete with each other in temporal terms.

What is lacking, of course, in our system is a demonstration that DNA is being repaired functionally. Thus far, no one has worked out the relationship of incorporation studies, gradient studies, and functional assessments of repair to underlying biochemical processes. Our system provides a starting point for such studies, for it allows a study of such processes in a simplified, primarily nondividing cell population. It is only with the use of such a system which clearly separates replicative synthesis (and its enzymatic machinery) from the series of processes thought to be related to repair that any real understanding of repair at the molecular level may be achieved in mammalian systems. Until we are able to answer such questions as what kinds of damage may be repaired, how much damage may be repaired, and what is the nature of the repaired area (does it necessarily have to be a faithful copy of the complementary strand?), we will be unable to deal with cellular concepts like the relation of cell damage to malignant transformation and cell death.

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Fig. 1. Autoradiograph of control lymphocytes cultured for 3 hr in 1 mM HU and 5 μCi TdR-3H per ml for 3 hr. Note single heavily labeled cell. Anucleate cells are red blood cells. H & E, × 900.

Fig. 2. Autoradiograph of lymphocytes pretreated for 1 hr in 0.1 mM MMS and cultured in 10 mM HU and 5 μCi TdR-3H per ml for 3 hr. Most of the lymphocytes are lightly labeled. H & E, × 900.

Fig. 3. Representative low-power electron micrograph of lymphocytes cultured for 24 hr. Note numerous cytoplasmic processes and normal cell organelles and chromatin pattern. Compare with Ref. 9. × 9,300.

Fig. 4. Damaged cell from culture treated with 0.01 mM HN2 for 1 hr and cultured for 15 hr in the presence of 10 mM HU. Note loss of cytoplasmic processes, large blebs between the inner and outer nuclear membranes, and clumping of chromatin. Compare with Ref. 36. × 8,800.

Fig. 5. Damaged cell from culture treated for 1 hr with 0.1 mM MMS and cultured as in Fig. 4. Note that changes are similar to those seen in Fig. 4. × 9,300.

Fig. 6. Cell from preparation treated for 1 hr with 0.1 mM N-acetoxy-AAF and cultured as in Fig. 4. Note relatively normal morphology. Only a few cells showed changes similar to those seen with HN2 (Fig. 4) and MMS (Fig. 5). × 10,500.
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