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INCREASED SENSITIVITY OF MAMMALIAN CELL CULTURES TO RADIOMIMETIC ALKYLATING AGENTS FOLLOWING INCORPORATION OF 5-BROMODEOXYURIDINE INTO CELLULAR DNA

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Abstract—Cultures *in vitro* of a murine mast cell tumor were incubated with 5-bromodeoxyuridine in order to incorporate 5-bromouracil into cellular DNA. Cells containing 5-bromouracil in their DNA exhibited an increased sensitivity to two biological alkylating agents, mechlorethamine (nitrogen mustard, HN2) and dimethylmyleran. This increase in sensitivity, however, was lower than the corresponding increase in radiosensitivity. Sensitivity to increased incubation temperatures was not significantly changed by the pretreatment of cultures with 5-bromodeoxyuridine. It is concluded that DNA represents the primary target for the action of these alkylating agents on cell reproduction. The results obtained suggest, however, that the chemical alterations of DNA caused by mechlorethamine, dimethylmyleran and X-rays may be different.

SEVERAL cytotoxic alkylating agents are used extensively in the treatment of certain forms of neoplastic disease. Therefore, the mechanism of action of these carcinostatic drugs is of considerable interest and has been dealt with in numerous studies.[1] Since biological alkylating agents react with many different cellular constituents, however, it has been difficult to decide which type of reaction is responsible for the cytotoxic effects of these inhibitors. Several findings have made it appear likely that DNA represents the primary target, i.e. that chemical alteration of DNA causes the effects on cellular reproduction which are observed after exposure to an alkylating agent.[2] Thus, the well-known mutagenic effects of this group of compounds have pointed to the possibility that the effects on cell proliferation also were due to an interaction with DNA.[3] Furthermore, the high *in vitro* sensitivity of transforming DNA preparations and DNA viruses to biological alkylating agents suggested that inactivation of cellular DNA may be responsible for subsequent proliferative arrest.[4] In addition, the observation that difunctional alkylating agents, as compared with monofunctional compounds, are in general more efficient[5-7] and are capable of producing chromosome damage including chromosome bridges,[8] has supported the assumption that crosslinking of DNA may cause the inhibition of cell reproduction.

Many of the effects of biological alkylating agents are similar to those exerted by ionizing radiation. Again, the problem of the primary target of ionizing radiation with respect to its inhibitory effects on cell division is complicated by the fact that chemical alterations of many different cellular constituents are taking place before the inhibition of cell reproduction becomes apparent. It has been shown repeatedly, however that the radiosensitivity of mammalian cells in culture and of other forms of life is

greatly enhanced if part of the thymine residues in DNA are replaced by 5-bromouracil.[9–11] Since 5-bromodeoxyuridine (BUDR) is incorporated exclusively into DNA,[12, 13] these results support the interpretation that damage to DNA is responsible for the lethal effects of radiation at the cellular level.[14, 15]

In the studies presented in this paper the sensitivity of mammalian cells in culture to two radiomimetic alkylating agents and to X-rays was determined following incorporation of BUDR into cellular DNA. Furthermore, for reasons of comparison, the sensitivity to increased temperatures of cells containing BUDR in their DNA was studied, because a short-time incubation at high temperatures appeared to produce cellular damage without any alteration of DNA.

MATERIALS AND METHODS

a. *Culture techniques*

Cultures *in vitro* of a transplantable murine mast cell tumor (cell line P-815-X2) were used. This cell line was derived from the original P-815 tumor[16] by selection and two consecutive cloning procedures.[17, 18] The culture techniques and the medium have been previously described.[17, 19] Cell reproduction in the cultures was determined by haemocytometer counts. In the medium used, average cell doubling times of about 15 hr were observed. As described before,[17] the P-815-X2 cells do not attach to the surface of culture vessels. In order to determine the number of viable cells after exposure of cultures to an alkylating agent, to X-rays or to increased incubation temperatures, the cultures were grown, after appropriate dilution with "cloning medium", in fibrin gels, as described before.[20] The colonies which developed from the individual cells were counted 5–8 days later.

b. *Incorporation of* BUDR *into cellular* DNA *and determination of sensitivity to alkylating agents*

Three cultures containing approximately 5×10^6 cells in 50 ml of medium were prepared and incubated either with BUDR at concentrations of 0·01 μmole/ml and 0·03 μmole/ml, or without BUDR, respectively. Every 24 hr, cell multiplication was determined, and the cells were centrifuged and resuspended, at the original cell density of 10^5 cells/ml, in 50 ml of fresh medium containing BUDR at the same concentration as before. After 3 days, the cells were centrifuged, resuspended and incubated for 2 hr in 50 ml of medium containing no BUDR. Subsequently the number of cells/ml was determined, and aliquots of 4 ml of each culture were incubated with various concentrations of mechlorethamine or dimethylmyleran. At the same time, mechlorethamine or dimethylmyleran were added to aliquots of the "cloning medium" (prewarmed at 37°) at the same concentrations as to the cell suspensions. Two to four hours later, the cell suspensions containing mechlorethamine or dimethylmyleran were diluted appropriately with cloning medium containing identical concentrations of the inhibitor (final cell densities: 50 cells/12 ml, 500 cells/12 ml, 5000 cells/12 ml, 50,000 cells/12 ml). Twelve millilitres of these diluted cell suspensions were then mixed with 3 ml of a fibrinogen solution (5 mg fibrinogen/ml) to obtain a gel, as described before.[20] The number of colonies which developed from the individual surviving cells was counted 5–8 days later.

c. *Determination of radiosensitivity*

After incubation for 3 days in media containing BUDR at the concentrations

mentioned above, the cells were centrifuged and resuspended in 50 ml of medium containing no BUDR. Two hours later, 20 ml of each culture were placed in a plastic Petri dish and irradiated at room temperature with X-rays (200 kVp, 20 mA; filtered by 0·5 mm Cu; dose rate 140 r/min., measured in air). After exposure to 0, 100, 200, 300, 400, 500 and 600 r, aliquots of 2 ml were withdrawn, diluted with cloning medium and mixed with fibrinogen as described under b. Similarly, the colonies developing from the individual surviving cells were counted 5 to 8 days later.

d. *Determination of sensitivity to increased incubation temperatures*

After incubation for 3 days in media containing BUDR at the concentrations mentioned above, the cells were centrifuged and resuspended in 50 ml of medium containing no BUDR. Two hours later, 4 ml of each culture were placed in a culture tube and incubated during 15 min in a water bath at 37°. This procedure was repeated with new aliquots of 4 ml at incubation temperatures of 43°, 44°, 45° and 46°, respectively. Following incubation at these temperatures, the cell suspensions were diluted with cloning medium and mixed with fibrinogen as described under b. Similarly, the colonies developing from the individual surviving cells were counted 5–8 days later.

e. *Inhibitors*

BUDR was obtained from Schwarz BioResearch Inc. Dimethylmyleran was kindly supplied by Dr. P. Alexander, Chester Beatty Institute, London. This inhibitor was dissolved in water no more than 13 min before being added to the cell suspensions and to the aliquots of "cloning medium". Mechlorethamine was used in the form of a commercially available preparation ("Dichloren", CIBA), and was dissolved in 2 mM HCl, again not earlier than 13 min before being added to the cell suspensions and to the "cloning medium". The aliquots of a solution containing the inhibitor at any one concentration were added to the three different cell suspensions within a time interval of only a few seconds.

f. *Determination of the extent of replacement of thymine by 5-bromouracil in cellular* DNA

Three cultures containing approximately 25×10^6 cells in 250 ml were incubated either with BUDR at concentrations of 0·01 μmole/ml and 0·03 μmole/ml, or without BUDR, respectively, as described under b. Extraction of DNA from the cells after 3 days incubation in medium containing BUDR was carried out using the method of Schmidt and Thannhauser[21] modified by Schneider.[22] After extraction of the acid-soluble fraction, lipids and RNA, the DNA was extracted with hot (90°) 5% trichloroacetic acid and further hydrolysed with 6 N HCl as described by Djordjevic and Szybalski[9] in order to obtain the free purine and pyrimidine bases. The HCl-free DNA hydrolysate was subjected to 2-dimensional paper chromatography, and the relative amounts of thymine and 5-bromouracil were determined according to the method of Djordjevic and Szybalski.[9]

RESULTS

a. *Growth rate in the presence of different concentrations of* BUDR

Cellular multiplication rates at different concentrations of BUDR are presented in Fig. 1. The data indicate that BUDR at a concentration of 0·01 μmole/ml produced a

small reduction in multiplication rate on the third day of incubation only, while the presence of BUDR at 0·03 μmole/ml resulted in a more marked inhibition of cell reproduction starting already on the second day. Even at this higher concentration of BUDR, however, cell multiplication continued at a slow rate, at least up to 72 hr. Since the total number of cell doublings during the 3 days of incubation is between

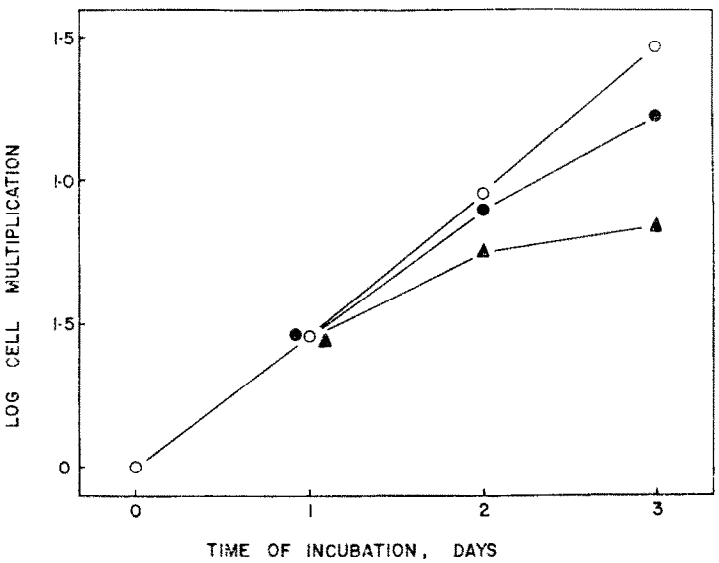

FIG. 1. Cellular multiplication in P-815 cell cultures during incubation with different concentrations of BUDR.

○——○ Cultures incubated in the absence of BUDR.
●——● Cultures incubated with 0·01 μmole/ml of BUDR.
▲——▲ Cultures incubated with 0·03 μmole/ml of BUDR.
The results represent the mean values from fifteen independent experiments.

2·8 and 4·9, it is reasonable to assume that, under the conditions of the experiments, BUDR is incorporated more or less uniformly into both strands of cellular DNA, i.e. that only few DNA strands are left which contain no BUDR.

b. *Extent of incorporation of* BUDR *into cellular* DNA

The results of the analysis of DNA extracted from the cells after incubation for 3 days in media containing BUDR are presented in Table 1. It can be seen that BUDR is incorporated into the DNA to a considerable extent, and the values obtained are in the same order of magnitude as those described before.[9, 13] The small variations between the results of different experiments indicate that the conditions of culture in the presence of BUDR are sufficiently reproducible to allow direct comparison of the effects caused by alkylating agents, X-rays and increased incubation temperatures.

c. *Cloning efficiency after incorporation of* BUDR *into cellular* DNA

Cell cultures were incubated either with BUDR at 0·01 and 0·03 μmole/ml, or without BUDR, during 3 days. Subsequently, the cells were incubated during 2 hr

in BUDR-free medium in order to test the sensitivity to X-rays, alkylating agents or increased temperatures. The colony yields from the three cultures under control conditions, i.e. without treatment of the cells by these different cytotoxic agents, are presented in Table 1. Because no attempt was made to determine the cell number per

TABLE 1. INCORPORATION OF BUDR INTO CELLULAR DNA AND CLONING EFFICIENCY OBSERVED FOLLOWING INCUBATION OF P-815 CELL CULTURES DURING 3 DAYS WITH DIFFERENT CONCENTRATIONS OF BUDR

BUDR concentration (mM)	Thymidine replaced by BUDR* (%)	S.E.	Cloning efficiency† (%)	S.E.
0	—	—	96·2	±24·5
0·01	37·3	±2·3	76·4	±19·0
0·03	53·4	±4·5	33·6	±22·4

* The replacement of thymidine by BUDR was determined following hydrolysis of DNA and chromatographic separation of DNA bases. The figures for thymidine replacement by BUDR represent the mean values from four independent experiments.

† The figures represent the mean values from fourteen independent experiments.

ml in the three cultures very accurately, a considerable variation of the cloning efficiency between the individual experiments was obtained, as seen from the standard error. The mean values, however, indicate that the cloning efficiency of cells from cultures incubated without BUDR was close to 100 per cent. Incubation in the presence of BUDR led to a decrease in cloning efficiency which was more marked at the higher concentration of this analogue.

d. *Increase in radiosensitivity after incorporation of* BUDR *into cellular* DNA

The results obtained after incubation of cells during 3 days in the presence or absence of BUDR are presented in Fig. 2. It can be seen that replacement of thymine by 5-bromouracil in cellular DNA results in a marked increase in radiosensitivity, in agreement with earlier reports.[9–11]

e. *Sensitivity to mechlorethamine and dimethylmyleran after incorporation of* BUDR *into cellular* DNA

Cell cultures were incubated either with BUDR at 0·01 and 0·03 μmole/ml, or with no BUDR, during 3 days. Subsequently the sensitivity to different concentrations of mechlorethamine or dimethylmyleran, respectively, was determined by cloning of cells as described under Methods. The sensitivity to dimethylmyleran was studied in two experiments which gave essentially the same results. The data given in Fig. 3 represent the average values of these two experiments. On the other hand, the results on the sensitivity to mechlorethamine were somewhat more variable. The data of Fig. 4 represent again average values of two experiments. In one of these, incubation of cells with BUDR at 0·03 μmole/ml produced a more pronounced increase in sensitivity than with BUDR at 0·01 μmole/ml, while in the other experiment the lower BUDR-concentration had a more marked effect. In a third experiment, only BUDR at 0·03 μmole/ml had a sensitizing effect, while the values obtained after

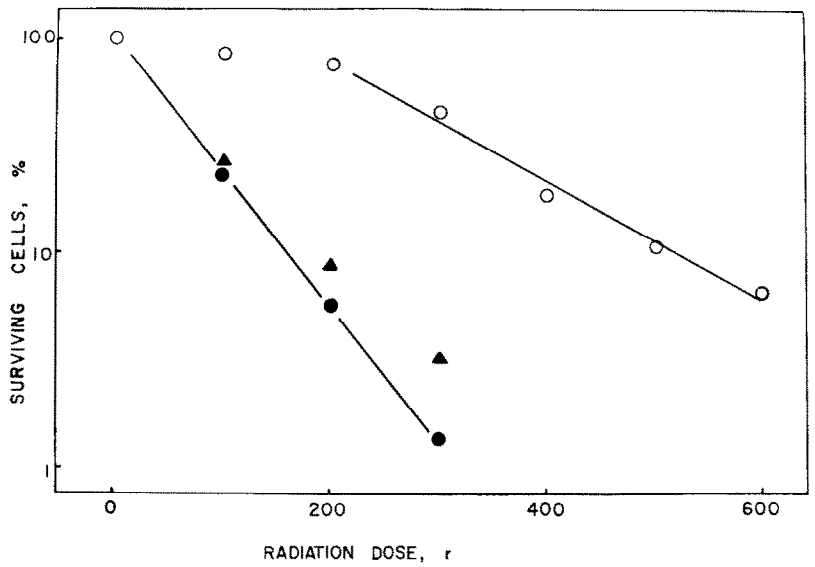

FIG. 2. Radiosensitivity of P-815 cells following incorporation of BUDR into cellular DNA.
○——○ Cultures preincubated in the absence of BUDR.
●——● Cultures preincubated during 3 days with 0·01 μmole/ml of BUDR.
▲——▲ Cultures preincubated during 3 days with 0·03 μmole/ml of BUDR.
The colony yield as obtained without irradiation was used to express "100 per cent surviving cells" for all three cultures, irrespective of the absolute cloning efficiency.

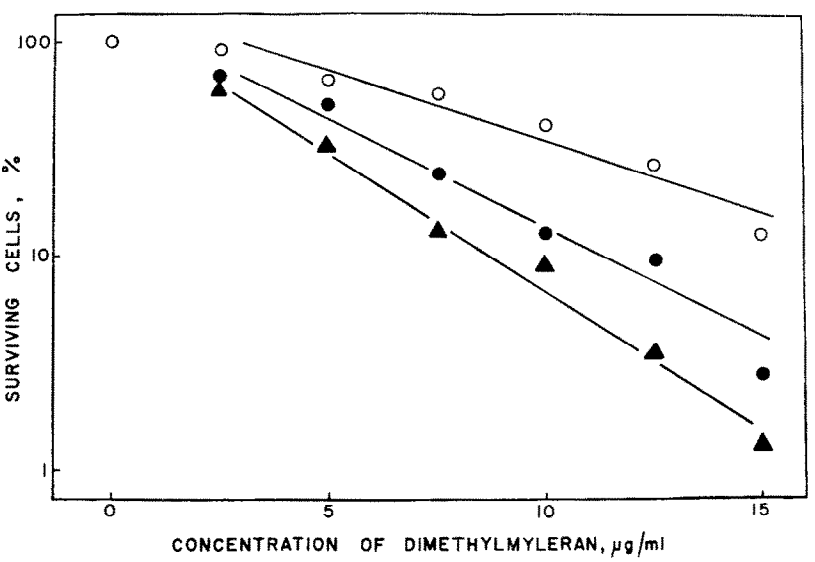

FIG. 3. Sensitivity of P-815 cells to dimethylmyleran following incorporation of BUDR into cellular DNA.
○——○ Cultures preincubated in the absence of BUDR.
●——● Cultures preincubated during 3 days with 0·01 μmole/ml of BUDR.
▲——▲ Cultures preincubated during 3 days with 0·03 μmole/ml of BUDR.
The colony yield as obtained without exposure to dimethylmyleran was used to express "100 per cent surviving cells" for all three cultures, irrespective of the absolute cloning efficiency.

incubation with BUDR at 0·01 μmole/ml were practically identical to those of the controls preincubated without BUDR.

It can be seen from Figs. 3 and 4 that the cells containing BUDR in their DNA exhibit a greater sensitivity to both mechlorethamine and dimethylmyleran, as compared to control cells. It should be noted, however, that the increase in sensitivity to these chemicals induced by the incorporation of BUDR into DNA is considerably

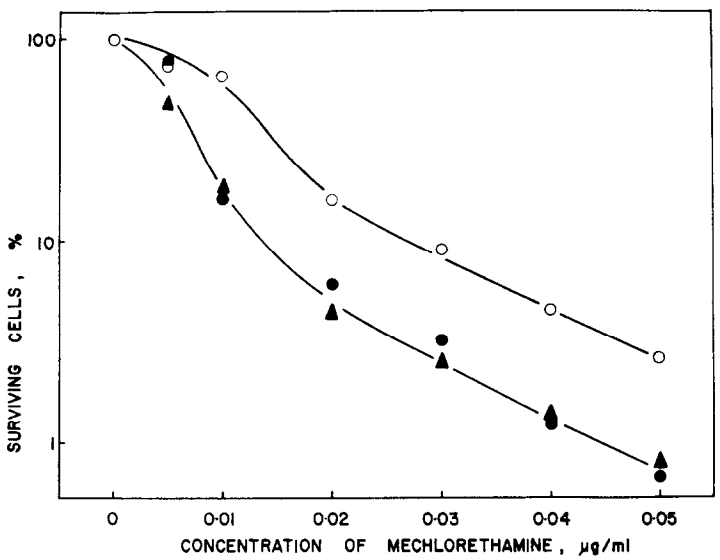

FIG. 4. Sensitivity of P-815 cells to mechlorethamine following incorporation of BUDR into cellular DNA.

○——○ Cultures preincubated in the absence of BUDR.
●——● Cultures preincubated during 3 days with 0·01 μmole/ml of BUDR.
▲——▲ Cultures preincubated during 3 days with 0·03 μmole/ml of BUDR.

The colony yield as obtained without exposure to mechlorethamine was used to express "100 per cent surviving cells" for all three cultures, irrespective of the absolute cloning efficiency.

smaller than the corresponding increase in radiosensitivity, as presented in Fig. 2. Furthermore, although the experimental conditions were identical, the shape of the curves expressing the action of dimethylmyleran is different from that for mechlorethamine.

f. *Sensitivity to increased incubation temperatures after incorporation of* BUDR *into cellular* DNA

Cell cultures were incubated either with BUDR at 0·01 and 0·03 μmole/ml, or with no BUDR, during 3 days. Subsequently the sensitivity to incubation at temperatures of 43–46° during 15 min was determined by cloning of cells as described under Methods. Two experiments were performed and gave essentially the same results. The data given in Fig. 5 represent the average values of these two experiments. It can be seen that replacement of thymine by 5-bromouracil does not significantly change the sensitivity of the cells to increased incubation temperatures.

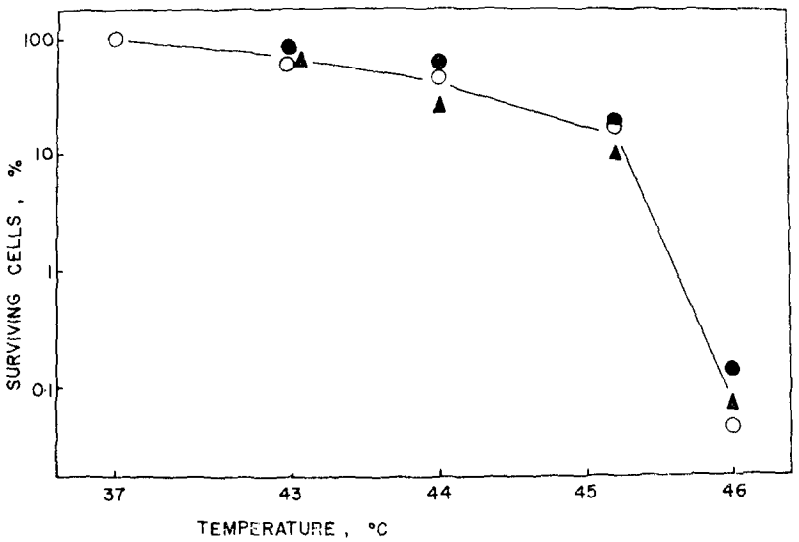

FIG. 5. Sensitivity of P-815 cells to incubation at increased temperatures during 15 min following incorporation of BUDR into cellular DNA.

○——○ Cultures preincubated in the absence of BUDR.

●——● Cultures preincubated during 3 days with 0·01 μmole/ml of BUDR.

▲——▲ Cultures preincubated during 3 days with 0·03 μmole/ml of BUDR.

The colony yield as obtained without exposure to increased temperatures was used to express "100 per cent surviving cells" for all three cultures, irrespective of the absolute cloning efficiency.

DISCUSSION

It has been reported that both mechlorethamine and dimethylmyleran have half lives of the order of 30 min under the conditions of cell culture at 37°.[23] They appear, therefore, to be suitable for the comparative studies described in this paper. Because of this short half life, it seems justified to assume that during the incubation period of 2–4 hr, most of the alkylating agent present in the medium is inactivated. Furthermore, at the time of their addition to the cell cultures, mechlorethamine or dimethylmyleran were added also to the "cloning medium" which 2–4 hr later served to dilute the cell suspensions. Therefore, dilution of the cell suspensions during the cloning procedure did not change the concentration of the inhibitors, except for a 20 per cent decrease due to the addition of fibrinogen solution.

As shown in Figs. 2–4, incorporation of BUDR into cellular DNA consistently resulted in an increased sensitivity of the cells to both mechlorethamine and dimethylmyleran as well as in an increased radiosensitivity. This is in agreement with other similarities between the effects of X-rays and biological alkylating agents. For instance, the biochemical events and giant cell formation following treatment of cell cultures with mechlorethamine closely resemble those observed after irradiation with X-rays.[24–27] Furthermore, cross-resistance to u.v. light and to nitrogen mustard has been described for *E. coli*,[28] and in general cross-resistance between different types of biological alkylating agents is observed.[29]

The reactions of biological alkylating agents with DNA have been studied extensively, and it was shown that mustards react preferentially with the nitrogen in position 7 of the guanine moiety, thus causing the formation of crosslinks between

two DNA strands.[2, 30–32] Furthermore, it was found that *E. coli* strains resistant to biological alkylating agents were capable of repairing the damaged parts of their DNA, while sensitive strains were not.[33] This mechanism of resistance, however, has not yet been confirmed for mammalian systems. Such studies on the chemical alterations of certain cell constituents by these inhibitors may answer the question as to the cellular material which is most sensitive to alkylation. They cannot be used, however, to decide which is the primary target of these inhibitors with respect to subsequent arrest of cell proliferation. This view is supported by the finding that different mustards at concentrations which cause the same degree of DNA alkylation may have markedly different effects on cell viability.[34] Furthermore, it has been shown that myleran causes much less cross-linking of DNA than nitrogen mustards at doses sufficient for inhibition of cell division.[35]

It can be seen in Fig. 5 that incorporation of BUDR into cellular DNA has no effect on the sensitivity of the cells to increased temperatures. It is indeed to be expected that certain cellular constituents such as enzymes and other proteins are more sensitive than DNA to increased temperatures. DNA, therefore, cannot represent the primary target for the effects of elevated incubation temperatures on the capacity of the cells to multiply. The finding that BUDR incorporation into cellular DNA has no effect on temperature sensitivity is in agreement with this concept and supports the conclusion that incubation of cells with BUDR specifically modifies the properties of cellular DNA. The results presented in Figs. 2–4, therefore, indicate that DNA represents the primary target for the effects of mechlorethamine, dimethylmyleran as well as X-rays on the capacity of the cells to multiply.

The mechanism of the sensitizing effect of BUDR has not yet been elucidated definitely. Lett *et al.*[36] have described experiments with two strains of lymphoma cells which differ in radiosensitivity because of a different capacity to repair radiation damage, and their results led to the interpretation that incorporation of bromouracil inhibits post-irradiation recovery processes. On the other hand, studies by Kim *et al.*[37] on the recovery of mammalian cells from X-irradiation, measuring survival of cells as a function of time interval between two X-ray doses, led to the conclusion that pretreatment with BUDR does not alter recovery from radiation damage. Therefore, although recovery from the effects of alkylating agents similar to that from X-ray damage has been demonstrated for microorganisms,[33, 38, 39] the question as to the effect of BUDR incorporation on repair processes still remains open,[40] and the data reported in this paper do not allow any conclusion in this respect.

It has been shown that the sensitivity of mammalian cells in culture to nitrogen and sulphur mustards[41] as well as to ionizing radiation[42, 43] changes during the division cycle. Furthermore, the increase in radiosensitivity following BUDR incorporation is dependent on the phase of the cell cycle in which the cells are irradiated.[44] As shown in Fig. 1, the doubling time of the cultures is increased following incorporation of BUDR into DNA. It cannot be excluded, therefore, that some of the phases of the division cycle may be prolonged preferentially by this treatment, and that a higher proportion of the cell population may be found in a phase of the cycle which confers an increased sensitivity of cellular DNA to X-rays or alkylating agents.

The typical shape of the curves of Fig. 4 has been described previously by Marin and Levis[45] and attributed to partial restoration of cell viability at higher population densities. These authors, in their studies on the combined effects of X-rays and

mechlorethamine, concluded that the mechanisms of action of these agents on cell multiplication are related to each other, but not identical. This view is supported by our observations that the sensitizing effect of BUDR incorporation is demonstrable for X-rays as well as for the alkylating agents studied, but that it is more marked for X-rays than for mechlorethamine or dimethylmyleran. Furthermore, the finding that usually no cross-resistance between X-rays and nitrogen mustards is observed[45] points to a difference in mechanism of action.

Finally, a comparison of Figs. 3 and 4 leads to the conclusion that there are differences between the mechanism of action of mechlorethamine and dimethylmyleran: the curves obtained with dimethylmyleran represent more or less straight lines with an initial shoulder, while the curves for mechlorethamine are more complex. Furthermore, the sensitizing effects of a previous BUDR incorporation appear to increase with the concentration of dimethylmyleran used, while the sensitization to mechlorethamine remains stable over most of the range of concentrations studied. Other differences in response to mechlorethamine and dimethylmyleran have been reported previously.[23]

In summary, the finding that partial replacement of thymine by 5-bromouracil in cellular DNA causes an increase in sensitivity of mammalian cells to mechlorethamine, dimethylmyleran and X-rays, leads to the conclusion that DNA is the primary target for the action of these cytotoxic agents on cell reproduction. The quantitative aspects of the sensitization by BUDR, however, indicate certain differences between the effects of mechlorethamine, dimethylmyleran, and X-rays. These differences suggest that the chemical alterations of DNA produced by X-rays, mechlorethamine and dimethylmyleran are not identical.

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