The Sensitivity of Cultured Mammalian Cells in Different Stages of the Division Cycle to Nitrogen and Sulfur Mustards*

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SUMMARY

Strain L mouse fibroblasts growing in vitro were exposed to varying concentrations of nitrogen and sulfur mustards, and the surviving fraction of cells was measured by their ability to form macroscopically visible colonies. Nitrogen mustard required 3 hours but sulfur mustard less than 1 hour to exert maximum killing effect. The mean lethal dose (D₃₇) for nitrogen mustard acting for 1 hour on an exponentially growing culture was 0.16 μg/ml. The D₃₇ for the uninterrupted action of sulfur mustard on exponentially growing cells was 0.12 μg/ml. Partially synchronously growing cultures were prepared by the addition of 5-fluoro-2'-deoxyuridine, followed 16 hours later by the addition of thymidine. Cell samples were taken from the synchronized culture at various times, and their sensitivity to the mustards was ascertained. With nitrogen mustard the D₃₇ obtained for cells in the period of DNA synthesis was 0.08 μg/ml. The D₃₇ for Gₛ cells was 0.18 μg/ml. Cells in the G₁ stage were also less sensitive than those in the period of DNA synthesis, but it was not possible to obtain a quantitative value. Sulfur mustard gave similar results.

The studies of Rueckert and Mueller (10) on the interruption of DNA synthesis and division in strain HeLa cells by amethopterin or 5-fluoro-2'-deoxyuridine (FUDR) and their resumption after addition of thymidine have paved the way for many intriguing studies with cultured mammalian cells, because when the cells begin to divide again they are partially synchronized. We have exploited this "synchronization" to determine whether cells in various phases of the division cycle differ in their sensitivity to nitrogen and sulfur mustards. Our results indicate that interphase L cells, as measured by their colony-forming capacity, are more sensitive during the period of DNA synthesis than in the other intermitotic stages.

MATERIALS AND METHODS

Cells.†—A clonally isolated subline of Earle's L cells (mouse fibroblasts) was used in all experiments. The line selected multiplied exponentially in a thymidine-free medium with a doubling time of about 20 hours. When plated as described below it formed well defined, compact colonies with an efficiency always greater than 50 per cent and most often of 80—90 per cent. The cells were grown routinely in suspension cultures at 37° C., with either "fast" roller tubes or spinner flasks. Growth medium consisted of CMRL 1066 containing 5 per cent or 10 per cent horse serum and antibiotics, with thymidine and coenzyme concentrate omitted. Cell counts were made with an electronic cell counter (Coulter Electronics Co., Chicago) which had been previously standardized by hemocytometer counts. Viable cell counts were obtained by plating appropriate numbers of cells in culture dishes. After 9—11 days at 37° C. in an atmosphere containing 5 per cent CO₂ the colonies were stained and counted. The medium used for plates was as previously described but contained 8 per cent horse serum and 8 per cent fetal calf serum.

"Synchronization" of cell growth.—Exponentially growing cells were diluted to a count of about 1 X 10⁶ cells per ml. and 5-fluoro-2'-deoxyuridine²

The FUDR was a gift from Hoffman La Roche, Montreal, Canada.

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‡ The following technics are described in detail in R. C. Parker, Methods of Tissue Culture, 3d ed. New York: Paul B. Hoeber, Inc., 1961.

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(FUDR) was added to give a concentration of $2 \times 10^{-7}$ M. Sixteen hours later thymidine (TDR) was added to give a concentration of 20 $\mu$g/ml.

**Nitrogen and sulfur mustards.**—For each experiment nitrogen mustard (HN$_2$; bis-[\(\beta\)-chloroethyl]methylamine) in the form of the hydrochloride was dissolved in 0.01 N HCl and was kept ice-cold during an experiment; or, if the experiment lasted several hours, the solutions were kept frozen at about $-26^\circ$ C. until required. Sulfur mustard (bis-[\(\beta\)-chloroethyl]sulfide) in 0.01 N or 0.01 N HCl decomposed too rapidly for use even when kept at $0^\circ$ C.; therefore, various organic solvents were tested for their suitability, and methanol was chosen. A stock solution of sulfur mustard in methanol containing 5 mg. mustard per ml. was kept at $-26^\circ$ C. Before use further dilutions with methanol were made, and these working solutions were kept at $0^\circ$ C. or $-26^\circ$ C. as previously described. The concentrations of the nitrogen and sulfur mustard solutions were checked periodically by reaction with excess thiosulfate at $37^\circ$ C., followed by back titration with iodine.

**Exposure of cells to mustard.**—Nitrogen mustard in 0.01 N HCl was added to a roller tube culture containing about $1 \times 10^8$ cells per ml. After the desired incubation period at $37^\circ$ C. the cells were quickly diluted and plated. The addition of 0.1 ml. of 0.01 N HCl to 10 ml. of culture had no effect on cell survival. Sulfur mustard in methanol was added directly to culture dishes in which the cells had already been plated. The dish contained 5 ml. of medium, and the amount of methanol solution added was always 0.05 ml. This concentration of methanol killed about 10 per cent of the cells. Methanol was always added to the control dishes, and the survival of mustard-treated cells was expressed as a percentage of the control values.

**RESULTS**

Chart 1 shows the survival curves obtained by exposing L cells to varying concentrations of HN$_2$ for different periods of time at $37^\circ$ C. When the HN$_2$ was not diluted out but was allowed to remain in the medium the survival data corresponded to an exposure period of about 8 hours. Thus, under our experimental conditions HN$_2$ continued to exert a noticeable effect on the survival of L cells for 8 hours. In a similar type of experiment with sulfur mustard the maximum killing effect was obtained within 1 hour. This comparison of the two mustards suggests that the sulfur mustard is more reactive than HN$_2$; presumably most of the mustard is consumed by reaction with constituents of the medium. The use of sulfur mustard is therefore technically superior when brief exposures to mustard are required. The requirement for brief exposures is obvious when cells are to be exposed as they move through the various phases of the division cycle. For quantitative comparison the mean lethal dose ($D_{37}$) may be used. This is the dose increment in the exponential part of the curve that will kill 63 per cent of the population (8). When HN$_2$ acted for 1 hour, the $D_{37}$ was 0.16 $\mu$g/ml; for HN$_2$ with uninterrupted action the $D_{37}$
immediately after addition of mustard. This factor was of some concern to us when we were attempting to standardize the exposure conditions.

In Chart 3 the heavy dashed line represents normal exponential growth of an untreated culture. The curve shows the course of cellular multiplication following the addition of FUDR to exponentially dividing cells and subsequently after the

addition of thymidine (TDR). The "synchrony" obtained by this method is obviously imperfect, but samples can be chosen in which a large proportion of the cells is in the S or G2 or G1 stage. Stage S is the period of DNA synthesis; G2 occurs after DNA synthesis is completed and before mitosis; G1 occurs after mitosis and before DNA synthesis (6). The numbered arrows 1–7 show when samples were taken to obtain cells in various stages of the division cycle. From the course of multiplication seen in Chart 3 and from the work of Till et al. in which the position and extent of DNA synthesis was determined for this type of "synchronized" culture, the following remarks may be made about the cell samples. In sample 1 no DNA synthesis is going on; the cells are poised, ready to commence DNA synthesis immediately upon the addition of thymidine. More will be said about these cells in the "Discussion." In the other samples there is

always a greater or lesser proportion of the cells synthesizing DNA. In samples 2 and 3, 90 per cent and 70 per cent, respectively, of the cells are synthesizing DNA. In samples 4 and 5, 20 per cent and 10 per cent, respectively, of the cells are synthesizing DNA. These samples, especially number 5, may be said, therefore, to contain mainly G2 cells. In both samples 6 and 7 ca. 50 per cent of the cells are synthesizing DNA. The other 50 per cent are G1 cells.

DISCUSSION

Our results (Charts 1 and 2) demonstrate that, when exponentially dividing L cells are exposed to HN2 or sulfur mustard, the survival curves are of the sigmoid-exponential type. A similar type of survival curve has been reported by Alexander and Mikulski (1) for the action of HN2 or dimethyl myleran on an established line of lymphoblasts (L5178Y). The points of their curves were obtained by extrapolating to zero time the linear portions of the growth curves of cells treated with the alkylating agents. The extrapolated values were considered to measure the number of surviving cells. However, in the case of HN2 at least, where there is an immediate and pronounced division delay, the extrapolated values give a low estimate of the number of surviving cells. Thus, the lymphoblast line was made to appear more sensitive to HN2 than the L cell. A sigmoid-exponential survival curve is also obtained when mammalian cells are x-irradiated (5, 12). The shape of the x-ray survival curve can be explained by assuming it to result from the exponential inactivation of a number of independent targets in the cell, each of which must be inactivated in order for the cell to be killed (8). However, the inactivation by mustards is more complicated. In the usual type of x-ray survival curve the dose rate is constant; but with mustard, which is continuously being consumed by reaction with constituents of the medium, the effective dose at each point in the survival curve is delivered to the cells at a continuously decreasing rate.

As regards the survival curves obtained with "synchronized" cells, it is not presently possible to state categorically whether the observed differences in sensitivity to mustards are real functions of the stage of the division cycle or merely artifacts arising from the "synchronizing" treatment. Evidence for the reality of these differences can be drawn from the work of several investigators who have determined the sensitivity of interphase cells to x-rays. Terashima and Tolmach (11) obtained "synchronously" dividing HeLa cells by shaking them off plastic dishes. At the time of division they are less firmly attached. During interphase cells in S were more sensitive than cells in G1 and G2, which had comparable sensitivities. Das and Albert (3) measured the sensitivity of interphase bean and onion root cells to chromosome breakage by x-rays. Their cells were not synchronized, but the position of the cells in the division cycle at the time of irradiation was determined by following the incorporation of tritiated thymidine. They too found that S cells were the most sensitive but were unable to find any difference in sensitivity between G1 and G2 cells. Dewey and Humphrey (4), using mouse L cells growing in vitro and mouse ascites tumor cells growing both in vivo and in vitro, found that cells in the S stage of the cycle sustained the most chromosome damage when anaphase cells of the first division after x-radiation were examined. However, when cultured Chinese hamster cells were used, G2 cells were found to be the most sensitive (7). Thus, there is much evidence to show that interphase cells vary in their sensitivity to x-rays with their position in the division cycle. It seems probable that our results with nitrogen and sulfur mustards are similar expressions of a varying sensitivity.

The cells in sample 1 deserve comment. One might anticipate that 15 hours after the addition of FUDR the population would contain mostly G1 cells. Till et al.4 have found that, when a tracer amount of tritium-labeled thymidine is added to cells treated 15 hours previously with FUDR, 90 per cent of the cell population begins DNA synthesis immediately. Mueller et al. (9) have recently reported a similar finding. It would appear that in the FUDR-treated cells some of the steps toward DNA synthesis have already been taken and that the addition of thymidine allows this partially finished process to be completed. This conclusion has also been reached by Mueller et al. (9). Thus, the cells in sample 1 (before thymidine is added) resemble S cells rather than G1 cells. The heightened sensitivity obtained for the cells in sample 1 (curve 1, Chart 4) is in accord with this reasoning.

If cells in the various phases of the division cycle indeed exhibit different sensitivities to mustard, the phenomenon remains to be explained. DNA has always held a prominent position in the...
mechanisms proposed for the damaging effects of x-rays and alkylating agents. The possibility must be considered that cells in G₂, which are least sensitive to mustard, have a lower sensitivity because they contain twice as much DNA as cells in other stages of the cycle. If this were the case, one would expect to find that the number of "hits" required to kill a cell would be greater for G₂ cells, since either of the two complements of DNA in the cell at that time should suffice to initiate a clone if it survived. The extrapolation number of a survival curve is often taken as an index of the number of hits needed to kill the sensitive organism (8). It can be seen from Chart 4 that the extrapolation number of the survival curve for sample 5 does, in fact, appear to be higher than that of the other samples. The slope of the exponential part of the survival curve, or $D_0$, is considered to represent a measure of the rate of reaction of mustard with sensitive material, presumably DNA. This rate would be expected to be the same no matter what quantity of DNA was present in the cell, provided mustard was present in excess. The fact that the survival curves eventually become exponential strongly suggests that mustard was in excess under the conditions of these experiments. Because the slopes of the curves for samples 1, 2, and 3 are steeper than any of the other curves one might postulate that, during the synthetic period, DNA is in a form more susceptible to reaction with mustards. It does not seem probable that the DNA synthetic process is especially liable to attack, because various workers (1, 2) have demonstrated that DNA synthesis continues unimpaired for at least one cell division following treatment with mustards. Perhaps there is a sensitive process not directly related to DNA synthesis that is impaired. These problems should be susceptible to attack with labeled mustard. We are currently engaged in ascertaining whether whole interphase cells and nuclei vary with the position in the division cycle in the degree to which they take up mustard and whether the DNA and other fractions from these cells vary in their reaction with mustard.

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**REFERENCES**


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