X-RAYS AND NITROGEN MUSTARD: INDEPENDENT ACTION IN CHINESE HAMSTER CELLS

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ABSTRACT We have studied the combined effects of X-irradiation and nitrogen mustard treatment on the colony-forming ability of Chinese hamster cells. In contrast to X-irradiation, nitrogen mustard acting by itself yields an exponential dose-effect relationship. Moreover, whether delivered in immediate sequence or after varying intervals, nitrogen mustard treatment does not affect radiation survival and vice versa. These results, plus the lack of any indication of the repair of sublethal nitrogen mustard damage, are consistent with independent but different modes of action at the same site(s), or action at different sites.

INTRODUCTION

To increase our understanding of how ionizing radiation acts in cells, in past work we studied the repair of sublethal X-ray damage and the effects some metabolic inhibitors have on this repair (1–3). Of the various materials tested (2), only actinomycin D showed a clear indication of an ability to interact with X-ray damage. This was true when measured either relative to single (4) or fractionated X-ray doses (1–3). The inhibition properties of actinomycin D, relative to RNA and DNA synthesis (5), stem from its ability to penetrate cell membanes and to bind to DNA (6–8) which it appears to do almost exclusively in mammalian cells (9). Particularly in view of its binding specificity for DNA, we concluded that actinomycin D and radiation interact probably because they act at the same locus and that in mammalian cells this locus consists of, or at least intimately involves, the DNA of the cell.

To help strengthen this view, we sought further evidence for a radiation-DNA interaction. Bifunctional alkylating agents, like nitrogen mustard, are known to produce cross-links in complementary strands of DNA (e.g., see reference 10) and in so doing they are thought to kill cells because they impair the normal functions of replication and/or transcription. In view of the genomic specificity indicated by these suggestions and observations, it seemed to us possible, if not likely, that lesions resulting from irradiation would interact with those resulting from alkyla-

tion. To our surprise, we found that X-rays and nitrogen mustard appear to act independently, from which one might conclude that these agents produce lesions at different loci. As we will show, however, the detailed natures of the damaging processes of these two agents are consistent nevertheless with a common site of action.

MATERIALS AND METHODS

Since our materials and methods are essentially the same as those we have used for a number of studies, we only briefly review them here.

Cells and Their Cultivation

We have used a number of subclones of a clonal line of Chinese hamster cells designated originally by us *V79-1*. This we isolated from line *V*, a line of female lung fibroblasts originally placed in culture by Ford and Yerganian (11). On glass or plastic surfaces, these cells double in number in 8–9 hr, have generally a spindle shape morphology, and produce tight, dense colonies about 2 mm in diameter in 8 days (untreated controls).

Our techniques for cultivating these cells and assessing survival by colony formation have been described (12, 13). Using a modified Eagle's medium (14) to which we added 15% fetal calf serum, cells plated in appropriate numbers in 9 cm petri dishes were incubated overnight in a CO_2 incubator before an experiment was started. We used this procedure to help insure that the distribution of cells throughout their growth cycle (i.e., the *age-density* distribution) was close to that resulting from asynchronous, log-phase growth and hence, fairly reproducible from experiment-to-experiment. During this overnight growth period, initially single cells formed micro-colonies of an average of about three cells. Our experiments were started, therefore, with populations of microcolonies rather than single cells. Since the cells we used survive radiation (13) and nitrogen mustard treatment independently,[1] multiplicities greater than one introduce no serious complications of interpretation as has been shown elsewhere (see chapters 2 and 3, reference 15).

Following a given treatment sequence, cells were incubated long enough for a maximum yield of colonies. These were stained with methylene blue and counted. Results are expressed as the fraction of cells surviving a given treatment relative to the number plated from the starting cell suspensions which were able to form colonies. (The percentage of colony formers in the starting suspension is designated the *plating efficiency*, PE.) In the figures to follow, $\bar{N}$ stands for the average multiplicity at the start of the experiment and was determined in each case from measurements of the increase in the number of colonies obtained when the cells in microcolonies were respread by trypsinization. Below surviving fractions of $\sim$0.2, the effect of multiplicity is simply to shift survival upward by $\bar{N}$ (15).

Irradiation and Nitrogen Mustard Treatment

As in earlier studies (12), we used 55 Kv X-rays at a dose rate of 722 rads/min. Because this radiation is readily absorbed by condensed matter, cells were exposed after removal of the overlying medium and while held in a fixture (for CO_2 and humidity control) having a cellophane window a few thousandths of an inch thick. Following exposure, medium was returned to the dishes.

[1] Sakamoto, K., and M. M. Elkind. Unpublished data.

For nitrogen mustard treatment, we first prepared a 200.0 μg/ml stock of $CH_3N(C_2H_4Cl)_2$ dissolved in 0.01 N HCl which was kept frozen between experiments. For use, appropriate stocks 10–20 times higher than the final concentrations required were prepared in buffer [Dulbecco's saline (16) plus 1% medium]. These were kept at ice temperature and while they remained potent for longer periods, they were usually discarded after 1 hr. Nitrogen mustard exposures were effected by adding 1.0 ml aliquots of an appropriate drug solution to 9.0 ml or 19.0 ml of medium at 37°C for 30 min. After sucking out the treatment medium, cells were rinsed once with buffer and refed medium at 37°C for colony formation.

Synchronization

Most of our experiments were performed with synchronized cells. For this, we used hydroxyurea, a well known inhibitor of DNA synthesis (e.g., see reference 17). This drug, while toxic to cells in the DNA synthetic phase S, permits those in the post-DNA synthesis period G_2 and mitosis to divide and join the cells in the pre-DNA synthesis period G_1 (18–20) where they are held up by the hydroxyurea. Thus, as we have shown (3, 21), during the course of a few hours of exposure to millimolar concentrations of hydroxyurea, an initially asynchronous population of Chinese hamster cells becomes synchronized with respect to colony formers at the G_1-S border. Replacing the hydroxyurea-containing medium by fresh medium permits cells to progress through the remainder of their age cycle (S, G_2, and mitosis), divide, and progress through the succeeding interphase period (3, 21). 3 hr after the removal of the hydroxyurea, cells are about halfway through their S phase and in another 3½–5 hr, they are in G_2-mitosis.

The survival curves dealing with synchronized cells start from 0.4 to 0.5 surviving fraction. This reduction is due to S cell killing but is less than the proportion of S cells in an asynchronous population because not all microcolonies which contain S cells consist of only S cells.

In spite of attempts to reproduce methods from experiment-to-experiment some variations cannot be avoided. To minimize interpretive ambiguities which might result from these, in each of the figures to follow, we present data all of which were obtained with the same starting cell suspension and with the same media and other materials.

RESULTS

Combined Radiation Plus Nitrogen Mustard, Asynchronous Cells

Using a subclone designated *V79-753B*, in Fig. 1 we show the effect that nitrogen mustard has on the X-ray survival curve of asynchronous cells. The closed circles trace the survival to graded X-ray doses alone of a population of microcolonies of average multiplicity 3.1. The adjustment of this curve to that for single cells is shown by the thin continuous line starting from 3.1 (see reference 15); alternatively, this latter curve could have been drawn from surviving fraction 1.0 in which case its terminal portion would be displaced downward by the multiplicity factor. This curve is typical of those observed with this and other cell lines in that it consists of a broad shoulder followed by an exponential region.

From visible examination alone, it is clear that the straight line region of the X-ray survival curve in Fig. 1 extrapolates to a value on the ordinate considerably more than 3.1 which, while indicative of its broad shoulder, relative to mechanism

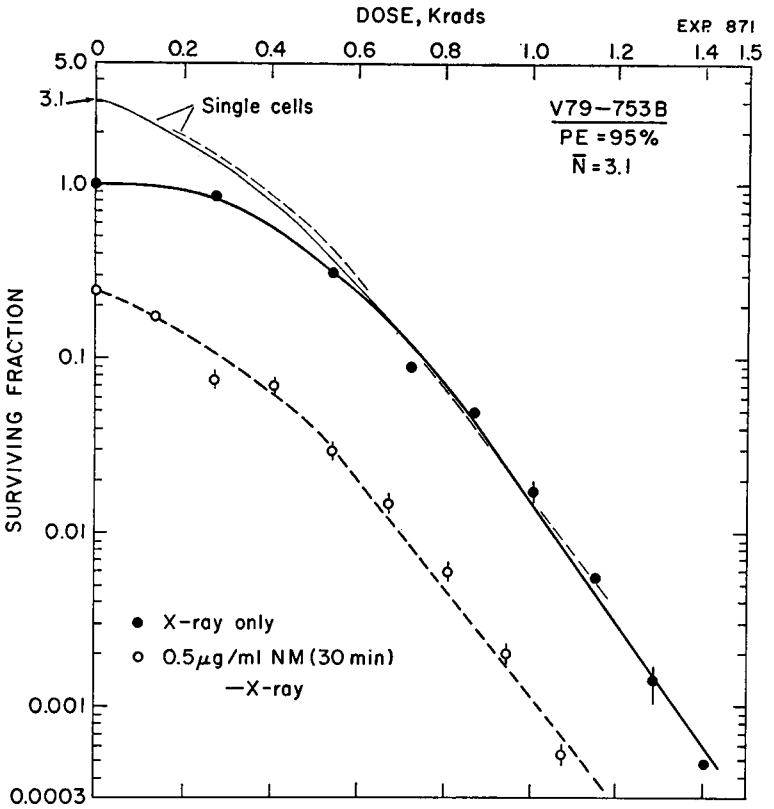

FIGURE 1 The effect of pretreatment with nitrogen mustard (NM) on the X-ray survival of Chinese hamster cells. *V79-753B* is the clone of cells used; PE = plating efficiency; and $\underline{\bar{N}}$ is the multiplicity at the start of the experiment. (Uncertainties, standard errors, are shown where larger than points as plotted.)

means that damage must be accumulated in cells for the registration of a lethal effect (15). Hence, a surviving cell is one which contains sublethal damage; after doses in the shoulder region a less than saturation level of sublethal damage is present and for doses along the straight line portion of the curve, surviving cells are very likely saturated with sublethal damage (15). Curves which are exponential and therefore extrapolate to the origin *may* result from single hit-to-kill inactivation in which case a cell may survive after any dose without having any sublethal damage relative to the end point (15).

Also shown in Fig. 1 is a test to see if nitrogen mustard has any effect on a cell's capacity to survive X-irradiation. The open circles show the result of 0.5 μg/ml of drug (30 min) followed by graded X-ray doses. To insure that an effect would be seen if one is present, we used a dose of nitrogen mustard sufficient to produce, by itself, a substantial degree of cell killing. The residual multiplicity at a survival of ~0.24 is small and hence, we see, relative to the origin for single cells (i.e., 3.1),

that 0.5 μg/ml nitrogen mustard kills more than 90% of the population. In spite of this, however, when the combined treatment curve is redrawn from 3.1 on the ordinate (with residual multiplicity after nitrogen mustard treatment accounted for), the latter curve and the "X-ray only" curves are practically superimposed. In contrast to a similar treatment sequence involving actinomycin D instead of nitrogen mustard (1, 3, 4), it appears in Fig. 1 that pretreatment with this alkylating agent has essentially no effect on X-ray survival.

Since our earlier studies suggested a difference only in degree between lethal and sublethal X-ray damage (4), we tested further the implication in Fig. 1 that nitrogen mustard has no effect on a cell's capacity for sublethal damage (i.e., no effect on shoulder width). The two-dose fractionation technique is one which we have shown (see reference 1 or 12) may be used to test for repair of sublethal X-ray damage and hence, we examined the effect of nitrogen mustard on the survival kinetics observed with fractionation.

The continuous line in Fig. 2 shows a pattern typical of the net survival observed with X-ray fractionation; the abscissa is the period between the dose fractions shown. As we have described elsewhere in detail (e.g., reference 1), the prompt initial increase in survival is due mainly to repair of sublethal damage (in S cells) and the minimum at about 6 hr shows the combined result of repair in first dose survivors plus their progression into age states of greater X-ray sensitivity (i.e., G_2, mitosis, and G_1). The time to reach the minimum is longer than that correspond-

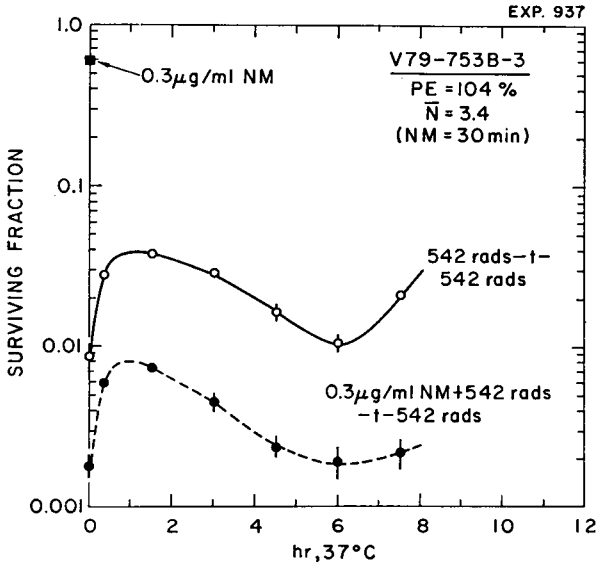

FIGURE 2 The effect of pretreatment with nitrogen mustard (NM) on the X-ray fractionation survival of asynchronous Chinese hamster cells. The survival due to nitrogen mustard alone is shown by the square on the ordinate. (Other details as in Fig. 1.)

ing to the age interval from just S to G_2 because of the delay in cell aging resulting from the first dose. Also shown in Fig. 2 is the resulting two-dose curve when cells are treated with nitrogen mustard before the first dose only. 0.3 μg/ml was used, the survival corresponding to which is shown on the ordinate; for single cells this would correspond to 0.24 surviving fraction. The origin of the X-ray fractionation curve is shifted downward by the amount of cell killing resulting from the nitrogen mustard pretreatment (as would be expected from Fig. 1), but the shape of the fractionation curve thereafter is essentially unchanged. After the minimum, the dashed curve appears to rise more slowly as might be expected if nitrogen mustard introduces some delay in progression and division in addition to that due to the first X-ray dose.

Thus, the results in Figs. 1 and 2 are consistent with no effect of nitrogen mustard on either lethal or sublethal X-ray damage. In Fig. 1, single-dose X-ray survival is unaffected and similarly in Fig. 2 for two-dose survival. Nitrogen mustard pretreatment appears to affect neither a cell's capacity for sublethal damage (Fig. 1) nor its ability to repair sublethal damage (Fig. 2).

Combined Radiation Plus Nitrogen Mustard, Synchronized Cells

To insure that our interpretation of the results thus far is substantially correct, we must examine the effects of combined treatment with synchronized cells. One reason for this, at the least, follows from the fact that we know that as cells age in their growth cycle, their ability to survive X-irradiation varies. Mammalian cells in general have radiation *age-response* functions which have structure (i.e., are nondegenerate; see chapters 2 and 3, reference 15) and since bifunctional alkylating agents are considered to be radiomimetic, it follows that the age-response pattern after nitrogen mustard treatment might also be nondegenerate. And if this is true, the possibility must be considered that the correct interpretation of results like those in Figs. 1 and 2 requires that an accounting be made of age-specific effects.

In Fig. 3 we show the single-dose age-response patterns for X-irradiation and nitrogen mustard treatment after cells were synchronized by exposure to hydroxyurea (2 mM, 3½ hr). [In a separate study, we will present more complete data on the age-variation in survival properties of Chinese hamster cells treated with nitrogen mustard[2].] Zero hours on the abscissa is the time when the medium containing hydroxyurea was removed. We know, from the results of Sinclair and Morton (22), that the X-ray survival maximum occurs in the latter part of the S phase—thus, this curve serves here to indicate the age of cells in their cycle when treated with nitrogen mustard as well as serving for comparison with the nitrogen mustard age-response pattern.

It is evident in Fig. 3, that the nitrogen mustard single-dose age-response curve has structure, structure which is similar in timing to that for X-rays. Although this

[2] Sakamoto, K., and M. M. Elkind. In preparation.

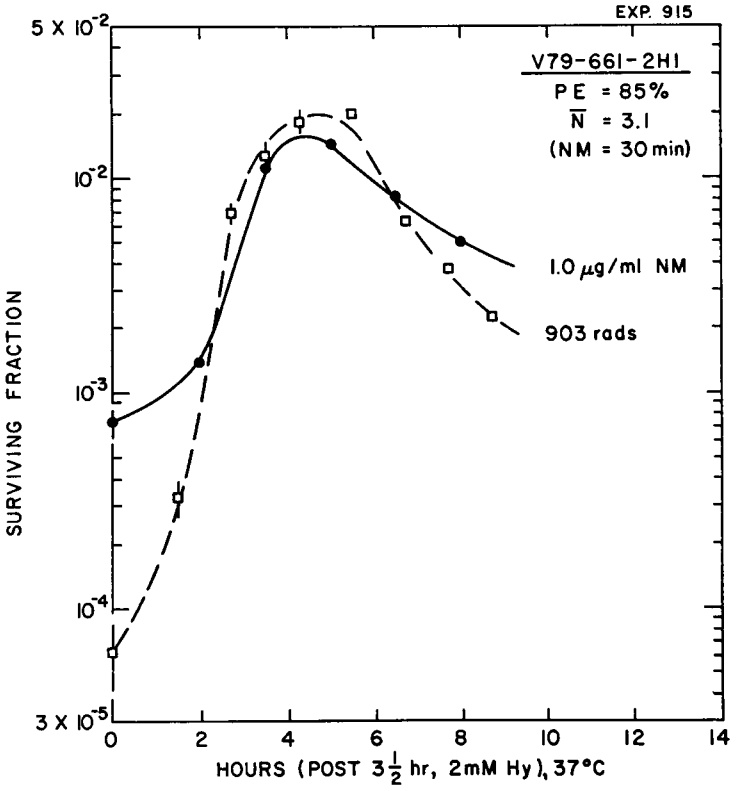

FIGURE 3 Age-response patterns for X-ray and nitrogen mustard (NM) treatment of Chinese hamster cells synchronized with hydroxyurea (Hy). The abscissa represents the progression of cells through their age cycle starting at the G_1-S border at zero hours. The midpoint of division would be at about 8 hr. (Other details as in earlier figures.)

similarity breaks down when survival curves at different ages are compared (e.g., see Figs. 4 and 5), it means at least that pretreating an asynchronous population with one agent does not select for cells inherently sensitive to the other.

Using synchronized cells of an age close to their survival maximum, in Fig. 4 we examine the effect of pretreatment, and posttreatment, with nitrogen mustard on the X-ray survival curve. The squares trace the single-dose survival of cells close to their most resistant age, late S. The residual multiplicity after synchronization was measured by trypsinization and with this value the X-ray survival curve for single cells was determined as in Fig. 1. As shown, the effect is to shift the origin to $\sim$1.2 relative survival.

The circles on the ordinate correspond to nitrogen mustard treatment *only* and as such, they should superimpose. However, for the closed circle the protocol required that the medium be removed and fresh medium added back just prior to the addition of the drug. This was done because before each X-ray dose, our prac-

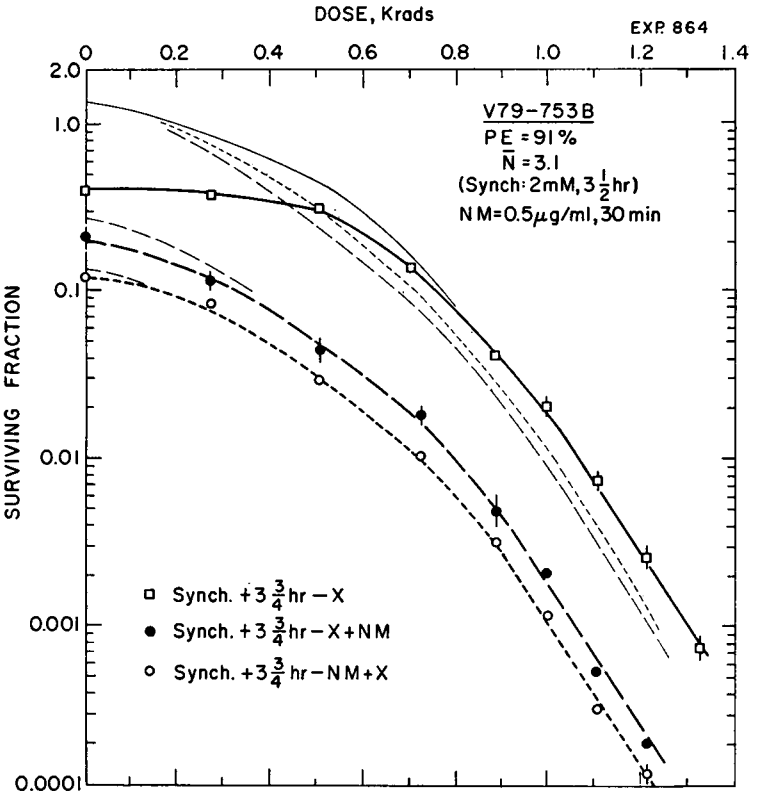

FIGURE 4 X-ray only and nitrogen mustard (NM) plus X-ray survival curves of late S Chinese hamster cells. The "thin" curves relative to the corresponding "thick" ones result when multiplicity is accounted for by shifting the origins upward. (Other details as in earlier figures.)

tice is to remove the medium as explained earlier. For the open circles, nitrogen mustard was simply added to the medium in which the cells had aged for 3¾ hr after synchronization. In a separate experiment, we found that changing to fresh medium just prior to the addition of the drug has the effect of reducing the final slope of the nitrogen mustard survival curve to a small extent. For present purposes, however, the point we note is that the closed circles trace a curve uniformly displaced upward from that traced by the open circles because of the extra change in medium involved in the former.

Returning to the question of damage interaction and the results in Fig. 4, we see that when both combined treatment curves are plotted from the origin for single cells, they almost overlap the X-ray only curve. There is at most a 50–100 rads reduction in the shoulder width of the combined treatment curves, a reduction quite small compared to that produced by pretreatment with an equivalent killing dose of actinomycin D (1, 3, 4).

To see if the effects of nitrogen mustard and X-rays are reciprocal relative to late S cells, we show in Fig. 5 an experiment similar to the preceding one. Here, however, we examine the effect of a pre- or posttreatment with a single X-ray dose relative to graded drug exposures. (Note that a medium change was introduced before *all* nitrogen mustard additions.) There are two principal points in this figure. First, in contrast to the X-ray survival of late S cells (Fig. 4), their survival after nitrogen mustard is almost exponential. The closed circle on the ordinate shows the relative survival obtained when cells are respread (for multiplicity estimation) by trypsinization after synchronization. Relative to this point as an origin, the nitrogen mustard only curve is almost exponential. The second point refers to the combined treatment data. These show essentially no effect of the order of treatment and no effect on the nitrogen mustard single-treatment curve.

Thus, as far as treatments in quick succession and late S cells are concerned, nitrogen mustard and X-irradiation produce effects which appear to act independently.

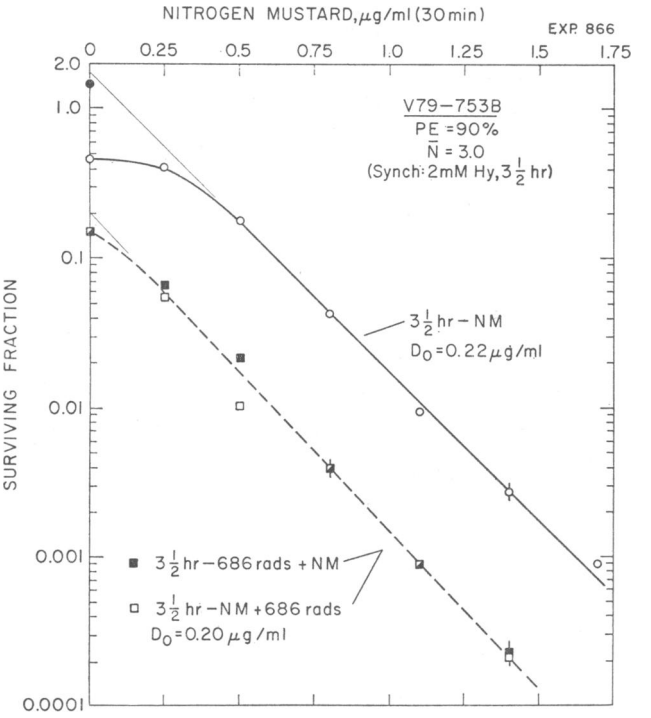

FIGURE 5 Nitrogen mustard single treatments, and X-ray plus nitrogen mustard combined treatment survival curves for late S Chinese hamster cells. D_0 is the dose, nitrogen mustard in this case, required to reduce survival by a factor of $1/e$ in the exponential region of a curve. (Other details as in earlier figures.)

Thus far, our results are that nitrogen mustard and radiation fail to show evidence of interactive damage when assessed relative to single as well as two-dose effects. Since we knew from earlier studies (see reference 1), that the onset of sublethal X-ray damage repair can be traced using a fractionation technique, we employed this same method for combined treatment fractionation.

The reasoning underlying these experiments is the following. In an X-ray dose fractionation sequence, we test for the repair of sublethal damage registered by the first dose by measuring how much of this damage remains in time to interact with a second dose. This procedure has permitted us to demonstrate a time-dependent loss of an interaction between two X-ray doses as well as between an X-ray dose followed by a treatment with actinomycin D (3, 4, 21). The loss of an interaction results from a repair of sublethal damage and hence, when a radiation-drug sequence is fractionated, we have a way of inquiring whether or not drug damage interacts with the sublethal X-ray damage which is being repaired.

As indicated by the radiation data in Fig. 3, late S cells are the principal survivors

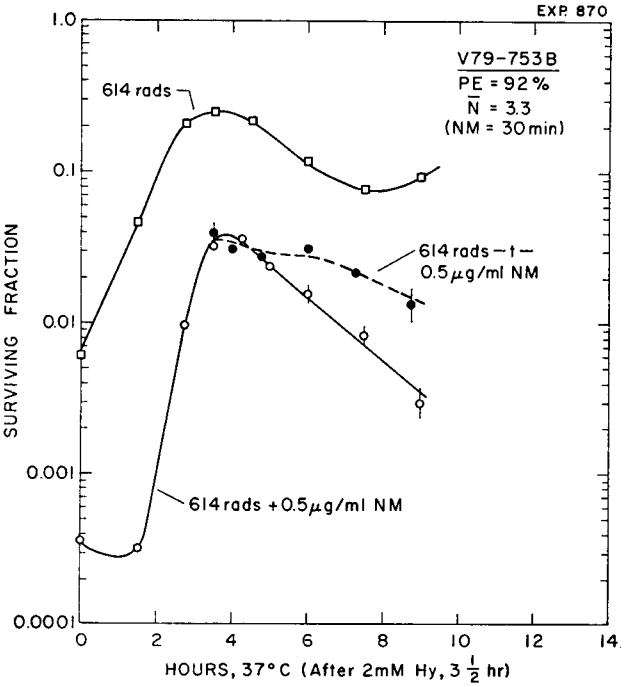

FIGURE 6 X-ray and combined treatment age-response functions for synchronized Chinese hamster cells. The combined treatments consisted of 614 rads followed promptly by 0.5 μg/ml nitrogen mustard (NM), open circles; or 614 rads at 3½ hr followed by the same drug treatment at varying intervals thereafter (closed circles). (Other details as in earlier figures.)

after an acute X-ray exposure which has the effect, therefore, of partially synchronizing an asynchronous population. S cells also comprise most of an asynchronous Chinese hamster cell population which is a second reason for our being concerned mainly with this moiety. Fig. 6 shows to begin with a typical single-dose X-ray age-response pattern (open squares) for cells synchronized with hydroxyurea. The open circles trace what is in effect a combined treatment, single-dose age-response pattern. At each time indicated, 614 rads was followed promptly by a 0.5 μg/ml nitrogen mustard exposure. The initial dip in the latter curve results from the fact that there are a small proportion of G_2 and/or mitotic cells in the predominantly G_1 population after $3\frac{1}{2}$ hr of hydroxyurea treatment[2]. G_2-mitotic cells, compared to G_1 cells, are more resistant to X-rays as well as nitrogen mustard. After irradiation, therefore, the proportion of these cells among the survivors is increased and hence, survival after combined treatment initially does not rise rapidly because G_2 cells progress into G_1 where they first become more sensitive.

Our main interest in Fig. 6 concerns late S cells (age equivalent $\sim 3\frac{1}{2}$ hr) which show a maximum in their combined treatment curve as we would expect they should judging from the results in Fig. 3. The dashed curve in Fig. 6 shows the result when

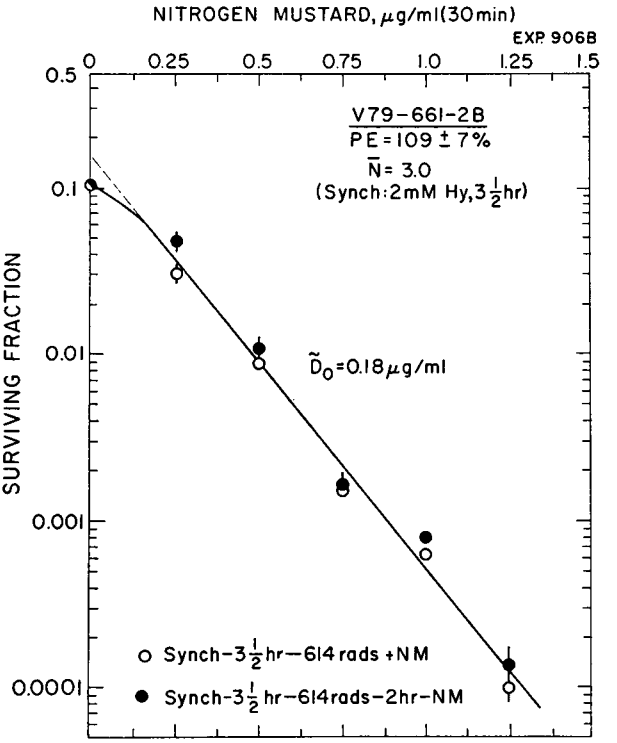

FIGURE 7 Fractionation combined treatment survival curves for synchronized Chinese hamster cells. The treatment sequences are indicated.

the combined treatment is fractionated; that is, 614 rads is given at 3½ hr and this is followed at varying intervals by 0.5 μg/ml nitrogen mustard. In contrast to the prompt increase in fractionation survival which results when a two X-ray dose fractionation is started with late S cells (e.g., references 1 and 4), the dashed curve in Fig. 6 is flat and drops slightly.

To understand more completely the meaning of this result, in Fig. 7 we show the nitrogen mustard survival curves of cells treated in late S with the same X-ray dose. The open circles resulted when drug treatment promptly followed X-ray treatment and the closed circles, after the same X-ray dose, are for cells incubated for 2 hr before nitrogen mustard treatment. These data show that the reason for the almost horizontal fractionation curve in Fig. 6 is that in 2 hr, at least, the cells surviving the X-ray treatment do not change in their responsiveness to nitrogen mustard. This is in sharp contrast to similar fractionation measurements made with X-ray plus

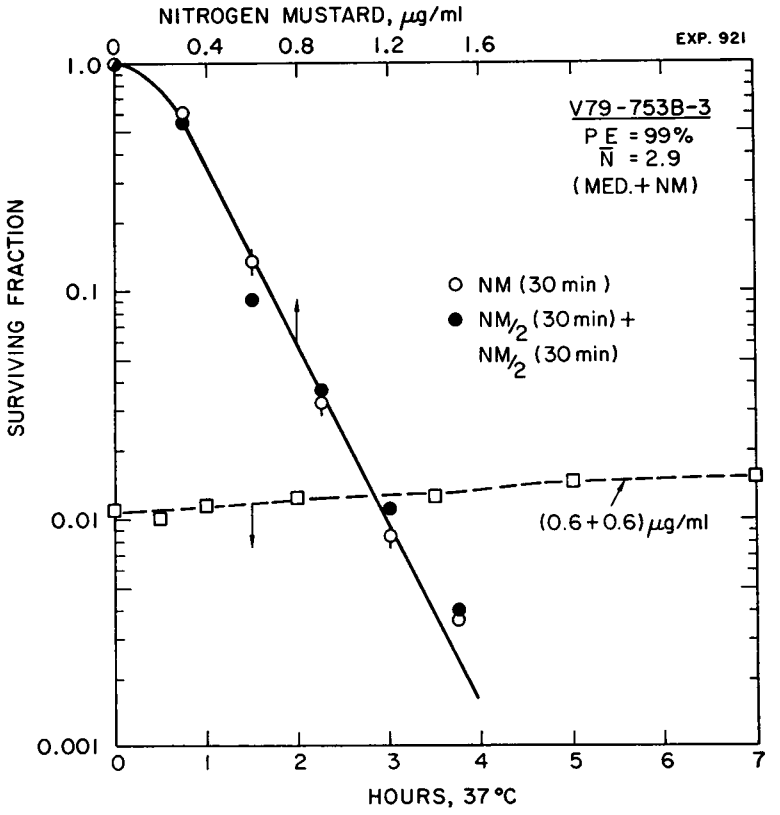

FIGURE 8 Survival and fractionation results with exposure of asynchronous Chinese hamster cells to nitrogen mustard (NM) (arrows indicate appropriate abscissae). The double-dose exposures traced by the closed circles serve as a control for the fractionation data (squares) because they indicate that the survival curve is not changed by treating sequentially (no interval between doses). (Other details as in earlier figures.)

X-ray or X-ray plus actinomycin D (3, 4, 21). In the latter instances, 2 hr gives rise to a large increase in capacity for sublethal damage as a consequence of the repair process that sets in immediately after the first X-ray dose. The effect of this repair is to shift upward the exponential portions of fractionation survival curves. The results in Figs. 6 and 7 suggest that no change in interactive ability occurs in the interval between irradiation and drug treatment. Although the dashed curve in Fig. 6 does not superimpose on the combined treatment curve, we suspect that this is because of the delay in aging resulting from the X-ray exposure.

Fractionation, Nitrogen Mustard Only

Having found no evidence for a radiation-drug interaction, for completeness we examined nitrogen mustard fractionation by itself. Fig. 8, for asynchronous cells in microcolonies ($\bar{N}$ = 2.9), shows survival curves and fractionation data. With respect to the upper abscissa, the circles trace survival curves; the open circles for single 30 min exposures and the closed circles for successive 30 min exposures.

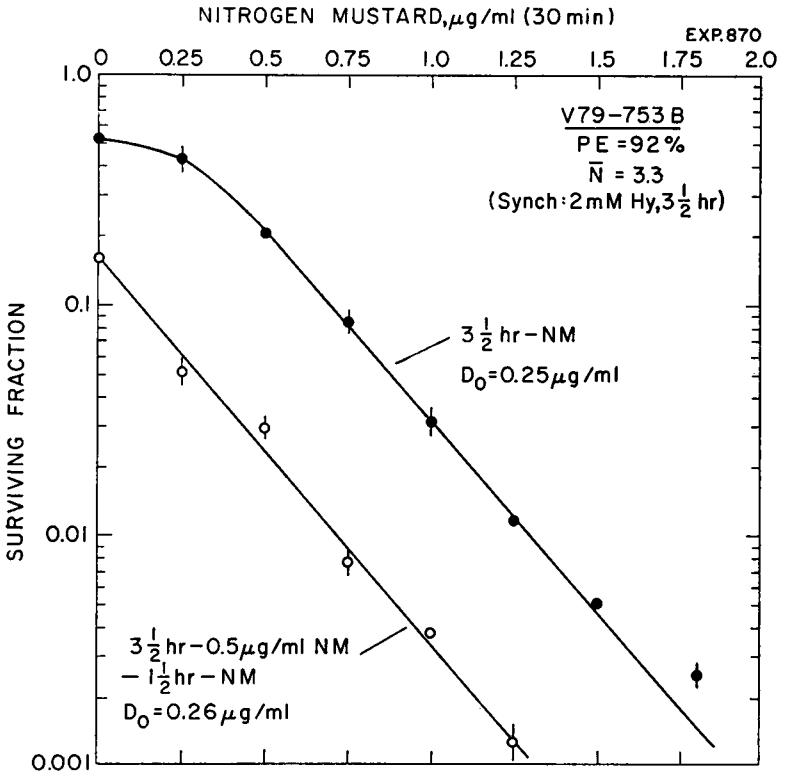

FIGURE 9 Single and fractionation treatment of synchronized Chinese hamster cells in late S. The doses for the open circles are the second doses 1½ hr after 0.5 μg/ml. (Other details as in earlier figures.)

With respect to the lower abscissa, the squares trace the net survival to two nitrogen mustard treatments separated by varying intervals. In contrast to the radiation fractionation results (e.g., Fig. 2), these data suggest that nitrogen mustard damage does not interact with itself.

To insure that this view is correct—at least for late S cells and an interval long enough to show substantial repair in the case of X-rays—in Fig. 9 we show a fractionation experiment similar to that in Fig. 7 but for nitrogen mustard treatment only. The drug survival curve of late S cells is traced by the closed circles; the fractionation curve by the open circles. As in Fig. 5, the single-treatment curve has a shoulder due essentially entirely to multiplicity. After a fractionation interval of 1½ hr, those late S cells which survived a first treatment of 0.5 μg/ml of nitrogen mustard survive graded second doses as though no fraction interval had intervened.

In analogy with our analysis relative to radiation results, the data in these last two figures indicate that cells cannot repair sublethal nitrogen mustard damage. This is, however, quite reasonable since the survival curve of single cells treated with this drug—asynchronous cells (Fig. 8), late S cells (Figs. 5 and 9), and G_2 cells[2]—is very close to exponential. Hence, these results present an internally consistent picture; cells surviving nitrogen mustard treatment do not repair sublethal damage because they do not contain such damage.

DISCUSSION

The principal observations we report here are: that nitrogen mustard has little or no effect on survival after single X-ray doses and vice versa; that nitrogen mustard damage does not interact with sublethal X-ray damage; and that no indication of a requirement for sublethal nitrogen mustard damage and/or its repair can be demonstrated. These observations were made principally with late S cells but probably can be taken to apply to S cells in general. We have also observed similar results with G_2 cells (to be reported elsewhere). While it is possible that age intervals exist in the growth cycle of Chinese hamster cells to which our conclusions may not apply, since the S phase occupies about 60% of the age cycle we may concern ourselves at least with a large fraction of the cycle. Added to this is the important fact that when asynchronous cells are treated witb moderate to large doses of X-rays or nitrogen mustard, initially late S cells are the main survivors (Fig. 3) and thus dominate the response of the population as a whole.

In contrast to our findings with combined radiation and actinomycin D treatments, we might conclude that nitrogen mustard and radiation act on different sites or molecules. Such a conclusion, however, would be inconsistent with the radiomimetic properties attributed to this drug (10, 23, 24). To be sure, if radiation damage consists of one or another form of molecular degradation due to bond breakage associated with energy absorption events, we would not expect alkylation and radiation lesions to be equivalent in all respects even though some bond break-

age results from alkylation. Still, some indication of damage interaction seems reasonable to expect if the sites of interaction are the same (e.g., DNA and/or chromatin). We keep open, therefore, the possibility that our results show that nitrogen mustard and radiation do not produce lesions critical for cell proliferation in the same place.

Two features relative to nitrogen mustard cell killing alone make clear an alternate possibility consistent with a common site of drug-radiation action. The first is the close to exponential nature of the nitrogen mustard single-dose survival curve which raises tbe possibility of a single hit-to-kill inactivation mechanism. And the second is the lack of a change in survival characteristics with fractionation of drug treatment, an observation which makes it more likely that single-hit inactivation is involved. These two features strongly imply that a sublethal component of injury is not involved in nitrogen mustard cell killing.

If, then, nitrogen mustard and radiation differ qualitatively in their modes of action in respect to an involvement of damage accumulation in cell killing, an internally consistent picture can be offered within the constraint of a common locus of action. In contrast to radiation damage, a single lesion registered in a cell by nitrogen mustard is lethal and hence, a cell surviving an exposure to this drug is not affected at all with respect to its division capability. This means that nitrogen mustard survivors will survive irradiation as though they had received no prior treatment. Conversely, while a cell surviving irradiation is sublethally damaged, any subsequent damage due to nitrogen mustard is adequate to kill it. Hence, X-ray survivors—regardless of the status of their sublethal damage—survive nitrogen mustard exposure as though they had received no prior treatment. [The foregoing statements are similar in mechanistic content to those which apply to combined high plus low linear energy transfer radiation treatments (e.g., reference 25).]

This picture points up the possibility that our observation of a *lack* of an interaction between nitrogen mustard and radiation damage is nevertheless consistent with a common locus of action. The lesion due to this alkylating agent may be in or associated with a cell's DNA—as our other results indicate for X-ray lesions (3, 4, 21)—and still may not interact with radiation damage because of essential differences in the damage resulting from these agents. Thus, we may have here an example of independent actions involving the same target molecule.

We are indebted to the Damon Runyon Memorial Fund for Cancer Research for fellowship support for Dr. K. Sakamoto during the course of this work.

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Received for publication 5 February 1969.

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