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DIFFERENCES IN THE RESPONSE OF LEUKAEMIA CELLS IN TISSUE CULTURE TO NITROGEN MUSTARD AND TO DIMETHYL MYLERAN

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Abstract—Two chemically different types of biologically active (radiomimetic) alkylating agents, i.e. nitrogen mustard (HN2) and dimethyl myleran, are shown to produce very different patterns of response in leukaemia cells grown as isolated cells in tissue culture. HN2 immediately stops division, while following dimethyl myleran the cells divide at least once before growth stops. The appearance of the cells is also different as is the shape of the curve relating dose with survival (i.e. ability to proliferate indefinitely). For HN2 the relationship between log survivors and dose is nearly linear, whereas with dimethyl myleran there is a marked threshold. It is suggested that the initial chemical reactions that are responsible for these effects on growth are different possibly because different sites are alkylated. The effects of dimethyl myleran simulate more closely those of X-rays than do those of HN2.

THE radiomimetic activity (i.e. the ability to act as a mutagen and carcinogen, to inhibit cell division, to kill lymphocytes and to cause chromosomal aberrations by acting on the cell during interphase) of the mustards, ethyleneimines, epoxides and esters of methane sulphonic acid is undoubtedly associated with their ability to alkylate, under physiological conditions, molecules within the cell.[1] There are however, many sites available for alkylation and it is possible that different alkylating agents may show a different pattern of reaction and the biological effects they produce will not be identical. The striking similarity in the biological end-effects made it tempting to postulate an identical mechanism at the cellular level for all these substances and to ascribe differences in their effects in mammalian systems to processes of transport and distribution.[2, 3] On the other hand, Elson[4] has consistently advocated that the differences in the blood picture seen after exposure to myleran and nitrogen mustard indicated a difference in mechanism at the cellular level and this view found support in a quantitative study of the bone-marrow[5] of treated rats. Alexander and Lett[6] were led to the same conclusion from biochemical investigations on the effect of these two types of alkylating agents.

In the present investigation the effect of HN2, $(CH_3N.(CH_2CH_2Cl)_2)$, and dimethyl myleran, $(CH_3SO_2.O.CH(CH_3).CH_2.CH_2.CH(CH_3).O.SO_2.CH_3)$, was studied on the multiplication of mouse leukaemia cells growing as isolated cells in tissue culture.[7] Dimethyl myleran was chosen instead of myleran, $(CH_3.SO_2.O.(CH_2)_4.O.SO_2CH_3)$, because its rate of reaction is much greater and its half life under physiological conditions is 37 min[8] as opposed to many hours for myleran.* In its

* A direct comparison is not possible since dimethyl myleran reacts by an SN1 mechanism, so that its rate of disappearance by alkylation or hydrolysis is independent of the presence of dissolved substances, while myleran reacts by an SN2 mechanism when the rate depends on the amount of alkylatable substrate present.[1]

biological effects dimethyl myleran is extremely similar to myleran[9] and shows the same selectivity for myeloid cells that distinguishes the latter from mustards. The half-life of HN2 in the tissue culture medium is less than $\frac{1}{2}$ hr. The rapid action of these substances ensures that the time of treatment of the cells (i.e. the time they are exposed to alkylation) is relatively short and eliminates the need to wash the cells free from reagent.

EXPERIMENTAL

L5178 Y Lymphoblasts, strain specific to DBA/2 mice (originating as a cloned derivative of an ascitic form of L 5178 supplied by L. W. Law of the National Cancer

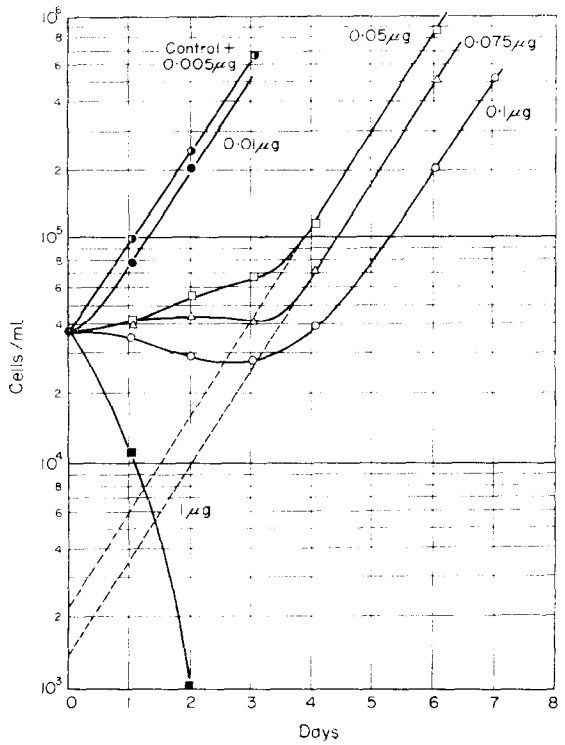

FIG. 1. Growth of leukaemia cells in culture containing different quantities of HN2 ($CH_3.N.(CH_2CH_2Cl)_2$. The concentrations are in μg HN2/ml. The dotted line shows the method of obtaining the fraction of cells of the original inoculum that retain capacity for indefinite proliferation.

Institute, Bethesda) were grown in the medium and by the techniques developed by Fischer.[7]* The cells were periodically transplanted into mice to ensure that they retained their strain specificity. In culture, they grow almost wholly as near diploid cells with a generation-time that varied from 12 to 18 hr, depending on the batch of medium.

In most of our experiments an initial inoculum of from 2 to 10 × 10^4 cells/ml were used. The culture was diluted when the population approached 10^6 ml. Figs. 1 and 2

* We are greatly indebted to Dr. G. A. Fischer of the Department of Pharmacology, Yale University School of Medicine, for giving us a culture and for much help.

show that the rate of growth of the untreated cultures is accurately logarthmic. Cells were counted in a haemocytometer. The distribution of cell sizes was determined with a "Coulter Counter" in which changes in electrical resistance are used to count and to size particles in suspension.

RESULTS

Growth curves

Comparison of Figs. 1 and 2 shows a most striking difference between the action of these two cytotoxic agents. HN2 stops cell division immediately. Microscopic examination of the culture in the first 24 hr fails to reveal any cell degeneration and the constancy of the cell numbers can therefore be attributed to cessation of division and is not due to compensation of reproduction and distribution. After 40 hr cell degeneration is seen and the cell count now represents a balance between degeneration and production of new cells. With dimethyl myleran, even with very substantial doses, at least one division occurs before growth ceases in the cultures. With low doses of dimethyl myleran, the rate of cell division is initially slowed down to a small extent

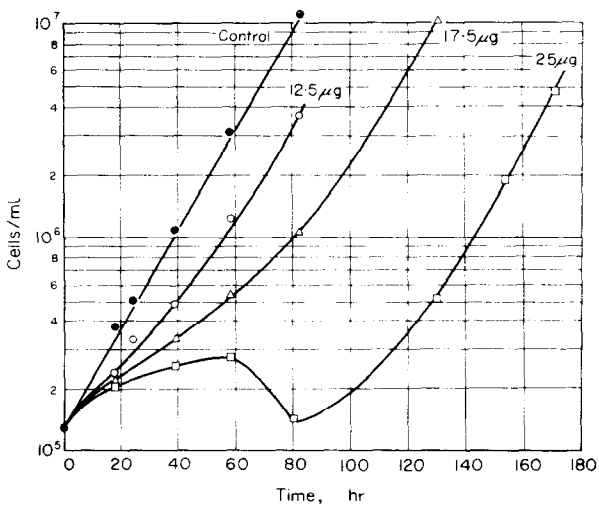

FIG. 2. Growth of leukaemia cells in tissue culture containing different quantities of dimethyl myleran ($CH_3.SO_2.O.CH(CH_3).(CH_2)_2.CH(CH_3).O.SO_2.CH_3$). The concentrations are in μg/ml.

only, so that there is almost no difference between the treated and the control cultures after 18 hr. Only on the second day can the effect of the cytotoxic agent be observed in that no further increase in cell numbers occurs and grossly abnormal cells are seen. With higher doses of dimethyl myleran the mitotic rate is reduced immediately, but the cells still divide at least once and abnormal cells are only seen after the initial population has doubled.

Following this initial period of growth, cell degeneration occurs and fragmentation is obvious after 40 hr. The actual cell count is then approximately constant (see Fig. 2), but after this a new population of cells makes its appearance and cell numbers increase at the same rate as with the controls. This, apparently stationary, phase also occurs after treatment with HN2, but in this case it sets in immediately after treatment. The exact time sequence depends on the dose of cytotoxic agent that is used.

Dose–response relationship

The new population of cells arises, we assume, from those cells that were not damaged by treatment with the alkylating agents. The number of these so-called survivors can be obtained by extrapolating the growth curves of the new population to zero-time (see dotted lines in Fig. 1).

This method of determining the fraction of the original population which has been damaged in such a way that it does not give rise to fully viable daughter cells is open to criticism on the grounds that the drugs may induce a temporary mitotic arrest from which the cells recover completely. If this occurs then the percentage of cells that have lost their capacity to multiply indefinitely, would be less than the value

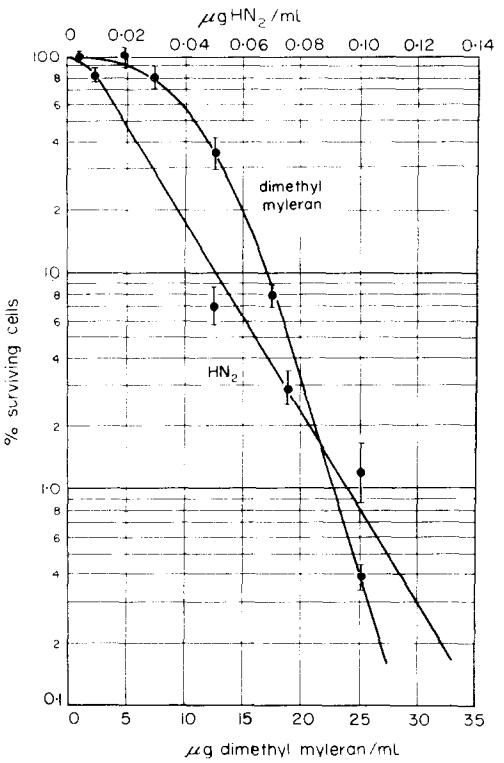

FIG. 3.Relationship between percentage of cells surviving (i.e. percentage of cells in original inoculum that retained ability to divide normally) and concentration of alkylating agents added to the tissue culture medium.

calculated by the extrapolation technique. We feel, however, that this objection is not valid, since small doses of dimethyl myleran cause almost no immediate reduction in the rate of cell division, although more than 50 per cent of the cells can be seen to be abnormal at 48 hr after treatment. These lymphoblasts would appear to be relatively resistant to the induction of mitotic delay which seems to occur only after sufficient damage has been done to render the cell incapable of repeated cell division.

Fig. 3 shows the dose–response curve determined by the extrapolation technique. Again, there is a qualitative difference between the two alkylating agents. The curve

of log survivors against dose is almost linear for HN2 and there is only a barely perceptible threshold. If one cared to express this curve according to "target theory" it would be 1·3 hit. However, this close approximation to a single hit inactivation curve cannot be interpreted along the line that one target molecule has to be inactivated.[10]

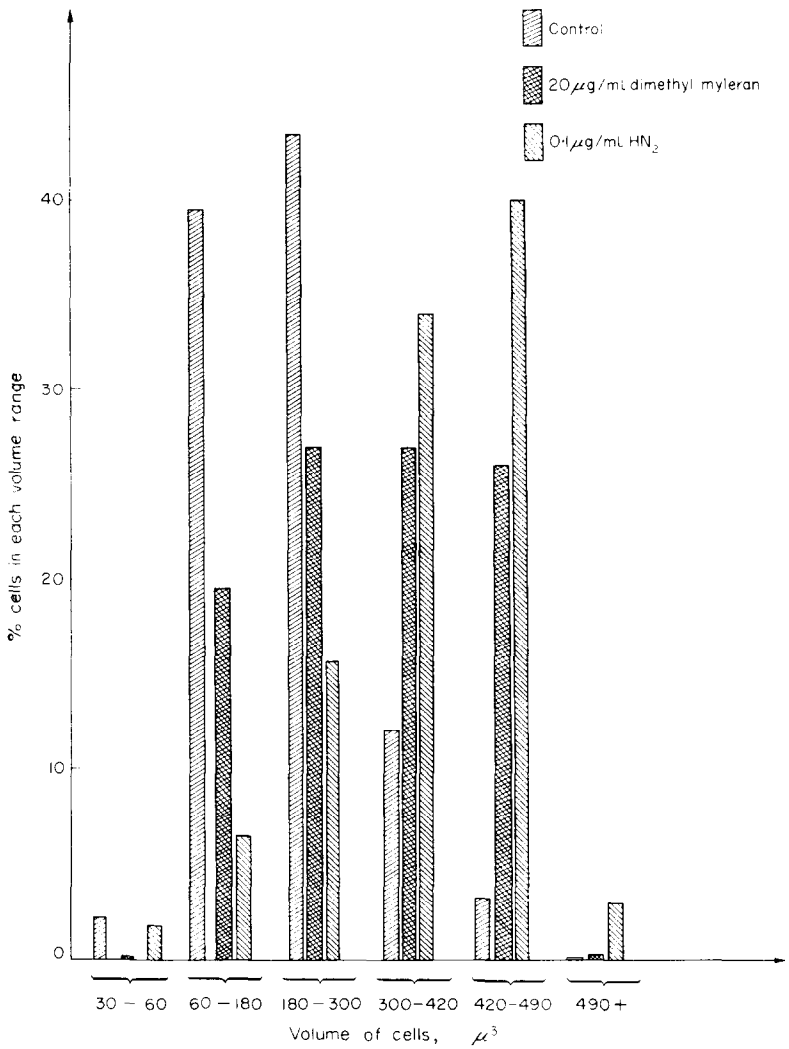

FIG. 4. Size distribution of leukaemia cells following treatment with dimethyl myleran and HN2.

From a practical point of view this type of dose–response curve indicates that the dose of HN2 needed to eradicate leukaemia *in vivo* (i.e. the dose needed to prevent every cell from proliferating) will depend on the number of cells present in the animal.

With dimethyl myleran the graph of the logarithm of survivors against dose is far from linear and shows a pronounced threshold (in the terminology of the target theory it would be 3·5 hit). In this respect dimethyl myleran shows a more typical

pharmacological response. Inspection of Fig. 3 shows that a value for the relative effectiveness of the two agents on a weight-basis depends on the level of cell "killing" at which the comparison is made. The difference in lethality is much greater at higher levels of survival.

Appearance of alkylated cells

Cells treated with dimethyl myleran appear quite normal under the microscope until they have divided at least once, when some of them turn into giant cells similar to those described by Puck and Marcus[11] in Hela cell cultures that have been irradiated with X-rays. Both the nucleus and cytoplasm increase in diameter to more than double to give cells having ten to fifty times the volume of the untreated. However,

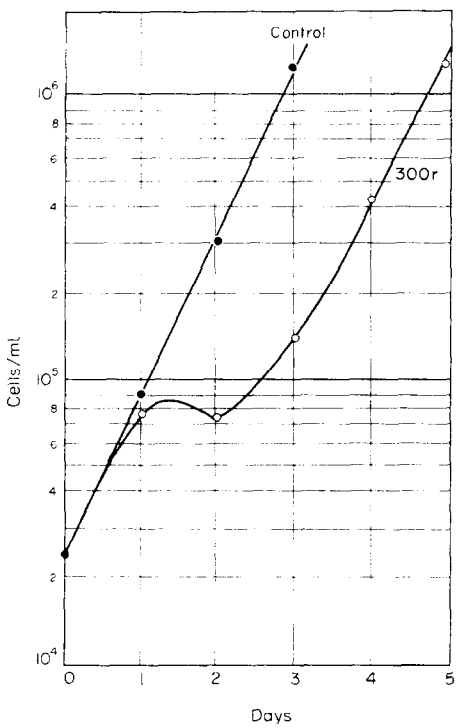

FIG. 5. Effect of a dose of 300 r of X-rays (220 kpV, 270 r/min) on the growth of a suspension of leukaemia cells. (To ensure that the suspension was not depleted of oxygen, an inoculum from a concentrated cell suspension was added to fresh medium immediately before irradiation.)

by no means all the cells that are "killed" turn into giants; 20 μg/ml gives rise to less than 15 per cent giants even though 95 per cent of the cells are killed. These giant cells are very fragile and degenerate after a few days.

After treatment with HN2, the cells already show abnormal features after 24 hr—in particular, the appearance of dark granules. The cell size increases after division has been stopped but the giant cells typical of dimethyl myleran are not seen. The HN2 cells degenerate more quickly and at all times the cells look quite different from those treated with dimethyl myleran. The immediate cessation of cell division by

HN2 would also not appear to be due to interference with DNA synthesis as this continues apparently normally for almost a day. Detailed biochemical investigations are now being made on the cells treated with these two alkylating agents.

Using the "Coulter Counter", changes in the size distribution following treatment can be seen (see Fig. 4). After 18 hr the volume of the HN2 treated cells is more than doubled and only 20 per cent of the cells are within the size range of the control cells. At later periods, fragments appear from degenerating cells but the size of the cells does not increase further.

With dimethyl myleran, a change in the size distribution is seen after 18 hr though to a much less extent. After 40 hr the distribution moves further to the right, but it does not peak at a high value as with HN2. A small tail of giant cells which are noticeable under the microscope become detectable in the "Coulter Counter" after 40 hr.

DISCUSSION

In its effect on the rate of cell division for these leukaemia cells dimethyl myleran is truly radiomimetic. With doses of X-rays up to 300 r—when 90 per cent of the cells are prevented from indefinite multiplication—growth in the culture only stops after the population has doubled (see Fig. 5).[12] The shape of the dose–response curve and the appearance of the pathological cells under the microscope is also extremely similar for dimethyl myleran and for X-rays.[11] The changes produced by HN2, on the other hand, are entirely different. It causes immediate cessation of cell division only seen after more than 2000 r, but even then the cells differ in appearance from those exposed to radiation, which even at these doses leads to the formation of giants.

After treatment with a nitrogen mustard, changes have been detected in the DNA of a variety of cells[2, 6, 13] which suggest that different DNA molecules have been joined together (cross-linked) by alkylation through the two centres present in the mustard molecule. It seems plausible that cross-linking of this kind will prevent cell division and that it is this reaction which is responsible for the immediate inhibition of growth. Dimethyl myleran has also been shown to cross-link the DNA within the nucleus,[13] but only at extremely high doses. For this reason Alexander and Lett[6] were led to suggest that at the chemical level the mode of action of the myleran type of compounds might be different from that of the mustards. Perhaps alkylation of a site other than the DNA is responsible and acid groups in internal cell barriers have been proposed.[6]

It is not clear whether the delay that is seen after treatment with dimethyl myleran is due to the fact that a cell division is necessary before the injury manifests itself e.g. by chromosome deletion effects[14] or whether metabolism alone is sufficient to develop the injury.

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