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EFFECTS OF NITROGEN MUSTARD ON PROTEIN AND NUCLEIC ACID SYNTHESIS IN MOUSE FIBROBLASTS GROWING *IN VITRO*

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Abstract—Nitrogen mustard, added at 0·1 μg/ml to the culture medium of mouse fibroblasts growing *in vitro*, led to rapid cessation of cell division and of DNA synthesis. RNA and protein synthesis continued at a rapid rate for several days, leading to greatly enlarged cells. Although the accumulation of DNA showed an abrupt halt shortly after nitrogen mustard addition, the inhibited cells nevertheless had up to twice the amount of DNA found in a normal, rapidly growing cell. Cells whose growth was limited by depriving them of an essential amino acid, leucine, showed no such effect. It is suggested that nitrogen mustard halts the division process at a time after the replication of DNA that occurs in preparation for division.

INTRODUCTION

THE growth-inhibitory and cytotoxic effects of nitrogen mustard (HN2)* have been widely believed to be due to blocking of DNA synthesis, perhaps by a process of cross-linking.[1, 2] Bodenstein and Kondritzer[3] demonstrated a block of DNA synthesis in salamander embryos exposed to HN2, while RNA accumulation continued to occur in a normal manner. Goldthwait[4] obtained similar results, studying the incorporation of ^{14}C-formate or ^{15}N-adenine into RNA and DNA of rat intestine. Herriott[5] has also obtained evidence, utilizing *E. coli*, that HN2-blocked cell division and DNA synthesis without interfering with RNA synthesis. Nevertheless, some question remains[6] as to whether the effects of HN2 on mammalian systems can be ascribed solely to an effect on DNA synthesis. It also seems clear that while polyfunctional alkylating agents are more potent than monofunctional agents with respect to growth-inhibitory activity,[7] monofunctional alkylating agents are at least as potent for other effects, such as mutagenesis.[8] Since the monofunctional compounds could not be participating in cross-linking reactions, mutagenic effects, at any rate, could not be explained by a cross-linking of DNA molecules.

In an effort to obtain quantitative data on the effects of HN2 on a rapidly growing mammalian cell, the effects of the drug were studied using mouse fibroblasts (Earle's "L" cell) growing *in vitro*. Cell counts, protein, RNA and DNA synthesis were examined.

METHODS

The procedures used were similar to those described by Saltzman.[9] Replicate cell inocula were prepared in 8-oz prescription bottles, stoppered with silicone stoppers,

* Abbreviations: HN2, nitrogen mustard; DNA, deoxynucleic acid; RNA, ribonucleic acid.

with 25 ml of Eagle's medium [10] containing 10 per cent beef serum. For analysis, the supernatant medium was poured off, and the adherent cells were gently rinsed with two 10-ml portions of a balanced salt solution. Individual cell counts were obtained on three bottles in each experimental group by scraping the cells in 10 ml of filtered 0·9% saline containing 0·1% trypsin, pipetting up and down vigorously a few times to disperse the cells, and immediately counting the cells in a Coulter electronic cell counter. The saline suspension was diluted ten-fold for counting when necessary. This procedure, using a weak concentration of trypsin, was found (by microscopic examination) to give well-dispersed, single cells with no signs of cell lysis. The entire scraping and counting procedure required less than 2 min.

Assays for protein, RNA and DNA were obtained from other bottles. The rinsed bottles were chilled to 3–5 °C, and the contents of from three to twenty bottles were scraped with successive 5-ml portions of ice-cold 8% perchloric acid. The acid-insoluble material in each group was pooled and centrifuged in the cold, and the supernatant solution discarded. Lipids were removed from the cold acid-insoluble material by extracting successively with 10-ml portions of alcohol–water (4:1), alcohol–ether (3:1), and ether. The residue was then treated with 3 ml of 6% perchloric acid for 20 min at 90 °C to hydrolyse the purine components of the nucleic acids. After cooling, the tubes were centrifuged and the hot acid-soluble fraction was separated from the residue.

Protein was determined on the residue after an alkaline digestion by the procedure of Oyama and Eagle.[11] Ribose was determined in an aliquot of the hot acid-soluble supernatant solution using the orcinol reaction of Volkin and Cohn.[12] Deoxyribose was determined in an aliquot utilizing the procedure described by Burton.[13] Since deoxyribose contributes a small amount of color to the orcinol reaction (10 μmoles of deoxyribose is equivalent to 0·66 μmoles of ribose), the ribose determinations were corrected by an appropriate amount. All colorimetric determinations were performed in duplicate.

RESULTS

The effects of various concentrations of HN2 on rapidly growing fibroblasts are indicated in Fig. 1. In the absence of drug these cells multiplied five to six-fold over a 3-day period. Nitrogen mustard* at 0·1 μg/ml limited cellular proliferation to about 50 per cent of the untreated cell count in this experiment. If HN2 was preincubated with medium for 12 hr before adding the medium to the cells, the drug lost its growth-inhibitory effect (Table 1). On the basis of these results, it was decided to study the effects of 0·1 μg/ml of the drug on the synthesis of protein, RNA and DNA.

Eighty-two replicate bottles were inoculated with cells, and after 2 days of growth in the normal medium, the medium was replaced with either normal medium, or medium containing HN2 at 0·1 μg/ml. The bottles were incubated for an additional 3 days at 37 °C. Cell counts were obtained daily from three bottles in each group. Different numbers of bottles were used for the chemical determinations, the contents of twenty bottles being pooled on day 1, while three bottles were sufficient on day 5.

* The nitrogen mustard used was triturated mechlorethamine hydrochloride (Merck, Sharp & Dohme). It was dissolved in water, diluted, and added to the medium immediately before use.

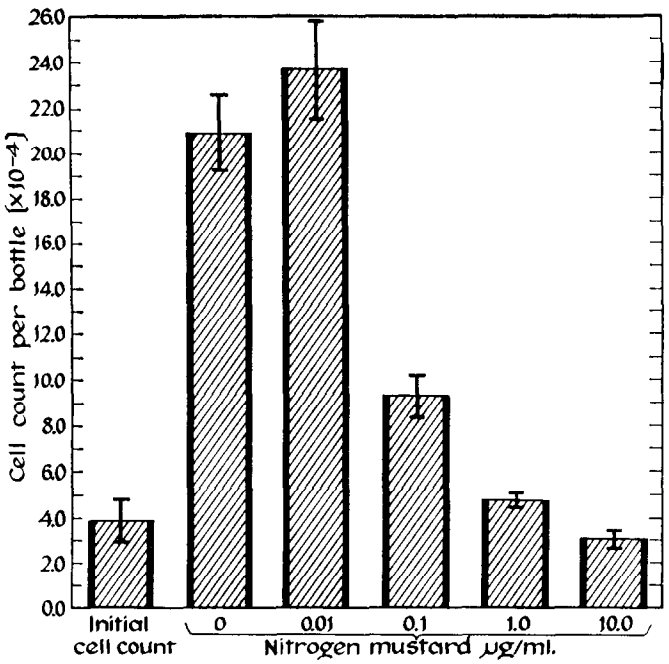

FIG. 1. Growth of fibroblasts after 3 days in varying concentrations of nitrogen mustard. Cells were inoculated into 8-oz prescription bottles, as described in the text. After 1 day, the supernatant medium was replaced with medium containing nitrogen mustard. The number of cells present in the bottles after 3 days in the experimental media was determined. The height of the bar represents the mean of of a group of three replicate bottles; standard errors of the means are also given.

TABLE 1. THE EFFECT OF PREINCUBATION OF NITROGEN MUSTARD WITH CULTURE MEDIUM ON THE GROWTH OF MOUSE FIBROBLASTS *in vitro*

Treatment	Cell count $\times 10^{-5}$
Initial inoculum	0·99 ± 0·04
Control, no drug	25·1 ± 6·9
HN2, 0·1 µg/ml	3·9 ± 0·4
1·0 µg/ml	1·6 ± 0·1
10 µg/ml	1·2 ± 0·1
HN2, 0·1 µg/ml, preincubated	19·6 ± 3·7
1·0 µg/ml, preincubated	19·8 ± 2·2
10 µg/ml, preincubated	19·6 ± 3·9

Prescription bottles were inoculated with mouse fibroblasts in Eagle's medium and on the following day, the medium was changed to one containing freshly added nitrogen mustard at the specified concentrations. Other bottles received medium containing the same concentrations of HN2, except that it was added to the medium on the previous day and allowed to incubate overnight at room temperature. Results are expressed as the mean cell count per bottle, ± standard error, obtained 6 days after the inoculation. There were three bottles in each group. The initial inoculum refers to the cell count obtained on the day after inoculation.

Fig. 2 demonstrates that the accumulation of protein and RNA in each bottle continue at about the normal rate after HN2 addition, while the increase in both cell count and DNA showed an abrupt halt after 1 day on the drug. The data are presented in terms of amounts of protein, RNA, and DNA per 10^5 cells in Table 2. There is a

large, and continuing, increase in ribose and protein per cell in the drug-treated group; at the same time these cells contain twice the normal complement of DNA, which does not increase further. The difference in the mean DNA content per 10^5 cells of the treated and untreated groups is significant at the 2 per cent level, as calculated by the Students *t*-test.

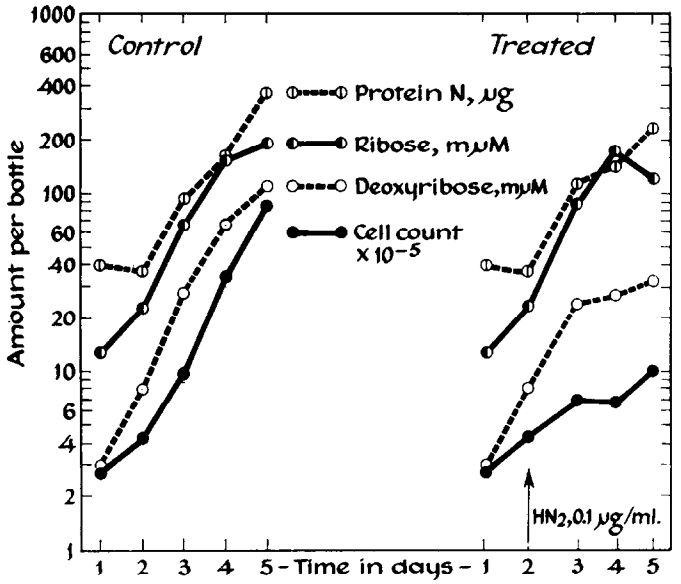

FIG. 2. Cell count, protein, ribose, and deoxyribose assays per bottle during a 5-day growth period in normal Eagle's medium and during a 3-day growth period in medium containing 0·1 μg of HN2 per ml. The cell count represents the mean of three bottles. Values for protein, ribose, and deoxyribose are the average of duplicate determinations performed on pooled bottles as described in the text.

TABLE 2. EFFECTS OF NITROGEN MUSTARD ON CELL COUNTS, RNA, PROTEIN AND DNA ACCUMULATION OF MOUSE FIBROBLASTS

Day	Untreated				HN2, 0·1 μg/ml, day 2			
	Cell count × 10^{-5}	Per 10^5 cells			Cell count × 10^{-5}	Per 10^5 cells		
		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)
1	2·78±0·30	4·7	1·1	14·2				
2	4·31±0·20	5·2	1·8	8·4				
3	9·83±0·90	6·8	2·8	10·2	6·86±1·0	12·9	3·5	16·4
4	34·7 ±1·0	4·4	1·9	4·7	6·78±0·9	25·6	3·9	25·4
5	86·9 ±4·0	2·2	1·3	3·4	10·0 ±0·4	12·1	3·3	23·2
Mean ± s.e.			1·8±0·3				3·6±0·2	

Eighty-two replicate bottles were inoculated with cells and cell counts and chemical assays were obtained on subsequent days as described in the text. Two days after inoculation, the medium was replaced in all remaining bottles, and nitrogen mustard added to half the bottles. The medium was replaced in all bottles on day 4 with the appropriate fresh medium.

Photographs of typical normal cells, and cells from an HN2-treated culture, are given in Fig. 3. It can be seen that the drug-treated cell has a large accumulation of protoplasm and enlarged nucleoli in comparison with normal, rapidly growing cells.

Since it seemed important to determine if the doubled amount of DNA found in the drug-treated cells was a specific effect of nitrogen mustard, or was merely a reflexion of the halt in growth, cellular growth was limited by depriving a growing

TABLE 3. EFFECTS OF LEUCINE-DEPRIVATION ON CELL COUNTS, RNA, PROTEIN AND DNA ACCUMULATION OF MOUSE FIBROBLASTS

Day	Untreated				No leucine			
	Cell count $\times 10^{-5}$	Per 10^5 cells			Cell count $\times 10^{-5}$	Per 10^5 cells		
		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)		Ribose (mμmoles)	Deoxy-ribose (mμmoles)	Protein N (μg)
1	2·01±0·10	6·3	2·7	11·3				
2	5·13±0·13	5·9	2·5	6·6				
3	10·2 ±0·4	5·9	2·9	5·7	10·6±0·3	5·7	2·9	4·7
4	25·5 ±0·8	5·5	2·4	3·1	15·4±0·4	6·5	2·9	4·6
5	63·5 ±2·6	3·3	2·0	2·9	18·1±1·4	6·5	1·8	3·6
Mean ± s.e.			2·5±0·2				2·5±0·4	

One hundred and three replicate bottles were inoculated with cells and cell counts and chemical assays were obtained on subsequent days as described in the text. Two days after inoculation, the normal medium was replaced with a fresh medium containing dialysed serum, and no leucine. Half the bottles (the untreated group) were supplemented with l-leucine at the usual concentration (0·1 millimolar).

culture of an essential amino acid, leucine. The results of this experiment appear in Table 3. It is apparent that when the amount of leucine in the culture medium is restricted, the cells shortly fail to grow; the cell count fails to increase normally, DNA synthesis halts, and RNA and protein synthesis also cease. Of more direct interest, however, is the finding that the DNA content per cell of cultures deprived of leucine is essentially the same as the DNA content of normal, rapidly growing cells.

Additional experiments with nitrogen mustard have confirmed the effects previously described. It should be noted, however, that the concentration of drug employed is critical. At concentrations lower than 0·1 μg/ml, little or no effect is observed. At higher concentrations (0·2 or 0·3 μg/ml), protein and RNA synthesis are also depressed, and cell lysis eventually occurs. Under these conditions, however, the DNA content per 10^5 cells remains higher than normal, at about 3·6 mμmoles of deoxyribose. The mean DNA content of normal, rapidly growing cells has been found, in later experiments, to be quite constant at about 2·4 mμmoles per 10^5 cells. The low value of 1·8 mμmoles reported in Table 2 (untreated cells) has not been seen in subsequent experiments, although the DNA content of HN2-treated cells has been observed (three experiments) to remain at 3·6 mμmoles.

The decline in protein content per cell, as log phase growth proceeds (Table 2 and, more typically, Table 3, untreated cells), is quite regularly observed and has been discussed by others.[9]

DISCUSSION

The data presented in Table 1 indicate that nitrogen mustard, and not one of its degradation products, is responsible for the growth-inhibitory effect reported here. Furthermore, it is clear that HN2 is not reacting with some component of the medium to produce its effect. The effects of low concentrations of nitrogen mustard, therefore, are a rapid cessation of cell division and DNA synthesis, but RNA and protein synthesis continues for several days at about the normal rate. Cells inhibited with HN2, therefore, had several-fold the normal complement of RNA and protein, and were much larger than normal, rapidly growing cells. While DNA synthesis showed an abrupt halt shortly after HN2 addition, these inhibited cells nevertheless had up to twice the normal amount of DNA found in a rapidly growing cell. The increased amount of DNA per cell after HN2 addition is interpreted to mean that these cells synthesize DNA up to the pre-mitotic amount, and then DNA synthesis ceases. Cells whose growth was limited by leucine-deprivation, on the other hand, had the same amount of DNA per cell as was found in a rapidly growing culture. These effects of HN2 are similar to the effects of X-radiation on mouse fibroblasts, as described by Whitfield and Rixon.[14] These authors observed a "piling up" of DNA at the pre-mitotic amount, and little effect on protein and RNA synthesis. Caspersson *et al.*, in a microspectrophotometric study of single ascites cells after X-radiation, also found an accumulation of cells containing the pre-mitotic amount of DNA.

This evidence suggests that nitrogen mustard does not interfere with the functional aspects of DNA metabolism, at least insofar as they are reflected in RNA and protein synthesis. It also suggests that DNA replication occurred, giving rise to cells containing the pre-mitotic amount of DNA, but final separation of the DNA into daughter chromosomes and cellular division no longer occurred. Undoubtedly the cells will develop into typical "giant cells", widely reported[16] to occur after nitrogen mustard treatment or radiation. Cobb[16] has specifically examined the cytological effects of HN2 on mammalian cells growing *in vitro*, and readily observes giant cell formation. She also reports observing "a suppression of mitosis and aberrant and sticky chromosomes".

If current theories of DNA replication,[17] involving unwinding of a parent polynucleotide helix with the simultaneous formation of two daughter helices, are correct, then it is hard to see how a simple cross-linking of DNA molecules could lead to the observed results. If DNA strands were cross-linked, and therefore unable to separate, one would expect a complete cessation of DNA synthesis as well as cell division. On the other hand, if cross-linking of chromosomal material occurred at an early prophase stage of division, such that chromatids perhaps could not condense or separate, one might expect to see a pre-mitotic value of DNA, as was actually observed. Such a scheme of cross-linking might possibly involve DNA–DNA interaction, as originally proposed by Goldacre *et al.*,[1] and Alexander and Lett.[2] However, it seems equally possible, and perhaps more likely, that DNA-nucleoprotein cross-linking could be responsible for the observed results. In fact, any interference with an early step in the mitotic process could give rise to these effects.

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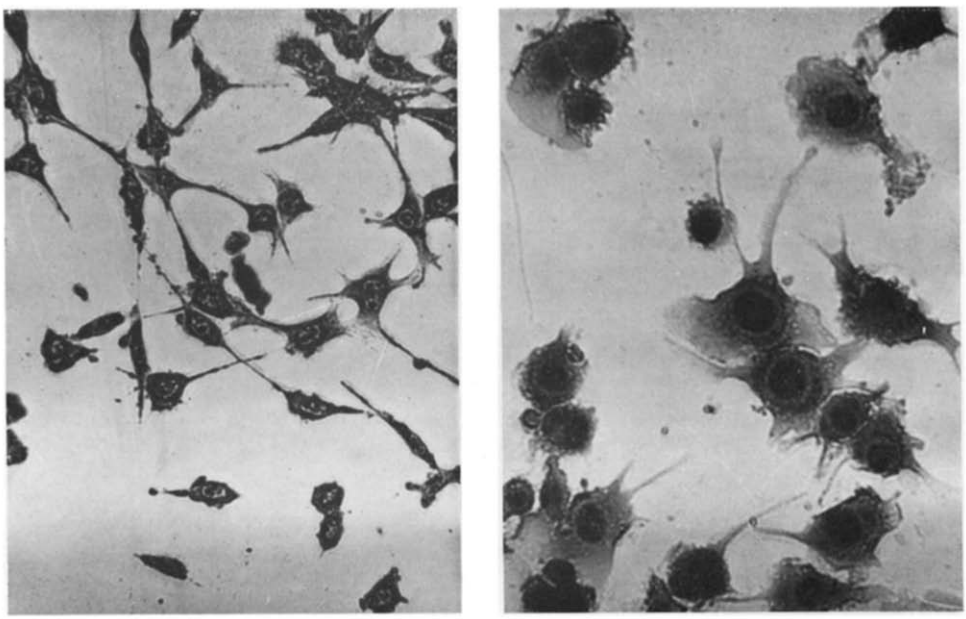

FIG. 3. Left: Normal fibroblasts, growing on microscope slides in Blake bottles. After 5 days of culture, the slide was removed, rinsed with a buffered saline solution and fixed in methanol. The slide was lightly stained with Giemsa solution. × 53·5.

Right: Same as above, but incubated for the final 2 days in medium containing 0·1 μg of HN2 per ml.

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