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Effect of a nitrogen mustard on amino acid incorporation by thymus cell fractions*

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NITROGEN mustards have been shown to alkylate nucleic acids and proteins and to inhibit protein synthesis. Studies of the mechanism of action of alkylating agents have been reviewed by Wheeler[1] and Brown.[2] Brookes and Lawley have studied the alkylation and subsequent changes in structure of DNA.[3] Rutman *et al.*[4] have discussed the alkylation of protein, DNA, RNA, and lipid constituents of the cell. However, the mechanism of inhibition of protein synthesis is not known, nor whether the inhibition is a primary or secondary effect.

In a previous report the effect *in vitro* of whole-body X-irradiation on amino acid incorporation by isolated thymus nuclei and cytoplasmic fractions was described.[5] Incorporation by the intact nuclei was inhibited at 4 hr, but the isolated cytoplasmic ribosomal fractions showed no inhibition of amino acid incorporation until 6 hr after irradiation. It was also demonstrated that an early manifestation of nuclear damage was the presence of DNA material in the isolated cytoplasmic fractions.[5, 6] The presence of DNA material in the cytoplasmic fractions after treatment with a nitrogen mustard was also reported.[5] The effects of a nitrogen mustard on the incorporation of amino acid by the thymus nuclei and cytoplasmic fractions have been studied. A comparison of the effects of X-irradiation and a radiomimetic agent on these systems was made in order to attempt to distinguish a similar or dissimilar mode of action.

In all experiments, male rabbits 10 to 12 weeks old were used. Treated animals received an intravenous injection of the nitrogen mustard, Mustargen [methyl-bis(β-chloroethyl)amine hydrochloride; Merck Sharp and Dohme]. Mustargen was dissolved in 0·04 M sodium phosphate buffer, pH 7·4, at a concentration of 4 mg/ml. Injections were given within 1 min of dissolving the mustard. Final pH of the solution was 6·6. Animals were given 4 mg Mustargen/kg body weight. Control animals were injected with buffer containing 40 mg NaCl/ml to make it comparable to the concentration of NaCl in the dissolved Mustargen preparation.

At 4, 6, 8, 12, and 18 hr after treatment, control and treated animals were decapitated and the thymuses removed and chilled. All further operations were carried out at 4°. The thymuses from four to six animals were pooled in the respective groups. All experiments have been repeated a minimum of three times with essentially the same results.

The nuclei, cytoplasmic microsomal and ribosomal fractions, and 5·1P fraction were prepared as described previously.[5, 7] Conditions for incorporation *in vitro* of leucine-1-^{14}C by the various fractions were those used in previous studies.[5, 7]

Incubations were terminated by addition of an equal volume of 10% perchloric acid. The precipitates were washed with cold and hot perchloric acid, ethanol, and ether. Protein residues were plated on tared planchets and counted in a thin-window, low-background counter, for a sufficient time to obtain 5 per cent statistical accuracy. All calculations are corrected for both self-absorption and counting efficiency.

Fractions for nucleic acid analysis were precipitated and washed with cold 4% perchloric acid, 95% ethanol, absolute ethanol, and ethyl ether. The RNA and DNA content of the dried protein–nucleic acid residues was determined by using the methods of Webb and Levy.[8, 9]

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The results of the effects of nitrogen mustard injection on incorporation *in vitro* of labeled leucine by the thymus fractions at various times after administration are summarized in Table 1. Per cent inhibition was calculated by taking the ratio of the difference between control and treated specific activities over the control value. Inhibition of incorporation into the intact nuclei is evident at 4 hr with an increase to 72 per cent inhibition by 18 hr after treatment.

TABLE 1. EFFECT OF NITROGEN MUSTARD ON INCORPORATION *in vitro* OF LEUCINE-1-^{14}C INTO THYMUS CELL FRACTIONS

Fraction	Hours after treatment				
	4	6	8	12	18
Nuclei	24	22	31	40	72
Microsomal	none	40	55	55	85
Ribosomal	none	none	29	50	85

Results expressed as per cent inhibition.

At 4 hr after injection of nitrogen mustard, no inhibition of amino acid incorporation by the isolated cytoplasmic microsomal or ribosomal fractions is seen. At 6 hr after treatment, inhibition is seen with the microsomal but not the ribosomal fraction. By 8 hr, incorporation by both fractions is inhibited, with practically complete loss of biological activity by 18 hr after injection.

Previous work on effects of X-irradiation on these fractions had shown the presence of DNA (or nucleohistone) breakdown products in the microsomal and 5·1P fractions from treated tissue.[5] Similar observations are seen after treatment of the animals with the nitrogen mustard (Table 2). The

TABLE 2. EFFECT OF MUSTARGEN ON DNA CONTENT OF CYTOPLASMIC FRACTIONS

Fraction	Hours after treatment					
	0	4	6	8	12	18
Microsome	0·86	2·0	7·1	13·2	32	2·1
5·1P	0·84	1·2	5·0	12·4	20	5·5

Results expressed as per cent dry weight.

data demonstrate increasing contamination of these fractions with the DNA material with increasing time after treatment. By 18 hr there is a decrease which probably reflects the clearing of cell debris that takes place at later times.

As was shown previously in the X-irradiation studies,[5] the DNA found in the microsomal fraction is removed by the deoxycholate treatment in the preparation of the ribosomal fraction. It is evident that the inhibition of incorporation noted with the microsomal fraction at 6 hr is only an apparent inhibition, because of the inert material found in the fraction after treatment. This material is removed by the deoxycholate treatment, and no inhibition is noted when incorporation is carried out with the ribosomal fraction. Data obtained from a typical experiment at 6 hr after treatment are shown in Table 3.

As found in the X-irradiation studies,[5] when the specific activities of SRNA were calculated from data obtained by colorimetric analysis for RNA concentrations rather than by ultraviolet absorption, no significant effect was found on labeling of SRNA. Values obtained for RNA concentration based on ultraviolet absorption also reflect the DNA that is found in the 5·1P fraction from treated tissue.

The studies reported here for the nitrogen mustard and the earlier studies of X-irradiation[5] indicate that the ability of thymus nuclei to incorporate amino acids is affected before any change is noted in the ability of isolated cytoplasmic fractions to incorporate labeled amino acid. Results with both agents also indicate gross changes in nuclear structure with labilization of the structure of the deoxyribonucleoprotein. Alkylation of DNA, RNA, protein, and lipid of cells[3, 4] have been observed.

The data reported here would indicate that alkylation results in disruption of nuclear structure and inhibition of amino acid incorporation. At early times, when nuclear structure and function are affected, no inhibition of biological activity of the cytoplasmic ribosomal fraction is noted. It therefore appears that the structure of the biologically active ribonucleoprotein particles is not altered by

TABLE 3. EFFECT OF MUSTARGEN ON INCORPORATION OF LEUCINE-1-^{14}C INTO THYMUS CELL FRACTIONS AT 6 HOURS AFTER TREATMENT

Fraction	Control	Treated
	(counts/min/mg protein residue	
Nuclei	1910	1510
Microsomal	58	32
Ribosomal	77	81

alkylation. Whether the effect of alkylating agents on nuclear structure and function is a primary result of alkylation of the DNA is still to be determined.

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