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EFFECTS OF NITROGEN MUSTARDS ON THE INCORPORATION OF AMINO ACIDS INTO THE PROTEINS OF TUMORS AND OTHER TISSUES; THE MUSTARD DERIVATIVE OF SERINE*

WILLIAM L. NYHAN† and EVANNE LOH HOEHN-SARIC‡

Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, Md., U.S.A.

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Abstract—The effects of O-[N-di-(2-chloroethyl)carbamoyl]-DL-serine, a nitrogen mustard derivative of serine, on the incorporation of amino acids into proteins have been studied in rats bearing the Walker 256 carcinosarcoma. The intraperitoneal injection of 1,500 mg of the compound/kg regularly induced carcinostasis and occasionally produced complete regression of the tumor, while producing minimal systemic toxicity. These effects were accompanied by an inhibition of the incorporation of amino acids into the protein of the tumor. The degree of inhibition was always greater in the nuclear proteins than in the cytoplasmic proteins, suggesting a predominantly antinuclear action which is consistent with biologic information about alkylating agents. It is proposed that these findings are related to the mechanism of action of these compounds. The inhibition observed after administration of serine mustard occurred only in the tumor, while other mustards such as HN2 and uracil mustard have been shown to inhibit these processes in a variety of nontumor tissues. Serine mustard was as effective in the inhibition of the formation of proteins from methionine as from serine. These data suggest that the compound acts not as an analogue inhibitor but as an alkylating agent with altered tissue specificity.

RECENT experiments have indicated that certain alkylating agents are capable of inhibiting the incorporation of amino acids into the proteins of transplantable tumors *in vivo*.[1] A pattern of inhibition accompanied the carcinostatic effect induced by these agents, in which the labeling of nuclear proteins was suppressed to a greater degree than that of cytoplasmic proteins. These studies were carried out with methyl-*bis*-(2-chloroethyl)amine (HN2) and the mustard derivatives of alanine and phenylalanine[1] [N-*bis*-(2-chloroethyl)alanine and *p*-N-*bis*-(2-chloroethyl)-amino-L-phenylalanine], as well as the mustard derivative of uracil[2] [5-*bis*-(2-chloroethyl)aminouracil]. Inhibition was produced by the amino acid mustards only in the tumor. However, the administration of HN2 was followed by effects on a number of nontumor tissues, and uracil mustard induced pronounced inhibition of the incorporation of amino acids into the proteins of the spleen as well as of the tumor. These data have suggested that the use of amino acid and other molecules as carriers on which the active alkylating group is superimposed confers some change in tissue specificity. On the other hand, there

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was no evidence that the mustard derivatives of alanine or phenylalanine acted as amino acid analogue inhibitors, for they were as effective in inhibiting the incorporation of structurally unrelated amino acids, such as methionine or lysine, as of the corresponding amino acids, alanine or phenylalanine.

Nevertheless it seemed possible that other amino acid mustards might produce effects which were amino acid-specific, and the tissue specificity observed provided a stimulus for further exploration of the mustard derivatives of amino acids. These experiments were designed to investigate the effects of the mustard derivative of serine. This compound, O-[N-di-(2-chloroethyl)carbamoyl]-DL-serine, is an alkylating agent of novel structure, in which the amino acid is joined to the mustard moiety through the carbamoyl group. It provides a model for mustard derivatives of proteins bearing multiple *bis*-(2-chloroethyl) carbamoyl groups on the serine hydroxyls.[3] In these experiments carcinostasis was induced by a single intraperitoneal injection of the mustard in rats bearing the Walker 256 carcinosarcoma. At intervals thereafter the rats were injected with ^{35}S-labeled methionine or ^{14}C-labeled serine, and incorporation into the protein fractions of various tissues was studied. It was found that the mustard derivative of serine produced significant inhibition of the incorporation of amino acids into the protein of the tumor. Evidence was obtained that this compound, like the mustard derivatives of alanine and phenylalanine, acted not as an amino acid analogue but as an alkylating agent with altered tissue specificity.

MATERIALS AND METHODS

The animals used in these experiments were male rats obtained from the Charles River Breeding Laboratories (Charles River SD), weighing 180 to 320 g, and fed *ad libitum* on Purina laboratory chow. The Walker 256 carcinosarcoma was implanted subcutaneously 5 to 10 days prior to the experiment. The specific activity of L-serine-U-^{14}C (Nuclear Chicago Corp.) was 3 to 6 mc/mmole; that of DL-methionine-^{35}S (Volk) was initially 40 mc/mmole, and appropriate corrections were made for isotopic decay. The instruments used for the measurement of radioactivity (Nuclear Chicago Corp.) record 1 μc as 7×10^5 cpm.

The O-[N-di-(2-chloroethyl)carbamoyl]-DL-serine was synthesized at the Chester Beatty Institute.*[4] This compound is unusual among nitrogen mustards for its extraordinarily low toxicity. Whereas the LD_{50} for HN2 in the rat is 1 mg/kg, the serine mustard is not toxic at 5,000 mg/kg, and 10,000 mg/kg provides an LD_{100}.[4] Similar data were obtained in this laboratory; however, extensive toxicity studies could not be carried out because of the large drain such doses put on available supplies of the compound. A single intraperitoneal dose of 1,500 mg/kg was employed in all experiments except those in which the effects of dose were under study. This dose appeared to be smaller than the 0·4 LD_{50} doses of HN2 and other mustards employed in previous experiments, but it regularly produced carcinostasis for a period of 8 to 16 days, the end point sought in the therapeutic considerations of previous experiments.[1] In some animals not used for metabolic studies complete regressions of tumor were observed, a result that has not been obtained in this laboratory with doses of HN2, alanine mustard, or phenylalanine mustard of this order of magnitude. The metabolic

* The compound was synthesized by F. Bergel and R. Wade,[3] and generously provided by Professor Bergel and Dr. J. L. Everett. Supplies of the compound were also kindly made available by Dr. Ronald B. Ross of the Cancer Chemotherapy National Service Center.

effects of the mustard were studied at intervals of 2, 5 and 15 days after its injection. The average size of the tumor employed in these experiments was 1 g. and each rat usually had four implants of such tumors. The experiment was designed to employ control tumors of the same size and appearance as those obtained after treatment; and since treatment produced carcinostasis throughout the period of study, it appeared appropriate to use as controls tumors studied at the time the treated animals were treated. This design was chosen rather than seeking controls 2 to 5 days later when the treated rats were studied for, with growth, these appear to be less appropriate controls, and alterations due to hemorrhage and necrosis begin to appear. Nevertheless, the growth rates of untreated tumors are linear during the period of study, and the rates of incorporation of a variety of amino acids into the proteins of the tumor have been found not to differ over 7 days studied. In each experiment the animals were injected intraperitoneally with 6 μc of the tracer per 100 g body weight. One hour thereafter the animals were killed; tissues were removed, homogenized, and separated into nuclear, mitochondrial, microsomal, and cytoplasmic supernatant fractions as previously described.[1] The histones were extracted from the nuclear fraction by stirring in 0·25 N HCl for 8 to 10 hr. The portion of the nuclear fraction insoluble in HCl was fractionated into alkali-soluble (HCl-2) and alkali-insoluble (HCl-1) proteins using 0·1 N NaOH.[1] The various fractions were precipitated with perchloric acid,[1] and the proteins were isolated, plated, and assayed for radioactivity.[5] In experiments with labeled serine there is a possibility, not present in the case of methionine or the other amino acids studied,[1] of incorporation into the purines and pyrimidines of the nucleic acids and acid-soluble nucleotides. This would not be expected to influence these data, for the method of extraction[1] in perchloric acid at room temperature extracts nucleotides and RNA and would be expected to convert much of the DNA to apurinic products. Nevertheless, the question has been investigated by extraction with hot trichloracetic acid of proteins obtained from rats given L-serine-U-^{14}C. No radioactivity was found in the extract of any fraction, indicating that the radioactivity assayed in the plated proteins was in protein only.

RESULTS

The incorporation of serine into proteins. The effects of the mustard derivative of serine on the incorporation of L-serine-U-^{14}C into the proteins of the Walker tumor are indicated in Fig. 1. Significant inhibition ($P < 0{\cdot}05$) was observed as early as 48 hr after the administration of the mustard. Kinetic studies indicated progressive decrease in specific activity and, by 5 days after injection, statistically significant ($P < 0{\cdot}01$ to $0{\cdot}05$) inhibition was demonstrated in the whole homogenate and in each of the fractions except that of the mitochondria. The pattern of inhibition was similar to that previously observed with other mustards. At 5 days the specific activities of the microsomes and mitochondria were 61 and 70% of control values, while those of the histones and the nuclear HCl-2 fraction were suppressed to 47 and 44% of the control values. The activity of the anionic nuclear proteins in the control system is also indicated by the fact that before treatment the specific activity of the HCl-2. fraction was exceeded only by that of the microsomes.

Treatment with the nitrogen mustard derivative of serine did not result in changes in the incorporation of serine into the proteins of the kidney, liver, bone marrow, or plasma (Table 1). Similar data were obtained in the spleen and pancreas.

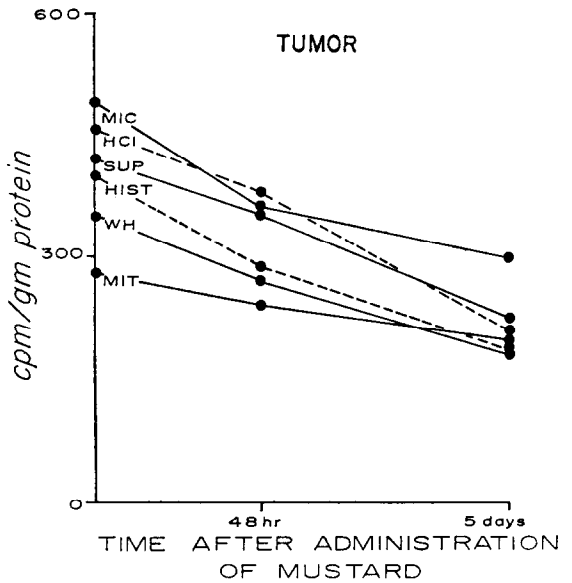

FIG. 1. Effects of serine mustard on the incorporation of L-serine-U-^{14}C into the proteins of the tumor. Abbreviations employed in this and subsequent figures include: WH, whole homogenate; MIT, mitochondria; MIC, microsomes; SUP, supernatant proteins; HIST, histones; HCl nuclear HCl-2 fraction. In all figures the data plotted are the means of three to six experiments; cf. Table 2.

TABLE 1. EFFECTS OF SERINE MUSTARD ON SERINE-U-^{14}C INCORPORATION INTO PROTEINS*

	Control	Treated (time after administration of mustard)	
		48 hr	5 days
	(cpm/mg)	(cpm/mg)	
Liver			
Whole homogenate	350 ± 51 (4)	328 ± 68 (3)	321 ± 57 (3)
Microsomes	697 ± 120 (4)	635 ± 114 (3)	592 ± 143 (3)
Mitochondria	333 ± 27 (3)	371 ± 107 (3)	344 ± 127 (3)
Supernatant	261 ± 16 (4)	266 ± 28 (3)	237 ± 63 (3)
Nuclear HCl-2	274 ± 23 (4)	230 ± 64 (3)	281 ± 97 (3)
Histones	235 ± 33 (4)	227 ± 45 (3)	231 ± 51 (3)
Kidney			
Whole homogenate	157 ± 6 (4)	170 ± 12 (3)	151 ± 22 (3)
Microsomes	227 ± 8 (4)	279 ± 23 (3)	191 ± 13 (3)
Mitochondria	154 ± 4 (4)	229 ± 57 (3)	139 ± 7 (3)
Supernatant	195 ± 12 (4)	243 ± 23 (3)	163 ± 12 (3)
Nuclear HCl-2	130 ± 4 (4)	137 ± 6 (3)	109 ± 8 (3)
Histones	119 ± 7 (4)	124 ± 3 (3)	104 ± 10 (3)
Marrow	406 ± 54 (4)	594 ± 40 (4)	432 ± 44 (3)
Plasma	480 ± 86 (4)	559 ± 118 (4)	429 ± 91 (3)

* In this table and in Table 2 the data presented are the mean specific activities of the proteins ± the standard error. The number of animals is shown in parentheses.

The incorporation of methionine into proteins. The effects of serine mustard on the incorporation of ^{35}S-labeled methionine into tissue proteins are indicated in Table 2, and the kinetics of the inhibition process are illustrated through 15 days in Fig. 2. In the tumor the specific activity of the nuclear HCl-2 fraction before treatment was

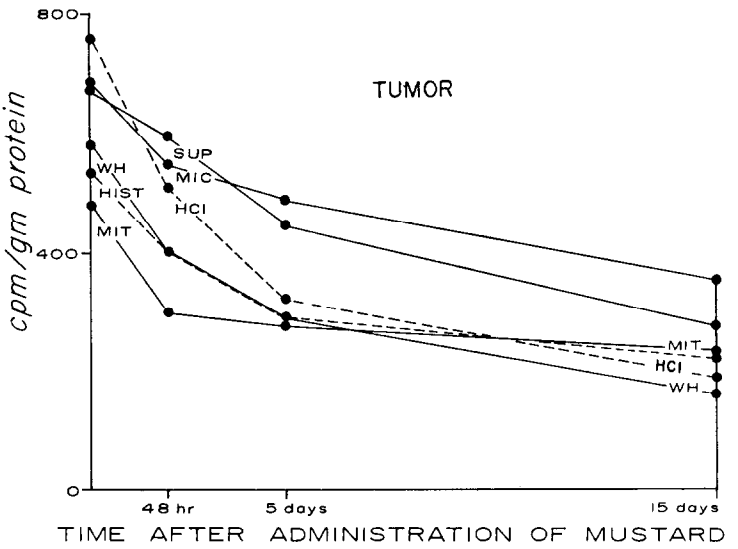

FIG. 2. Effects of serine mustard on ^{35}S-methionine incorporation.

TABLE 2. EFFECTS OF SERINE MUSTARD ON ^{35}S-METHIONINE INCORPORATION INTO PROTEINS

	Control (cpm/mg)	Treated (time after administration of mustard) 48 hr (cpm/mg)	5 days (cpm/mg)
Tumor			
Whole homogenate	581 ± 38 (5)	401 ± 52 (3)*	294 ± 22 (6)‡
Microsomes	680 ± 58 (5)	544 ± 107 (3)	489 ± 59 (6)
Mitochondria	481 ± 59 (6)	299 ± 37 (3)*	275 ± 30 (5)*
Supernatant	668 ± 55 (5)	597 ± 46 (3)	449 ± 40 (6)*
Nuclear HCl	756 ± 66 (6)	507 ± 83 (3)*	321 ± 40 (6)‡
Histones	530 ± 46 (6)	403 ± 38 (3)*	290 ± 30 (6)†
Liver			
Whole homogenate	492 ± 37 (6)	729 ± 61 (3)*	636 ± 55 (4)
Microsomes	984 ± 77 (6)	1,276 ± 88 (3)*	1,239 ± 98 (4)
Mitochondria	461 ± 57 (6)	665 ± 75 (3)	502 ± 28 (4)
Supernatant	387 ± 32 (6)	636 ± 102 (3)	474 ± 30 (4)
Nuclear HCl	427 ± 43 (3)	888 ± 276 (3)	420 ± 26 (4)
Histones	294 ± 25 (6)	468 ± 85 (3)	383 ± 22 (4)
Kidney			
Whole homogenate	614 ± 43 (6)	610 ± 107 (3)	606 ± 57 (6)
Microsomes	879 ± 84 (6)	978 ± 123 (3)	937 ± 70 (6)
Mitochondria	623 ± 90 (6)	672 ± 103 (3)	562 ± 45 (6)
Supernatant	620 ± 43 (6)	699 ± 48 (3)	720 ± 62 (6)
Nuclear HCl	518 ± 44 (5)	559 ± 95 (3)	521 ± 50 (6)
Histones	390 ± 43 (6)	501 ± 46 (3)	402 ± 44 (6)
Marrow	524 ± 48 (6)	773 ± 244 (3)	655 ± 60 (6)
Plasma	582 ± 76 (6)	745 ± 47 (3)	638 ± 82 (5)

These data were compared (t test)
* $P < 0.5$; † $P < 0.01$; ‡ $P < 0.001$

greater than that of any other tissue fraction. The rapid fall in the specific activity of the proteins of this fraction after treatment was particularly striking. As in the case when serine was the tracer, significant inhibition could be demonstrated in some fractions within 48 hr of the administration of the mustard. In most fractions these changes were progressive over the next three days, so that by 5 days the specific activities of the proteins of the nuclear HCl-2 fraction and the whole homogenate were even more strikingly different from control values ($P < 0{\cdot}001$) than in the experiments with ^{14}C-labeled serine. Thus, there was no evidence that the inhibition observed was directed against the metabolism of serine with any specificity.* The pattern of inhibition observed was that of more prominent effects in nuclear than in cytoplasmic proteins

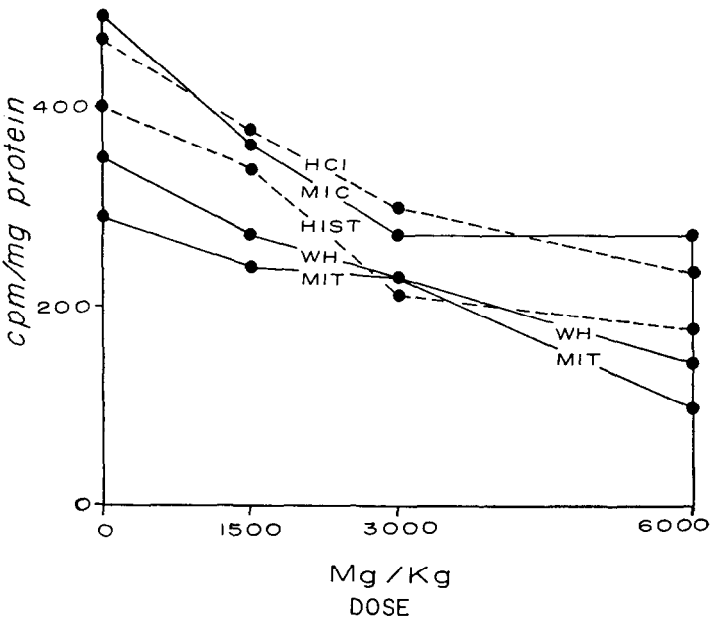

FIG. 3. Serine mustard dose response.

The rate of change over the next 10 days was slower, and by this time the differences between the specific activities of cytoplasmic and nuclear proteins were less striking, possibly reflecting the regression of tumor apparent by this time. At 15 days the specific activities of all the proteins of the tumor were suppressed to at least 50% of control values, and the specific activity of the nuclear HCl-2 fraction was 25% of control. Among the nontumor tissues (Table 2) inhibition was not observed. An increase in

* Biologic experiments have been carried out with the assistance of Dr. Hans Helge in order to investigate further the possibility of amino acid analogue effects in the action of serine mustard. In these experiments attempts have been made to antagonize the antitumor effect of serine mustard by the administration of serine. Rats bearing well-established Walker tumors were pretreated with serine, by the administration of 250 mg/kg intraperitoneally twice a day. Then they were given 1,200 mg serine mustard/kg and 250 mg serine/kg simultaneously. Thereafter they received 250 mg/kg twice a day on alternate days for six doses. Three similar groups of rats, were untreated controls and received the mustard only or received the serine only. With doses of serine higher than this rats died with ascites, large edematous kidneys, and increased organ weight in the liver, heart, lungs and kidneys.[8] The animals were weighed, and their tumors were measured with calipers every 2 days. The administration of serine was ineffective in altering either the toxic effects of the compound, as indicated by loss of weight, or its effects in inhibiting the growth of the tumor. These data were interpreted as confirming the biochemical experiments in indicating the absence of analogue activity.

the specific activities of proteins of the liver was noted, particularly in the earlier experiments. Similar results have been obtained in experiments with cyclophosphamide[6] and in studies of the incorporation of uracil-2-^{14}C into RNA in animals treated with uracil mustard.[7]

Dose response data for serine mustard were obtained with ^{35}S-labeled methionine (Fig. 3). There was some question whether all of the highest dose was absorbed from the peritoneum by the time the animals were killed at 48 hr after injection. However, the decline in specific activity with increasing dose was essentially linear.

DISCUSSION

Elucidation of the mechanism of action of the alkylating agents is complicated by the very high reactivity of these compounds. They react readily with a wide variety of compounds found in biologic systems,[9] and in suitable doses they produce a variety of pharmacologic effects.[10] Many of the early experiments on the biochemical effects of mustards reflect the use of very high concentrations *in vitro*. On the other hand, studies of the biological effects of these agents, such as those of Friedenwald and Buschke[11] and Koller and Casarini,[12] clearly showed that cytologic abnormalities occurred in the nuclei of dividing cells in the presence of concentrations of mustards considerably lower than the LD_{50} range, which had no effects on the respiration or glycolysis of these cells. In relating biochemical effects produced by these compounds to the mechanisms of biologic action, this predominantly antinuclear activity should be reflected, and effects should be demonstrable *in vivo* at low doses. The compound used in these experiments is remarkable for its extremely low toxicity and the absence of measurable effects on nontumor tissues even in the presence of doses that cause complete regression of the tumor. It is evident that the antitumor effect of the serine mustard is associated with a pronounced inhibition of the incorporation of amino acids into the protein of the tumor. Furthermore, the suppression of protein labeling which followed treatment was more pronounced in the nuclear proteins than in those of the cytoplasmic fractions. These data are consistent with those obtained with other mustards. In studies with phenylalanine mustard[1] it was shown that the inhibition occurred considerably earlier in the anionic nuclear proteins (HCl-2) than in any other protein fraction. In addition, the development of resistance on the part of a tumor accompanies a loss of the effects of the agent on the incorporation of amino acids into tumor protein.[6] These observations indicate that the effects of the alkylating agents on the formation of proteins from amino acids are indeed related to the mechanisms of biologic action. The anionic proteins of the nucleus may be primary sites for the action of these compounds.

The recent demonstration by Brookes and Lawley[13] of the alkylation by sulfur mustard of the N-7 of guanine has provided direct evidence of the reaction of mustards with nucleic acids in biologic systems. Steele[14] on the other hand, has presented evidence that suggests cross alkylation between nucleic acids and proteins. Such an effect or a direct alkylation of nuclear proteins, might secondarily alter the synthesis of nucleic acids. Alternatively, an altered alkylated nucleic acid might affect the synthesis of proteins.

The degree of inhibition observed after the administration of the serine mustard was quite similar whether the process was studied with labeled serine or labeled methionine. It was concluded that this compound, like the derivatives of alanine and

phenylalanine, was not acting as an amino acid analogue inhibitor. This conclusion was supported by biologic experiments in which the administration of serine did not protect the tumor against the serine mustard. The effects of serine mustard were also consistent with those of alanine and phenylalanine mustards in producing inhibition only in the tumor. Such evidence of tissue specificity has not been seen with HN2 or with uracil mustard. It is suggested that the use of the amino acid molecule as a carrier for the mustard group is responsible for an alteration in tissue specificity, which could represent differences in permeability or differences of metabolism among the tissues exposed. Further exploration of the amino acid as a carrier for cytotoxic groups, particularly in the form of larger molecules, peptides, and proteins[4, 5] may yield agents of heightened specificity and clinical utility.

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