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EFFECTS OF 4(5)-(3,3-DIMETHYL-1-TRIAZENO)-IMIDAZOLE-5(4)-CARBOXAMIDE (NSC 45388) IN PROLIFERATING RAT TISSUES*

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Abstract—Some effects of 4(5)-(3,3-dimethyl-1-triazeno)imidazole-5(4)-carboxamide (DIC) on normal proliferating tissues in rats were described. DIC caused marked but transient inhibition of labeled thymidine incorporation into DNA and presumably DNA synthesis of thymus, regenerating liver, spleen and small intestine. The inhibition was reversible and its duration dose dependent. When incorporation of precursors into DNA was markedly inhibited, no consistent concomitant effect on incorporation of precursors into RNA or protein was observed. Karyorrhectic cells appeared in the intestinal crypts as well as signs of necrosis in the thymus after administration of 125 mg/kg of DIC, but no significant pathologic changes were found in the spleen or regenerating liver. The relationship between selective inhibition of DNA synthesis and cytotoxic effect was observed.

4(5)-(3,3-DIMETHYL-1-triazeno)imidazole-5(4)-carboxamide (NSC 45388, DIC) was one of a number of disubstituted triazenoimidazole derivatives shown to have antitumor activity in mice[1–5] and immunosuppressive activity in rats.[6] DIC has since demonstrated significant antitumor activity against various human neoplasms[7–10] with a more pronounced effect on malignant melanoma.[8–14]

The clinical pharmacology of DIC in man was reported.[14,15] The metabolism of DIC in man and rats was studied. Illustration of a known pathway of DIC metabolism was presented by Skibba and Bryan.[16] DIC undergoes *N*-demethylation to 4(5)-(3-methyl-1-triazeno)imidazole-5(4)-carboxamide (MIC) in rats and man.[17,18] MIC spontaneously decomposes at physiologic pH to yield 4-aminoimidazole-5-carboxamide (AIC) and a possible methylating intermediate.[19,20] After the administration of DIC to patients with cancer, a pronounced increase in the urinary excretion of AIC was observed.[21–23] AIC was identified as a metabolite of DIC through the use of DIC-2-^{14}C.[23] Methylation of nucleic acids of rats and urinary excretion of ^{14}C-labeled 7-methylguanine by rats and man after administration of ^{14}C-methyl-DIC was reported.[16] *N*-dealkylation of 1-aryl-3,3-dialkyltriazenes was described by Preussmann *et al.*[24,25] and suggested as a mechanism for the conversion of these triazeno compounds to alkylating intermediates to explain their carcinogenic activity. DIC has since been found to be a very potent carcinogen for the rat.[26]

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The mechanism for the antitumor activity of DIC remains unknown. DIC was synthesized because of the instability of the parent compound, 4(5)-diazoimidazole-5(4)-carboxamide (Diazo-ICA), which was shown to have antitumor activity.[1,2] DIC was proposed to act as a latent form of Diazo-ICA.[3] Studies by Hano *et al.*,[20] Yamamoto,[27] and Saunders and Schultz[28] showed that an active form of DIC and other triazeno compounds for cytotoxicity *in vitro*[20] and antibacterial activity[27,28] was Diazo-ICA. The monomethyl derivative, MIC, has shown cytotoxic activity *in vitro*[20] and antitumor activity in mice.[19] Metabolic activation would then be a probable requirement for the activity of DIC.

The present communication reports the effect of DIC on nucleic acid and protein synthesis and its relationship to cytoxicity in proliferating rat tissues.

MATERIALS AND METHODS

DIC was provided by the Clinical Branch, Collaborative Research, Nationa Cancer Institute, U.S. Public Health Service, in vials suitable for human use. [2-^{14}C]-Thymidine (51·8 mc/m-mole), [6-^{14}C]-orotic acid (36·5 mc/m-mole), and L-[^{14}C]-leucine (316 mc/m-mole) (random labeled) purchased from Schwarz Bioresearch, Inc., Orangeburg, N.Y., served as precursor materials and indicators of DNA, RNA and protein synthesis respectively.

Male Sprague–Dawley rats, 190–200 g (Sprague–Dawley Co, Madison, Wis.) underwent partial hepatectomy[29] 24 hr prior to i.p. administration of DIC (7·5 or 25 mg). Radioactive precursor (5 μc) was administered, i.p., 20 min before sacrifice by decapitation under ether anesthesia. The liver, spleen, thymus and distal 4–6 cm of the small intestine were immediately excised, washed in ice cold isotonic saline and extracted for DNA, RNA and protein according to Munro[30] except that the final DNA extract was obtained by heating in 0·5 N perchloric acid at 90° for 20 min. DNA was assayed by the diphenylamine method of Burton,[31] RNA as given by Munro[30] and protein by the method of Lowry *et al.*[32] Aliquots of the final hydrolyzed radioactive samples were counted in ANPO* as previously described.[33] The same tissues were obtained for study by light microscopy at 2, 4, 6, 8, 12 and 24 hr after injection of 7·5 or 25 mg of DIC. Tissues were processed and stained with hematoxylin and eosin as described.[34]

The dosages of DIC utilized in this study were selected on the basis of the data of Freireich *et al.*[35] comparing the toxicity of anti-cancer agents in various animals and man. A dose of 7·5 mg in the rat approximated the dose employed in clinical trials,[5,9] whereas a human dose equivalent to the 25-mg rat dose would be too high for human use.

RESULTS

Incorporation of DNA, RNA and protein precursors. At 1 hr after injection of DIC (7·5 or 25 mg), the incorporation of thymidine-2-^{14}C into DNA of regenerating liver, spleen, small intestine or thymus was depressed to $<$ 10 per cent of controls (Figs. 1 and 2). Recovery to $>$ 50 per cent of controls occurred within 6 hr in the group receiving 7·5 mg (Fig. 1), whereas the depression in the group receiving 25 mg was longer lasting (Fig. 2).

* ANPO: a medium containing 259·2 g naphthalene, 18·4 g of 2,5-diphenyloxazole, 0·1839 g of α-naphthylphenyloxazole, 1400 ml of xylene, 1400 ml of dioxane and 840 ml of ethanol.

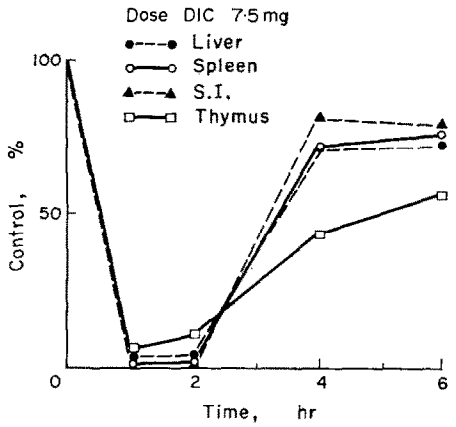

FIG. 1. Effect of DIC on incorporation of the thymidine into DNA as per cent of control specific activity. (Ave. control specific activities of all experimental points for each tissue DNA in dis./min per milligram were: liver, 9600; spleen, 4400; small intestine 14,800 and thymus, 340.) Each point represents the average of two pairs of animals, each pair being a separate experiment with a control and treated rat. The points are plotted at the time of killing, 20 min after injection of labeled precursor. The abbreviations used in Fig. 1 and the other figures are: Tdr, thymidine; S.I., small intestine.

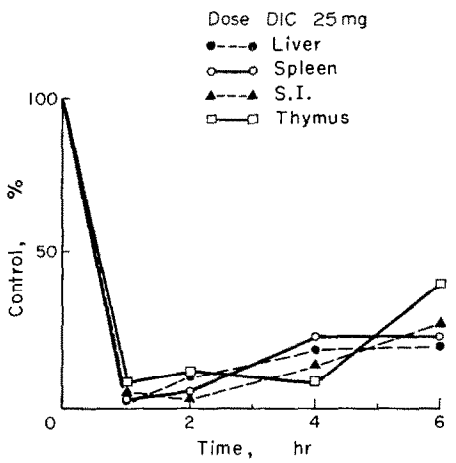

FIG. 2. Effects of DIC on the incorporation of thymidine into DNA. Each point was plotted as in Fig. 1 with the same representation. (Ave. control specific activities in dis./min per milligram were: liver, 7900; spleen, 3600; S.I., 13,600 and thymus, 290.)

Incorporation of ^{14}C-orotic acid into RNA after injection of DIC showed somewhat different results (Figs. 3 and 4). After administration of 25 mg of DIC, no consistent effect of DIC on RNA synthesis in the four proliferating tissues was apparent (Fig. 3), although transient inhibition occurred in each tissue studied. At 1 hr after injection of 7·5 mg of DIC, ^{14}C-orotic acid uptake into thymus decreased to < 60 per cent of control, but rapid recovery ensued (Fig. 4).

At 1 hr after injection of 25 mg of DIC, the incorporation of ^{14}C-leucine into protein was depressed to < 40 per cent in the small intestine and < 50 per cent in the

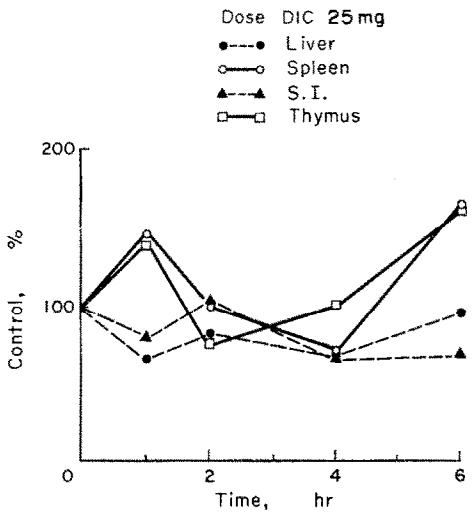

FIG. 3. Effects of DIC on the incorporation of orotic acid into RNA. Each point was plotted as in Fig. 1 with the same representation. (Ave. control specific activities in dis./min per milligram were: liver, 64,500; spleen, 600; S.I., 500 and thymus, 350.)

thymus, with the small intestine showing rapid recovery and the thymus recovering only slowly (Fig. 5). There was also transient inhibition of protein synthesis in regenerating liver and spleen (Fig. 5). After administration of 7·5 mg DIC, there was no consistent effect on the uptake of ^{14}C-leucine by these proliferating tissues (Fig. 6), except that during the 6-hr study period protein synthesis decreased in each tissue with the nadirs occurring at different times. The greatest decrease from that of normals occurred in the spleen and small intestine.

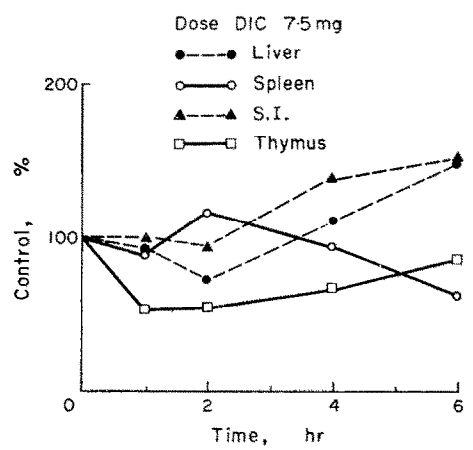

FIG. 4. Effects of DIC on the incorporation of orotic acid into RNA. Each point was plotted as in Fig. 1 with the same representation. (Ave. control specific activities in dis./min per milligram were: liver, 127,800; spleen, 1100; S.I., 700 and thymus, 1020.)

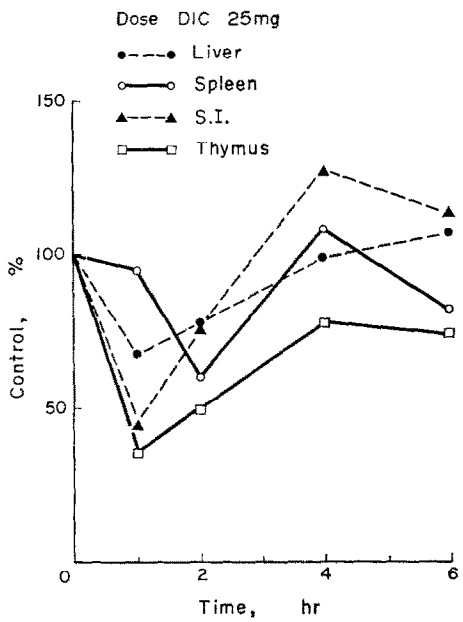

FIG. 5. Effects of DIC on the incorporation of leucine into protein. Each point was plotted as in Fig. 1 with the same representation. (Ave. control specific activities in dis./min per milligram were: liver, 1600; spleen, 1020; S.I., 1350 and thymus, 650.)

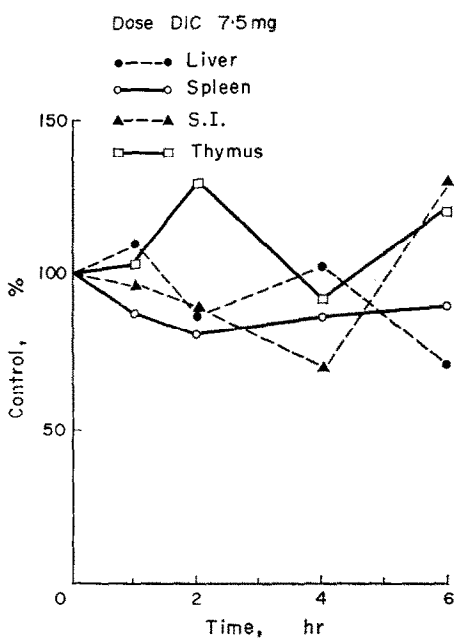

FIG. 6. Effects of DIC on the incorporation of leucine into protein. Each point was plotted as in Fig. 1 with the same representation. (Ave. control specific activities in dis./min per milligram were: liver, 2100; spleen, 1200; S.I., 2200 and thymus, 390.)

Comparing all tissues and all three precursor experiments at both dosage levels, one consistent effect of DIC appears to be that it depresses incorporation of ^{14}C-thymidine into DNA.

Lesions in proliferating tissues. After a single dose of 25 mg of DIC, moderate cellular damage was observed in the crypt epithelium of the small intestine. The first changes were seen at 6 hr with a few karyorrhectic cells per crypt, but at 8 hr there was obvious karyorrhexis. Thereafter, dead cells steadily decreased in number, and by 24 hr the duodenal crypts appeared normal. Changes in the thymus after administration of 25 mg of DIC were seen at 4 and 6 hr and were characterized by granular basophilic particles which may represent nuclear debris due to necrobiotic changes. These changes were noted at 8 hr, but at 12 and 24 hr the thymus of the treated animal appeared normal. No significant pathologic changes were found in the cells of the spleen or regenerating liver. There was no evidence of cellular damage to the four tissues examined during the 24-hr period after injection of 7·5 mg of DIC.

DISCUSSION

The results described demonstrate that DIC *in vivo* inhibited thymidine uptake into DNA and presumably DNA synthesis in normal proliferating tissues of the rat such as thymus, regenerating liver, spleen and small intestine. When incorporation of precursors into DNA was markedly inhibited, no consistent concomitant effect on incorporation of precursors into RNA or protein was observed except for the transient inhibition of protein synthesis observed after administration of 25 mg of DIC (Fig. 6). DIC also induced necrosis in intestinal crypt epithelium and necrotic changes in the thymus. This represents the first report of a selective effect of DIC on DNA synthesis. The relationship between selective inhibition of DNA synthesis and cytotoxic effect was observed.

These effects of DIC *in vivo* are similar to the effects of Diazo-ICA *in vitro* on *Escherichia coli*.[27] Yamamoto[27] reported that Diazo-ICA primarily inhibited DNA synthesis but not RNA and protein synthesis in *E. coli*. Diazo-ICA, the immediate photodecomposition product of DIC, was found to be the active form of DIC which was inhibitory to *Bacillus subtilis*.[28] Studies *in vitro* utilizing leukemia L1210 cells showed that DIC adversely affected RNA and protein synthesis more than DNA biosynthesis and that *in vivo* the mean content of DNA per cell increased after exposure to DIC.[36] This effect *in vitro* was enhanced by light.[36] The mechanism whereby Diazo-ICA exerts its effect may be through its reaction with sulfhydryl groups in biological preparations.[37] Sulfhydryl compounds had a protective effect on bacteria exposed to Diazo-ICA.[27,28]

The findings presented are consistent with the studies on bacteria[27,28] and with cytotoxicity studies utilizing Ehrlich Ascites carcinoma cells,[20] but not with results *in vitro* utilizing L1210 cells.[36] This activity of DIC *in vivo* is not unlike that of hydroxyurea on regenerating liver.[38,39] Inhibition of growth of mammalian cells by hydroxyurea appears to be predominantly due to its capacity to interfere with the formation of DNA at concentrations of drug which produce little or no inhibition of the incorporation of precursors into RNA or protein.[40] Hydroxyurea has cell cycle activity.[40] It was demonstrated that DIC produced a first-order kinetic reduction in viable L1210 cells *in vitro* as measured by animal bioassay, a result presented as evidence against

any cell-cycle specific activity of DIC.[41] Peters and McGeer[42] demonstrated inhibition of *de novo* purine biosynthesis by diazo analogues of AIC similar to Diazo-ICA.

These seemingly conflicting data could be the result of the experimental cell system used or perhaps the results *in vivo* reported here stem from metabolic conversion of DIC to an active metabolite. DIC, MIC, and Diazo-ICA could all be affecting macromolecular synthesis together *in vivo*. Further studies *in vitro* designed to compare the effects of each of these chemicals on dividing cells are in progress.

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