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EFFECT OF STEROID HORMONE ON ACTIVATION OF ENDOXAN (CYCLOPHOSPHAMIDE)

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Abstract—An effect of steroid hormone on activation of Endoxan (cyclophosphamide) by the liver *in vitro* and *in vivo* is reported. When Endoxan was incubated with rat liver slices, indicated that the addition of prednisolone caused a definite inhibition of Endoxan activation. The *in vivo* experiments, in which serum concentrations of Endoxan after i.p. administrations in normal rats were determined by colorimetric method, demonstrated that the simultaneous administration of prednisolone with Endoxan prevented Endoxan activation *in vivo* and caused a significant reduction of the concentration ratios of active to inactive Endoxan in sera. The results obtained from these experiments indicate that the activation of Endoxan by the liver appears to be inhibited by steroid hormone (prednisolone).

ENDOXAN is a latent alkylating agent, which is almost inactive *in vitro*, and the agent becomes biologically active *in vivo* only on appropriate activation, which is thought to be accomplished by the liver.

Attention has been directed to the combined administration of various cytostatic agents in order to obtain good effects by chemotherapy of malignant tumors. Steroid hormones have antitumor effect and, alone or combined with the other cytostatic agents, are used for the treatment of malignant tumors, particularly leukaemia. The present investigation deals with the effect of steroid hormone on activation of Endoxan and was designed to elucidate the mechanism.

The following types of experiments were performed:

(1) The effect of prednisolone on activation of Endoxan by rat liver slices.
(2) The effect of prednisolone-pretreatment of liver slices on activation of Endoxan by the liver slices.
(3) The direct actions of prednisolone on active metabolites and inactive form of Endoxan.
(4) The effect of simultaneous administration of prednisolone on activation of Endoxan *in vivo*.

MATERIALS AND METHODS

Animals

Male Donryu rats, weighing 150–200 g, were employed in this study.

Analytical method:

The concentration of activated Endoxan was determined photometrically by using γ-(*p*-nitrobenzyl)pyridine (NBP) which measures alkylating capacity. This method is

an adaptation of that originally described by Epstein[1] for analyses of alkylating agents and later modified by Friedman[2]. Recently, Morita *et al.*[3] have extended the application of this method to the analysis of Endoxan and unmetabolized inactive form of Endoxan, which has no alkylating activity, was measured as well as active metabolites, which have alkylating activities. The details of the procedure are given in Fig. 1.

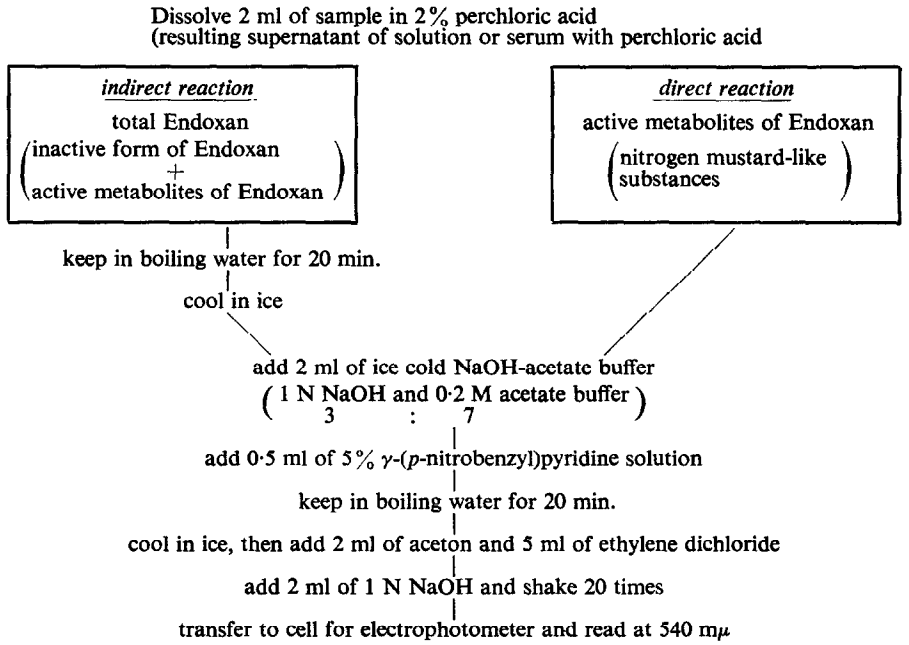

FIG. 1. Analytical procedures of Endoxan (Morita *et al.*[3])

Experimental procedures

(1) Endoxan was incubated with rat liver slices. The incubation system included 500 μg of Endoxan, 1·5 g (wet weight) of liver slices and 5 ml of Krebs–Ringer phosphate buffer solution (KRPB) (pH 7·4). The rat liver slices were prepared by Stadie–Riggs' slicer with 500 μ thickness. The temperature was 37° and the time of incubation 1 hr. After the incubation, the active metabolites of Endoxan (NBP-positive substances) were measured. The effect of prednisolone on activation of Endoxan by the liver was investigated by adding of several doses of prednisolone to the incubation mixture.

(2) After the liver slices were incubated with 500 μg of prednisolone and 5 ml of KRPB at 37° for 1 hr, they were washed gently 3 times with 5 ml of KRPB, and were reincubated with 500 μg of Endoxan and 5 ml of KRPB at 37° for 1 hr. The active metabolites of Endoxan were then measured. The control liver slices were preincubated in 5 ml of KRPB without prednisolone.

(3) KRPB solution, which contained the active metabolites and inactive form of Endoxan, was incubated with or without prednisolone 100 μg/ml with gentle shaking at 37°, and the changes in concentration of Endoxan (active metabolites and inactive form of Endoxan) were measured at intervals of 30 min.

(4) Endoxan was dissolved in 2% (w/v) isotonic sodium chloride. Prednisolone was dissolved in 1% (w/v) physiological saline. After Endoxan 100 mg/kg of body weight and prednisolone 100 mg/kg were simultaneously injected i.p., animals were sacrificed at varying intervals of posttreatment, and their blood was collected by exsanguination. The serum concentrations of Endoxan (active metabolites and inactive form of Endoxan) were measured and compared with those of the control animals, which were injected the Endoxan solutions and the physiologic saline solution without prednisolone.

RESULTS

The effects of prednisolone on activation of Endoxan by liver slices are illustrated in Fig. 2. Prednisolone inhibited the activation of Endoxan by the liver slices.

Preincubation of liver slices with prednisolone had inhibitory effect on the Endoxan activation by the liver slices, and the active metabolites of Endoxan were formed only 81·3 per cent of the control. The results indicate that prednisolone may inhibit Endoxan activation through the liver.

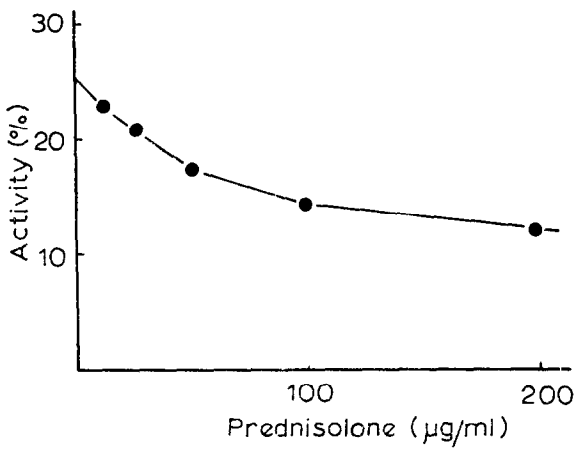

FIG. 2. Inhibitory effect of prednisolone on activation of Endoxan in male rat liver slices.

The decrease of Endoxan with time is almost linear for at least 1 hr (Fig. 3), and that of the active metabolites of Endoxan was more rapid than that of total Endoxan (active metabolites + inactive form of Endoxan). Addition of prednisolone had no effect on the rate. The result suggested that prednisolone has no direct action on the active metabolites and inactive form of Endoxan.

Fig. 4 shows the mean values of serum concentrations of Endoxan after intraperitoneal administrations. Serum concentration of total Endoxan, which was found to be high at 30 min after administration, decreased steeply for about 2 hr, and then fell off gradually. The concentration of active metabolites, which was lower initially, reached the highest level 1 hr after administration, and then decreased with time. The ratio of concentration of active metabolites/concentration of total Endoxan 1 hr after administration is 60–70 per cent.

Simultaneous intraperitoneal injection of prednisolone with Endoxan prevented the Endoxan activation. The activation rates of prednisolone-injected animals were

lower than the controls and the highest level of the concentration of the active metabolites in serum did not reach that of the controls. The decreases of the serum concentrations of active metabolites and total Endoxan, of the prednisolone-injected animals curved more slowly than the controls, and, after 2 hr, the serum concentrations of active metabolites and total Endoxan, were higher than the controls. The

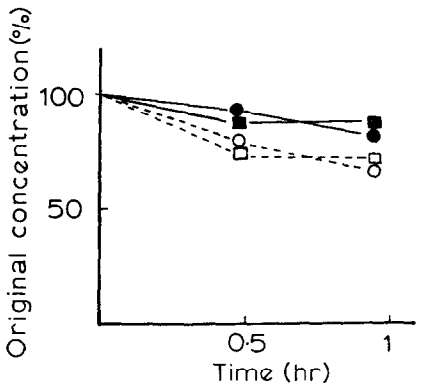

FIG. 3. Influence of predinisolone on concentration changes of Endoxan with time.

Total Endoxan ●—● Control
Active metabolites ○---○ Control
□---⊡ with prednisolone

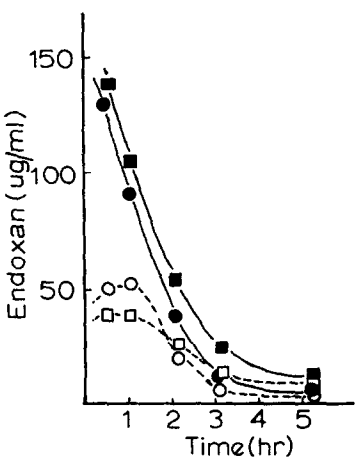

FIG. 4. Serum concentrations of Endoxan in normal and prednisolone-administered male rat. Endoxan and prednisolone were administered i.p. Each point is the average of five rats.

Total Endoxan ●—● Control
Active metabolites ○---○ Control
⊡---□ with prednisolone

influence of prednisolone on the absorption of Endoxan from the peritoneal cavity was thought to be negligible, for there was little difference between the initial serum concentrations of total Endoxan of the prednisolone-injected animals and those of the controls.

DISCUSSION

Endoxan was synthetized by Arnold, Bourseaux and Brock[5] as a model for an enzyme-activated antitumor agent following the observation that nitrogen mustard is essentially detoxified by *N*-phosphorylation. It shows little cytostatic activity *in vitro* and becomes biologically active only upon appropriate activation. Endoxan was believed to be activated in tumor cells by enzymatic hydrolysis, but there is no evidence for the presence of the necessary enzymes in tumor cells. Brock and Hohorst[6,7] reported Endoxan to be stable when incubated *in vitro* in suspensions of Yoshida sarcoma cells. Since Foley *et al.*[8] suggested that Endoxan is activated by the liver, the view has been widely held that Endoxan is activated primarily by the liver and not by tumor.[9] However, the exact mechanism of activation of Endoxan still remains obscure.

Recently, interest has developed in $NADPH_2$ dependent enzyme system in liver microsomes as the enzymes for Endoxan activation, since the demonstration by Brock and Hohorst[6,7] that Endoxan activation is carried out by liver microsomes in the presences of $NADPH_2$ and oxygen. Druckrey *et al.*[10] and Rauen *et al.*[11] observed that large doses of NBP-positive substances, which are regarded as active metabolites of Endoxan, were formed from Endoxan by incubation with Udenfriend's hydroxylase (oxygenase) model[12] in phosphate buffer (pH 7·3) at 37°. Several investigators have shown the induction of increased activity of microsomal drug-oxidizing enzymes of the liver by phenobarbital, and Rauen *et al.*[13] and Tochino *et al.*[14] demonstrated that pretreatment of rat with phenobarbital produced markedly increased blood levels of the alkylating activity of Endoxan.

The active metabolites of Endoxan may be produced from Endoxan by catabolic oxidation, and the $NADPH_2$ dependent drug-metabolizing (oxidizing) enzymes in the liver microsomes is intimately related to this reaction. SKF 535A, which inhibits microsomal drug-metabolizing enzymes, prevents activation of Endoxan. The liver of carp, which lacks this enzyme system, does not activate Endoxan.[7] Furthermore, Endoxan activation does not occur under anerobic condition, and the effects of oxygen tensions on Endoxan activation under atmospheric and hyperbaric conditions have been reported.[15] Orrenius *et al.*[16, 17] demonstrated that $NADPH_2$-cytochrome C reductase and the hemoprotein, known as the CO-binding pigment (P 450),are components of the enzyme system.

In recent years, evidence has been obtained that steroid hormones are normally involved in oxidative drug-metabolizing enzymes in liver microsomes and that drugs, like phenobarbital, which stimulate the microsomal oxidation, also stimulate the microsomal hydroxylation of steroids. Conney *et al.*[18] and Bernstein and Bhavnani[19] reported that treatment of guinea pigs with phenobarbital causes a marked increase in the activity of the enzyme system in liver microsomes that hydroxylates cortisol.

Our observations in this study clearly demonstrate that steroid hormone (prednisoline) inhibits Endoxan activation by the liver. Combined with previous reports, this suggests that the inhibition takes place at the level of $NADPH_2$ dependent enzyme system. The simplest interpretation of the inhibition seems to be that two processes, prednisolone-hydroxylation and Endoxan-activation, compete for the $NADPH_2$ dependent drug-metabolizing enzyme system. Orrenius *et al.*[16] demonstrated that drugs undergoing oxidative demethylation strongly inhibit $NADPH_2$ dependent peroxidation of lipids in microsomes, and suggested that the inhibition probably is

the result of a competition between the two processes for the $NADPH_2$ dependent enzyme system. Gerhardt and Lutzman[20] investigated the effects of Endoxan on the metabolism of cortisone in rat liver and postulated that Endoxan inhibits the catabolic metabolism of cortisone in the liver. Recently, Wada *et al.*[21] reported that steroid hormone inhibits the activity of microsomal drug-hydroxylation, e.g. hydroxylation of aniline and demethylation of aminopyrine.

But the relationship between Endoxan and the $NADPH_2$ dependent enzyme system may be more complicated. Gerhardt and Lutzman[20] reported that larger amounts of active metabolites of Endoxan inhibit the catabolic metabolism of cortisone in the liver more strongly. This indicates that the active metabolites of Endoxan have inhibitory effects on the activity of the $NADPH_2$ dependent enzyme system and it seems likely that the $NADPH_2$ dependent enzyme system not only activates Endoxan and produces the active metabolites, but also the activity of the enzyme system is inhibited by the active metabolites of Endoxan, and that there exists a negative feed back mechanism in Endoxan activation by the $NADPH_2$ dependent enzyme system in the liver. This relation is schematically illustrated in Fig. 5.

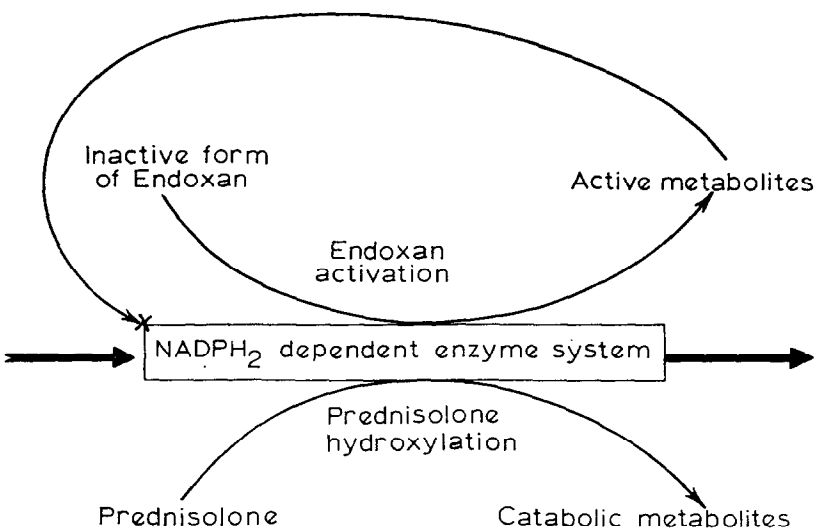

FIG. 5. Schematic representation of competition between Endoxan-activation and prednisolone-hydroxylation and of the relation between Endoxan-activation and $NADPH_2$ dependent drug-metabolizing enzyme system.

Recently, attention has been directed largely to the treatment of malignant tumor by combined administration of various cytostatic agents. Steroid hormone is used not only for the treatment of malignant tumor as an antitumor agent with or without combination of other cytostatic agents, but also for the treatment of agranulocytosis caused by chemotherapy. Furthermore, steroid hormone is often used for the treatment of brain oedema caused by chemotherapy of brain tumor. The results obtained in this study may have a bearing on the cytostatic activity by the combined administrations of Endoxan and steroid hormone for the treatment of malignant tumors.

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