IMMUNODEPRESSIVE EFFECT OF CYCLOPHOSPHAMIDE ACTIVATED *in vitro* BY LIVER MICROSOMES FROM MICE OF DIFFERENT LINES

L. Yu. Telegin, G. F. Zhirnov, A. V. Mazurov, and L. A. Pevnitskii

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In a previous paper [7] describing analysis of differences in sensitivity of mice of different lines to the immunodepressive action of cyclophosphamide (CP) *in vivo* the writers suggested that differences in the action of CP may depend on the character of metabolism of the compound in the liver of different lines of mice. CP is converted into its active form as a result of interaction with cytochrome P-450 — the terminal oxidase of the NADPH-dependent enzyme system of the membranes of the endoplasmic reticulum of liver cells [13]. As a result of the hydroxylation reaction an oxidized metabolite of CP is formed, namely 4-hydroxycyclophosphamide, with which the biological action of the compound is associated [11, 14]. It has also been shown [7] that the rate of oxidation of CP by liver microsomes differs in mice of different lines.

The object of this investigation was to study the immunodepressive action of cyclophosphamide when oxidized by liver microsomes of mice of different lines.

EXPERIMENTAL METHOD

Male CBA, BALB/c, and DBA/2 mice weighing 18-25 g were obtained from the Stolbovaya Nursery, Academy of Medical Sciences of the USSR. The CP used in the experiments was the Soviet preparation cyclophosphan. Microsomes were isolated from the mouse liver by differential centrifugation [3]. To remove blood present as an impurity, the microsomes were resuspended in isolation medium and resedimented by centrifugation under the same conditions (105,000g, 60 min, 4°C). The content of microsomal protein was determined by Lowry's method [16] in the presence of 0.15% sodium deoxycholate. The velocity of hydroxylation of CP was determined by polarography [10] on the LP-17 polarograph (Czechoslovakia) with a platinum electrode of closed type. The spectrophotometric measurements were made on an Aminco-DW-2 spectrophotometer (USA). Cytochrome P-450 was determined by the method in [17]. Binding spectra of CP with cytochrome P-450 were recorded as described previously [18]. Binding constants (K_S) and maximal amplitudes of spectral changes (ΔA_{max}) were determined from Lineweaver–Burk plots. Coefficients of linear regression equations describing these graphs were obtained on the HP 9830B computer (USA).

CP was activated *in vitro* in a Vibrotherm apparatus (Hungary) at 37°C. The incubation mixture contained (in 1 ml): 50 mM phosphate buffer, (pH 7.4), mouse liver microsomes equivalent to a protein content of 3 mg, 150 mM KCl, 10 mM $MgCl_2$, 0.6 mM EDTA, 5 mM CP. The reaction was initiated by the addition of 3 mM NADPH and was stopped by placing the samples on an ice bath and adding 4 ml of cold incubation medium.

To obtain supernatant containing active metabolites of CP the microsomes were sedimented at 105,000g for 1 h. The supernatants were used to determine their immunodepressive activity. For this purpose, a cell suspension was prepared from spleens of intact CNA mice in medium No. 199 with antibiotics 100 i.u. each and incubated for 1 h at 37°C in the presence of supernatant, previously sterilized by filtration through a millipore filter (pore diameter 0.45 μ). The incubation mixture contained 125 million spleen cells and 0.427 ml of supernatant in 1 ml. The cells were then washed once with medium No. 199, cooled to 4°C, and injected intravenously into syngeneic mice in a dose of 50 million together with 4×10^8

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TABLE 1. Rate of Hydroxylation of CP, K_s, ΔA_{max}, and Content of Cytochrome P-450 in Liver Microsomes of Mice of Different Lines (mean results of 5-7 experiments)

Line of mice	Rate of hydroxylation of Cl, nmoles O_2 absorbed/mg microsomal protein*	Cytochrome P-450 content, nmoles/mg microsomal protein	K_s, mM^2	ΔA_{max}, in optical density units/nmole cytochrome P-450†
BALB/c:				
NADPH	17,3±0,549	1,026±0,050	5,8	0,0098
CP	25,5±0,672			
CBA:				
NADPH	11,0±0,218	0,941±0,044	5,1	0,0050
CP	15,7±0,252			
DBA/2:				
NADPH	9,3±0,252	0,810±0,073	4,2	0,0069
CP	13,0±0,218			

*Values of rate of O_2 consumption after addition of NADPH and after addition of CP are given.
†Significance of differences ($P < 0.05$) determined by Wilcoxon's rank sum test.

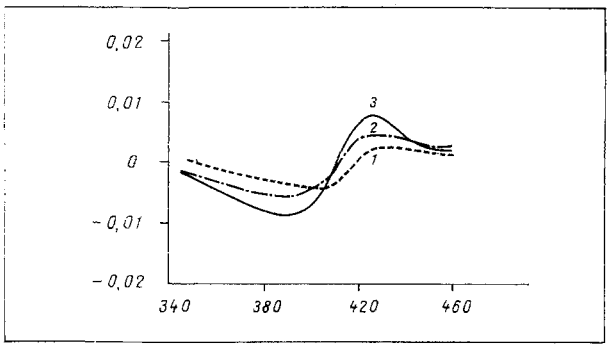

Fig. 1

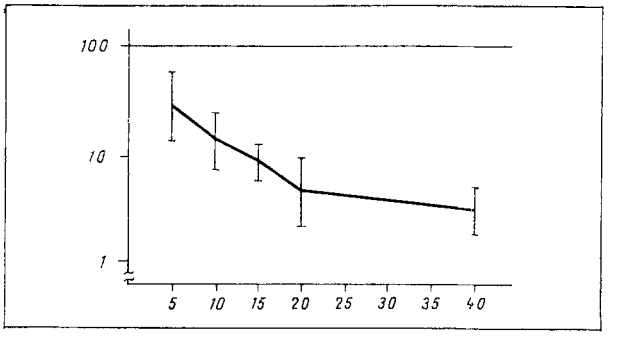

Fig. 2

Fig. 1. Binding spectra of CP with microsomes from BALB/c (3), DBA/2 (2), and CBA (1) mice. Abscissa, wavelength (in nm); ordinate, optical density units. Incubation mixture contained: 2 nmoles microsomal cytochrome P-450 (2-4 mg/ml microsomal protein), 50 mM Tris-HCl buffer, pH 7.4, and 7 mM CP.

Fig. 2. Dependence of immunodepressive action of CP activated by liver microsomes of BALB/c mice on duration of activation. Abscissa, duration of incubation of CP with microsomes and other components of activating mixture (in min); ordinate, number of antibody-forming cells in spleen of recipient mice (in percent of control).

sheep's erythrocytes. The recipient mice received CP in a dose of 200 mg/kg 3-4 h before injection of the cells in order to suppress their own immunoreactivity [1]. The number of 19S antibody-forming cells in the recipients' spleens was determined 5 days later by the local hemolysis in agar method [15]. The alkylating activity of the CP metabolites in the supernatant was determined by the NBP test [12], modified as described previously [4].

The significance of differences was determined by parametric and nonparametric statistical methods (Student's t test, Wilcoxon's rank sum test [2, 8]).

EXPERIMENTAL RESULTS

As Table 1 shows, the rate of hydroxylation of CP depends on the cytochrome P-450 content. In microsomes of BALB/c mice the rate of hydroxylation of CP and the cytochrome P-450 content were significantly higher than in CBA/2 mice ($P < 0.001$ and $P = 0.043$ respectively). CBA mice occupied an intermediate position with respect to these indices: The rate of hydroxylation of CP differed significantly from values recorded in mice of the other two lines, whereas there were no significant differences in the content of cytochrome P-450.

The next step was to determine whether correlation exists between the rate of hydroxylation of CP, the cytochrome P-450 content, and affinity of CP for cytochrome P-450 in microsomes of mice of different lines.

TABLE 2. Immunodepressive Action of CP, Activated by Liver Microsomes from Different Lines of Mice

Line of mice donating microsomes	Alkylating activity in supernatant, optical density, units/ml	Number of antibody-forming cells in recipients' spleen*	P
BALB/c	0,094	185—(67—515) $n=11$	0,424
CBA	0.070	302—(128—714) $n=12$	0,0014
DBA/2	0,052	900—(663—1219) $n=12$	
Control†	—	11270—(9488—13390) $n=12$	

*Geometric means, confidence intervals at $P < 0.05$.

†Normal spleen cells of CBA mice in medium No. 199 with antibiotics. Preliminary experiments showed that CP activation medium (supernatant after incubation of microsomes without CP but with the remaining components of the incubation mixture, supernatant after incubation of CP with the remaining components but without microsomes) did not affect the immune response of CBA mouse spleen cells.

It will be clear from Fig. 1 that CP, on binding with cytochrome P-450 of BALB/c and DBA/2 mice, induced spectral changes with a maximum and minimum at 424 and 388 nm respectively. The minimum for CBA mice was shifted to 405 nm. Spectral changes of this type (type II) are characteristic of binding spectra of nitrogen-containing compounds with cytochrome P-450 [18]. The values of spectral K_S and ΔA_{max} given in Table 1 are parameters of a Michaelis' equation describing dependence of ΔA_{max} on CP concentration. The values of K_S did not differ significantly, indicating equal affinity of CP for cytochrome P-450 from mice of different lines. The absence of correlation between affinity of CP for cytochrome P-450 and the rate of its hydroxylation is evidently due to the fact that substrate binding is a rapid reaction [5] and does not limit the overall velocity of the hydroxylation reactions.

Meanwhile the value of ΔA_{max} was significantly higher in BALB/c mice than in mice of the other two lines, in which ΔA_{max} was practically the same. The difference in the intensity of the spectral changes may be due to the different orientation of the binding site relative to the heme iron of cytochrome P-450. These differences evidently indicate the nonidentity of structure of the cytochromes P-450 in the microsomes of these lines of mice. It can accordingly be postulated that differences in the rate of hydroxylation of CP in the microsomes of mice of different lines are due not only to a difference in the content of cytochrome P-450, but also to differences in its structure.

Since the rate of oxidation of CP by microsomes of mice of different lines was not identical, it was suggested that the immunodepressive effect of CP oxidized *in vitro* would also differ. To detect interlinear differences in depression of the immune response, the time of oxidation of CP by microsomes at which comparative analysis could be carried out had to be chosen. The relationship between the immunodepressive effect and the duration of incubation of CP with liver microsomes from mice of one line (BALB/c) is shown in Fig. 2.

As Fig. 2 shows, after incubation for 20 min the accumulation of active products was so great that it caused strong inhibition of the immune response. With a further increase in the duration of incubation, depression of the immune response remained virtually unchanged, in good agreement with the results of measurement of alkylating activity in the corresponding supernatant. On the basis of these data 15 min was chosen as the incubation time of CP with microsomes from different lines of mice.

The results of experiments to study immunodepressive activity of supernatant from mice of different genotypes are given in Table 2. The lowest value of both these indices was characteristic of DBA/2 mice.

The results thus show that liver microsomes from BALB/c mice metabolize CP more effectively *in vitro* than those of DBA/2 mice. This corresponds to a more intensive accumulation of products capable of depressing the immune response of cells in adoptive transfer.

Despite the high metabolic activity of the microsomal apparatus of BALB/c mice in relation to CP, mice of this line are more resistant to the immunodepressive action of CP than mice of other lines [7]. This shows that the final effect of the action of the immunodepressant, namely depression of the immune response, is determined not only by the features of its activation, but also by subsequent events: the kinetics of entry of metabolites into the blood stream, their distribution in the body, the character of their interaction with target cells. These parameters differ in mice of different genotypes [6, 9], evidence of the multiplicity of levels of genetic control over the immunodepression process.

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