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PHARMACOKINETIC ASPECTS OF THE IMMUNODEPRESSIVE ACTION OF CYCLOPHOSPHAMIDE

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The alkylating and immunodepressive activity of the serum of CBA, BALB/c, and DBA/2 mice after administration of cyclophosphamide was studied. Interlinear differences were found in these parameters, but no direct correlation could be shown to exist between them. The DBA/2 mice, which were most sensitive to the immunodepressive action of cyclophosphamide, had the highest serum immunodepressive activity.

KEY WORDS: cyclophosphamide; metabolism; immunodepression; genotype.

A previous investigation [5] showed that mice of different lines are unequally sensitive to the immunodepressive action of cyclophosphamide (CP). All stages of the pharmacokinetic process determining the fate of a drug in the body are considered to "take place by means of specific and nonspecific enzymes whose synthesis is unquestionably under genetic control" [2]. It can therefore be tentatively suggested that differences found in the immunodepressive activity of CP are connected with differences in its pharmacokinetics in mice of different genotypes. This suggestion is supported by earlier observations [5] showing differences in the rate of oxidative hydroxylation of CP in mice of different lines.

CP is an alkylating agent but, unlike many other immunodepressants of this group, in the intact state it has virtually no cytotoxic activity. It owes its biological effect to the formation of active metabolites, produced as a result of activation of the substance by an NADPH-dependent enzyme system of the endoplasmic reticulum of the liver [10]. It was accordingly decided to study the alkylating and immunodepressive activity of the blood serum, i.e., indices reflecting the formation of active CP metabolites in the body, in mice of different lines.

EXPERIMENTAL METHOD

Male CBA, BALB/c, and DBA/2 mice weighing 18-25 g (from the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR) were used. The Soviet preparation cyclophosphan was used as CP. The alkylating activity of CP metabolites was determined in the blood serum of the mice at various times after intraperitoneal injection of CP. Each sample consisted of pooled sera from three mice. The NBP-test [9] by the method described previously [4], with slight modifications, was used for the determination. The immunodepressive activity of the serum was tested by a method developed by the writer for mice. CBA mice were sensitized intravenously with 10^6 sheep's red cells (SRBC) 7 days before the experiment. A cell suspension was prepared from the spleens of these mice in medium 199 with antibiotics (100 units penicillin and 100 units streptomycin to 1 ml), which was incubated for 1 h at 37°C in the presence of serum from mice receiving CP ("active"

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TABLE 1. Alkylating Activity of Blood Serum of Mice of Various Lines after Injection of 200 mg/kg CP

Line of mice	Time of investigation after injection of CP, min		
	10	30	90
BALB/c	0,85	1,24	0,40
CBA	0,73	1,07	0,26
DBA/2	0,76	1,06	0,25

Legend: 1. Mean results of three experiments given. 2. Here and in Table 2, alkylating activity expressed in optical density units/ml. 3. Significance of differences determined by nonparametric criteria (see "Experimental Method").

serum). The incubation mixture (1 ml) contained 125 million spleen cells and 0.5 ml of active serum. The cells were then washed once with medium 199 cooled to 4°C and injected intravenously into syngeneic mice in a dose of 50 million cells together with 4×10^8 SRBC. The recipient mice had received CP 3-4 h previously in a dose of 200 mg/kg in order to suppress their own immunoreactivity [1]. The number of 19S-antibody-forming cells in the recipients' spleen was determined 5 days later by the method of local hemolysis in agar [11]. The content of immunodepressive metabolites of CP in the serum was judged from the degree of depression of the immune response of the transplanted cells compared with the control (incubation of cells with normal mouse serum).

The significance of differences was determined by parametric and nonparametric statistical methods (Student's t-test, criterion of signs, Wilcoxon's two-sample test [3, 6]).

EXPERIMENTAL RESULTS

The results of the investigation of the alkylating activity of serum taken from mice at various times after injection of 200 mg/kg CP are given in Table 1.

The alkylating activity of the serum reached a considerable level 10 min after injection of the compound. The concentration of alkylating metabolites in the serum reached a maximum after about 30 min, and after 1.5 h their concentration fell significantly (the results of intermediate times of determination are not given in Table 1). Statistical analysis showed that the alkylating activity of the serum of BALB/c mice was significantly ($0.01 < P < 0.05$) higher than that of CBA and DBA/2 mice, in which it was equal ($P > 0.05$).

In the next series of experiments the immunodepressive activity of the blood serum of mice of different lines was tested after injection of CP in a dose of 200 mg/kg. Serum was taken 30 min after injection of CP, i.e., at the time of the highest level of alkylating products in the blood. The experiments were repeated 3 times and showed that the immunodepressive activity of the serum in DBA/2 and BALB/c mice was significantly ($P < 0.001$) higher than in CBA mice (the immune response in the test system was depressed to 35.3×1.2, 40.4×1.3, and $86.9 \times 1.1\%$ of the control level). At the same time it was found that DBA/2 mouse serum was slightly but significantly ($P < 0.05$) more active than BALB/c serum.

Comparison of the alkylating and immunodepressive activity of sera from the various lines of mice showed no parallel trend of these parameters. For instance, DBA/2 mice, in which the concentration of alkylating products was the same as in CBA mice, had much higher immunodepressive activity of their serum. On the other hand, in BALB/c mice, with the highest level of alkylating metabolites of CP, the blood serum had weaker immunodepressive action than that of DBA/2 mice.

In the final series of experiments the alkylating and immunodepressive activity of the serum of mice receiving different doses of CP was compared. BALB/c mice received injections of 200 and 800 mg/kg CP; blood was taken 30 min later and the alkylating activity of the sera and their action on the immune response of spleen cells of intact BALB/c mice were determined in adoptive transfer. Before incubation with the cells, the serum of the mice receiving CP in a dose of 800 mg/kg, with higher alkylating activity than serum of mice receiving only 200 mg/kg, was diluted with normal mouse serum so that the concentrations of alkylating products were the same in the two incubation mixtures. The results are given in Table 2. They show that, despite

TABLE 2. Alkylating and Immunodepressive Activity of Mouse Blood Serum after Administration of Different Doses of CP

Expt.	Dose of CP injected into mouse serum donors, mg/kg	Alkylating activity of incubation mixture	Number of antibody-forming cells in spleen of recipient mice*	P
1	800	0,94	33 (16–68) $n=5$	<0,001
	200	0,94	1 012 (690–1 483) $n=6$	<0,001
	Control	—	16 520 (13 270–20 560) $n=6$	
2	800	0,98	26 (16–41) $n=5$	<0,001
	200	0,98	646 (367–1 135) $n=6$	<0.001
	Control	—	10 190 (5 902–17 580) $n=5$	

*Geometric mean values and (in parentheses) confidence intervals at $P \leq 0.05$.

the equal alkylating activity, the serum of mice receiving CP in a dose of 800 mg/kg had a much stronger immunodepressive action.

These results, together with those published previously [5], lead to the following conclusion. It was shown previously [5] that the rate of oxidative hydroxylation of CP by the liver microsomes of BALB/c mice is almost 4 times greater than in CBA and DBA/2 mice. This finding is confirmed by the results of the present investigation showing that BALB/c mice have the highest serum alkylating activity during the first few hours after administration of CP. Nevertheless, it was the DBA/2 mice which had the greatest sensitivity to the immunodepressive action of CP [5], and at the same time, their serum had the highest immunodepressive activity. The results of the present experiments thus point to direct dependence of the immunodepressive effect of CP on the level of its active metabolites with immunodepressive activity in the blood serum. At the same time, the present experiments showed that there is no parallel between the alkylating activity of CP metabolites and their action on immunocompetent cells. This fact is to some degree paradoxical, for it is the alkylating process that is considered to be responsible for the cytotoxic action of alkylating agents [8, 10]. The most likely explanation is that the immunodepressive action is due to other, weakly alkylating CP metabolites [12], the accumulation of which in the body obeys a different kinetics and depends both on the dose of CP given and on the genotype of the animals.

In this connection it is interesting to note that in a study of the mutagenic activity of the blood serum of mice receiving high doses of CP [7] its alkylating activity was virtually unchanged despite an increase in the dose of CP from 400 to 1000 mg/kg, whereas the cytogenetic effect increased directly proportionally to the dose of CP given. The identification of these metabolites and the study of their pharmacodynamics in animals of different genotypes will be the subject of future research.

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MECHANISMS OF HYPOREACTIVITY IN MICE AFTER INJECTION OF LYSED ERYTHROCYTE ANTIGEN

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A state of specific hyporeactivity to sheep's red cells (SRBC) was induced in mice by injection of hemolyzed SRBC. Blocking serum factor in these mice was shown not to be identical in the character of its action with antired-cell antibodies, but to be probably an antigen–antibody complex. After combined injection of hemolyzed SRBC and cyclophosphamide (CP) into mice production of blocking serum factor was suppressed but the reactivity of the mice to SRBC was considerably reduced. It is suggested that in this case inactivation of the immunocompetent cells took place through the combined action of CP and SRBC antigen in a nonimmunogenic form.

KEY WORDS: antigen of hemolyzed red cells; serum blocking factor; cyclophosphamide; suppression of immune response.

Hemolyzed sheep's red cells (SRBC) obtained by treating SRBC with distilled water, followed by ultracentrifugation, if injected into mice, induce a state of hyporeactivity to SRBC [1, 2]. In our own experiments [1], unlike those of Auerbach et al. [3, 4], the blood serum of mice treated with hemolysate possessed blocking activity, which disappeared after absorption with native SRBC.

The object of this investigation was to study the factors determining the state of reduced reactivity arising as a result of injection of hemolyzed SRBC into mice.

EXPERIMENTAL METHOD

The method of obtaining the hemolyzed SRBC was described previously [1]. Male CBA, (CBA × C57BL/6)F_1, and (DBA/2 × C57BL/6)F_1 mice weighing 20-26 g were obtained from the "Stolbovaya" nursery, Academy of Medical Sciences of the USSR. SRBC hemolysate was injected either in a dose of 0.5 ml daily on 5 successive days or as a single dose 2.5 ml intraperitoneally (both methods were equally effective).

The reactivity of the animals was determined from the number of 19S-antibody-forming cells (AFC) in the spleen on the 5th day after intraperitoneal injection of 2×10^8 SRBC (the method of local hemolysis in agar [7]).

Antired-cell sera were prepared from the blood of mice immunized singly or repeatedly with SRBC. The sera were inactivated at 56°C for 30 min and were kept at −20°C before use.

The results were subjected to statistical analysis by Student's t-test.

EXPERIMENTAL RESULTS

The SRBC hemolysate had very low immunogenicity and, consequently, it induced only weak production of 19S-AFC (in these experiments on average 200 AFC per spleen, i.e., only 2 or 3 times more than the

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