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Inhibition of Cellular Immune Reactions by Cyclophosphamide Analogues Ifosfamide and Trofosfamide. I. Mixed Lymphocyte Reaction (37816)

R. G. DEVLIN, N. L. SCHWARTZ, AND P. E. BARONOWSKY
(Introduced by W. A. Zygmunt)
Department of Biochemistry, Mead Johnson Research Center, Evansville, Indiana 47721

Ifosfamide and trofosfamide are analogs of cyclophosphamide (1, 2) which suppress humoral immune reactions in mice (3–5) and rats (4, 5) in a dose-related manner. Ifosfamide also prolonged skin allograft survival in rats, but in this case a dose–response relationship could not be confirmed statistically (6).

The mixed lymphocyte reaction (MLR) is a convenient test system in which cellular reactions involved in the initiation of allograft rejection can be investigated *in vitro* (7). The purpose of the present work was to determine whether ifosfamide and trofosfamide are capable of suppressing the MLR in a dose-related fashion. Since ifosfamide, like cyclophosphamide, is activated by an enzyme system present in liver microsomes (8–10), we have devised an *"in vivo—in vitro"* MLR test system in which animals are treated with the test drugs *in vivo* 4 hr before their splenic lymphocytes are harvested and prepared for culture *in vitro*.

Methods. Mice. BALB/c (H-2^d) and CBA/J (H-2^k) strains of mice (Jackson Labs, Bar Harbor, ME) were used for these experiments.

Drugs. Cyclophosphamide was furnished by the Mead Johnson Co. Ifosfamide and trofosfamide were obtained from Asta-Werke A. G., Chemische Fabrik, Brackwede, West Germany. All drugs were suspended in 0.5% methocel in water and were injected intraperitoneally into groups of 4 CBA/J mice. Four hours later, the mice were sacrificed and their spleens excised and prepared for tissue culture.

Tissue Culture. Spleens from the 4 mice in each group were pooled and teased through 80-mesh screen to obtain cell suspensions. The cells were then prepared for culture and mixed lymphocyte reactions (MLRs) according to the method of Gazit and Harris (11). On the third day of culture, cells were pulsed with tritiated thymidine (1 μCi/ml). Eighteen to twenty-four hours later, cells were washed and extracted with 5% trichloroacetic acid. Acid-insoluble material was dissolved in NCS tissue solubilizer (Amersham/Searle), and incorporated tritium was measured in a liquid scintillation counter as previously described (11). In this system, spleen cells from drug-treated CBA/J mice are tested for their ability to react to BALB/c spleen cells pretreated *in vitro* with 25 μg/ml of mitomycin C (a mitotic inhibitor).

Results. When spleen cells from normal CBA/J mice were cultured with mitomycin-treated BALB/c spleen cells, a vigorous synergistic uptake in ^{3}H-thymidine by CBA/J cells was seen (Table I). This increased incorporation of radioisotope was severely inhibited by ifosfamide, trofosfamide, or cyclophosphamide injected 4 hr before sacrifice. At a dose of 100 mg/kg intraperitoneally, there was no significant difference between the suppression caused by cyclophosphamide and that caused by either ifosfamide or trofosfamide (90% suppression with all 3 drugs).

In Table II are shown the effects of in-

TABLE I. Effect of Ifosfamide (I), Trofosfamide (T), and Cyclophosphamide (CP) on ^{3}H-Thymidine Uptake by CBA/J Cells Reacting Against Mitomycin-Treated BALB/c Cells (a MLR).

Strain of spleen cells added to culture tubes	^{3}H-Thymidine incorporation Mean dpm ($\times 10^3$)
CBA/J[a]	4.5
BALB/c mito[a,b]	0.4
CBA/J + BALB/c mito	115.0
CBA/J I[a,c]	7.3
CBA/J I + BALB/c mito	11.8
CBA/J T[a,d]	5.4
CBA/J T + BALB/c mito	9.2
CBA/J CP[a,e]	4.8
CBA/J CP + BALB/c mito	8.8

[a] Culture contains only cells of one strain.

[b] BALB/c cells were treated with mitomycin C to prevent incorporation of ^{3}H-thymidine into DNA.

[c] These mice received ip ifosfamide at a dose of 100 mg/kg.

[d] These mice received ip trofosfamide at a dose of 100 mg/kg.

[e] These mice received ip cyclophosphamide at a dose of 100 mg/kg.

jecting varying doses of ifosfamide into mice 4 hr before sacrifice and collection of spleen cells for MLRs. The analysis of variance indicated a significant fit (.05 level) of the data to the regression line, and the ED_{50} of ifosfamide was determined to be 62 mg/kg

TABLE II. Determination of ED_{50} for Ifosfamide in the MLR.

Strain of spleen cells added to culture tubes	^{3}H-Thymidine incorporation Mean dpm ($\times 10^3$)
CBA/J	15.6
BALB/c mito[a]	0.7
CBA/J + BALB/c mito[a]	150.4
CBA/J[b] 81	4.8
CBA/J[b] 81 + BALB/c mito	13.2
CBA/J 54	10.7
CBA/J 54 + BALB/c mito	103.7
CBA/J 36	10.3
CBA/J 36 + BALB/c mito	143.4
CBA/J 24	5.9
CBA/J 24 + BALB/c mito	155.5
CBA/J 16	16.0
CBA/J 16 + BALB/c mito	165.6

[a] BALB/c cells were treated with mitomycin C to prevent incorporation of ^{3}H-thymidine into DNA.

[b] Mice were injected with ifosfamide intraperitoneally at a dose of 81 mg/kg.

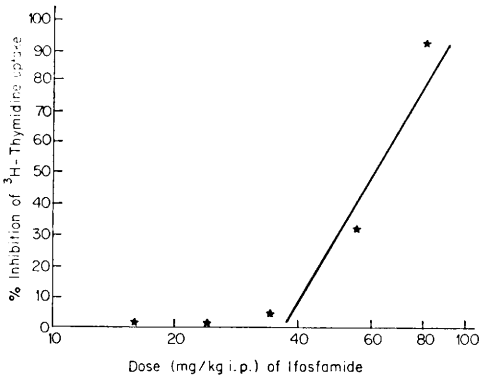

FIG. 1. Spleen cells from CBA/J mice react against mitomycin-treated BALB/c cells by incorporating ^{3}H-thymidine. Injection of ifosfamide resulted in a dose-dependent reduction of ^{3}H-thymidine uptake by the CBA/J cells.

(Fig. 1). The differences seen in ^{3}H-thymidine incorporation of CBA/J cells from each treatment group cultured alone are probably due to a combination of drug toxicity and the variability which is often seen in cultures of one cell type alone (8).

We were unable to determine an ED_{50} for trofosfamide in this assay. In three different experiments, trofosfamide suppressed the MLR, but there was no reproducible dose-related response. We attributed this lack of dose response to the extreme insolubility of this drug and/or to the fact that only 4 hr had elapsed between the time of drug administration and sacrifice.

Discussion. The cyclophosphamide analogues ifosfamide and trofosfamide suppressed the cellular immune response exhibited in the MLR. The ED_{50} of ifosfamide was found to be 62 mg/kg which is about twice as high as that of cyclophosphamide (30 mg/kg) in this assay (unpublished data). Consistent with this result are the findings of Brock *et al.* that after doses of ifosfamide in rats and man, the peaks of alkylating activity in blood serum were about one-half those after equivalent doses of cyclophosphamide (2, 9).

Harrison and Fuquay reported that cyclophosphamide consistently showed slightly better immunosuppression of humoral antibody production in mice and rats than did ifosfamide or trofosfamide when all three compounds were administered on an equiva-

lent weight basis (5). Potel and Brock chose equivalent fractions of the LD_{50} as the doses for each drug (4). Under these conditions, cyclophosphamide was superior to ifosfamide and trofosfamide in suppression of humoral antibody production against *Brucella* sp in rats, but ifosfamide appeared somewhat superior in mice with heterologous erythrocytes as the antigen. Potel and Brock concluded that while cyclophosphamide and ifosfamide are potent immunosuppressive agents, trofosfamide appeared to be less well-suited for this purpose because of increased toxicity (4).

The immunosuppressive effectiveness of ifosfamide and trofosfamide is thus established in the case of humoral antibody production. With regard to cell-mediated immune responses, Adelsberger and Deicher reported that ifosfamide showed good immunosuppressive activity against the skin allograft rejection reaction in rats, but this response was not adequately dose related (6). In our test system, both cyclophosphamide and ifosfamide inhibited the initiation of a cell-mediated immune response in a dose-related manner. Under these conditions, cyclophosphamide was more effective than ifosfamide and trofosfamide, but the possible consequences of varying the dosing schedule were not explored.

Summary. The cyclophosphamide analogues ifosfamide and trofosfamide suppressed the mixed lymphocyte reaction in mice to about the same extent as did cyclophosphamide when the dose of the three drugs was 100 mg/kg intraperitoneally. The suppressive activity of ifosfamide was dose dependent, and the ED_{50} dose was 62 mg/kg intraperitoneally, twice that of cyclophosphamide. The activity of trofosfamide was not adequately dose dependent.

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