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Suppression of Spleen Lymphocyte Mitogenesis in Mice Injected with Platinum Compounds*

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Abstract—*The effect of i.v. injection of four platinum compounds, cis-dichlorodiammineplatinum, dichloro-1,2-benzenediamine-N,N'-platinum, cis-dichlorobis (cyclohexylamine)platinum, and cis-dichlorobis (cyclopentylamine) platinum, on murine T and B-splenic lymphocyte function was assessed by polyclonal activation with the selective T- and B-lymphocyte mitogens, concanavalin A and lipopolysaccharide, respectively. Results from both single dose, time course analyses and dose–response studies indicated that the platinum compounds were more toxic to T-lymphocyte function than to B-lymphocyte function.*

INTRODUCTION

SINCE Rosenberg *et al.* [1] first described the antitumour properties of *cis*-dichlorodiammineplatinum (NSC 119875) (DDP) against the sarcoma 180 tumor in mice, many different platinum compounds have been synthesized that also demonstrate antitumor activity in animals [2–4]. Except for DDP, relatively little information exists on the effects of these agents on the hemopoietic system. Thompson and Gale [5] observed a decrease in circulating reticulocytes and lymphocytes in rats after administration of 1–3 mg/kg of DDP. Khan and Hill [6] demonstrated that this same drug suppressed antibody plaque-forming cells in mice when given in a single dose within 2 days before or after sensitization with sheep red blood cells. Both Khan and Hill [7] and Howle *et al.* [8] demonstrated that DDP inhibited phytohemagglutinin-induced stimulation of lymphocytes. DDP has also been shown to suppress adjuvant-induced arthritis in rats [9] and to inhibit human and murine colony-forming cells, *in vitro* [10].

In the present study, we examined the effect that DDP and 3 of its analogs (see Fig. 1), dichloro-1,2-benzenediamine-N,N'-platinum (NSC 170895; DBP), *cis*-dichlorobis(cyclohexylamine)platinum (NSC 170899; DBCH), and *cis*-dichlorobis (cyclopentylamine)platinum (NSC 170898; DBCP), had on murine T and B-splenic lymphocyte function. These 3 analogs (Fig. 1) have been reported [2] to possess greater antitumor activity against a mouse plasma cell tumor than the parent compound, DDP. In general, the results from the studies reported in this paper suggested that the 4 platinum compounds inhibited splenic T-lymphocyte function to a greater extent than splenic B-lymphocyte function as assessed by polyclonal activation with selective T- and B-lymphocyte mitogens, concanavalin A (Con A) [11,12] and lipopolysaccharide (LPS) [12, 13], respectively.

MATERIALS AND METHODS

Platinum compounds

cis-Dichlorodiammineplatinum (II) (NSC 119875), *cis*-dichloro-1,2-benzenediamineplatinum (II) (NSC 170895), *cis*-dichlorobis(cyclopentylamine)platinum (II) (NSC 170898) and *cis*-dichlorobis-(cyclohexylamine) platinum (II) (NSC 170899) were kindly donated by Dr. Harry B. Wood of the National Cancer

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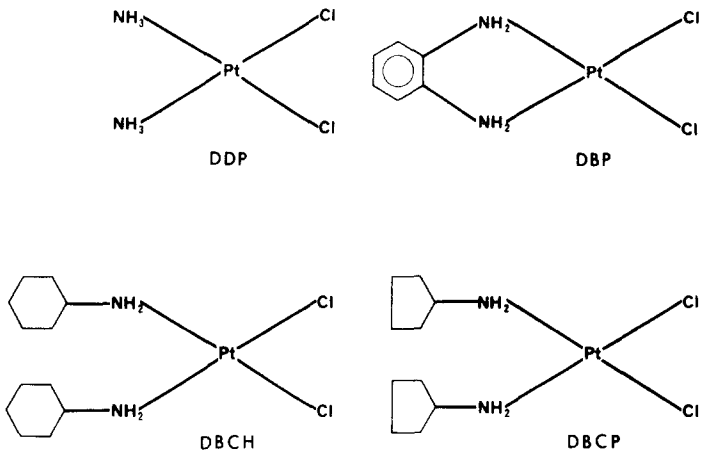

Fig. 1. Chemical structures of cis-*dichlorodiammineplatinum (DDP), dichloro-1,2-benzenediamine-N,N'-platinum (DBP),* cis-*dichlorobis(cyclohexylamine) platinum (DBCH), and* cis-*dichlorobis(cyclopentylamine)platinum* (DBCP).

Institute, Bethesda, Maryland. The dichlorodiammineplatinum agent was supplied as a powder that also contained mannitol and sodium chloride and was reconstituted with water before i.v. injection into mice. The other three agents were suspended by sonification in 6% polyvinylpyrrolidine (General Aniline and Film Corp., New York, N.Y.) in 0.9% saline immediately preceding i.v. injection.

Mice

Female BDF_1 (C57BL/6 × DBA/2) mice were obtained from Jackson Laboratories, Bar Harbor, Me. All mice used in this study were 8–24 weeks old.

Tissue culture media

Concentrates of Eagle's minimum essential medium (MEM) and Hank's balanced salt solution (HBSS) were obtained from Grand Island Biological Co., Grand Island, N.Y. MEM was supplemented with (mg/liter) glutamine (Sigma), 146; sodium pyruvate (Sigma), 100; serine (Sigma), 21; and asparagine (Sigma), 20. Fetal calf serum was obtained from Kansas City Biological Inc., Lenexa, Kan. 2-Mercaptoethanol (2-ME) was purchased from Sigma, St. Louis, Mo.

Mitogen assays

BDF_1 spleen cells (2×10^6 cells/ml) were cultured in Linbro 24-well culture trays (Linbro Chemical Co., New Haven, Ct.) in 1.0 ml of supplemented MEM containing 7.5% fetal calf serum, 5×10^{-5} M 2-ME, 100 units/ml of penicillin and 100 μg/ml of streptomycin per well. Two and a half μg/ml of Con A (Sigma, St. Louis, Mo.) or 10 μg/ml of *Salmonellatyphosa* LPS (DIFCO), Detroit, Mich.) was added to stimulate T or B cells, respectively. The trays were placed in airtight tissue culture boxes containing a water vessel to increase humidity, gassed with trio gas (10% CO_2, 7% O_2, 83% N_2) and incubated at 37°C. After 48 hr each culture received 1 μCi of ^{3}H-thymidine (sp. act. = 6.7 Ci/mM, New England Nuclear) and 24 hr later the incorporation of ^{3}H-thymidine into DNA was assessed by the method of Uyeki *et al.* (14). The filters containing radioactive material were placed in vials containing Aquasol (New England Nuclear) scintillation fluid and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Expression of data

Results for the day 1, 3 and 7 studies are expressed as a ratio of average experimental response per organ/average control response per organ with actual control values for spleen cellularity and mitogen responses in counts/min also listed. Results for the dose–response studies are expressed as an average experimental to average control ratio both per culture and per organ and as counts/min ± S.E. for both per culture and per organ response. (Per organ responses were calculated as follows: Con A response per organ = counts/min per 2×10^6 cells plated × cellularity ÷ 2×10^6 cells). Cellularity refers to the average spleen nucleated cell count from each group of mice.

Statistics

Values are expressed as a ratio of average experimental response/average control response and, in the dose–response studies, as the actual average response in counts/min or

No. of cells observed ± the S.E. Significance was determined from the original data at the 5% level of significance using a one-way ANOVA and Duncan's multiple range test.

RESULTS

Groups of BDF_1 mice (4 per group) were injected through the tail vein 1, 3 or 7 days before sacrifice with either a single dose of a platinum compound or with the suspending agent. Spleen cells harvested from control or drug-treated mice were plated at 2×10^6 cells per culture for the mitogen assays (Figs. 2–5). After the injection of 6.5 mg/kg of DDP, spleen cellularity was suppressed on all 3 days examined (Fig. 2). Cellularity was lowest when examined 3 days after injection as shown by the experimental/control response ratio (E/C ratio) of 0.56. Injection of DDP suppressed equally the T and B lymphocyte mitogen responses per organ when examined 1 and 3 days later. When examined 7 days after drug administration, the Con A and LPS responses as well as spleen cellularity had recovered toward control values (E/C ratios of 0.87, 0.78 and 0.84, respectively).

Injection of 16mg/kg of DBP caused a minor reduction in spleen cellularity 1 and 3 days later while complete recovery of cellularity was observed 7 days after injection (Fig. 3). Both T- and B-lymphocyte responses to mitogens were below an E/C ratio of 0.45 when examined 1 and 3 days after injection. One week after injection of DBP the T-lymphocyte response to Con A was even more suppressed as indicated by an E/C ratio of 0.22. Meanwhile, the B-lymphocyte response to LPS had recovered toward control values (E/C ratio of 0.82).

As shown in Fig. 4, injection of 100 mg/kg of DBCH caused little reduction in spleen cellularity or in the B-lymphocyte response when examined on any of the days indicated. In contrast, the T- lymphocyte response to Con A was significantly reduced 1 day after drug injection and this response remained low when examined 3 days after drug injection (E/C ratio of 0.61). However, by the 7th day after injection of DBCH the T- lymphocyte response had returned to control values.

Injection of 60 mg/kg of DBCP caused a reduction in spleen cellularity that was comparable with the reduction produced by DBP in that the average E/C ratio was never below 0.70 on any of the indicated days after drug injection (Fig. 5) Both the T- and B-lymphocyte mitogen responses were progressively inhibited after injection of DBCP when examined on the days indicated with the lowest responses observed 7 days after drug injection (E/C ratios of 0.19 and 0.23 for T- and B-lymphocyte responses, respectively). This pattern of recovery for DBCP differed from the observed recovery pattern of the other 3 platinum compounds in that one or

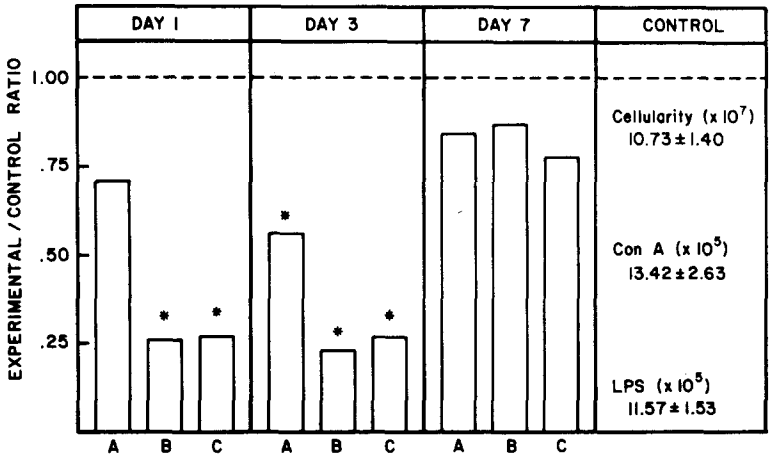

Fig. 2. Effects of 6.5 mg/kg *of DDP on spleen cellularity and mitogen activation were assessed on the days indicated after i.v. injection into* BDF_1 *mice (4 per group). Data are presented graphically as a ratio of average experimental responses per organ/average control responses per organ. Actual average control values* ± S.E. *are given in the last coloumn. Bar graph A represents the change in spleen cellularity; bar graph B represents the response to Con A stimulation; bar graph C represents the response to LPS stimulation. An asterisk denotes statistical significance at the* 5% *level of significance.*

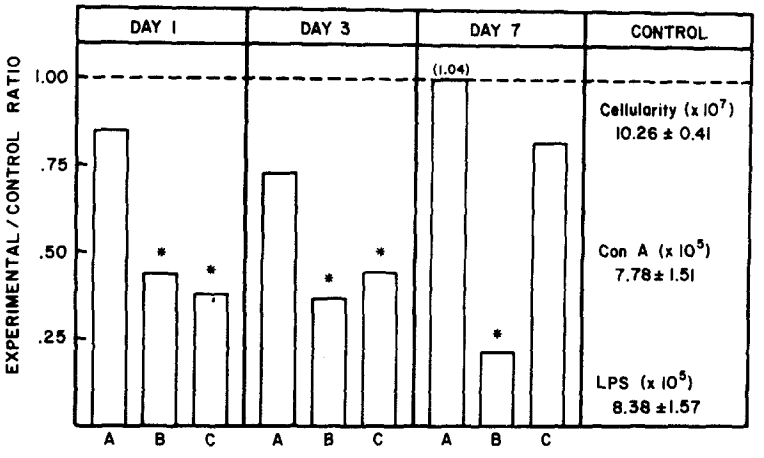

Fig. 3. Effects of 16 mg/kg *of DBP on spleen cellularity and mitogen activation was assessed on the days indicated after i.v. injection into* BDF_1 *mice (4 per group). Legend is the same as for Fig. 2.*

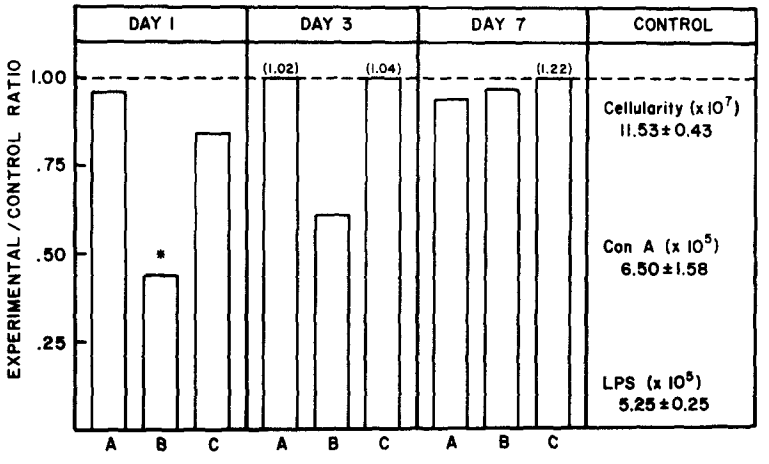

Fig. 4. Effects of 100 mg/kg *of DBCH on spleen cellularity and mitogen activation was assessed on the days indicated after i.v. injection into* BDF_1 *mice (4 per group). Legend is the same as for Fig. 2.*

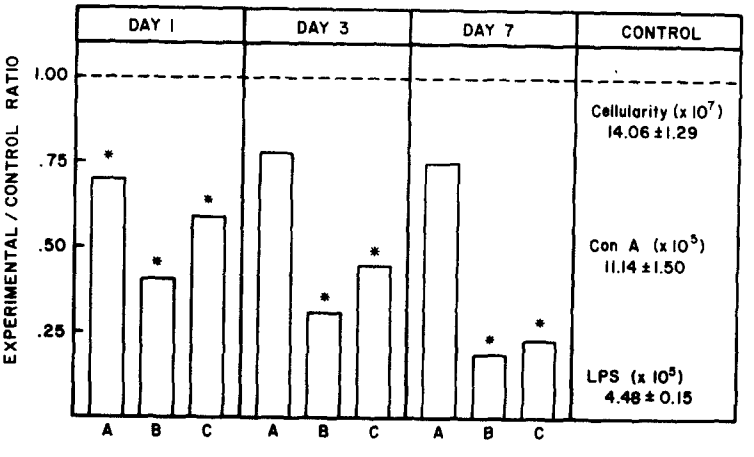

Fig. 5. Effects of 60 mg/kg *of DBCP on spleen cellularity and mitogen activation was assessed on the days indicated after i.v. injection into* BDF_1 *mice (4 per group). Legend is the same as for Fig. 2.*

both mitogen responses were observed to recover 1 week after injection of either DDP, DBP or DBCH.

Upon evaluation of the results from the single dose studies, it appeared that all 4 platinum analogs might inhibit the recovery of T-lymphocyte function more than the recovery of B-lymphocyte function. To test this observation further, dose–response studies were performed to determine if this selective recovery would occur over a range of doses for each platinum compound. Previous studies with immunosuppressants in our laboratory have shown that spleen cellularity is usually at its nadir 3 days after a single injection of an immunosuppressive agent (unpublished results). Therefore, we chose 3 days after drug administration as the time period in which to examine the differential recovery of T- and B-lymphocyte function. For the dose–response experiments, groups of 4 BDF_1 mice were injected i.v. 3 days before sacrifice with either suspending agent or different doses of a platinum compound as described in Figs. 6–9. As depicted in Fig. 6, the 3 highest doses of DDP significantly reduced spleen cellularity and both the per culture and per organ T-lymphocyte responses to Con A. In contrast, the B-lymphocyte response to LPS per culture was not significantly reduced with any of the doses injected but due to the marked decrease in cellularity, the B-lymphocyte response to LPS per organ was significantly reduced with the 2 highest doses of DDP. Thus, upon comparing both the per culture and per organ results, it was observed that DDP decreased the number of both B- and T-lymphocytes in the spleen at this time but proportionally reduced T-lymphocyte function more than B-lymphocyte function.

A variable reduction in spleen cellularity was observed after injection of doses of DBP with a significant reduction observed at only 1 dose (8 mg/kg) (Fig. 7). The T-lymphocyte response to Con A was significantly reduced in a dose-dependent fashion with all doses examined both on a per culture and a per organ basis. The B-lymphocyte response to LPS calculated per organ was significantly reduced with all doses examined while the per culture response was significantly reduced only with the highest dose injected. These

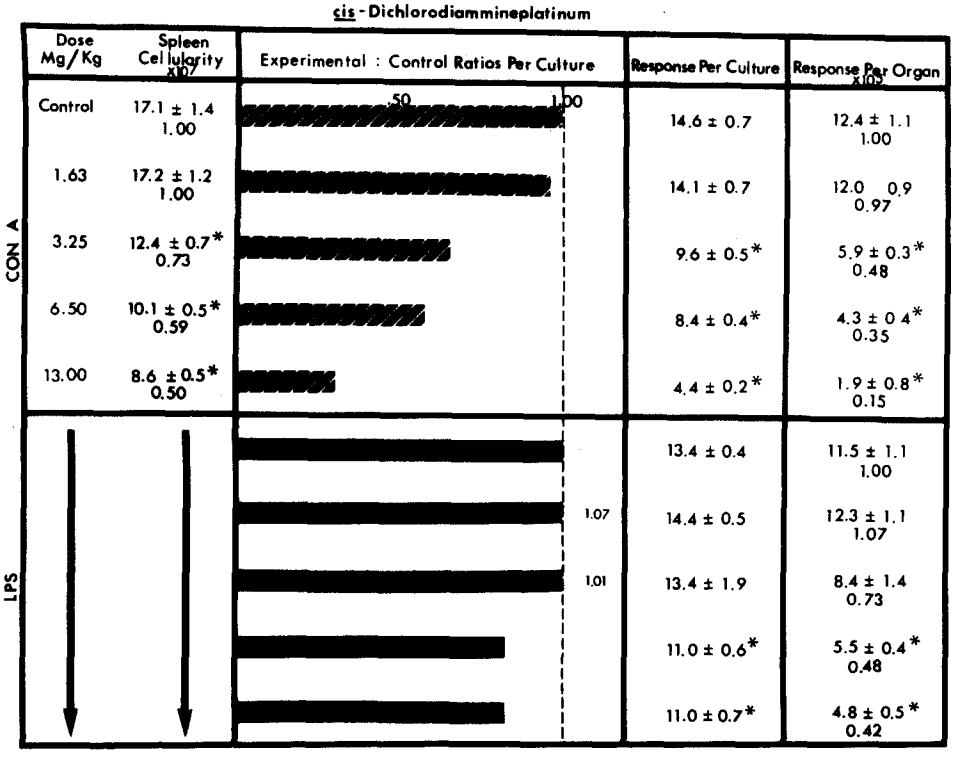

Fig. 6. Dose–response effect of DDP on mitogen activation and spleen cellularity was determined 3 days after i.v. injection into BDF_1 mice (4 per group). Data are presented graphically as the ratio of the average experimental response per culture/average control response per culture and in table form as the actual average response ± S.E. per organ. Decimal fractions listed below some responses represent the ratio of average experimental response/average control response for that parameter. An asterisk denotes significance at the 5% level of significance.

results demonstrated that DBP, like DDP, also reduced T-lymphocyte function more than B-lymphocyte function. DBCH did not significantly reduce spleen cellularity with any of the doses when examined 3 days after injection (Fig. 8). However, both the Con A and LPS responses per culture and per organ were inhibited with all doses. The T-lymphocyte response to Con A was inhibited more than the B-lymphocyte response to LPS at 6.4, 40 and 100 mg/kg. These results indicated that even with minimal toxic effects on spleen cellularity 3 days after injection, organ only with 80 and 160 mg/kg. These results showed that, even though the absolute number of mitogen responsive lymphocytes decreased at all doses, the relative proportion of lymphocytes responding to Con A compared with control increased at low doses of DBCP and decreased with higher doses.

DISCUSSION

cis-Dichlorodiammineplatinum (DDP) has been the most thoroughly investigated com-

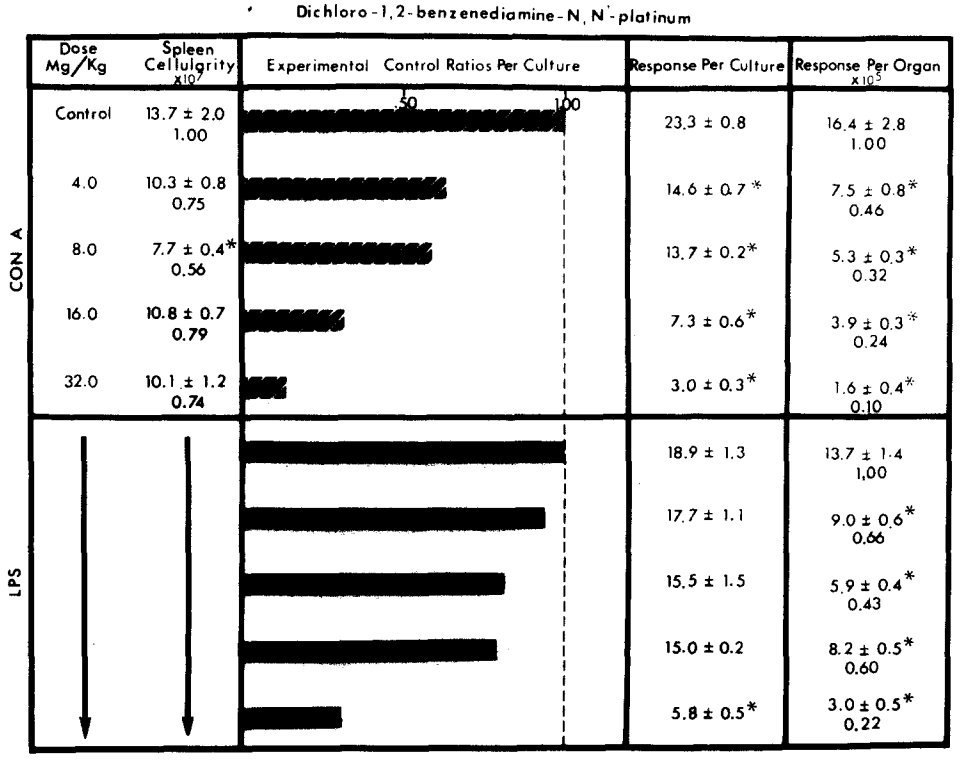

Fig. 7. Dose–response effect of DBP on mitogen activation and spleen cellularity was determined 3 days after i.v. injection into BDF_1 mice (4 per group). Legend is the same as for Fig. 6.

there was a selective and marked reduction in the ability of splenic lymphocytes to respond either to Con A or LPS.

DBCP significantly reduced spleen cellularity with all doses 3 days after injection (Fig. 9). The Con A response per culture was inhibited only after injection with 80 and 160 mg/kg but due to the decrease in spleen cellularity, the Con A response per organ was significantly reduced with all doses. The inhibition of the B-lymphocyte responses to LPS stimulation both per organ and per culture paralleled the inhibition of the T-lymphocyte responses to Con A with each dose; however, the LPS response was reduced significantly per culture only with 160 mg/kg and per

pound among the platinum complexes and has undergone extensive clinical investigation against a variety of tumors (for a review see Rozencweig *et al.*, [15]. DDP has also been shown to be cytotoxic to a human lymphoma cell line, *in vitro* [16, 17], and to be an immunosuppressant, *in vivo* [6, 8, 9], with its mechanism of action postulated to be similar to that of the alkylating agents [18, 19]. Preliminary studies with second-generation platinum complexes of DDP have produced a number of drugs with antitumor activity and some of these have extremely low toxicity [2]. As of the present, very little information exists on the toxicity of these second-generation drugs toward the immune system; this infor-

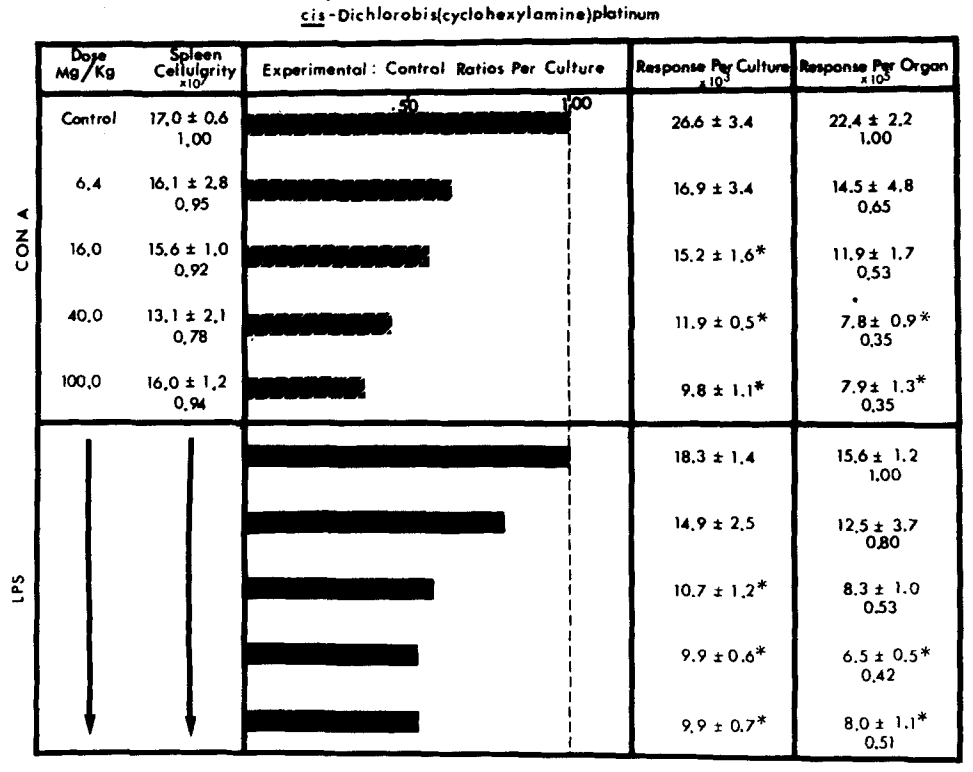

Fig. 8. Dose–response effect of DBCH on mitogen activation and spleen cellularity was determined 3 days after i.v. injection into BDF_1 mice (4 per group). Legend is the same as for Fig. 6.

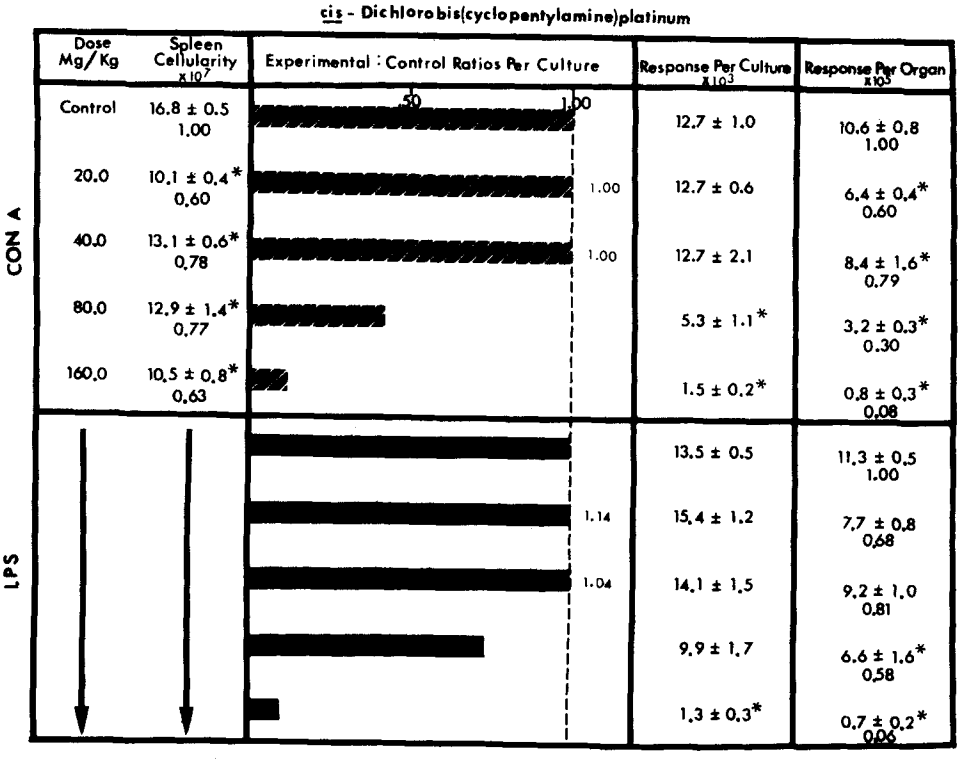

Fig. 9. Dose–response effect of DBCP on mitogen activation and spleen cellularity was determined 3 days after i.v. injection into BDF_1 mice (4 per group). Legend is the same as for Fig. 6.

mation would be useful in deciding which agents merit clinical trials.

Our purpose, therefore, was to compare the effects of i.v. administration of DDP with the effects of DBP, DBCH and DBCP on splenic T- and B-lymphocyte function in mice. These three analogs of DDP were chosen for study because Connors and co-workers [2] found that these agents had higher therapeutic indexes against a mouse plasma cell tumor than did DDP. Doses of each drug used in these studies were, for the most part, given in concentrations that were above those shown to produce complete plasma cell tumor repression in mice and below the LD_{50} in mice [2]. Function of splenic T- and B-lymphocytes was determined after i.v. injection of the platinum compounds by assessing the amount of activation in lymphocyte cultures selectively stimulated by Con A or LPS. One of us [20] has previously shown, using X-irradiation, that this method accurately assesses inhibition of T- or B-lymphocyte function.

As depicted in Figs. 2–5, the time course analysis of lymphocyte function after a single dose of each of the platinum compounds in question demonstrated that T-cell function was usually suppressed to an equal or greater extent than B- cell function at the time intervals investigated. Since this unique pattern of suppression and recovery of lymphocytic function observed for each platinum compound may be due totally or at least in part to the dose of the drug chosen, a dose–response analysis was performed with each of the 4 platinum compounds. As discussed previously, day 3 was chosen to evaluate splenic lymphocyte function in groups of mice previously given increasing doses of one of the four platinum compounds. Figure 10 summarizes the results of these experiments which demonstrated that 3 days after injection the T-lymphocyte recovery was more depressed than the B-lymphocyte recovery with almost all doses of the 4 platinum compounds. The greayest differential toxicity occured with doses of DDP and DBP. This phenomenon was also apparent after injection of most doses of DBCH and DBCP although the differences in inhibition between T- and B-lymphocyte func-

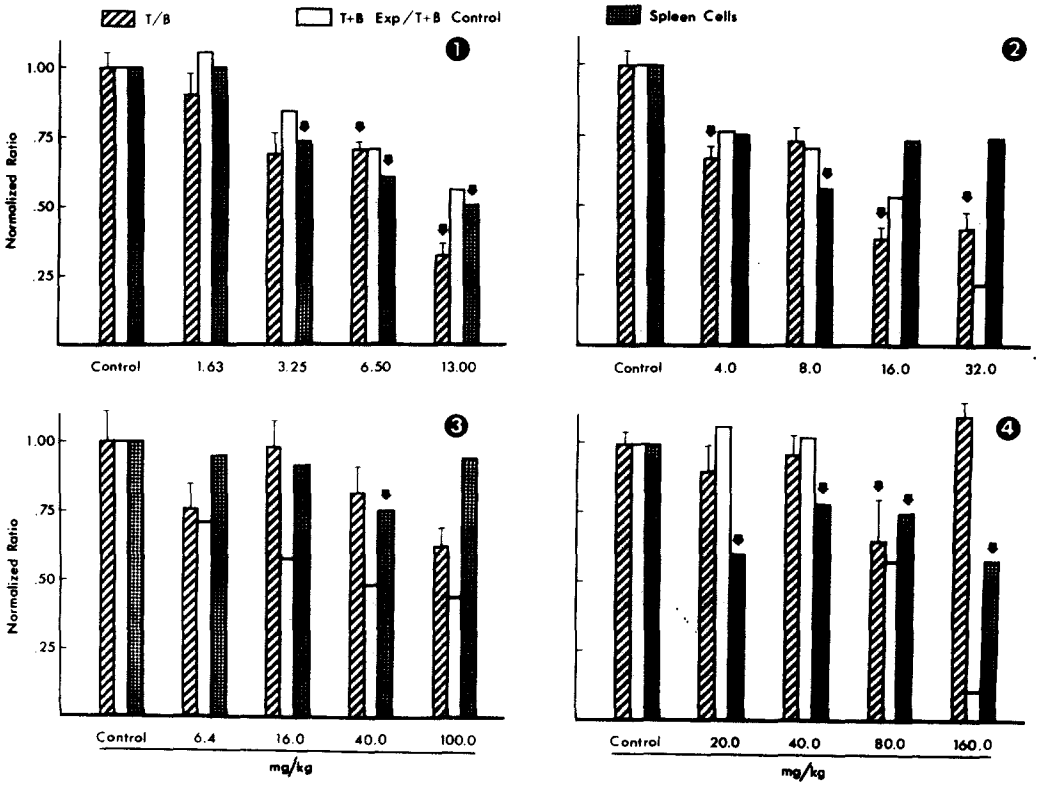

Fig. 10. Summary of the dose–response effects assessed 3 days after i.v. injection into BDF1 mice for each of the 4 platinum compounds. Slashed column represents the ratio of the average T-lymphocyte response to Con A vs average B-lymphocyte response to LPS. Open column is a ratio of the average T- and B-lymphocyte responses to mitogen in experimental cultures vs average T- and B-lymphocyte mitogen responses in control cultures and indicates the degree of suppression of lymphocyte function. Stippled column is the ratio of average spleen cellularity in control mice. An arrow represents significance at the 5% level of significance. Number 1 equals DDP; No. 2 = DBP; No. 3 = DBCH; and No. 4 = DBCP.

tion were not as great as with the first 2 compounds. It should be pointed out that although DBCH and DBCP were less toxic to spleen cellularity and bone marrow cellularity (unpublished results) than DDP or DBP, these compounds were effective immunosuppressants as indicated by the decrease in total mitogen responsiveness of the spleen lymphocytes taken from mice injected with these agents (T and B experimental/T and B control); immunosuppression by platinum complexes was also verified by using the Mishell–Dutton system to detect formation of antibody-producing cells *in vitro* (unpublished results). These results contrast with the observation by Speer and co-workers [3] which suggested that DBCP might not be immunosuppressive.

The selective toxicity of the platinum compounds for T-lymphocytes observed in this study is not unique among organometallic complexes. As reviewed by Vos [21], organotin compounds also cause a reduced responsiveness of peripheral T-lymphocytes without compromising B-lymphocytes or macrophage responses. Reasons for the differential effect of organometallic compounds on T- and B-lymphocyte function are unknown and we can only speculate on the mechanisms involved. For example, T-lymphocytes may possess an inherent susceptibility that makes them more sensitive to the platinum compounds, as is the case for B-lymphocytes treated with X-irradiation [20] or certain doses of cyclophosphamide [22, 23]. A greater decrease in T-lymphocyte function relative to B-lymphocyte function may also be observed after administration of the platinum complexes if the recovery of B-lymphopoiesis occurred more rapidly than the recovery of T-lymphopoiesis. If this differential recovery in lymphopoiesis occurred, it could be related to the effects that the platinum compounds have on the lymphopoietic inductive microenvironment. Pharmacokinetic distribution of the platinum compounds could also in part account for the greater suppression of T-lymphocyte function than B-lymphocyte function. This is particularly true for the 4 compounds in this study since DDP is water soluble while DBP, DBCH and DBCP are insoluble and must be injected in suspension. These insoluble drugs were injected i.v. as an aqueous suspension in polyvinylpyrrolidine (PVP). This mode of injection circumvents the depot formation that occurs if the solution is injected intramuscularly. We assume that after i.v. injection these drugs have a reticulo-endothelial distribution due to the particulate nature of the platinum-PVP preparations. Further studies must be performed to determine what effect PVP and other suspending agents have on both the antitumor efficacy and hemopoietic toxicity of certain platinum compounds.

Although the platinum compounds have been shown in mice to be effective against a variety of tumors, in human chemotherapy combination regimens are the rule. This is done in an attempt to obtain synergistic antitumor action and also to reduce patient toxicity by using doses that are smaller in combination therapy than when used alone [24]. Because the results of our studies demonstrated that the platinum compounds preferentially inhibit splenic T-lymphocyte function, it would be of interest to study this phenomenon in mice treated with a platinum agent in combination with cyclophosphamide or X-irradiation. As mentioned earlier, these agents can preferentially inhibit B-lymphocyte function. By alternating the sequence of administration of these agents, it may be possible to selectively spare lymphocyte populations while maintaining sustained antitumor efficacy. Gale and co-workers [24, 25] have already set a precedent in antitumor therapy for the use of platinum compounds in combination with cyclophosphamide. They tested 6 platinum compounds in combination with cyclophosphamide and other antitumor agents against L1210 leukemia in BDF_1 mice and noted an impressive synergism when cyclophosphamide was used in combination with each platinum complex.

In conclusion, we have observed that the platinum complexes tested were more toxic to splenic T-lymphocyte function than splenic B-lymphocyte function. We have also demonstrated that DBCH and DBCP were considerably less cytotoxic to bone marrow nucleated cells (unpublished results) and spleen nucleated cells than were DDP and DBP, yet DBCH and DBCP effectively inhibited T- and B-lymphocyte function. Collectively, the results of these experiments demonstrated that the type of immunosuppression produced by the platinum compounds is markedly different from the type of inhibition caused by such commonly used immunosuppressants as cyclophosphamide [22, 23] or X-irradiation [20]. Because of this unique suppression, we believe further clinical testing of these compounds as potential antitumor agents is warranted.

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