Cis-Dichlorodiammineplatinum [II]: Inhibition of Nucleic Acid Synthesis in Lymphocytes Stimulated with Phytohemagglutinin[1] (35675)

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cis-Dichlorodiammineplatinum(II) (*cis*-[Pt $(NH_3)_2Cl_2]^0$) has been shown to have an impressive chemotherapeutic action against the Ehrlich ascites carcinoma (1), leukemia L1210 (2), sarcoma 180 (2, 3), and virus-induced reticulum cell sarcoma (4). Studies of the mode of action of this compound using Ehrlich ascites carcinoma cells showed that it effected a selective and persistent inhibition of deoxyribonucleic acid (DNA) synthesis *in vivo*. The rates of synthesis of ribonucleic acid (RNA) and protein were also depressed in the first 24 hr after treatment, but returned to virtually control values by 96 hr after a single injection. The depression of the rate of DNA synthesis was irreversible during the period of observation (1).

Peripheral blood studies of young rats receiving single or multiple injections of *cis*-$[Pt(NH_3)_2Cl_2]^0$ revealed a diminution of the number of circulating lymphocytes and reticulocytes without a concomitant reduction of the number of granulocytes or erythrocytes. Upon sacrifice and necropsy of treated animals there was observed a marked depletion of the cell population of the thymic cortex (5). The present study was consequently initiated to determine the effects of *cis*-$[Pt(NH_3)_2Cl_2]^0$ on nucleic acid synthesis in human peripheral lymphocytes under the mitogenic stimulus of phytohemagglutinin (PHA) *in vitro*, as well as to assess any cytological aberrations coincident with such treatment.

Materials and Methods. *cis*-$[Pt(NH_3)_2Cl_2]^0$ was synthesized by the method of Kauffman and Cowan (6). Elemental analysis (Galbraith Laboratories) substantiated the empirical formula, and activity of the compound was confirmed by biological assay using *Escherichia coli* B (7). Solutions of the platinum complex were prepared immediately prior to use.

Venous blood from presumably normal donors was defibrinated with glass beads and the lymphocytes were separated as described by Ohno and Hersh (8). Culture medium was Eagle's minimum essential medium with Hanks' balanced salt solution (MEM) supplemented with L-glutamine (2.0 m*M*), autochthonous serum (20%), $NaHCO_3$ (1.7 m*M*), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Difco PHA-P, 0.005 ml/ml of culture medium, served as the mitogenic stimulus. Cultures were incubated at 37° in an atmosphere of 5% CO_2–95% air without agitation for 2 or 3 days, this interval being varied according to experimental design.

Rates of synthesis of DNA, RNA, and protein were measured by the respective rates of incorporation of thymidine–methyl-^{3}H, uridine-5-^{3}H, and L-leucine-^{14}C (New England Nuclear Corp.) into the acid-insoluble fraction of the cells. The labeling period was the terminal 2 hr of incubation, and the final activity of each isotopically labeled precursor was 1.0 μCi/ml. Following the labeling period, cold 0.9% NaCl was added to each tube, the cultures were centrifuged, and the supernatant solution was discarded. Nucleic acid and protein were then precipitated by the addition of cold 5% trichloroacetic acid (TCA). After three washings with cold 5%

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TCA by sedimentation and resuspension, the acid-insoluble fraction was solubilized in 1.0 *M* hydroxide of Hyamine in methanol. Each solution was transferred quantitatively to a toluene-based phosphor (PPO-POPOP), and radioactivity was measured with a liquid scintillation spectrometer (Mark I, Nuclear Chicago Corp.).

To obtain specimens for electron microscopy, cells were harvested by centrifugation after 24 or 48 hr incubation in the presence of the platinum compound at 3×10^{-6} *M* or 3×10^{-5} *M*. The primary fixative was phosphate-buffered 6% glutaraldehyde. Post-fixation in phosphate-buffered 1% OsO_4 was followed by staining in 1% uranyl magnesium acetate, and cells were embedded in Epon. Sections were cut with an LKB Ultrotome using a DuPont diamond knife, and were visualized with an RCA EMU3-H electron microscope.

Results. *cis*-$[Pt(NH_3)_2Cl_2]^0$ was a potent inhibitor of the PHA-induced blastogenic response of cultured human peripheral lymphocytes. The dose-response relationship of this action is shown in Fig. 1, and is altogether compatible with a typical response of a population containing a standard distribution of cells with varying sensitivities. In the experiment shown in Fig. 1, the cells were incubated with the platinum complex for 24 hr prior to addition of tritiated thymidine; extension of the exposure period to 48 hr before pulse labeling did not appreciably alter the extent of inhibition obtained. The concentrations of *cis*-$[Pt(NH_3)_2Cl_2]^0$ which conferred 50 and 95% inhibition were calculated by the method of least squares to be 10^{-6} *M* and 2.3×10^{-5} *M*, respectively; these values were highly reproducible.

Relative selectivity of the action of *cis*-$[Pt(NH_3)_2Cl_2]^0$ against DNA synthesis as compared with the synthesis of RNA was found to be influenced by both concentration of inhibitor and duration of exposure of cells to inhibitor prior to the pulse period (Fig. 2). Virtually no degree of selectivity was evident at 10^{-4} *M*, regardless of the brevity of exposure. At 3×10^{-5} *M*, however, some selectivity of action against DNA synthesis emerged, but only following relatively short

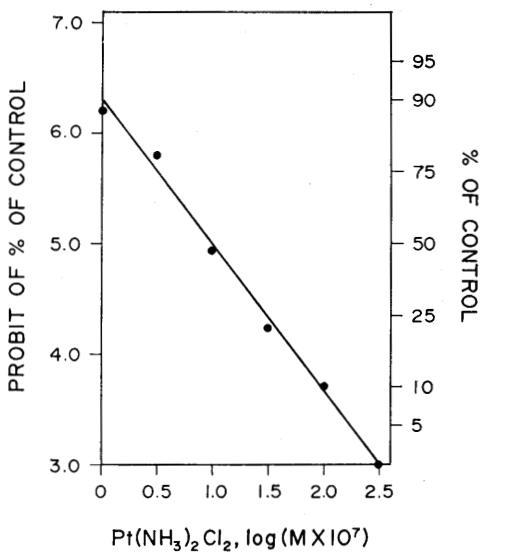


FIG. 1. Rate of deoxyribonucleic acid synthesis in phytohemagglutinin-stimulated human lymphocytes as a function of *cis*-$[Pt(NH_3)_2Cl_2]^0$ concentration. Phytohemagglutinin was added at 0 time, the platinum complex was added at 24 hr, thymidine–methyl-^3H was added at 46 hr, and the reaction was terminated at 48 hr. All inhibitor concentrations were multiplied by 10^7 to avoid negative logarithms, and the line was calculated by the method of least squares.

preincubation periods. At pharmacologically realistic concentrations of 10^{-5} *M* and below, the greater extent of inhibition of DNA synthesis attained as compared with RNA synthesis was quite striking, and was altogether compatible with data obtained when using the Ehrlich ascites carcinoma *in vivo* as a model system (1).

The time-dependent onset of inhibition of DNA synthesis and the persistence of this action following a single injection of *cis*-$[Pt(NH_3)_2Cl_2]^0$ into tumor-bearing mice (1) prompted an examination of the degree of reversibility of this action in lymphocytes. Replicate cultures were prepared with PHA added at 0 time. At 24 hr, *cis*-$[Pt(NH_3)_2Cl_2]^0$, 3×10^{-6} *M*, was added to one-half of the tubes. The other half served as controls. At 48 hr, one-third of the control and platinum-containing tubes received thymidine–methyl-^3H, the cells were labeled for 2 hr, and processed for liquid

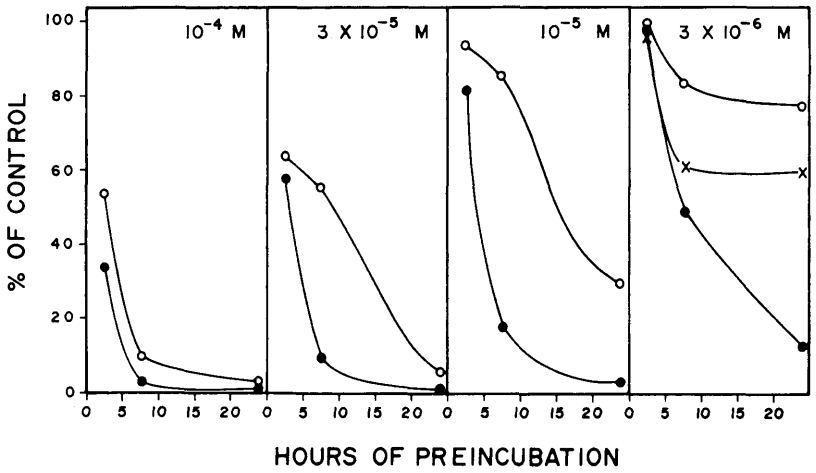


FIG. 2. Influence of various concentrations of *cis*-$[Pt(NH_3)_2Cl_2]^0$ and duration of preincubation with the inhibitor on the rates of synthesis of (●) deoxyribonucleic acid, (○) ribonucleic acid, and (×) protein in phytohemagglutinin-stimulated human lymphocytes.

scintillation counting. This established the degree of inhibition extant after 24 hr of exposure. Cells in one-third of the remaining tubes, both control and experimental, were washed three times with fresh medium to remove any diffusible inhibitor, the original volume of medium was restored, and incubation was resumed for another 24 hr. The remaining one-third of the control and experimental cultures were not washed, but merely incubated for a total period equivalent to that of the washed cells. Following the terminal 2-hr labeling period, tabulation of the extent of inhibition attained under each of the three conditions revealed that no amelioration whatsoever of the inhibitory action on DNA synthesis was achieved by washing the cells (Table I), indicative of an intimate binding of the platinum complex, or a modified species thereof, to the biological receptor(s) under these experimental conditions.

In spite of the profound suppression of DNA and RNA synthesis induced by *cis*-$[Pt(NH_3)_2Cl_2]^0$ at a concentration of 3×10^{-5} *M*, no cytologic alterations of the cells could be detected at the ultrastructural level following either 24 or 48 hr incubation with this concentration. Cultures representing inhibitor concentrations of 3×10^{-6} *M* or 3×10^{-5} *M* and incubated for 24 or 48 hr contained a small proportion of cells in vari-

TABLE I. Persistence of the Inhibitory Action of *cis*-$[Pt(NH_3)_2Cl_2]^0$ on Deoxyribonucleic Acid Synthesis in Phytohemagglutinin-Stimulated Human Lymphocytes after Washing and Resuspending the Cells in Fresh Medium Devoid of the Inhibitor.[a]

Total incubation, (hr)	Washed at 48 hr	*cis*-$[Pt(NH_3)_2Cl_2]^0$ 3×10^{-6} *M*[b]	Average cpm	% Inhibition
48	—	—	5278	
48	—	+	1282	76
72	—	—	5052	
72	—	+	1133	78
72	+	—	8805	
72	+	+	1592	82

[a] Phytohemagglutinin was added at 0 time.
[b] Added at 24 hr.

ous stages of degeneration; however, in no case did the relative proportion of these exceed that observed in control cells. Despite the diversity of combinations of morphological features observed in both experimental and control cultures, all cytologic elements resolvable at the ultrastructural level in the experimental cells were seen in the control specimens. Representative cells are shown in Figs. 3 and 4.

Discussion. The time-dependent onset of inhibitory action of *cis*-$[Pt(NH_3)_2Cl_2]^0$ against DNA synthesis, previously described in a murine tumor system *in vivo* (1) is shown by the present work to exist also in a human lymphocyte population *in vitro*. A fundamental query to be raised concerns the manner in which this gradual manifestation of pharmacologic activity is related to the molecular mechanism of action of this compound. It was proposed earlier (1) that even though the delayed response of DNA synthesis in Ehrlich carcinoma cells may be merely a reflection of a slow rate of penetration of the lipid-insoluble complex through the cell membrane, an alternate hypothesis could just as well invoke a 2-stage transformation

$$\text{(a)}\quad A \rightarrow B \rightarrow C,$$

where A is *cis*-$[Pt(NH_3)_2Cl_2]^0$, B is an intermediate with nonselective action against DNA, RNA, and protein synthesis, and C is an agent with selective and highly persistent action against DNA synthesis. The present study indicates that whatever conversion(s) may be necessary for biological activity do not require the intact animal, but may be elicited by the enzyme(s) of the single cell. Alternatively, of course, the proposed conversion(s) may be completely nonenzymatic in nature.

With regard to likely transformations which this complex may undergo, either enzymatically or nonenzymatically, the most logical 2-step conversion would be

$$\text{(b)}\quad Pt(NH_3)_2Cl_2 \rightarrow Pt(NH_3)_2Cl^+ + Cl^-,$$
$$\text{(c)}\quad Pt(NH_3)_2Cl^+ \rightarrow Pt(NH_3)_2^{2+} + Cl^-.$$

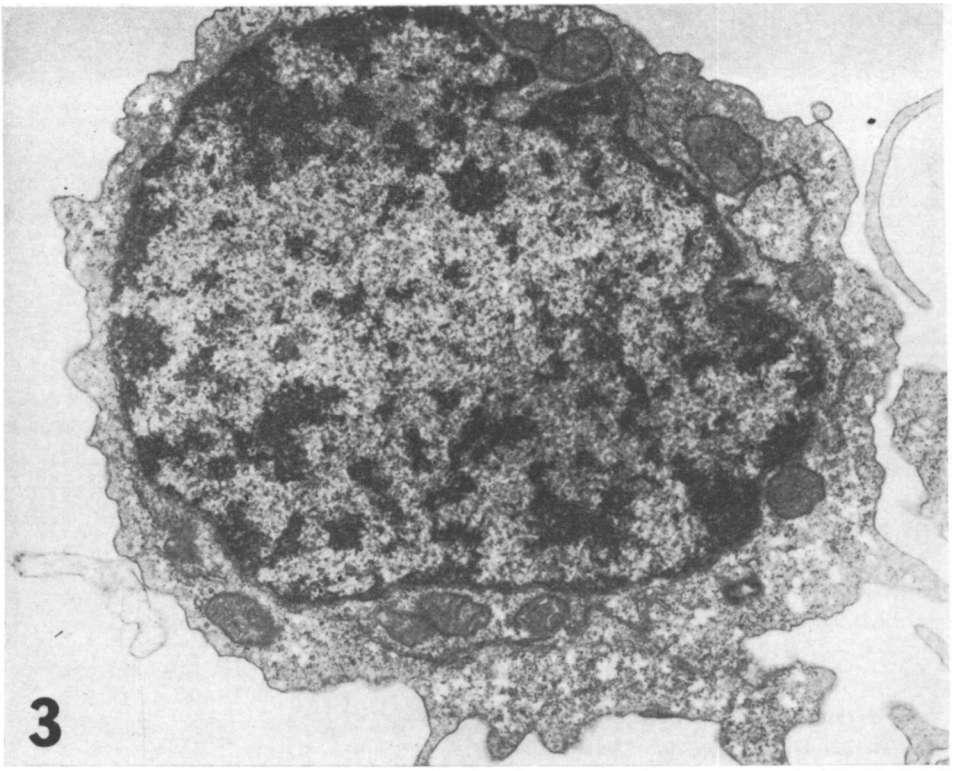
Since platinum is a heavy metal, it would be expected to be quite reactive with a number of cellular functional groups, notably by forming covalent bonds with nitrogen and sulfur. The monofunctional species arising from reaction (b) above would be expected to interact with nucleophilic centers such as —N= and -S- in a unity ratio, with the equilibrium of the interaction governed by the law of mass action

$$\text{(d)}\quad K_{\text{binding}} = \frac{[Pt(NH_3)_2ClX^+]}{[Pt(NH_3)_2Cl^+][X]},$$

where X = any cellular nucleophile. It is thus tentatively proposed that at high initial concentrations of *cis*-$[Pt(NH_3)_2Cl_2]^0$, there is, after an interval required for step (b) above, sufficient monofunctional binding to produce the early nonselective inhibition reported in the Ehrlich ascites carcinoma *in vivo* (1), and which was also observed with high inhibitor concentrations in the present *in vitro* study. At lower concentrations and after a period of time sufficient for reaction (c) above to ensue, the more stable bifunctional binding established between $Pt(NH_3)_2^{2+}$ and two closely adjacent nucleophilic centers would be manifest.

A striking parallelism can be seen between this postulated action mechanism and that of the bifunctional alkylating agents. Further, such a proposal is not incompatible with the prolonged duration of action noted in both the earlier (1) and the current study. At a recent international invitational symposium on the biological actions of selected platinum compounds held at Michigan State University, H. Harder (personal communication) stated that his work suggested such a possibility, and T. A. Connors (personal communication) reported that a line of the Walker 256 carcinoma with an induced resistance to *p*-bis-(β-chloroethyl)-aminophenylalanine also became resistant to *cis*-$[Pt(NH_3)_2Cl_2]^0$.

Thus, the experiments described here offer a possible pharmacological basis for the peripheral lymphocytopenia and marked depletion of thymic lymphocytes in rats treated with this compound (5). Since the response of the PHA-stimulated lymphocyte culture system is considered to be a reliable measure of the effects of potential immunosuppressive agents on the lymphoid organ (8), it seems that *cis*-$[Pt(NH_3)_2Cl_2]^0$ should receive consideration as a potentially useful suppressor



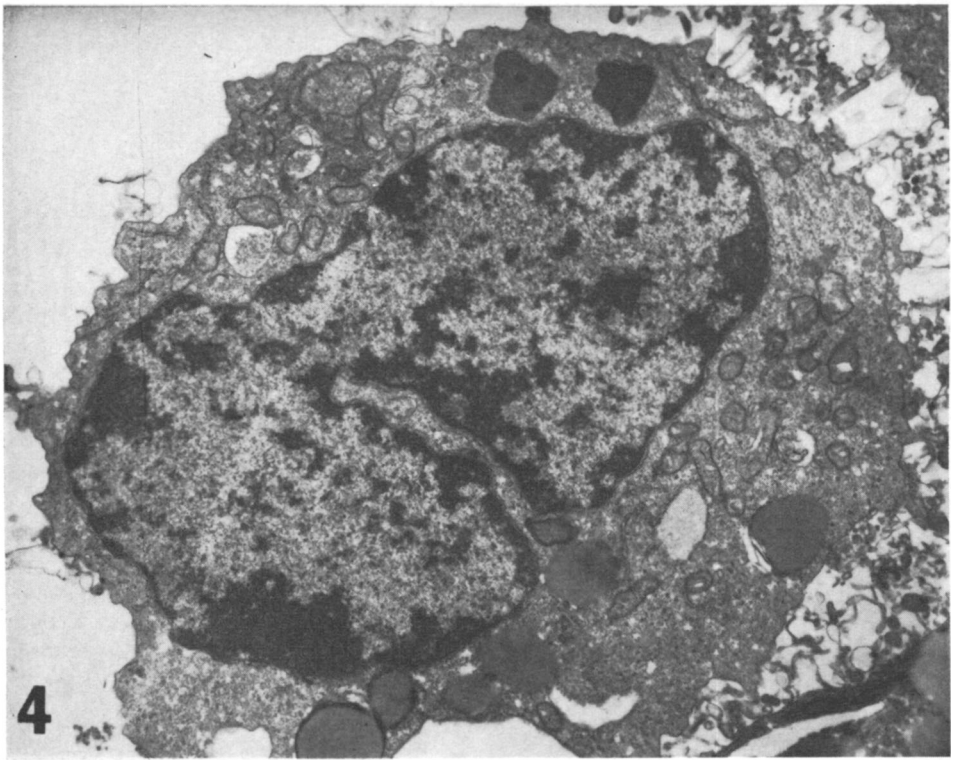


FIG. 3. Representative lymphocyte from control culture (×11,900).

FIG. 4. Representative lymphocyte cultured for 48 hr in the presence of *cis*-$[Pt(NH_3)_2Cl_2]^0$ at 3×10^{-5} *M* (×11,900).

of immune reactions.

Summary. *cis*-Dichlorodiammineplatinum (II) (*cis*-$[Pt(NH_3)_2Cl_2]^0$) is a potent and probably irreversible inhibitor of deoxyribonucleic acid synthesis in cultures of human lymphocytes stimulated with phytohemagglutinin mitogen. At low concentration (3×10^{-6} *M*), selectivity for this process is quite evident; rates of synthesis of ribonucleic acid and protein are only slightly diminished. No resolvable cytologic lesion coincident with the action of the inhibitor was observed by electron microscopy. It was proposed that *cis*-$[Pt(NH_3)_2Cl_2]^0$ may undergo two sequential transformations, with the loss of one Cl^- ion at each step, and that the resultant platinum species may act bifunctionally to cross-link adjacent nucleophilic centers through covalent binding.

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