THE RELATIONSHIP BETWEEN cis-Pt (II) AND HOST IMMUNE RESPONSE IN A SYNGENEIC SYSTEM

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

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1977

ABSTRACT

THE RELATIONSHIP BETWEEN *cis*-Pt(II) AND HOST IMMUNE RESPONSE IN A SYNGENEIC SYSTEM

By

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The exact mode of action of antitumor platinum compounds has not been determined, although much research has been devoted to this subject. One hypothesis, the enhanced antigenicity hypothesis, suggests that these antineoplastic agents [particularly *cis*-dichlorodiammineplatinum(II): *cis*-Pt(II)] function by enhancing the expression of tumor specific antigens on tumor cell surfaces, thereby allowing the intact immune system to attack and destroy the tumor. This study was undertaken to evaluate the relationship between the host immune response and the antitumor activity of *cis*-Pt(II).

Immunocompetent DBA/2 female mice were x-irradiated with 700 rads whole-body x-irradiation (to suppress the immune system), injected with 4×10^6 P388 lymphoma cells, and treated with *cis*-Pt(II). Comparing the growth curve of the P388 cells in this syngeneic system with that in various control groups, e.g., mice with tumor alone, mice with x-ray and tumor, and mice with tumor and *cis*-Pt(II),

insight into the relationship between the immune system and *cis*-Pt(II) was obtained.

The results of this study indicate that the ability of *cis*-Pt(II) to kill P388 tumor cells in this syngeneic system is not undermined by suppression of the immune response (as determined by the hemolytic plaque assay). Tumor cells are destroyed with equal or increased efficiency in *cis*-Pt(II)-treated, x-irradiated mice than in nonirradiated, tumored mice treated with *cis*-Pt(II).

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1977

Dedicated to Joan and Elizabeth

to whom I owe everything

ACKNOWLEDGEMENTS

I wish to thank Dr. Barnett Rosenberg, my research advisor, for his constant support of this research with regard to the facilities and research materials provided. His assistance and advice are greatly appreciated.

I cannot begin to express my appreciation to Loretta VanCamp, not only for her expert technical advice and assistance, but also for the many things she taught me that were non-science related. She was thoughtful and considerate of her fellow workers, and always managed a smile even in the most distressing situations.

Special thanks to Martha Thomas, my academic advisor and friend, for supplying some of the equipment for this research, and for putting up with me in trying times.

Deep appreciation is due to Dr. John Dunkel, who was always willing to unselfishly give of himself and his time to listen to my problems.

Thanks also to Dr. Harold Miller for his advice concerning the immunological aspects of this research, and to Barbara Laughter for teaching me the hemolytic plaque assay.

Appreciation is also expressed to Ann Grimes and Linda Glover for their assistance with this research project.

Special thanks and deep appreciation to my wife, Joan, for her support and love in difficult times.

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INTRODUCTION

The treatment of malignant disease by chemotherapy has been used increasingly for the past several years. Many effective chemotherapeutic agents with different modes of action have been utilized efficiently against different tumor types, and now are important tools in the oncologist's armamentarium.

Experimental evidence indicates that coordination complexes of platinum are potent antineoplastic agents. The most widely studied of these complexes, *cis*-dichlorodiammineplatinum(II): *cis*-Pt(II), has been shown to be a potent, broad spectrum antitumor compound in experimental animals and human patients.

Although a great deal of time and effort has been spent attempting to delineate the mode of action of these complexes, the exact mechanism remains an enigma. One hypothesis of particular interest suggests that the antitumor activity of platinum compounds is related to their ability to interact with the tumor-host immune system in such a way that the expression and recognition of tumor specific antigens is enhanced. This enhanced antigenicity hypothesis was proposed to interject specificity into the mode of action of these compounds. This was needed because, relative to nonneoplastic tissues (*in vivo*),

there is no selective uptake of these platinum complexes by neoplastic tissue.

This study was undertaken to examine the relationship between the host immune system and the antitumor activity of *cis*-Pt(II) in a syngeneic animal-tumor model. By modulating the immunocompetence of host mice, it is hoped that the validity of the enhanced antigenicity hypothesis can be tested.

LITERATURE REVIEW

The biological activity of platinum complexes has been known since its serendipitous discovery by Rosenberg and VanCamp in 1965 (36). They were studying the electric field effects on cultured *Escherichia coli* when they noticed that the bacteria were growing into long, filamentous rods, an indication that mitotic division had ceased. Attempts to isolate the component(s) responsible for the mitotic inhibition were successful (35), and it was found that with low frequency alternating current applied across the platinum electrodes, approximately 8 ppm of platinum went into solution in the steady state, forming what was then thought to be the $[PtCL_6]^{=}$ complex.

It was later observed (by L. VanCamp) that if solutions of the complex were allowed to stand for a few days their ability to induce filamentous growth in *Escherichia coli* increased. That is, some sort of photochemical change occurred which produced compounds more effective in inhibiting mitotic division. Subsequently, it was shown that when solutions of the doubly negative complex ($[PtCL_6]^{=}$), which is actually a bacteriocide, were irradiated by light, one or more ligand chloride ions was replaced by an ammonia group from the bacterial medium (see reactions 1 and 2). Replacement of one chloride ion produced the singly negative

$[PtCl_5NH_3]^-$ complex, which has little or no effect on *E. coli*. The neutral $[PtCl_4(NH_3)_2]^0$ complex, formed by the replacement of two chloride ions is the most stable form, and has no inhibitory effect on growth at concentrations where it completely inhibits *cell division*.

1) $[PtCl_6]^{-2} + NH_4 \xrightarrow{h\nu} [PtCl_5NH_3]^- + Cl^- + H^+$

2) $[PtCl_5NH_3]^- + NH_4 \xrightarrow{h\nu} [PtCl_4(NH_3)_2]^0 + Cl^- + H^+$

A variety of agents may cause filamentous growth in *Escherichia coli* (16). Cytokinesis in bacteria subjected to these agents, e.g., radiation, pressure, or temperature changes, may be reinitiated by treatment with pantoyl lactone, divalent cations or a temperature of 42° Centigrade. Rosenberg et al. (33) showed that the inhibitory effect of platinum on cytokinesis in bacteria could *not* be reversed by treatment with these agents and only by removal of the neutral platinum complex ($[PtCl_4(NH_3)_2]^0$) was cross-septation noticed. In addition, it was shown that only Gram negative bacilli were sensitive to the platinum complex with regard to its "antimitotic" activity.

Research to determine the antitumor activity of platinum compounds was completed in 1969 when Rosenberg et al. (34,37) reported that certain of the platinum complexes were effective inhibitors of L1210 leukemia and S-180 solid tumor in mice. Subsequent research indicated that platinum complexes (particularly *cis*-tetrachlorodiammineplatinum(IV) and *cis*-dichlorodiammineplatinum(II)

[*cis*-Pt(II)] were effective broad spectrum antitumor agents in a variety of hosts, against virally induced, chemically induced, and transplantable tumors. (See reference 30, page 401, for a review of this antitumor activity.)

In addition to studies involving the above mentioned tumors in experimental animals, numerous clinical trials in human patients have been completed. These trials have established the current mode of administration of *cis*-Pt(II) as well as its toxic effects on nonneoplastic tissue. While excellent results were obtained with *cis*-Pt(II) against testicular tumors (11,12), a considerable number of different tumor types have been shown to be sensitive to the drug as well. Thus, as in experimental animals, *cis*-Pt(II) has a wide range of antineoplastic activity in human patients (20,30).

The relationship between the structure and activity of platinum complexes has been studied by Cleare and Hoeschele (6). They observed a marked dissimilarity in the antitumor activity of *cis*- and *trans*-platinum analogues. When *trans* isomers exist, they were found to be inactive in comparison to the active *cis* isomer. The *trans* isomers are *more* reactive chemically and the *trans* isomer of $[Pt(NH_3)_2Cl_2]$ aquates four times faster and undergoes ammonation thirty times faster than the *cis* form (7). The authors suggested that the *trans* isomers apparently react with a wider variety of molecules than the *cis* form, hence decreasing the specificity of their action. This was suggested by ^{195m}Pt-labelled $[Pt(NH_3)_2Cl_2]$ excretion

studies which showed a higher initial excretion rate for the *cis* isomer. The whole-body retention of both isomers after five days was, however, comparable (approximately 20%) (6).

Replacement of the chloride ions in *cis*-$[Pt(NH_3)_2Cl_2]$ with other anions greatly affected the toxic and antitumor properties of the complex. Groups which were loosely bound to platinum, e.g., NO_3^-, H_2O, exhibited little or no antitumor activity and were highly toxic. Ions, e.g., Cl^- and Br^-, with an intermediate lability (less readily replaced by H_2O: reactions 3 and 4), demonstrated good antitumor activity with minimal toxicity, while those which were bound tightly to platinum, e.g., SCN^-, NO_2, and CN^-, exhibited no activity whatsoever, and little toxicity. It was suggested that readily replaced ligands react rapidly and nonspecifically *in vivo*, leaving little compound to react at sites where antitumor activity takes place. Complexes with tightly bound groups which reach the proper sites interact only minimally and have no antitumor activity.

3) $[Pt(II)(NH_3)_2Cl_2]^0 + H_2O \rightleftharpoons [Pt(II)(NH_3)_2Cl(H_2O)]^+ + Cl^-$

4) $[Pt(II)(NH_3)_2Cl(H_2O)]^+ + H_2O \rightleftharpoons [Pt(II)(NH_3)_2(H_2O)_2]^{++} + Cl^-$

Variation of the ammine group in $[Pt(II)(NH_3)_2Cl_2]$ modified the antitumor activity of the complex by virtue

of their differing steric, electronic and basic properties, perhaps by interfering with its ability to hydrogen bond to receptor biomolecules. This was suggested because increased alkyl substitution of the hydrogens in NH_3 led to decreased antineoplastic activity.

The charge of the platinum complex greatly affects its antitumor properties. To date, only neutral species have demonstrated activity. This is probably related to membrane transport functions and/or to the body's ability to excrete charged molecules (increased H_2O solubility) at a faster rate than noncharged ones.

From the experimental work cited above, Cleare and Hoeschele (5,6) have established a set of "empirical rules" that are essential for antitumor activity of platinum compounds. They are: (1) The complex should be neutral and (2) contain a pair of *cis* leaving groups. (3) The "window of liability" for the leaving groups should be centered on the chloride ion. (4) The "carrier ligands" should be strongly bonded, relatively inert ligands, preferentially amines. (5) The complexes are square planar or octahedral in configuration.

As early as 1970 data were being reported with regard to the mode of action of antitumor platinum complexes. Harder and Rosenberg (10) studied the effects of these compounds on DNA, RNA, and protein syntheses by measuring the incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-L-leucine in human amniotic AV_3 cells in tissue culture. They showed that at a concentration of 5 μM *cis*-Pt(II)

^{3}H-thymidine incorporation was inhibited, while ^{3}H-uridine and ^{3}H-L-leucine incorporation was affected only minimally. They stated that at concentrations of 5 μM *cis*-Pt(II) and below, DNA synthesis was selectively inhibited. At 25 μM (which is equivalent to a therapeutic dose of 8.0 mg/kg in mouse-tumor systems), DNA synthesis was more rapidly inhibited than RNA or protein synthesis: at 24 hours all three were completely inhibited.

A selective and *persistent* inhibition of DNA synthesis by *cis*-Pt(II) was demonstrated by Howle and Gale, working with Ehrlich ascites tumor cells (14). Based on the incorporation rates of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-L-leucine, they observed an initial inhibition of all three labelled precursors with a maximum depression at six to twelve hours after injection of *cis*-Pt(II). After this time, however, the rates of RNA and protein synthesis returned to normal levels, while the rate of DNA synthesis remained inhibited for 96 hours. The authors suggested a conversion of the injected neutral platinum species ($[cis\text{-}Pt(NH_3)_2Cl_2]^0$) into a metabolically active complex, which is responsible for the observed effects.

The interaction of *cis*-Pt(II) with DNA has been demonstrated by a number of researchers. Horacek and Drobnik (13) observed a shift in the UV absorption maximum of calf thymus DNA from 259 nm to 264 nm, after treatment with *cis*-Pt(II). The shift was minimized by increasing the concentration of chloride ions, hence decreasing the concentration of positively charged aqua complexes as in

equations 3 and 4. This had the effect of decreasing the affinity of the platinum complexes for DNA, by decreasing the probability of nucleophilic attack. The authors showed that the reaction of *cis*-Pt(II) facilitates renaturation and suggested that it may be due to interstrand cross-linking, an effect commonly observed with bifunctional alkylating agents.

Roberts and Pascoe (27) proposed that the antitumor activity of platinum complexes was the result of their ability to form crosslinks between complementary DNA strands. They developed an unequivocal method to demonstrate the crosslinking ability of these complexes. They reacted "heavy" single stranded DNA (made by growing cells in the presence of 5-bromo 2'-deoxyuridine:BUdR) with "light" single stranded DNA in the presence of *cis*-Pt(II) to produce "hybrid" double stranded DNA. After density gradient centrifugation with cesium chloride, and spectroscopic analysis, the presence of the crosslinked hybrid species could be determined. Working with the DNA from HeLa cells, they showed that hybridization did occur after treatment with *cis*-Pt(II) in a fashion similar to mustard gas, an agent known for its ability to crosslink DNA. By radioactively labelling the "heavy" DNA, quantitation of the extent of crosslinking was accomplished. It was found that the degree of crosslinking (by both *cis*- and *trans*-isomers of dichlorodiammineplatinum(II)) was directly dependent on the dose of the platinum complex. The authors suggested that the crosslinking occurred between the amino

groups of adjacent base pairs, particularly on guanine, adenine, and cytosine.

Mansy et al. (22) reported that guanosine, adenine, and cytidine were the bases that react with platinum compounds most readily. Based on UV spectrophotometric analysis at varying pH's, they suggested that the *cis* isomer (with two *cis* leaving groups) of dichlorodiammineplatinum(II) forms a bidentate chelate between the 6-NH_2 and either the N-1 or N-7 of adenosine and between the 4-NH_2 and N-3 of cytidine, while both isomers interact monofunctionally with the N-7 of guanosine. Uridine and thymidine were not bound by platinum. The distance between the chloride leaving groups is 3.3 Å (41), which is very close to the base stacking distance of 3.4 Å in the Watson Crick DNA model. The authors concluded that when adenosine and cytidine have the correct steric arrangement (as in DNA) they will form bidentate chelates with *cis*-Pt(II).

Shooter et al. (38) were able to show that interstrand platinum crosslinks between complementary DNA strands were of little importance in the inactivation of T7 bacteriophage. Munchausen (25), working with transforming DNA in *Hemophilus influenzae*, concluded that interstrand crosslinks with platinum complexes were too infrequent to account for the loss of transforming ability. However, she added that the integration of platinum-bound transforming DNA into the host genome was inhibited at a rate parallel to the loss of transforming ability. Hence,

the ability to form *intrastrand* crosslinks may play a role in the activity of platinum compounds as suggested by Thomson (40), Harder (9), and Rosenberg (32).

Guanosine has been implicated as the preferential binding site for platinum complexes (21,28,39). Recently, data obtained by x-ray photoelectron spectroscopy was presented which showed the involvement of the 0-6 of guanine in *cis*-Pt(II) binding (23). It was stated that the specificity of *cis*-Pt(II) could be explained by the bidentate chelate formed between the N-7 and 0-6 of guanine. Following DNA replication, this could lead to a base substitution mutation, and mispairing of bases in subsequent DNA strands.

An earlier hypothesis proposed by Rosenberg (29,30, 31,32) implicated the host immune response in the anti-tumor activity of platinum complexes. That is, because there is no selective uptake of these complexes by neoplastic tissues (42), their specific antineoplastic activity could not be explained. By imposing the host immune response into the mechanism of action, specificity could be obtained by virtue of the inherent specificity of immunocompetent cells for tumor specific antigens.

This "enhanced antigenicity" hypothesis has been suggested by data from viral transformation and immunological studies. Resolva (26) demonstrated that antitumor platinum complexes were potent inducers of lysogenic bacteria. That is, after incorporation of viral DNA (which was subsequently repressed) into the host genome,

treatment with *cis*-Pt(II) derepressed the viral genome, causing production of viral particles, and cell lysis. This was similar to other agents, e.g., radiation, x-rays, chemical carcinogens, etc., which likewise caused derepression of viral DNA and cytolysis. Vonka et al. (43) also showed that treatment of cultured human lymphocytes (transformed by Epstein-Barr virus) with *cis*-Pt(II) resulted in increased numbers of new virally-coded proteins on the cell surfaces.

In immunological studies (8) injecting hydrocortisone (a nonspecific immunosuppressant) into Swiss white mice with S-180 solid tumors (an allogeneic system) decreased the number of cures by 50% over controls after treatment with *cis*-Pt(II). In a syngeneic system (S-180 tumors in BALB/c mice) injection of Zymosan (a nonspecific immunostimulant) prior to treatment with *cis*-Pt(II) resulted in a 50% cure rate. The cure rate for mice without Zymosan stimulation was 0%.

Ironically, most platinum complexes with antitumor activity have demonstrated immunodepressant characteristics. *cis*-Pt(II) decreased the number of plaque forming cells in spleens removed from mice sensitized with sheep red blood cells (1,17). Brambilla et al. (3) observed a significant depression in skin allograft rejection (graft-vs-host) in mice after treatment with therapeutic levels of *cis*-Pt(II). Maximum effect was observed when the drug was administered 24 hours prior to grafting. Other research has shown that *cis*-Pt(II) inhibits nucleic acid synthesis

in lymphocytes stimulated with phytohemagglutinin (15) and suppresses lymphocyte blastogenesis in man (18).

MATERIALS AND METHODS

Experimental Animals

In all phases of this experiment, 9 week old female DBA/2 mice were used. They were obtained from Jackson Laboratory (Bar Harbor, Maine) and maintained in the laboratory of Dr. Barnett Rosenberg in the Biophysics Department. During the testing, eight mice were housed per cage and given food and water as required. To increase the life span of the mice following x-irradiation, acidified-chlorinated water was supplied to all mice. This was prepared by adding 4.0 ml concentrated hydrochloric acid and 4.5 ml bleach to 3.78 liters water.

Drugs

The *cis*-dichlorodiammineplatinum(II) [*cis*-Pt(II)] used in this experiment was made available by the Biophysics Department at Michigan State University. It was prepared for injection by mixing it with sterile physiological saline not more than 20 hours prior to the final injections. The mice were weighed and the *cis*-Pt(II) was administered by intraperitoneal injections (i.p.) on a multiple dose schedule, consisting of 1.5 mg/kg *cis*-Pt(II) every five hours for 25 hours. The total *cis*-Pt(II) concentration per mouse was 7.5 mg/kg body weight.

X-Irradiation

The source of x-irradiation was supplied by Dr. Ulreh Mostosky of the Veterinary Clinical Center, Michigan State University. Whole-body x-irradiation equivalent to 700 rads was applied to the mice by using 1 mm copper at 300 mVolts and 200 mAmps. The total dose was divided into four segments of 175 rads each, with an exposure time of 8.9 minutes per segment. After each time interval the mice were rotated in such a way as to insure uniform irradiation from mouse to mouse.

Tumor

The P388 tumor is a lymphoma originally induced in 1956 with methylcholanthrene. It grows in the ascites form, and is syngeneic at the H-2 locus with DBA/2 mice (4). The tumor was obtained from the A.D. Little Company in Cambridge, Massachusetts, and maintained in female DBA/2 mice by transfer every eight days. The transfer mice were killed by cervical dislocation eight days after injection of the tumor. The ascitic fluid was removed under sterile conditions, was mixed with sterile physiological saline (0.85% NaCl), and centrifuged at 500 rpm for five minutes at 4°C. The supernatant fluid was discarded to remove undesirable material, e.g., connective tissue and red blood cells.* The tumor cells were washed

*Mice with excessively bloody ascites were not used as transfer animals. Another mouse was selected and sacrificed.

repeatedly by this method until no red blood cells remained. The remaining P388 cells were then suspended in sterile saline, and a hemacytometer was used to determine their concentration in the suspension. The cells were then diluted (again with sterile saline) to a concentration of 4×10^6 cells in 0.2 ml. Two-tenths milliliter of the suspension was injected (i.p.) into the DBA/2 mice after cleansing the injection site with 70% ethyl alcohol. All mice developed the tumor, as evidenced by the profuse ascites observed eight days later.

Tumor Cell Quantitation

On given days, randomly selected mice from all experimental and control groups were sacrificed, and the ascitic fluid removed. Physiological saline was used to thoroughly wash the peritoneal cavity, i.e., until the washings were clear. The total volume of fluid was noted and an aliquot was diluted (1:500) in an autodiluter. This diluted specimen was then used for counting in a Coulter counter (model B).* The *total number of cells* present in the ascitic fluid was determined by multiplying the number of cells counted per milliliter of sample times the dilution factor times the total ascitic fluid volume. To establish the total *number of P388 cells* per mouse, a drop of the fluid was smeared on albuminized microscope slides, was fixed with 95% ethyl alcohol, and stained by the Papanicolaou

*Coulter Electronics Corporation, Hialeah, Florida.

technique. The slides were then examined and a differential cell count performed. In this manner the relative number of P388 cells was determined. This number, when expressed as a percentage and multiplied by the total number of cells present in the ascitic fluid, equals the total number of tumor cells present.

Ascitic fluids with detectable numbers of red blood cells after dilution were treated with Zap-Isoton, the standard red cell lysing agent used for Coulter counting. It had no effect on the tumor cells.

Experimental Scheme

Randomly selected female DBA/2 mice were arbitrarily divided into two groups: irradiated and nonirradiated. The irradiated group received 700 rads whole-body x-irradiation on day -1. The following day (day 0), both groups of mice were injected with 4×10^6 P388 cells. On the day following tumor cell injection (day 1) both groups (irradiated and nonirradiated) were again divided (randomly) with roughly half of the irradiated and nonirradiated groups each getting multiple injections of *cis*-Pt(II). Thus, four groups of animals were produced. The first group (untreated control) was injected with only 4×10^6 P388 cells. The second group was x-irradiated and injected with 4×10^6 P388 cells. Group three was injected with 4×10^6 P388 cells, and 5 x 1.5 mg/kg *cis*-Pt(II). The fourth group was x-irradiated and injected with 4×10^6 P388 cells and 5 x 1.5 mg/kg *cis*-Pt(II) (experimental group).

Beginning on day 1 (after three *cis*-Pt(II) injections) three animals from each group were sacrificed (by cervical dislocation) and the ascitic fluid removed. The total number of P388 tumor cells was determined as described above, and the values averaged for the three mice. This was continued until the mice were depleted.

Hemolytic Plaque Assay

The hemolytic plaque assay (modified Jerne plaque assay) was used to determine the efficiency of the x-irradiation to eradicate the immune response in the host mice. A set of mice other than those used in the actual experiments was randomly divided into two groups: irradiated and nonirradiated. The irradiated group was again divided into two groups. One group received 700 rads whole-body x-irradiation, while the other received 700 rads x-irradiation, 4×10^6 P388 cells (i.p.) and five injections (i.p.) of 1.5 mg/kg *cis*-Pt(II).* The nonirradiated DBA/2 mice were separated into three groups, one of which served as a normal control. Of the two remaining groups, one was injected with 4×10^6 P388 cells (i.p.) and five injections of 1.5 mg/kg *cis*-Pt(II), while the other received only 4×10^6 P388 cells. Several hours following injection of the P388 cells, 5×10^8 sheep red blood cells (SRBC's) were injected into the lateral tail

*The same scheme of x-irradiation followed by tumor cells followed by *cis*-Pt(II) that was used for the experimental mice was employed for the hemolytic plaque assay.

vein of the mice. Four days following injection of the SRBC's, all mice were sacrificed, their spleens were removed, and the assay performed (19).

RESULTS

Trial 1

The Growth Curve of P388 Cells in DBA/2 Female Mice

Nine week old female DBA/2 mice were injected (i.p.) with 4 x 10^6 P388 lymphoma cells on day 0. Beginning on day 1 and on each day thereafter, 3 mice were sacrificed and the average number of tumor cells in each mouse quantitated as described above. The average number of P388 cells per day is presented in Table I. A plot of these data as a function of time is shown in Figure 1.

The mean day of death for this group of mice was 10.1 days.

The Growth Curve of P388 Cells in X-Irradiated DBA/2 Female Mice

Seven hundred rads whole-body x-irradiation was administered to nine week old female DBA/2 mice on day -1. On day 0 they received 4 x 10^6 P388 cells by i.p. injection.

The mean day of death for these mice was 6.3 days, which is consistent with the results of other x-irradiation studies (24). No animal died from tumor burden, even though sizable ascitic fluids were observed in all mice. Death was probably due to x-irradiation and subsequent bacterial

Table I. Number of P388 cells in DBA/2 female mice: Trial 1

Time in days	Avg. number tumor cells in mice	Standard deviation
1	5.53×10^6	2.69×10^5
2	2.06×10^7	2.65×10^5
3	6.68×10^7	2.28×10^7
4	2.35×10^8	4.76×10^7
5	3.06×10^8	4.36×10^7
6	5.25×10^8	5.97×10^7
7	6.09×10^8	5.71×10^7
8	8.47×10^8	8.75×10^7

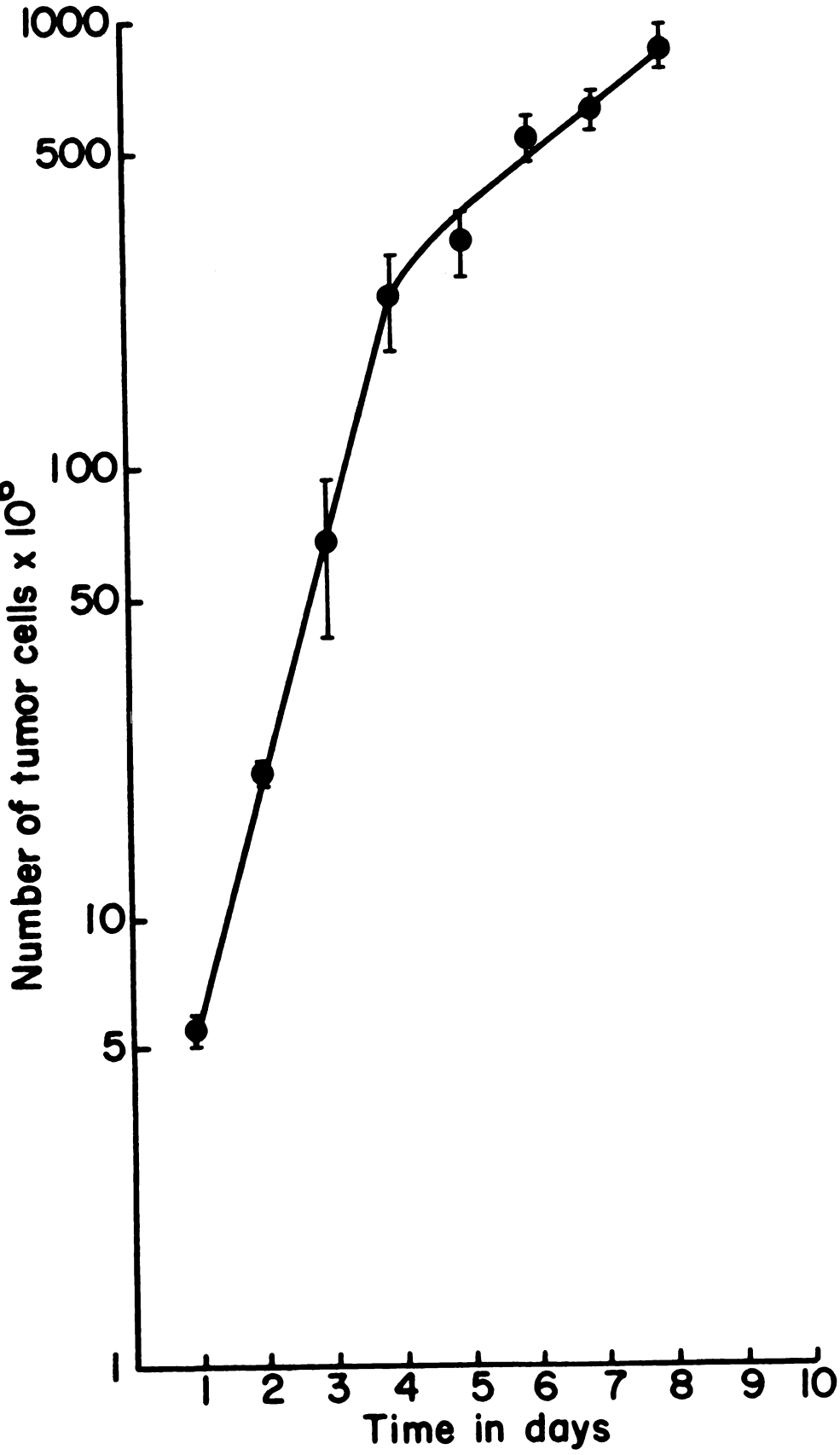

Figure 1. Growth curve of P388 cells in DBA/2 female mice: Trial 1.

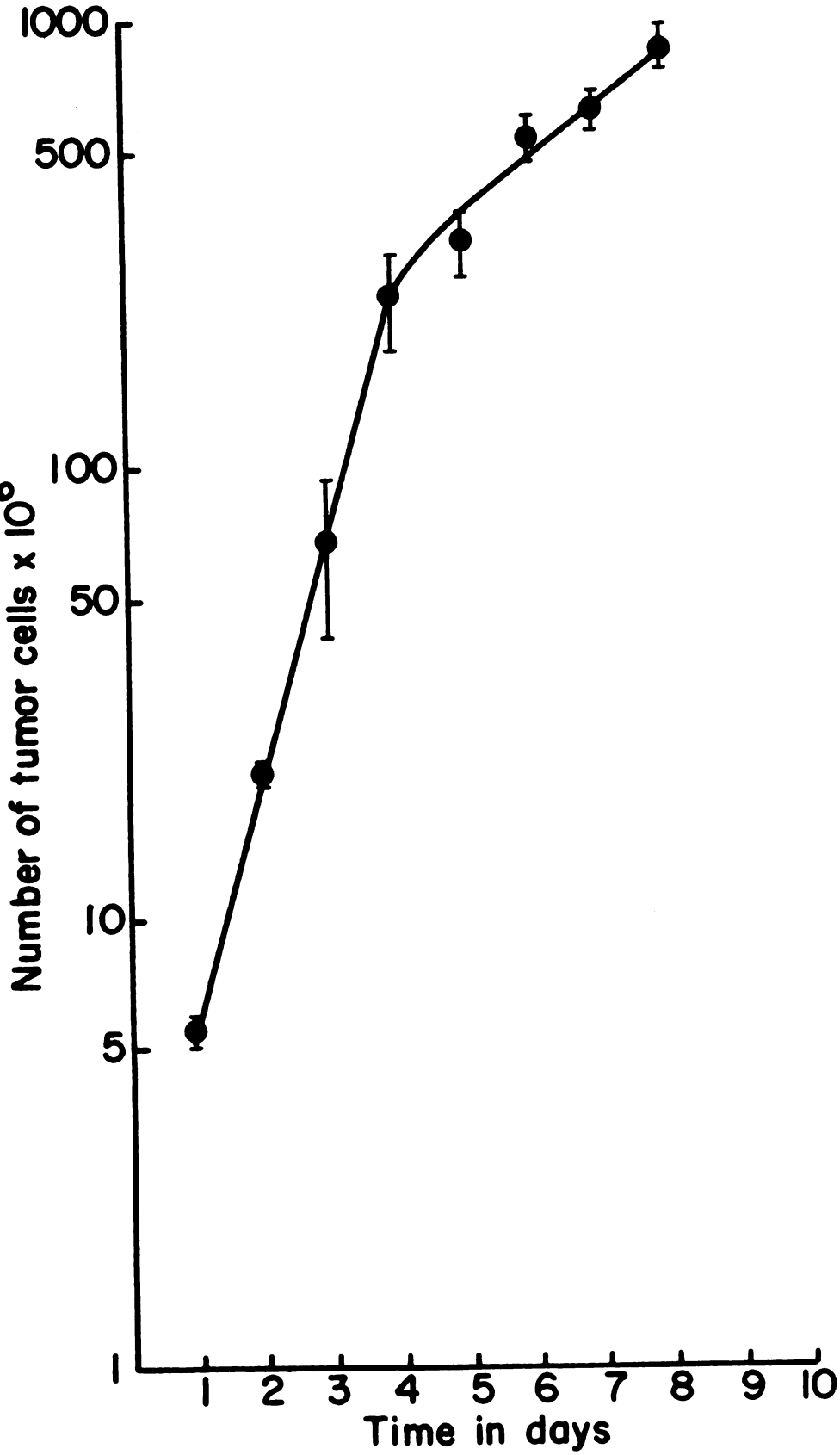

Figure 1. Growth curve of P388 cells in DBA/2 female mice: Trial 1.

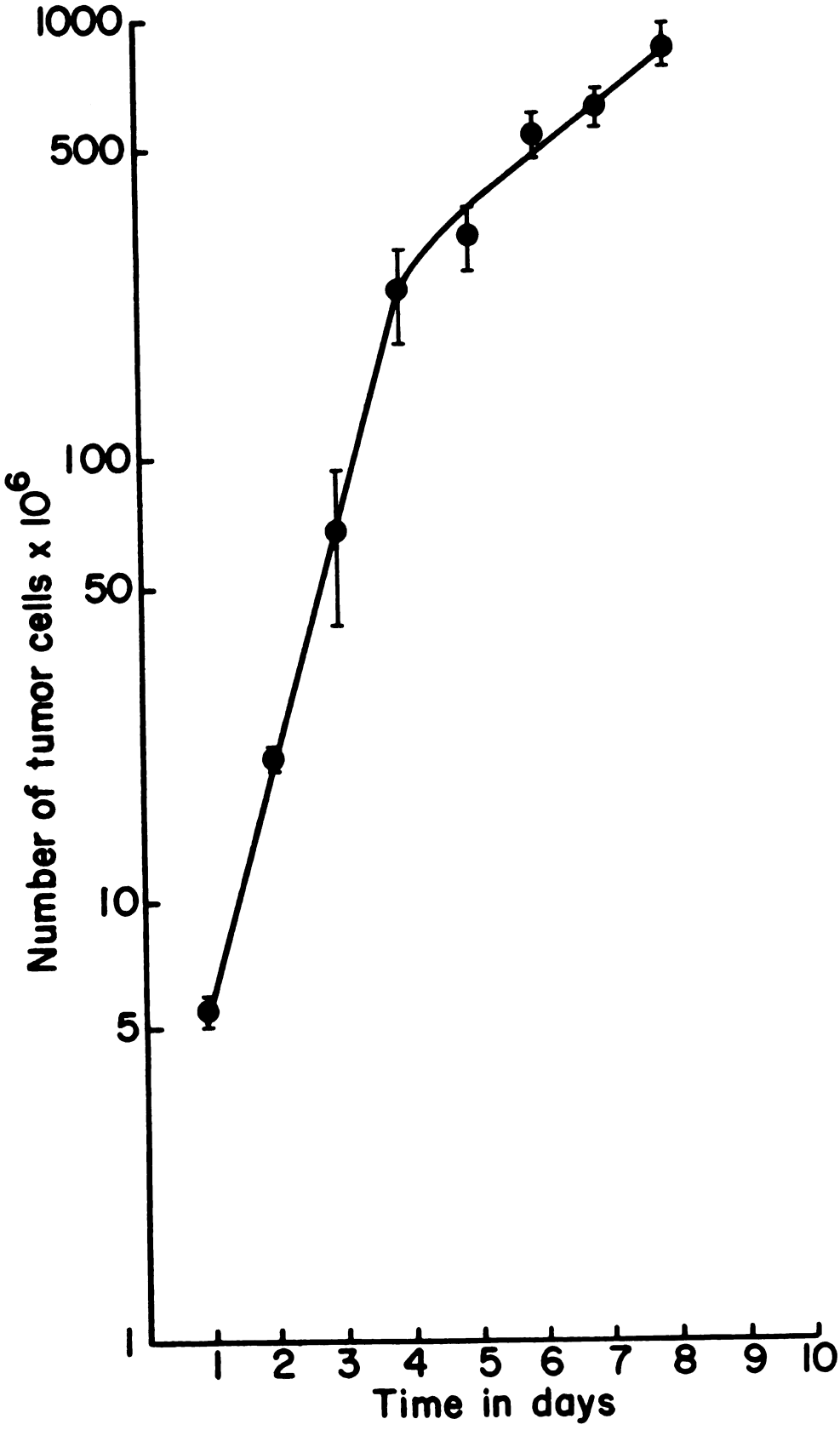

Figure 1. Growth curve of P388 cells in DBA/2 female mice: Trial 1.

infection, although the exact cause of death was not determined.

The average number of P388 cells per day is presented in Table II. These data are shown graphically in Figure 2.

The Growth Curve of P388 Cells in DBA/2 Female Mice Treated with 5 x 1.5 mg/kg *cis*-Pt(II)

Nine week old female DBA/2 mice were injected (i.p.) with 4 x 10^6 P388 cells on day 0 and 5 x 1.5 mg/kg *cis*-Pt(II) on day 1.

The mean day of death for these animals was 24.9 days. No mouse in this group died before day 17, and the last three mice died on day 30. No cures were observed, and most of the mice eventually showed evidence of ascites by day 15.

The therapeutic effectiveness of *cis*-Pt(II) on the P388 lymphoma can be established by calculating the increased life span (ILS) of the treated animals as compared with the untreated animals. Thus, the ILS for this tumor-treatment system was 146%.

The average number of P388 cells per day is presented in Table III. A plot of the average number of P388 cells as a function of time is shown in Figure 3.

The Growth Curve of P388 Cells in X-Irradiated DBA/2 Female Mice Treated with 5 x 1.5 mg/kg *cis*-Pt(II)

X-irradiated DBA/2 mice injected with 4 x 10^6 P388 lymphoma cells (day 0) and treated with 5 x 1.5 mg/kg

Table II. Number of P388 cells in irradiated DBA/2 female mice: Trial 1

Time in days	Avg. number tumor cells in mice	Standard deviation
1	3.40×10^6	4.55×10^5
2	7.86×10^6	3.60×10^6
3	1.91×10^7	7.00×10^6
4	9.76×10^7	6.46×10^7
5	1.18×10^8	4.63×10^7
6	1.68×10^8	1.87×10^7
7	--	--
8	--	--

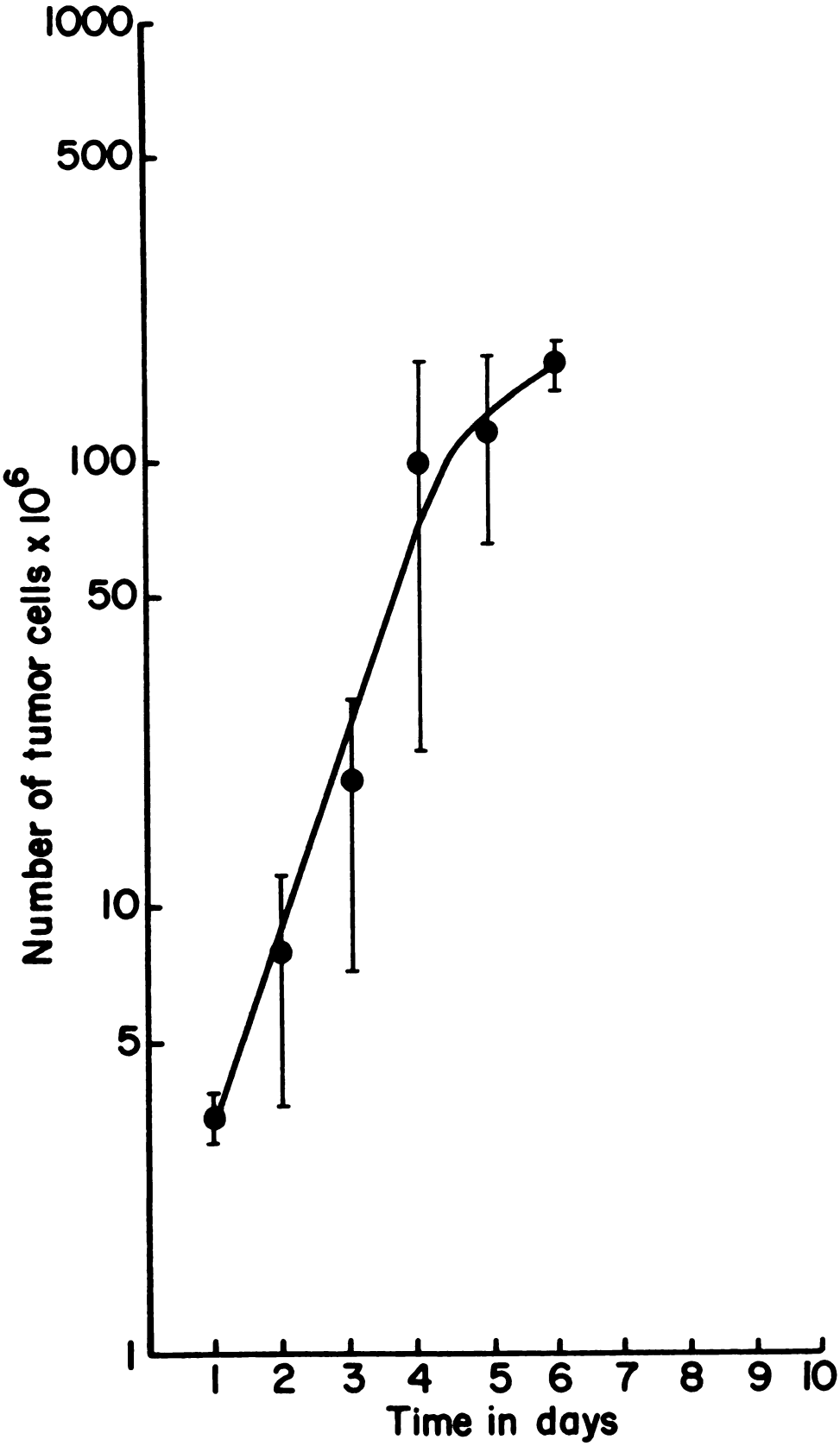

Figure 2. Growth curve of P388 cells in irradiated DBA/2 female mice: Trial 1.

Table III. Number of P388 cells in DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 1

Time in days	Avg. number tumor cells in mice	Standard deviation
1	6.41×10^6	2.29×10^6
2	4.21×10^6	1.32×10^6
3	2.89×10^6	8.52×10^5
4	2.51×10^6	8.50×10^5
5	2.12×10^6	1.06×10^6
6	2.36×10^6	9.47×10^5
7	2.29×10^6	1.34×10^6
8	2.11×10^6	5.37×10^5

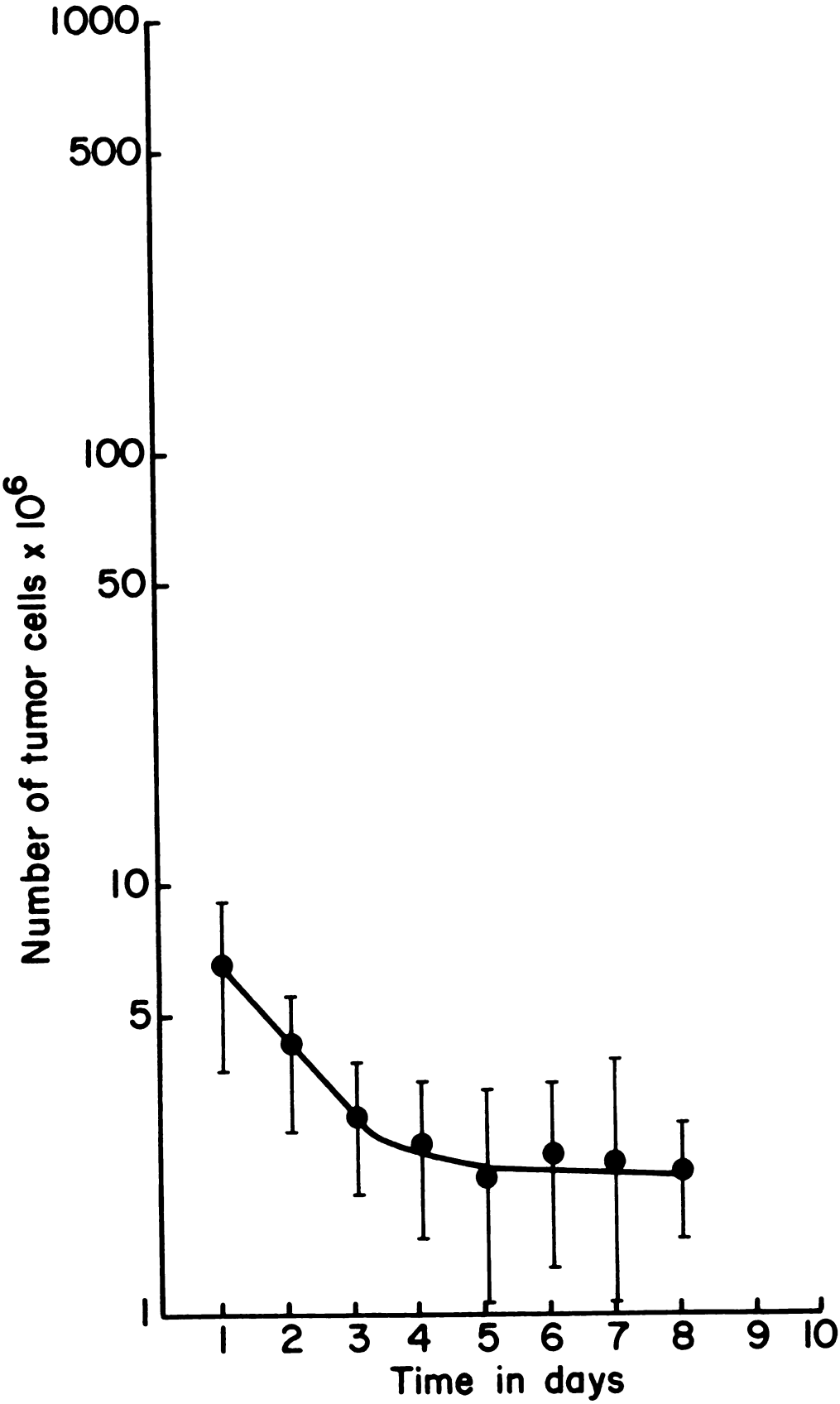

Figure 3. Growth curve of P388 cells in DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 1.

cis-Pt(II) had a mean day of death of 5.5 days. This value was similar to that for irradiated tumored mice without *cis*-Pt(II). No deaths were caused by tumor.

The average number of P388 cells per day is presented in Table IV. These data are shown graphically in Figure 4.

Trial 2

The experimental scheme used in Trial 1 was repeated exactly for Trial 2, and the results of this trial are presented below.

The Growth Curve of P388 Cells in DBA/2 Female Mice

The mean day of death of DBA/2 mice injected with 4×10^6 P388 cells (day 0) was 9.8 days, with a range of 8 to 12 days. Table V shows the average number of P388 cells per day. A graphic representation of these data is shown in Figure 5. For comparison's sake, Figure 6 is a composite of the graphs in Figures 1 and 5.

The Growth Curve of P388 Cells in X-Irradiated DBA/2 Female Mice

X-irradiated DBA/2 female mice injected with 4×10^6 P388 cells had a mean day of death of 6.5 days. Two animals died on day 5, three died on day 6, and nine mice died on day 7. As in Trial 1, no animals died from tumor burden, even though ascites was prominent.

The average number of P388 cells per day is presented in Table VI. A plot of these data as a function of time

Table IV. Number of P388 cells in irradiated DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 1

Time in days	Avg. number tumor cells in mice	Standard deviation
1	4.94×10^6	1.08×10^6
2	2.23×10^6	1.01×10^6
3	8.91×10^5	2.90×10^5
4	3.01×10^5	1.87×10^5
5	2.71×10^5	2.59×10^5
6	2.11×10^5	1.67×10^5
7	--	--
8	--	--

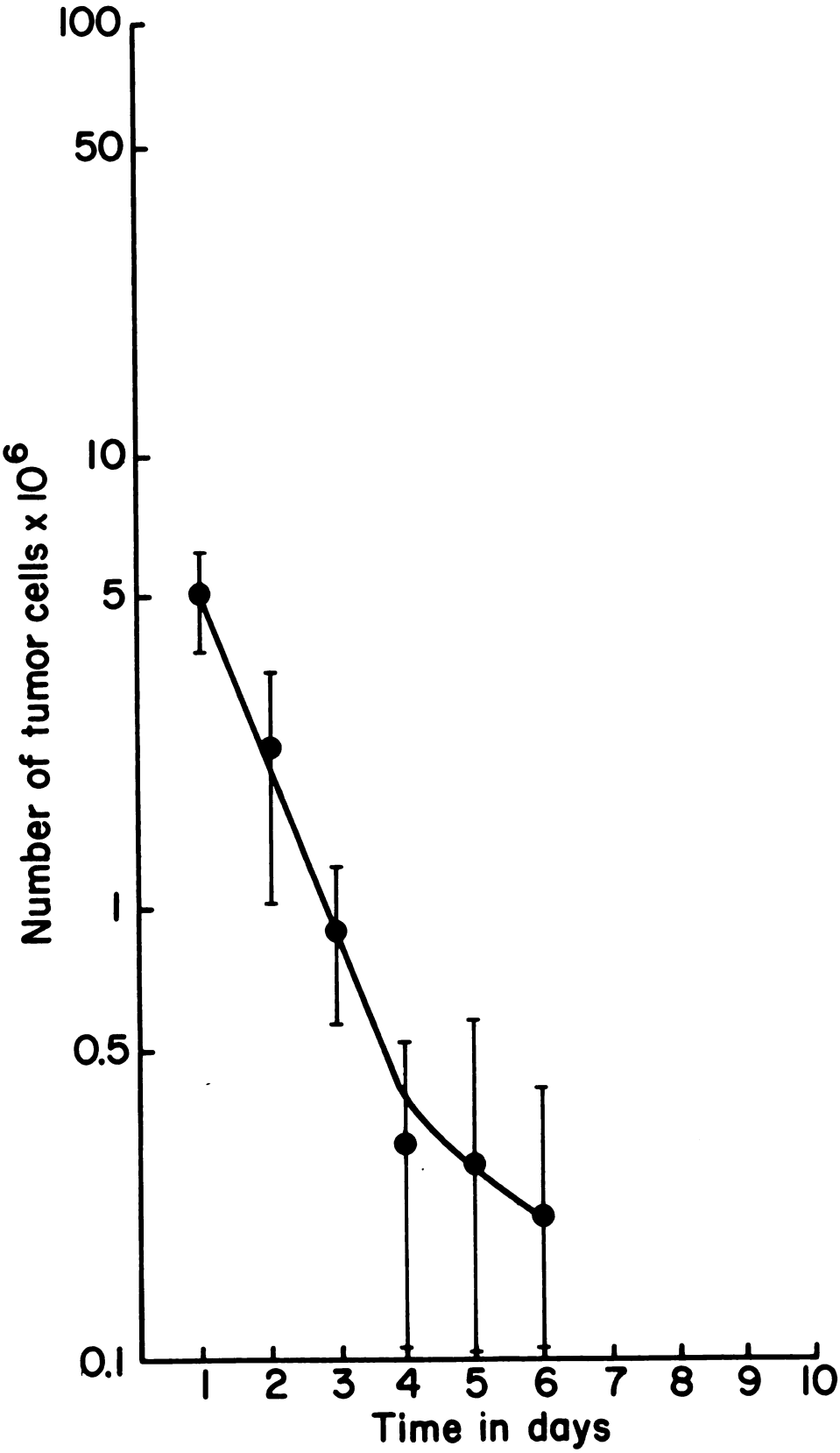

Figure 4. Growth curve of P388 cells in irradiated DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 1.

Table V. Number of P388 cells in DBA/2 female mice: Trial 2

Time in days	Avg. number tumor cells in mice	Standard deviation
1	1.12×10^7	1.58×10^6
2	3.25×10^7	1.07×10^7
3	1.10×10^8	6.23×10^7
4	2.76×10^8	5.23×10^7
5	4.56×10^8	5.46×10^7
6	6.57×10^8	1.09×10^8
7	9.12×10^8	1.55×10^8
8	1.04×10^9	1.33×10^9

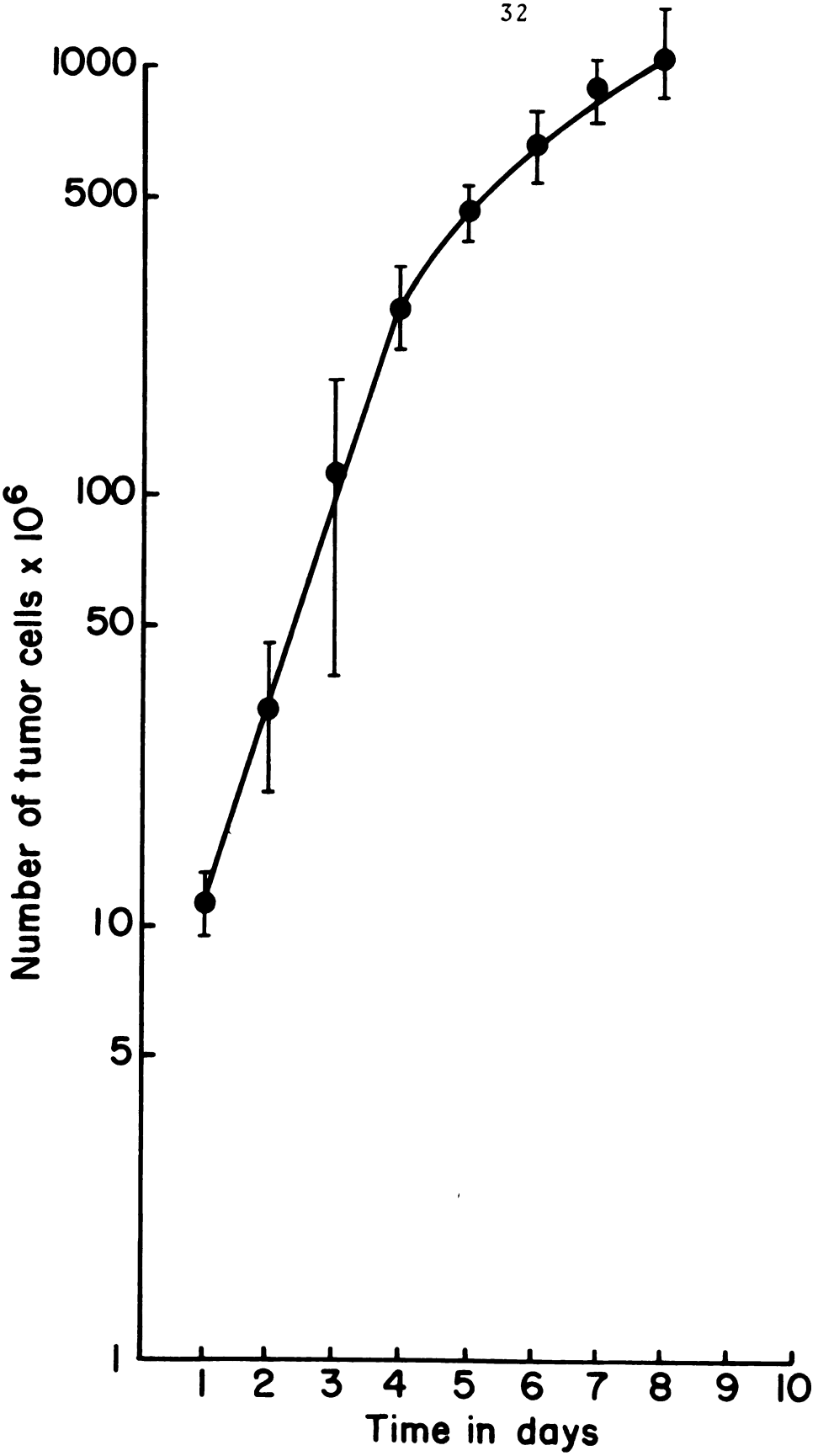

Figure 5. Growth curve of P388 cells in DBA/2 female mice: Trial 2.

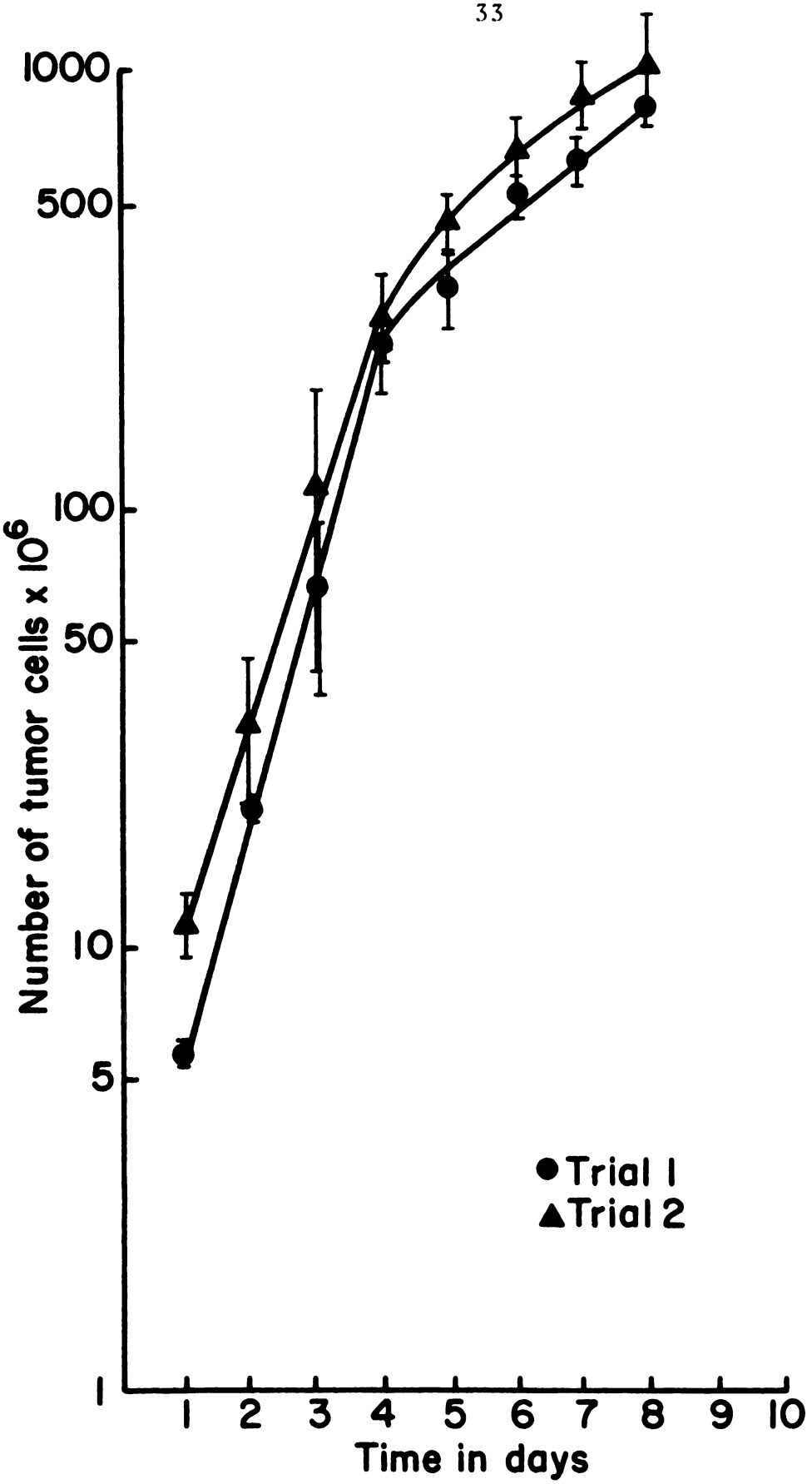

Figure 6. Growth curves of P388 cells in DBA/2 female mice: Trials 1 and 2.

Table VI. Number of P388 cells in irradiated DBA/2 female mice: Trial 2

Time in days	Avg. number tumor cells in mice	Standard deviation
1	7.29×10^6	4.44×10^6
2	1.59×10^7	4.53×10^6
3	6.21×10^7	8.41×10^6
4	2.58×10^8	6.16×10^7
5	3.51×10^8	7.91×10^7
6	5.17×10^8	3.22×10^7
7	5.01×10^8	3.41×10^8
8	--	--

is shown in Figure 7. Figure 8 is a composite of Figures 2 and 7.

The Growth Curve of P388 Cells in DBA/2 Female Mice Treated with 5 x 1.5 mg/kg *cis*-Pt(II)

The mean day of death of mice injected with 4 x 10^6 P388 cells and treated with 5 x 1.5 mg/kg *cis*-Pt(II) was 22.2 days. This value, as well as the calculated ILS of 127%, is consistent with the results of Trial 1.

The average number of P388 cells per day is presented in Table VII, and a graph of these data is shown in Figure 9. Figure 10 is a composite of Figures 3 and 9.

The Growth Curve of P388 Cells in X-Irradiated DBA/2 Female Mice Treated with 5 x 1.5 mg/kg *cis*-Pt(II)

X-irradiated DBA/2 mice injected with 4 x 10^6 P388 cells and treated with 5 x 1.5 mg/kg *cis*-Pt(II) had a mean day of death of 6.0 days. It is consistent with the mean day of death reported for Trial 1. Again, no animal died from tumor burden.

The average number of P388 cells per day is presented in Table VIII. These data are presented graphically in Figure 11, and a composite of this graph and Figure 4 is shown in Figure 12.

The Number of Plaque Forming Cells in Spleens from DBA/2 Mice Sensitized with Sheep Red Blood Cells

DBA/2 mice, treated as described in the materials and methods section, were injected with 5 x 10^8 sheep red blood

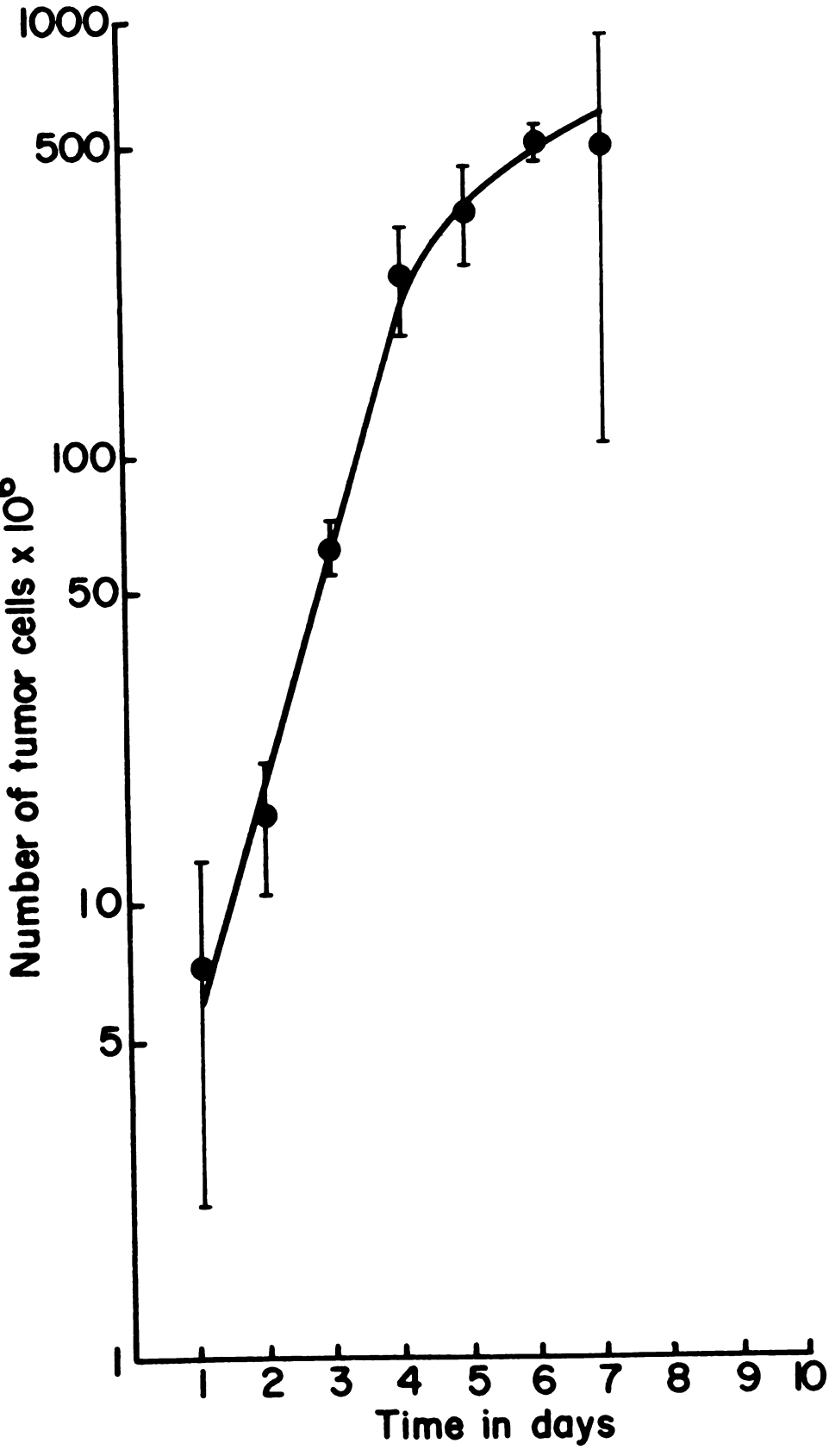

Figure 7. Growth curve of P388 cells in irradiated DBA/2 female mice: Trial 2.

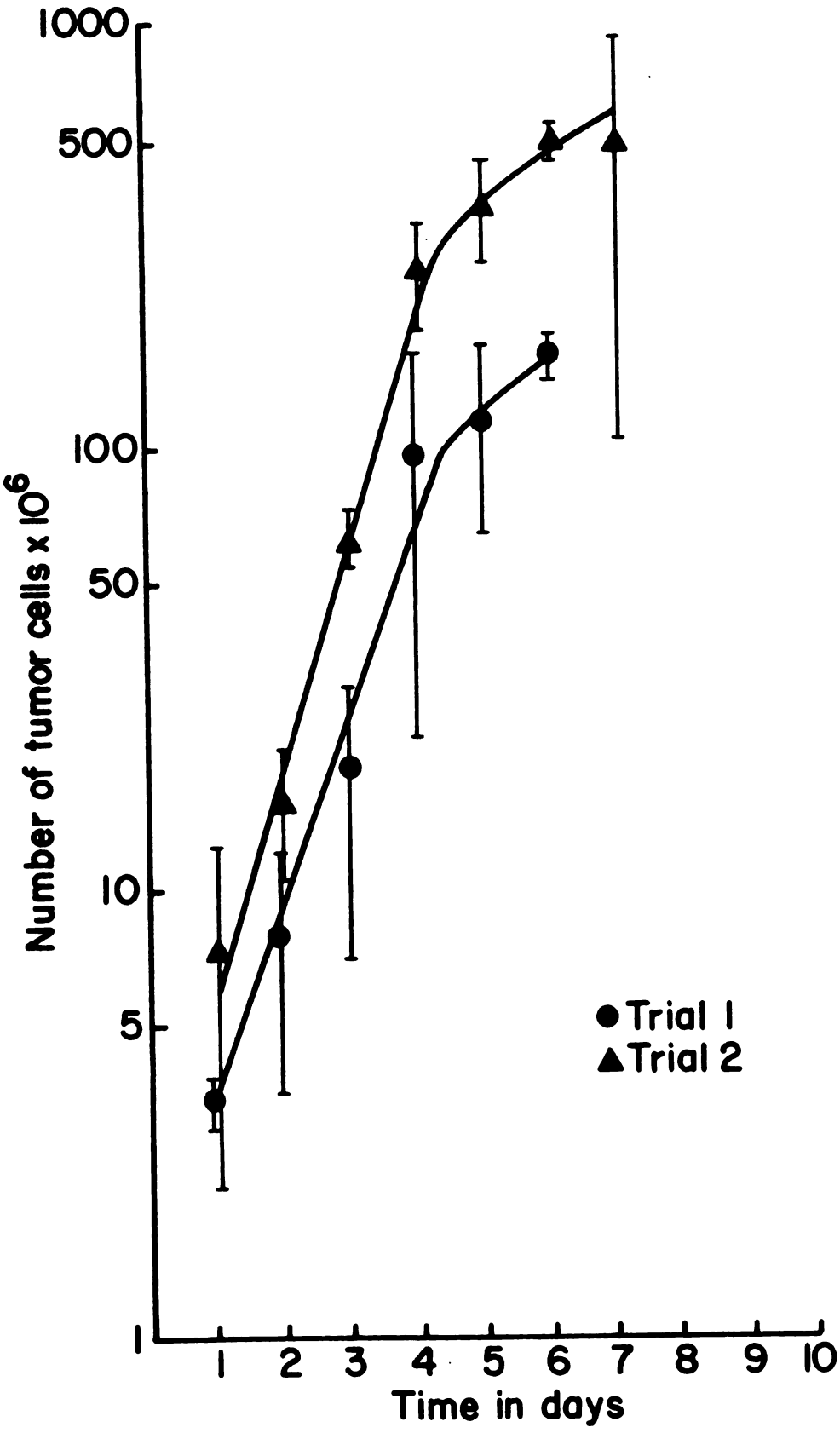

Figure 8. Growth curves of P388 cells in irradiated DBA/2 female mice: Trials 1 and 2.

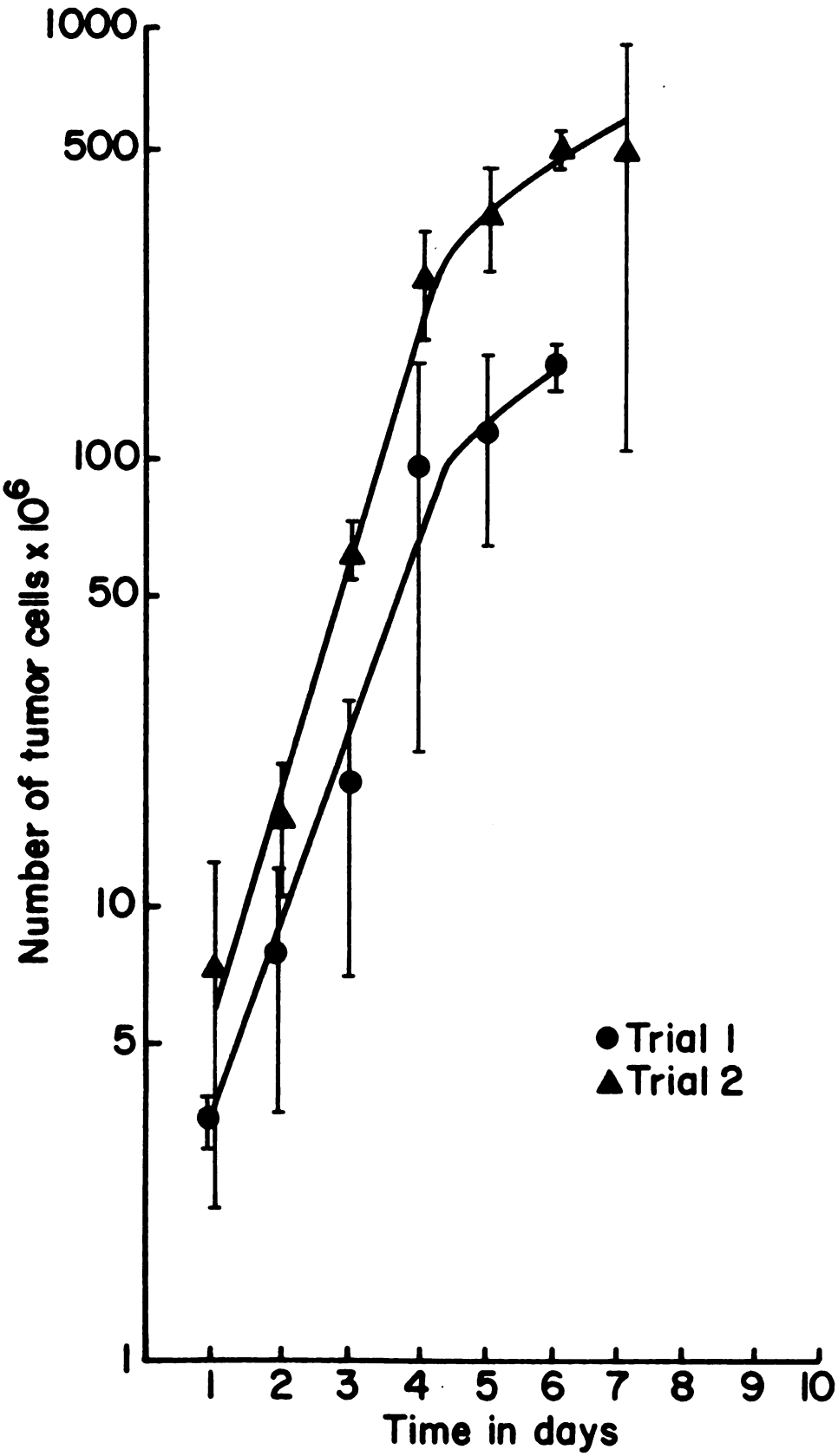

Figure 8. Growth curves of P388 cells in irradiated DBA/2 female mice: Trials 1 and 2.

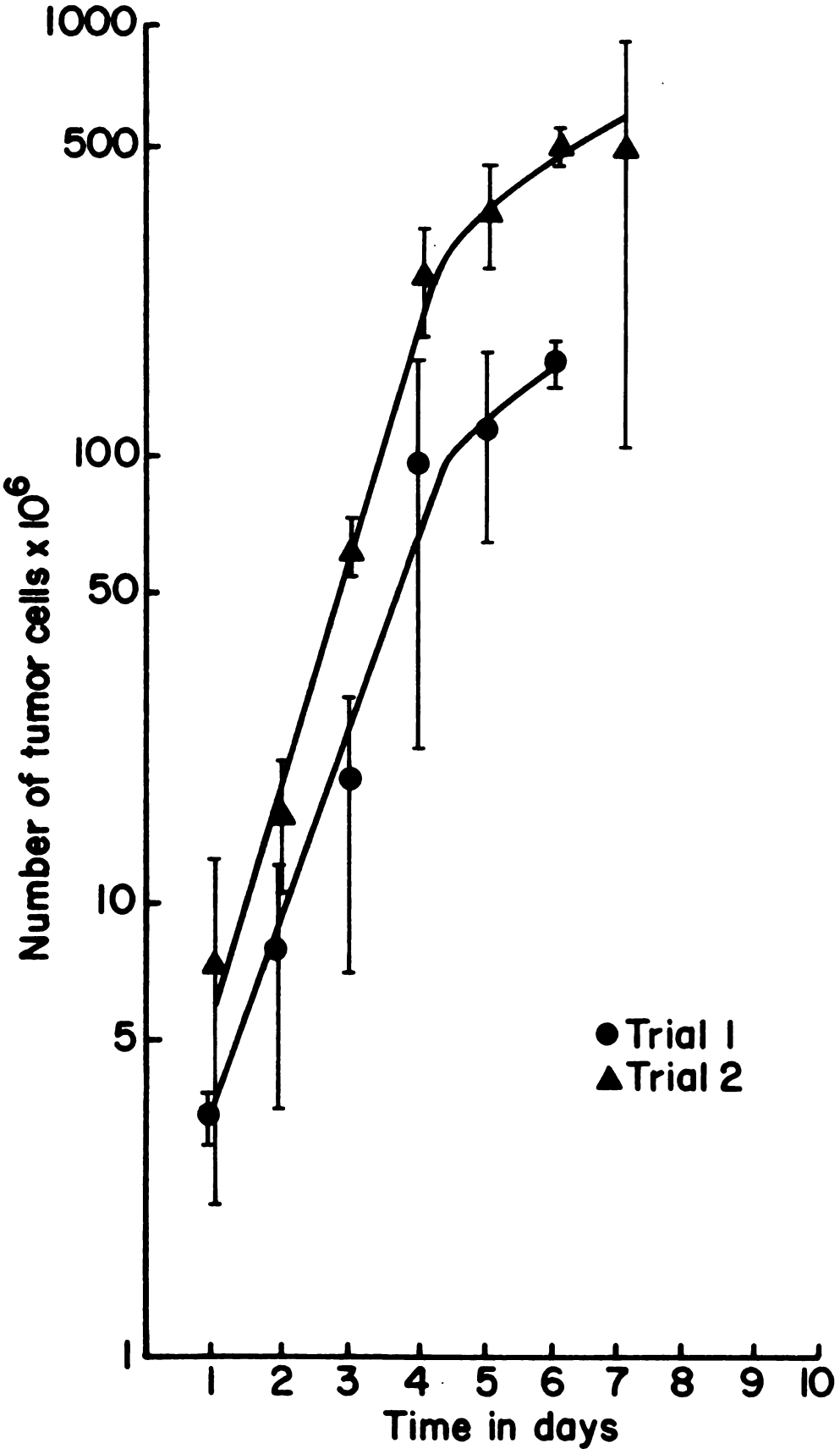

Figure 8. Growth curves of P388 cells in irradiated DBA/2 female mice: Trials 1 and 2.

Table VII. Number of P388 cells in DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 2

Time in days	Avg. number tumor cells in mice	Standard deviation
1	6.47×10^6	1.96×10^6
2	9.21×10^6	4.65×10^6
3	7.22×10^6	3.29×10^6
4	5.25×10^6	6.10×10^5
5	4.59×10^6	2.72×10^6
6	3.61×10^6	8.34×10^5
7	4.22×10^6	3.41×10^6
8	2.76×10^6	1.80×10^6

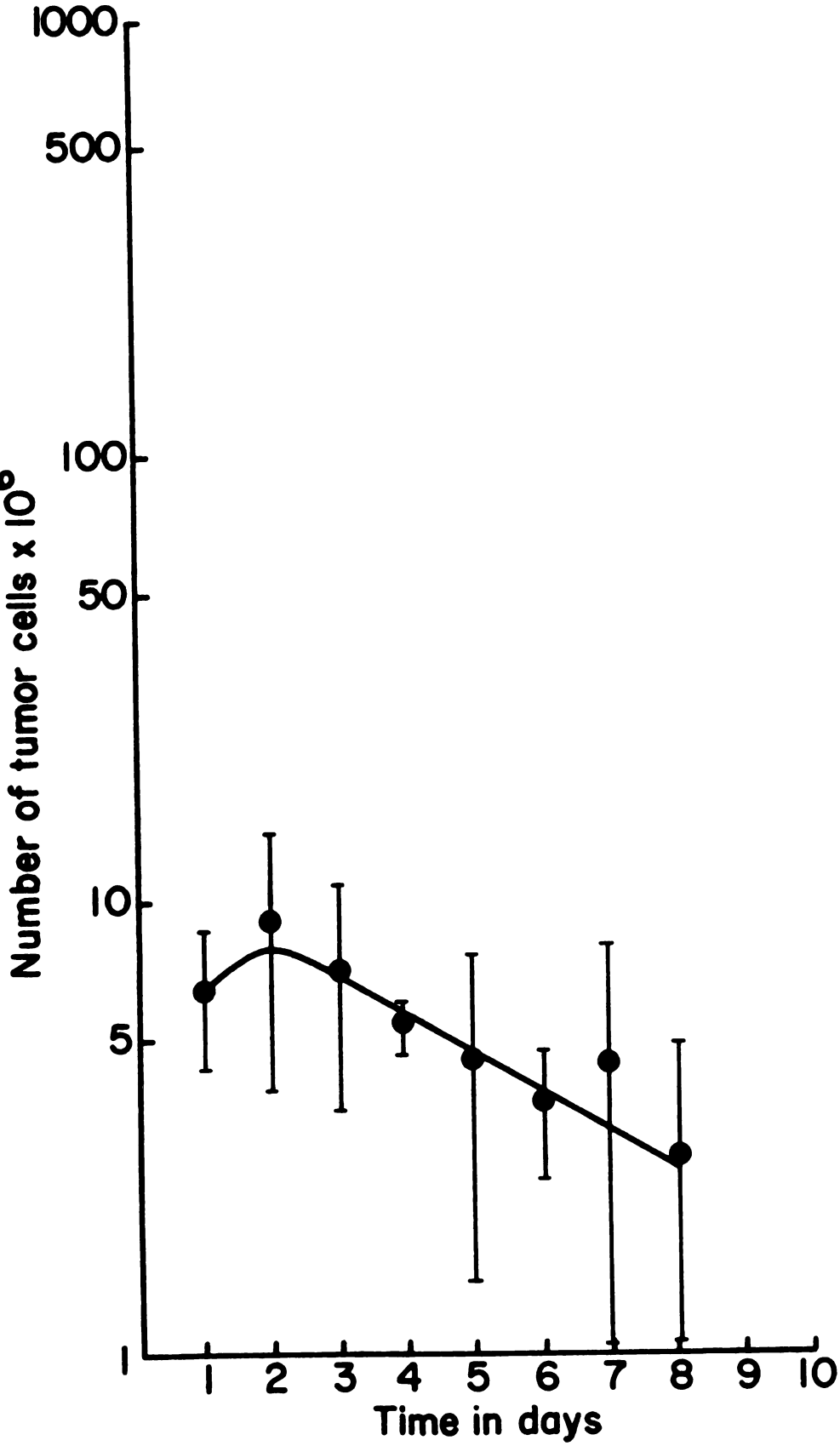

Figure 9. Growth curve of P388 cells in DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 2.

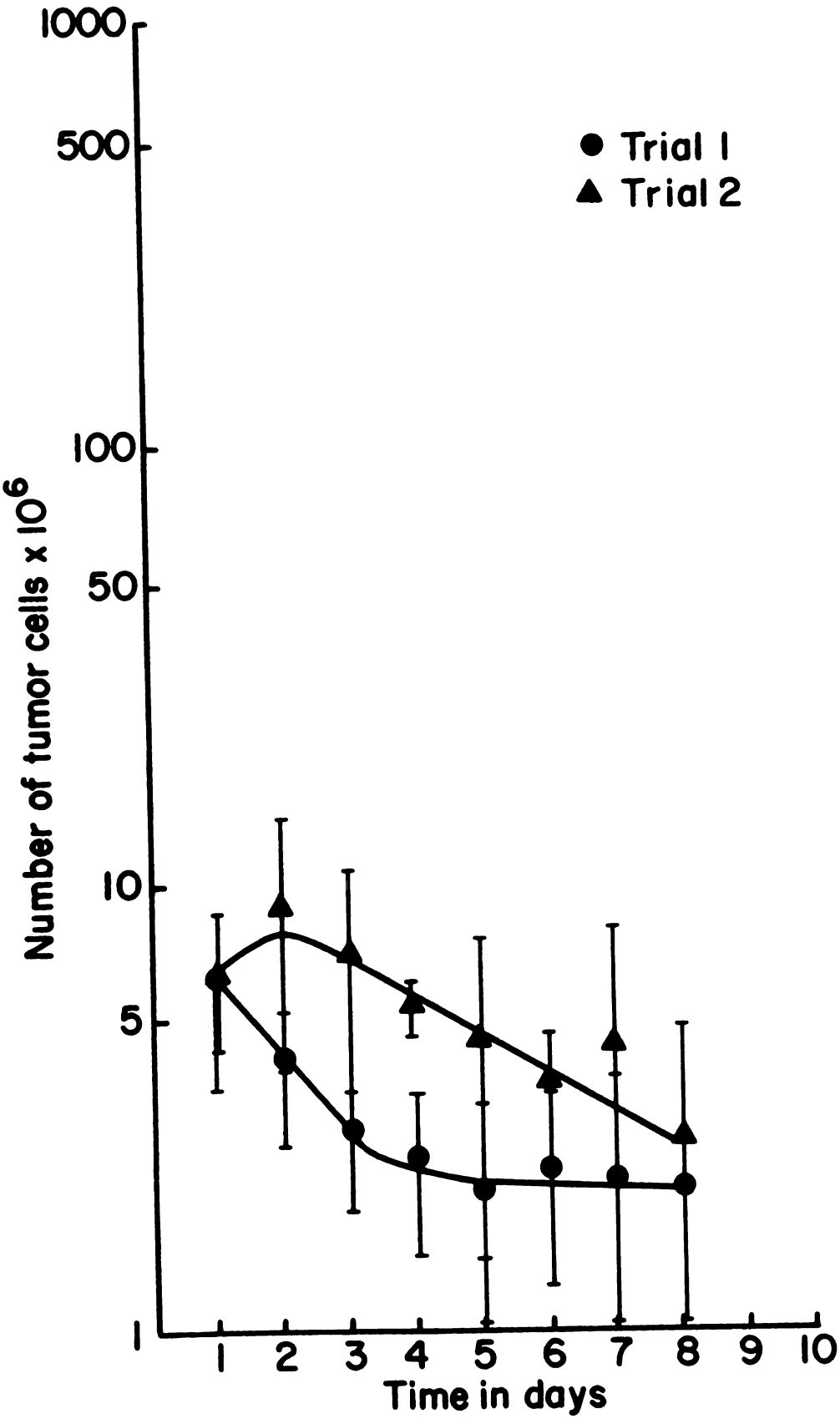

Figure 10. Growth curves of P388 cells in DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trials 1 and 2.

Table VIII. Number of P388 cells in irradiated DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 2

Time in days	Avg. number tumor cells in mice	Standard deviation
1	5.45×10^6	8.73×10^5
2	4.79×10^6	7.93×10^5
3	1.95×10^6	9.36×10^5
4	7.00×10^5	2.90×10^5
5	6.01×10^5	2.10×10^5
6	6.75×10^5	9.29×10^4
7	3.45×10^5	1.41×10^5
8	--	--

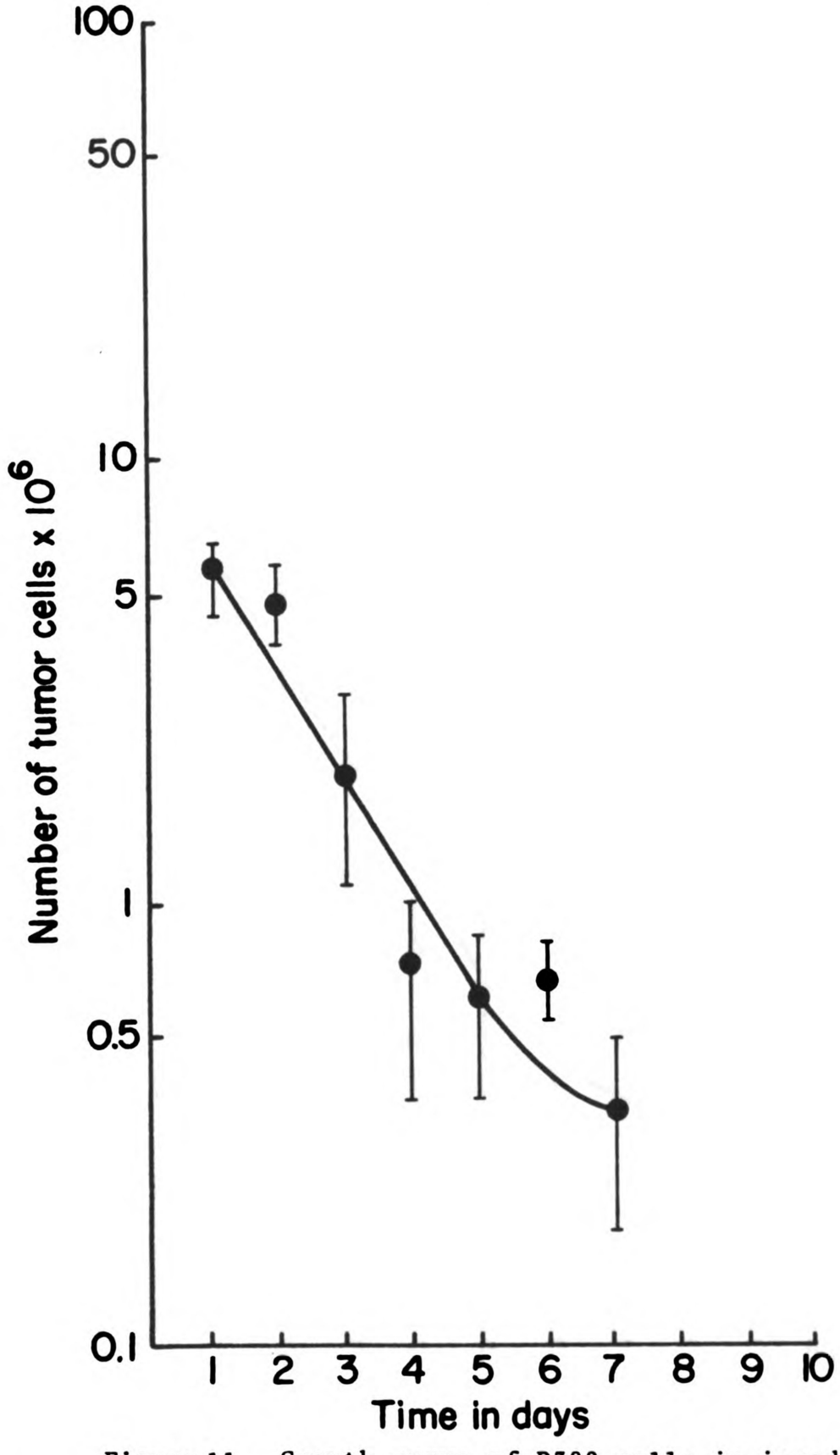

Figure 11. Growth curve of P388 cells in irradiated DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trial 2.

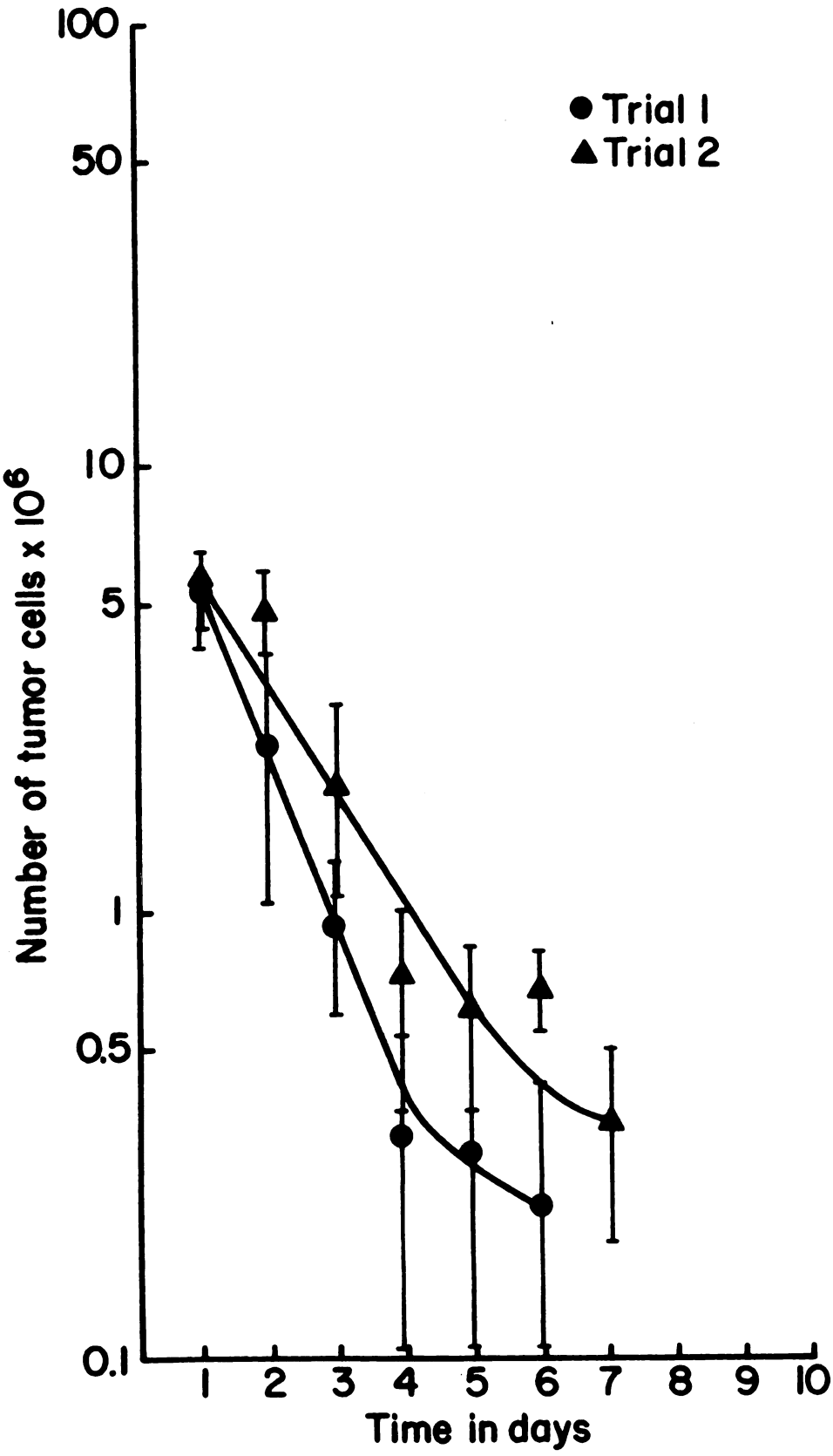

Figure 12. Growth curves of P388 cells in irradiated DBA/2 female mice treated with 5 x 1.5 mg/kg *cis*-Pt(II): Trials 1 and 2.

cells on day 0. They were sacrificed on day 4 and their spleens were removed. The hemolytic plaque assay was performed and the number of plaque forming cells (PFC) per spleen was determined. The results of this assay are shown in Table IX.

The difference in numbers of plaque forming cells between control and nonirradiated groups was not significant.

Table IX. Antibody response in spleens as measured by the hemolytic plaque assay

Experimental group	Number of mice	Number of direct plaque forming cells/spleen
Control	4	$2.44 \times 10^3 \pm 1.34 \times 10^3$
X-irradiation	6	0
X-irradiation + P388 cells + *cis*-Pt(II)	6	0
P388 cells + *cis*-Pt(II)	5	$1.21 \times 10^3 \pm 4.94 \times 10^2$
P388 cells	3	$3.92 \times 10^3 \pm 1.09 \times 10^3$

DISCUSSION

In a syngeneic host-tumor system such as the one used in this study, one would expect a "linear" increase in the number of tumor cells as a function of time, and no spontaneous cures due to H-2 incompatibility. Figure 1, the standard growth curve for the P388 lymphoma in immunocompetent DBA/2 female mice, indicates that this mouse-tumor system is usable as a positive control for further studies.

Figure 2 demonstrates the effect of prior x-irradiation on the growth of P388 cells. While the absolute number of tumor cells per day in this group of mice is consistently lower than for corresponding days in Figure 1 (for an undetermined reason), the slopes of both curves are approximately the same. This indicates that 700 rads x-irradiation when administered prior to injection of tumor cells has little effect on their rate of proliferation.

The efficiency of *cis*-Pt(II) to destroy P388 cells in this syngeneic system is shown in Figure 3. On day 1, an average of 6.41×10^6 P388 cells were present. This number decreases steadily until the fourth day, when the number of tumor cells plateaus. Comparing this graph with those in Figures 1 and 2, it is evident that a dosage of 1.5 mg/kg *cis*-Pt(II) when administered in 5 i.p. injections

over a period of 25 hours is effective against the P388 lymphoma.

As reported in the results section of this thesis, the ILS for this group of mice was 146%.

As shown in Table IX, mice which received 700 rads whole-body x-irradiation were devoid of the ability to produce antibodies to a challenge of sheep red blood cells. Figure 4 demonstrates graphically the ability of *cis*-Pt(II) to eradicate tumor cells in immunologically depressed mice. In this group of mice there is a parallel if not greater effect of *cis*-Pt(II) on the number of P388 cells per day than in the previous group of immunocompetent mice (Figure 3). In fact, a more precipitous decrease in the number of tumor cells can be seen in Figure 4. As reported by Zak (44), when 700 rads x-ray were administered *prior* to *cis*-Pt(II), there was a slight additive effect of their toxicities. This additive effect could account for the greater decrease in the number of P388 cells in these mice.

Examining the graphs of the data for Trial 2 (Figures 5, 7, 9 and 11) and comparing these with the graphs for Trial 1 indicates that there is a high degree of reproducibility in the numbers of tumor cells per day per group of mice. This reproducibility suggests that the methods used in this study are reliable, and that the conclusions drawn from the data are significant.

Scrutinizing the composite graphs (Figures 6, 8, 10 and 12) demonstrates an interesting feature. Note that in

Figure 6 the number of P388 cells per day for Trial 1 is consistently below that for corresponding days in Trial 2. The same phenomenon can be seen in all subsequent composite figures, and it is of particular significance in Figure 10. (The ILS for Trial 2 is also lower than that for Trial 1: 127% and 146%, respectively.) The number of tumor cells in this group of mice on day 1 is 6.47×10^6, and 9.21×10^6 on day 2. This modest increase in the number of cells suggests that there may be a relationship between the total *absolute* number of tumor cells injected on day 0 and the dosage of *cis*-Pt(II) required to substantially reduce their numbers. That is, is the initial effectiveness of the drug dependent upon tumor mass (numbers of tumor cells)? While research examining this relationship must be completed for verification, I propose that a larger number of P388 cells was injected on day 0 in Trial 2 (compared with Trial 1) and that perhaps this could explain the transient increase in the number of tumor cells (in this and other studies) shortly after treatment with *cis*-Pt(II).

With regard to the syngeneic animal tumor system that was used in this study, it is unlikely that the antitumor activity of *cis*-Pt(II) is related to enhancement of tumor specific antigens (TSA's). By choosing a syngeneic experimental system, bias was placed in *favor* of the enhanced antigenicity hypothesis. That is, by not using an allogeneic tumor-mouse system the possibility of interference by H-2 histocompatibility antigens was diminished. Hence,

if immune enhancement by *cis*-Pt(II) were to occur, it would be directed at TSA's and not H-2 antigens.

if immune enhancement by *cis*-Pt(II) were to occur, it would be directed at TSA's and not H-2 antigens.

CONCLUSIONS

By comparing the growth curves of P388 cells in untreated control mice with the growth curve with P388 cells in x-irradiated mice treated with *cis*-Pt(II), insight into the relationship between the host immune system and the antitumor activity of *cis*-Pt(II) was obtained. Based upon the data presented in this study, it is clear that the antineoplastic property of this compound is *not* related to its ability to enhance expression and recognition of tumor specific antigens as proposed by the enhanced antigenicity hypothesis.

It has been shown that *cis*-Pt(II) is effective in reducing the number of P388 cells in immunocompetent DBA/2 mice (Figure 10). If this activity is dependent upon an intact host immune response (which can be modulated by x-irradiation: Table IX), then the growth curve of P388 cells in x-irradiated *cis*-Pt(II) treated mice (Figure 12) should resemble that of control groups (Figures 6 and 8). What was observed instead was a more rapid decline in the number of tumor cells per day. Thus, there is no dependence of *cis*-Pt(II) on the immune response to effect initial tumor cell death in this system.

This does not imply that in immunocompetent animals *cis*-Pt(II) does not function by reducing the tumor burden

to such a level that the immune response can "handle" the tumor load and effect a cure. In fact, it would be surprising if this were not the case.

It has been suggested (2) that an increase in the number of tumor cells following *cis*-Pt(II) injection was related to host intervention, thus implicating the immune system as inherent in the mode of action of *cis*-Pt(II). It is conceivable, however, that these transient increases in the number of tumor cells can be explained by the possible relationship between initial tumor mass (burden) and dose of *cis*-Pt(II). This is suggested as an alternative explanation. To examine this proposition, a study similar to this one must be done on an allogeneic mouse-tumor system using different quantities of tumor cells relative to the dose of platinum. This could establish a dose-response relationship and could provide insight into the reason for the transient increase in cell numbers as described above.

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VITA

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After attending summer classes at the Kellogg Biological Station in Hickory Corners, Michigan, he enlisted in the U.S. Army. In 1973 he was honor graduate of the U.S. Army School of Cytotechnology (ASCP) and was subsequently stationed at Tripler Army Medical Center (TAMC), Honolulu, Hawaii. While assigned at TAMC, he worked in the clinical cytology laboratory and was responsible for teaching cytology to resident pathologists and staff personnel.

Upon returning to the mainland as a civilian in August, 1975, he enrolled in the Clinical Laboratory Science program at Michigan State University as a Master of Science candidate. He was lead instructor of cytotechnology at the Lansing Community College School of Cytotechnology from September, 1975, to June, 1977.

The author married Joan DeVregud on the 29th of December, 1972, and their first child, Elizabeth Marie, was born on January 11, 1977.