THE ROLE OF HOST DEFENSES IN CIS-DICHLORODIAMMINEPLATINUM(II) MEDIATED REGRESSIONS OF SARCOMA 180 IN MICE

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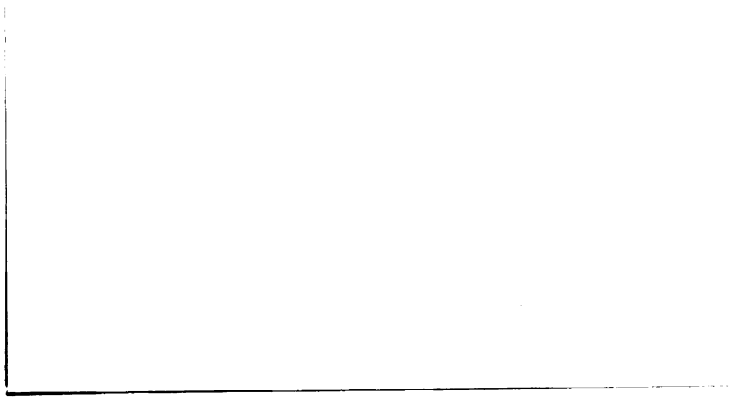

ABSTRACT

THE ROLE OF HOST DEFENSES IN CIS-DICHLORODIAMMINEPLATINUM(II) MEDIATED REGRESSIONS OF SARCOMA-180 IN MICE

By

Philip B. Conran

The role of host defenses in mediating the regression of Sarcoma 180 (S-180) tumors in mice treated with cis-Dichlorodiammineplatinum(II) [cis -Pt(II)] was investigated.

It was found that the marked antitumor efficacy of cis-Pt(II) against S-180 implanted in Swiss mice was reduced when hydrocortisone (HC), an immunosuppressive drug, was administered 7 days before, 6 hours after or 7 days after the platinum compound. This reduction was most dramatic when HC was administered 7 days before or 6 hours after the platinum compound.

In another series of experiments it was found that cis-Pt(II) was ineffective in promoting regressions of S-180 implanted in BALB/c mice. The administration of zymosan, a nonspecific immune stimulant, in combination with cis-Pt(II), however, promoted significant numbers of tumor regressions. This was particularly true if the zymosan was administered on day 1 of tumor growth followed in 7 days by a single injection of cis-Pt(II).

From the results of the previously described experiments it was concluded that the antitumor efficacy of cis-Pt(II) is, at least in part, dependent on an active host response directed against the tumor.

The immunologic integrity of BALB/c mice treated with a combination of zymosan and cis-Pt(II) was studied. Humoral antibody production was evaluated by the agarose slide technique using sheep red blood cells as antigen. There was virtually no difference in spleen cell plaque forming ability between control and treated animals when the antigen was administered on day 14 of the experiment. When the antigen was administered on day 21 of the experiment, however, those animals treated with a combination of zymosan and cis-Pt(II) had significantly higher numbers of plaque forming spleen cells than the saline treated animals.

Similar studies were performed on animals bearing S-180. When antigen was injected 14 days after tumor implantation, plaque forming cells were found in significantly higher numbers in the animals treated with zymosan or saline than in those treated with cis-Pt(II) or cis-Pt(II) plus zymosan. When antigen was administered on day 21 of the experiment, however, those animals treated with cis-Pt(II) or cis-Pt(II) plus zymosan had significantly higher numbers of plaque forming cells than the other two groups. Thus it was concluded that, depending on the time of antigen administration, treatment with cis-Pt(II) or zymosan plus cis-Pt(II) may have some stimulatory effect on humoral antibody responses.

Cell mediated immune responses were evaluated by skin allograft rejection. Allografts were rejected earlier in animals bearing S-180 and treated with a combination of zymosan and cis-Pt(II). Consequently,

it was concluded that combination therapy may stimulate cell mediated immune responses.

Selected tissues from animals bearing S-180 and treated with saline, zymosan, cis-Pt(II) or a combination of zymosan and cis-Pt(II) were evaluated histologically. Spleens and regional lymph nodes from all groups were markedly hyperplastic, particularly in the marginal zones of the lymphoid follicles. The thymuses of animals with regressing tumors had a normal appearing architecture with a somewhat hyperplastic cortex. In contrast, the thymuses of those animals with non-regressing tumors were atrophic and the demarcation between cortex and medulla was obscured.

Regressing tumors, regardless of treatment, were characterized by marked lymphocytic infiltration and were surrounded by a proliferating fibrous capsule similar to that seen in homograft rejection. This suggested that ultimate tumor regression may be mediated via immunologic mechanisms rather than a specific attack on tumor cells by the therapeutic agents.

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Philip B. Conran

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To all cancer patients

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INTRODUCTION

The role of host defense mechanisms in promoting the regression of tumors in animals treated with cis-Dichlorodiammineplatinum(II) [cis-Pt(II)] has recently become of great interest. Rosenberg and VanCamp (1970) reported that treatment with cis-Pt(II) caused regressions in 63-100% of Swiss mice bearing advanced Sarcoma 180 (S-180). Generally, S-180 will regress spontaneously in 0-25% of the Swiss mice in which it is implanted (Mihich, 1969). This suggests that there are marked histocompatibility differences between the tumor and the host and that host responses against the tumor are vigorous.

It has been observed that various cancer chemotherapeutic agents are less effective when the tumor and host are histocompatible (Bradner and Pindell, 1965). In addition it has also been noted that there is a reduction in chemotherapeutic efficacy if host responses are purposely suppressed (Ferrer and Mihich, 1967; Tarnowski and Stock, 1957; Mihich and Nichol, 1959; Martin *et al.*, 1962).

On the other hand, numerous investigators have reported that the efficacy of cancer chemotherapeutic agents can be enhanced by using specific or nonspecific immunostimulation in combination with the chemical agents (Martin *et al.*, 1962; Martin *et al.*, 1964; Mathé, 1970; Kalpaktsoglou and Good, 1970; Martin *et al.*, 1970; Fugmann *et al.*, 1970; Esber *et al.*, 1972; Fass and Fefer, 1972; Pearson *et al.*, 1972).

Since cis-Pt(II) is a relatively new drug and not related chemically to any cancer chemotherapeutic agents presently in use, a series of experiments was performed to ascertain what effect suppression or stimulation of host defenses would have on its efficacy as a cancer chemotherapeutic agent.

LITERATURE REVIEW

Host Defenses Against Neoplasia

It has been postulated that the development of neoplasms is due to a breakdown in immunologic surveillance (Keast, 1970). Although this hypothesis must be kept in perspective because of the many factors involved in the development of neoplasia, it is of interest to note that cancer in man and animals develops in the extremes of life when the immune system is maturing or when it is weakened by thymic atrophy. In concert with this observation is the fact that the incidence of neoplastic diseases is documented as being 100 times greater in immunologically deficient or immunosuppressed indivuals than in the normal population (Fahey, 1971). Data from animal studies are equally convincing since it has been shown that animals which have undergone neonatal thymectomy, radiation treatment, treatment with antilymphocyte serum or treatment with immunosuppressive drugs have an increased incidence of neoplastic diseases and are more susceptible to the implantation of tumors (McMichael, 1967; Allison, 1970; Doll and Kinlen, 1970; Klein, 1970; Law, 1970; Fahey, 1971; Kreider *et al.*, 1971). Contrasted to this, both specific and nonspecific stimulation of host defenses have, in some instances, had a marked influence on the induction time, growth and persistence of experimental tumors as well as promoting reduction of tumor mass and prolongation of remission time in spontaneous neoplasms (Klein, 1969; Alexander, 1970; Mathé,

1970; Bernstein *et al.*, 1971; Humphrey *et al.*, 1971a; Humphrey *et al.*, 1971b; Morton *et al.*, 1971).

One could also assume that the reported spontaneous regression of autochthonous tumors in man and animals may also be immunologically mediated (Sumner and Foraker, 1960; Everson and Cole, 1966; Fefer *et al.*, 1968; Bell, 1970; Kreider *et al.*, 1971).

In accordance with the hypothesis that immunologic factors play a role in mediating the regression of neoplasms, Doniach *et al.* (1958) and Klein (1969) reported that two of the most antigenic tumors known, Burkett's lymphoma and choriocarcinoma, are also extremely responsive to chemotherapy and immunotherapy.

The revitalization of interest in tumor immunology came in 1953 when the results of a series of experiments were reported by Foley. Prior to this time the lack of successful immunization attempts and the paucity of standardized laboratory animals led many investigators to believe that nothing fruitful could be gained from studying immunologic responses to tumors. Foley, however, found that if he ligated isografted, methylcholanthrene-induced sarcomas in C3H mice he could induce necrosis in the tumors. More importantly, subsequent rechallenge of the mice with live pieces of the same tumor resulted in rejection of the implants. Since these animals did not reject implants of other tumors, it was apparent that a tumor specific immunity had developed.

In recent years tumor specific transplantation antigens (TSTA) have been demonstrated in a wide array of neoplasms including those induced by chemicals, physical agents and both DNA and RNA oncogenic viruses. These TSTA are antigens which are capable of inducing rejection responses in syngeneic hosts in a preimmunization-viable cell challenge type of experiment (Klein, 1969). For the most part

virally induced TSTA are common to all tumors produced by the same virus regardless of morphological appearance or strain of animal in which it arises. With DNA viruses, e.g., Polyoma, SV40 and Shope papilloma, the TSTA are virus related but distinct from virus specific antigens since they can be demonstrated after the infecting virus is no longer present. Evidence for separate virus related TSTA versus virally specific antigens is lacking in RNA viruses, however, since tumor cells continue to shed viral particles (Pessens, 1970; Law, 1969).

The tumors induced by chemical or physical agents have TSTA which are tumor specific but not carcinogen specific. Thus there is virtually no cross-reactivity even when tumors are morphologically identical and arise in a single animal. This difference in specificity between the TSTA of chemically induced and virally induced tumors may, however, be more spurious than real (Klein, 1969). Recently Morton *et al.* (1969) demonstrated antigens which were specific for individual spontaneous mammary tumors. These tumors arose in mice which carried mammary tumor virus and were presumed to be virally induced. Their antigenicity and growth behavior patterns, however, mimicked chemically induced tumors. On the other hand, G antigen (Gross virus) has been detected in methylcholanthrene induced sarcomas which, as Old and Boyse (1965) pointed out, considerably weakens the argument that viruses are not involved in chemically induced tumor systems.

Although these TSTA are capable of immunizing hosts against subsequent implantations of the same or similar tumors, their overall ability to promote effective immunologic responses against these tumors is questionable. Law (1969) summarized their biological activities by declaring that those antigens which are located on the surface of tumor cells, e.g., mammary tumor virus and murine sarcoma

virus, are of the histocompatibility type and are ostensibly of potential importance in relation to contact inhibition, cell division and immunologic reactions. Those antigens which are located intracellularly and which have been demonstrated by complement fixation or immunofluorescence, however, are of questionable significance as defense mechanisms.

Many neoplasms of man have also been shown to have what appears to be tumor-related antigens. However, some of these antigens can be associated with non-neoplastic fetal cells as well. Tumors in which antigenicity can be demonstrated are: Burkett's lymphoma, choriocarcinoma, nasopharyngeal carcinoma, melanoma, neuroblastoma, colonic carcinomas and osteogenic sarcoma. Some of these antigens are intracytoplasmic, while others are bound to cell membranes (Klein, 1969; Fairly, 1970; Pessens, 1970; Thompson *et al.*, 1969; Morton *et al.*, 1971; Oettgen *et al.*, 1971). In most of these cases cytotoxic antibodies are found in the sera of patients bearing these tumors. In many instances the antibodies cross-react with similar tumor cells from other patients indicating that there may be common antigenicity between certain tumor types (Hellstrom *et al.*, 1968; Pessens, 1970; Morton *et al.*, 1971). In addition, lymphocytes from patients bearing these tumors have been shown to be cytotoxic for their tumors as well as for neoplastic cells of patients bearing tumors of the same class (Hellstrom *et al.*, 1968; Pessens, 1970; Hellstrom, 1971; Oettgen *et al.*, 1971).

Aside from tumor-specific antigens, there are other indications of active host responses against tumors. One of the most obvious is the fact that regional lymph nodes draining neoplastic sites become hyperplastic resembling the reactions seen in nodes responding to non-neoplastic antigenic stimuli. This hyperplastic response is found in patients bearing primary tumors without metastatic lesions. It has

also been observed that the presence of lymphocytic infiltrates in neoplasms is associated with a more favorable prognosis (Alexander *et al.*, 1966).

Another indication of host reactivity is the development of concomitant immunity. This type of immunity is defined as the ability of the immunologic resistance of the host to destroy small implants of tumors even though large tumors, and in some cases metastatic lesions, are growing progressively in the host (Klein, 1969). The importance of concomitant immunity was emphasized by the independent experiments of Gershon *et al.* (1968) and Crile and Deodhar (1971). Gershon and his co-workers found that a normally nonmetastasizing lymphoma of hamsters did metastasize if the primary tumor was removed. Their experiment indicated that removal of the primary tumor 7 days after transplantation led to a rapid decrease in immunity, the production of enhancing antibodies and the appearance of metastatic nodules. The metastatic lesions were thought to be derived from preexisting tumor cells in the blood and lymphoid tissues.

Crile and Deodhar (1971) utilized two different tumor systems in their experiments studying concomitant immunity. In one experiment, S-180 was implanted in the legs of mice. They found that if the tumor bearing leg was amputated 10 days after implantation, 60% of the animals were susceptible to tumor challenge 4 days later. If, on the other hand, the tumors were irradiated but not removed, the animals were immune to challenge for 3 weeks. The investigators suggested that the irradiated tumor cells were capable of absorbing enhancement antibody, thus maintaining the state of concomitant immunity. They also suggested that the continued antigenic stimulation produced by the irradiated tumor cells may have helped to sustain cell-mediated immunity.

In a second experiment, the same workers found that the incidence of pulmonary metastasis of Lewis T241 fibrosarcoma in mice was higher when the foot bearing the primary tumor was amputated. The incidence of metastasis was reduced when the tumor bearing foot was irradiated but not amputated. It was suggested that the release of tumor antigens from the irradiated tumor may have increased immunity which in turn was responsible for destroying metastatic progenitors.

It was previously mentioned that both humoral and cell mediated immune responses have been observed in a vast array of neoplastic diseases of both animals and man. Fairly (1970) suggested that circulating antibodies, even if cytotoxic, have little effect on solid tumors but may prevent blood borne metastasis. This postulation is suggested by the fact that many neoplasms metastasize to local lymph nodes where they remain for some time before spreading via the circulatory system. This postulate might also explain why neoplasms metastasize by local lymphatics when antibodies are present but via the blood when they are absent.

Alexander (1970) summarized the effector mechanisms which are recognized in immunological reactions against neoplasms *in vivo*. He suggested that humoral antibodies, probably in conjunction with complement, are capable of destroying tumor cells, assuming they have adequate access to them. In concert with these are cytotoxic lymphocytes which infiltrate the neoplasms like a homograft and macrophages which may be coated with cytophilic antibody which destroy tumor cells by direct contact.

Utilization of Sarcoma 180 in Cancer Chemotherapy and Immunotherapy

Sarcoma 180 (Crocker Sarcoma-180, Crocker tumor 180, Mouse Sarcoma 180) arose spontaneously in the right axillary region of a white male mouse necropsied in 1914. It was initially described as a carcinoma. However, the tissue of origin was not cited. With serial transplantation, the histologic pattern was altered so that, by 1919, the tumor was described as a sarcoma. Stewart *et al.* (1956) suggested that the tumor be classified as undifferentiated because of the anaplastic appearance of the cells and lack of any specific histologic pattern.

Sarcoma 180 has little strain specificity and, for the most part, grows well in a number of mouse strains. For this reason it is often referred to as a "nonspecific" tumor. The untreated tumor kills its host in 4 to 5 weeks. Generally the rate of spontaneous regression varies from 5-25% (Stewart *et al.*, 1956; Mihich, 1969; Sellei *et al.*, 1970). Snell *et al.* (1953) reported that inbred strains of mice carrying the $H\text{-}2^d$ histocompatibility locus, e.g., BALB, BALB/c, DBA/2, had fewer spontaneous regressions than those carrying other H-2 genes. They explained this on the basis that the tumor may have arisen in mice of the genotype $H\text{-}2^d/H\text{-}2^d$.

The H-2 locus is one of a number of loci determining susceptibility or resistance to transplants. It is a particularly strong locus, however, since when tumor and host differ at this locus a powerful deterrent against progressive tumor growth is evoked. The fact that the proposed $H\text{-}2^d$ origin still prevails in S-180 after many years of transplantation in innumerable host suggests that the "nonspecific" nature of the tumor may have occurred by an increase in virulence which allowed it to overpower histocompatibility differences

rather than by attenuation or modulation of histocompatibility genes (Snell *et al.*, 1953).

Due to its lack of specificity, S-180 has been used as a cancer chemotherapy screening tumor for many years. It has the disadvantage of being only moderately sensitive to chemotherapeutic agents. Complete regressions are usually attained in a low percentage of cases and mostly by using doses very close to toxic levels (Issekutz, 1969). Sellei *et al.* (1970) reported that nitrogen mustard, Degranol, Mitomen and Sarcolysin, which are normally high in effectiveness in other tumors, had only moderate effectiveness against S-180 and that they produced excessive weight loss, indicating toxicity. Myleran, Mannagranol and Colchicine were ineffective against the tumor. Azaserine, which was very effective in inhibiting this tumor, failed to have similar favorable effects on other experimental tumors and was found to be of no value in clinical practice. Although Sellei stated that an effect by Mercaptopurine cannot be detected against S-180, Ferrer and Mihich (1967) found that 6-mercaptopurine (6-MP) could promote cures in 42-52% of animals.

It appears that a crucial issue in the success or failure of chemotherapy against S-180 is the strain of mice in which it is implanted. Bradner and Pindell (1965) found that, by implanting the tumor in DBA/2 mice, which are compatible with S-180 at the $H\text{-}2^d$ locus, they could reduce the chemotherapeutic efficacy of 6-MP, Actinogan and Phleomycin. The same drugs were effective in promoting regressions of S-180 when it was implanted in Swiss Ha/ICR mice, a noncompatible host.

Sarcoma 180 does not lend itself to sophisticated immunologic study because of its lack of specificity. It does appear that its growth and ultimate disposition, however, are regulated by host

responses. Old *et al.* (1961) found that phagocytic activity increased and splenomegaly occurred in Swiss mice in which S-180 was implanted. These changes occurred relatively early but disappeared prior to the death of the animal. The same authors noted similar enhanced phagocytic activity and splenomegaly when they injected the supernatant from spleen homogenates taken from animals bearing S-180. Although they could not induce tumors with this material, the effect on the reticulo-endothelial system (RES) suggested that a transmissible agent might be associated with this tumor.

Mihich and Nichol (1959) and Mihich (1962) found that spontaneous regression rates of S-180 increased markedly in mice fed a diet deficient in vitamin B_6. They attributed this effect to an alteration in the production of humoral enhancement antibodies. Similar increases in spontaneous regressions were found by Old *et al.* (1962), Ferrer (1968) and Ferrer and Mihich (1968) when mice were splenectomized prior to tumor implantation. Since the spleen is the major contributor of humoral antibody in the rodent, the authors concluded that splenectomy served to decrease the quantity of enhancement antibody formed. These same investigators noted that the efficacy of chemotherapeutic agents and nonspecific immunostimulants was enhanced when prior splenectomies were performed. Ferrer and Mihich (1968) found that the incidence of tumor regressions in animals fed vitamin B_6 deficient diets or treated with 6-MP or Kethoxal-Bis (Thiosemicarbazone) (KTS) was greatly reduced by active immunization with frozen-thawed tumor cells or by passive transfer of specific hyperimmune serum. They attributed this to the development of an enhancement phenomenon.

While splenectomies enhanced the efficiency of various chemotherapeutic agents, neonatal thymectomy was found to reduce the

chemotherapeutic effects of 6-MP and KTS (Ferrer and Mihich, 1967). It was concluded that removing the thymus caused cell-mediated immune responses to be greatly impaired.

Besides the use of chemotherapeutic agents, numerous investigators have studied the effects of nonspecific immunostimulation in promoting the regression of S-180. Certain agents capable of stimulating the RES, such as Bacillus Calmette Guerin (Old *et al.*, 1962), Zymosan (Bradner and Pindell, 1965), *Bacillus pertussis* lipopolysaccharide (Malkiel and Hargis, 1961), lipopolysaccharide from *Proteus vulgaris* (Mizuno *et al.*, 1963), Lentinan (Maeda and Chihara, 1971) and various other polysaccharides from plants and yeasts (Fukuoka *et al.*, 1968; Kamasuka *et al.*, 1968; Suzuki *et al.*, 1969; Komatsu *et al.*, 1969; Tanaka, 1967) were either capable of inhibiting the growth of S-180 or were capable of promoting tumor regressions. Lemperle (1966) demonstrated that the combination of immunizing mice with killed S-180 tumor cells and stimulating the RES with restin, a crystalized lipid fraction of shark liver, or glucan, the active polysaccharide fraction of zymosan, rendered mice significantly resistant to the growth of S-180.

The Use of Platinum Compounds in Cancer Chemotherapy

In 1965, Rosenberg *et al.* reported that *Escherichia coli* underwent filamentous growth (elongation) but would not divide when under the influence of electric current delivered by platinum electrodes. After a number of experiments, it was disclosed that several new species of platinum salts were generated by the electrical current. Further investigation indicated that many of these salts were capable of inducing bacterial elongation when added to their growth medium

(Rosenberg *et al.*, 1967). In 1969, Rosenberg *et al.* reported that one of these salts, cis-Dichlorodiammineplatinum(II) [cis-Pt(II)] was capable of retarding the growth of S-180 and mouse leukemia L1210. An additional report by Rosenberg and VanCamp (1970) indicated that delayed cis-Pt(II) treatment was capable of promoting regressions of S-180 in 63-100% of the animals treated.

Since the original reports of Rosenberg, oncostatic properties of cis-Pt(II) have been demonstrated against the Ehrlich's Ascites tumor (Howle and Gale, 1970), L1210 leukemia when combined with other drugs (Speer *et al.*, 1971; Vanditti, 1971; Sirica *et al.*, 1971; Woodman *et al.*, 1971), Rous Sarcoma (Hinz, 1970), Dunning ascitic leukemia and Walker 256 carcinosarcoma (Kociba *et al.*, 1970), chemically induced myeloid and lymphatic leukemias (Leonard *et al.*, 1971), mouse reticulum cell sarcoma (Talley, 1970), chemically induced rat mammary carcinoma (Welsch, 1971) and a variety of other tumor systems such as Lewis Lung carcinoma, B-16 melanocarcinoma, P388 leukemia and ADJ-PC6A plasma cell tumor (Rosenberg, 1971).

The drug is presently in the early phases of testing against human neoplasms, and its activities against some of these have been reported (Speer *et al.*, 1971; Talley *et al.*, 1972).

The toxic properties associated with cis-Pt(II) are particularly pronounced in tissues having rapidly dividing cellular constituents, e.g., gastrointestinal tract, lymphoid organs, bone marrow. Kociba and Sleight (1971) observed that, although erythrocyte numbers, packed cell volume and hemoglobin concentration remained normal, there was panleukocytopenia, reticulocytopenia and depressed numbers of platelets in rats 2 to 4 days after treatment. There were also decreases in the levels of serum proteins. Thompson and Gale (1970, 1971) reported

depression in hematopoiesis as well as a reticulocytopenia and lymphopenia in rats. Histologically, rats and mice treated with cis-Pt(II) were characterized by thymic and splenic atrophy, denudation of intestinal epithelium and acute nephrosis (Kociba *et al.*, 1970; Thompson and Gale, 1970, 1971; Toth-Allen, 1970; Leonard *et al.*, 1971).

Since the organs most sensitive to the cytotoxic properties of cis-Pt(II) are those with rapidly dividing cells, the ability of the drug to suppress immune responses has been studied. Richardson (1969) observed that cis-Pt(II) suppressed the formation of antibodies in dispersed spleen cells stimulated *in vitro* with *Brucella abortus* antigen. In comparing it to 4 commonly used immunosuppressive drugs, cyclophosphamide, metotrexate, 6-MP and puromycin, she found that the concentrations of cis-Pt(II) required to suppress antibody formation were lower than any of the latter drugs. Khan and Hill (1971) and Howle *et al.* (1971) found it to be a potent inhibitor of phytohemagglutinin-induced mitogenesis of human lymphocytes. Berenbaum (1971) and Khan and Hill (1971) reported that 10 mg per Kg doses of cis-Pt(II) reduced the number of antibody forming cells in the spleens of mice immunized with sheep erythrocytes. Khan and Hill observed significant reduction when the drug was administered at any time between 2 days before and 2 days after antigenic stimulation. They considered the drug to be a potent immunosuppressive. Berenbaum, on the other hand, noted that maximum immunosuppression was attained 2 days after antigenic stimulation and that administration of the drug prior to or concurrent with the antigen was ineffective. In comparing cis-Pt(II) to cyclophosphamide, he concluded that cis-Pt(II) was a weak immunosuppressor at therapeutic levels.

The platinum compound has also been shown to have an ability to suppress cell-mediated immunity. Khan and Hill (1971, 1972) reported that it inhibited graft versus host responses and prolonged the life of skin allografts in mice.

It has been proposed that cis-Pt(II) mimicks the alkalating agents in regard to its antitumor activities. Connors (1971) found that it was active against 2 tumors which are sensitive to an alkylating agent, melphalan. On the other hand, its antitumor activity was markedly diminished against a tumor resistant to alkylating agents, e.g., Walker R. tumor. Similarities were also seen in the ability of cysteine and other nucleophiles to reduce the toxicity of both alkylating agents and the platinum compound.

Harder and Rosenberg (1970) and Howle and Gale (1970) found that cis-Pt(II) inhibited DNA synthesis primarily and RNA and protein synthesis secondarily. The effects on DNA were considered to be direct since the synthesis of DNA precursors and the ability of the precursors to enter cells were not impaired (Harder and Rosenberg, 1970).

Roberts and Pascoe (1972) compared the mechanisms of action of cis-Pt(II) and mustard gas. The results of their experiments indicated that both compounds selectively inhibited DNA synthesis while showing no effects on gross RNA and protein synthesis. They also indicated that the effect on DNA was due to a direct reaction rather than interference with the enzymes involved in DNA synthesis. The authors proposed that, like mustard gas, cis-Pt(II) produced an inter-strand cross-linking reaction in the DNA molecule. They suggested that likely regions for this cross-linking to occur would be between the 6-amino groups of adenosine in an adenosine phosphate thymidine sequence, the

2-amino groups of guanine in the narrow groove and the 6-amino groups of cytosine in the wide groove of DNA in a cytidine phosphate guanosine sequence.

Although the available evidence suggests that cross-linking of complimentary strands of DNA is a mechanism by which cis-Pt(II) exerts its biological activity, it still remains to be proven that this is the principal way in which it produces cytotoxicity.

The Properties of Zymosan and its Use as an Immunostimulant

Zymosan is an insoluble polysaccharide of yeast cell walls. Chemically it is composed of approximately 50-60% glucan, 16-20% mannan, 13-17% protein, 6-7% lipid, 3.0-3.5% ash and less than 1% chitin (Fitzpatrick and DiCarlo, 1964). It was concluded that the active component of zymosan is glucan and that its functions may depend upon the specific configurations and spatial arrangements of its sugar residues (Fitzpatrick and DeCarlo, 1964). In concert with this hypothesis were the findings of Sakai *et al.* (1968), who found that glucans composed of beta-(1-3)-linked D-glucose residues were more active against neoplasms than those with alpha linkages. Diller *et al.* (1963) found hydroglucan superior to zymosan in promoting regressions of S-180 and S-37. Suzuki *et al.* (1969), on the other hand, demonstrated that the oncostatic properties of mannan fractions from *Saccharomyces cerevisiae* were superior to the glucan fractions.

The administration of zymosan causes marked reticuloendothelial (R.E.) activity. Machado *et al.* (1968) observed that low doses, i.e., 2 to 4 mg per Kg per day for 6 days, caused marked Kupffer cell and reticuloendothelial cell hyperplasia in the liver. The R.E. cells proliferated and formed nodules. There were also increases in the

numbers of lymphocytes, neutrophils and monocytes. The spleen was characterized by marked proliferation of R.E. cells and macrophages in the red pulp and marginal zones of the lymphoid follicles. Occasionally, multinucleated giant cells were also observed.

Zymosan inactivates the third component of complement (C'3) and in so doing causes reduction in the levels of properdin. Properdin is a heat labile beta globulin in serum which is important in natural resistance. In combination with complement it enhanced phagocytosis, inactivates certain viruses, e.g., Newcastle virus, is bactericidal to some gram negative bacteria, e.g., *Shigella dysenteriae* and *Bacillus subtilis*; lyses certain unsensitized but abnormal erythrocytes, e.g., in paroxysmal nocturnal hemoglobinuria; and protects against the lethal effects of whole body irradiation (Wardlaw and Pillemer, 1956; Fitzpatrick and DeCarlo, 1964). The reduction of properdin levels by zymosan are dose dependent and apparently transient in nature. Pillemer and Ross (1955) found that the injection of low doses of zymosan, i.e., 5 mg per Kg, produced a precipitous drop in properdin levels within 1 to 2 hours after injection followed in 2 to 14 days by a marked rise which increased to 200 to 300% above that of control levels. At high doses, i.e., 25 to 125 mg per Kg, the properdin levels decreased to levels lower than that found with the 5 mg per Kg dose and within 6 to 10 days were 25% lower than controls.

Zymosan was reported to enhance hemolysin titers in rats when given 48 hours before, at the same time as or 48 hours after antigen administration (Cutler, 1959). This adjuvant effect was most pronounced when the zymosan was administered in low doses and 48 hours following antigen administration.

Blattberg (1957) reported the production of heat stable agglutinins in response to zymosan in rabbits. The best results were obtained when it was combined with adjuvants since zymosan alone elicited very little antibody response. He also observed that it was capable of increasing the bactericidal activity of rabbit sera for *Escherichia coli* B. It was proposed that zymosan and *E. coli* B were related antigenically and that antibodies produced against zymosan might cross-react with similar antigenic determinants found on the bacteria. In light of the experiments cited by Pillemer and Ross (1955), however, Blattberg may have been observing an effect due to enhanced properdin levels rather than a cross-reacting antibody response.

Zymosan has a pronounced stimulatory effect on the reticuloendothelial system (RES). Cutler (1960) demonstrated that, within 48 hours after an intravenous injection of 3 mg, the phagocytic index in rats rose to 6 times that of normal values. This stimulation persisted for 6 weeks. Shinichiro and Shinoki (1968) observed that zymosan was not only capable of causing increases in the phagocytic index, but also in the levels of subcutaneous pre-histiocytes.

The activity of zymosan against neoplasms, both by itself and in combination with chemotherapeutic agents, has been studied by numerous investigators. Modica (1958) observed that 1 mg administered before, during or after the application of 3,4-benzopyrene to rats prolonged the latent period of tumor growth and the survival time of the host. Larger doses, however, hastened tumor appearance and shortened survival time. Bradner *et al.* (1958) studied the effects of low and high doses of zymosan against S-180 in Swiss mice. At doses between 10 and 160 mg per Kg given on day 1 of tumor growth, they found an average survival rate of 6% over the controls. Doses below and above these levels

yielded survival rates comparable to untreated controls. If 20 mg per Kg were administered on the first day of tumor growth, 50% regressions were obtained. Multiple dose schedules were most satisfactory when low doses were administered, i.e., 5 mg or 20 mg per Kg. These multiple doses, however, were no more effective than equivalent amounts of zymosan given in a single dose. Generally, large amounts, i.e., 320 mg per Kg, appeared to be deleterious, and large doses following small doses abrogated antitumor activity.

Old *et al.* (1960) found that 1 mg of zymosan injected intravenously in Swiss mice 1 week prior to implantation of S-180 yielded better protection than zymosan injected on the day of tumor challenge.

Diller *et al.* (1963) compared zymosan and hydroglucan. With these products there were regressions in 90-95% of animals in which Sarcoma-37 or S-180 had been implanted and 83% of animals in which Krebs-2 carcinoma had been implanted. They found hydroglucan to be superior to zymosan and suggested that the intravenous route of administration was superior to any others.

Shinichiro and Shinoki (1968) observed that zymosan was effective in promoting inhibition of rat Ascites Hepatoma (AH-130). Since the most effective oncostatic results were found when it was administered 3 days prior to tumor implantation, they concluded that the effect of zymosan was mediated via enhanced host responses rather than a direct effect on the tumor.

Bradner and Pindell (1965) compared the ability of zymosan, 6-MP, actinogan, phleomycin and a pyridoxine-deficient diet to promote regression in S-180 in Swiss Ha/ICR or DBA/2 mice. They found that all treatments were capable of increasing the incidence of recovery in the Swiss mice. However, only zymosan given intravenously at 50 mg

per Kg increased recovery from S-180 when the tumor was grown in DBA/2 mice.

Zymosan has also been shown to potentiate the antitumor effects of various chemotherapeutic agents and/or surgery. Martin *et al.* (1962) reported that the efficacy of both 6-MP and cyclophosphamide against Adenocarcinoma R.C. was enhanced when zymosan was used in combination with the two drugs. It was also observed that a combination of zymosan and 6-MP was far superior to either compound alone in promoting regressions of S-180. Again the reduction of tumor size by surgery potentiated the success of combination therapy even more. Martin *et al.* (1964) found that the combination of zymosan, surgery and cyclophosphamide was significantly more effective in reducing the recurrence of spontaneous mammary tumors than surgery or cyclophosphamide alone or in combination. Martin *et al.* (1970) observed that zymosan in combination with 4 chemotherapeutic agents, i.e., Streptomigran, cyclophosphamide and Mitomycin C, effected local cures in 54% of the animals treated. Local cures were defined as a lack of tumor recurrence. The 4 chemotherapeutic agents plus surgery, on the other hand, effected local cures in only 15-39%.

The Use of Steroid Hormones as Immunosuppressive Drugs

Hydrocortisone (HC) is a steroid hormone with glucocorticoid activity. The effects of this drug are basically the same as cortisone, but HC is more potent. There are numerous physiological activities attributed to HC. However, for the purpose of this manuscript, the immunosuppressive and anti-inflammatory effects will be emphasized.

Kass and Finland (1953) reported atrophy of lymphoid tissues and a reduced concentration of pentose nucleic acids after the

administration of cortisone and HC. They also reported that, after allotypic erythrocytes were injected into rabbits treated with HC, they were observed in lymph node macrophages for as long as 10 days. In untreated rabbits the erythrocytes were generally eliminated within 48 hours. Thus, it was suggested that the immunosuppressive effects of cortisone and HC may be due to an interference with lymphocyte nucleic acid metabolism as well as interference with the mechanism by which cells dispose of phagocytized material.

Snell (1962) reported that low doses of HC stimulated the phagocytic index but that high doses blocked phagocytosis by interfering with the mechanism by which phagocytized material is eliminated. Lurie (1962) also reported depressed RES activity after treatment with HC.

The ability of HC to suppress antibody formation mimics irradiation (Taliaferro, 1957; Berenbaum, 1967). It was found that maximum antibody suppression could be obtained in the rat when the drug was administered prior to and/or on the same day as antigen administration. It was also reported that the drug suppressed anamnestic responses but had no effect on the biologic half life of antibodies in passively immunized animals. Finally, hemolysin production could be restored after cortisone treatment by the administration of thymus or spleen cells.

Schlesinger (1967) reviewed the effects of corticosteroid hormones on the antigenicity of tissue. Allogeneic skin grafts of mice and rats treated with HC were rejected much later than those from untreated controls. It was also reported that the involution of the thymus in HC treated mice was accompanied by loss of thymus-distinctive serological properties. This loss is similar to that observed in the involuted thymus of tumor-bearing mice. As an example, the reactivity of thymus

cells of A and SJL/J strains of mice to TL antibody disappeared within a day after the subcutaneous administration of 1 mg of HC, and no antibody was detected for 6 days. Concomitantly, the thymus cells also lost their sensitivity to the cytotoxic effects of guinea pig serum, but their reactivity to H-2 antibodies was unaltered. After the 6-day period, the thymus cells returned to normal. It was suggested that these antigenic changes observed in the thymus of tumor-bearing and HC-treated animals might be due to: 1) a selective detrimental effect on thymus cells possessing distinctive serological properties, leaving a cell population devoid of these properties, 2) inhibition of the induction of thymus-distinctive properties in stem cells entering the thymus, or 3) an effect on the genetic regulatory mechanism of thymus cells. This latter possibility is based on the fact that HC inhibits purine nucleotide and protein synthesis in the thymus.

Corticosteroids have been reported to render animals more susceptible to oncogenesis. This is believed to be due to their ability to depress host responses. The onset of regressions of tumors initiated with fibroma virus in rabbits was delayed in animals treated with prednisone, and antibodies to fibroma virus were delayed in appearance and at reduced titers (Bergman *et al.*, 1962). Hurst (1964) reported that cortisone enhanced the growth of Shope fibroma but did not greatly inhibit the development of antibodies to the virus. The tumors in treated animals grew one week longer and were twice the size of the tumors in untreated animals. Methylprednisolone has been shown to reduce the regression rates of Shope papilloma in rabbits and prolong the period of tumor growth in rats (McMichael, 1967; Kreider *et al.*, 1971). Shachat *et al.* (1968) observed that mice pretreated with

cortisone had an increased susceptibility to oncogenesis with Maloney murine sarcoma virus.

The administration of corticosteroids in conjunction with cancer chemotherapeutic agents and/or nonspecific immunostimulants has been reported to reduce the therapeutic efficacy of the latter. Hydrocortisone blocked the anti-tumor activity of zymosan in promoting regressions of S-180 in Swiss mice (Bradner and Clarke, 1959). Cortisone has been reported to reduce the number of regressions of S-180 in animals fed a diet deficient in vitamin B_6 (Stoerck, 1954; Mihich and Nichol, 1959), reduce the efficacy of 6-MP, Puromycin, cyclophosphamide, surgery and zymosan against various transplantable tumors (Tarnowski and Stock, 1957; Martin *et al.*, 1962) and decrease the number of local cures of spontaneous murine mammary carcinomas when used in conjunction with Streptonigrin, Thioguanine, cyclophosphamide, Mitomycin C and Actinomycin D (Fugmann *et al.*, 1970).

MATERIALS AND METHODS

General Plan

A three-phase study was undertaken to investigate the role of host defenses in promoting tumor regression in animals treated with cis-Pt(II). Phase one consisted of treating Swiss mice bearing S-180 with cis-Pt(II) and hydrocortisone acetate, an immunosuppressive drug. Phase two consisted of treating BALB/c mice with cis-Pt(II) and zymosan, an immunostimulant. Phase three consisted of evaluating the integrity of host defenses in BALB/c mice treated with a combination of cis-Pt(II) and zymosan. The agarose-slide technique was used to evaluate humoral antibody production, and the ability to reject skin allografts was used to evaluate cell-mediated immune responses. Some of these animals were killed on day 21 of the experiment, and selected tissues were evaluated histologically.

Source of Animals

Female Swiss mice, 8 weeks of age, were procured from a commercial supplier.* Female BALB/c mice, 8 weeks of age, and DBA/2 female mice, 8 weeks of age, were obtained from a second commercial supplier.** The latter 2 strains of mice are inbred and bear the H-2^d histocompatibility locus.

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*Spartan Research Animals, Haslett, Michigan.

**Simonsen Laboratories, Gilroy, California.

Maintenance of Animals

All mice, with the exception of those used in the allograft experiments, were maintained in the Biophysics Department animal quarters. The animals were maintained on a commercial diet* and water *ad libitum*. Animal care was provided by Biophysics Department personnel.

The animals utilized for skin allograft rejection experiments were maintained in quarters provided by the Michigan State University Center for Laboratory Animal Resources (CLAR) and cared for by CLAR personnel.

A Montadale wether lamb was maintained at Michigan State University's Veterinary Research Barn No. 1 as a source for sheep erythrocytes (SRBC) and was cared for by the Department of Large Animal Surgery and Medicine personnel.

The housing and care of animals was performed in accordance with the standards promulgated by the National Society of Medical Research.

Preparation of cis-Pt(II) Compound

The compound cis-Pt(II) was synthesized and purified by a Biophysics Department chemist.** Sterile saline (0.85% NaCl) was used in the preparation of all aqueous solutions of cis-Pt(II), and all solutions were prepared within 1 hour of the time of injection. The compound was administered via the intraperitoneal (IP) route in all experiments.

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*Zinn's Feed, A. K. Zinn & Company, Battle Creek, Michigan.

**Dr. James Hoeschele.

Preparation of Hydrocortisone

A saline suspension of hydrocortisone acetate (HC) was purchased from a commercial supplier.* The product was used as provided by the supplier and was administered via the subcutaneous (SC) route. All injections were given immediately posterior to the right humerus.

Preparation of Zymosan

Zymosan from *Saccharomyces cerevisiae* yeast was purchased from a commercial supplier.** Sterile saline was used in the preparation of all aqueous suspensions of zymosan, and all suspensions were boiled in a water bath for 1 hour prior to the time of injection. Zymosan was administered via the IP route in all experiments.

Transplantation of Sarcoma 180

Sarcoma 180 (S-180) was maintained in Swiss mice by weekly transfer. The tumors used in BALB/c mice originated from Swiss mice and the number of transfers in the latter strain was recorded in each experiment.

The S-180 tumors were implanted according to Cancer Chemotherapy National Service Center protocols (1962). According to these methods, animals bearing 8- to 16-day-old implants of S-180 were killed by cervical dislocation. The left axillary region was swabbed with 80% ethyl alcohol and the tumor was carefully and aseptically dissected away from the overlying skin and adjacent normal tissues. The tumor was placed in a sterile 100 x 15 mm Petri dish containing Chloromycetin, and necrotic and hemorrhagic regions were extirpated. Portions of the

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*Hydrocortone^{R} acetate, Lot No. 0324N, Merck, Sharp and Dohme Division of Merck and Company, Incorporated, West Point, Pennsylvania 19486.

**Zymosan, Lot No. 9913-0100 and 71C-1370, Sigma Chemical Company, 3500 DeKalb Street, St. Louis, Missouri 63118.

remaining tumor were cut into 1-3 mm segments, placed in a 13-gauge trocar and implanted subcutaneously in the region of the left axilla of the recipients. Prior to implantation, the left axillary regions were swabbed with 80% ethyl alcohol. The day of tumor implantation was recorded as day 0. The tumors were measured with calipers at weekly intervals. The measurements included 2 diameters at right angles to each other, the results being expressed as the average of the two in millimeters. Regressions were defined as complete disappearance of tumors without reappearance in a period of 90 days.

Humoral Antibody Assay

A protocol for the performance of the agarose-slide technique, a modification of Jerne's plaque counting technique, was used (Plotz *et al.*, 1968).*

Each week 25 ml of SRBC were collected and placed in 25 ml of Alsever's Solution (Carpenter, 1965). The resultant suspensions were stored at 4°C for 2 weeks prior to use. Animals to be evaluated were given an IP injection of 3×10^8 SRBC which were washed 3 times in .01M phosphate buffered saline (PBS) at pH 7.0. The PBS was freshly prepared prior to each experiment. On the fifth day after injection, the animals were killed by decapitation. After exsanguination the spleens were removed and placed in sterile 100 x 15 mm Petri dishes and stored on ice. The individual spleens were then forced through fine stainless steel mesh (faucet aerators) with a pestle. The homogenate was taken up through a 22-gauge hypodermic needle into a

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*Provided by Dr. Harold C. Miller, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan.

tuberculin syringe containing 1 ml of Eagle's Minimum Essential Medium (MEM)* (pH 6.9 to 7.2). The splenic material was then forced back and forth through the needle until it flowed freely. A 27-gauge hypodermic needle was attached to the syringe, and the forcing procedure was repeated a number of times. Since there was a certain amount of fluid loss during this procedure, at its termination the fluid volume was restored to 1 ml with fresh MEM. The homogenates were then placed in 100 x 16 mm screw cap test tubes and stored on ice. The spleen cells were quantitated on an electronic counter.**

A 1% solution of agarose*** was prepared in distilled water and melted on a hot plate. Upon melting, an equal volume of 2 times concentrated MEM, prewarmed to 56°C, was added. Thus the final concentration of agarose was 0.5%.

Using plastic pipettes, a 0.4 ml aliquot of the 0.5% agarose-MEM solution was delivered into Wasserman tubes located in a 48-50°C water bath. A 0.05 ml aliquot of 20% SRBC previously washed 3 times in PBS was suspended in MEM and delivered into the tubes as well. Finally, in rapid succession the tubes were removed from the bath, a 0.1 ml aliquot of spleen cells diluted 1:100 with MEM was added, the suspensions were gently agitated on a Vortex mixer and the suspensions were then poured on glass microscope slides which had previously been coated with 0.1% agarose.

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*Grand Island Biological Company, Grand Island, New York, Catalog No. F-15.

**Coulter Counter Model B, Coulter Electronics, Hialeah, Florida.

***Agarose-L'Industrie Biologique Francaise S.A., Distributed by Fisher Scientific Company, 15800 West McNichols Road, Detroit, Michigan 48235.

Upon solidification, slides were placed face down on trays which were specially made for these experiments by a member of the Biophysics Department.* Briefly, the trays consisted of a flat sheet of plexiglass 9" x 17" x 1/4". In the middle and along both sides 1/4" from the edge, glass microscope slides were mounted extending the full length of the sheet. The slides were glued to the surface with epoxy resin. Narrow strips of plexiglass 1/2" x 17" x 1/4" were then glued in the middle of the glass slides forming 2 separate compartments on each tray. When the slides containing the agarose-spleen cell mixture were placed on the tray, their ends were in contact with the glass slides of the tray. As a result the agarose-spleen cell mixture was suspended approximately 1 mm from the floor of the tray. Each tray held 24 slides.

Once placed on the trays, the slides were incubated at 37°C in a humid environment consisting of 95% air and 5% CO_2. After this initial incubation, the trays were removed, and 2 ml of a 1:10 dilution of guinea pig complement** in MEM were pipetted into each tray compartment so that the agarose-spleen cell mixture was immersed. They were then re-incubated at 37°C for an additional 2 to 3 hours.

At the termination of the experiment the complement was poured off the slides, and the plaques were quantitated using bright indirect light and the unaided eye.

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*Mr. George Moldovan.

**Guinea Pig Complement-Microbiological Associates, Bethesda, Maryland, Catalog No. 30-956.

Cell Mediated Immunity Assay

Skin allograft rejection was selected as the means to evaluate cell-mediated immune responses. The skin grafting technique used was a modification of that described by Wexler (1970). Female DBA/2 mice, 4 months of age, were used as donors. They were anesthetized with methoxyflurane* and the hair was clipped with scissors from the lateral abdomen and back. The area was then shaved with a straight razor.** The shaved skin was incised with iris scissors commencing immediately lateral to the linea alba on one side. The remaining dissection was alternately performed with iris scissors and a scalpel. Skin from the lateral abdomen on both sides and back was removed in one sheet. The donor animal was killed by cervical dislocation at the termination of the dissection. The skin was placed in a sterile 100 x 15 mm Petri dish with the dermis side up, and all remaining subcutaneous tissues were removed with iris scissors. Rectangular grafts, 5 x 5 mm, were cut from the skin sheet. The grafts were placed in a sterile 100 x 15 mm Petri dish containing 150,000 IU of procaine penicillin G*** and 250 mg dihydrostreptomycin sulphate*** in a total volume of 6 ml. Petri dishes were then placed on ice for the remainder of the experiment.

Recipient female BALB/c mice, 10 weeks of age, were anesthetized with methoxyflurane. Hair was then clipped and shaved from a region on the right side extending from the tuber coxae to the thoracic inlet

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*Metofane^{R}, Pitman-Moore, Incorporated, Fort Washington, Pennsylvania 19034.

**Weck Hair Shaper, Edward Weck and Company, L.I. City, New York 11101.

***W. A. Butler Company, Columbus, Ohio 43201.

and from the vertebral transverse processes to the linea alba. The area was swabbed with 80% ethyl alcohol. A linear incision was then made parallel to and equidistant from the transverse processes and the linea alba. The incision was 15 mm in length and extended to the costal arch. Subcutaneous tissue superior to the incision was separated from the overlying skin by blunt dissection. The skin in this region was then elevated with forceps, and the skin graft was placed dermis side down in the undermined region, making sure that the graft remained flat. The overlying skin was then pulled over the graft, and the incision was closed with wound clips.*

Seven days after grafting, the skin covering the grafts was incised and dissected away. The animals were observed daily. A superficial layer of desquamation usually formed over the graft area in the days following separation of the protective body skin covering. This layer usually sloughed off prior to graft rejection. When the entire graft was dark and had lost its pliability, graft rejection was considered complete.

Treatment of Animals with Combined cis-Pt(II) and Hydrocortisone (HC)

Two experiments were performed to ascertain what effect immunosuppression with HC would have on the anti-tumor properties of cis-Pt(II). Segments of S-180 tumors, 8 to 10 days old, were implanted in 248 female Swiss mice eight weeks of age. The treatment schedules and doses are listed in Table 1.

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*Autoclips^{R}, Clay-Adams, Incorporated, New York 10, New York.

Table 1. Treatment schedule for combined hydrocortisone and cis-Pt(II) therapy in Swiss mice bearing S-180

	Treatment	Dose	Day of Treatment	Number of Animals
1.	Saline	0.2 ml	1, 8 and 14	
		0.5 ml	8	38
2.	cis-Pt(II)	8 mg/Kg	8	34
3.	Hydrocortisone	150 mg/Kg	1	40
4.	Hydrocortisone	150 mg/Kg	8	24
5.	Hydrocortisone	150 mg/Kg	15	24
6.	Hydrocortisone	150 mg/Kg	1	
	cis-Pt(II)	8 mg/Kg	8	40
7.	cis-Pt(II)	8 mg/Kg	8	
	Hydrocortisone	150 mg/Kg	8	24
8.	cis-Pt(II)	8 mg/Kg	8	
	Hydrocortisone	150 mg/Kg	15	24

The animals serving as controls received sterile saline in an equivalent volume as the drug being administered and via the same route, e.g., 0.5 ml cis-Pt(II) IP versus 0.5 ml saline IP. When cis-Pt(II) and HC were administered on the same day, e.g., group 6, the cis-Pt(II) was administered 6 hours prior to the HC. All animals which did not have palpable tumors by day 15 of the experiment and those dying prior to that time were eliminated from the experiment.

Treatment of Animals with Combined cis-Pt(II) and Zymosan

Four experiments were performed in an attempt to ascertain what effect the administration of zymosan on day 1 of tumor growth would have on the anti-tumor efficacy of cis-Pt(II). Segments of S-180 tumors,

8 to 12 days old, were implanted in 384 female BALB/c mice 8 to 12 weeks of age. Depending on the experiment, the tumors had been transferred at weekly intervals in BALB/c mice 3, 8, 26 or 38 times. The treatment schedules and doses are listed in Table 2.

Table 2. Treatment schedule for combined zymosan and *cis*-Pt(II) therapy in BALB/c mice bearing S-180

	Treatment	Dose	Day of Treatment	Number of Animals
1.	Saline	0.2 ml 0.5 ml	1 8	87
2.	*cis*-Pt(II)	7 mg/Kg	8	85
3.	Zymosan	50 mg/Kg	1	65
4.	Zymosan	75 mg/Kg	1	20
5.	Zymosan	100 mg/Kg	1	20
6.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	67
7.	Zymosan *cis*-Pt(II)	75 mg/Kg 7 mg/Kg	1 8	20
8.	Zymosan *cis*-Pt(II)	100 mg/Kg 7 mg/Kg	1 8	20

The animals in the control groups received sterile saline via the IP route and at a volume equivalent to the drug being administered on each treatment day. All animals not bearing palpable tumors by day 15 of the experiment and those dying prior to that time were eliminated from the experiment.

The animals were weighed on day 1 and twice weekly thereafter through day 21 of the experiment. Tumors were measured at weekly

intervals. The diameters of the tumors were measured at right angles to each other, and the average of these 2 measurements was expressed in millimeters.

Timing Experiment 1

Three experiments were performed in an attempt to ascertain what influence the day of zymosan administration had on the anti-tumor efficacy of cis-Pt(II). In these experiments the zymosan was given prior to the platinum compound.

Segments of S-180 tumors, 8 to 11 days old, were implanted in 300 female BALB/c mice, 8 to 9 weeks of age. These tumors had been transferred at weekly intervals in BALB/c mice 3, 12 or 15 times. The treatment schedules and doses are listed in Table 3.

The control animals were treated, all animals were weighed and the tumors measured as previously described. All animals not bearing palpable tumors by day 15 and those dying prior to that time were eliminated from the experiment.

Timing Experiment 2

Two experiments were performed in order to ascertain what influence the administration of zymosan concomitant with and after the cis-Pt(II) would have on the anti-tumor efficacy of the latter drug. Segments of S-180 tumors, 11 to 12 days old, were implanted in 120 female BALB/c mice, 8 to 11 weeks of age. These tumors had been transferred at weekly intervals in BALB/c mice 3 or 18 times. The treatment schedule and doses are listed in Table 4.

The control animals were treated, all animals were weighed and the tumors were measured as previously described. All animals not

Table 3. Treatment schedule for combined zymosan and *cis*-Pt(II) therapy in BALB/c mice bearing S-180. Timing study 1

	Treatment	Dose	Day of Treatment	Number of Animals
1.	Saline	0.2 ml 0.5 ml	1,2,4 and 6 8	30
2.	*cis*-Pt(II)	7 mg/Kg	8	30
3.	Zymosan	50 mg/Kg	1	30
4.	Zymosan	50 mg/Kg	2	30
5.	Zymosan	50 mg/Kg	4	30
6.	Zymosan	50 mg/Kg	6	30
7.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	30
8.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	2 8	30
9.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	4 8	30
10.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	6 8	30

Table 4. Treatment schedule for combined zymosan and cis-Pt(II) therapy in BALB/c mice bearing S-180. Timing study 2

	Treatment	Dose	Day of Treatment	Number of Animals
1.	Saline	0.5 ml 0.2 ml	8 8,10,12 and 14	10
2.	cis-Pt(II)	7 mg/Kg	8	10
3.	Zymosan	50 mg/Kg	8	10
4.	Zymosan	50 mg/Kg	10	10
5.	Zymosan	50 mg/Kg	12	10
6.	Zymosan	50 mg/Kg	14	20
7.	cis-Pt(II) Zymosan	7 mg/Kg 50 mg/Kg	8 8	10
8.	cis-Pt(II) Zymosan	7 mg/Kg 50 mg/Kg	8 10	10
9.	cis-Pt(II) Zymosan	50 mg/Kg 7 mg/Kg	12 8	10
10.	cis-Pt(II) Zymosan	7 mg/Kg 50 mg/Kg	8 14	20

bearing palpable tumors by day 15 and those dying prior to that time were eliminated from the experiment.

Timing Experiment 3

A single experiment was performed in an attempt to ascertain what effect preimmunization and multiple injections of zymosan would have on the anti-tumor efficacy of cis-Pt(II). In addition multiple low dose injections of cis-Pt(II) were evaluated.

Segments of an S-180 tumor, 8 days old, were implanted in 110 female BALB/c mice, 10 weeks of age. The tumor had been transferred 23 times in BALB/c mice at weekly intervals. Segments from a 10-day-old S-180 tumor which had been transferred 3 times in BALB/c mice were implanted in 10 female BALB/c mice 10 weeks old. The treatment schedules and doses are listed in Table 5.

All animals were weighed, and the tumors were measured as previously described. All animals not bearing palpable tumors by day 15 and those dying prior to that time were eliminated from the study.

Evaluation of the Integrity of Host Defenses

Evaluation of Humoral Antibody Response

A total of 115 female BALB/c mice, 8 weeks of age, was used to evaluate the humoral antibody response in animals treated with a combinaion of zymosan and cis-Pt(II), but not bearing tumors. The treatment schedule, day of antigen administration and the day on which the agarose-slide technique was performed are listed in Table 6.

A total of 112 female BALB/c mice, 8 weeks of age, was used to evaluate the humoral antibody response in animals bearing S-180 and

Table 5. Treatment schedule for combined zymosan and cis-Pt(II) therapy in BALB/c mice bearing S-180. Timing study 3

	Treatment	Dose	Day of Treatment	Number of Animals
1.	cis-Pt(II)	1 mg/Kg	1-7	10
2.	cis-Pt(II)*	5 mg/Kg	1 and 6	10
3.	cis-Pt(II)	7 mg/Kg	8	10
4.	Zymosan	50 mg/Kg	-7	10
5.	Zymosan	50 mg/Kg	1	10
6.	Zymosan	50 mg/Kg	14	10
7.	Zymosan	50 mg/Kg	1 and 14	10
8.	Zymosan cis-Pt(II)	50 mg/Kg 7 mg/Kg	-7 8	10
9.	Zymosan cis-Pt(II)	50 mg/Kg 1 mg/Kg	-7 1-7	10
10.	Zymosan cis-Pt(II)	50 mg/Kg 1 mg/Kg	1 1-7	10
11.	Zymosan cis-Pt(II)	50 mg/Kg 7 mg/Kg	1 and 14 8	10

*Tumor transferred in BALB/c mice 3 times at weekly intervals.

Table 6. Treatment schedule for evaluation of humoral antibody responses in BALB/c mice treated with zymosan and cis-Pt(II) but not bearing tumors

	Treatment	Dose	Day of Treatment	Day Antigen Administered	Day Agarose-Slide Technique Performed
1.	Saline (10)*	0.2 ml	1		
		0.5 ml	8	14	19
2.	Zymosan (11)	50 mg/Kg	1	14	19
3.	cis-Pt(II) (13)	7 mg/Kg	8	14	19
4.	Zymosan	50 mg/Kg	1		
	cis-Pt(II) (12)	7 mg/Kg	8	14	19
5.	Saline (18)	0.2 ml	1		
		0.5 ml	8	21	26
6.	Zymosan (17)	50 mg/Kg	1	21	26
7.	cis-Pt(II) (17)	7 mg/Kg	8	21	26
8.	Zymosan	50 mg/Kg	1		
	cis-Pt(II) (17)	7 mg/Kg	8	21	26

*Numbers in parentheses are numbers of animals per group.

treated with a combination of zymosan and cis-Pt(II). The mice were implanted with segments of an S-180 tumor 12 days of age. The treatment schedule, day of antigen administration and the day on which the agarose-slide technique was performed are listed in Table 7.

Evaluation of Cell Mediated Immune Responses

A total of 85 female, BALB/c mice, 8 weeks of age, was used to evaluate cell mediated immune responses in animals treated with a combination of zymosan and cis-Pt(II), but not bearing tumors. The treatment schedule and day on which skin allografts were applied are listed in Table 8.

One hundred four female BALB/c mice, 8 weeks of age, were used to evaluate cell mediated immune responses in animals bearing S-180 tumors and treated with a combination of zymosan and cis-Pt(II). Segments from S-180 tumors 12 days of age were used to initiate tumor growth. The treatment schedule and day on which skin allografts were applied are listed in Table 9.

Animals that died prior to the complete rejection of allografts were eliminated from the experiment.

Microscopic Examination of Tissues

Segments of an S-180 tumor, 12 days of age, were implanted in 20 female BALB/c mice 8 weeks old. The animals were divided into 4 equal groups and treated with saline, zymosan, cis-Pt(II) or combinations of zymosan and cis-Pt(II). On the 21st day of tumor growth, the mice were killed by cervical dislocation. Selected tissues including spleen, thymus, regional lymph nodes and tumors were removed and fixed in 10% neutral buffered formalin. Tissue sections were cut at 6 microns, stained with hematoxylin and eosin and evaluated histologically.

Table 7. Treatment schedule for evaluation of humoral antibody responses in BALB/c mice bearing S-180 tumors and treated with zymosan and cis-Pt(II)

	Treatment	Dose	Day of Treatment	Day Antigen Administered	Day Agarose-Slide Technique Performed
1.	Saline (12)*	0.2 ml 0.5 ml	1 8	14	19
2.	Zymosan (12)	50 mg/Kg	1	14	19
3.	cis-Pt(II) (12)	7 mg/Kg	8	14	19
4.	Zymosan cis-Pt(II) (12)	50 mg/Kg 7 mg/Kg	1 8	14	19
5.	Saline (12)	0.2 ml 0.5 ml	1 8	21	26
6.	Zymosan (13)	50 mg/Kg	1	21	26
7.	cis-Pt(II) (13)	7 mg/Kg	8	21	26
8.	Zymosan cis-Pt(II) (26)	50 mg/Kg 7 mg/Kg	1 8	21	26

*Numbers in parentheses are numbers of animals per group.

Table 8. Treatment schedule for evaluation of cell-mediated immune responses in BALB/c mice treated with zymosan and *cis*-Pt(II) but not bearing tumors

	Treatment	Dose	Day of Treatment	Day Allograft Applied	Number of Animals
1.	Saline	0.2 ml 0.5 ml	1 8	14	10
2.	Zymosan	50 mg/Kg	1	14	7
3.	*cis*-Pt(II)	7 mg/Kg	8	14	10
4.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	14	8
5.	Saline	0.2 ml 0.5 ml	1 8	21	11
6.	Zymosan	50 mg/Kg	1	21	9
7.	*cis*-Pt(II)	7 mg/Kg	8	21	10
8.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	21	10

Table 9. Treatment schedule for evaluation of cell-mediated immune responses in BALB/c mice bearing S-180 tumors and treated with zymosan and cis-Pt(II)

	Treatment	Dose	Day of Treatment	Day Allograft Applied	Number of Animals
1.	Saline	0.2 ml 0.5 ml	1 8	14	26
2.	Zymosan	50 mg/Kg	1	14	26
3.	cis-Pt(II)	7 mg/Kg	8	14	26
4.	Zymosan cis-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	14	26

Statistical Analysis

The 1 way analysis of variance was used to analyze all data and the method of Scheffe was used to establish confidence limits (Lewis, 1966; Scheffe, 1961).

RESULTS

Combined cis-Pt(II) and Hydrocortisone Therapy

The results of combined cis-Pt(II) and hydrocortisone therapy are summarized in Table 10. The cis-Pt(II) was capable of eliciting regressions in 19/33 (57%) of the animals treated. The administration of HC 7 days prior to or 6 hours after the platinum compound, however, markedly reduced its anti-tumor activity, e.g., 11/48 (23%) and 5/22 (23%). Administering the HC 7 days after cis-Pt(II) reduced the number of tumor regressions to 8/19 (42%).

Statistically significant differences in mean life span (MLS) were observed between certain treatment groups (Table 10).

Combined Zymosan and cis-Pt(II) Therapy

The results of the initial experiments utilizing combined zymosan and cis-Pt(II) therapy are summarized in Table 11. There were no spontaneous regressions in the control animals, nor were any regressions observed in those animals treated with cis-Pt(II) alone. Regressions did occur, however, in those animals treated with 50, 75, or 100 mg per Kg of zymosan on day 1 of tumor growth, e.g., 2/61 (3%), 4/17 (24%) and 1/17 (6%), respectively.

The most successful treatment regimen was the administration of 50 mg per Kg of zymosan on day 1 of tumor growth followed by 7 mg per Kg of cis-Pt(II) on day 8, e.g., 30/64 (47%). Doses of 75 or 100 mg per Kg of zymosan on day 1 of tumor growth followed by 7 mg per Kg of

Table 10. Results of combined cis-Pt(II) and hydrocortisone therapy in Swiss mice bearing S-180

	Treatment	Dose	Day of Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
1.	Saline	0.2 ml	1, 8 and 15		
		0.5 ml	8	6/37 (17%)	25 ± 10
2.	cis-Pt(II)	8 mg/Kg	8	19/33 (57%)	32 ± 10[c]
3.	Hydrocortisone	150 mg/Kg	1	9/36 (25%)	28 ± 9
4.	Hydrocortisone	150 mg/Kg	8	1/18 (5.6%)	30 ± 10[d]
5.	Hydrocortisone	150 mg/Kg	15	3/22 (13.8%)	28 ± 7
6.	Hydrocortisone	150 mg/Kg	1		
	cis-Pt(II)	8 mg/Kg	8	11/48 (23%)	30 ± 8
7.	cis-Pt(II)	8 mg/Kg	8		
	Hydrocortisone	150 mg/Kg	8	5/22 (23%)	28 ± 5
8.	cis-Pt(II)	8 mg/Kg	8		
	Hydrocortisone	150 mg/Kg	15	8/19 (42%)	29 ± 11

a. Number of tumor regressions/total number of animals; b. S.E. - Standard error of the mean; c. Significantly ($P<0.03$) greater than mean life span of saline treatment group 1; d. Significantly ($P<0.04$) greater than mean life span of saline treatment group 1.

Table 11. Results of combined zymosan and cis-Pt(II) therapy in BALB/c mice bearing S-180

	Treatment	Dose	Day of Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
1.	Saline	0.2 ml 0.5 ml	1 8	0/86 (0%)	30 ± 5
2.	cis-Pt(II)	7 mg/Kg	8	0/84 (0%)	33 ± 5[c,e]
3.	Zymosan	50 mg/Kg	1	2/61 (3%)	29 ± 6
4.	Zymosan	75 mg/Kg	1	4/17 (24%)	31 ± 7
5.	Zymosan	100 mg/Kg	1	1/17 (6%)	31 ± 3
6.	Zymosan cis-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	30/64 (47%)	37 ± 7[d,e,f]
7.	Zymosan cis-Pt(II)	75 mg/Kg 7 mg/Kg	1 8	4/18 (22%)	35 ± 8[g]
8.	Zymosan cis-Pt(II)	100 mg/Kg 7 mg/Kg	1 8	2/17 (12%)	32 ± 7

a. Number of tumor regressions/total number of animals; b. S.E. - Standard error of the mean; c. Significantly ($P<0.001$) greater than mean life span of saline treatment group 1; d. Significantly ($P<0.0005$) greater than mean life span of saline treatment group 1; e. Significantly ($P<0.0005$) greater than mean life span of zymosan treatment group 3; f. Significantly ($P<0.01$) greater than mean life span of cis-Pt(II) treatment group 2; g. Significantly ($P<0.002$) greater than mean life span of saline treatment group 1.

cis-Pt(II) on day 8 resulted in regression rates which were comparable to those achieved when zymosan was administered alone at the same dose, e.g., 4/18 (22%) and 2/17 (12%), respectively.

There was a statistically significant increase in MLS between certain treatment groups (Table 11).

The results of the initial timing experiments utilizing combined zymosan and cis-Pt(II) therapy are summarized in Table 12. There were no spontaneous tumor regressions in the control animals nor any regressions in those animals treated with cis-Pt(II) alone.

Statistically significant differences in MLS were noted between certain treatment groups.

The results of the second timing experiment in which zymosan was administered at the same time or after cis-Pt(II) are summarized in Table 13. Tumor regressions were only noted in 2 groups, i.e., zymosan plus cis-Pt(II) on day 8 and cis-Pt(II) on day 8 plus zymosan on day 14.

Statistically significant differences in MLS were noted between certain treatment groups.

The results of the third timing experiment utilizing combined zymosan and cis-Pt(II) therapy are summarized in Table 14. Tumor regressions were only noted in 2 groups, i.e., zymosan on day -7 plus cis-Pt(II) on days 1 through 7 and zymosan on days 1 and 14 plus cis-Pt(II) on day 8.

There was a marked prolongation of MLS in those animals treated with cis-Pt(II) on days 1 and 6.

The rate of tumor growth in animals treated with a combination of zymosan and cis-Pt(II) is illustrated in Figure 1. These measurements include animals from all experiments in which zymosan was administered at 50 mg per Kg on day 1 and cis-Pt(II) at 7 mg per Kg on day 8.

Table 12. Results of combined zymosan and cis-Pt(II) therapy in BALB/c mice bearing S-180. Timing study 1

	Treatment	Dose	Day of Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
1.	Saline	0.2 ml 0.5 ml	1, 2, 4 and 6 8	0/27 (0%)	26 ± 5
2.	cis-Pt(II)	7 mg/Kg	8	0/25 (0%)	32 ± 6[c,e,h,i,j]
3.	Zymosan	50 mg/Kg	1	1/27 (4%)	25 ± 6
4.	Zymosan	50 mg/Kg	2	1/28 (4%)	28 ± 8
5.	Zymosan	50 mg/Kg	4	0/29 (0%)	30 ± 8[j]
6.	Zymosan	50 mg/Kg	6	0/30 (0%)	25 ± 5
7.	Zymosan cis-Pt(II)	50 mg/Kg 7 mg/Kg	1 8	2/29 (3%)	31 ± 9[d,f]
8.	Zymosan cis-Pt(II)	50 mg/Kg 7 mg/Kg	2 8	2/30 (3%)	29 ± 6[g]

Table 12 (cont'd.)

Treatment	Dose	Day of Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
9. Zymosan	50 mg/Kg	4		
cis-Pt(II)	7 mg/Kg	8	1/29 (3%)	29 ± 8
10. Zymosan	50 mg/Kg	6		
cis-Pt(II)	7 mg/Kg	8	5/24 (21%)	35 ± 8[k,l]

a. Number of tumor regressions/total number of animals; b. S.E. - Standard error of the mean; c. Significantly ($P<0.0005$) greater than mean life span of saline treatment group 1; d. Significantly ($P<0.007$) greater than mean life span of saline treatment group 1; e. Significantly ($P<0.0005$) greater than mean life span of zymosan treatment group 3; f. Significantly ($P<0.001$) greater than mean life span of zymosan treatment group 3; g. Significantly ($P<0.05$) greater than mean life span of saline treatment group 1; h. Significantly ($P<0.01$) greater than mean life span of zymosan treatment group 4; i. Significantly ($P<0.05$) greater than mean life span of zymosan plus *cis*-Pt(II) treatment group 8; j. Significantly ($P<0.04$) greater than mean life span of saline treatment group 1; k. Significantly ($P<0.0005$) greater than mean life span of saline treatment group 1; l. Significantly ($P<0.0005$) greater than mean life span of zymosan treatment group 6.

Table 13. Results of combined zymosan and cis-Pt(II) therapy in BALB/c mice bearing S-180. Timing study 2

	Treatment	Dose	Day of Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
1.	Saline	0.2 ml 0.5 ml	8, 10, 12 and 14 8	0/6 (0%)	25 ± 1
2.	cis-Pt(II)	7 mg/Kg	8	0/8 (0%)	30 ± 6[d,h]
3.	Zymosan	50 mg/Kg	8	0/9 (0%)	22 ± 4
4.	Zymosan	50 mg/Kg	10	0/9 (0%)	25 ± 5
5.	Zymosan	50 mg/Kg	12	0/7 (0%)	28 ± 8
6.	Zymosan	50 mg/Kg	14	0/17 (0%)	31 ± 3[k]
7.	cis-Pt(II) Zymosan	7 mg/Kg 50 mg/Kg	8 8	1/6 (17%)	35 ± 0[c,e,f]
8.	cis-Pt(II) Zymosan	7 mg/Kg 50 mg/Kg	8 10	0/8 (0%)	38 ± 1[g,i,j]

Table 13 (cont'd.)

Treatment	Dose	Day of Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
9. cis-Pt(II)	7 mg/Kg	8		
Zymosan	50 mg/Kg	12	0/9 (0%)	27 ± 7
10. cis-Pt(II)	7 mg/Kg	8		
Zymosan	50 mg/Kg	14	2/19 (11%)	30 ± 5[k]

a. Number of tumor regressions/total number of animals; b. S. E. - Standard error of the mean; c. Significantly ($P<0.001$) greater than mean life span of saline treatment group 1; d. Significantly ($P<0.002$) greater than mean life span of zymosan treatment group 3; e. Significantly ($P<0.0005$) greater than mean life span of zymosan treatment group 3; f. Significantly ($P<0.03$) greater than mean life span of cis-Pt(II) treatment group 2; g. Significantly ($P<0.0005$) greater than mean life span of saline treatment group 1; h. Significantly ($P<0.03$) greater than mean life span of zymosan treatment group 4; j. Significantly ($P<0.001$) greater than mean life span of cis-Pt(II) treatment group 2; k. Significantly ($P<0.02$) greater than mean life span of saline treatment group 1.

Table 14. Results of combined zymosan and cis-Pt(II) therapy in BALB/c mice bearing S-180. Timing study 3

	Treatment	Dose	Day and Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
1.	cis-Pt(II)	1 mg/Kg	1-7	0/10 (0%)	29 ± 3
2.	cis-Pt(II)	5 mg/Kg	1 and 6	0/7 (0%)	43 ± 6[g]
3.	cis-Pt(II)	7 mg/Kg	8	0/10 (0%)	33 ± 2[d,e]
4.	Zymosan	50 mg/Kg	-7	0/10 (0%)	29 ± 7[f]
5.	Zymosan	50 mg/Kg	1	0/9 (0%)	30 ± 7[f]
6.	Zymosan	50 mg/Kg	14	0/10 (0%)	29 ± 3[f]
7.	Zymosan	50 mg/Kg	1 and 14	0/10 (0%)	21 ± 5
8.	Zymosan cis-Pt(II)	50 mg/Kg 1 mg/Kg	-7 1-7	1/10 (10%)	29 ± 7

Table 14 (cont'd.)

	Treatment	Dose	Day and Treatment	Tumor Regressions at 90 Days (%)[a]	Life Span of Non-Survivors (Days) Mean ± S.E.[b]
9.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	-7 8	0/10 (0%)	32 ± 7[c]
10.	Zymosan *cis*-Pt(II)	50 mg/Kg 1 mg/Kg	1 1-7	0/10 (0%)	32 ± 9
11.	Zymosan *cis*-Pt(II)	50 mg/Kg 7 mg/Kg	1 and 14 8	3/10 (30%)	29 ± 5

a. Number of regressions/total number of animals; b. S.E. - Standard error of the mean; c. Significantly ($P<0.001$) greater than mean life span of zymosan treatment group 7; d. Significantly ($P<0.0005$) greater than mean life span of zymosan treatment group 7; e. Significantly ($P<0.04$) greater than mean life span of zymosan plus *cis*-Pt(II) treatment group 11; f. Significantly ($P<0.002$) greater than mean life span of zymosan treatment group 7; g. Significantly ($P<0.003$-<0.0005) greater than mean life span of all other groups.

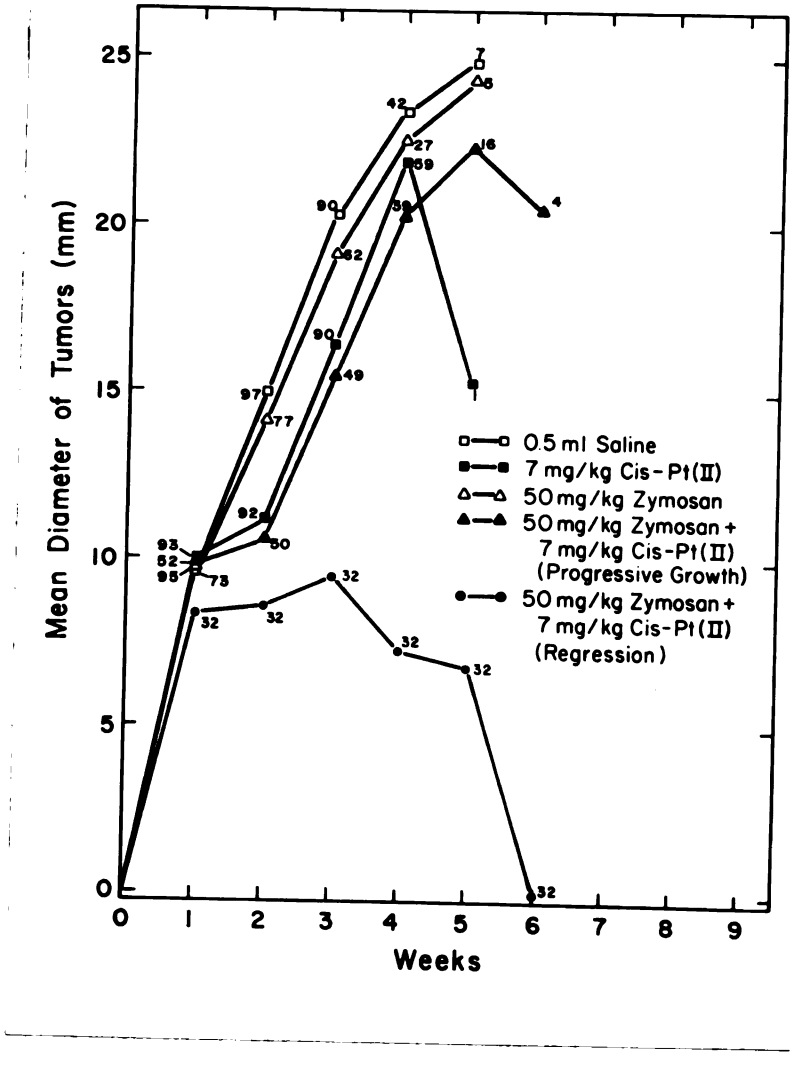

Figure 1. Growth of S-180 in BALB/c mice treated with combinations of zymosan and cis-Pt(II). Numbers of surviving mice are indicated by the number at each point.

The tumors in animals of all treated groups were significantly smaller than those of control animals on days 14 and 21 ($P<0.03$ to 0.0005). On day 7, however, only those animals treated with zymosan on day 1 differed significantly from control animals ($P<0.04$). The tumors from animals treated with cis-Pt(II) and those treated with combined zymosan and cis-Pt(II) were significantly smaller on days 14 and 21 than those treated with zymosan alone ($P<0.0005$). Those animals treated with combined zymosan and cis-Pt(II), and whose tumors ultimately regressed, had significantly smaller tumors than all other groups on days 14 and 21 ($P<0.008$ to 0.0005).

The changes in body weight in animals from all experiments in which 50 mg per Kg of zymosan was administered on day 1 and 7 mg per Kg of cis-Pt(II) was administered on day 8 are illustrated in Figure 2. The administration of zymosan on day 1 caused a significant ($P<0.02$), but transient, loss in body weight by day 4. The weight changes in these animals then paralleled that of the control animals until day 21. At this point they again weighed less than the control animals ($P<0.02$). The animals treated with cis-Pt(II) on day 8 exhibited a precipitous weight loss after treatment. The body weights remained significantly lower ($P<0.0005$) than the control animals and those treated with zymosan through day 21.

The animals treated with combined zymosan and cis-Pt(II) were divided into 2 groups: those with progressively growing tumors and those with regressing tumors. In the group with progressively growing tumors, the weight changes paralleled the cis-Pt(II) group, but were not as marked. Their weight loss by days 14 and 21 was significantly ($P<0.0005$) greater than in the control animals and those treated with zymosan. On day 17 the group treated with both compounds weighed

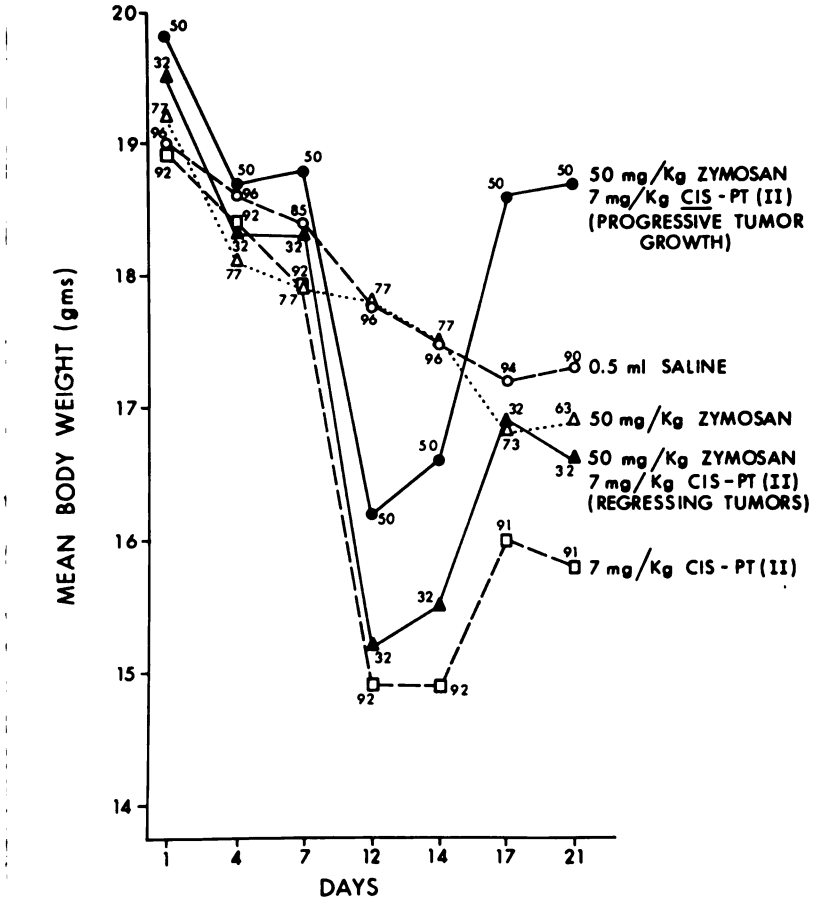

Figure 2. Changes in body weight in BALB/c mice bearing S-180 and treated with combinations of zymosan and cis-Pt(II).

essentially the same as the other 2 groups, while on day 21 those treated with both compounds weighed the same as the zymosan group but significantly less ($P<0.02$) than the saline controls.

Those animals treated with combination zymosan and cis-Pt(II) and whose tumors regressed showed transient weight loss by days 12 and 14. By day 17, however, their average weight was greater than any other group ($P<0.0005$). The weight loss by days 12 and 14 in this group was significantly less ($P<0.004$ to 0.0005) than those treated with cis-Pt(II) and those treated with both compounds but whose tumors were progressive in growth.

The Evaluation of Host Defenses in Animals Treated with Combinations of Zymosan and cis-Pt(II)

Evaluation of Humoral Antibody Response

The ability of animals treated with combinations of zymosan and cis-Pt(II), but not bearing S-180, to produce antibody to SRBC's is summarized in Table 15. There were no differences in the groups to which the antigen was administered on day 14. When the antigen was administered on day 21, however, the animals treated with combinations of zymosan and cis-Pt(II) produced significantly higher ($P<0.009$) numbers of plaque-forming spleen cells than the control animals.

The ability of animals bearing S-180 and treated with combinations of zymosan and cis-Pt(II) to produce antibody to SRBC's is summarized in Table 16. In the group of animals in which the antigen was administered on day 14, the control animals produced significantly greater numbers of plaques than any of the treatment groups. Those animals treated with zymosan produced significantly more plaques than those treated with combinations of the 2 compounds but not more than those treated with cis-Pt(II) alone.

Table 15. Results of evaluation of humoral antibody responses in BALB/c mice treated with zymosan and cis-Pt(II), but not bearing tumors

	Treatment	Dose	Day of Treatment	Day Antigen Administered	Day Agarose-slide Technique Performed	Number of Plaques Mean ± S.E.[b,c]
1.	Saline (10)[a]	0.2 ml 0.5 ml	1 8	14	19	255 ± 128
2.	Zymosan (11)	50 mg/Kg	1	14	19	283 ± 81
3.	cis-Pt(II) (13)	7 mg/Kg	8	14	19	364 ± 364
4.	Zymosan cis-Pt(II) (12)	50 mg/Kg 7 mg/Kg	1 8	14	19	251 ± 259
5.	Saline (18)	0.2 ml 0.5 ml	1 8	21	26	169 ± 68
6.	Zymosan (17)	50 mg/Kg	1	21	26	197 ± 88
7.	cis-Pt(II) (17)	7 mg/Kg	8	21	26	211 ± 101
8.	Zymosan cis-Pt(II) (17)	50 mg/Kg 7 mg/Kg	1 8	21	26	254 ± 108[d]

a. Numbers in parentheses are numbers of animals per group; b. Plaques quantitated per 10^6 spleen cells; c. S. E. - Standard error of the mean; d. Significantly ($P<0.009$) greater than values of saline treatment group 5.

Table 16. Results of evaluation of humoral antibody response in BALB/c mice bearing S-180 and treated with zymosan and cis-Pt(II)

	Treatment	Dose	Day of Treatment	Day Antigen Administered	Day Agarose-slide Technique Performed	Number of Plaques Mean ± S.E.[b,c]
1.	Saline (12)[a]	0.2 ml 0.5 ml	1 8	14	19	364 ± 159[f,g,h]
2.	Zymosan (12)	50 mg/Kg	1	14	19	227 ± 129[i]
3.	cis-Pt(II) (12)	7 mg/Kg	8	14	19	194 ± 146
4.	Zymosan cis-Pt(II) (12)	50 mg/Kg 7 mg/Kg	1 8	14	19	189 ± 87
5.	Saline (12)	0.2 ml 0.5 ml	1 8	21	26	64 ± 85
6.	Zymosan (13)	50 mg/Kg	1	21	26	165 ± 122
7.	cis-Pt(II) (13)	7 mg/Kg	8	21	26	249 ± 193[j]

Table 16 (cont'd.)

Treatment	Dose	Day of Treatment	Day Antigen Administered	Day Agarose-slide Technique Performed	Number of Plaques Mean ± S.E.[b,c]
8. Zymosan[d]	50 mg/Kg	1			
cis-Pt(II) (18)	7 mg/Kg	8	21	26	239 ± 197[k]
9. Zymosan[e]	50 mg/Kg	1			
cis-Pt(II) (8)	7 mg/Kg	8	21	26	321 ± 129[l,m]

a. Numbers in parentheses are numbers of animals per group; b. Plaques quantitated per 10^6 spleen cells; c. S.E. - Standard error of the mean; d. Animals with progressively growing tumors; e. Animals with regressing tumors; f. Significantly ($P<0.02$) greater than zymosan treatment group 2; g. Significantly ($P<0.003$) greater than cis-Pt(II) treatment group 3; h. Significantly ($P<0.0005$) greater than zymosan plus cis-Pt(II) treatment group 4; i. Significantly ($P<0.03$) greater than zymosan plus cis-Pt(II) treatment group 4; j. Significantly ($P<0.006$) greater than saline treatment group 5; k. Significantly ($P<0.005$) greater than saline treatment group 5; l. Significantly ($P<0.0005$) greater than saline treatment group 5; m. Significantly ($P<0.02$) greater than zymosan treatment group 6.

In the group in which the antigen was given on day 21, the animals treated with combinations of the 2 drugs and whose tumors were regressing produced significantly greater numbers of plaques than either the control animals or those treated with zymosan. The animals treated with cis-Pt(II) and those treated with combinations of the 2 drugs with nonregressing tumors produced greater numbers of plaques than the controls. There was no significant difference between the numbers of plaques produced by either combination therapy group and those treated with cis-Pt(II) alone.

Evaluation of Cell Mediated Immune Responses

In animals not bearing S-180, there was no significant difference in the time of skin allograft rejection between treatment groups (Table 17).

In those animals bearing S-180, the animals treated with combinations of the 2 compounds rejected allografts earlier than the controls or other treatment groups. This enhanced time of rejection, however, was independent of the fate of the tumor (Table 18).

Microscopic Evaluation of Selected Tissues

Spleen

The spleens of the animals evaluated were characterized by marginal hyperplasia of lymphoid follicles. This change was characterized by proliferation of what appeared to be immature lymphocytes on the margins of the follicles. Numerous degenerating cells and occasional macrophages were also noted in those proliferative zones. The central artery of each follicle was generally surrounded by a thin halo of

Table 17. Results of evaluation of cell-mediated immune responses in BALB/c mice treated with zymosan and *cis*-Pt(II), but not bearing tumors

Treatment	Dose	Day of Treatment	Day of Allografting	Day of Allograft Rejection Mean ± S.E.[b]
1. Saline (10)[a]	0.2 ml	1		
	0.5 ml	8	14	18 ± 4
2. Zymosan (7)	50 mg/Kg	1	14	18 ± 4
3. *cis*-Pt(II) (10)	7 mg/Kg	8	14	18 ± 4
4. Zymosan	50 mg/Kg	1		
cis-Pt(II) (7)	7 mg/Kg	8	14	19 ± 4
5. Saline (11)	0.2 ml	1		
	0.5 ml	8	21	14 ± 4
6. Zymosan (8)	50 mg/Kg	1	21	17 ± 2
7. *cis*-Pt(II) (10)	7 mg/Kg	8	21	16 ± 3
8. Zymosan	50 mg/Kg	1		
cis-Pt(II) (9)	7 mg/Kg	8	21	16 ± 5

a. Numbers in parentheses are numbers of animals per group; b. S. E. - Standard error of the mean.

Table 18. Results of evaluation of cell-mediated immune responses in BALB/c mice bearing S-180 and treated with zymosan and cis-Pt(II)

Treatment	Dose	Day of Treatment	Day of Allografting	Day of Allograft Rejection Mean ± S.E.[b]
1. Saline (8)[a]	0.2 ml 0.5 ml	1 8	14	17 ± 3
2. Zymosan (17)	50 mg/Kg	1	14	16 ± 3
3. cis-Pt(II) (17)	7 mg/Kg	8	14	18 ± 4
4. Zymosan[c] cis-Pt(II) (5)	50 mg/Kg 7 mg/Kg	1 8	14	13 ± 3[f]
5. Zymosan[d] cis-Pt(II) (5)	50 mg/Kg 7 mg/Kg	1 8	14	14 ± 3[e]

a. Numbers in parentheses are numbers of animals per group; b. S.E. - Standard error of the mean; c. Animals bearing progressively growing tumors; d. Animals with regressing tumors; e. Significantly ($P<0.02$) shorter rejection time than cis-Pt(II) treatment group 3; f. Significantly ($P<0.05$) shorter rejection time than cis-Pt(II) treatment group 3.

mature lymphocytes. This hyperplastic change appeared to be independent of the type of treatment or the disposition of the tumor.

Thymus

In those animals in which tumors were regressing, the thymus was composed of a wide cortical region of lymphocytes and the normal complement of epithelial cells in the medullary region (Figure 3). The thymuses of animals with progressively growing tumors were consistently atrophic. They were characterized by disarranged cortical lymphocytes, many of which were degenerate. The medullary region was virtually nonexistent (Figure 4).

Lymph Nodes

The regional lymph nodes of all groups were generally hyperplastic (Figure 5). The medullary regions were composed of an admixture of lymphocytes, plasma cells and macrophages. Occasionally, macrophages and lymphocytes were noted in the medullary sinusoids (Figure 6).

In one of the control animals the tumor had metastasized to the regional lymph node, displacing the normal follicular architecture (Figures 7 and 8).

Tumors

The microscopic changes in regressing tumors were characterized by degeneration of tumor cells, infiltration of the tumor by lymphocytes and plasma cells and the formation of a fibrous capsule on the periphery of the tumor (Figures 9 and 10).

In progressively growing tumors, there were multifocal regions of necrosis, but the majority of the tumor cells appeared healthy, and lymphocyte infiltration and/or fibroplasia were virtually nonexistent (Figures 11 and 12).

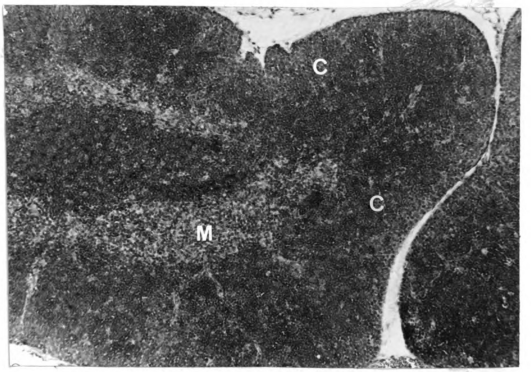

Figure 3. Thymus from BALB/c mouse treated with zymosan and bearing a regressing tumor. Note the wide cortical region (C) composed of lymphocytes and the more pale staining medulla (M) composed of epithelial cells. Hematoxylin and eosin. x 100.

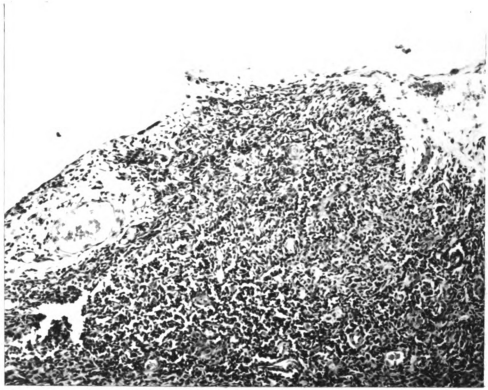

Figure 4. Thymus from BALB/c mouse treated with cis-Pt(II) and bearing a progressively growing tumor. The thymus is atrophic and has lost its normal architecture. Note absence of medulla. Hematoxylin and eosin. x 100.

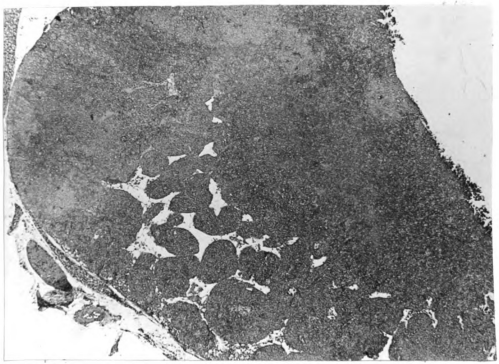

Figure 5. Axillary lymph node from BALB/c mouse treated with zymosan plus cis-Pt(II). There is marked hyperplasia of both the cortex and medulla. Hematoxylin and eosin. x 40.

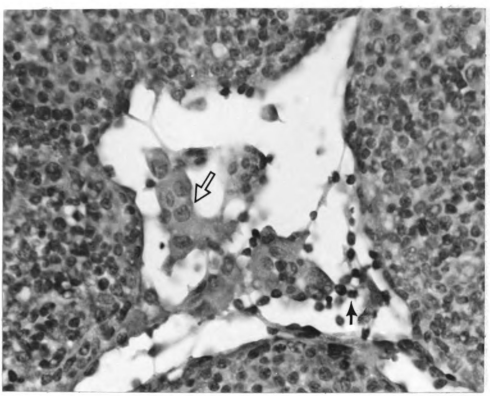

Figure 6. Higher magnification of the medulla of the lymph node in Figure 5. Note macrophages (blunt arrow) and lymphocytes (arrow) in medullary sinuses. Hematoxylin and eosin. x 400.

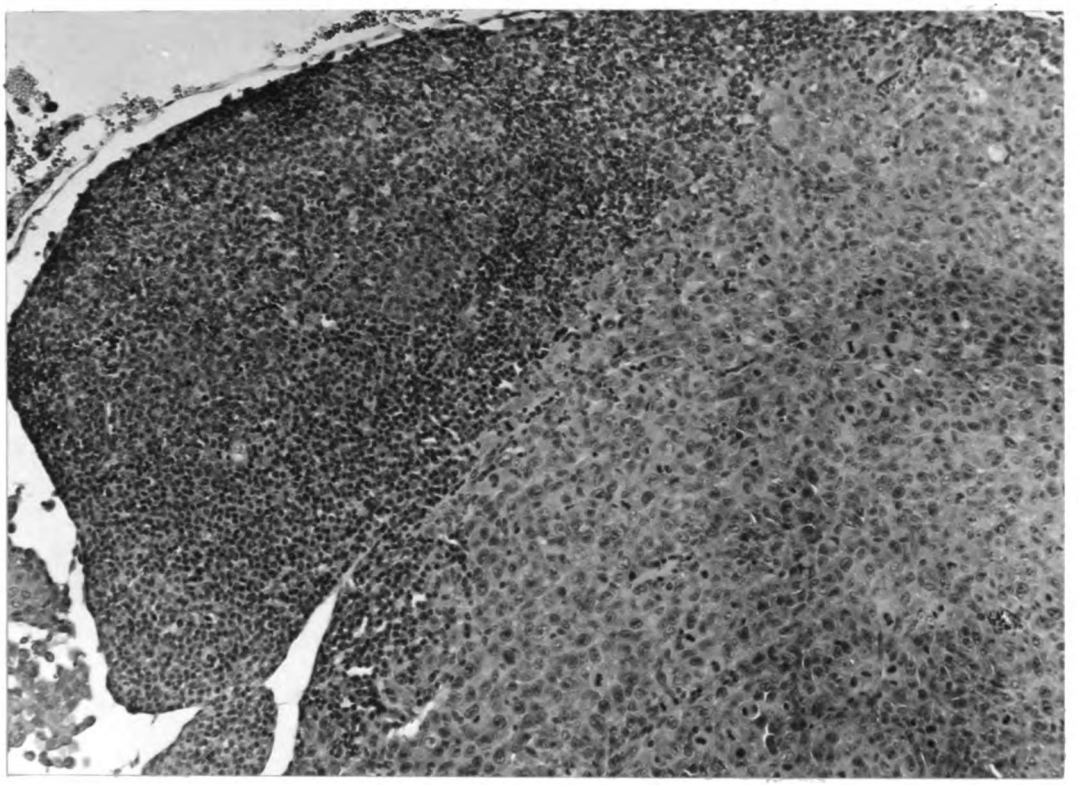

Figure 7. Axillary lymph node from BALB/c mouse treated with saline and containing metastatic tumor cells. Note sheets of tumor cells compressing cortical lymphocytes toward the periphery. Hematoxylin and eosin. x 100.

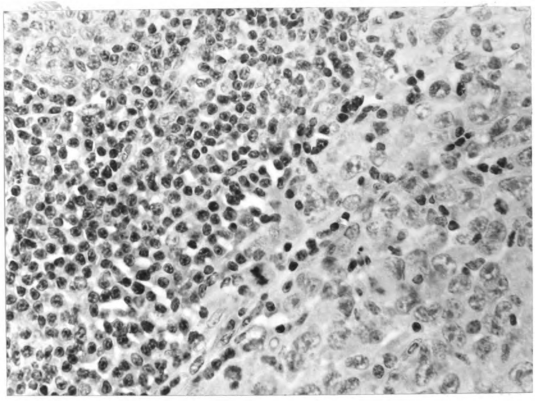

Figure 8. Higher magnification of a portion of the lymph node in Figure 7. Note tumor cells compressing and infiltrating cortical lymphocytes. Hematoxylin and eosin. x 400.

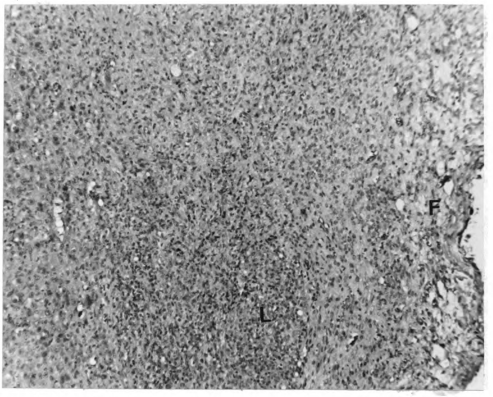

Figure 9. Regressing tumor from BALB/c mouse treated with zymosan. There is proliferation of a fibrous capsule (F) on the periphery and marked lymphocytic infiltration (L). Hematoxylin and eosin. x 100.

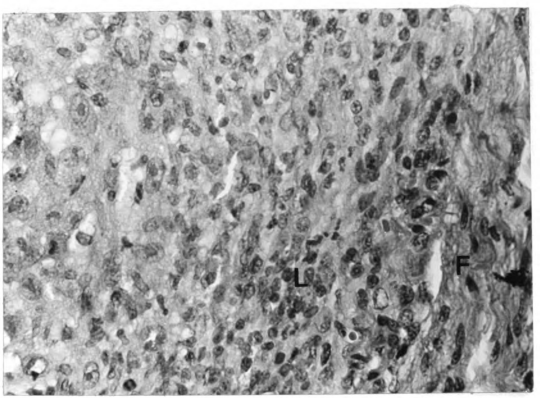

Figure 10. Higher magnification of a portion of the tumor in Figure 9. Note fibrous tissue (F) on periphery and lymphocytes (L) infiltrating tumor cells. Hematoxylin and eosin. x 400.

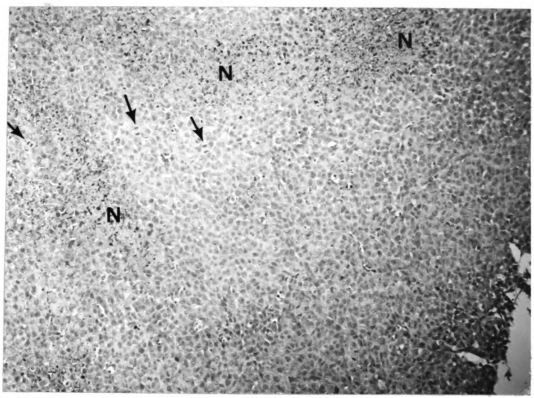

Figure 11. Progressively growing tumor from BALB/c mouse treated with cis-Pt(II). It is characterized by sheets of proliferating neoplastic cells, numerous mitotic figures (arrows) and multifocal areas of necrosis (N). Hematoxylin and eosin. x 100.

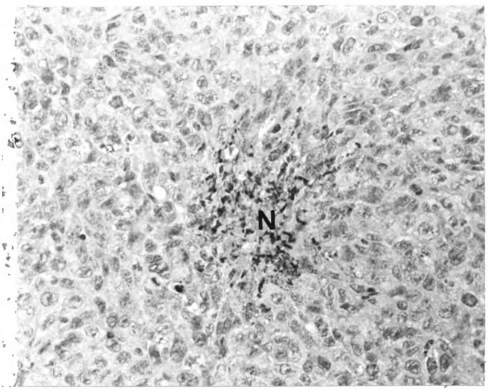

Figure 12. Higher magnification of a portion of the tumor in Figure 11. Note area of necrosis (N) surrounded by pleomorphic, neoplastic cells. Hematoxylin and eosin. x 200.

DISCUSSION

The results of these experiments indicate that the anti-tumor activity of cis-Pt(II) is, at least in part, dependent upon active host responses. In the first series of experiments hydrocortisone was administered in combination with the platinum compound to ascertain if immunosuppression would abrogate the anti-tumor efficacy of the latter drug. A host of investigators have reported that immunosuppression with corticosteroids decreases the anti-tumor efficacy of various chemotherapeutic and/or immunotherapeutic compounds when given before (Fugmann *et al.*, 1970), at the same time as (Martin *et al.*, 1962; Fugmann *et al.*, 1970) and/or after the administration of these compounds (Stoerck, 1954; Tarnowski and Stock, 1957; Mihich and Nichol, 1959; Bradner and Clarke, 1959; Fugmann *et al.*, 1970). Similarly, the experiments reported here indicate that the suppression of host responses with hydrocortisone either before, concomitant with or after cis-Pt(II) markedly diminish the anti-tumor effects of the latter drug. The greatest abrogation occurred when hydrocortisone was administered 7 days prior to or 6 hours after the cis-Pt(II). In both instances the number of tumor regressions was reduced to less than 50% of those in mice treated with cis-Pt(II) alone. When hydrocortisone was administered 7 days after the cis-Pt(II), the anti-tumor efficacy of the drug was diminished by 26%. This indicates that the later immunosuppression occurs after cis-Pt(II) administration

the less effective it is in diminishing the anti-tumor activities of the drug.

It is difficult to ascribe a specific mechanism by which immunosuppression with hydrocortisone interferes with the ability of cis-Pt(II) to promote regressions. As has been previously described, the steroids are known to suppress humoral antibody synthesis and depress cell mediated immunity. It is significant to note that the administration of the steroid on day 1 or day 8 of tumor growth caused the greatest retardation of cis-Pt(II) activity. It is during this time that host responses are initiated against the tumor. VanCamp (personal communication) demonstrated that concomitant immunity does not develop until day 9 of S-180 growth. Thus, the decrease in tumor regressions observed when hydrocortisone was administered 7 days prior to the cis-Pt(II) may be explained on the basis that the steroids suppressed the host responses against the tumor in their initial stages.

When hydrocortisone was administered 6 hours after the cis-Pt(II) the anti-tumor efficacy of the latter drug was again markedly diminished. This might be explained on the basis that both drugs have immunosuppressive capabilities and that the combination of the two suppressed host responses to a point where they were ultimately less effective in promoting tumor regressions. Although unmentioned by previous investigators using similar treatment regimens, one cannot rule out the possibility that, since the cis-Pt(II) and hydrocortisone were given on the same day, there might have been a chemical antagonism between the two drugs which in turn nullified the effects of the platinum compound. Without the use of sophisticated chemical analysis, this problem remains unresolved.

The fact that the administration of hydrocortisone one week after cis-Pt(II) was less effective in reducing the number of tumor regressions parallels the observations of Bradner and Clarke (1959). They found that the administration of hydrocortisone on day 13 of tumor growth was less effective in blocking the anti-tumor activity of zymosan than if it were administered earlier. They concluded that by day 13, host responses to the tumor were well under way and less susceptible to immunosuppression.

The specific mechanism by which hydrocortisone depresses the anti-tumor efficacy of cis-Pt(II) is at best speculative. It is readily apparent, however, that the oncostatic properties of cis-Pt(II), like many cancer chemotherapeutic agents, are markedly diminished by the immunosuppressive effects of hydrocortisone.

The results of the next series of experiments reinforce the hypothesis that cis-Pt(II) requires active host responses in order to promote regressions of S-180. In these experiments it was found that the platinum compound was ineffectual in promoting regressions of S-180 implanted in BALB/c mice. In this particular system the host and tumor have been reported to be histocompatible (Snell *et al.*, 1953) and, consequently, host responses against the tumor are not vigorous. Of particular interest in these experiments, however, was the potentiation of anti-tumor activity by combining cis-Pt(II), an immunosuppressive drug, and zymosan, an immunostimulant.

Bradner and Pindell (1965) reported that zymosan was markedly effective in promoting regressions of S-180 implanted in DBA/2 mice, a histocompatible system, but chemotherapeutic agents were not. In the experiments reported here, zymosan was found to be minimally successful, but in the proper temporal relationship, the combination

of zymosan and cis-Pt(II) appeared to elicit a moderate anti-tumor effect. Although some regressions were noted with various time intervals between zymosan and the platinum compound, the most consistently successful treatment regimen was found when 50 mg per Kg of zymosan was administered on day 1 of tumor growth followed by 7 mg per Kg of cis-Pt(II) on day 8. The variation in success of this regimen, and possibly the others as well, was ostensibly dependent on numerous factors such as age of animals, number of tumor transfers, size of the tumor on the day of cis-Pt(II) administration, as well as the time interval between zymosan and cis-Pt(II)

In general the combination therapy, although promoting moderate numbers of tumor regressions, did not appear to have any significant effect in prolonging the MLS of non-surviving animals. This is in agreement with the work of Bradner *et al.* (1958), who found that Swiss mice bearing S-180 and treated with zymosan either exhibited tumor regressions or died at essentially the same time as control animals. These authors also reported that the tumors of non-surviving zymosan-treated animals grew at essentially the same rate as those in control animals. In the experiments reported here, essentially the same phenomenon occurred. Those animals treated with zymosan and whose tumors did not regress had tumor growth curves which were essentially the same as in the untreated control animals (Figure 1). Similarly, those mice treated with combinations of zymosan and cis-Pt(II), but whose tumors did not regress, had tumor growth curves that paralleled those of animals treated with cis-Pt(II) alone (Figure 1).

In evaluating the body weight curves (Figure 2), it was noted that the animals treated with cis-Pt(II) had a transient, but marked,

loss in body weight immediately following treatment. This appears to be typical of cis-Pt(II) therapy since other investigators have made similar observations (Rosenberg and VanCamp, 1970). Although exhibiting a similar pattern of weight loss, the animals treated with zymosan and cis-Pt(II) lost significantly less weight than those treated with cis-Pt(II) alone. The animals whose tumors eventually regressed were generally in better health, and this could explain their greater tolerance of the cis-Pt(II). On the other hand, those animals with progressively growing tumors also demonstrated less weight loss than those animals treated with the platinum compound alone. This would suggest that pre-treatment with zymosan protected the host from the subsequent toxic effects of cis-Pt(II) to some degree. Fitzpatrick and DeCarlo (1964) reported that zymosan could decrease the lethal effects of total-body irradiation. They observed that, if zymosan was given prior to the irradiation of cancer patients, there was faster recovery from the radiation therapy and better hematopoietic recovery. This was believed to be due to an increased function of the RES. They made no mention of protection against weight loss, however.

The relationship between zymosan and cis-Pt(II) in promoting tumor regressions is unknown. Zymosan is known to stimulate the production of macrophages, increase phagocytosis, elevate properdin levels and have an adjuvant effect on the production of antibodies to various antigens. Cis-Pt(II) exhibits cytotoxic effects against neoplastic cells but has immunosuppressive capabilities. Martin *et al.* (1964) suggested that the synergism between zymosan and cyclophosphamide in reducing the recurrence rate of spontaneous mammary tumors in mice was based on the cross-reactivity between antibodies produced by zymosan and antigenic determinants on tumor cells. This cross-reactivity was

believed to alter the integrity of the tumor cells, rendering them more susceptible to the cytotoxic action of the cyclophosphamide. Since cis-Pt(II) is thought to resemble the alkylating agents in mechanism of action, this hypothesis deserves mention.

In the experiments reported in this manuscript, it was observed that occasional regressions were obtained with zymosan alone whereas cis-Pt(II) was completely ineffective. Consequently it would appear that a more likely possibility would be that the platinum compound altered the integrity of the tumor cells making them more susceptible to zymosan stimulated host responses.

The means by which zymosan stimulates host defenses to ultimately mediate tumor regressions is presently unknown. Bradner *et al.* (1958) reported that zymosan promoted tumor regressions in Swiss mice through the medium of host defense mechanism rather than by direct inhibitory action of the tumor. This conclusion was based on the following evidence: 1) the effect (tumor regression) was considerably delayed beyond the time the treatment was administered; 2) the response was quantal in nature, as opposed to the overall tumor suppressive action seen with many chemical agents; 3) zymosan was more effective at low doses than at high doses; 4) zymosan was not effective against S-180 in tissue culture. In comparing Bradner's observations with those reported here with combination zymosan and cis-Pt(II) therapy, one notes definite similarities with regard to the delay in tumor regressions and the quantal nature of these regressions.

Bradner and his co-workers ruled out the possibility of immediate direct stimulation of anti-tumor antibody by zymosan because treatment prior to tumor implantation, i.e., before the host had experienced tumor antigen, was still successful in promoting what appeared to be

tumor specific immunity. They also observed that the tumor loss effect could be abrogated within 48 hours by giving an initial low dose of zymosan followed by a high dose. The reversal phenomenon was considered to be too rapid for a typical acquired antibody reaction. Finally, they reported that animals receiving large doses of zymosan did not have a significantly higher rate of tumor regressions than untreated control animals. Thus it was hypothesized that the anti-tumor activities of low doses of zymosan were not due to the production of anti-tumor antibodies but rather to the production of some intermediary which altered the balance between tumor and host, shifting it in favor of the host. They speculated that this intermediary might be properdin.

Numerous investigators have attempted to correlate properdin levels with anti-tumor activity, some even before Bradner's publication. Southam and Pillemer (1957) discovered that patients with advanced cancer had low properdin levels and readily accepted cancer cell homografts. Normal individuals who were capable of rejecting cancer cell homografts, however, had normal properdin levels. These investigators were quick to point out, however, that the ability to reject cancer homografts in no way reflected the defense mechanism which controls the growth of spontaneous tumors. While Southam and Pillemer worked with human patients, Herbut and Kraemer (1956) found that colonic carcinomas taken from humans could be transplanted into rats if multiple injections of zymosan were given prior to transplantation. They speculated that the zymosan reduced the levels of properdin leading to a loss of natural resistance to the heterografts. When properdin levels were quantitated, however, they were found to be so variable that the investigators concluded that natural resistance to

the transplanted tumor was not mediated through the properdin system (Herbut *et al.*, 1958). In addition to the transplantation experiments, some investigators attempted to elucidate the effect of properdin on tumor cells both *in vivo* and *in vitro* (Sekiguchi *et al.*, 1962; Diller *et al.*, 1963; Tokunaga *et al.*, 1962). From these studies it was generally concluded that properdin was not a significant factor in determining the outcome of neoplastic disease.

Another possible mechanism by which zymosan promotes tumor regressions may be in its ability to stimulate the proliferation of macrophages. Alexander (1970) hypothesized that tumors are not rejected because there are too few suitable macrophages to produce appropriate levels of antitumor cytophilic antibody. He based his hypothesis on the fact that many skin tumors have disappeared if inflammation was induced in the vicinity of the tumor. Zymosan is known to be capable of causing macrophage proliferation and enhancing the phagocytic index in animals and humans bearing advanced tumors (Diller *et al.*, 1963; Kampschmidt and Upchurch, 1968). Kampschmidt and Upchurch (1968) reported that zymosan was capable of markedly stimulating the reticuloendothelial system of tumor-bearing rats. Consequently, the authors suggested that, with appropriate stimulation, the normally depressed RES of tumor-bearing animals could be stimulated. Shinichiro and Shinoki (1968) suggested that the enhanced production of phagocytic cells in the peritoneal cavity after zymosan administration was responsible for the ultimate regression of a rat ascites tumor. They found that transfer of these cells to unsensitized animals inhibited subsequent tumor implants. It was also noted that, at least in their experiments, the number of macrophages was

less important than the time at which they were given, e.g., 3 days prior to tumor implantation was most successful.

A third possible mechanism by which zymosan might promote tumor regressions is through the production of antibodies which, in turn, cross-react with tumor antigens (Martin *et al.*, 1964). Allegedly, heat stable agglutinins were produced against zymosan when it was injected in combination with an adjuvant into rabbits (Blattberg, 1957). It was also observed that zymosan enhanced the bactericidal activity of rabbit serum against *E. coli* B, and it was suggested that antibodies produced against zymosan might cross-react with antigenic determinants on the bacteria.

Finally, zymosan may act as an adjuvant, enhancing the titers of anti-tumor antibodies. Cutler (1959) reported that zymosan enhanced hemolysin in rats particularly when administered 48 hours before, at the same time as, or 48 hours after antigen administration.

As has been previously mentioned, cis-Pt(II), while demonstrating marked cytotoxicity for tumor cells, has also been shown to be an immunosuppressor. This is not surprising since virtually all cancer chemotherapeutic agents usually suppress immune responses. Recently, however, it has been reported that under certain conditions, many of these agents can, in fact, enhance immune responses. Buskirk *et al.* (1965) reported that cytarabine and 5-Fluoro-2-deoxyuridine (FUDR) cause immunologic enhancement at high doses given as a single dose but suppression at low doses given daily. He also observed that uracil mustard and KTS were not immunosuppressors when given at therapeutic doses. Chanmougan and Schwartz (1966) demonstrated that there was enhancement of immune response after the termination of a one-week treatment course of 6MP. Uracil mustard at therapeutic levels and

x-irradiation have also shown immunologic enhancement (Haines *et al.*, 1967; Taliaferro, 1964). Schwartz (1967) reported that 6-MP, prednisone and amethopterin could, under given circumstances, aggravate the graft versus host reaction. This led him to suggest that virtually all cytotoxic materials can enhance immunologic responses.

The mechanism by which these cytotoxic drugs elicit immunostimulation is obscure. It has been suggested, however, that the nucleic acids released from injured cells may stimulate lymphoid tissues which in turn enhance antibody formation (Chanmougin and Schwartz, 1966). Stolfi *et al.* (1971) proposed that the success of cancer chemotherapeutic agents in promoting tumor regressions may, in fact, have an immunologic basis. They suggested that, concomitant with tumor cell destruction, there is a drug-induced lymphoreticular depression followed by a period of lymphocytic propagation. Due to the tumoricidal activity of the drugs, large amounts of tumor cell antigens should be available during this latter period. Thus, conceivably, the proliferating and differentiating lymphocytes could become immunologically committed to these antigens at this time.

Currently, the subject of chemoimmunotherapy, i.e., combination chemotherapy and specific or nonspecific immunotherapy, is of great interest to the cancer therapist. Mathé (1971) recently reviewed the rationale behind its use. He cited numerous experiments in which various chemotherapeutic agents were used in combination with both specific and nonspecific immunostimulants. In virtually all of the experiments, the combined therapy elicited greater numbers of cures or extended the life span to a far greater degree than either chemotherapy or immunotherapy alone. Mathé suggested that the a priori fear that chemotherapeutic agents would negate the effects of immunostimulation

was not justified if the temporal relationship between the drugs and immunostimulants were appropriate. As an example, it was found that chemotherapeutic agents are much more immunosuppressive if given in daily doses than if administered as one injection. The effects of appropriate timing were demonstrated by Chanmougin and Schwartz (1966), who found that if rabbits were treated with 6-MP and then rested for 5 days they would produce hyperimmune responses to antigen. Currie and Bayshawe (1970) demonstrated the importance of timing between the adjuvant (*Corynebacterium pavum*) and the chemotherapeutic agent (cyclophosphamide). They found that giving the adjuvant 12 days after a single dose of cyclophosphamide resulted in complete and lasting regressions in 70% of the animals but that the results were negative if the interval between adjuvant and drug was shortened or lengthened.

Alexander (1970) concluded that, if immunotherapy was to be effective, the tumor size must be reduced since immunotherapy is most effective when relatively few tumor cells are present. Thus it was suggested that chemotherapy should precede immunotherapy. Mathé (1970) appeared to be in complete agreement with this philosophy and, in fact, stated that immunotherapy followed by chemotherapy is not theoretically recommended. He based this on the premise that immunotherapy makes lymphocytes commence cyclic division, making them more susceptible to destruction by chemotherapy. He noted that administration of *Corynebacterium pavum* and BCG prior to chemotherapy was generally ineffective.

The results of the experiments reported here appear to be in direct conflict with this notion. The most successful mode of therapy was when zymosan preceded cis-Pt(II). In concert with these observations were those of Sokoloff *et al.* (1961). They found that zymosan

administered prior to Mitomycin C considerably increased the inhibitory effects of the drug against Sarcoma 180. This was not the case, however, when zymosan was administered at the same time as or after the drug. Since S-180, a nonspecific transplantable tumor, was used in both the experiments reported here and in Sokoloff's experiments, it could be concluded that there are behavioral differences between transplantable tumors and syngeniec or autochthonous tumors. The credibility of this argument breaks down, however, with the report of Martin *et al.* (1964). These investigators found that the most effective treatment regimen in decreasing the recurrence rates of spontaneous mammary tumors in mice consisted of a combination of surgery, cyclophosphamide therapy and zymosan. Of particular interest is the fact that the initial injection of zymosan was always given prior to surgery and the cyclophosphamide, i.e., days -9 to -11, and that two other injections were administered during the period of cyclophosphamide therapy. More recently Martin *et al.* (1970) found that zymosan enhanced the cure rates of spontaneous murine mammary tumors when used in combination with surgery and 4 chemotherapeutic agents, i.e., Streptonigrin, Thioguanine, Endoxan and Mitomycin C. Zymosan was administered in 5 injections: the first 3 days prior to surgery and the others at 14-day intervals. The last 4 injections were during the period in which the chemotherapeutic agents were being used. Consequently, it would appear that at least with zymosan, treatment prior to and/or during chemotherapy is of value.

Taking into consideration the properties of zymosan and cis-Pt(II), the following hypothesis as to their combined mechanism of action can be made. It is proposed that, although zymosan is capable of increasing properdin levels, stimulating the RES and/or producing

cross-reacting antibodies, these augmentors of host responses are insufficient to promote tumor regressions in the majority of animals treated with zymosan alone. The addition of cis-Pt(II) to animals previously treated with zymosan, however, may alter the integrity of the tumor rendering it more susceptible to subsequent attack by zymosan stimulated host defenses.

Although varying numbers of tumor regressions were observed with virtually all combinations of zymosan and cis-Pt(II) therapy, the most consistently successful treatment regimen was found when zymosan was administered 7 days prior to the platinum compound. This suggests that, during this 7-day period, a population of lymphoid cells is produced which are less susceptible to the immunosuppressive effects of cis-Pt(II). The platinum compound is known to exert its maximum immunosuppressive effects within 2 days after antigen administration (Berenbaum, 1971). Consequently, the shorter the interval between the administration of the compounds, the greater the chance of suppressing the immune responses stimulated by zymosan. In fact, experiments utilizing shorter intervals were less successful.

Since S-180 is a nonspecific tumor, attempts to evaluate tumor specific immunologic responses were considered inapplicable. Consequently, the integrity of host responses in animals treated with combinations of zymosan and cis-Pt(II) were evaluated indirectly by measuring responses to SRBC's and skin allograft rejection times. The results of the experiments measuring host responses to SRBC's were of some interest. In the studies in which the treated animals did not bear tumors, the only significant difference noted was that between animals receiving combination therapy and those receiving saline. This difference was only present in those animals who

received antigen on day 21 (Table 15). This suggests that there was a slight enhancement of immune responses with combined therapy as contrasted to zymosan or cis-Pt(II) alone.

The results of those experiments involving animals bearing S-180 were even more rewarding. When the antigen was administered on day 14, those animals treated with saline or zymosan produced plaque-forming spleen cells far in excess of those treated with cis-Pt(II) or combinations of the two compounds. This indicated that: 1) the immunosuppressive capabilities of cis-Pt(II) were still prevalent at 6 days after treatment and 2) pre-treatment with zymosan did not protect against the immunosuppressive effects of cis-Pt(II). When the antigen was administered on day 21, however, the results were completely reversed. Those animals treated with saline or zymosan had marked reduction in plaque numbers. This was particularly true in the former group. Those animals treated with cis-Pt(II) or combinations of the two drugs, on the other hand, had an enhanced plaque-forming ability as compared to the other groups. This was in spite of progressively growing tumors which, in their own right, are believed to be immunosuppressive (Esber *et al.*, 1972). It is of interest to note that the enhancement in response to SRBC's was observed in both mice treated with cis-Pt(II) and those treated with zymosan and cis-Pt(II) and that there were no significant differences between the two treatment groups. The enhancement with the drug alone is suggestive of that noted with other chemotherapeutic agents previously described. The fact that combined therapy did not elicit greater responses in those animals with progressively growing tumors is probably based on the status of the tumor. The animals that did not respond favorably to the treatment in regard to either the tumor

or the SRBC antigen might be considered "poor reactors." On the other hand, those animals whose tumors were regressing appeared to respond more favorably to the antigen. However, one cannot rule out the possibility that the enhanced response to SRBC's in this latter group may have been due to a nonspecific response. Since these animals were responding favorably to the tumor, it would be suspected that their RES activity might be accelerated; thus, their response to SRBC's might be enhanced as well.

When cell mediated immunity was evaluated, a different set of responses was observed. In these experiments the only treatment to accelerate skin allograft rejection was the combination of zymosan and cis-Pt(II). This acceleration was noted only in those animals bearing S-180. In these experiments it appeared that combination therapy was capable of enhancing allograft rejection, and the status of the tumor did not appear to play a significant role. Because of the number of animals dying prior to graft rejection, however, the significance of these data is questionable.

The histologic changes in the lymphoid organs and tumors taken from animals treated with saline, zymosan, cis-Pt(II) or combinations of zymosan and cis-Pt(II) were nonspecific. They were dependent on the status of the tumor and independent of the type of treatment. The hyperplastic response of spleens and regional lymph nodes was marked in all groups and indicated that, even in the controls, the S-180 tumor evoked a host response. In those animals with progressively growing tumors, the thymuses were atrophic. On the other hand, those animals with regressing tumors had normal appearing thymuses. Of particular interest were the cellular reactions in regressing tumors. These were compatible with those found in a homograft rejection

irrespective of treatment. Consequently, it was concluded that the success of the therapeutic regimen was not dependent upon the specificity of the treatment, but rather the ability of the drug or combination of drugs to alter the balance between the tumor and host so that it was favorable to the host.

SUMMARY

The role of host defenses in mediating the regression of Sarcoma 180 (S-180) tumors in mice treated with cis-Dichlorodiammineplatinum(II) [cis-Pt(II)] was investigated.

It was found that the marked anti-tumor efficacy of cis-Pt(II) against S-180 implanted in Swiss mice was reduced when hydrocortisone (HC), an immunosuppressive drug, was administered 7 days before, 6 hours after or 7 days after the platinum compound. This reduction was most dramatic when HC was administered 7 days before or 6 hours after the platinum compound.

In another series of experiments it was found that cis-Pt(II) was ineffective in promoting regressions of S-180 implanted in BALB/c mice. The administration of zymosan, a nonspecific immune stimulant, in combination with cis-Pt(II), however, promoted significant numbers of tumor regressions. This was particularly true if the zymosan was administered on day 1 of tumor growth followed in 7 days by a single injection of cis-Pt(II).

From the results of the previously described experiments it was concluded that the anti-tumor efficacy of cis-Pt(II) is, at least in part, dependent on an active host response directed against the tumor.

The immunologic integrity of BALB/c mice treated with a combination of zymosan and cis-Pt(II) was studied. Humoral antibody production was evaluated by the agarose-slide technique using sheep red blood cells as antigen. There was virtually no difference in spleen

cell plaque forming ability between control and treated animals when the antigen was administered on day 14 of the experiment. When the antigen was administered on day 21 of the experiment, however, those animals treated with a combination of zymosan and cis-Pt(II) had significantly higher numbers of plaque forming spleen cells than the saline treated animals.

Similar studies were performed on animals bearing S-180. When antigen was injected 14 days after tumor implantation, plaque forming cells were found in significantly higher numbers in the animals treated with zymosan or saline than in those treated with cis-Pt(II) or cis-Pt(II) plus zymosan. When antigen was administered on day 21 of the experiment, however, those animals treated with cis-Pt(II) or cis-Pt(II) plus zymosan had significantly higher numbers of plaque-forming cells than the other two groups. Thus it was concluded that, depending on the time of antigen administration, treatment with cis-Pt(II) or zymosan plus cis-Pt(II) may have some stimulatory effect on humoral antibody responses.

Cell mediated immune responses were evaluated by skin allograft rejection. Allografts were rejected earlier in animals bearing S-180 and treated with a combination of zymosan and cis-Pt(II). Consequently, it was concluded that combination therapy may stimulate cell mediated immune responses.

Selected tissues from animals bearing S-180 and treated with saline, zymosan, cis-Pt(II) or a combination of zymosan and cis-Pt(II) were evaluated histologically. Spleens and regional lymph nodes from all groups were markedly hyperplastic, particularly in the marginal zones of the lymphoid follicles. The thymuses of animals with regressing tumors had a normal appearing architecture with a somewhat hyperplastic cortex. In contrast, the thymuses of those animals with

nonregressing tumors were atrophic and the demarcation between cortex and medulla was obscured.

Regressing tumors, regardless of treatment, were characterized by marked lymphocytic infiltration and were surrounded by a proliferating fibrous capsule similar to that seen in homograft rejection. This suggested that ultimate tumor regression may be mediated via immunologic mechanisms rather than a specific attack on tumor cells by the therapeutic agents.

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