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Rescue of rats from large dose cyclophosphamide toxicity using protein A

Prasanta K. Ray[1], Mariam Dohadwala[2], Santu K. Bandyopadhyay[2], Pramook Canchanapan[2], and Denise McLaughlin[2]

[1] Industrial Toxicology Research Center, Lucknow – 226001, India
[2] Department of Surgery, Microbiology and Immunology, The Medical College of Pennsylvania and Hospital, 3300 Henry Avenue, Philadelphia, PA 19129, USA

Summary. *Cyclophosphamide (Cy) is widely used as an effective cytotoxic drug, but its use is limited because of its toxicity. In this report, we describe for the first time the ability of purified protein A (P) of Staphylococcus aureus to reduce Cy-induced toxicity in rats. Protein A-treated animals recover quickly from the toxic effects of Cy. The antitumor property of Cy is not reduced in the P + Cy group. In fact, the latter showed a persistent decrease in tumor volume compared with the Cy group. Protein A may prove to be an effective agent in increasing the therapeutic index of Cy.*

Introduction

Cyclophosphamide (Cy), an alkylating agent, has been found to be effective against a variety of tumors [6]. Because of its ability to cause immunosuppression, it is also used in organ transplant recipients and in the treatment of autoimmune diseases [11–13]. Because of the toxicity of Cy, particularly when it is used at a high dose, its use is limited. Animal studies [1, 4] have indicated that Cy at a high dose is accompanied by severe toxicity, such as hematopoietic depression, nausea, hemorrhagic cystitis, urotoxicity, and toxicity to the microsomal mixed function oxidase (MFO) system, leading to the death of the animals [14, 20]. Several studies have been done in attempts to find some protection against Cy-induced toxicity [4, 8, 15, 18, 24], but great disparities exist regarding their usefulness [1, 17, 19].

In this report, we provide evidence that rats can be protected from acute toxicity syndromes associated with high-dose Cy by IV infusion of purified protein A (P) of *Staphylococcus aureus* Cowan I. It is also gratifying to note that protein A does not alter the antitumor effect of Cy, as was tested in a chemically induced rat mammary adenocarcinoma model. We propose that P can rescue rats from the toxicity associated with high-dose Cy with no loss of the cytoreductive capacity of Cy.

Materials and methods

Animals. For this study female Sprague-Dawley rats (200 ± 15 g) were obtained from Charles River Laboratories, Mass.

Tumors. Primary mammary adenocarcinomas were induced in female Sprague-Dawley rats using dimethylbenzanthracene (DMBA) according to the method described previously [21]. At the time of experiment the average body weight of the animals was 225 ± 25 g.

Cyclophosphamide. Cyclophosphamide (Cy) was obtained from Mead Johnson Pharmaceutical Co., Evansville, Ind. It was solubilized in normal saline at 37° C and was injected (200 mg/kg) IV into rats through the tail vein. This dose of Cy produced about 67% death in normal rats. For rats with DMBA-induced mammary tumors we used a somewhat reduced dose of Cy (180 mg/kg), which caused 30% death in tumor-bearing rats, since the higher dose (200 mg/kg) showed more mortality in tumor-bearing rats than in normal animals.

Protein A. Freeze-dried powder of protein A (P) (5 mg/vial) was obtained from Pharmacia Fine Chemicals, Stockholm, Sweden. A solution of P was prepared in normal saline. Aliquots of this solution were stored below −60° C. Each day one tube was thawed and used. Protein A used in this study was highly purified. Protein A was purified by affinity chromatography using IgG coupled to Sepharose. The purity of the product was checked by polyacrylamide gel electrophoresis and immunoelectrophoresis. To study the effect of P to rescue animals from high-dose Cy toxicity, the following protocol was used. Five groups (6–7 animals/group) of female Sprague-Dawley rats (200 ± 15 g, Charles River Laboratories, Mass) were inoculated with Cy, Cy + P, P + Cy, P, or nothing (3 animals/group). Animals in the first group received Cy (200 mg/kg) IV through the tail vein on day 1. This group (6 rats) is designated Cy. The group (6 rats) which received one inoculation of Cy (200 mg/kg) on day 1 and P (60 μg/kg) also on day 1, and then P being continued for 2 weeks (2 inoculations/week) is designated Cy + P. The group (6 rats) that received first P inoculations for a period of 2 weeks (2 inoculations/week) and then one inoculation of Cy at the end of 2 weeks of P inoculations is designated P + Cy. The experiment was designed in such a way that Cy could be administered on the same day in all the three groups receiving Cy (Cy, Cy + P, P + Cy). Protein A (60 μg/kg) was given twice a week for 2 weeks in all the groups receiving P (Cy + P, P + Cy, P). This dose of P was chosen because both lower doses (< 60 μg/kg) and higher doses (> 60 μg/kg) did not show similar results. Body weights and survival of animals were recorded periodically.

Peripheral and bone marrow WBC counts. Whole blood (0.1 ml) was collected by cutting the tail vein and was

Offprint requests to: P. K. Ray

heparinized. The WBCs were counted on a hemocytometer using 2% acetic acid solution as the diluent to hemolyze the red blood cells. The results are expressed as mean percentages of untreated control values ± SD (n = 3 or more). The average WBC counts for the untreated control group was 10.07 ± 2.1 × 10^6/ml of blood.

Bone marrow WBCs were counted by flushing (X3) the cells from a measured (2.2 cm) portion of the left tibia using Hank's balanced salt solution (HBSS, Gibco, New York). The cells were washed (1,500 rpm, 10 min) with the medium (X3) and then suspended in a known volume of HBSS. The WBCs were counted in the same manner as described above. The results are expressed as mean percentages of untreated control values ± standard deviation (mean ± SD) (n = 3 or more). The total bone marrow WBC level in untreated controls was 64.8 ± 4.0 × 10^6.

Differential cell counts were done by smearing 50 µl heparinized blood on a glass slide using the cytocentrifuge technique. The slides were stained with Wright's stain (Camco Quick Stain) and were counted under an oil immersion lens.

Statistical analyses of the data were done by one-way, nonrepeated measures (ANOVA) with Newman-Keuls post-hocs.

Antitumor effect of Cy and Cy + P. Two groups (Cy, P + Cy) of tumor-bearing rats (10 animals/group) matched for their tumor volumes were given Cy (180 mg/kg) IV on day 1. The second group (P + Cy) received protein A (50 µg/kg) for 14 days (2 inoculations/week) prior to Cy inoculation on day 1. Tumor volumes were determined weekly for a period of 3 weeks and the number of animals remaining alive at the end of every week were scored.

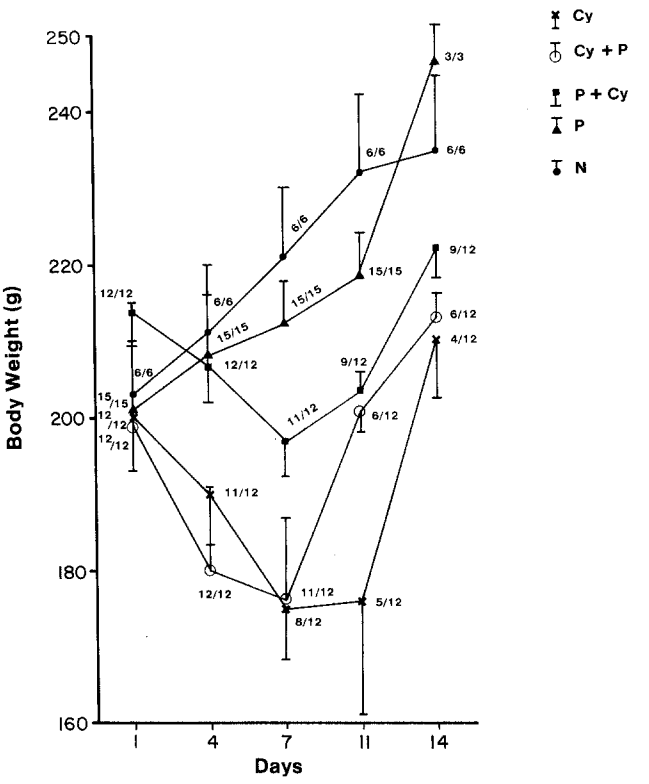

Fig. 1. Effect of Cy, Cy + P, P + Cy, and P on the body weight and survival of female Sprague-Dawley rats. Note the improved survivability (*figures in parentheses*) and body weight increase in Cy + P and P + Cy groups from day 11 onwards. Normal (N) and P groups continue to show increase in their weights

Results and discussion

The pooled data from two separate experiments are presented in Fig. 1. An increase in survival and body weight was observed in Cy + P and P + Cy groups from day 11 onwards. When the majority (67%) of animals in the Cy group died by the 14th day the death rate was 50% in the Cy + P group, and 25% in the P + Cy group, respectively (Fig. 1). Animals receiving only P did not show any mortality or loss of body weight, indicating that P itself is not toxic. Statistical analysis of the survival data on day 14 showed a significant difference ($P < 0.05$ by the chi-square test) between the Cy and the P + Cy groups. However, the difference between the Cy and Cy + P groups was not significant. Data on the decrease in body weight on day 11 were analyzed by Student's t-test, and both Cy + P and P + Cy groups were found to be significantly ($P < 0.05$) different from the Cy group.

Animals belonging to the only P and the untreated control groups gained body weight during the course of the experiment. By day 14, all surviving animals had made up primary loss of body weight and remained alive. When compared with the weights of various organs from age-matched normal rats, a marked increase in the weights of urinary bladder (100%), kidney (46%), and liver (27%) were observed in all the Cy-inoculated animals (Cy, Cy + P, P + Cy) up until day 11. Simultaneously, decreases in the weights of the thymus (26%–83%) and spleen (from no change to 60% decrease) were noted in all groups receiving Cy. Such initial changes in organ weight in Cy + P and P + Cy groups reverted to the normal level by day 14. The protein A group did not show any appreciable change in organ weights. These data suggest that P may not act by abrogating the cytotoxic effect of Cy.

Periodic peripheral and bone marrow WBC counts were taken on different days. All the groups receiving Cy (Cy, Cy + P, P + Cy) showed a marked depletion in WBC counts up to day 7 after Cy inoculation. At this time the cell numbers were too few to count. This also indicates that the cytoreductive ability of Cy was not reduced by P. On day 9, WBC counts in all the groups receiving Cy were possible and showed a similar pattern of depletion in all the groups. WBC counts on day 9 in the Cy group were therefore considered as the baseline showing maximal depletion for comparison purposes. The peripheral WBC counts showed an obvious ($P < 0.001$) regeneration in both Cy + P and P + Cy groups on day 11 (Fig. 2). A significant ($P < 0.0001$) recovery from the initial depletion of WBC counts in the bone marrow was observed on day 11 in both Cy + P and P + Cy groups (Fig. 2). Differential peripheral WBC counts done on day 11 showed a marked depression in lymphocyte count in both Cy + P and P + Cy groups on day 11, although they showed significant ($P < 0.002$) recovery compared with the Cy group. Interestingly, there was a marked increase ($P < 0.0001$) in polymorphonuclear leukocyte (Segs) counts in both Cy + P and P + Cy groups compared with other groups (Fig. 2). Monocyte counts in these two groups also showed an appreciable increase (Fig. 2), but the data did not appear to be statistically significant.

Infection associated with granulocytopenia is a major cause of mortality in patients with cancer [3]. Therefore, development of therapeutic maneuvers to minimize the duration of granulocytopenia induced by various chemotherapeutic agents should be beneficial. Our data (Fig. 2) show

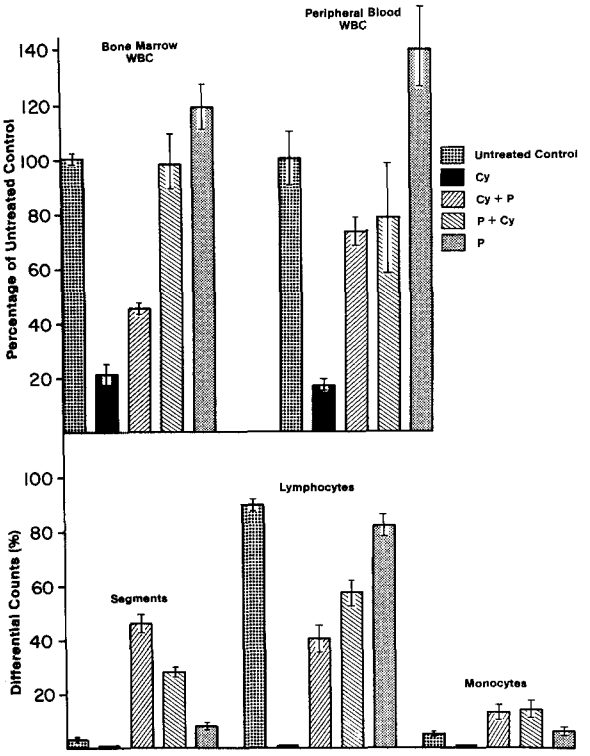

Fig. 2. Effect of Cy, Cy + P, P + Cy, and P on peripheral blood and bone marrow white blood cell counts. Differential counts are also shown

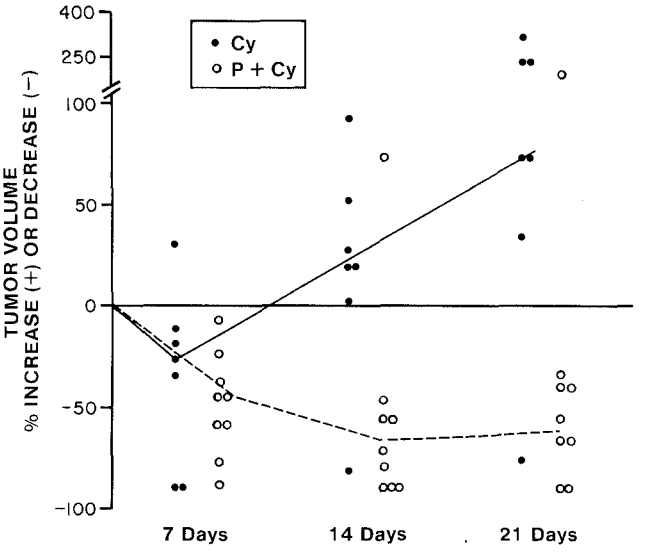

Fig. 3. Effect of Cy and P + Cy on the growth of dimethylbenzanthracene (DMBA) induced rat mammary adenocarcinomas

markedly accelerated recovery of the peripheral granulocyte count from Cy-induced leukopenia, suggesting that protein A may be useful in protecting the host from leukopenia caused by Cy. These data also provide a possible explanation why survivability of animals was better in P + Cy group than the Cy group (Fig. 1).

To answer the question as to whether P treatment interferes with the antitumor property of Cy, an experiment was carried out in two groups of mammary tumor-bearing rats [21]. To keep the majority of tumor-bearing animals alive, an LD_{30} dose of Cy (180 mg/kg) was used. The P dose was also reduced to 50 μg/kg in this experiment. The first group received only Cy, while the second group received P for 2 weeks and then Cy. Cy inoculation was given on the same day in both the groups. The data are shown in Fig. 3. It is apparent that at the end of the 1st week the extent of tumor regression was similar in both the groups (Cy, P + Cy). During the 2nd week, the Cy group showed recurrent growth of tumors while the P + Cy group continued to show tumor regression. The situation remained the same during the 3rd week of follow-up. Thus, administration of P in Cy-inoculated animals appears to be therapeutically beneficial.

Cyclophosphamide is metabolically transformed by the enzymes of the hepatic MFO system into an active alkylating form, phosphoramide mustard, and an acute toxic aldehyde form, acrolein [23]. Acrolein is devoid of alkylating activity but it is responsible for the urotoxicity of Cy [7] and also for the inactivation of the MFO system [4, 8, 18, 24]. In the present study, urinary blood (hematurea) was obtained up to 7–9 days in the Cy groups, as against only 3–4 days in the P + Cy group. Thus, hematurea was less severe in the P + Cy group than in other Cy-treated groups. In an accompanying paper [10] we reported that protein A administration in Cy-inoculated animals helped them to regenerate the activity of the enzymes of the hepatic MFO system (aryl hydrocarbon hydroxylase, aminopyrine demethylase, and cytochrome P-450) faster than the Cy group, although the initial depletion of the enzyme activity up to day 4 was at the same level in all the groups (Cy, Cy + P, and P + Cy). These data suggest that protein A treatment of Cy-inoculated animals is associated with an accelerated regeneration of the biochemical moieties depleted by Cy. Protein A is a potent immunomodulator [5, 9, 21, 22]. The immunopotentiating ability of P may be related to the early regeneration of the damage caused by Cy helping the animals to remain alive. Other bacterial immunomodulators such as BCG [16] and *Corynebacterium parvum* [2] have also been reported to provide added benefit when used in combination with Cy. More research is needed to understand the exact mechanism of P to rescue rats from high-dose Cy toxicity. Whatever the mechanism, it is interesting to note that P does not interfere with the cytoreductive effect of Cy, raising the possibility of its use in experimental cancer therapy.

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