Cancer Chemother Pharmacol (1985) 14: 135–138

Cancer Chemotherapy and Pharmacology

© Springer-Verlag 1985

In vivo protection by protein A of hepatic microsomal mixed function oxygenase system of cyclophosphamide-treated rats

Mariam Dohadwala[1] and Prasanta K. Ray[1,2]

[1] Department of Surgery, Microbiology and Immunology, The Medical College of Pennsylvania and Hospital, Philadelphia, PA 19129, USA
[2] Industrial Toxicology Research Centre, Lucknow-226001, India

Summary. *At a high dose, cyclophosphamide (Cy, 200 mg/kg) causes depression of the enzyme activity of the hepatic mixed function oxygenase (MFO) system in Sprague-Dawley rats. The present report provides evidence for the early regeneration of the depleted enzyme activity in Cy-treated rats by purified protein A (P) of Staphylococcus aureus. Enzymes of the MFO system, such as aminopyrine demethylase and aryl hydrocarbon hydroxylase, were assayed and the content of cytochrome P-450 was determined. Inoculation of P (60 μg/kg) prior to Cy inoculation provides a better effect than P administration after Cy. The exact mechanism of P action is unknown. P-treated animals appear to have an ability to repair the damage caused by the toxic metabolites of Cy earlier than those in the Cy group. This property of protein A may become useful in accelerated regeneration of the enzyme activity in the hepatic MFO system following the toxic insult of Cy metabolites.*

Introduction

Cyclophosphamide, 2H-1,3,2-oxazaphosphorine, 2-bis(2-chloroethyl)aminotetrahydro-2-oxide (Cy) is widely used as an anticancer drug [5, 9, 15, 16, 20, 32]. Cy is also used as an immunosuppressive agent in organ transplant recipients and in the treatment of autoimmune diseases [4, 8, 10–12, 34]. Cyclophosphamide, being inactive in its native form, requires activation by the hepatic microsomal mixed function oxygenase (MFO) system [33] to be converted into phosphoramide mustard, an alkylating agent, and an acute toxic aldehyde form, acrolein. The toxicity associated with high or repeated low doses of Cy include: hematopoietic depression, nausea, hemorrhagic cystitis, urotoxicity, and denaturation of the enzymes of the hepatic microsomal MFO system [9, 20].

The MFO system is necessary for the metabolism and activation of drugs, including Cy [7]. The interaction of acrolein with critical sulfhydryl groups of the enzymes of the MFO system has been proposed as a potential mechanism for the inactivation of the MFO system [23]. The protection of MFO system may prove to be helpful in enhancing the drug-metabolizing ability of the host. In the course of our studies on the antitumor properties of purified protein A of *Staphylococcus aureus* [1, 25, 26–28], we have observed that protein A-treated rats are better protected against lethal doses of Cy [29]. In the present paper we report that protein A administration to Cy-treated rats gives them the capacity for accelerated regeneration of activity of the hepatic microsomal enzymes of the MFO system.

Offprint requests to: P. K. Ray

Materials and methods

Chemicals. Cyclophosphamide (Cytoxan) was obtained from Mead Johnson Pharmaceutical Company, Evansville, Ind. DL-Isocitrate, isocitrate dehydrogenese (type IV), 4-dimethyl-aminoantipyrene (aminopyrine), benzo(a)pyrene, and sodium dithionite were all purchased from Sigma Chemical Co., St. Louis, Mo. NADP and NADPH were purchased from Aldrich Chemical Co., Milwaukee, Wisc, and Boehringer Mannheim, Mannheim, West Germany, respectively. Purified protein A was purchased from Pharmacia Fine Chemicals, Stockholm, Sweden. A solution of protein A was prepared in normal saline. Aliquots were stored at below −60° C for future use. Each day one sample was thawed and used. Protein A used in this study was described by the manufacturer as a highly purified preparation. Protein A was purified to homogeneity by affinity chromatography using IgG coupled to sepharose, and the purity of the product was checked by polyacrylamide gel electrophoresis and immunoelectrophoresis.

Animal treatment. Female Sprague-Dawley rats (200 ± 15 g, Charles River Laboratories, Mass) were divided into five groups (8–10 animals/group). One group was inoculated with Cy (200 mg/kg) solubilized in normal saline at 37° C, the second group (Cy + P) received two separate inoculations of Cy and protein A (60 μg/kg) on day 1 and then protein A twice a week for 2 weeks; the third group (P + Cy) received protein A twice a week for 14 days and then Cy on day 1; the fourth group (P) received protein A twice a week for 2 weeks; and the fifth group received no treatment. Both Cy and P were inoculated IV into the tail vein. The Cy was inoculated on the same day (day 1) in all groups (Cy, Cy + P, P + Cy).

Preparation of microsomes. Hepatic microsomes were isolated from the 105,000 *g* supernatant fraction of liver homogenates according to the method of Berrigan et al. [3]. To minimize the adsorption at 420 nm due to hemoglobin, the microsomal preparation was made in 1.15% KCl solution for the determination of cytochrome P-450 content [24]. Two enzymes of the MFO system, i.e., aminopyrine demethylase and aryl hydrocarbon hydroxylase, were assayed using microsomal preparations in 0.25 *M* sucrose solution [3]. The concentration of proteins in the microsomal preparation was determined by

the method of Lowry et al. [21], bovine serum albumin being used as standard.

Enzyme assay. Hepatic aryl hydrocarbon hydroxylase and aminopyrine demethylase were assayed by the methods of Nebert and Gelboin [23] and Gurtoo et al. [13], respectively. The phenolic metabolites formed during the aryl hydrocarbon hydroxylase reaction were quantified following a method of activation at 396 nm and fluorescence at 522 nm [23]. Fluorescence was compared with a standard curve obtained with 3-hydroxy benzo(a)pyrene supplied by courtesy of Dr A.C. Capomacchia, University of Georgia. A Turner spectrofluorometer (model 430) was used in these studies, and was calibrated with a quinine sulfate standard before and during each determination. Cytochrome P-450 was determined by difference spectroscopy using a Beckman trace III spectrophotometer (model 25), following the method of Omura and Sato [24]. The cytochrome P-450 content was calculated using an extinction coefficient of 91 nM^{-1} cm^{-1} [22].

Results and discussion

All the animals (8–10/group) receiving Cy (200 mg/kg), Cy and P (60 μg/kg; P being administered either prior to or following Cy inoculation), P, and no treatment were sacrificed on day 4 or day 11 after Cy inoculation. Livers of these animals (3 or more) were taken out, pooled, and their microsomes prepared. This microsomal preparation was then used for the assay of the enzymes and cytochrome P-450 content as described above. Triplicate samples ($n = 3$) were analyzed and their averages taken. Results are presented as percentages of the activity in untreated controls. The results described in Fig. 1 show a 50%–54% decrease in activity of the enzyme, aminopyrene demethylase, in the Cy- and (Cy + P)-treated groups on day 4. However, the depletion of the activity of this enzyme was less prominent in the P + Cy group (31% decrease) than the other two Cy groups (Cy and Cy + P). On day 11 the demethylase activity was reversed to normal or near normal in all P groups. The reversal was more obvious in the P + Cy group (13% more than normal) than in either the Cy or the Cy + P groups. The P group even showed an increase of 33% above the normal value at this time.

The activity of aryl hydrocarbon hydroxylase was found to be grossly affected by Cy (Fig. 2) compared with the depletion of aminopyrene demethylase activity. On day 4 in all the Cy groups (Cy, Cy + P, P + Cy), the aryl hydrocarbon hydroxylase activity was decreased up to 80% of normal values. An initial loss of about 40% of this enzyme activity was observed in the protein A group as well. However, on day 11, all the groups showed regeneration of this enzyme activity. Both P + Cy and P groups showed more pronounced regeneration than did the Cy- and (Cy + P)-treated groups.

The content of cytochrome P-450 was decreased by 25%–40% in Cy + P and Cy groups on day 4 compared with untreated normals (Fig. 3). The level of cytochrome P-450 was back to normal in all P-treated groups (Cy + P, P + Cy, and P) on day 11, while the Cy group still showed a 32% decrease in the cytochrome P-450 content compared with normals.

Our data indicate that protein A administration helped in the accelerated regeneration of the enzyme activity of the hepatic MFO system following its depletion by Cy metabolites. The regeneration starts to become evident as early as day 4 (Figs. 1–3) and is obvious by day 11 (Figs. 1–3).

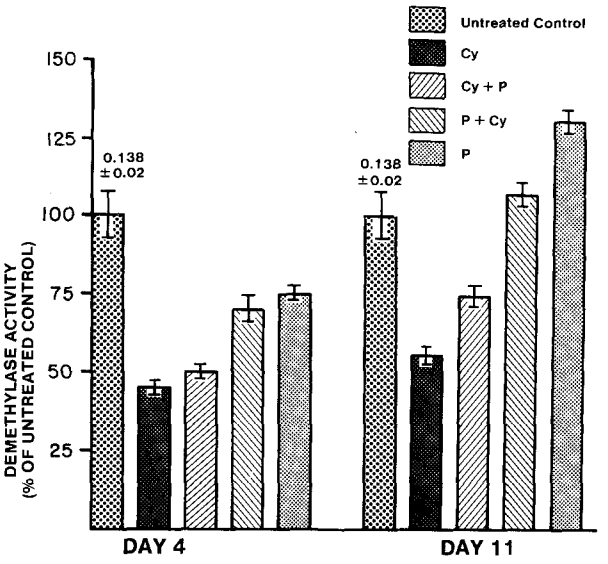

Fig. 1. Protection by protein A (P) from high-dose cyclophosphamide (Cy)-induced depression of aminopyrine demethylase (AD) activity. In addition to untreated controls, adult female Sprague-Dawley rats were treated with Cy and/or P. Rats were sacrificed on day 4 or 11 to isolate hepatic microsomes. AD activity of microsomal preparation was determined. Results are expressed as percent of the levels in untreated controls ± standard deviation ($n = 3$). AD activity of the untreated control group was 0.138 ± 0.02 mmol HCHO formed/mg protein/h. Results similar to those reported here were obtained in repeat experiments

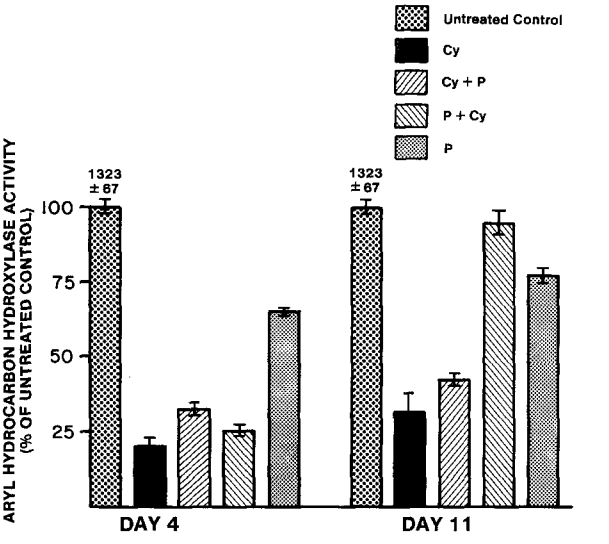

Fig. 2. Protection by protein A (P) from high-dose cyclophosphamide (Cy)-induced depression of aryl hydrocarbon hydroxylase (AHH) activity. Microsomal preparations were used to determine the AHH activity. Results are expressed as percent of levels in untreated controls ± SD ($n = 3$). AHH activity of untreated control group was 1,323 ± 67 pmol equivalent of 3-OH benzo(a)pyrene formed/mg protein/10 min. Results similar to those reported here were obtained in repeat experiments

Acrolein, a toxic metabolite of Cy, has been reported to be responsible for denaturation of the enzymes of the MFO system in vivo [14]. P somehow rescues the animals from the toxic effects of acrolein. The P does not appear to exert its effect directly on the toxic metabolites of Cy, since the

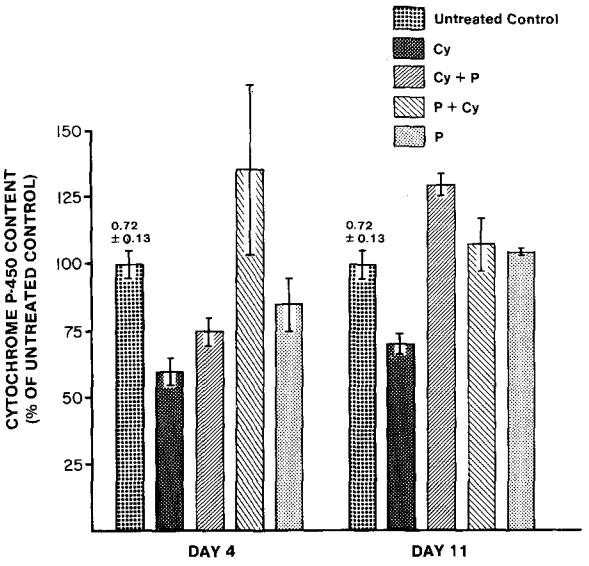

Fig. 3. Protection by protein A (P) from high-dose cyclophosphamide (Cy)-induced depression of cytochrome P-450 content. Hepatic microsomal preparations were used to assay the content of cytochrome P-450. The cytochrome P-450 content of the microsomal preparation of untreated rats was 0.72 ± 0.13 nmol/mg protein. The results are expressed as percent of levels in untreated controls ± SD ($n = 3$). In repeat experiments, similar results were obtained

depletion of the enzyme activity of the MFO system during the early phase (day 4) seems to be similar in all the Cy groups (Cy, Cy + P, P + Cy). Therefore, the P effect appears to be mediated through some other mechanism inducing an accelerated recovery of the depleted enzyme activity in all P-treated groups (Cy + P, P + Cy, P). Our subsequent studies on in vitro ^{14}C-cyclophosphamide metabolism using a hepatic microsomal preparation from normal rat showed that P does not affect ^{14}C-cyclophosphamide conversion to ^{14}C-acrolein (Ray et al. unpublished observation). We have also observed that P does not directly interfere in the acrolein-induced denaturation of cytochrome P-450 activity studied in vitro (Ray et al., unpublished observation). Thus P cannot alter the metabolic conversion of Cy to acrolein, nor does it directly react on acrolein, diminishing its activity.

However, the exact mechanism of protein A action is unknown. Protein A is a glycoprotein of *Staphylococcus aureus* having diverse immunologic properties [6, 8, 25–31], including antitumor properties [1, 25–28]. Other bacterial immunomodulators, such as BCG and *Corynebacterium parvum,* have also been reported to have the ability to modify Cy effects in animals [2, 17]. Our preliminary studies have indicated that protein A can rescue animals from dying of severe toxicity associated with high dose of Cy (200 mg/kg) [29]. At this high dose, most (66%) of the Cy-treated rats died within 7–12 days, whereas the majority (75%) of the (P + Cy)-treated rats remained alive [29]. In the present study, the same group (P + Cy) showed the greatest capacity for accelerated regeneration of the depleted hepatic microsomal enzyme activity. We have also observed that hematologic toxicity rendered by Cy at this high dose (200 mg/kg) can be reversed in an accelerated manner by prior inoculation of protein A to the host [29, 30]. Peripheral blood differential counts suggested an appreciable increase in polymorphonuclear neutrophils in (P + Cy)-inoculated animals [30]. It may be mentioned, however, that at lower doses of Cy (50 and 100 mg/kg) the toxicity is less and the protein A effect is more obvious. Thus, it appears that the protection of rats from the lethality of Cy treatment by protein A may be related to a number of mechanisms [29, 30].

Cy is a widely-used anticancer drug [5, 9, 15, 16, 20, 32]. We have observed that protein A does not alter the antitumor activity of Cy [29]. Thus, it appears that the use of protein A in combination with Cy may be helpful to decrease the toxicity of Cy and thus increase the therapeutic index of Cy.

Acknowledgements. This work was supported by grants from W.W. Smith Charitable Trust, R.J. Reynolds Industries, Inc. and Fannie E. Rippel Foundation. The authors are grateful to Drs J.G. Bassett and D.R. Cooper for their continued interest.

References

1. Bandyopadhyay S, Mobini J, Ray PK (1983) Intravenous infusion of purified protein A causes regression of rat mammary adenocarcinomas. Fed Proc 42: 2270
2. Bernard F, Nurten G (1979) Further observations on the inhibition of tumor growth by *Corynebacterium parvum* with cyclophosphamide. X. Effect of treatment on tumor cell kinetics in mice. J Natl Cancer Inst 62: 1545
3. Berrigan MJ, Gurtoo HL, Sharma SD, Struck RF, Marinello AJ (1980) Protection by *N*-acetylcysteine of cyclophosphamide metabolism-related in vivo denaturation of cytochrome P-450. Biochem Biophys Res Commun 93: 797
4. Brock N, Stekar J, Pohl J, Niemeyer U, Scheffler G (1979) Acrolein the causative factor of urotoxic side-effects of cyclophosphamide, ifosfamide, trofusfamide and sufosfamide. Arzneimittelforsch 29: 659
5. Carter SK (1972) Single and combination nonhormonal chemotherapy in breast cancer. Cancer 30: 1543
6. Catalona WJ, Ratliff TL, McCool RE (1981) Interferon induced by *S. aureus* protein A augments natural killing and ADCC. Nature 291: 77
7. Cooper DY, Levin S, Narasimhula S, Rosenthal O (1965) Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. Science 147: 400
8. Cowan FM, Klein DL, Armstrong GR, Pearson JW (1979) Augmentation and inhibition of delayed hypersensitivity by *Staphylococcus aureus* protein A. Biomedicine 30: 241
9. Fernandez CH, Lindberg RD, Sutow WW, Samuels ML (1979) Localized Ewig's sarcoma-treatment and results. Cancer 34: 143
10. Friedman RM, Myles A, Colvin M (1979) Cyclophosphamide and related phosphoramide mustards – current status and future prospects. Adv Cancer Chemother 1: 143
11. Gerber NL, Steinberg AD (1976) Clinical use of immunosuppressive drugs (I). Drugs 11: 14
12. Gerber NL, Steinberg AD (1976) Clinical use of immunosuppressive drugs (II). Drugs 11: 90
13. Gurtoo HL, Gessner T, Culliton H (1976) Studies of the effects of cyclophosphamide, vincristine, and prednisone on some hepatic oxidations and conjugations. Cancer Treat Rep 60: 1285
14. Gurtoo HL, Marinello A, Struck RF, Paul B, Dahms R (1981) Studies on the mechanism of denaturation of cytochrome P-450 by cyclophosphamide and its metabolites. J Biol Chem 256: 11691
15. Heyn RM, Hollan R, Newton WA, Jr, Tefft M, Breslow N, Hartman JR (1974) The role of combined chemotherapy in the treatment of rhabdomyosarcoma in children. Cancer 34: 2128
16. Humphrey RL, Kvols LK, Braine HF, Abelhoff MD (1973) High-dose cytoxan therapy of poor-risk myeloma and Waldenstrom's macroglobulinemia. Proc Am Assoc Cancer Res 14: 54
17. Ladisch S, Reaman GH, Poplack DG (1979) Bacillus calmette-Guérin enhancement of colony-stimulating activity and

myeloid colony formation following administration of cyclophosphamide. Cancer Res 39: 2544
18. Lares RK, Penner JA (1971) Thrombocytopenic purpura treated successfully with cyclophosphamide. J Am Med Assoc 215: 445
19. Lenhard RE Jr (1976) Prentice RL, Owens AH Jr, Bakemeier R, Horton JH, Shnider BI, Stolback L, Bernard CW, Carbone PP (1976) Combination chemotherapy of the malignant lymphomas-A controlled clinical trial. Cancer 38: 1052
20. Livingston RB, Carter SK (1970) Single agents in cancer chemotherapy. Plenum, New York, p 28
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265
22. Masters BSS, Okita RT (1980) The history, properties and function of NADPH-cytochrome P-450 reductase. Pharmacol Ther [B] 9: 227
23. Nebert DW, Gelboin HV Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. J Biol Chem 243: 6242
24. Omura R, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. J Biol Chem 239: 2370
25. Ray PK (1982) Suppressor control as a modality of cancer treatment. Perspectives and prospects in the immunotherapy of malignant disease. Plasma Ther Transfus Technol 3: 101
26. Ray PK, Bandyopadhyay SK (1983) Inhibition of rat mammary tumor growth by purified protein A – a potential anti-tumor agent. Immunol Commun 12: 453
27. Ray PK, Bandyopadhyay S (1984) Antitumor activity with non-toxic doses of protein A. Cancer Immunol Immunother (in press)
28. Ray PK, Mohammed J, Allen P, Raychaudhuri S, Dohadwala M, Bandyopadhyay S, Mark R (1984a) Effect of frequency of plasma adsorption over protein A-containing *Staphylococcus aureus* on regression of rat mammary adenocarcinomas: Modification of antitumor immune response and tumor histopathology. J Biol Resp Modif 3: 39
29. Ray PK, Dohadwala M, Bandyopadhyay SK, Canchanapan P, McLaughlin D (1984b) Rescue of rats from large-dose cyclophosphamide toxicity using protein A. Cancer Chemother Pharmacol 14: 59–62
30. Ray PK, Bandyopadhyay S, Dohadwala M, Canchanapan P (1984c) Reversal of cyclophosphamide-induced leukopenia by protein A. Biomed Pharmacother (in press)
31. Sakane T, Green I (1978) Protein A from *Staphylococcus aureus* – A mitogen for human T-lymphocytes and B-lymphocytes but not for L-lymphocytes. J Immunol 120: 302
32. Santo GW, Sesenbrenner LL, Anderson PN, Burke PJ, Klein DL, Slavin RE, Schacter B, Borgaonkar DS (1976) HL-A identical marrow transplants in aplastic anemia, acute leukemia and lymphosarcoma employing cyclophosphamide. Transplant Proc 8: 607
33. Sladek NE (1971) Metabolism of cyclophosphamide by rat hepatic microsomes. Cancer Res 31: 901
34. Zinke H, Woods JE (1977) Donor pretreatment in cadaver renal transplantation. Surg Gynecol Obstet 145: 183

Received January 6, 1984/Accepted August 1, 1984