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MODULATION OF BENZENE INDUCED TOXICITY BY PROTEIN A

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Abstract—Administration of benzene (i.p. 1.0 mL/kg body weight) for 3 consecutive days produced leucopenia and lymphocytopenia in female albino rats. In addition, the total iron content, lipid peroxidation and superoxide dismutase activity of the liver and bone marrow were significantly ($P < 0.001$) increased. Low molecular weight (LMW) bleomycin-detectable iron accumulated only in bone marrow. Prior administration of Protein A (PA), a multipotent immunostimulant and interferon inducer (60 μg/kg body weight, i.v. twice weekly for 2 weeks), ameliorated most of the adverse effects of benzene. PA restored the changes in hepatic histological architecture, reversed leucopenia and superoxide dismutase activity, lipid peroxidation, total iron content and LMW iron content of bone marrow were normalized. Isozymes of glutathione-*S*-transferase (α, π, μ) which decreased following benzene exposure increased in PA pretreated benzene exposed rats. This study suggests that pretreatment with PA modulates the toxicity of benzene.

Benzene is a ubiquitous environmental pollutant, myelotoxic agent and human carcinogen [1–3]. Chronic exposure to benzene results in bone marrow depression resulting in pancytopenia, aplastic anemia and acute myelogenous leukemia [4, 5]. Exposure to benzene induces immunosuppression and genotoxic effects such as chromosome aberrations [6]. Benzene is a multipotent carcinogen, as it produces a variety of neoplasias in various animal species including zymbal gland carcinomas, carcinomas of oral and nasal cavities, skin and mammary glands [7]. Benzene metabolites induce sister chromatid exchanges in lymphocytes and also inhibit the proliferation and differentiation of lectin stimulated lymphocytes in culture and interfere with microtubule assembly [8].

Lewis *et al.* [9] and Schlosser *et al.* [10] have observed that benzene metabolites have potent toxic effects on macrophage function and activation, which may play a significant role in haemotoxicity. Short term exposure to benzene depresses mitogen induced blastogenesis of both B and T lymphocytes in mice [11]. Benzene also induces a delay in cell mediated immune response. Kalf *et al.* [12] observed prevention of benzene myelotoxicity by indomethacin, a nonsteroidal anti-inflammatory drug.

It was reported earlier that the synthetic immunostimulant and interferon inducer poly-inosinic-cytidilic acid (Poly IC†) [13] or a fungal immunostimulant and interferon inducer 6MFA from *Aspergillus ochraceous* [14] can modulate benzene toxicity. Protein A (PA) is a glycoprotein (M_r 43) present on the cell surface of *Staphylococcus aureus* Cowan I and has the property of binding to the Fc part of immunoglobulin G (IgG) and immune complexes [15]. PA is a multipotent immunostimulant with the ability to induce interferon and potentiate NK cell activity [16]. PA administration resulted in the amelioration of toxicity of carbon tetrachloride and bacterial endotoxin [17, 18] and this paper describes its effects on benzene toxicity.

MATERIALS AND METHODS

PA was purchased from Pharmacia (Piscataway, NJ, U.S.A.) (No. 17-0770-01).

Female albino rats (Swiss Wistar) bred at ITRC Animal House, weighing approx. 100 ± 10 g and maintained on a standard Lipton diet were used throughout the experiments. The animals were divided into four groups of six and treated as follows: Group 1. Normal saline control, injected sterile normal saline. Group 2. Benzene group was injected (i.p.) with benzene (1.0 mL/kg body weight) daily for 3 consecutive days. Group 3. PA (60 μg/kg body weight) in normal saline was injected i.v. twice weekly for 2 weeks. Group 4. PA (60 μg/kg body weight) administered i.v. twice weekly for 2 weeks and then injected with benzene (1.0 mL/kg body weight) i.p. one injection daily for 3 consecutive days. A dose of 60 μg/kg body weight PA was used because in earlier studies it had produced significant biological effects [17].

All the animals were killed by cervical dislocation 24 hr after receiving the last injection of benzene. Blood was collected from the jugular vein for enumeration of total leucocytes as well as differential leucocyte counts after staining with Leishman's stain.

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† Abbreviations: δ-ALA, δ-amino levulinic acid; GST, glutathione-*S*-transferase; Ig, immunoglobin; IL, interleukins; LMW, low molecular weight; 6MFA, 6th mycelial fraction acetone acronymed 6MFA, given to the interferon-inducing antiviral agent isolated from fungus *Aspergillus ochraceous* ATCC 28706; PA, protein A; PFC, plaque forming cells; Poly IC, poly inosinic-cytidilic acid; SOD, superoxide dismutase; SRBC, sheep red blood cells H and E, haemotoxylene and Eosin stain; NK, natural killer.

Table 1. Effect of PA on the levels of hepatic and bone marrow lipid peroxidation of rats treated with three i.p. injections of benzene (1.0 mL/kg body wt)

Treatment	Lipid peroxidation (nmol TBARS formed/30 min/mg protein) Liver	Bone marrow
None	0.098 ± 0.002	0.150 ± 0.002
Benzene	0.39 ± 0.008†	0.22 ± 0.004*
Benzene + PA	0.099 ± 0.025‡	0.152 ± 0.002§
PA	0.09 ± 0.016	0.145 ± 0.03

* $P < 0.001$; † $P < 0.002$; as evaluated by Student's *t*-test, when compared with control.
‡ $P < 0.001$; § $P < 0.02$; when compared with benzene treated rats.

Table 2. Effect of PA on the iron content of the liver and bone marrow of rats exposed to three i.p. injections of benzene (1.0 mL/kg body wt)

	Liver		Bone marrow	
	Total Fe (μg/g wt tissue)	Bleomycin Fe (μg/mg protein)	Total Fe (μg/g wt tissue)	Bleomycin Fe (μg/mg protein)
Control	124 ± 7	ND	158.5 ± 7	ND
Benzene	172 ± 8*	ND	190 ± 8*	0.041 ± 0.001*
Benzene + PA	117.7 ± 6†	ND	160 ± 6‡	0.01 ± 0.001†
PA	119.7 ± 5	ND	149 ± 8	ND

ND, not detectable.
* $P < 0.001$; when compared with control as evaluated by Student's *t*-test.
† $P < 0.001$; ‡ $P < 0.01$; when compared with benzene treated group of animals.

A second set of animals containing four groups identical to the previous set were injected i.p. with sheep red blood cells (SRBC) (1×10^9 cells per 0.2 mL per 100 g body wt) 24 hr before the last PA injection. An additional fifth group of animals exposed to SRBC and normal saline, was used as a SRBC control for measuring haemagglutinin titre.

Histological studies. Routine autopsy was performed on all the animals. Organs such as liver, thymus, spleen and kidney were collected for organ weights. Portions of the tissues were fixed in 10% buffered formaline and Bouin's fluid and then later processed for microscopical examination using routine microtomy and staining procedures [19].

Estimation of tissue iron content. Tissue iron content was estimated according to the method of Thompson and Blanchflower [20] and finally measured in a DC plasma Emission Spectrophotometer (Beckman Model Spectrospan V). Low molecular weight (LMW) bleomycin-detectable iron was estimated by the method of Pandya *et al.* [21].

Assay of lipid peroxidation. Tissues were homogenized in 0.15 M KCl and malonaldehyde levels as thiobarbituric acid reactants (TBARS) indicating the formation of lipid peroxides were estimated at 535 nm according to the procedure of Utley *et al.* [22].

Purification and assay of hepatic and bone marrow superoxide dismutase. The procedure followed for the preparation of mitochondria was that of Weisiger and Fridovich [23] as modified by Pandya *et al.* [14]. Pooled samples of marrow were extracted from the femurs of at least four animals and homogenized in a balanced salt solution containing 0.01% D-glucose, 50 μM calcium chloride, 0.1 mM magnesium chloride, 14.5 mM Tris and 0.54 mM potassium chloride, as described by Hanson *et al.* [24]. The enzyme assay was carried out according to the procedure of Misra and Fridovich [25]. The assay system consisted of 1.0 mL carbonate buffer (0.05 M; pH 10.2), 0.2 mL EDTA (1×10^{-4} M), 2.0 mL epinephrine solution (3×10^{-4} M) and 0.1 mL enzyme solution. Protein content was measured according to the method of Lowry *et al.* [26].

Haemagglutinating antibody response. Sera samples were collected on the fifth day after SRBC administration from animals receiving different treatments. The test was performed essentially according to the method described by Dusre *et al.* [27] with some modifications as described by Pandya *et al.* [14]. End points were taken as partial but definite agglutination of SRBC and recorded after 90 min of incubation at room temperature.

Glutathione-S-transferase (GST). Livers were excised and washed with ice cold saline. A 20% (w/v) homogenate was prepared in ice cold 50 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose in a Potter–Elvehjem type glass homogenizer. The liver homogenates were centrifuged at 9000 *g* for 20 min at 4° and the resulting supernatant (S-9 fraction) was

Table 3. Effect of PA on the activity of hepatic and bone marrow SOD activity of rats treated with three injections of benzene (1.0 mL/kg body wt)

Group	Liver		Bone marrow	
	Mitochondrial enzyme	Post mitochondrial enzyme	Mitochondrial enzyme	Post mitochondrial enzyme
Control	2.69 ± 0.20	3.10 ± 0.55	2.49 ± 0.20	2.88 ± 0.44
Benzene	0.67 ± 0.15*	1.58 ± 0.25	0.21 ± 0.14*	0.66 ± 0.17*
Benzene + PA	3.14 ± 0.142†	2.6 ± 0.19‡	0.97 ± 0.06†	3.31 ± 0.28†
PA	3.14 ± 0.35	3.5 ± 0.31	2.60 ± 0.25	3.17 ± 0.32

Activity expressed as μM adrenochrome formed per min 25 μg protein.
The values are evaluated using $E_{max} = 4.01 \times 10^{-3} M^{-1} cm^{-1}$. Background epinephrine oxidation = 4.75 μmol/min.
* $P < 0.001$; when compared to control.
† $P < 0.001$; ‡ $P < 0.05$; when compared with benzene treated rats.

Table 4. Total and differential counts in peripheral blood of female albino rats exposed to three i.p. injections of benzene (1.0 mL/kg body wt) and pretreated with PA

	Leucocyte counts Total ($\times 10^3/\mu L$)	Differential leucocyte counts Polymorph (%)	Lymphocytes (%)	Monocytes (%)	Esinophils (%)
Control	13.8 ± 1	23.0 ± 1.5	76.0 ± 1.9	0.5 ± 0.2	0.5 ± 0.25
Benzene	7.1 ± 0.5*	26.5 ± 2.6	70.3 ± 0.4	1.5 ± 0.5	1.5 ± 0.6
Benzene + PA	12.95 ± 0.4†	24.5 ± 2.5	75.3 ± 3	0.5	1.0 ± 0.8
PA	13.9 ± 0.8	23.5 ± 1.2	75.5 ± 2.0	0	1.1 ± 0.7

* $P < 0.001$; when compared with control.
† $P < 0.001$; when compared with benzene treated.

used for GST assay according to the procedure of Habig *et al.* [28] using chloro-2,4-dinitrobenzene (CDNB) as a substrate.

GST isoenzymes. Primary antibodies raised against purified GST isoenzymes, i.e. human placental π, human liver-α and human liver-μ classes, were generous gifts from Prof. Yogesh C. Awasthi (Department of Human Genetics, University of Galveston, TX, U.S.A.). The preparation, purification and characterization of the antibodies has been described by Dwivedi *et al.* [29]. Secondary goat anti-rabbit-IgG antibodies, coupled with horseradish peroxidase, were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

For the dot blot immunobinding assay the protein samples (i.e. 50, 100 and 150 μg) of the S-9 fraction were spotted onto nitrocellulose paper sheets using a Minipold apparatus (Schleicher and Schuell, West Germany) and air dried. Cross reactive proteins were detected immunochemically as described earlier by Young and Davis [30], with some modifications. Briefly, nitrocellulose sheets, containing spots of S-9 protein samples were incubated with either of the primary antibodies, i.e. anti-human placental GST-π (1:3000 dilution), anti-human liver GST-α (1:300 dilution) and anti-human liver GST-μ (1:750 dilution) followed by horseradish peroxidase coupled with secondary antibodies (1:1000 dilutions). The bound antibodies were visualized by incubating the nitrocellulose sheets with diaminobenzidine and hydrogen peroxide. An EC densitometer, coupled to a Hewett Packard integrator, was used for the quantification of the dots during this experiment.

RESULTS

Tissue iron content, lipid peroxidation and superoxide dismutase (SOD) activity in liver and bone marrow cells

Treatment of animals with benzene caused a significant increase in the iron content and lipid peroxidation of the liver and bone marrow (Tables 1 and 2). Pretreatment with PA normalized these. LMW bleomycin-detectable iron significantly increased in bone marrow of the benzene treated rats. However, pretreatment with PA normalized it. SOD activity showed significant enhancement upon exposure to benzene alone. Prior exposure to PA resulted in reversal of the benzene effect on SOD activity (Table 3).

Haematology

The exposure of rats to benzene brought about a significant decrease in the total leucocyte counts in the peripheral blood as well as a significant decrease in the number of lymphocytes ($P < 0.001$). However, pretreatment with PA brought these levels to normal (Table 4).

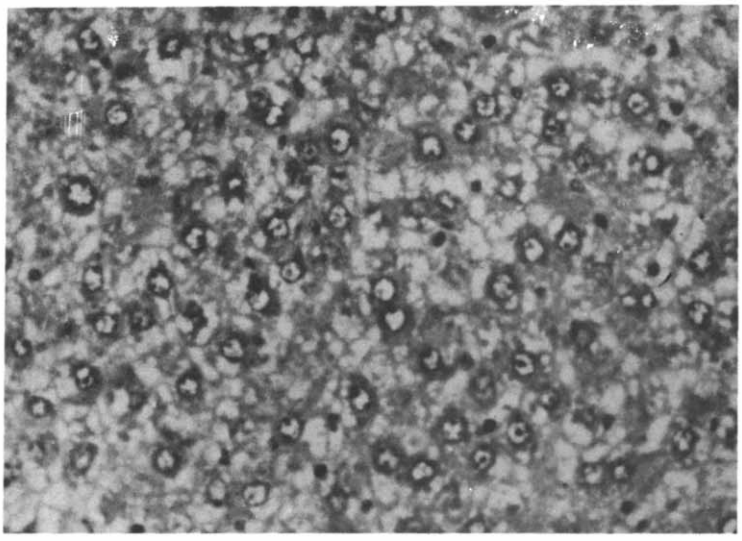

Fig. 1. Section of rat liver after three i.p. injections of benzene. Hepatic lobule showing wide spread fatty changes (H and E × 290).

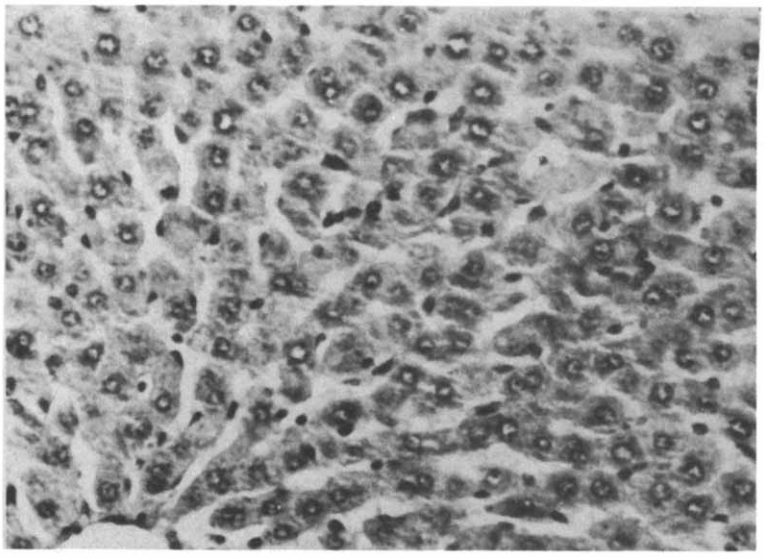

Fig. 2. Section of liver pretreated with PA followed by three i.p. injections of benzene. Section showing normal hepatic architecture (H and E × 290).

Organ weights and histopathological findings

Benzene treatment resulted in a decrease in gross organ weight of thymus and spleen. Prior exposure of animals to PA restored the weights of thymus to some extent (data not given).

Amongst the histopathological findings, liver presented widespread fatty changes of moderate to severe degree as evidenced by clear vacuoles in hepatic cells of benzene-treated animals. However, there was hardly any evidence of nuclear changes in hepatic cells. Portal triad areas and sinusoids were within the limits of histological variation. Similarly, spleen and thymus did not show any significant change in their architecture. However, the liver from PA plus benzene-treated animals presented almost normal architecture which closely resembled PA alone. Other organs, such as thymus, spleen and peripheral and regional lymph nodes from the latter two groups, showed no histological changes (Figs 1–3).

Haemagglutinating antibody response

The serum haemagglutinating SRBC antibody

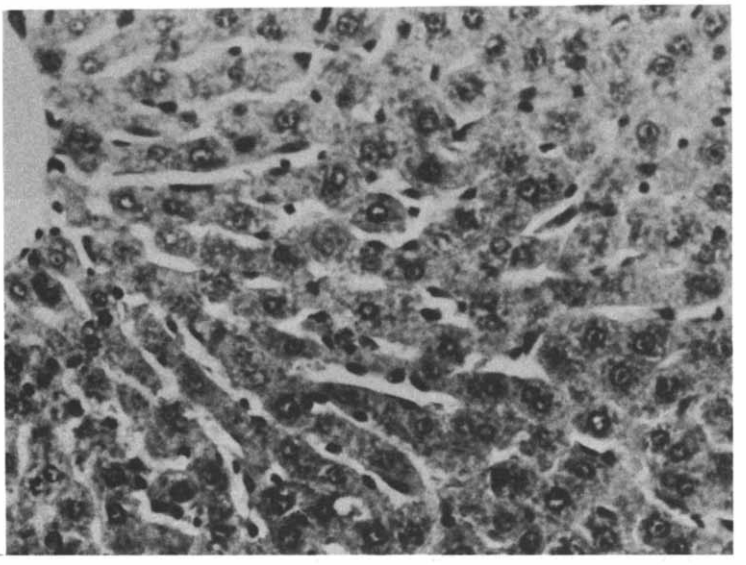

Fig. 3. Section of rat liver treated with PA showing characteristic normal hepatic paranchyma and hepatocyte (H and E × 290).

Table 5. Haemagglutinating SRBC antibody titres of rats exposed to three i.p. injections of benzene (1.0 mL/kg body wt) and pretreated with PA

Group	I	II	III	IV	V	VI	Mean ± SE	
Saline	0	0	0	0	0	0		
SRBC + saline	64	128	128	64	64	64	85.3 ± 13.5	(B)
SRBC + saline + benzene	32	64	4	32	16	8	26 ± 9.0	(A)
SRBC + benzene + PA	128	256	256	128	128	128	170.66 ± 27.0	(C)
SRBC + PA	1024	1024	572	256	1024	1024	811 ± 139	(D)

Significance: P values were determined by Student's *t*-test. A vs B = $P < 0.01$. B vs C = $P < 0.001$. B vs D = not significant.

Note: I–VI serum samples were obtained from animals injected with different batches of SRBC.

Table 6. Effect of benzene administration and PA treatment of rats on hepatic GST activity

Treatment	GST (nmol conjugate formed/ min/mg protein)
Control	684.8 ± 26
Protein A	797 ± 29*
Benzene	602.0 ± 27†
Protein A + benzene	654.8 ± 25

* $P < 0.02$; † $P < 0.05$.

titre in benzene-treated animals was considerably reduced on the fifth day and pretreatment with PA to benzene-exposed animals raised the serum titre (Table 5).

GST

The total hepatic GST activity increased after PA administration. There was a decrease in total hepatic GST activity after benzene administration, which was reversed by PA treatment (Table 6).

Isoenzyme analysis

The antibodies raised against various isoenzymes of GST used in dot blot immunobinding assays revealed that in untreated animals the levels of GST-α were present in higher amounts, as compared to those of GST-π and GST-μ isoenzymes. Benzene caused a reduction in the levels of GST-α (16%), GST-π (29%) and GST-μ (4%) in rat liver. PA induced increases of GST-α by 2.60, of GST-π by 5.25 and of GST-μ by 5.7 times (Fig. 4). PA administered during benzene toxicity showed higher levels of GST-α 1.82, GST-π 1.16 and GST-μ 2.87 times as compared to that in the controls. The former are reduced because of the toxic insult by benzene.

DISCUSSION

The present study demonstrates that treatment of rats with PA before benzene exposure results in normalization of benzene induced SOD activity and lipid peroxidation of the liver and bone marrow. The accumulation of total iron in liver and bone marrow as well as LMW bleomycin-detectable iron in bone marrow, observed in the benzene group,

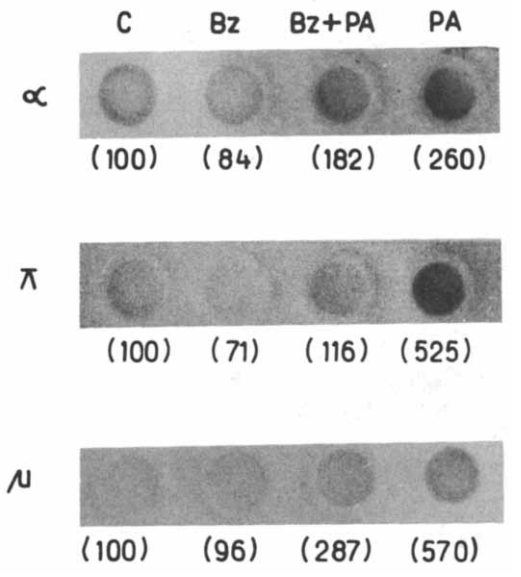

Fig. 4. Dot blot immunoassay of post mitochondrial supernatant fractions, obtained from control (C), benzene toxicity (Bz), benzene + PA treated (Bz + PA) and PA treated, animals, using anti GST isoenzyme (π), anti GST isoenzyme (α) anti GST isoenzyme (μ) antibodies. Experiments were repeated three times using different concentrations of sample protein (50–150 μg) each time. Results of a typical experiment (with 100 μg sample protein) are given. Numbers in parentheses represent per cent intensity levels of immunoreactive protein, taking control values as 100%.

were normalized by PA. Hepatic GST activity was higher as a result of PA treatment. Our earlier studies with interferon inducers like 6MFA [14] and Poly IC [13] indicated that both ameliorate benzene toxicity.

Benzene exposure leads to anaemia, lymphocytopenia and neutropenia in mice as observed by Snyder *et al.* [31]. Our studies also showed that rats exposed to benzene developed leukopenia which could be ameliorated by pretreatment with PA. Histopathological changes observed during benzene exposure can also be prevented by PA treatment. Wierda *et al.* [32] observed that exposure of mice to benzene inhibited lymphocyte (T & B) mitogenesis and also decreased antibody titre and plaque forming cells (PFC). The decrease in antibody titre to SRBC and reduced PFC (IgM) in groups of rats exposed to benzene are in agreement with their observations. The possible target cells of benzene toxicity are macrophages, which provide polypeptide growth factors required for proliferation, development and survival progenitor lineages. Lewis *et al.* [9] noted that benzoquinone, hydroquinone and benzenetriol, all benzene metabolites, inhibited priming of interferon for cytolysis [33]. It has been widely reported [34–36] that interferon (or interferon inducing agents) depresses the mixed function oxidase system and decreases cytochrome P450 haemoprotein level which will reduce the formation of cytotoxic metabolite(s) of benzene.

GST isoenzymes (α, π, μ) were decreased after benzene exposure and restored by PA. Dwivedi *et al.* [29] observed that PA causes an induction of GST-π. It is possible that PA induces GST-π via increased interferon levels. The mechanism of induction of GST by PA is not understood. The increase of GST isoenzymes induced by PA either alone or even following benzene treatment suggests that this activity may play a role in ameliorating the toxicity of benzene. Benzene epoxide and benzene dihydro-diol epoxide-2 [1] are excreted as GSH conjugates.

The following hypothesis on putative mechanism of benzene induced toxicity and its modulation by PA, 6MFA [14] or Poly IC [13] is presented in Fig. 5. It has been observed that benzene inhibits δ-amino levulinic acid (δ-ALA) dehydratase [37] and decreases haem regulatory pool as observed by

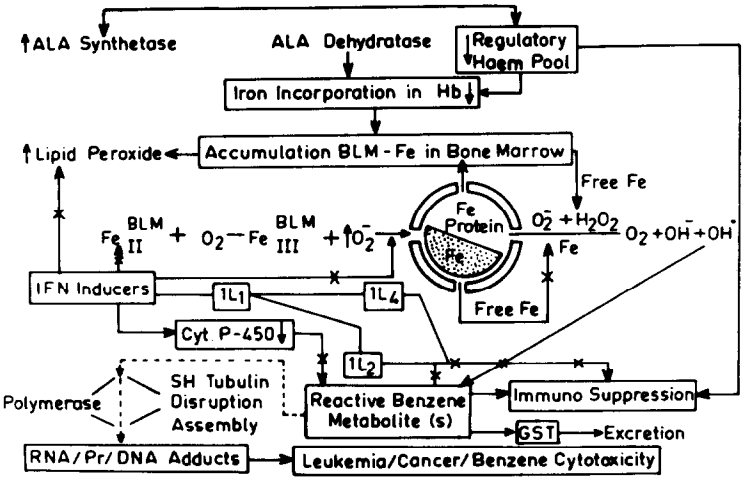

Fig. 5. Putative mechanism of benzene toxicity and its amelioration.

Siddiqui *et al.* [38] which may lead to a decrease in iron incorporation in haemoglobin [39, 40]. As a consequence, LMW catalytically active iron accumulates [21] in bone marrow. The accumulated LMW iron can react with superoxide anions leading to formation of ·OH radicals by Fenton reaction as observed by Khan *et al.* [41]. The hydroxyl radicals may lead to the formation of reactive metabolite(s) of benzene. Interferon inducers can reverse immunosuppressive effects as well as biochemical, histopathological and haematological alterations, ultimately reducing myelotoxicity. Oxidative stress induced during benzene toxicity is ameliorated as evident from the decrease in lipid peroxidation and SOD activity. PA increases specific GST isozyme suggesting decreased retention of cytotoxic metabolites like epoxides and diol-epoxides and this could be one of the ways by which PA mediates its antitoxic properties. Benzene metabolites (like hydroquinone, benzoquinone) can prevent maturation of B lymphocytes from pre-B lymphocytes by inhibiting production or release of IL-4 from stromal cells. Recently MacEachern and Laskin [42] reported increased production of tumour necrosis factor α and IL-1 by bone marrow leukocytes following benzene treatment of mice. Altered cytokine levels may have a profound effect on haemopoesis and can play a significant role in benzene induced cytotoxicity and its prevention by PA treatment. Macrophage IL-1 is needed for the release of IL-4 [43]. Cheung *et al.* [44] observed that benzene metabolites (*p*-benzoquinone, catechol or hydroquinone) significantly inhibited γ-interferon production and IL-2 production in cultures of spleen cells. PA is known to induce interferon [16] which can depress cytochrome P450 levels [36] leading to a decrease in the generation of toxic metabolite(s) of benzene. This may be one of the key factors in abrogating its toxicity.

The protective potency of interferon inducers like PA against benzene toxicity and toxicity of various other chemicals suggests that immuno-modulation therapy may have significant importance in ameliorating the toxicity of chemicals.

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