Original research paper

Black Tea-Induced Cellular Survival: Evidence for Reduced Toxicity and Enhanced Immunity in Mice Under Stress

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ABSTRACT

It is known that during disease conditions like cancer, due to the disease itself or due to continuous exposure to various toxic drugs, the intrinsic regulatory machinery, e.g., immune and detoxification systems, of the host becomes jeopardized. Thus the drug has to be withdrawn to prevent further immunosuppression and toxicity in tumor-bearer, resulting in total failure of the treatment. It is, therefore, of utmost necessity to search for a biological response modifier that can restore the immunopharmacological balance of the host. Many dietary constituents are chemopreventive in animal models. It is already recognized that black tea, the popular beverage, has both anticancer and immunostimultory effects. In the current study the role of black tea in reducing toxicity and ameliorating immunosuppression in mice bearing Ehrlich's Ascites carcinoma (EAC) has been elaborated. Our results showed that black tea delivers the immune system of the host from its suppressed condition as caused by the developing tumor. It has also been demonstrated that this popular beverage is not toxic by itself, additionally it regresses the tumor-induced toxicity in the host and improves the blood parameters. All these findings indicate that black tea plays an important role in protecting the intrinsic defense machineries of the tumor bearer. These results may help in increasing the likelihood of success in chemoprevention trials and in providing dietary advice to the general population to ultimately optimize the chances of preventing cancer.

Keywords : cancer; toxicity; immunity; mice; tumor bearer dietary constituents; immuno-stimulation; chemopreveion.

Introduction

Stress responses are known to cause massive immunosuppression and toxicity. Stress can be of various kinds, e.g., diseases, toxic chemicals, drugs, environmental pollutants, psychological etc. It has been recognized that stress can even lead to fatal diseases like cancer which in turn induce stress in the host thereby causing immunesuppression and toxicity (Khonina *et al.,* 2002, Pollock RE, Roth JA, 1989). Increased production of immunosuppressive interleukin-10 with simultaneous suppression of IL-2 thereby leading to severe immunodepletion in cancer patients have also been reported (Neuner *et al.,* 2002). Moreover, antitumor treatments can also have a detrimental impact on host immunity and detoxification systems (Celio *et al*., 2002).

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During the regimen of cancer chemotherapy, cancer itself as well as various popular and effective drugs in use these days exert concurrent toxic manifestations including oxidative stress, liver damage, hemotoxicity and immunosuppression in the tumor bearer (Gopalakrishna and Jaken, 2000; Barone *et al.,* 2003). As a result, hepatotoxic marker enzymes like serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase (ALP) activities are often elevated (Meriggi and Forni, 2002). Status of antioxidant enzymes, i.e., Superoxide dismutase (SOD), Catalase, glutathione-S-transferases (GST), peroxidases etc., which protect cells against oxidative damage, is almost invariably altered during carcinogenesis as well as during drug therapy (Marx, 2001). Moreover, most of the cancer drugs have been reported to cause simultaneous apoptosis in splenocytes of the tumor bearer (Ferraro *et al*., 2000). Hemotoxicity, resulting in anemia, neutropenia etc., is another cancer-associated

effect (Chen *et al*., 2003). All these information indicate that cancer itself as well as the existing cancer drugs, besides regressing tumor, causes general toxicity and immunosuppression of the tumor-bearing host. Therefore, identification of any agent that would have effective tumor killing ability as well as minimum side effects and associated toxicity is of great importance from the point of view of successful cancer therapy.

Recently, considerable attention has been focussed on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing the process of multistage carcinogenesis without harming the normal systems of the cancer bearer. Wide arrays of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anti-carcinogenic and anti-mutagenic effects. The majority of these naturally occurring phenolic compounds retain anti-oxidative and anti-inflammatory properties, which appear to contribute to their chemopreventive activity. Some of them have already recognized for their anti-toxic effected towards the normal cells of the host (Chung *et al*., 2001).

It is recognized that next to water, tea is the most ancient and widely consumed beverage in the world. Among different types of tea, anti-tumor effect of green tea has gained lots of attention so far as the scientific reports are concerned (Gupta *et al*, 2001). Black tea also has diverse biomodulatory activities and has already been reported to have apoptogenic effect towards the cancer cells (Bhattacharyya *et al*., 2003). However, besides some scattered reports and supporting epidemological evidences on the protective role of black tea, the detail molecular mechanisms underlying the effect of the anti-cancer dose of black tea on the normal systems of the cancer bearer is still the Cinderella of investigation although the worldwide production and consumption of black tea far exceeds that of green tea (Bickers and Athur, 2000). Here we report that the anti-cancer dose of this popular beverage can ameliorate EAC-induced immunosuppression, liver toxicity and hemotoxicity in mice, thereby indicating that it can be a good candidate as a safe and potential anti-cancer agent.

Methods

Tea preparation and treatment – To determine an effective dose for our pilot studies on the anti-tumor effect of black tea, we prepared different doses of black tea extracts by brewing required amounts of black tea leaves in hot water. The extracts were given ad-libitum to mice instead of drinking water (Bhattacharyya *et al.*, 2003).

Mice and tumor models – All animal experiments were performed following "Principles of laboratory animal care" (NIH publication No. 85-23, revised in 1985) as well as specific Indian laws on "Protection of Animals" under the prevision of authorized investigators. Swiss albino mice (~20 g each) were randomly divided into different groups including (i) normal set (non-tumor-bearing), (ii) tumor-bearing set (which were intra-peritoneally injected with $1x10^5$ exponentially grown EACs and (iii) black tea-treated tumor-bearing set. Untreated mice received drinking water instead of black tea.

Splenic cell count in tumor-bearing mice – One week prior to EAC inoculation black tea extract treatment was started. Spleens from Swiss mice were removed on 21st day after EAC inoculation and cell suspension was made in RPMI 1640 medium. Red blood cells were sedimented by keeping the tubes containing cell suspension at 45-degree angle for 30 min at 4^{o}C. The top supernatant containing leukocyte-rich cell population was then allowed to adhere in petridishes at 37^{o}C for 1 h. The non-adherent cell populations were collected and subjected to Ficol-hypaque (Pharmacia Fine Chemicals) density gradient separation to obtain lymphocyte-rich population (Pal *et al.*, 2001; Das *et al.*, 2002). Viable cells were counted in haemocytometer by trypan blue exclusion test.

Cell cycle analysis by flowcytometry— Cell cycle analysis was performed as already described (Bhattacharyya *et al.*, 2003). In brief,

for the determination of cell-cycle phase distribution of nuclear DNA, splenic lymphocytes (1 x 10^6 cells in each case) as harvested from normal or black tea treated EAC bearing Swiss albino mice were fixed with 3% p-formaldehyde for 30 min, trypsinized, and nuclear DNA was labeled with propidium iodide (PI, 125 µg/ml) after RNase treatment using Cycle TEST PLUS DNA reagent kit (Becton Dickinson Immunocytometry system). Cell-cycle phase distribution of nuclear DNA was determined on FACS Calibur using CellQuest software (Becton Dickinson), fluorescence (FL2-A) detector equipped with 488 nm Argon laser light source and 623 nm band pass filter (linear scale, Becton Dickinson). Total 10,000 events were acquired for analysis. Simultest LeucoGATE was used to reduce debris, monocytes, granulocytes or other contamination, if any, and analysis of flowcytometric data was performed using ModFit software (Becton Dickinson).

Liver toxicity test –

Assay of alkaline phosphatase and billirubin: During the treatment, on day 21 blood samples were drawn from the supraorbital sinuses from the mice and serum was separated for the estimation of alkaline phosphatase (ALP) and bilirubin according to standard protocols (Talib, 1999).

Assays of SGOT and SGPT: The marker serum-enzymes of liver toxicity, e.g., serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) were assayed in our tumor-bearing mice model with or without black tea extract treatment following the method protocol used by Talib *et al*. (1999).

Determination of Hematological profile– Before sacrificing the animals, blood was collected from the retro-orbital plexus. Total RBC, WBC and hemoglobin concentrations were evaluated by standard methods. The smear was prepared from fresh blood for differential leukocyte counts in the light microscope using oil immersion lens. Trypan blue dye exclusion test was performed to determine viability of cells.

Results

Effect of black tea on splenic cell count in EAC bearing mice – The effect of black tea extract on the number of splenic lymphocytes of tumor-bearing mice is shown in Figure-1. It was observed that tumor burden caused depletion of immune cells of the host in case of splenic lymphocytes within three weeks of tumor inoculation. Interestingly, black tea extract treatment caused restoration of the depressed cell number in a dose-dependent manner (Bhattacharyya *et al.* 2003). These results indicate that black tea extract could restore the immune system of tumor-bearing host even from an immunosuppressed condition.

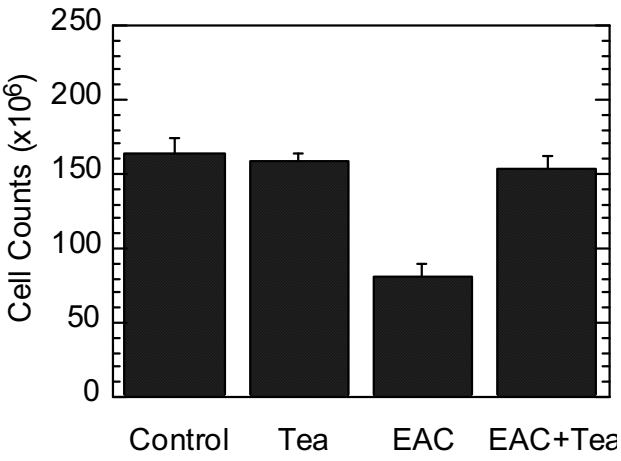

Fig. 1. Effect of black tea on splenic cell count in Ehrlich's ascites carcinomabearing mice.

Amelioration of tumor-induced splenic apoptosis by black tea— As shown in Figure 2, the control non-tumor bearing mice indicated that 1.1% of splenic lymphocytes were in sub-G0/G1 of the cell cycle. Black tea itself had no significant effect on the cell cycle pattern of splenocytes from non-tumor bearing animals. However, tumor burden caused severe increase in the content of nuclear DNA in hypoploid region. Interestingly, the black tea treatment caused reversal of immunosuppressive status of splenic lymphocytes to the normal status (Fig. 2).

Changes in the hematological pattern following black tea treatment— As demonstrated in Figure-5, in untreated tumor

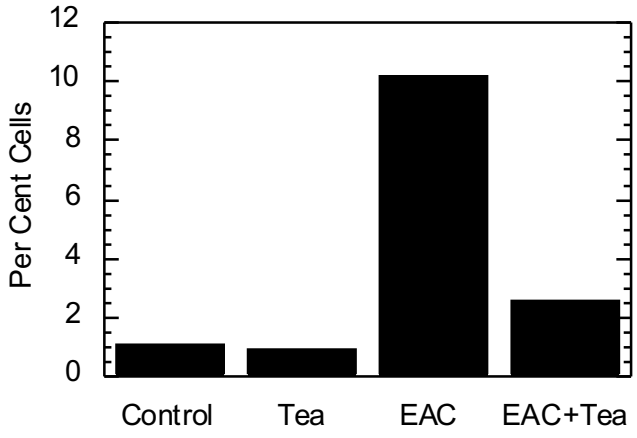

Fig.-2. Amelioration of tumor-induced splenic cell apoptosis by black tea.

bearing mice, development of anemia was apparent from distinctively lowered RBC count and hemoglobin content as compared to the normal non-tumor bearing animals. An increase was also observed in peripheral leukocyte count. Differential distribution of leukocytes revealed tumor-associated leukocytosis. However, black tea could rescue the tumor-bearing host from EAC-induced hemotoxicity (Fig. 3).

Effect of black tea on tumor-induced hepatic toxicity—To evaluate the effect of anti-tumor dose of black tea (Bhattacharyya et al., 2003) on tumor-induced liver toxicity, we measured the activities of different marker enzymes of liver toxicity. As shown in Figure 4, activities of SGOT, SGPT and AP were significantly increased in EAC-bearing mice serum in comparison to normal mice. This indicates that tumor itself renders toxic insult to these mice. Treatment with the anti-tumor dose, i.e., 2.5%, could reduce the toxicity levels almost to the normal ones. Black tea itself had no effect on the host system when treated to normal mice like other traditional drugs (Fig. 4).

Black tea: effect on billirubin profile in EAC bearing mice–Total serum bilirubin is an important index for detecting hemolytic damage. In our experimental system, due to EAC burden, the level of serum billirubin became high in comparison to the normal level (Figure. 5). Black tea treatment could revert back the level to almost normal one. Importantly, black tea itself showed no significant change in billirubin status from the normal level.

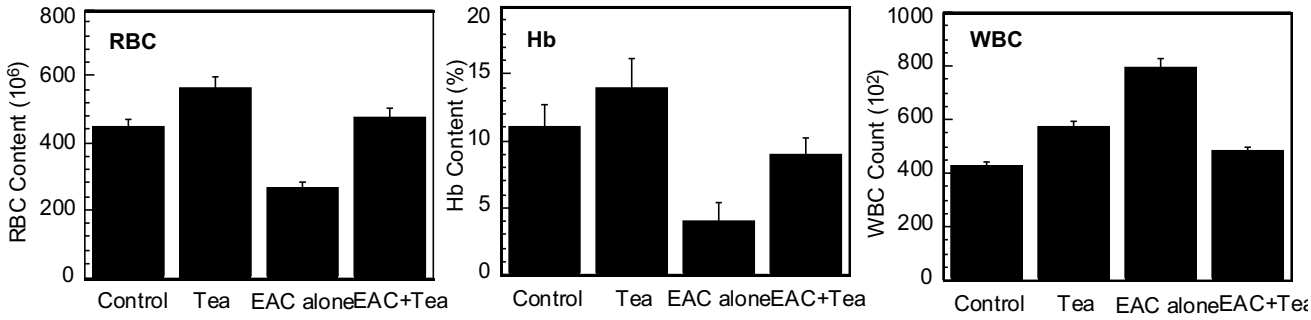

Fig.3. Changes in the hematological pattern following black tea treatment.

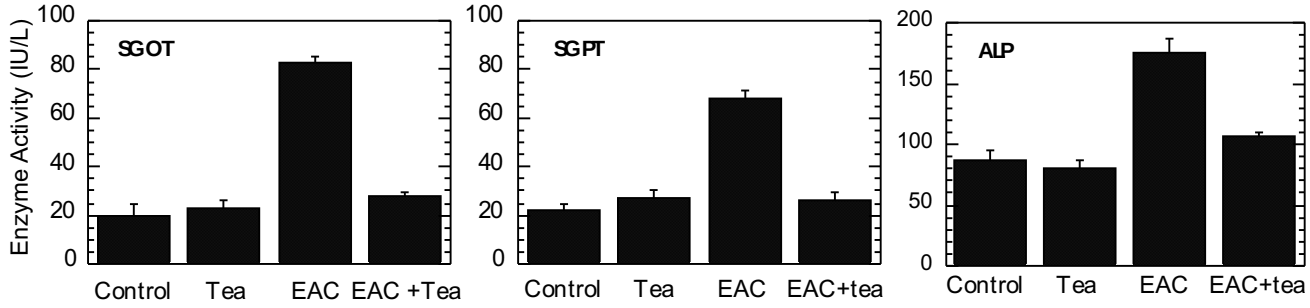

Fig.4. Tumor-induced hepatic toxicity and its regression by black tea.

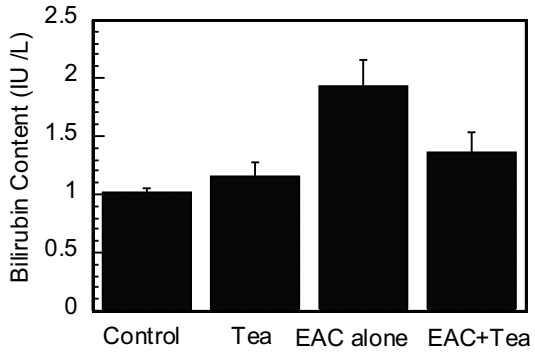

Fig.5. Effect of black tea on billirubin profile in Ehrlich's ascites carcinoma-bearing mice.

Discussion

Black tea has been reported to cause anti-tumor effect in a range of cell types (Bhattacharyya *et al.*, 2003). It also inhibits ornithin decarboxylase, one of the important markers of toxicity (Liang *et al*., 2002). Thus it is important to determine whether its effectiveness as a chemopreventive agent is a reflection of its multiple modes of action or whether it will be more effective in humans as individual agents or as a part of foodstuff from which it is derived.

Our previous results demonstrated that oral administration of black tea could induce apoptosis in tumor cells and we have also worked out the molecular mechanisms thereof (Bhattacharyya *et al*., 2003). That report also showed that the black tea treated tumor-bearing mice had a better survival advantage over their non-treated counterparts (Bhattacharyya *et al*., 2003). Here we report that in EAC bearing mice, there is severe immunosuppression due to the tumor burden. In fact, our results showed that there was tumor-induced apoptosis of splenic lymphocytes as confirmed by the formation of sub-G0/G1 hypoploid peak in cell cycle phase distribution of lymphocyte DNA. Interestingly, besides killing the cancer cells (Bhattacharyya *et al*., 2003), the immunosuppressive effect of the developing tumor on the splenic lymphocytes was brought back to almost normal conditions by black tea treatment by inhibiting tumor-induced splenic cell apoptosis as shown by marked decrease in sub-G0/G1 phases compared to the untreated control. Reports regarding nontoxic therapies using tea of different lifestyle-related diseases, including cancer, (Sueoka *et al.*, 2001; Myers, 1999; Gensler *et al*, 1996) as well as the protection of immunosuppression by this popular beverage (Katiyar *et al*., 2001; Katiyar and Mukhtar, 2001) are already there. However, most of these studies have been done using green tea and its polyphenols. Since consumption of black tea far exceeds that of green tea all over the world, our results will not only add new knowledge regarding the potentiality of this black tea as a immunopotentiating, anti-cancer agent but also may help in designing a well acceptable dietary therapy of cancer in future.

In addition anemia is a common complication of malignancies. Cancer-related anemia may occur as a direct effect of the neoplasm, it may be due to products of the cancer (Kyle *et al.,* 2003), or it may develop as a result of the cancer treatment itself (Pisch *et al.,* 2002). In many cancer patients, the causative mechanism of anemia is incompletely defined; thus, the term "anemia of chronic disease" is used. Impairment of iron metabolism and depressed erythropoiesis constitute primary hallmarks as well as the basis for anemia in cancer patients (Kim *et al*., 2002). Our results lead us to the conclusion that black tea treatment significantly upholds the suppressed haematopoetic system of the tumor bearer.

In case of cancer chemotherapy, various popular and effective drugs exert concurrent toxic manifestations including oxidative stress and liver damage in the tumor bearer. As a result SGOT, SGPT and AP are elevated in serum (Bhimani *et al*., 1993). It is interesting to note that each organism has the built-in capacity to tolerate stress up to a certain limit. In a developing tumor the host is subjected to a wide variety of stress ranging from the tumor itself to drug toxicity. Ultimately, the increase in SGOT and SGPT activities of the host gets manifested (Su *et al.,* 2003)**.** We have found that in untreated EAC bearing mice, levels of serum SGOT, SGPT, ALP as well as billirubin were high and were brought down to the normal levels by the continued treatment of black tea. Reversal of tumor-

induced hepatotoxicity by black tea indicates its role as a scavenger that provides the hepatoprotective effect to the host to fight the toxicity generated by the developing tumor. Such protective effect of black tea may be due to the healing of liver parenchyma and regeneration of hepatic cells. The protective effect of green tea and its polyphenols has already been well studied (Edenharder *et al.,* 2002; Kamat and Lamm, 2002)**.** However, while it has been suggested that black and green tea have mostly similar actions so far as their anti-tumor and anti-toxic effects are concerned, very few reports have attempted to study the mechanisms of black tea action (Weisburger and Chung, 2002, Catterall *et al.*, 1998). Our results directly showing the detoxifying effect of black tea will therefore be of immense help in enriching the knowledge so far as the designing of a potential anti-toxic anti-cancer drug is concerned.

Above discussion signifies the role of black tea in ameliorating immunosuppression and toxicity in the tumor-bearing host. These findings in conjunction with our previous report lead us to conclude that by using black tea, the tumor-affected defense system of the host can be regenerated as a result of which dietary therapy of cancer can be made possible without harming the normal cells of the host.

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