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Article

Neoplastic and Hematological Effects of Endosulfan and Bleomycin in the Swiss Albino Mice *Mus musculus*

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Effects of endosulfan (EN), an insecticide, and bleomycin (BL), an antibiotic, on the body weight in the normal mice, and the *in vivo* cell growth, tumor weight, and hematological parameters of the Ehrlich ascites carcinoma (EAC) cell-bearing Swiss albino mice *Mus musculus* were evaluated. EN and BL were respectively administered orally and intraperitoneally to the experimental mice; the control group consisted of EAC cell-bearing untreated mice only. EN reduced the body weight in normal mice, whereas BL resulted in a steady body weight compared to the control. EN increased the EAC cell count significantly by reducing the growth of normal viable cells. In contrast, BL reduced the cell count by increasing the proportion of viable cells in the body. The tumor weights induced by EN were significantly higher than those of the EAC control and the BL-treated animals. In comparisons with the control and the BL mice, hematological parameters such as hemoglobin (%) and the number of RBC and lymphocytes were lowered, while counts of WBC, neutrophils, and monocytes were elevated after EN treatments. These results show that BL is capable of reducing the EN-induced neoplastic and haematological alterations in the mice under laboratory conditions.

Key words: *bleomycin, EAC cells, endosulfan, hematological parameters, Swiss albino mice, tumor growth*

EN is a broad-spectrum, chlorinated hydrocarbon insecticide as well as an acaricide widely used for the control of agricultural insects and mites on fields, fruits, and vegetable crops [Naqvi and Vaishnavi, 1993]. Residual amounts of EN and other pesticides detected in the soil, water bodies, vegetables, grains, and other food products [IARC, 1983; Smith, 1991; USEPA, 2002; WHO, 2002] raised great concerns on the environmental pollution. Recognized as a persistent organic pollutant having variable half-life ranging from 3 days to about 5 months in water [Howard, 1991], EN shows high levels of bio-availability that can be bio-accumulated and bio-magnificated in the food chain [Bhalerao and Puranic, 2007; Rivas *et al*., 2007; Tan *et al*., 2007].

Earlier works on animals indicate that EN toxicity may be influenced by the species tested and the level of protein in the diet [Smith, 1991]. Metabolites of EN have the ability to cause cellular changes through stimulation of the central nervous system, and the organs most likely to be affected include kidneys, liver, parathyroid glands, and blood [ATSDR, 2000]. EN has been shown to be highly to moderately toxic on fishes [Johnson and Finley, 1980; Siang *et al*., 2007], birds [Hill and Camardese, 1986], and bees [Kidd and James, 1991; USNLM, 1995]. Moreover, chronic toxicity, reproductive abnormalities, and carcinogenicity of this compound in mice and rats [NCI, 1978; Hurt, 1991; Hack *et al*., 1995; Manzula *et al*., 2000; Paul *et al*., 2000; Dalsenter *et al*., 2003] and EN exposure in human male children have been shown to delay sexual maturity and interfere with the sex hormone synthesis [Saiyed *et al*., 2003].

BL is a glycosylated linear nonribosomal peptide

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Abbreviations: BL, bleomycin; EAC, ehrlich ascites carcinoma; EN, endosulfan; RBC, red blood cell; WBC, white blood cell

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antibiotic used in the treatment of Hodgkin lymphoma, squamous cell carcinomas, testicular cancer, pleurodesis, and plantar warts [Claussen and Long, 1999]. This antibiotic acts through the induction of the DNA strand breaks, and is categorized as one of the cytotoxic and/or anti-tumor antibiotics [BNF, 2006] and generally does not suppress the production of blood cells in the bone marrow [Sweetman, 2006].

Keeping the features of the two above compounds in mind, the present investigation was designed to focus on the neoplastic and toxicological effects of EN, accompanied by the evaluation of the probable antagonistic effects of BL on the EAC cell-bearing mice.

Materials and Methods

Experimental animals and care. Swiss albino mice of 6-8 weeks of age, each weighing 20-25 g, were collected from International Center for Diarrheal Disease Research, Bangladesh (ICDDRB). They were kept in iron cages (45 cm×30 cm×30 cm) with sawdust bedding, which was replaced once a week. Water and standard mouse pellets procured from ICDDRB were supplied *ad libitum*. A constant room temperature of 28-30°C and a controlled light: dark cycle (14 h:10 h) were maintained in the laboratory.

Chemicals and reagents. Technical grade EN ($C_9H_6Cl_6O_3S$, 97% pure) and BL used in this study were obtained from Sigma-Aldrich (Dorset, UK). Trypan blue was purchased from Sigma (St. Louis, MO). All other chemicals and reagents used were of high purity and analytical grade.

Experimental tumor model. Transplantable EAC cells used in this work were obtained from Indian Institute of Chemical Biology (IICB), Kolkata 700032, West Bengal, India, and were maintained in our laboratory mice by intraperitoneal (i. p.) transplantation.

Transplantation of ascitic tumor. Ascitic fluids were drawn out from different EAC-bearing mice at the respective log-phases of the tumor cells. A 5-mL syringe fitted with a 20-gauge needle was used for the cell aspiration. The freshly drawn fluid was diluted with the normal saline, and the number of tumor cells was adjusted to approximately 2×10^6 cells/mL by counting the cells with a hemocytometer. The viability of EAC cells was checked by trypan blue dye (0.4%) exclusion assay. Cell samples showing above 90% viability were used for transplantation. Tumor suspension of 1 mL was injected intraperitoneally to each mouse. Strict aseptic conditions were maintained throughout the transplantation process.

***In vivo* assessment of EN and BL.** Neoplastic activity of the test compounds was determined *in vivo* by measuring their effects on the body weight, viable cell growth, weight of the tumor cells, and the hematological parameters of the EAC cell-bearing mice.

Working schedule. Five groups of mice, EAC control, EAC+$EN_{1.25}$, EAC+$EN_{2.50}$ EAC+$EN_{5.00}$, and EAC+$BL_{3.00,}$ were used. Groups 2-4 respectively received 1.25, 2.50, and 5.00 mg/kg EN, whereas group 5 intraperitoneally received 3.00 mg/kg BL. For therapeutic evaluations, 140×10^4 EAC cells per mouse were inoculated into each group of mice on day 0. Treatments were started after 24 h of tumor inoculation and continued for 5 days. Mice were sacrificed on day 7, and total intraperitoneal tumor cells were harvested using normal saline. Viable cells were stained with trypan blue and were counted with a hemocytometer. Percentage of the viable cell growth was estimated using the following formula:

Cell count of $EAC_{0.00}$−cell count of treatment group÷ cell count of $EAC_{0.00}\times100$.

Tumor weight of the mice was recorded on each alternate day from 2 to 20 days. Hematological parameters *viz*, counts of RBC, WBC, lymphocytes, neutrophils, and monocytes, and % hemoglobin in the experimental mice were measured on day 12 following EN and BL treatments.

Statistical analyses. The data obtained for body weight, viable cell growth, tumor weight, and hematological parameters were subjected to statistical analyses for interpretations. Mean±SE for each parameter was calculated for performing one way analysis of variances (ANOVA), followed by LSD test for multiple comparisons. A statistical package (SPSS version 11.0) was used for the data analysis.

Results

Body weight. Effects of EN and BL on the body weight of normal healthy mice are presented in Table 1. Compared to the EAC control and the BL-treated mice, the body weights of the EN-treated mice were reduced significantly ($F_{4,10}=3.51$, $p<0.05$) with no significant difference shown among the groups. Thus, the body weight-gaining effect of BL on the normal mice appeared to be antagonistic to that of EN.

Cell growth. Table 2 shows the EAC cell counts and corresponding growths of the viable cells in the EAC cell-bearing mice following EN and BL treatments. EN significantly enhanced the number of EAC cells on day 7 after treatment, accompanied by a simultaneous decrease in the number of the viable cells, thus suggesting that the cell growth inhibition by EN is significantly different

Table 1. EN- and BL-induced changes in the body weight of normal mice

Treatment groups	Body weight (g) on day 0	Body weight (g) on day 6
Normal	27.03±1.53	32.14±1.42[a]
Normal+$EN_{1.25}$	25.50±0.29	24.87±0.35[b]
Normal+$EN_{2.50}$	25.90±0.67	24.83±0.73[b]
Normal+$EN_{5.00}$	27.50±0.29	26.07±0.37[ab]
Normal+$BL_{3.00}$	25.87±0.37	30.72±1.03[a]

EN=endosulfan, BL=bleomycin; subscripts followed by abbreviations are doses (mg/kg). Dissimilar superscript letters in the same column indicate significant differences by LSD at $p<0.05$.

Table 2. *In vivo* cell growth in EAC cell-bearing mice after EN and BL treatments

Treatment groups	EAC cell counts ($\times10^7$)* on day 6 after tumor cell inoculation	Cell growth (% viable cells)
$EAC_{0.00}$	6.92±0.44[a]	-
EAC+ $EN_{1.25}$	1.33±0.7[b]	80.78[a]
EAC+ $EN_{2.50}$	1.56±0.03[b]	77.46[a]
EAC+ $EN_{5.00}$	1.94±0.04[c]	71.97[b]
EAC+$BL_{3.00}$	0.38±0.42[d]	94.51[c]

*Cell count of $EAC_{0.00}$ −cell count of treatment group÷ cell count of $EAC_{0.00}\times100$; EAC=Ehrlich ascites carcinoma; EN=endosulfan, BL=bleomycin. Subscripts followed by abbreviations denote doses (mg/kg). Dissimilar superscript letters in the same column indicate significant differences by LSD at $p<0.05$.

from that in the untreated and BL-treated mice ($p<0.05$). These data clearly demonstrated the detrimental role of EN in the mice under study.

Tumor weight. The neoplastic effect of EN on EAC mice is apparent from the results shown in Fig. 1. Compared with the EAC control group, in which the tumor weight increased by 31.75% over a period of 20 days, EN-treated mice (EAC+EN 1.23, 2.50, 5.0) exhibited progressive increases in the tumor weights respectively by 34.85, 36.62, and 44.00% over the same period. BL-treated mice, on the other hand, showed only 15.58% increase in tumor weight, significantly lower than that of the EAC mice. This result corresponds with the cell growth data mentioned above.

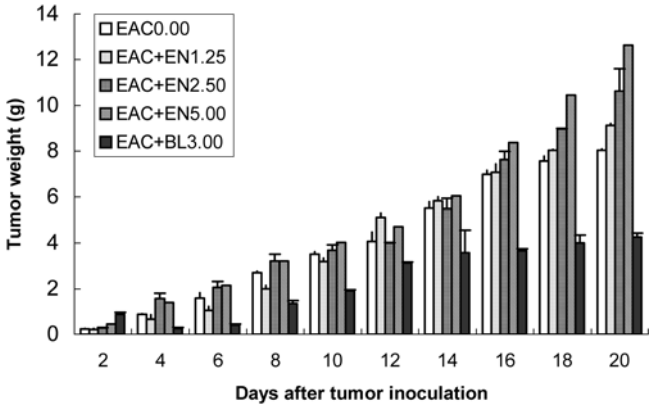

Fig. 1. EN- and BL-induced changes in the tumor weight in EAC cell-bearing mice over a period of 20 days.

Hematological parameters. With the enhanced growth of the tumor cells in the EN-treated mice, hematological parameters showed significant deviations from those of the EAC control and BL-treated groups (Table 3). EN decreased the numbers of RBC ($F_{4,10}$ =28.92, $p<0.001$), hemoglobin ($F_{4,10}$=12.65, $p<0.001$), and lymphocytes ($F_{4,10}$=70.53, $p<0.001$), but increased those of WBC ($F_{4,10}$=26.82, $p<0.001$), neutrophils ($F_{4,10}$= 33.40, $p<0.001$), and monotytes ($F_{4,10}$=63.79, $p<0.001$). In contrast, BL treatment significantly enhanced the numbers of RBC, hemoglobin, and lymphocytes with the corresponding depletion of the WBC, neutrophils, and monocytes in the experimental mice. The protective role of BL against the neoplastic and toxicological effects of EN is thus apparent from these results.

Table 3. EN- and BL-induced alterations in the hematological parameters of EAC cell-bearing mice on day 12 after tumor inoculation

Treatment groups	RBC (10^9 cells/mL)	WBC (10^6 ells/mL)	Hemoglobin (%)	Lymphocytes (%)	Neutrophils (%)	Monocytes (%)
$EAC_{0.00}$	2.93±0.03[a]	25.60±0.61[a]	9.42±0.09 [a]	45.33±0.67[a]	33.67±0.88[a]	12.00±0.58[a]
EAC+$EN_{1.25}$	2.60±0.02[b]	25.71±0.02[a]	9.02±0.07 [b]	45.67±0.88[a]	34.33±0.88[a]	8.00±0.58[b]
EAC+$EN_{2.50}$	2.19±0.01[c]	27.09±0.05[b]	7.62±0.08[c]	36.33±0.67[b]	46.67±0.88[b]	11.00±0.58[a]
EAC+$EN_{5.00}$	1.75±0.03[d]	29.76±0.14[c]	6.12±0.04[d]	28.33±0.33[c]	54.33±1.20[c]	18.33±0.33[c]
EAC+$BL_{3.00}$	8.70±0.01[e]	6.54±0.07[d]	14.19±0.10[e]	67.33±1.20[d]	21.67±1.20[d]	7.00±0.58[d]

EAC=Ehrlich ascites carcinoma; EN=endosulfan, BL=bleomycin. Subscripts followed by abbreviations denote doses (mg/kg). Dissimilar superscript letters in the same column indicate significant differences by LSD at $p<0.05$.

Discussion

The present results revealed that oral administration of EN causes a significant decrease in the body weight of the normal mice, accompanied by a significant reduction in the viable cell growth, increase in the tumor weight, and decrease in the number of RBC, hemoglobin percentage, and lymphocytes in the experimental EAC mice. BL, on the contrary, was found to increase the body weight as well as the viable cell growth, while reducing the tumor weight and the numbers of WBC, neutrophils, and monocytes. Thus, it is suggestive that BL is capable of reducing the EN-induced neoplastic and hematological toxicity in the Swiss albino mice.

Given the fact that EN is highly toxic via oral route in mice, in which the LD_{50} value has been estimated to be 7.36 mg/kg [Kidd and James, 1991; Smith, 1991], the present doses of 1.25, 2.50, and 5.00 mg/kg are likely to affect the nutrition as well as the body weight of the surviving adult mice. An alternative explanation would be that the EN-induced cardiotoxicity [Kalender *et al*., 2004] might have contributed to the weight loss of the mice under study. Similar to the normal and healthy mice, increased body weight of the BL-treated mice explains the anti-tumor property of the antibiotic [BNF, 2006].

EN poisoning in animals has been attributed to the stimulation of the central nervous system, where the metabolites of the compound show the ability to cause cellular changes [ATSDR, 2000]. This, coupled with the EN-induced changes in the blood chemistry [Smith, 1991; Sinha *et al*., 2001] and the vital organs, such as kidneys, liver, and parathyroid glands in the EAC mice, could have a significant impact on the proportion of the viable cell growth in the body. In contrast to the cell growth inhibition by the EN-treated mice, BL-treated ones showed a significant increase in the viable cells, because BL is reported to kill cancer cells by degrading their genetic materials (DNA) at the dividing phase of the cell life cycle [Sweetman, 2006].

The progressive increase of tumor weight in the EN-treated EAC mice over a period of 20 days supports the findings of Hack *et al*. [1995], who reported EN-induced chronic toxicity and carcinogenicity in mice and rats. This is contradictory to the earlier observations that EN alone is neither carcinogenic in mice and rats, nor does it cause increased incidence of tumors in mice [NCI, 1978; Takenada *et al*., 1983]. Possible explanation to this disagreement is that EN toxicity and carcinogenicity affecting the tumor weight may be influenced by the species and the level of protein in the diet [Smith, 1991].

Biochemical alterations in the blood of mice by EN [ATSDR, 2000], heavy metals [Sharma *et al*., 2005] or ONO-1301, a prostacyclin agonist [Murakami *et al*., 2006], raise concerns on the indiscriminate use of pesticides in the natural environment. EN-induced reduction in RBC, hemoglobin and lymphocytes in EAC mice is similar to the ONO-1301- [Murakami *et al*., 2006] and the mercury-mediated [Sharma *et al*., 2005] changes in the blood chemistry in mice. Our present results lend support to the earlier findings of Smith [1991] and ATSDR [2000], which emphasized that the main target organs of EN in animals include, among others, the immune system. Depletion of some vital hematological parameters following the EN treatments in our study accounts for other such physiological changes as loss in body weight and viable cells, but increase in EAC cell count and tumor weight. Because BL generally does not suppress the production of blood cells in the bone marrow [Sweetman, 2006], the protective role of this antibiotic against EAC mice became evident from the present data, where increases in the RBC, hemoglobin, and lymphocytes took place in the BL-treated mice.

The protective effect of BL on the EAC cell-bearing mice is somewhat similar to those reported by John *et al*. [2001] and Kalender *et al*. [2004], who demonstrated that vitamin E is effective against EN cardiotoxicity and free radical metabolism in rats. The present findings, therefore, are promising in the context that there are health and environment concerns due to the abundant usage of EN for the protection of a variety of crops. Judicious administration of BL in animals could protect them from EN toxicity hazards.

In conclusion, compared to the EN-induced neoplastic and hematological changes in the carcinoma cell-bearing Swiss albino mice, BL has been found to maintain normal body weight, reduce EAC cell counts, increase viable cell growth, lower tumor weight, and increase vital hematological parameters. Consequences of EN pollution in nature could thus be minimized by the appropriate applications of BL.

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