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Butylated Hydroxytoluene: Tumor-promoting Activity in an *in vitro* Two-stage Carcinogenesis Assay

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The antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are extensively used as additives in food for human consumption. The human consumption of phenolic antioxidants in the United States is of the order of magnitude of several mg per person a day. Several reports have delt with the capacity of BHA and BHT to act as inhibitors of carcinogenesis, and WATTENBERG (1980) has recently published an excellent review article on this topic.

The experimental procedure for testing the inhibition of carcinogen-induced neoplasia by BHA and BHT is usually to give the experimental animals the antioxidants either in the diet or by other methods prior to or at the same time as the carcinogen is given. Typical for most of these experiments is the termination of antioxidant treatment shortly after carcinogen administration. BHT and BHA seem to excert their antitumorigenic activity through the increased detoxification of carcinogens and/or through enzyme alternations which may result in decreased synthesis of essential metabolites of the carcinogen in question (LAM & WATTENBERG 1977; ULLAND et al. 1973).

BHT is only slightly toxic, and when tested in the Ames Salmonella typhimurium mutagenicicity test, no significant effect on the mutation frequence was observed (JONER 1977). SHELEF and CHIN (1980) have, however, reported that BHA and BHT can increase the number of revertants in experiments where the cells first were treated with aflatoxin B_1 and then with the antioxidant.

Since our environment contains a large variety of carcinogens, it may be possible that most people possess a pool of initiated cells as defined by BERENBLUM (1941). WITSCHI & LOCK (1978) have shown BHT to affect the formation of urethan-induced adenoma in mouse lung. Both the number of tumor bearing animals and the number of tumors formed per lung increased as a result of BHT treatment. These results

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are in contrast to the anticarcinogenic effects reported by WATTENBERG (1978), indicating that the time of BHT treatment is of crucial importance in these types of experiments.This report is a first attempt to study the possible role of BHT as a tumor promoter in an in vitro two-stage carcinogenesis test. The test cells used are the mouse embryo fibroblasts C3H/10T½ clone 8, first described by REZNIKOFF et al. (1973).

MATERIALS AND METHODS

The mouse embryo fibroblasts C3/10T½Cl8 were obtained from C. Heidelberger, University of Southern California, Los Angeles. The cells were grown in Basal Medium Eagle (BME-Gibco) supplemented with 10 % heat-inactivated foetal calf serum (Gibco) and incubated at 37°C in a humidified atmosphere of 5 % CO_2 in air. Plastic petri dishes were from Nunc, Denmark. 20-methylcholanthrene (MCA) was from Koch-Light Lab. Ltd., 12-0-tetradecanoyl phorbol-13-acetate (TPA) was purchased from P.L. Biochemicals Inc. Butylated hydroxytoluene was from Bayer Chemical Co.

The two-stage carcinogenesis assay was essentially as described by MONDAL et al. (1976), using C3H/10T½ cells between passage 7 and 12. The transformed cell foci were scored after fixation with methanol and staining with Gimsa. Only type III foci were scored. Several dishes contained more than one transformed focus, however, the frequency of transformation was calculated as percent dishes containing one or more foci of total number of dishes (MONDAL et al. 1976).

RESULTS

As shown in Table 1, BHT was not toxic to the C3H/10T½ cells over the concentration range 10^{-7} to 10^{-4}M. The relative plating efficiency of the cells was not influenced by BHT up to 10^{-4}M.

In vitro transformation experiments, using the mouse embryo fibroblasts C3H/10T½ clone 8 as test organism, showed high frequencies of transformation with a transforming dose of MCA (0.37 µM) and with a low initiating dose (0.037 µM) of MCA and repeated treatment with the tumor promoter TPA, Table 2. The negative control experiments groups 1 and 3 in Table 2

TABLE 1

Treatment	% Plating efficiency	% Relative plating efficiency
0.5 % acetone	18.5	100
10^{-7}M BHT	17.5	94.6
10^{-6}M "	16.6	89.7
10^{-5}M "	17.4	94.1
10^{-4}M "	17.9	96.8

200 cells were plated in 60 mm plastic dishes and incubated at 37 °C. 24 h after plating the medium was changed to one containing BHT at the concentrations indicated. 6 dishes were used for each BHT concentration. The cells were fixed and stained after 10 days as described in Methods.

showed no type III transformants. However, the test groups 5, 6, 7, and 8 all contained type III foci, suggesting that BHT under the present test conditions has the capacity to enhance the transformation frequency in the C3H/10T½ cells. Statistical analysis of the data showed that groups 1 and 6 different from one another, $p < 0.05$. 1 vs 7 gave $p < 0.07$, and group 1 vs 8 gave $p < 0.08$. There is not significant differance between group 1 and 5.

DISCUSSION

BHT and BHA are antioxidants widely used as food additives. In animal experiments both BHT and BHA have anti carcinogenic effect when administered to the experimental animals prior to carcinogen exposure (WATTENBERG 1978). JONER (1977) has shown that neither BHT nor BHA has any significant mutagenic activity in the Ames bacterial mutagenicity test. This is in support of the assumption that BHT and BHA are not carcinogenic and, therefore, should be safe to use as food additives. There is, however, no data available on the effect of these antioxidants in a two-stage carcinogenesis assay where the cocarcinogenic effect could be tested.

TABLE 2

Two-stage transformation of C3H/10T½ cells using 20-methylcholanthrene and butylated hydroxytoluene.

Treatment	% Relative plating efficiency	Total no. of dishes	Dishes with type III foci	% dishes with foci
0.5 % Acetone + 0.5 % Acetone	100	43	0	0
0.37 μM MCA + 0.5 % "	68.6	41	8	19.5
0.037μM " + 0.5 % "	79.1	40	0	0
0.037μM " + 0.17 μM TPA	75.0	30	13	43.3
0.5 % Acetone + 10 μM BHT	100.9	42	1	2.4
0.037μM MCA + 10 μM "	79.7	43	6	14.0
0.037μM " + 5 μM "	84.5	37	5	13.5
0.037μM " + 1 μM "	87.2	43	4	9.3

The assay was carried out as described in Materials and Methods. The TPA and BHT treatment was initiated 5 days after seeding the cells and lasted throughout the experiment. Medium was changed twice weekly. The cells were fixed and stained after six weeks. Only type III foci were scored.

Our present investigation deals with the possible activity of BHT as a tumor promoter. The data obtained are consistant with the assumption that BHT might act as a cocarcinogen. At all three concentrations of BHT in the two-stage transformation assay, a significant increase in transformation frequency was observed. In group 5 of Table 2 where noninitiated cells were treated for 5 weeks with 10 μM BHT, 1 dish of 42 dishes contained transformed foci. This is not statistically different from the control experiment. However, since the spontaneous transformation frequency of the C3H/10T½ cells is very low, probably less than 1 dish out of 100, a low carcinogenic potential of BHT can not be ruled out. Such a carcinogenic activity does not weaken the observation that BHT enhanced the transformation frequency in the MCA initiated C3H/10T½ cells.

Our present results are the first using an in vitro test system that shows BHT to increase the incidence of malignant transformation. The data for BHT are very similar to those obtained for saccharin by MONDAL el al. (1978), using identical test system. However, the potency on a molar basis, of saccharin as a cocarcinogen was approx. 1/1000 of the cocarcinogenic potency observed for BHT.

Since BHT is widely used in high amounts in various food products, our results should be taken as an indication that further study of the possible cocarcinogenic effect of BHT must be conducted.

Preliminary experiments with the related antioxidant BHA have also been carried out without obtaining conclusive evidence for BHA to possess cocarcinogenic potential. However, further studies with high concentrations of BHA must be performed before any conclutions may be made.

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