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Stimulation of DNA Synthesis in Mouse Lung Following Intraperitoneal Injection of Butylated Hydroxytoluene[1] (38418)

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In a recent paper, Marino and Mitchell (1) reported an interesting and so far undescribed effect of the antioxidant butylated hydroxytoluene (BHT) in mouse lung: Intraperitoneal injection of BHT in the dose range of 40–2500 mg/kg produced, within 3–5 days, a hypertrophy, hyperplasia, and general disorganization of the cellular components of the lung. Many alveolar cells became enlarged and there was proliferation and blebbing of the alveolar epithelium, accompanied by edema, capillary engorgement, and congestion. Lungs examined 7 days after BHT had a normal appearance.

The description of the histopathological lesions and the accompanying microphotographs suggested that BHT would stimulate cell proliferation and multiplication in mouse lung. The present experiments were designed to examine whether biochemical evidence for stimulated DNA synthesis by BHT in mouse lung could be obtained.

Material and Methods. Male Swiss–Webster mice (25–30 g) were housed on wood bedding in plastic cages. Butylated hydroxytoluene (Sigma Chemical Co.) was dissolved in corn oil and injected ip (0.2 ml); control animals received corn oil alone. The incorporation of thymidine into DNA was measured 1–9 days after BHT as follows: Ninety minutes before sacrifice, all animals received 1 μCi of thymidine-2-^{14}C ip (New England Nuclear, specific activity 54.7 mCi/mmole). The animals were killed by cervical dislocation and the two lungs were removed and weighed. The total lungs were homogenized in 5 ml of ice-cold water to which were added 2.5 ml of ice-cold 0.6 *N* $HClO_4$. An aliquot of the liver, the duodenum and proximate jejunum, the two kidneys and the spleen were also homogenized and acid-precipitated. After centrifugation, the supernatant was discarded and the pellets were washed three times with cold 0.2 *N* $HClO_4$ and extracted for 20 min at 70–80° with 4 ml of 0.5 *N* $HClO_4$. Two milliliters of extract were mixed with 10 ml of Aquasol (New England Nuclear) and 0.2–0.5 ml of extract were analyzed with Burton's modification of the diphenylamine method (2). Corrections for quenching were made by automatic external standardization. Results were calculated as dpm/mg of DNA and analyzed by Student's *t* test. *P* values of 0.05 or less were considered to be significant. For histology, tissue was fixed in 10% buffered formalin embedded in paraffin and stained with hematoxylin–eosin.

Results. Histopathological changes similar to the ones described by Marino and Mitchell (1) were observed in the lungs of mice 5 days after the injection of 125, 250, or 500 mg/kg of BHT. Injection of corn oil alone did not produce appreciable histological alterations in the lung. In treated animals, the alveolar septa were thickened, and, when examined at higher magnification, were found to contain many abnormally big cells with very large nuclei. In some cells, the nuclei stained deeply basophilic, whereas in others they were pale, almost devoid of chromatin, but with intensely stained nucleoli. However, mitotic figures were only rarely seen. Next, the 9-day LD_{50} was determined with the method of Litchfield and Wilcoxon (3). In the strain of mice used in our experiments, it was 400 mg/kg of BHT given ip (confidential limits: 210–760 mg/kg). In all subsequent experiments, the animals were injected with 400 mg/kg of BHT.

A small increase in total lung weight was observed as early as 2 days after BHT adminis-

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tration. Significantly heavier lungs than found in the corn-oil-injected control animals were observed between 3 and 5 days after BHT. Later, lung weights fell towards control values (Fig. 1). The DNA concentration per unit wet lung weight apparently did not change much over the same time period; only on Day 7 after BHT was there more DNA per gram of lung in the BHT-treated animals than in the control group (Table I). However, if the total amount of DNA per lung was calculated, it was found to increase steadily after Day 2 until it stayed about 1.6 times as high as in the lungs of control animals (Fig. 1). This increase in total DNA per lung was accompanied by an increased incorporation of thymidine into DNA (Fig. 2). Increased synthesis of DNA seemed to begin between 24 and 48 hr after BHT and reached peak values on Days 4 and 5; on Days 7 and 9, it fell back towards control values.

The incorporation of thymidine into DNA was also measured in the kidneys, spleen, liver, and the proximal portion of the small intestine. A statistically significant increase of thymidine incorporation into DNA was seen in the spleen on Day 1 and in liver and intestine on Day 9 after BHT. On the other hand, a significantly decreased incorporation was found in liver and kidney 5 days and in spleen 7 days after BHT. In all other cases there was no difference between treated and control animals. It was concluded that the few significant changes were apparently random events and not related directly to the action of BHT. Among the organs examined, lung was the only one where DNA synthesis was stimulated by BHT.

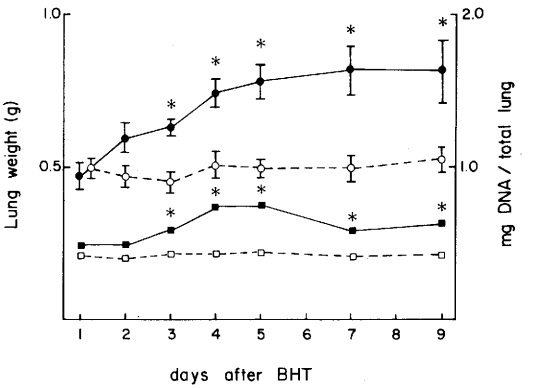

FIG. 1. Increase in total lung weight (experimental: ■; controls: □) and total DNA per lung (experimental: ●; controls: ○) at different days after BHT. Experimental animals received 400 mg/kg of BHT ip; controls were given corn oil alone. All values are given as means from 4 to 6 animals per group; SEM are indicated for total DNA. Values significantly different ($P < 0.05$) from corresponding controls are marked with an asterisk.

Discussion. There are several models of stimulated DNA synthesis available, in which usually quiescent cells can become actively proliferating when exposed to an appropriate stimulus. Such stimuli include surgical procedures, mechanical or chemical injuries, administration of drugs, hormones, and toxic agents. Organs in which cell division can be initiated include liver, kidney, spleen, several exocrine glands, and the lens (4). It was estab-

TABLE I. Concentration of DNA in Mouse Lung after BHT.[a]

Days after BHT	mg DNA/g lung[b,c] BHT	Control	*P*
1	4.32 ± 0.18 (6)	4.65 ± 0.26 (6)	NS[d]
2	4.73 ± 0.20 (6)	4.82 ± 0.19 (6)	NS
3	4.28 ± 0.05 (5)	4.20 ± 0.44 (6)	NS
4	4.21 ± 0.33 (6)	4.91 ± 0.26 (5)	NS
5	4.27 ± 0.40 (6)	4.53 ± 0.33 6)	NS
7	5.69 ± 0.26 (6)	4.44 ± 0.22 (5)	<0.05
9	5.12 ± 0.23 (5)	5.20 ± 0.22 (6)	NS

[a] 400 mg/kg of BHT ip; control animals received corn oil alone.
[b] Wet weight.
[c] Mean ± SEM; number of animals in parentheses.
[d] NS = not significant.

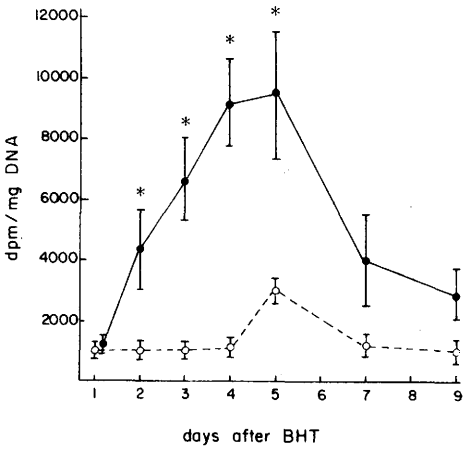

FIG. 2. Incorporation of ^{14}C-thymidine into pulmonary DNA. Experimental animals (●) received 400 mg/kg of BHT, controls (○) corn oil alone. Data are given as means ± SEM from 4 to 6 animals per group, and values significantly different ($P < 0.05$) from corresponding controls are marked with an asterisk.

lished already some time ago that lung cell growth can be stimulated by unilateral pneumonectomy (5, 6). However, very little information is available about the biochemical events preceding and/or accompanying stimulated DNA synthesis in the lung. One reason for this might be that unilateral pneumonectomy is technically not as easy to perform as is, for example, partial hepatectomy. The intraperitoneal injection of BHT seems to offer a convenient alternative to surgical removal of one lung: one single administration of this antioxidant produces, 2 days later, a burst of pulmonary DNA synthesis, accompanied by an increased lung weight and a net increase in total lung DNA. No similar effects are observed in liver, kidney, spleen, or gastrointestinal tract.

Deoxyribonucleic acid synthesis and cell proliferation can be easily stimulated in mouse lung with urethan (7, 8). However, there seem to exist several differences between urethan and BHT; although urethan produces 4 days after administration an increased incorporation of thymidine in the alveolar cells, it definitely inhibits DNA synthesis up to 3 days after injection (8). No such inhibition of DNA synthesis was seen after BHT. Following one single urethan injection, the number of cells increases progressively during the next 4 weeks (7). After BHT, stimulated DNA synthesis appears to be essentially terminated 7 days later. Marino and Mitchell (1) emphasized that lungs examined 7 days after 400 mg/kg looked completely normal again; whether their cell number was actually increased remains to be established in future quantitative histological examinations. However, it would seem that BHT does not trigger an event which leads to continuous cell proliferation as does urethan; BHT rather produces one single round of DNA synthesis. The results of BHT administration are very similar to the effects produced by partial hepatectomy in liver (9) or by isoproterenol in salivary glands (10). Finally it has to be pointed out that urethan is a well-known and potent carcinogen, whereas there is no evidence available which would demonstrate that BHT is carcinogenic in any organ of any animal species.

It is not yet known whether BHT incites only one particular pulmonary cell type to proliferate or whether increased DNA synthesis occurs in several different cell types of the pulmonary parenchyma. It also remains to be established whether BHT will stimulate pulmonary cell growth in animal species other than the mouse. Finally, it has to be pointed out that 400 mg/kg of BHT is a large dose compared to the estimated average daily intake of BHT by man [2 mg/day (11)]. On the other hand, 400 mg/kg represents a dose of 1.8 mmole/kg and is therefore of the same order of magnitude as the dose of isoproterenol used to stimulate DNA synthesis in salivary glands (1 mmole/kg).

Summary. Male Swiss-Webster mice were injected ip with 400 mg/kg of the antioxidant butylated hydroxytoluene (BHT). Between 2 and 5 days after BHT, there was a marked increase in the incorporation of thymidine into pulmonary DNA; 7 and 9 days after BHT, incorporation of thymidine fell towards values found in control animals. The increased incorporation of thymidine was accompanied by a net and significant increase in total lung weight and a persistent elevation of total lung DNA. Butylated hydroxytoluene did not stimulate DNA synthesis in liver, kidney, spleen, or gastrointestinal tract. Butylated hydroxytoluene could be used as a tool to study the biochemical events leading to or accompanying stimulated DNA synthesis and cell growth in lung.

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