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PAPER

VOLUMETRIC EVALUATION OF PULMONARY ALVEOLAR TOXICITY: THE BHT MODEL

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ABSTRACT

Butylated hydroxytoluene (BHT) provides a good model of toxicity to the pulmonary alveolar epithelium of mice. The volume fraction (V_V) of each epithelial cell type is determined by volumetric analysis, using point counting on electron micrographs of mice treated with a combination of BHT and oxygen. BHT is the major influence in inducing the degeneration of type I pneumocytes, early (days 1 to 3) after its administration. These lesions in turn trigger epithelial regeneration (at days 3 to 5), effected by type II pneumocytes. The most sensitive index of epithelial modifications is found in the V_V of the intermediate type cell, which represents the transformation of type II into type I cells. Addition of a high concentration of oxygen (95%) for 24 hours does not much enhance this toxicity, but hastens epithelial regeneration by the type II pneumocyte.

INTRODUCTION

Butylated hydroxytoluene, a chemical widely used as a food preservative and totally innocuous to man in such small quantities, is highly toxic to the rodent lung when administered in large doses. Marino and Mitchell (1972) first demonstrated its destructive effect on mice. Subsequent morphological and biochemical studies (Witschi and Saheb, 1974) localized these lesions to the pulmonary alveolar epithelium, more specifically to the type I pneumocyte.

After BHT stimulation, the development of alveolar lesions follows a reproducible, cell-specific time course (Hirai et al., 1977). This permits its use, in combination with a second pulmonary agressor, to look for the effects of the latter agent on an alveolar region already under pathological modification. We chose a short-term exposure to a high concentration of oxygen, normally inducing little change in the alveolus on its own, as this second agent.

The murine alveolar territory has not yet been fully characterized by stereological means, as were the human and the rat lungs through the pioneering work of Weibel. We hope that the definition of such volumetric parameters for cellular (and spacial) compartments of the alveolus, at rest and in toxic transformations, provide a basis of reference for future studies of pulmonary toxicology using the mouse model.

MATERIAL AND METHODS

Male Swiss-Webster white mice (18-22 gram body weight) were randomly assigned in experimental groups of 5 animals. Three parameters were investigated T, the treatment with the chemical agent BHT (2,6-di-tert-butyl-p-cresol, Sigma Chem Co, crystalline) or its corn oil vehicle control, was administered in a single injection i.p.: volume of injection 0,01 ml per gram body weight, concentration of BHT 400 mg per kg body weight (the LD_{50} dose). D, duration of the incubation period, was the time elapsed from injection of BHT to sacrifice of the animal, to allow for development of lesions: 1, 3 or 5 days post injection. E, exposure to a toxic gas, was effected during the final 24 hours of the incubation: exposure of groups of 10-12 mice, to 95% humidified oxygen at a rate of 3 liters/min, in 22 liters plexiglass inhalation cages; compressed air control, in the same exposure conditions.

At {time D 1, 3 and 5 days post injection} (± 1 hour) the animals were sacrificed as follows. After anaesthesia of the mouse, its trachea was cannulated to instillate in vivo, by gravity and through ventilatory efforts for 3 minutes, using a mixture of osmium tetroxide and glutaraldehyde in a 2:1 volume ratio. The left lung was minced with razor blades and blocks of tissue of similar size were prepare for embedding in Epon, for electron microscopic analysis. The right lung was retained for histological corro-

boration of lesions.

For each animal six blocks of tissue were sectionned to a preselected standard pyramidal surface (LKB Ultratome III, or Sorvall MT-1 ultramicrotomes) for electron microscopy (silver sections, 40-60 nm thickness) and placed on EM grids (copper, 300 mesh, LKB). From each block six randomly chosen fields were photographed at 5000x magnification (Philips 300 EM), enlarged to 13 000x and printed for analysis. Stereological analysis made use of a Carl Zeiss MOP 3 semi-automatic quantimeter, with a point counting grid showing a rectangular lattice and totalling 4520 points per micrograph. We measured the volume fraction V_V for each epithelial cell type, with reference to the total alveolar volume as observed on the micrograph. The data were treated according to the methods outlined by Weibel for point counting volumetry (1979a, 1979b). The results of 36 micrographs per animal were pooled into one experimental unit; five such units (animals) gave a group mean. To minimize observer-induced bias, all experimental manipulations involving an observer-generated decision was performed blind, and by one experimenter only: dicing of lung to obtain tissue blocks, random selection of these and preparation of block face for sectionning, photomicrography and final analysis. Stereological analysis was performed on all micrographs of animal randomly chosen from the available pool of subjects, and identified by a code number only.

No effort was made to determine the absolute volume of the whole lung in the various groups. We felt that the unreliable determination of this measure, in such an animal as the mouse, would decrease the precision of our observations of relative volume fraction. These relative V_V may not, then, be extended to describe absolute volumes in each group; they do, however, retain their full comparative value when opposed in different groups.

Homogeneity of variances was verified at both levels of data pooling. Analysis of variance (ANOVA) on the factorial design, and multiple means comparison (Student-Newmann-Kheuls) were performed using the a = 0,01 level of significance.

RESULTS

Type I pneumocyte

All experimental groups are compared both to the control groups at corresponding time (control) and to a mean value of these controls for all times considered (mean control). The analysis of variance for the type I pneumocyte, as developed in table 1, reveals significant differences for T, D or their interaction DT. The modifications in V_v are best illustrated in figure 1. Groups treated with BHT show a significant decrease in time: not yet evident at day 1, their V_v decreases to 77% of oil controls at day 3 (or 82% of mean oil controls), then to 64% 5 day of oil controls (62% of mean controls). Also at day 5, the BHT-O_2 group shows an increase over its BHT-air control of 31%. This BHT-air group represents 56% of day 5 oil controls (53% of mean controls), a further decrease from day 3, whereas BHT-O_2 only decreases to 73% of day 5 oil controls (71% of mean oil controls), a negligible difference from day 3.

Type II pneumocyte

Table 2 shows the analysis of variance pertaining to the type II pneumocyte. Some level of significance is barely reached with T (just over the 0,01 level), and with DT (a 0,05), especially at day 3. The histogram in figure 2 details its volume fractions. At day 1, the BHT set as a whole shows no difference from its oil controls. The BHT-O_2 component of this set, however, is much higher than its BHT-air control, 215% (or 2x↑), or the oil controls at day 1, 145% (mean oil controls, 146%); the BHT-air group is significantly lower than the day 1 oil control, 67% (68% of mean controls).

At day 3, the BHT groups show no differences among themselves, but a marked increase over their corresponding oil controls, 274% (or 2,7x↑), and overall oil controls, 196% (or 2x↑). At day 5, the high value reached by the oil-air group precludes its use as a proper control. The BHT set shows a net increase above the value of the overall oil controls, of 144% (or 1,4x↑). We also find a slight decrease from group BHT-air to group BHT-O_2 of 83%. Although no difference was noted in the ANOVA for exposure factor E, the groups making up the oil set show such a variation in V_v: at day 1, oil-O_2 surpasses oil-air by 124%; there is no difference at day 3; and at day 5, oil-O_2 decreases below oil-air, representing 75% of its control value.

Table 1. Analysis of variance for the type I pneumocyte

TYPE I EPITHELIAL CELL

SOURCE OF VARIANCE	SS	DF	MS	F	P
GROUPS	0.002600	11	0.000236	7.280	< 0.01
D	0.000892	2	0.000446	13.942	< 0.01
T	0.000738	1	0.000738	23.054	< 0.01
E	0.000002	1	0.000002	0.656	NS
DT	0.000796	2	0.000398	12.445	< 0.01
DE	0.000042	2	0.000021	0.656	NS
TE	0.000085	1	0.000085	2.667	NS
DTE	0.000045	2	0.000023	0.703	NS
RESIDUAL	0.001556	48	0.000032		
TOTAL	0.004156	59	0.000070		

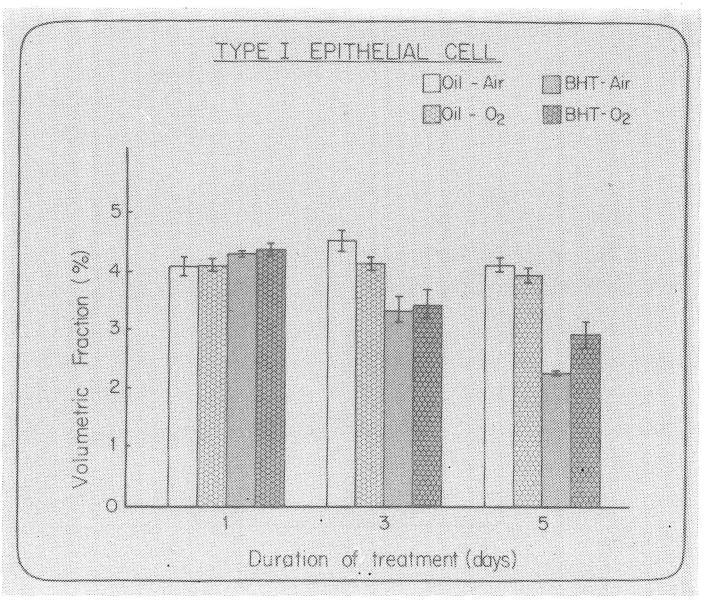

Figure 1. Histogram of the volume fraction mean per experimental group, for the type I pneumocyte

Table 2. Analysis of variance for the type II pneumocyte.

TYPE II EPITHELIAL CELL

SOURCE OF VARIANCE	SS	DF	MS	F	P
GROUPS	0.006212	11	0.000565	1.947	NS
D	0.000694	2	0.000347	1.196	NS
T	0.001988	1	0.001988	6.857	< 0.05
E	0.000039	1	0.000039	0.136	NS
DT	0.002389	2	0.001194	4.119	< 0.05
DE	0.000869	2	0.000435	1.498	NS
TE	0.00005	1	0.000051	0.176	NS
DTE	0.000182	2	0.000091	0.314	NS
RESIDUAL	0.013923	48	0.000290		
TOTAL	0.020135	59	0.000341		

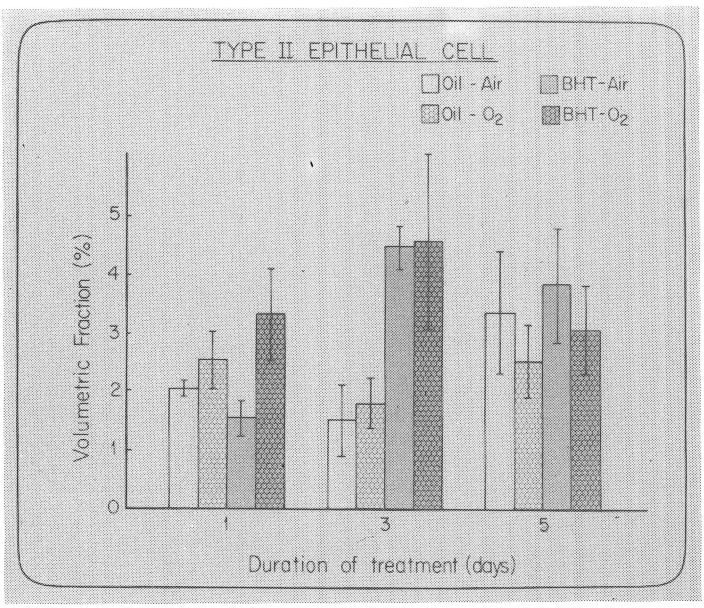

Figure 2. Histogram of the volume fraction mean per experimental group, for the type II pneumocyte.

Table 3. Analysis of variance for the intermediate type pneumocyte

INTERMEDIATE TYPE EPITHELIAL CELL

SOURCE OF VARIANCE	SS	DF	MS	F	P
GROUPS	0.014219	11	0.001293	12.725	< 0.01
D	0.006516	2	0.003288	32.368	< 0.01
T	0.005058	1	0.005058	49.792	< 0.01
E	0	1	0	0	NS
DT	0.002318	2	0.001159	11.409	< 0.01
DE	0.000004	2	0.000002	0.039	NS
TE	0.000209	1	0.000209	2.049	NS
DTE	0.000054	2	0.000027	0.266	NS
RESIDUAL	0.004876	48	0.000120		
TOTAL	0.019095	59	0.000324		

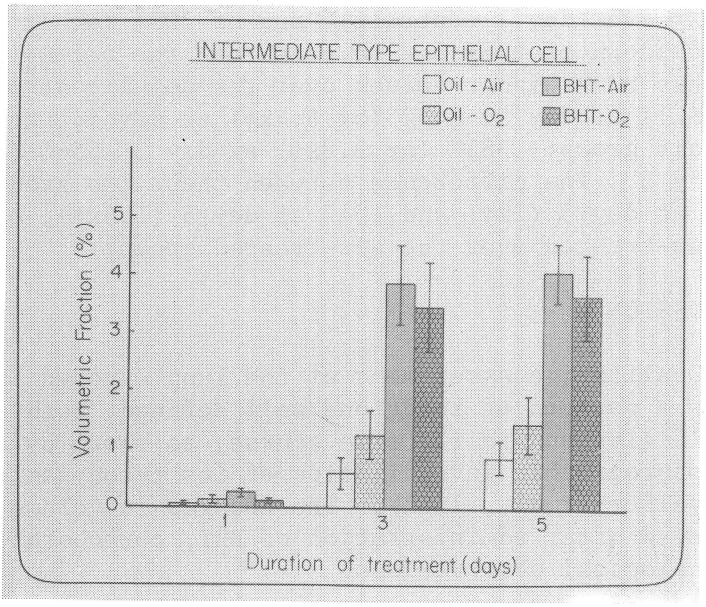

Figure 3. Histogram of the volume fraction mean per experimental group, for the intermediate type pneumocyte

Intermediate type cell

The analysis of variance for the intermediate type pneumocyte appears in table 3. Once again factors T, D and DT are significant. One might note in passing the very high F value in this ANOVA for parameters T and D, well below the 0,001 level. In figure 3, we can appreciate the marked modifications induced in the V_V of the intermediate type pneumocyte. At day 1, representing the control situation (non-stimulated lung), the four groups show a low V_V value, of 0,002. This is taken as the true control value for all subsequent comparisons of means. At day 3, all groups show a rise in V_V from day 1 controls, which is maintained at day 5. The BHT set increases at both times, well above the day 1 controls: 2292% (or 23x↑) at day 3, 2444% (or 24x↑) at day 5. It also keeps well above the oil set control at corresponding times: 392% (4x↑) at day 3, 348% (3,5x↑) at day 5. There is no difference between the two BHT-treated groups in this set.

A much smaller, but nonetheless significant increase in V_V is found in the oil set, representing exposure to gases alone. This is of the order of 584% for the oil set at day 3 (6x↑ over day 1 controls), and of 702% (or 7x↑) at day 5. A breakdown of this set reveals that the oil-O_2 group reaches much higher values over the day 1 controls: 787% (or 8x↑) at day 3, 844% (or 8,4x↑) at day 5; than does the oil-air group: 381% (or 3,8x↑) at day 3, 556% (or 5,6x↑) at day 5. The difference between these two groups is then 207% at day 3 (2x↑) and 151% at day 5 (1,5x↑), of the oxygen-treated over the air-treated group.

DISCUSSION

Qualitative assessment of the lung exposed to BHT reveals a pattern of reactions well defined, both in its cell specificity and in its time course, as shown by a recent radioautographic investigation of its cytodynamics (Chevalier et al., 1982). The changes in volume fractions observed after administration of BHT, corroborate these morphological observations.

In the type I pneumocyte, localized cytoplasmic blebbing at day 1, increases the V_V, though not significantly. Degeneration and destruction then set in, shown as a marked decrease in V_V, which is continued to day 5 inclusively.

The regenerative phase usually appears at a latter time, from days 5 to 7 post injury, and is reflected here in an increase in V_V in the BHT-O_2 group only (because of the earlier onset of regeneration for such combined treatment). Coincident with, and causally related to the loss of type I cells at day 3 is the onset of proliferation within the ranks of the type II pneumocyte (Williamson et al., 1978). Its V_V markedly increases from day 1, as type II cells are recruited into dividing, and come to occupy a major part of the septal surface in replacement of type I cells. After a few cycles of division, the process of dedifferentiation begins. The intermediate type cell is rarely seen in the healthy lung, as examplified by the V_V values at day 1 where the stimulus for epithelial regeneration has not yet taken effect. From day 3 to 5, as the type II population proliferates, the majority of the newly divided cells undergo a progressive transformation into intermediate type cells. These then differentiate into true, mature type I cells which we see reappearing at day 5 in the BHT-O_2 group.

Though toxic to the type I cell (Adamson and Bowden, 1974), oxygen is here a much less potent irritant than BHT. In the BHT-O_2 groups, its influence is mostly masked by the overwhelming toxicity of BHT. Only when used with oil does it show any effect. At day 3, the oil-O_2 group shows a lower V_V than its oil-air control for type I cells, an indication of added toxicity to the membranous pneumocyte. This difference is no longer evident at day 5. The mitotic effect of oxygen on the type II cell has been noted when used alone in high concentration and over extended periods of time (Haschek and Witschi, 1979). We note here a similar effect in the oil set, at days 1 and 3 (the excessively high value for oil-air at day 5 prevents any conclusion at this time). The same holds true for the intermediate type cell, which follows closely the modifications in type II cells.

The combined effect of BHT and oxygen does not show any increased toxicity to the type I cell. Rather, BHT alone provides the dominant toxic influence, and masks that of oxygen. The two irritants do, however, interact to stimulate the earlier onset of type II cell proliferation, as shown at day 1 for the BHT-O_2 group over the BHT-air control. At day 5, the BHT-O_2 group seems to have exhausted its potential for multiplication, and its value decreases

from the BHT-air control toward the oil control levels. There is no appreciable difference between these two groups when considering the intermediate type cell. However, the type I cell shows a renewal of its ranks, as newly differentiated cells join it after going through the complete regenerative cycle.

The modifications in volume fractions noted in all three epithelial cell types, closely parallel earlier morphological assessments (Hirai et al., 1977) (Williamson et al., 1978). Furthermore, the pattern of cell injury: type I degeneration, type II proliferation and dedifferentiation, intermediate type differentiation into new type I, resembles the normal process of epithelial turnover expected of the healthy lung, though in a more extensive and more rapid fashion. Thus the BHT model of epithelial injury calls upon no more than the usual mode of epithelial repair, leaving intact the architecture and functional anatomy in the alveolus.

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