ACTIVE OXYGEN GENERATION BY ALVEOLAR MACROPHAGE IN MICE TREATED WITH CARCINOGEN AND PROMOTER

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ABSTRACT—Glycerol has an action to enhance pulmonary tumorigenesis in mice treated with 4-nitroquinoline 1-oxide (4NQO). In order to evaluate factors contributing to enhancing effect of glycerol on 4NQO-induced pulmonary tumorigenesis, we selected alveolar macrophage (AM) as a source of active oxygen formation in the lung and investigated the effect of glycerol on active oxygen formation in AMs of mice treated with 4NQO in this study. AMs were stimulated with opsonized zymosan, and active oxygen formation in AMs after stimulation was examined. Continuous glycerol treatment within 4 weeks after 4NQO injection has no influence on the capacity of active oxygen generation in AMs (expressed as maximum count of chemiluminescence) and total amount of active oxygen generation in AMs (expressed as total count of chemiluminescence). These results suggest that active oxygen formation in AMs don't contribute to enhance 4NQO-induced pulmonary tumorigenesis in mice treated with glycerol.

KEY WORDS : Active oxygen, Alveolar macrophage, Lung tumorigenesis, Promotion, Mice

INTRODUCTION

Bronchiolar nonciliated (Clara) cell may be a possible progenitor cell of peripheral carcinoma in the lung (Kanisawa, 1982). Glycerol has been known to induce morphological changes in the Clara cell and stimulate the development of lung tumor in ddY mice treated with 4NQO (Inayama et al., 1986 ; Kitamura et al., 1987). It has been reported that oxidative stress caused by chemicals may play an important role in some cases of chemical-induced tumorigenesis (Cerutti, 1985). The increase of active oxygen formation can induce oxidative stress (Ames, 1989). The major sites responsible for active oxygen formation in a cell are mitochondria, microsomes and nuc-

leus (Nohl and Hegmer, 1978 ; Hilderbrandt and Roots, 1975 ; Bartoli et al., 1977). We found that continuous treatment of glycerol after 4NQO injection promoted active oxygen formation in pulmonary microsomes and nucleus, and this promotion might be associated with the enhancing effect of glycerol on pulmonary tumorigenesis (Yano and Ichikawa, 1992). In addition, phagocytes like macrophage have the strongest capacity on active oxygen formation of various cells and represent a major source of active oxygen (Lewis and Adams, 1986). Moreover, active oxygen generated from macrophage plays an important role in inducing some diseases including cancer (Yoshikawa et al., 1990). Therefore, we tested the effect of glycerol on the capacity and total amount of active oxygen formation by AMs in order to evaluate the contribution of this forma-

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tion by the cells to the enhancing effect of glycerol. As active oxygen in AMs stimulated with opsonized zymosan can be generated via a major pathway of respiratory burst which is related to produce active oxygen in phagocytes (Morel et al., 1991). It is also obvious that opsonized zymosan-stimulated active oxygen formation may reflect the functions of AMs in vivo more strictly than other stimulation (Hishinuma et al., 1989). Thus, opsonized zymosan was used as a stimulant to estimate active oxygen formation in AMs. Since glycerol had to be given orally for 4 weeks after 4NQO injection to enhance 4NQO-induced maximum pulmonary tumorigenesis (Inayama et al., 1986), we selected 2 and 4 weeks as the period of glycerol treatment after 4NQO injection.

MATERIALS AND METHODS

Animals : The animals used were 6-week-old male mice, specific pathogen free ddY strain (SLC Corp., Shizuoka, Japan). After 1 week acclimatization, healthy mice were used in this experiment. The mice were maintained under clean-rack systems at the temperature of $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with a 12 hour diurnal system. The mice were fed ad libitum on diet (CE-2, Japan Clea, Tokyo, Japan) and permitted sterilized water *ad libitum*.

Chemicals and Treatment : 4NQO (Tokyo Kasei Industry, Tokyo, Japan) was suspended in a mixture of olive oil and cholesterol (20:1, w/w). The mice were given 4NQO solution at the dose of 10 mg/kg body weight by a single subcutaneous injection on the first experimental day (Mori, 1962). Glycerol (Wako Pure Chemicals Industries, Osaka, Japan) (5%) was given instead of water *ad libitum*. At 2 and 4 weeks after 4NQO injection, all mice were killed by exsanguination of the abdominal artery under anesthesia with pentobarbital.

Cell preparation : The lungs were lavaged ten times *in situ* using Hank's balanced salt solution (phenol-red free) (Sigma, St. Louis, U. S. A.). AMs were prepared from this lavage according to a previous method (Reasor and Heyneman, 1983). AMs were more than 95% pure and 98% of the cells exclude trypan blue.

Assay method : Opsonized zymosan was prepared by a method using normal human serum (Wardlaw and Pillemer, 1956). For monitoring active oxygen formation by AMs challenged with opsonized zymosan, lucigenin (Boehringer, Mannheim, Germany)-amplified chemiluminescence was measured in a luminescence reader (Aloka, Tokyo, Japan) (Minkenberg and Ferber, 1984). The mixture for measuring chemiluminescence contained $200\mu\text{M}$ lucigenin, 1mg opsonized zymosan and 10^6 AM cells in 1ml Hank's balanced salt solution. The chemiluminescence measurement in this system was performed at 37°C for 30 minutes. The reaction in this system was started by the addition of opsonized zymosan.

Data analysis : Statistical comparison between 4NQO- and 4NQO-plus-glycerol-treated groups were performed by the use of Student's or Welch's t-test after analysis of variance.

RESULTS

There was no intergroup difference in terms of body weight change throughout this experimental period and AMs recovery from the lungs (table 1).

As shown in table 2, the capacity of active oxygen formation in AMs (expressed as maximum count) showed no difference between 4NQO- and 4NQO-plus-glycerol-treated groups at 2 and 4 weeks after 4NQO injection. Similarly, glycerol treatment had no influence on total amount of active oxygen formation in AMs (expressed as total count) (table 3). These results indicate that glycerol has no effect on active oxygen formation in AMs stimulated with opsonized zymosan.

DISCUSSION

In a previous report, the enhancement of 4NQO-induced pulmonary tumorigenesis with glycerol didn't correspond to the amount of the ultimate carcinogen 4-hydroxyaminoquinoline 1-oxide-DNA adduct in the lung (Nagahara et al., 1990). On the other hand, our previous report indicated that the enhancing effect of glycerol

Table 1. Data for initial and final body weights, and alveolar macrophage recovery.

Treatment & Period	Body weight (g)		AM cell recovery ($\times 10^6$ cells / kg B. W.)
	Initial	Final	
4NQO (2 weeks)	33.1 $\pm$ 0.5	37.8 $\pm$ 1.2	6.0 $\pm$ 0.8
4NQO + glycerol (2 weeks)	32.9 $\pm$ 1.2	38.2 $\pm$ 1.8	6.7 $\pm$ 1.6
4NQO (4 weeks)	32.2 $\pm$ 0.9	40.8 $\pm$ 2.0	7.5 $\pm$ 1.3
4NQO + glycerol (4 weeks)	31.9 $\pm$ 0.5	41.3 $\pm$ 2.5	7.2 $\pm$ 1.0

Values in body weight and AM cell recovery were obtained from 15 mice and 5 samples, respectively. One sample for measuring AM cell recovery was prepared from 3 mice. Value in parenthesis indicated glycerol-treated period after 4NQO injection.

Table 2. The alteration of maximum active oxygen formation (maximum count) by alveolar macrophages in response to glycerol.

Treatment Period (weeks)	Active oxygen formation (Kcount / min / 10^6 cells)		
	4NQO(A)	4NQO + glycerol (B)	(A/B)
2	0.76 $\pm$ 0.17	0.84 $\pm$ 0.21	(1.11)
4	0.67 $\pm$ 0.21	0.70 $\pm$ 0.13	(1.04)

Maximum active oxygen formation was expressed as a maximum count of chemiluminescence per minute recorded during chemiluminescence reaction of 30 minutes. Each value represents the mean $\pm$ S. E. from 5 samples. One sample was prepared from 3 mice.

Table 3. The alteration of total active oxygen formation (total count) by alveolar macrophages in response to glycerol.

Treatment period (weeks)	Active oxygen formation (Kcount / min / 10^6 cells)		
	4NQO (A)	4NQO $\pm$ glycerol (B)	(A/B)
2	23.72 $\pm$ 4.17	22.60 $\pm$ 5.62	(0.95)
4	20.87 $\pm$ 3.19	21.9 $\pm$ 15.24	(1.05)

Total active oxygen formation was expressed as a total count of chemiluminescence recorded during chemiluminescence reaction of 30 minutes. Each value represents the mean $\pm$ S. E. from 5 samples. One sample was prepared from 3 mice.

could be associated with the increase of active oxygen formation in pulmonary microsomes and nucleus (Yano and Ichikawa, 1992), and the level of lipid peroxidation in these fractions showed a significant increase by glycerol treatment after 4NQO injection (Yano et al., 1993). These data suggest that glycerol treatment can induce pulmonary oxidative stress due to the increase of active oxygen formation in microsomes and nucleus. In addition to this factor inducing oxidative stress, active oxygen generated from phagocytes like macrophage is considered to be an important factor which induces oxidative stress

(Yoshikawa et al., 1990). It has been also reported that asbestos-related stimulation of active oxygen generation by AMs via respiratory burst may be an important factor on asbestos-induced lung carcinogenesis (Hansen and Mossman, 1987). Therefore, we estimated the effect of glycerol treatment on active oxygen formation with AMs in the lung. However, glycerol treatment had no effect on active oxygen formation with AMs. This result suggest that this glycerol treatment may not stimulate *in vivo* active oxygen generation by AMs in 4NQO-treated mice. Therefore, being different from asbestos-induced

lung carcinogenesis, active oxygen formation from AMs via respiratory burst can not contribute to the enhancing effect of glycerol on 4NQO-induced lung tumorigenesis.

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