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Plasmodium berghei Infection in Mice: Effect of Low-Level Ozone Exposure

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Animal and human studies have accumulated that report extrapulmonary effects from inhaled ozone (O_3). Buckley et al. (1975) exposed human volunteers to 0.5 ppm O_3 for 2.75 h and demonstrated increases in red cell fragility, serum vitamin E levels, and red cell glucose-6-phosphate dehydrogenase (G-6-PD) activity. Decreases in red cell acetylcholinesterase (ACHase) activity and reduced glutathione (GSH) were also evident. Similar results were obtained by Hackney et al. (1977) who exposed human male volunteers to 0.37 ppm O_3 for 2.0 hours. Moore et al. (1981) exposed sheep to 0.25, 0.50, or 0.70 ppm of O_3 for 2.75 h and observed a decreased erythrocyte survival time as determined by CR-51 studies. Exposure of male Sprague-Dawley rats to 0.3 ppm O_3 for 3 h resulted in a time-delayed increase in serum and hepatic reduced ascorbic acid levels as compared to controls (Calabrese et al. 1983).

Although the mechanism of a toxicity to red blood cells is not clearly understood, Lamberts et al. (1964) demonstrated that inhalation of an O_3 containing atmosphere potentiates *in vitro* sphering of human erythrocytes. This and other work has led to the hypothesis that O_3 is radiomimetic and results in free radical formation (Stokinger 1965). Lipid peroxidation has also been found to be produced by radiation and is thought to result in the formation of free radicals (Desai and Tappel 1963). This suggests the possibility that one of the biological effects of O_3 inhalation is mediated by the interaction of O_3 with the soluble bonds of unsaturated fatty acids, resulting in lipid peroxidation. Goldstein and Balchum (1967) studied the effect of O_3 on lipid peroxidation in the human red blood cell and found that exposure to O_3 was associated with an increase in lipid peroxide formation, and that erythrocytes exposed to O_3 have increased osmotic fragility. This evidence suggests that peroxidation of unsaturated fatty acids in the RBC membrane due to free radical formation may occur upon O_3 exposure.

Current research, therefore, has demonstrated that ozone affects erythrocyte. It was the purpose of this research to demonstrate

the effects of O_3 exposure on the course of parasitemia in mice. A study was designed utilizing Plasmodium berghei, a murine malarial parasite host-specific for rodents, as the specific pathogen. The purpose of this project was to study mortality and percent parasitemia in the A/J mouse first infected with P. berghei and then exposed to low levels of ozone.

MATERIALS AND METHODS

Nine-month-old female A/J mice were maintained in individual stainless steel cages in a room with controlled temperature ($72^0 \pm 2^0$F) and lighting (12 h light and 12 h dark) until the beginning of the study. During the study, the mice were housed two per cage and were transferred to the laboratory setting to reduce the risk of malarial infection to the colony. The average temperature for the laboratory was $72^0 \pm 3^0$F, and the mice received approximately 10 hours of light and 14 hours of darkness during the incubation period. Mice were fed Purina Lab Chow and distilled water ad libitum, the same diet they have received since weaning.

Three mice infected with Plasmodium berghei (strain NK65) were received from the Department of Parasitology, University of North Carolina at Chapel Hill several weeks prior to the study. Infection with P. berghei is fatal to mice within 19 to 22 days after intraperitoneal introduction, so it was necessary to transfer the parasite via carrier hosts until the study began in order to keep the parasite strain alive. The eight female A/J mice used in this study were infected with P. berghei by intraperitoneal innoculation on day zero (0) of this study.

Eighteen days post-infection the mice injected with P. berghei were sacrificed by guillotine. The blood of each mouse was aseptically collected in a 1.5 ml microeppendorf tube with 100 µl heparin, agitated, and the blood of the four mice was then pooled and a red blood cell count was made to determine erythrocyte density using a Royco Model 920-A Cell Counter and Model 365-A Diluter (Royco Instruments, Inc., 141 Jefferson Drive, Menlo Park, CA 94025). Once the erythrocyte density was determined, the fresh blood sample was drawn and diluted with physiological saline PBS to yield a final erythrocyte density of 1.01×10^{-6} cells/mL whole blood. In order to innoculate each mouse with approximately 2.0×10^{-5} red blood cells, each mouse received an intraperitoneal innoculation of 0.2 mL infected mouse blood containing 2.02×10^5 erythrocytes. Innoculations were performed using a 1 ml tuberculin syringe. After infection, the mice were returned to their cages to rest 24 hours before the exposure regimen began.

Mice were placed in the individual stainless steel cages, and then randomly assigned to one of the two 69 by 69 cm stainless steel exposure chambers according to the design of Hinners et al. (1968). The two chambers were operated simultaneously with control mice (0.0 ppm O_3) in chamber 2 and test mice (0.30 ±

0.1 ppm O_3) in chamber 1. Ozone was produced by a silent arc generator and added to the filtered air entering the chamber. Sampling ports for drawing air for analyzers were located at the rear of the chamber at the same level as the cage shelves (breathing zone). The flow of temperature-controlled filtered air through the chamber was maintained at 2.5 ± 0.2 SCFM. The temperature of both chambers averaged 72 ± 3°F with a relative humidity of 34 ± 1%. Both chambers were analyzed sequentially at 3-minute intervals by drawing air samples through a sequential sampling system (Moore et al. 1979) and delivering the sample, first from one chamber and then the other, to an ultraviolet O_3 monitor. Consequently, the conditions to which the control and test mice were exposed during any single 3 hour exposure period were essentially identical except for the level of O_3 directed into chamber 1. Mice were exposed each day for 3 hours until death or approximately 2 to 3 weeks.

Blood smears were prepared from tailblood (1 drop) for each of the eight mice in the study on day zero (0) before infection and immediately after each exposure period; therefore, samples of tailblood were taken for slide preparation on days 0, 1, 4, 7, 10, 13, 16, and 19. Giemsa stain was used to demonstrate the presence of the parasite. Blood smear slides were stained according to the Thin Blood Film Method outlined in the Encyclopedia of Microscopic Stains, Gurr (1960). Slides were fixed and stained within two hours after the smears were made, subsequent to each exposure period. Examination of the slides was performed on a double blind basis. The identification of each mouse with respect to control or treatment group was unknown to the examiner. The order in which the slides were examined was randomized by mouse identification number and day of incubation.

Ten trial counts of total cell number (excluding white blood cells) and number of parasitized cells within the grid were made for each slide. The ten counts for each category were then summed individually. Dividing the total number of parasitized cells by the total number of red cells viewed under the grid yielded the percent parasitemia for the blood smear stained on each slide. This procedure was performed for each smear made during the study.

Mice were carefully monitored so that their approximate time of death could be determined. The time of death was recorded as having occurred during one of four six-hour time periods: 12 AM - 5:59 AM, 6 AM - 11:59 AM, 12 PM - 5:59 PM and 6 PM - 11:59 PM. The date was also recorded for each mouse in the study.

RESULTS AND DISCUSSION

Treatment mice exposed to 0.30 ppm O_3 died an average of 3.5 days earlier than control mice. Two of the ozone exposed mice died on day 17 and two on day 18, while all the control mice died on day 21 (Table 1).

Table 1. Mortality Schedule of Ozone-Exposed and Control Mice

Mouse Group	Days to Death (Post Infection)	Number of Deaths	Time Period of Deaths
-----	16	0	-----
Ozone Exposed	17	2	12 AM - 5:59 AM
Ozone Exposed	18	2	12 AM - 5:59 AM
-----	19	0	-----
-----	20	0	-----
Controls	21	4	12 AM - 5:59 AM

Mice in the treatment group exposed to 0.30 ppm O_3 showed statistically significant increases in number of parasitized cells ($p < 0.03$ to $p < 0.005$) during the entire study when compared to controls (Table 2).

The percent of parasitemia ranged from 5.75% at 4 days from innoculation to 30.88% at 16 days post-innoculation in ozone-exposed animals whereas controls showed a 3.45% to 17.13% parasitemia for the same time period.

Table 2. Percent Parasitemia for Ozone-Exposed and Control Mice Infected with P. berghei

	CONTROLS		OZONE EXPOSED (0.03 PPM)		
Days from Innoculation	Percent Parasitemia	σ	Percent Parasitemia	σ	P Value
4	3.45%	0.78	5.75%	2.60	<0.01
7	1.75%	1.10	3.60%	1.50	<0.005
10	7.08%	2.50	16.68%	2.70	<0.005
13	20.93%	2.60	24.53%	6.0	<0.03
16	17.13%	2.30	30.88%	5.7	<0.005
19	27.83%	7.98	**		

*Calculated as a mean from the values obtained for each of the four mice in each of the comparison groups.
**All of the ozone-exposed mice died by the beginning of day 18.
σ = standard deviation

Mice exposed to O_3 died earlier and exhibited parasitemia to

a significantly greater ($p < 0.005$) extent than observed for controls. The different parasitemia values obtained for the two comparison groups support the hypothesis that changes may occur in the erythrocyte membrane following exposure to low levels (0.30 ppm) of O_3. It is thought that such exposure to O_3 alters the mouse erythrocyte membrane in a manner that enhances cellular invasion by the parasite. The possibility of alteration of the erythrocyte membrane structure and/or metabolism by O_3 exposure is consistent with the findings of Goldstein et al. (1968, 1969) and Buckley et al. (1975) who reported increases in osmotic fragility for ozone-exposed human erythrocytes.

The mechanism of how the intraerythrocyte merozoite of P. berghei alters the permeability of mouse erythrocytes has not been elucidated. Cenedella et al. (1969) proposes that the parasite may obtain fatty acids from both the plasma and the host cell lipids. This apparently occurs during both the intraerythrocytic and extraerythrocytic stages of the growth of P.berghei in the white mouse. Infection with P. berghei accompanied by peroxidation of membrane lipids may contribute to a change in membrane protein and lipid composition which increases membrane permeability to P. berghei.

An alternate hypothesis is that O_3 inhalation causes an in vivo change in the red cell population by oxidant damage of the more susceptible older erythrocytes (Lamberts et al. 1964). The increased rate of splenic sequestration of these damaged cells reduces the mean age of the remaining red cell population (Moore et al. 1981; Calabrese et al. 1983). These young cells are reported to be more resistant to osmotic lysis (Cohen et al. 1976) than older erythrocytes. Such action by the spleen may induce reticulocytosis. Some strains of P. berghei in the white mouse have a requirement for reticulocytes (Ladda and Lalli 1966; Ott 1968) so that the parasitemia and reticulocyte count will rise together. Pollack et al. (1966) found this to be the case where P. berghei infected mice were exposed to oxidant agents (Sodium Nitrite, Alloxan, Phenylhydrazine HCl) which produce reticulocytosis. The mice exposed to these oxidative agents displayed an accelerated course from P. berghei infection with higher levels of parasitemia than controls, apparently due to the increased numbers of reticulocytes observed in the treatment group. It should be noted, however, that with some strains of P. berghei the percentage of immature cells does not rise, although there is obvious anemia of the infected animal (Kreier et al. 1972).

Statements about changes in the red cell population can only be made after sufficient histological and biochemical analyses have been performed. The results of this study are based solely upon histological examination and point to the need for future research directed at this interaction which is occurring at the subcellular level.

Extrapolation of these results to the metabolism of the

host-parasite relationships of P. falciparum and P. vivax in humans is unwarranted without further study. If we allow the premise that the laboratory experience with P. berghei is generalizable to human malarial infections, these findings may be of some import for the following hypothetical situations.

There are many areas of the world in which malaria is still a major cause of morbidity. Increasing industrialization in these countries may create atmospheric conditions containing photochemical oxidant compounds, including ozone in urban areas. These conditions could serve to exacerbate the malarial infection in laborers who migrate to the developing cities from rural areas seeking employment. This factor may be most relevant in areas where Chloroquine-resistant strains of P. falciparum exist. These strains are most prevalent at elevations of less than 1,500 feet in the following countries: Surinam, Venezuela, Colombia, Panama, Brazil, Malaysia, Vietnam and Thailand (C.D.C. 1980).

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