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OXYGEN INHIBITION OF GROWTH OF *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

GOTTLIEB, S. F. (Linde Division, Union Carbide Corporation, Tonawanda, N.Y.), NOEL R. ROSE, JEROME MAURIZI, AND EDWARD H. LANPHIER. Oxygen inhibition of growth of *Mycobacterium tuberculosis*. J. Bacteriol. **87**:838-843. 1964.—Continuous or intermittent exposure to 3 atm of oxygen in the presence or absence of 40 mm of Hg CO_2 resulted in marked delay or inhibition of growth of *Mycobacterium tuberculosis*. This inhibition was more pronounced with newly inoculated cultures, as compared to young, actively growing cultures. Oxygen inhibition of growth was observed on both Lowenstein-Jensen and blood agar media. Bacteriostatic effects of oxygen are a direct function of exposure time. A more marked effect of oxygen was observed in the presence of isoniazid, streptomycin, or *p*-aminosalicyclic acid.

Previous work revealed that oxygen at 1 atm adversely affects the growth of *Mycobacterium tuberculosis* (Moore and Williams, 1909; Adams, 1912; Novy and Soule, 1925; Knox et al., 1961). It was also found for other forms of life that an inverse relationship exists between the oxygen tension and the duration of exposure before the toxic effects of oxygen become manifest (Bean, 1945). On the basis of these data, Gottlieb (1963) postulated that oxygen under pressure, alone or in combination with drugs, may result in an efficacious treatment of mycobacterial infections. This series of experiments was undertaken to explore the possible relationship that may exist between exposure time and oxygen tension on the growth of the tubercle bacillus.

MATERIALS AND METHODS

The organism used in most of these studies was the H37Rv strain of *M. tuberculosis* var. *hominis* (obtained from the Division of Laboratories and Research, New York State Department of Health); other organisms used included both drug-sensitive and drug-resistant strains of *M. tuberculosis* isolated from sputa of patients. Commercially prepared Lowenstein-Jensen (L-J) tubed slants (BBL) and blood agar (B.A.) media (Difco Blood Agar Base, 3.0%; glycerol, 1.0 ml/100 ml; outdated blood-bank blood containing citrate, 30 ml/100 ml; and penicillin G to give a final concentration of 100 units per ml) were used throughout these experiments.

All media were inoculated with 0.1 ml of a 1:8 dilution of a bacterial suspension equivalent to McFarland's no. 1 standard. The tubes were plugged with cotton-wool and screw caps; the caps were loosened prior to insertion in the pressure chamber. The chamber was evacuated to a pressure of 126 mm of Hg and filled with the appropriate gas mixture [100% oxygen, 98.2% O_2 + 1.8% CO_2 (O_2-CO_2), or air]. This flushing procedure was executed five times before filling to 3 atm absolute (ata). Temperature was maintained at 35 ± 1 C by circulating water from a constant-temperature reservoir through copper coils situated in the chamber: temperature in the 1 atm incubator was 37 C. Tubes were removed at various intervals and incubated at 1 ata. In experiments in which tubes were not removed periodically, the chamber was flushed once daily by releasing the pressure, evacuating once, and refilling with the appropriate gas mixture. Experiments were performed in duplicate and triplicate.

RESULTS

The data in Table 1 reveal that a mixture of 98.2% O_2 and 1.8% CO_2 at 3 ata (2,240 mm of Hg O_2 + 40 mm of Hg CO_2) markedly delayed the emergence of visible growth of the H37Rv strain of *M. tuberculosis* on both L-J and B.A. media. It was also observed that increased dura-

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TABLE 1. *Effect of continuous exposure to 2,240 mm of Hg O_2 plus 40 mm of Hg CO_2 on the growth of Mycobacterium tuberculosis H37Rv**

Hr of exposure	Medium†	No. of days after inoculation											
		6	7	9	11	15	17	21	23	25	27	30	34
0	B.A.	0	6	17	+	++	+++	++++					
	L-J	0	8	22	+	++	+++	++++					
24	B.A.	0	0	6	14	23	+	+++	++++				
	L-J	0	0	3	11	23	+	+++	++++				
48	B.A.	0	0	0	7	13	20	++	+++	++++			
	L-J	0	0	0	9	24	24	++	+++	++++			
72	B.A.	0	0	0	0	0	2	23	+	++	++++		
	L-J	0	0	0	0	3	9	+	++	+++	++++		
96	B.A.	0	0	0	0	0	1	6	13	25	+	+++	+++
	L-J	0	0	0	0	0	2	12	20	+	++	+++	+++
120	B.A.	0	0	0	0	0	0	4	8	13	20	+	+++
	L-J	0	0	0	0	0	0	8	12	19	+	+	+++

* Initially growth was expressed as the number of colonies on the slant; when colony counts could no longer be obtained, growth was recorded on the basis of an arbitrary plus scale ranging from + to ++++. The results are the average of three tubes.

† B.A. and L-J refer to blood agar and Lowenstein-Jensen media, respectively.

tion of exposure resulted in a more marked delay of growth. Similar data were obtained with 3 ata of 100% oxygen. At exposure times greater than 120 hr, 3 ata of 100% oxygen exerted a bactericidal effect, as evidenced by the limited growth (1 to 15 colonies) that occurred on incubation after removal from the chamber; continued exposure to 3 ata of 100% oxygen for 264 hr resulted in complete inhibition of growth; growth was not observed even after prolonged incubation following removal from the chamber.

In other experiments, media inoculated with the standard suspension were permitted to incubate under air at 1 ata until microcolonies appeared (approximately 10 days), and were then exposed to 3 ata of oxygen for varying lengths of time. Similar, but less pronounced, inhibition was obtained on these young, actively growing cultures as compared to freshly inoculated cultures. The growth of three strains of *M. tuberculosis* freshly isolated from sputa was also found to be inhibited by the O_2-CO_2 mixture at 3 ata.

Exposure of uninoculated L-J and B.A. media to 3 ata of 100% O_2 or to 3 ata of the O_2-CO_2 mixture for 120 to 144 hr did not result in any alteration of the media which would adversely affect subsequent growth of this organism. The rate of growth on the exposed media was identical to that obtained on unexposed media.

The inhibition of growth was due to the increased oxygen tension and not to the pressure per se. This was shown by the nearly identical growth rates obtained when comparing the growth of H37Rv on L-J and B.A. media in air at 3 ata with that obtained in air at 1 ata. That this inhibition was not due to other possible differences in growth conditions within the pressure chamber, as compared to the incubator, was demonstrated by comparing the growth curves obtained with organisms incubated in the chamber at 1 ata for 240 hr with similar organisms grown in the incubator. Initiation of growth in the chamber on B.A. and L-J media occurred simultaneously with growth on similar media in the incubator.

The inhibitory effects of oxygen under pressure were not limited just to H37Rv; atypical mycobacteria of the scotochromogenic and

TABLE 2. *Effect of continuous exposure to 2,240 mm of Hg O_2 plus 40 mm of Hg CO_2 on the growth of atypical mycobacteria**

Organism	Hr of exposure	No. of days after inoculation					
		8	13	20	24	41	50
Scotochromogen	0	0	Slight	++++	Heavily pigmented		
	96	0	0	0	Slight	+	+++
Battey type	0	0	Slight	++	++++		
	96	0	0	0	Slight	+++	++++

* Grown on Lowenstein-Jensen medium. Growth expressed in same manner as explained in Table 1. "Slight" refers to an amount of growth less than +1.

TABLE 3. *Effect of intermittent exposure to 2,240 mm of Hg O_2 plus 40 mm of Hg CO_2 on the growth of Mycobacterium tuberculosis**

Organism	No. of exposures	No. of days after inoculation																				
		6	7	8	9	10	11	13	14	16	20	21	22	23	24	25	28	29	30	31	32	35
H37Rv	0	0	2	4	6	6	8	18	+	++	+++	+++	++++									
	2	0	0	0	1	2	8	13	23	+	++	++	+++	+++	++++							
	4	0	0	0	1	1	2	4	6	10	+	++	++	++	++	++	+++	+++	++++			
	8	0	0	0	0	1	2	3	5	9	+	+	++	++	+++	+++	++++					
	10	0	0	0	0	0	1	2	4	7	14	+	++	++	+++	+++	+++	++++				
Clinical specimen	0	0	0	0	0	0	0	2	4	11	+	+	++	++	+++	+++	+++	++++	++++			
	2	0	0	0	0	0	0	0	1	1	4	6	8	8	16	22	+	++				
	4	0	0	0	0	0	0	0	0	0	9	18	+		+	+	++			++++		
	8	0	0	0	0	0	0	0	0	0	0	0	3		8	10	23	+	+	+	++	++
	10	0	0	0	0	0	0	0	0	0	0	0	3		9	10	18	+	+	+	++	+++

* Grown on Lowenstein-Jensen medium. Growth expressed in the same manner as explained in Table 1. Exposures consisted of 3 atm, 3 hr, twice daily; a 4-hr interval separated the two daily exposures.

Battey types also were adversely affected (Table 2).

A study was undertaken next to ascertain the effects of intermittent exposure of *M. tuberculosis* to increased oxygen tensions. H37Rv and one strain freshly isolated from sputum were subjected to 3 ata of the O_2-CO_2 mixture for 3 hr twice daily. Between exposures the organisms were incubated in 1 ata of air. It was found (Table 3) that even intermittent exposure delayed the emergence of growth of these two strains of *M. tuberculosis*. The freshly isolated strain of tubercle bacilli appeared to be more sensitive to the effects of intermittent oxygen exposure than was H37Rv, a well-adapted laboratory strain. Additional experiments were conducted in which H37Rv was subjected to 2 ata of the O_2-CO_2 mixture for 2 hr twice daily. A slight delay in the emergence of growth was noted only after ten exposures.

The question of oxygen enhancement of, or interference with, drug action presented itself (Gottlieb, 1963). To explore these possibilities, experiments were performed in which drug-containing media were inoculated with freshly isolated cultures of *M. tuberculosis* or atypical mycobacteria. *M. tuberculosis* was exposed ten times to the O_2-CO_2 mixture for 3 hr twice daily, with a 4-hr interval separating the two daily exposures; during this interval, the organisms were subjected to air at 1 ata. The freshly isolated scotochromogen was exposed to the same gas mixture continuously for 72 hr before being removed from the chamber. The data presented in Tables 4 and 5 are suggestive of a possible synergistic effect between oxygen and the drugs tested.

DISCUSSION

The data presented reveal that the longer *M. tuberculosis* H37Rv is exposed to oxygen under pressure the more pronounced is the delay before

TABLE 4. *Effect of intermittent exposure to 2,240 mm of Hg O_2 plus 40 mm of Hg CO_2 on the growth of Mycobacterium tuberculosis (clinical specimen) in the presence and absence of drugs**

Drug†	No. of exposures	No. of days after inoculation												
		11	13	14	16	20	21	22	23	24	25	28	29	35
None	0	0	2	4	11	+	+	++	++	+++	+++	+++	++++	++++
	10	0	0	0	0	0	0	3		9	10	18	+	+++
PAS, 1.0 μg/ml	0	0	2	4	6	10	15	+	+	++	+++	+++	++++	++++
	10	0	0	0	0	1	1	2		5	6	13	18	+
INH, 0.2 μg/ml	0	0	3	6	15	+	+	++	++	+++	+++	++++		
	10	0	0	0	0	1	1	2		5	6	8	13	+
SM, 3.5 μg/ml	0	0	2	4	8	20	+	++	++	+++	+++	++++		
	10	0	0	0	0	0	0	1		2	4	7	10	+

* Grown on Lowenstein-Jensen medium. Growth expressed in the same manner as in Table 1. See text for details of exposure.

† Abbreviations: PAS, *p*-aminosalicylic acid; INH, isonicotinic acid hydrazide; SM, streptomyicn.

the onset of growth. This oxygen effect—delay of emergence of growth—is independent of the growth medium, as evidenced by the fact that similar oxygen effects were obtained on both L-J and B.A. media and that this effect does not appear to be related to the phenomenon observed by Cahn-Bronner (1940). We found that the adverse effects of oxygen on growth are manifested under conditions in which there is a deficiency of carbon, but not under conditions in which nitrogen is limiting. The fact that other mycobacteria, drug-sensitive and drug-resistant strains of *M. tuberculosis* as well as various atypical strains, show the oxygen effect indicates that this phenomenon is not peculiar to a particular mycobacterial strain but is of a more general nature.

The retardation of emergence of growth observed in the intermittent oxygen-exposure studies at 3 ata indicates the powerful deleterious effects of oxygen on mycobacteria. It is interesting to note that the twice daily 3-hr exposure of 3 ata exerted a noticeable oxygen effect, whereas the twice daily 2-hr exposure of 2 ata did not. These data suggest the existence of a critical relationship between the partial pressure of oxygen and the exposure time before deleterious effects of oxygen become manifest.

Data obtained from the intermittent oxygen exposures tend to indicate a greater susceptibility to the noxious effects of oxygen for the pathogenic mycobacteria than for the non-

TABLE 5. *Effect of 72-hr exposure to 2,240 mm of Hg O_2 plus 40 mm of Hg CO_2, in the presence and absence of drugs, on the growth of an atypical mycobacterium (scotochromogen)**

Drug†	Hr of exposure	No. of days after inoculation						
		11	14	23	35	42	46	51
None	0	0	Slight	++++				
	72	0	0	Slight	5	9	+	+
PAS, 1.0 μg/ml	0	0	0	2	3	5	5	5
	72	0	0	0	0	0	0	0
INH, 0.2 μg/ml	0	0	Slight	++++				
	72	0	0	0	Slight		+	+
SM, 3.5 μg/ml	0	0	0	0	2		+	+
	72	0	0	0	0		0	0

* Grown on Lowenstein-Jensen medium. Growth expressed in the same manner as explained in Table 1. "Slight" refers to an amount of growth less than +1.

† Abbreviations: PAS, *p*-aminosalicylic acid; INH, isonicotinic acid hydrazide; SM, streptomycin.

pathogens (Table 3). Knox et al. (1961) reported that slower growing strains of mycobacteria (BCG, *M. tuberculosis*, chromogenic acid-fast bacilli) are more susceptible to oxygen at 1 ata than are the faster growing saprophytic strains (*M. phlei*, *M. stercoris*, and *M. smegmatis*). On

the basis of these data it is suggested that mycobacterial responsiveness to high oxygen tensions should be explored as a possible additional differential physiological parameter. Perhaps such investigations should be broadened to include other microbial genera.

At the present time, it is not known whether the enhanced effectiveness of various antituberculosis drugs in the presence of oxygen (or the increased effectiveness of oxygen in the presence of the drugs) is due to oxygen's altering the responsiveness of the organisms to the drugs or the drugs' altering the responsiveness of the organism to oxygen. Our data do not permit differentiation between an additive or synergistic effect between oxygen and the drugs nor a concise differentiation between bacteriostatic and bactericidal effects of oxygen, either in the presence or absence of drugs. Some of the data presented strongly indicate that oxygen exerts a bactericidal effect. Bactericidal effects of oxygen on mycobacteria were observed by Moore and Williams (1909) and Knox et al. (1961), but not by Adams (1912) or Novy and Soule (1925).

The fact that oxygen exerts profound effects on various mycobacteria, as evidenced by the delay in the emergence of growth and increased susceptibility to certain drugs, leads one to wonder whether exposure to high partial pressures of oxygen, in the presence or absence of drugs, also may not alter the pathogenicity of these organisms.

One of the foremost theories concerning the adverse effects of oxygen on living systems pertains to the oxidation of sulfhydryl groups. It is known that one of the requirements for cell division for metozoa as well as protista is a high concentration of available sulfhydryl groups (Barron, 1953; Pine, 1954). In bacteria, cellular reducing activity and sulfhydryl content are maximal during the logarithmic phase of growth (Kopper, 1952; Mortenson and Beinert, 1953). These data imply that substances which interfere with the accumulation of sulfhydryl groups would exert marked growth-inhibitory properties. Also, there is the further implication that, all things being equal, these substances would be most effective in organisms in the lag phase of growth because that would be the time that metabolism would be shifting toward the rapid production and accumulation of sulfhydryl groups. Part of the deleterious effects of oxygen may possibly be ascribed to oxidation of sulfhydryl groups.

As might be predicted theoretically, oxygen appears to exert a more pronounced effect on mycobacteria in the lag phase, as opposed to the logarithmic phase of growth. This conclusion is deduced from the data in which it was shown that the oxygen-induced retardation of emergence of growth was more pronounced with newly inoculated cultures as compared to the delay observed when young, actively growing cultures were exposed to the high oxygen tensions.

Our data not only are of theoretical interest but also are suggestive of certain practical applications. At the present time, the available evidence indicates that human lungs are more resistant to the noxious effects of oxygen than are the lungs of the commonly employed laboratory animals, including primates. These animals, in all likelihood, would succumb to oxygen toxicity before any beneficial effects of oxygen on the disease process would be noted. The in vitro data obtained from the intermittent-exposure experiments reported herein are suggestive of a satisfactory therapeutic index for high-pressure oxygen for man, and thereby strongly indicate that serious consideration should be given to a clinical evaluation of the use of oxygen under pressure for the treatment of tuberculosis and possibly other mycobacterial infections.

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