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Effects of anaerobic and microaerophilic conditions of extraction and incubation on the survival of Treponema pallidum in vitro

G H W WONG, B M STEINER, AND S R GRAVES

From the Department of Microbiology, Monash University Medical School, Alfred Hospital, Prahran, Victoria, Australia

SUMMARY *Treponema pallidum* extracted from infected rabbit testes under anaerobic conditions survived longer in vitro than those extracted under aerobic conditions. Anaerobically extracted treponemes were incubated anaerobically for 0, 12, 24, 36, or 48 hours and then exposed to microaerophilic conditions (3% oxygen) for further incubation. Treponemes transferred to microaerophilic conditions after 36 or 48 hours' anaerobic incubation maintained significantly greater viability compared with those kept under constant microaerophilic conditions, although there was no difference after 12 or 24 hours. *T pallidum* incubated under constant anaerobic conditions, however, usually maintained greater viability than those kept under constant microaerophilic conditions.

These results suggest that *T pallidum* is sensitive to oxygen toxicity both during initial extraction from orchitic rabbit testes and subsequent incubation in vitro. In the latter case, it can be partially protected by a period of anaerobic incubation in vitro, before exposure to microaerophilic conditions.

Introduction

Treponema pallidum has little ability to handle oxygen toxicity in vitro. Although high concentrations of oxygen enhanced the metabolism of *T pallidum* in vitro,[3 4] prolonged survival and growth have been observed only under low oxygen concentrations[5 6] and electronegative redox potentials.[1]

The survival of *T pallidum* in vitro has been prolonged by several methods—presumably by reducing the toxicity of oxygen—such as the addition of reducing agents,[7-9] co-incubation with tissue culture cells,[9] and the addition of oxygen-protective enzymes (unpublished data).

In this paper we compared the survival of *T pallidum* in vitro after either anaerobic or aerobic extraction from infected rabbit testes on the assumption that oxygen toxicity was minimal in the former case. We also tested the effect of a variable period of incubation under anaerobic conditions before the treponemes were exposed to 3% oxygen to determine whether they were more sensitive to oxygen toxicity immediately after extraction from infected rabbit testes or at sometime later.

Address for reprints: Dr G H W Wong, Department of Microbiology, Monash University Medical School, Alfred Hospital, Prahran, Victoria, Australia 3181

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Materials and methods

The Nichols strain of *T pallidum* was maintained by intratesticular passage in rabbits. *T pallidum* was harvested within two days of orchitis appearing in the rabbit, which was killed by an intravenous injection of 3 ml sodium pentobarbitone (200 mg/ml). The testes were removed aseptically and cut into longitudinal and transverse slices with sterile scissors.

AEROBIC EXTRACTION

After initial slicing, the testes were further cut and minced in a sterile Petri dish under aerobic conditions in a laminar flow cabinet. The first extract, containing large amounts of tissue debris and erythrocytes, was discarded. The minced testes were then eluted with reduced medium (30 ml) and the extraction completed within 30-60 minutes. When the yield of treponemes was very low or the testicular tissue haemorrhagic or both, the treponemes were not

used, since they were often found to be of low viability.

ANAEROBIC EXTRACTION

The slices of testicular tissue were placed in a bottle containing reduced medium (30-50 ml) and then flushed with sterile oxygen-free nitrogen. The treponemes were eluted by gentle agitation for 30-60 minutes. The extract was then separated from the testicular tissue and counts made in a bacterial counting chamber by darkfield microscopy. The treponemes obtained by the two extraction methods were adjusted to the same concentration before inoculation into media.

A previously published medium,[1] with 2 mmol/l dithiothreitol instead of sodium thioglycollate was used. The final pH was $7 \cdot 3 \pm 0 \cdot 1$, the redox potential -350 ± 50 mV (Ecal), and the incubation temperature 34°C. Anaerobic and microaerophilic incubation have been described.[1]

Percentage motility based on counts of 50-100 treponemes by darkfield microscopy and virulence in rabbits, as shown by the latent period before lesions appeared after intradermal inoculation,[1] were used for determining the viability of *T pallidum* in vitro. Student's *t* test was used to compare the differences between the data.

Results

COMPARISON OF EXTRACTION METHODS ON RETENTION OF VIABILITY AFTER MICROAEROPHILIC INCUBATION

No difference was detected between anaerobically and aerobically extracted treponemes after 24 and 48 hours' incubation. Both the percentage motilities of the cultures and their latent periods for lesion development in rabbits were similar (data not shown). However, significant differences were observed after 72 and 96 hours' incubation in vitro (table I). The anaerobically extracted treponemes maintained significantly higher percentage motility and virulence (shorter latent periods) than aerobically extracted treponemes.

SURVIVAL OF ANAEROBICALLY EXTRACTED TREPONEMES AFTER MICROAEROPHILIC INCUBATION

After anaerobic extraction treponemes were maintained under anaerobiosis for different periods (0, 12, 24, 36, and 48 hours) before incubation in 3% oxygen in nitrogen. The controls were treponemes incubated either anaerobically or under 3% oxygen. After 12 or 24 hours' anaerobic incubation, followed by microaerophilic incubation, there was no significant difference in treponemal survival compared with either control group. However, treponemes held for 36 or 48 hours anaerobically and then exposed to 3% oxygen survived significantly longer, as measured by percentage motility (table II), than those exposed to constant 3% oxygen when sampled at 60, 72, 96, and 120 hours after harvesting. The latent periods of lesion formation in rabbits from treponemes incubated anaerobically for 36 or 48 hours were also significantly shorter than those from treponemes incubated under constant 3% oxygen (table III). Treponemal survival was not significantly enhanced compared with those in the constant anaerobic control group. Treponemes usually maintained viability longer under constant anaerobic conditions than under constant microaerophilic (3% O_2) conditions during prolonged incubation in vitro (tables II and III).

Discussion

The anaerobically extracted treponemes survived longer in vitro than those extracted aerobically, suggesting that *T pallidum* is sensitive to oxygen during the extraction process. Oxygen-derived species (superoxide free radical, hydroxyl free radical, singlet oxygen, and hydrogen peroxide) may act directly or indirectly on: (a) the DNA synthetic complex,[10] the treponemes possibly having lost the ability to initiate or continue DNA synthesis in vitro; (b) the extracellular layer,[11] resulting in loss of virulence in vitro; or (c) the membranes and enzymes,[12] causing a loss of biochemical function in vitro.

TABLE I *Comparison of the effect of anaerobic and aerobic extraction of T pallidum from infected tissue on the survival of motile and virulent treponemes after incubation in vitro*

	% Motility* (±SD) after extraction:			Latent period† (days±SD) after extraction:			
Incubation period (hours)	*Anaerobic*	*Aerobic*	*P value*	*Experiment*	*Anaerobic*	*Aerobic*	*P value*
72	55·2±7·6	28·0±13·6	<0·01	1	8·5±1·1	12·1±1·0	<0·001
				2	14·5±0·8	17·3±1·0	<0·001
96	24·3±11·9	6·0±2·8	<0·05	1	15·7±0·8	20·0±0	<0·001
				2	16·6±0·5	18·0±0·8	<0·02

*Mean of counts of 200-600 treponemes in 4-6 samples (50-100 treponemes/sample) from two independent experiments.
†Time between rabbit inoculation and appearance of lesions (mean of 10 lesions for each experiment).
P = probability; P<0·05 is significant (Student's *t* test).

TABLE II *Effect of 36 or 48 hours' anaerobic incubation followed by incubation under 3% oxygen on the viability of T pallidum as measured by the retention of motility*

	% Motility† (±SD) after different incubation periods in vitro (h):							
	Experiment 1			Experiment 2			Experiment 3	
Incubation conditions	72	96	120	72	96	120	60	72
Constant 3% O_2 (control)	56±4	29±5	20±1	50±4	35±2	20±3	11±1	6±4
Anaerobic (36 h)*	74±1 P<0·001	57±4 P<0·001	52±1 P<0·001	79±1 P<0·001	66±4 P<0·001	54±2 P<0·001	56±0 P<0·001	45±0·3 P<0·005
Anaerobic (48 h)*	66±4 P<0·01	63±4 P<0·001	57±3 P<0·001	74±1 P<0·001	62±3 P<0·001	47±4 P<0·001	45±2 P<0·005	36±6 P<0·05
Constant anaerobic (control)	64±4 P<0·05	34±5 NS	28±3 P<0·005	70±1 P<0·001	63±3 P<0·001	44±5 P<0·001	43±4 P<0·01	29±2 P<0·02

* *T pallidum* incubated anaerobically and then exposed to 3% O_2 at 34°C in vitro.
†Mean of counts of 100-300 treponemes in 2 or 3 samples (50-100 treponemes/sample) from duplicate or triplicate culture tubes.
P = probability; P<0·05 is significant (Student's *t* test). NS = not significant.

During aerobic extraction, the minced rabbit testes containing the treponemes were directly exposed to atmospheric oxygen. *T pallidum* appears to lack the means of detoxifying oxygen-derived species (unpublished data), thus possibly explaining the more rapid decrease in motility and virulence in vitro compared with anaerobically extracted treponemes (table I). The longer survival of the anaerobically extracted treponemes may have been due to: (a) the prevention of the direct reaction of oxygen with key cellular components of *T pallidum;* (b) a slowing of the rate of production of oxygen-derived free radicals from the medium components; or (c) prevention of a possible oxygen-mediated autolysis of host tissue cells.

Anaerobically harvested treponemes incubated anaerobically for 36 and 48 hours before being exposed to 3% oxygen survived longer than treponemes incubated under constant 3% oxygen (tables II and III) but not longer than the control group under constant anaerobic incubation. This may suggest that *T pallidum* is more sensitive to oxygen during early incubation in vitro.

It is assumed that treponemes which are freshly isolated from the rabbit host are metabolically highly competent. Their endogenous metabolism is presumably very high. A flavoprotein-based energy metabolism, which they are presumed to have,[13] would result in the production of large amounts of hydrogen peroxide.[14] This may explain their high sensitivity to oxygen shortly after extraction from tissue even in the presence of reducing agents. What the oxygen requirements are inside the host and how *T pallidum* avoids the toxicity of oxygen-generated reduction products are as yet unknown. In a recent report of growth of the organism in tissue culture[6] the oxygen concentration was 1·5%. Oxygen may play a very important role in the survival and growth

TABLE III *Effect of 36 or 48 hours' anaerobic incubation followed by incubation under 3% oxygen on the viability of T pallidum as measured by the retention of virulence (latent period of infection)*

	Latent period† (days ± SD) of infection after inoculation with treponemes incubated for (h):							
	Experiment 1			Experiment 2			Experiment 3	
Incubation conditions	72	96	120	72	96	120	60	72
Constant 3% O_2 (control)	7·3±0·5	11·4±0·5	13·5±0·7	10·4±1·4	14·6±1·0	17·0±0	12·6±1·1	11·2±1·4
Anaerobic (36 h)*	5·8±0·6 P<0·001	9·2±0·4 P<0·001	11·4±0·7 P<0·001	8·4±1·1 P<0·001	12·6±1·0 P<0·001	15·2±0·6 P<0·001	10·7±0·9 P<0·005	9·6±0·5 P<0·005
Anaerobic (48 h)*	6·3±0·7 P<0·01	9·6±0·5 P<0·001	11·6±0·7 P<0·001	9·1±1·4 P<0·05	12·6±0·9 P<0·001	15·5±0·5 P<0·001	10·5±1·8 P<0·01	9·8±0·8 P<0·025
Constant anaerobic (control)	6·8±1·6 NS	11·1±0·7 NS	13·0±0·7 NS	9·2±1·6 NS	12·8±1·1 P<0·001	15·8±0·6 P<0·001	11·0±1·5 P<0·005	9·4±0·5 P<0·005

* *T pallidum* incubated anaerobically and then exposed to 3% O_2 at 34°C in vitro.
†Time between inoculation of rabbits and appearance of lesions (mean of 10-12 lesions).
P = probability; P<0·05 is significant (Student's *t* test). NS = not significant.

of *T pallidum* in vitro,[3 4] but the sensitivity of *T pallidum* to oxygen during extraction from tissue and early incubation in vitro is also very important. Methods used to reduce oxygen toxicity during incubation in vitro may further improve the survival or growth, or both, of *T pallidum* in vitro.

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