The role of respiratory protection on increased survival of *Treponema pallidum* (Nichols) when cocultivated with mammalian cells *in vitro*

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The ability of mammalian cells in tissue culture to protect against oxygen toxicity for *Treponema pallidum* was examined. Addition of catalase to the incubation medium enhanced *T. pallidum* survival when co-incubation was carried out under aerobic conditions. When co-incubation was carried out under 3% oxygen, catalase had no enhancing effect on survival despite the fact it was still highly stimulatory when *T. pallidum* was incubated under 3% oxygen in the same medium with no tissue culture cells present. Inactivation of the catalase present endogenously in the mammalian cells by the addition of the catalase inhibitor 3-amino-1,2,4-triazole largely eliminated the enhancing effect of mammalian cells on the survival of *T. pallidum* under 3% oxygen. Increasing the oxygen consumption of the host mammalian cells with 0.1 m*M* 2,4-dinitrophenol enhanced *T. pallidum* under both aerobic and microaerobic conditions; a much greater effect was seen under aerobic conditions. The results indicated that mammalian cells offer significant protection against toxic oxygen reduction products for *T. pallidum in vitro* under microaerobic conditions.

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On a étudié la capacité de cellules de mammifères en culture de tissus à protéger *Treponema pallidum* contre l'effet toxique de l'oxygène. L'addition de catalase au milieu d'incubation augmente la survie de *T. pallidum* lorsque cette co-incubation est faite dans des conditions d'anaérobiose. Lorsque l'incubation se fait en présence de 3% d'oxygène, la catalase n'a pas d'effet favorisant la survie malgré le fait qu'elle soit encore fortement stimulante lorsque *T. pallidum* est incubé en présence de 3% d'oxygène dans le même milieu en absence de cellules de la culture de tissus. Lorsque la catalase présente de façon endogène dans les cellules de mammifères est inactivée par addition d'un inhibiteur de la catalase, le 3-amino-1,2,4-triazole, il y a inactivation de cette propriété des cellules de mammifères d'augmenter la survie de *T. pallidum* en présence de 3% d'oxygène. Le fait d'augmenter la consommation d'oxygène chez les cellules de mammifères avec du 2,4-dinitrophénol 0.1 m*M* favorise *T. pallidum* dans des conditions tant d'aérobiose que de microaérobiose. Ces résultats indiquent que, pour *T. pallidum in vitro* dans des conditions de microaérobiose, les cellules de mammifères offrent une protection significative contre l'effet toxique de produits de réduction de l'oxygène.

[Traduit par le journal]

Introduction

Recent success in achieving limited growth of *Treponema pallidum* in tissue culture has emphasized the obligate parasitic nature of *T. pallidum* (Fieldsteel *et al.* 1981, 1982). One possible way mammalian cells in tissue culture could promote *T. pallidum* survival is by acting to detoxify reduction products of oxygen which are formed in normal aerobic respiration. Reducing agents are known to be required for survival in a cell-free system (Nelson 1948; Weber 1960; Norris *et al.* 1978) and for survival and replication in tissue culture (Fitzgerald *et al.* 1977; Fieldsteel *et al.* 1982). Reducing agents may be acting to remove toxic oxygen products like hydrogen peroxide, which previous work in our laboratory has shown to be a major contributor to oxygen toxicity for *T. pallidum* (Steiner *et al.* 1984). Mammalian cells may function to carry out this same role *in vivo* and in tissue culture (Fitzgerald *et al.* 1976; Fieldsteel *et al.* 1977). In this paper we report on the ability of mammalian cells in tissue culture to protect *T. pallidum* against oxygen related loss of viability.

Materials and methods

Maintenance and harvesting of T. pallidum

Treponema pallidum was maintained by intratesticular inoculation in adult rabbits. Inflammatory reactions were reduced by injections of 4 mg of methylprednisolone acetate per kilogram body weight on days 2 and 9 after injection with *T. pallidum* (Brause *et al.* 1979). Rabbits were sacrificed by intravenous injection of 3 mL of sodium pentobarbitone (200 mg/mL) at 10–12 days after injection when orchitis was maximal. The testes were removed aseptically, minced, and

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placed in approximately 50 mL of Eagle's minimal essential medium (EMEM) with 10% fetal calf serum (FCS) and 10 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and shaken for 20 min. The suspension was centrifuged at 1000 × *g* for 10 min and the supernatant containing approximately 3 × 10^7 treponemes per millilitre, used as the inoculum.

Tissue culture techniques

Two mammalian cell types were used. Both were composed of cells which were of a large and regular shape making reproducible counting of attached treponemes possible. They were a rabbit testis cell culture (RT) isolated in this laboratory by the method of Fitzgerald *et al.* (1975) and a human skin tumor, C76/203, a gift from Professor E. Pihl, Pathology Department, Monash University. The rabbit testis cell culture was isolated from adult rabbit testes. The testes were cut, minced, and stirred in phosphate-buffered saline (PBS) containing 0.025% trypsin, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C for 30 min. The cells were collected by centrifugation at 1000 × *g* for 10 min and resuspended in EMEM with 20% FCS and 10 m*M* HEPES and antibiotics as above. Once the cell culture was established, it was maintained in EMEM supplemented with 10% FCS and 10 m*M* HEPES without antibiotics. The passage medium was used for experiments with both cell cultures. Studies on survival of *T. pallidum* in tissue culture were done in Leighton tubes containing glass cover slips. Mammalian cells were grown to approximately 50% confluence before the start of the experiment. The tissue cells were washed twice with medium before inoculation with *T. pallidum*. The treponemal inoculum consisted of an extract of syphilitic, orchitic rabbit testes which had been centrifuged at 1000 × *g* to remove rabbit tissue cells. Reducing agents are generally used in *T. pallidum* survival experiments. When used appropriately they extend survival by as much as 10-fold or greater (Norris *et al.* 1978). Since they probably cause this enhancement of *T. pallidum in vitro* survival by protecting against oxygen toxicity, they were excluded from all of the experiments reported here. Before addition of the treponemes to the mammalian cell cultures, the extract was divided into fractions and the appropriate amount of the filter-sterilized (0.45 μm, Amicon microporous filters) reagent being tested was added. Previous experiments had shown that 200–400 units/mL catalase gave optimal protection when *T. pallidum* was incubated under aerobic conditions without mammalian cells. Thus all experiments with catalase were performed with 400 units/mL of the enzyme in the medium. 3-Amino-1,2,4-triazole was used at 40 m*M* since this level gives an optimal inhibition of catalase (Margoliash *et al.* 1960). Assays performed on the cells and medium when known amounts of catalase were added indicated that 40 m*M* 3-amino-1,2,4-triazole inhibited catalase activity by 80–85% within the first 45 min after addition of the inhibitor. 2,4-Dinitrophenol was used at 0.1 m*M*. This level of the drug has been shown to allow growth of several *Bacteroides* species in tissue culture without killing the host mammalian cells (Matsuyama *et al.* 1982). Final concentrations of *T. pallidum* were adjusted to approximately 3 × 10^7/mL before the start of the experiment. Once the tissue cultures had been inoculated with *T. pallidum*, they were co-incubated at 34°C, either aerobically or microaerobically (3% oxygen) in a sealed hood (Steiner *et al.* 1981). Tubes were removed at the prescribed times and the survival of *T. pallidum* was determined.

Survival of T. pallidum

Survival of *T. pallidum* was measured by determining the percentage motility of the treponemes in the culture, and in some experiments, the virulence for rabbits measured by intradermal injection as previously described (Steiner *et al.* 1981). To obtain treponemes for virulence determinations attached treponemes and tissue cells were removed from the glass by gently scraping with a rubber policeman. The suspensions were pipetted several times to break the cell clumps and allow treponemes to detach from the tissue cells. This process was monitored by dark-field microscopy. The suspensions were then centrifuged at 1000 × *g* for 10 min to remove the tissue cells. The treponeme-containing supernatants (containing the treponemes which were either free in the medium or attached to tissue cells before the removal of the tissue cells) were then injected into four sites on the backs of two rabbits for each determination of virulence. Separate determinations were not performed on the attached and free treponemes because of difficulties arising from the large difference between attached and free treponemes (generally greater than 95% of the treponemes were unattached). To avoid biasing of results from dilution procedures, the virulence of the entire culture was determined instead.

Rabbits showed no inflammatory reaction to the supernatant without treponemes from the tissue culture controls. Motility of both unattached treponemes in the culture supernatant and treponemes attached to tissue cells were determined. At least 20 mammalian cells with attached treponemes were counted per sample.

Chemicals

Twice crystallized catalase (30 000 units/mg) and 3-amino-1,2,4-triazole were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. 2,4-Dinitrophenol was purchased from British Drug House (BDH). Tissue culture components were purchased from Gibco or C.S.L. (Commonwealth Serum Laboratories, Melbourne, Australia). All other chemicals were purchased from Sigma or BDH and were of analytical grade or higher.

Results

Effect of catalase on T. pallidum *survival and attachment in tissue culture*

Under aerobic conditions, catalase showed a stimulatory effect on survival of *T. pallidum* in tissue culture. This effect was seen with both the rabbit testis cell culture and human skin tumor C76/203 (Fig. 1). The addition of catalase had no effect on the level of *T. pallidum* attachment to mammalian cells (data not shown); the number of treponemes attached per tissue culture cell was dependent only on the concentration of the treponemal inoculum.

Under microaerophilic conditions (3% oxygen atmosphere), the enhancing effect of catalase was much less and not statistically significant when measured by

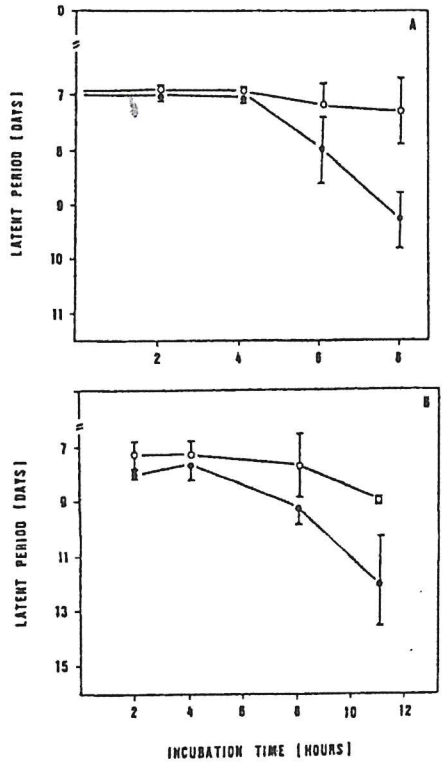

FIG. 1. Effect of catalase (400 units/mL) on the survival of *T. pallidum* co-incubated with (A) RT cells; and (B) C76/203 cells; under aerobic conditions. Survival was measured by virulence for rabbits expressed as latent period for infection leading to formation of syphilitic lesions. ○, Catalase present in medium used to co-incubate *T. pallidum* with mammalian cells; ●, no catalase present in medium. Medium was $EMEM_{10}$ with 10 m*M* HEPES.

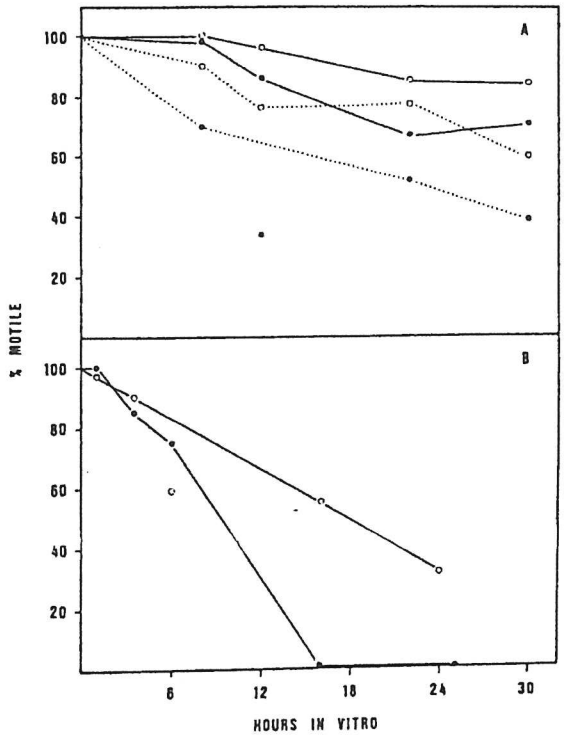

FIG. 2. The effect of catalase on the survival of *T. pallidum* under microaerobic (3% oxygen) conditions. (A) Effect of 400 units/mL of catalase on motility of *T. pallidum* incubated in the presence of rabbit testes cells *in vitro*. ●———●, Motility of *T. pallidum* attached to rabbit testes cells, no catalase present in medium; ●·····●, motility of *T. pallidum* free in medium, no catalase present in medium; ○———○, motility of *T. pallidum* attached to rabbit testes cells, 400 units/mL catalase in medium; ○·····○, motility of treponemes free in medium, 400 units/mL catalase in medium. (B) Effect of 200 units/mL catalase on motility of *T. pallidum* in the same medium without rabbit testes cells present. ●———●, No catalase in medium; and ○———○, 200 units/mL catalase in medium.

virulence. In comparison, catalase was still stimulatory to the survival of *T. pallidum* in a cell-free system under 3% oxygen. The results of an experiment comparing the effects of catalase in cell-free versus tissue culture systems are shown in Fig. 2. Whereas catalase doubled the time until 50% of the culture was immotile (MT_{50}) in a cell-free system, it had no effect when the treponemes were co-incubated with rabbit testes cells *in vitro* (Figs. 2A, 2B). These experiments were performed with rabbit testes cells; similar results were obtained when C76/203 cells were used (Fig. 3). These results suggest that the tissue cells are providing the protective effect against hydrogen peroxide toxicity that catalase supplies in a cell-free system. Other factors are probably also involved since survival is at least twice as long in the presence of tissue cells as with catalase alone. Survival of *T. pallidum* under a 3% oxygen atmosphere varies from two to five times as long as under 21% oxygen as measured by virulence or motility (Figs. 4 and 6).

The catalase inhibitor 3-amino-1,2,4-triazole had little effect on the survival of *T. pallidum* under aerobic conditions when cocultivated with C76/203 cells (Fig. 5A). It did not effect the treponemes in the absence of mammalian cells *in vitro*. Under microaerobic conditions (3% oxygen), the abrogation of catalase activity was detrimental to *T. pallidum* (Fig. 5B). In the presence of 3-amino-1,2,4-triazole under 3% oxygen, attached treponemes did not survive appreciably longer than unattached treponemes in the control culture (Fig. 5B).

Effect of 2,4-dinitrophenol on the survival of T. pallidum *in the presence of mammalian cells* in vitro

2,4-Dinitrophenol is a respiratory uncoupler which increases the consumption of oxygen by mammalian

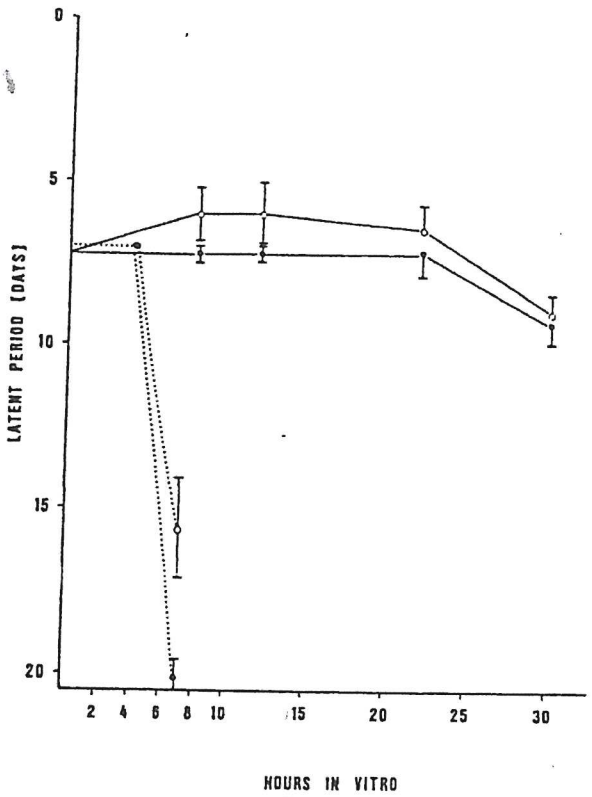

FIG. 3. Effect of catalase on the *in vitro* survival of *T. pallidum* under microaerobic conditions (3% oxygen). Survival was measured by virulence for rabbits expressed as latent period for infection leading to formation of syphilitic lesions. ●———●, Control, no catalase co-incubated with C76/203 cells; O———O, 400 units/mL catalase in medium co-incubated with C76/203 cells; ●·····●, control, no catalase in cell-free system; and O·····O, 200 units/mL catalase in medium, cell-free system.

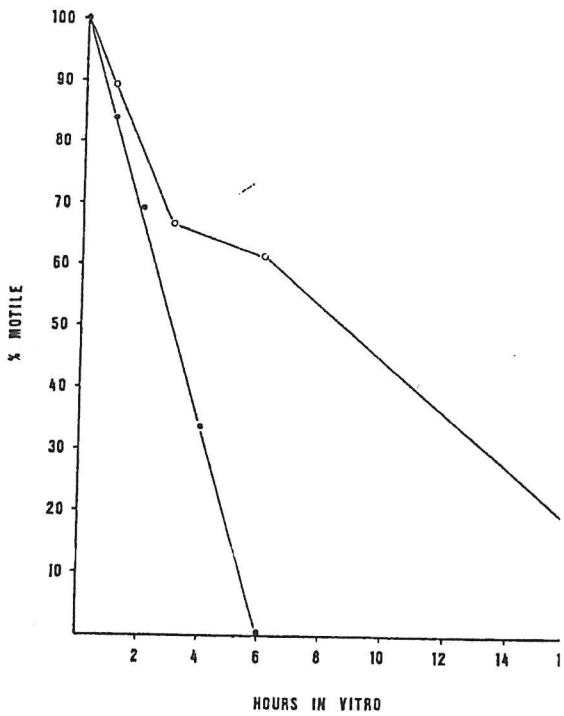

FIG. 4. Comparison of the survival of *T. pallidum* in mammalian cell-free system when incubated under aerob conditions (21% oxygen) and under microaerobic conditio (3% oxygen). Survival was monitored by determining tl motility of the *T. pallidum*. ●, Motility of *T. pallidu* cultures under aerobic conditions; O, motility of *T. pallidu* cultures under microaerobic conditions.

cells. When C76/203 cells were treated with 0.1 m*M* 2,4-dinitrophenol under aerobic conditions, *T. pallidum* survived much longer in their presence than in the presence of untreated cells (Fig. 6A). Several experiments were also run with the baby hamster kidney cell line (BHK). 2,4-Dinitrophenol showed no enhancing effect with this cell line. Either the drug or the treponemes appeared to be toxic for BHK cells as they became heavily vaculated shortly after addition of the two. The same toxic effect was observed in the culture in which only treponemes were added indicating the effect was due to treponemal toxicity. We and others have observed the toxic effect of high concentrations of *T. pallidum* for mammalian cells previously (Fitzgerald *et al.* 1982; Oakes *et al.* 1982; Wong *et al.* 1983). Further experiments were done exclusively with the C76/203 cell line. The drug had no enhancing effect on *T. pallidum* survival when tissue culture cells were not present under aerobic or microaerobic conditions; i cell-free culture treponemes in several experiment actually lost motility more rapidly in the presence of th drug. As can be seen in Fig. 6, survival of *T. pallidur* was much shorter in a mammalian cell-free medium; con trol and 2,4-dinitrophenol-containing cultures differe by 5% or less when mammalian cells were absent fron the medium. Under microaerobic conditions (3% oxygen in the presence of C76/203 cells, 2,4-dinitrophenol alsc enhanced survival; the drug lengthened survival in th treated cultures (Fig. 6A). The effect of 2,4-dinitropheno was far less under 3% oxygen than under 21% oxyger (compare Fig. 6A with Fig. 6B).

Discussion

In previous work we have found that *T. pallidum* is very sensitive to hydrogen peroxide and probably also tc the hydroxyl radical and singlet oxygen (Steiner *et al.* 1984). One of many possible reasons for the dependence of *T. pallidum* on mammalian cells may be to protect it from reactive oxygen species. We tested this hypothesis in several ways. We looked at the effect of exogenous catalase on the survival of *T. pallidum* in tissue culture. Under

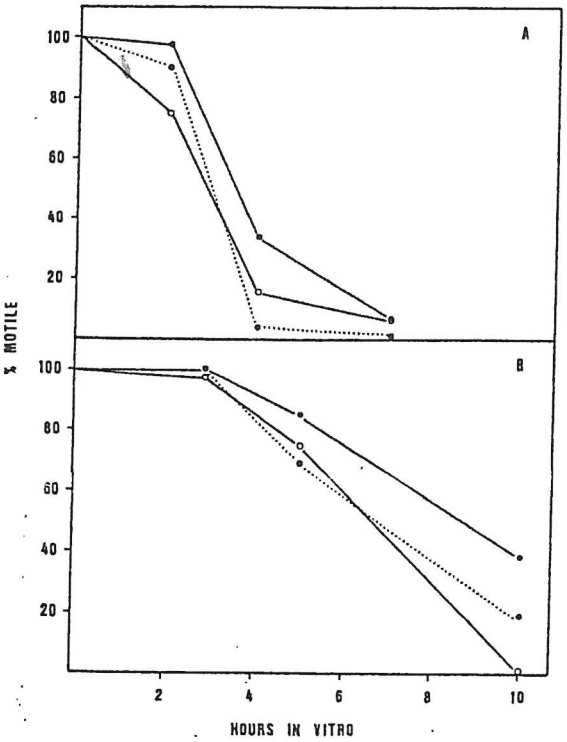

FIG. 5. Effect of 3-amino-1,2,4-triazole on the survival of *T. pallidum* cocultivated in the presence of C76/203 cells. 3-Amino-1,2,4-triazole was present at 40 m*M* in the experimental samples. ●———●, Motility of treponemes attached to C76/203 cells, no 3-amino-1,2,4-triazole in medium; O———O, motility of treponemes attached to C76/203 cells in the presence of 3-amino-1,2,4-triazole; and ●·····●, motility of *T. pallidum* found free in the medium in the control culture. (A) Under aerobic conditions: (B) under microaerobic conditions (3% oxygen).

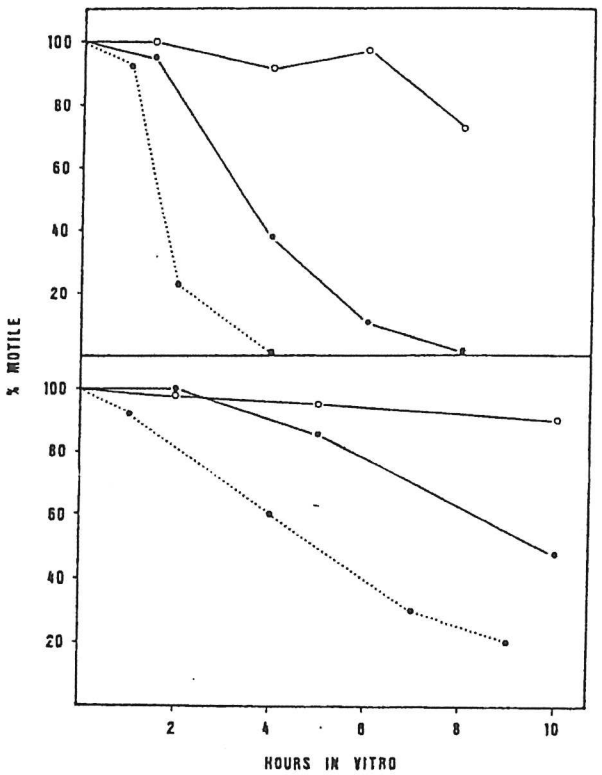

FIG. 6. Effect of 0.1 m*M* 2,4-dinitrophenol (2,4-DNP) on survival of *T. pallidum* cocultivated with C76/203 cells. ●———●, Motility of treponemes attached to C76/203 cells, no 2,4-DNP in medium; O———O, motility of treponemes attached to C76/203 cells with 0.1 m*M* 2,4-DNP in medium; ●·····●, motility of treponemes in a cell-free system, with or without 2,4-DNP present in medium. (A) Under aerobic conditions; (B) under microaerobic conditions (3% oxygen).

aerobic conditions a clear enhancement of treponemal survival was seen with catalase with both cell cultures tested (Fig. 1). Under microaerobic conditions the enhancing effect was slight or absent in the presence of mammalian cells but was still clearly present in an acellular medium (Figs. 3 and 4). Abrogation of endogenous catalase activity in the mammalian cells compromised their ability to protect *T. pallidum* under microaerobic conditions (Fig. 5). Increasing the basal oxygen consumption of the mammalian cells by addition of 2,4-dinitrophenol enhanced their ability to support *T. pallidum* survival under aerobic and to a lesser extent under 3% oxygen (Fig. 6). From these results we conclude that mammalian cells can protect *T. pallidum* from oxygen toxicity *in vitro*, especially under 3% oxygen. At atmospheric levels of oxygen, toxicity was too high to be relieved by the mammalian cells and experiments with catalase indicated that hydrogen peroxide, or products derived from it, are at least partly responsible for the oxygen toxicity for *T. pallidum* seen under aerobic conditions.

Possible limitations on the ability of tissue cells in culture to maintain *T. pallidum* viability may relate to the location of *T. pallidum in vitro* in comparison to *in vivo*. *In vivo T. pallidum* penetrates the ground substance between cells (Fitzgerald *et al.* 1979; Baker-Zander and Sell 1980) which may act to regulate oxygen diffusion. In a tissue culture system the treponemes are on the cell surface and directly exposed to the liquid environment, with the exception of a few that achieve intracellularity (Fitzgerald *et al.* 1975).

There are limitations to the detoxifying potential of catalase added to the medium and to the protection afforded by the intracellular catalase of mammalian cells. The major limitation is the enzyme's inability to penetrate the treponemal cell and detoxify internally generated hydrogen peroxide. Internally produced hydrogen peroxide may explain two well-known attri-

butes of *T. pallidum in vitro*: the requirement for low oxygen tensions and the presence of a reducing agent such as dithiothreitol (Norris *et al.* 1978; Fieldsteel *et al.* 1981). Fitzgerald *et al.* (1980) offered evidence that dithiothreitol was taken up by *T. pallidum in vitro*; thus it could detoxify internally generated hydrogen peroxide. Reducing agents can also function as hydrogen donors to organic radicals (Howard-Flanders 1960). Their ability to prevent strand breakage of DNA by reducing radicals formed by ionizing radiation is well documented (Ginsberg and Webster 1969; Lohman *et al.* 1970; Sawada and Okada 1970). Recent work in our laboratory has shown that low doses of hydrogen peroxide can cause single-stranded breakage in the DNA of *T. pallidum* (B. Steiner, G. H. W. Wong, P. Sutrave, and S. Graves, unpublished data). The requirement for reducing agents in the *in vitro* survival of *T. pallidum* may be due to their ability to reduce organic radicals and prevent DNA breakage or damage to other *T. pallidum* macromolecules.

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