The Effect of Reducing and Other Agents on the Motility of *Treponema pallidum* in an Acellular Culture Medium

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The maintenance of *Treponema pallidum* motility was investigated in an acellular medium based on *T. pallidum* immobilization test medium. The acellular medium contained cysteine, glutathione, thioglycollate and dithiothreitol as reducing agents and had a redox potential of -275 ± 25 mV at pH 7.3. In an atmosphere containing 3% O₂, motile treponemes survived four times longer when calf serum and bovine serum albumin were added to the medium. The selective omission of glutathione and, particularly, thioglycollate prolonged the survival of motile treponemes almost fivefold. In addition, stored medium, in which thioglycollate had become inactive, sustained motile treponemes for longer than did freshly prepared medium. Thus, thioglycollate is toxic for the organisms. It may be omitted from the medium because low redox potentials can be achieved without it.

INTRODUCTION

The effect of reducing agents on the motility of *Treponema pallidum* *in vitro* has been documented by various workers. Healthy human tissue *in vivo* in the presence of dissolved oxygen has a redox potential (E_h) of -40 mV at pH 7.2 (Shapiro, 1972). It may be assumed that as treponemal infection progresses to cause tissue necrosis, the E_h value will diminish with the development of optimal conditions for organism survival. Media with various E_h values, ranging from -10 to -408 mV, have been used for treponemal maintenance *in vitro* (Metzger & Smogór, 1966; Graves *et al.*, 1975; Sandok *et al.*, 1976), those with intermediate values apparently being the most successful. Freshly prepared medium used for the *T. pallidum* immobilization (TPI) test (Wilkinson *et al.*, 1972; Horvath & Etelka, 1973) has a redox potential of -210 ± 20 mV at pH 7.3 when finally diluted with complement and serum. It is difficult, however, to prepare media with low E_h values even by removing all traces of oxygen, and, as in the case of the TPI test medium, reducing agents have to be added – cysteine, thioglycollate and glutathione have been mainly used for this purpose (Graves *et al.*, 1975; Sandok *et al.*, 1976, 1978). In addition, dithiothreitol is known to keep these agents in a reduced state and has been found useful in maintaining treponemal motility (Fitzgerald *et al.*, 1977). We wondered, however, whether all the reducing agents were necessary for maintaining a low E_h value and whether some might even be toxic for the organisms, by virtue of disulphide groups released by oxidation. We therefore compared media which contained different reducing agents known to produce low redox potentials for their ability to maintain treponemal motility.

METHODS

Glassware. 'Universal' 20 ml screw-capped glass bottles were used as culture vessels. They were rinsed in a siliconizing solution of Repelcote (Hopkin & Williams) for 30 s to prevent attachment of the organisms to the glass, washed three times in distilled water and autoclaved for 15 min at 121 °C.

Medium. Double-strength medium was prepared as follows: to a 200 ml solution of five times concentrated Eagle's MEM and Earle's salts (Gibco Bio-Cult) was added L-glutamine (292 mg), N-2-hydroxyethyl-piperazine-N'-ethanesulphonic acid (HEPES, 7.14 g), cocarboxylase (4 mg), adenine (14 mg), cytosine (14 mg), uracil (14 mg), choline chloride (80 mg), 2-aminoethanol (0.08 ml) and fructose (1 g), all obtained from Sigma; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (200 mg), glucose (4 g), sodium pyruvate (120 mg), ascorbic acid (50 mg), KCl (260 mg), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (100 mg) and KH_2PO_4 (110 mg), all from BDH; $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (0.6 mg), from Hopkin & Williams; lactose (1 g), from Edward Gurr; gentamicin sulphate (100 mg), from Roussel Laboratories; MEM non-essential amino acids (100-fold concentrated solution, 30 ml), BME amino acids (100-fold concentrated solution, 10 ml) and MEM vitamins (100-fold concentrated solution, 10 ml) all from Gibco Bio-Cult; 1 M-NaOH (20 ml), from May & Baker. This solution was diluted to 500 ml with 130 ml of distilled deionized water prepared for injection (Antigen, Ireland) to produce basic double-strength medium. This was sterilized by filtration and stored at 4 °C.

Four reducing agents were used: L-cysteine. HCl, stored at room temperature, and glutathione (reduced), stored at 4 °C, were from BDH; sodium thioglycollate (batch nos. 66C-0120; 77C-0074), stored desiccated at -20 °C, and dithiothreitol, stored desiccated at 4 °C, were from Sigma. Each was dissolved in distilled water, sterilized by filtration and stored at -20 °C. To 5 ml of double strength medium in culture vessels was added 0.1 ml of each reducing agent as follows: L-cysteine. HCl, 120 mg ml⁻¹; glutathione, 120 mg ml⁻¹; thioglycollate, 200 mg ml⁻¹; dithiothreitol, 30 mg ml⁻¹. [In some experiments, not all of these reducing agents were added, as indicated in the text. Furthermore, foetal calf serum (FCS; Flow Laboratories) and bovine serum albumin (BSA; Armour Pharmaceutical Co.) were sometimes added.] The medium was then diluted to 9 ml with distilled deionized water, to produce complete medium, before inoculation of the treponemal suspension.

Preparation of *Treponema pallidum* inoculum. New Zealand White rabbits (2.5 to 3.0 kg) were anaesthetized with pentobarbitone given intravenously (1 mg per 33 g body weight). They were inoculated intratesticularly with viable *T. pallidum* organisms (Nichols virulent strain) and then intramuscularly with 0.2 ml methylprednisolone acetate (40 mg ml⁻¹). Seven d later both testes were removed aseptically, incised longitudinally, and rinsed twice with 5 ml of complete medium without thioglycollate in a 250 ml conical flask. After adding 10 ml of the same medium, the flask was flushed with 1 l of a mixture of 95 % N₂ and 5 % CO₂ (BOC, London), sealed and shaken for 1 h on a Griffin flask shaker at 22 °C. The suspension was then centrifuged at 450 g for 10 min at 22 °C and the supernatant medium was withdrawn; it contained a total of 3 × 10⁸ to 5 × 10⁸ treponemes ml⁻¹ and not more than 10⁸ tissue cells ml⁻¹. This concentration of treponemes was used in all experiments.

Measurement of redox potential. Redox potentials (E_h) were measured with a combination platinum electrode containing 3.8 M-KCl solution (EIL model 1144) connected to a pH meter (Kent pH meter 7010, EIL).

Estimation of number of treponemes. After gentle mixing, medium which contained treponemes was introduced into a Thoma-Weber dark-ground counting chamber. The number of motile and non-motile organisms in 25 squares, each containing 16 smaller squares, was counted to estimate the number of organisms ml⁻¹, and the number of motile organisms was expressed as a percentage of the total number of organisms. The times (T), measured from the start of an experiment, for survival (S) of 50 % of motile treponemes (ST₅₀) and 10 % of motile treponemes (ST₁₀) were determined.

Experimental procedure. Culture vessels containing 9 ml of complete medium were placed in Fildes-MacIntosh anaerobiosis jars. These were evacuated and filled twice with a mixture of 95 % N₂ and 5 % CO₂ and kept at 33 °C overnight. The pH of the medium was then readjusted to 7.3 with 1 M-NaOH and the E_h value was measured; invariably this was found to be -260 ± 10 mV. Treponemal suspension (1 ml) was added to the pre-reduced medium and the culture vessel was placed in an anaerobiosis jar. This was evacuated, filled twice with a mixture of 92 % N₂, 5 % CO₂ and 3 % O₂ (prepared by the Division of Anaesthesia, Clinical Research Centre) and incubated at 33 °C. Treponemal motility was monitored at intervals throughout an experiment and E_h values were determined at the beginning and end.

RESULTS

Effect of reducing agents on redox potential. In preliminary experiments, medium was divided into aliquots and various amounts of one or other of the three reducing agents normally found in TPI test medium were incorporated. The E_h values produced by the various reducing agents are shown in Fig. 1. Thioglycollate was less effective than cysteine or glutathione in producing a low redox potential (two different batches had similar effects); it was used at 2 mg ml⁻¹ in further experiments. Cysteine and glutathione produced

Fig. 1. Redox potentials produced by different concentrations of reducing agents: ○, sodium thioglycollate; ●, cysteine or glutathione.

Table 1. *Enhancement of treponemal motility by incorporation of serum additives into complete medium*

Treponemes were suspended at $3.6 \pm 0.5 \times 10^8$ ml⁻¹ in complete medium containing foetal calf serum (FCS) and bovine serum albumin (BSA) as indicated. The times from the start of the experiment at which 50% (ST₅₀) and 10% (ST₁₀) of the treponemes survived and remained motile were recorded.

Addition(s) to medium	ST ₅₀ (h)	ST ₁₀ (h)
None	11	21
FCS (10%)	15	50
FCS (10%)+BSA (0.5%)	16	80
FCS (20%)+BSA (0.5%)	52	98

similar E_h values; both these agents were used subsequently at 1.2 mg ml⁻¹. To keep these agents in a reduced form and maintain a low E_h value, dithiothreitol was added to the medium, initially at 0.3 mg ml⁻¹ and then daily at 0.15 mg ml⁻¹; this kept the redox potential at -275 ± 25 mV at pH 7.3.

Effect of serum and serum albumin on treponemal motility. The effect of adding 0.5% BSA and different concentrations of FCS to complete medium on the survival of motile treponemes was determined. The results (Table 1) indicate that the addition of 10% FCS increased the treponemal survival time, particularly the ST₁₀ which was more than doubled. The addition of 0.5% BSA to 10% FCS further increased the survival time and 20% FCS prolonged survival more than fourfold compared with that in medium without these additives.

Effect of reducing agents on treponemal motility. To test whether the reducing agents used in the complete medium were toxic, they were selectively omitted from medium containing 10% FCS and 0.5% BSA. As shown in Table 2, the motility of treponemes was prolonged when glutathione and, particularly, thioglycollate were omitted and a low E_h value was maintained with cysteine and dithiothreitol. Similar results were obtained with two different batches of thioglycollate and with treponemes from two different rabbits.

In addition, with only cysteine and dithiothreitol as reducing agents, the incorporation of 20% FCS had no greater enhancing effect on treponemal motility than 10% FCS. Thus, the ST₅₀ was 110 h in both cases, and the ST₁₀ was 141 h with 20% FCS and 144 h with 10% FCS.

Comparison of stored and freshly prepared media. Medium which contained 20% FCS,

Table 2. *Enhancement of treponemal motility by omission of various reducing agents from the medium*

The medium contained 10% foetal calf serum and 0.5% bovine serum albumin. One or more of the four reducing agents – L-cysteine.HCl (Cys), glutathione (GSH), thioglycollate (Thio) and dithiothreitol (DTT) – was omitted as indicated (–). ST₅₀ and ST₁₀ were determined as in Table 1.

Reducing agents present				ST ₅₀ (h)	ST ₁₀ (h)
Cys	GSH	Thio	DTT		
+	+	+	+	16	80
+	–	+	+	24	135
+	+	–	+	107	150
+	–	–	+	122	> 163

Fig. 2. Motility of *T. pallidum* in medium containing four reducing agents including thioglycollate: ○, medium stored at 4 °C for 10 d; ●, freshly prepared medium.

0.5% BSA and the four reducing agents was kept at 4 °C for 10 d. It was then compared with freshly prepared medium for its ability to maintain treponemal motility. As shown in Fig. 2, treponemal motility was maintained for a longer period in the stored medium, the ST₅₀ being 127 h compared with 40 h for fresh medium. It may be noted that the ST₅₀ in stored medium was similar to that in fresh medium from which thioglycollate had been omitted (Table 2).

DISCUSSION

In order to develop an acellular medium capable of supporting the motility of *T. pallidum* organisms for prolonged periods, we used the TPI test medium, which contains three reducing agents, as a guide. The possibility that not all the reducing agents were necessary was indicated by the results of preliminary experiments in which it was found that thioglycollate was less effective than either cysteine or glutathione in reducing the E_h value of the medium. It seemed, therefore, that thioglycollate might be omitted from the medium without adversely affecting treponemal survival. Indeed, omission of thioglycollate and, to a lesser extent, glutathione was not only compatible with maintaining a low redox potential but beneficial in that treponemal motility was enhanced. Thus, when thioglycollate was omitted, the ST₅₀ was increased more than fourfold, and when cysteine was used as the main reducing agent at a concentration of 2.4 mg ml⁻¹, the ST₅₀ was increased more than fivefold. Clearly, in the concentrations used in our experiments, thioglycollate is particularly toxic to the treponemes. This is not a chance phenomenon since it was observed with treponemes from various rabbits and with two different batches of thioglycollate. It is, therefore, difficult to reconcile with the observations of Sandok *et al.* (1976) who reported

that thioglycollate enhanced treponemal survival. However, their acellular medium was different from ours and did not contain dithiothreitol. In the absence of dithiothreitol, the thioglycollate may have been important in keeping a low E_h and so enhancing treponemal motility, toxicity being overcome by the ox serum ultrafiltrate used by Sandok and colleagues. This is supported by our finding that the toxicity of thioglycollate can be partially reversed by adding FCS and BSA.

Although the exact mechanism underlying toxicity is unknown, the long survival of motile treponemes in stored medium may indicate one of the factors responsible. In freshly prepared medium, thioglycollate becomes oxidized and we suggest that it is the released disulphide groups which are toxic to the organisms. During storage, the dithiothreitol reduces the disulphide groups and renders them harmless. Stored thioglycollate-containing medium compares favourably with fresh medium without thioglycollate in maintaining motility.

The main purpose of these experiments was to develop a medium in which the organisms would remain motile for a prolonged period. The studies were a prelude to more detailed investigations of the requirements for *in vitro* multiplication. We were therefore interested to observe that the length of treponemes increased two- to threefold after 30 h incubation in medium without thioglycollate, even though their numbers remained constant. It seems likely that the best way of inducing *T. pallidum* to multiply is to use tissue cultures or organ cultures supplemented by a simple acellular medium without thioglycollate.

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