Effect of four serum components on survival of *Treponema pallidum* and its attachment to rabbit cells in vitro

G H W WONG, B STEINER, AND S GRAVES

From the Department of Microbiology, Monash University, Clayton, Victoria, Australia

SUMMARY Transferrin and, to a lesser extent, insulin were two components of serum that enhanced the survival of *Treponema pallidum* in microaerophilic, acellular medium. These two components also enhanced the attachment of *T pallidum* to baby rabbit genital organ (BRGO) cells in both aerobic and microaerophilic tissue culture systems. Growth hormone and epidermal growth factor marginally enhanced the attachment of *T pallidum* to BRGO cells.

Introduction

Treponema pallidum rapidly attaches to mammalian cells in tissue culture, and this appears to be related to *T pallidum* pathogenesis.¹⁻³ Blockage of attachment by immune serum correlates with resistance to superinfection in syphilis.⁴ Modulation of host cell metabolism by changes in the concentration of serum during in vitro cultivation, or by the use of metabolic inhibitors, affects the relation between the parasite, *T pallidum*, and the host baby rabbit genital organ (BRGO) cells in vitro.⁵ In the report published here we examine the effect of four individual components of serum (transferrin, insulin, growth hormone, and epidermal growth factor) on the survival of *T pallidum* in vitro and on its attachment to BRGO cells.

Materials and methods

SOURCE OF *T PALLIDUM*

We propagated *T pallidum* (Nichols strain) in adult male rabbits as described previously.⁶ After the infected testes had been removed, they were sliced into pieces and extracted for 15-30 minutes in a 150 ml bottle containing 30-50 ml of medium under anaerobic conditions.⁷ The medium consisted of Eagle's minimum essential medium supplemented with 20% fetal calf serum, 10 mmol/l N-2-hydroxyethylpiperazine-N¹-2-ethanesulphonic acid (HEPES), and 0.5 mmol/l dithiothreitol. We then separated extracted treponemes from rabbit tissue cells by

centrifuging the mixture at $1000 \times g$ for 10 minutes. We measured the concentration of *T pallidum* by dark field microscopy using a bacterial counting chamber.

MEASURING MOTILITY RETENTION BY *T PALLIDUM*

We extracted *T pallidum* in serum free medium and added serum components as above. Incubation was microaerophilic at 34°C for 72 hours. *T pallidum* concentration was 7×10^6 /ml.

We examined 100 to 200 *T pallidum* from triplicate tubes at random by dark field microscopy, and recorded whether they were motile or non-motile.

TISSUE CULTURE CELLS

We used BRGO cells for all experiments. After their initial isolation the BRGO cells were maintained in Eagle's minimum essential medium with 10% fetal calf serum and 10 mmol/l HEPES without antibiotics at 37°C. We changed medium every three days and subcultured monolayers once they reached confluency after 14-21 days. We removed the monolayer by washing with phosphate buffered saline (PBS) and then with PBS containing 0.025% trypsin and 0.001% ethylenediaminetetra-acetic acid (EDTA). We resuspended the cells in maintenance media, which we replated in Falcon tissue culture flasks or milk dilution bottles for routine passage.

COINCUBATION OF *T PALLIDUM* AND BRGO CELLS

We seeded 1 to 5×10^4 BRGO cells into each Leighton tube containing a coverslip (8 × 32 mm) one to two days before starting the experiment. We then washed the cells with PBS and placed them in serum free medium containing insulin (Commonwealth Serum Laboratories, Parkville, Australia) 50 g/l, growth

Address for reprints: Dr S Graves, Department of Microbiology, La Trobe University, Bundoora, Victoria, Australia 3083

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hormone (Sigma, St Louis, USA) 50 mg/l, epidermal growth factor (Collaborative Research, Waltham, Massachusetts, USA), 100 mg/l, transferrin (Sigma) 50 mg/l, or a combination of all four factors. For comparison, we exposed BRGO cells to the same Eagle's minimum essential medium containing 10% fetal calf serum. We added *T pallidum* 4×10^7 /ml and incubated the cultures either aerobically for four hours or microaerophilically for 24 hours, after which time we assess their attachment.

MEASURING ATTACHMENT OF *T PALLIDUM* TO BRGO CELLS

We removed cover slips from the Leighton tubes, washed off unattached treponemes with medium, and counted the number of treponemes attached to each BRGO cell under dark field microscopy. We recorded those attached to 30 to 40 BRGO cells in duplicate or triplicate samples.

INCORPORATION OF TRITIATED URIDINE BY *T PALLIDUM*

We used tritiated uridine (^3H -uridine) 1 MBq/ml (20 $\mu\text{Ci}/\text{ml}$ per 2×10^7 *T pallidum* to measure ribonucleic acid (RNA) synthesis by *T pallidum* under cell free microaerophilic conditions. We extracted *T pallidum* micro-organisms in serum free medium, adjusted the concentration to 2×10^7 /ml, and allowed them to incorporate ^3H -uridine for 24 hours in the presence or absence of the four individual serum factors, all four factors combined, or 10% fetal calf serum. At the end of the labelling period we discarded the supernatant and solubilised the *T pallidum* with 150 μl of a mixture of 0.5 mol/l sodium hydroxide and 0.5% sodium dodecyl sulphate. Using a multiple cell culture harvester (Skatron, Norway), we washed the treponemes sequentially with trichloroacetic acid (5%), water, and methanol. We collected the precipitates on to glass fibre filters (Flow Laboratories), and measured radioactivity with a Packard Tricarb scintillation counter using 5 g/l of 2,5-diphenyloxazole (PPO) and 0.4 g/l of 1,4-bis [2-(3-methyl-5-phenyl-oxazolyl)]-benzene (dimethyl POPOP) in toluene as the scintillation fluid.

Results

RETENTION OF *T PALLIDUM* MOTILITY

Figure 1 shows that transferrin alone was as effective at retaining *T pallidum* motility as 10% fetal calf serum or all four serum factors combined. Insulin was less effective, and growth hormone and epidermal growth factor even less so. Boiling the combined growth factors for 30 minutes destroyed this ability.

FIG 1 Retention of motility of *Treponema pallidum* in serum free medium containing insulin, epidermal growth factor, growth hormone, transferrin, all four growth factors combined, combination of all four factors inactivated by boiling for 30 minutes, or 10% fetal calf serum after microaerophilic incubation for 72 hours at 34°C in cell free medium. Each bar represents standard deviation of 30-40 assessments.

TABLE Ribonucleic acid synthesis by *Treponema pallidum* as measured by uptake of tritiated uridine over 24 hours in the presence of four serum factors, individually or combined, or 10% fetal calf serum. Numbers are mean (SD) of 10 samples

Serum factor	Uptake of ^3H -uridine (counts per min/well)
No serum or serum factor	63 (26)
10% Fetal calf serum	143 (30)
Insulin	135 (27)
Transferrin	113 (41)
Epidermal growth factor	94 (15)
Growth hormone	69 (30)
All four serum factors combined	165 (32)
All four serum factors combined and inactivated	50 (11)

INCORPORATION OF ^3H -URIDINE BY *T PALLIDUM*

The table shows that insulin and all four serum factors combined permitted RNA synthesis equivalent to that obtained with 10% fetal calf serum. Transferrin, growth hormone, epidermal growth factor, and inactivated combined serum factors were less effective than 10% fetal calf serum.

ATTACHMENT OF *T PALLIDUM* TO BRGO CELLS

Figure 2 shows that all four serum factors enhanced *T pallidum* attachment, transferrin and insulin being superior to growth hormone and epidermal growth factor. The combination of all four factors combined was appreciably better than any individual factor, yet 10% fetal calf serum was the best. This was true in

FIG 2 Attachment of *Treponema pallidum* ($4 \times 10^7/\text{ml}$) to baby rabbit genital organ (BRGO) cells ($1 \times 10^4/\text{ml}$) in serum free medium containing insulin, epidermal growth factor, growth hormone, transferrin, all four growth factors combined, combination of all four factors inactivated by boiling for 30 minutes, or 10% fetal calf serum by (a) aerobic culture for four hours and (b) microaerophilic culture for 24 hours. Each bar represents standard deviation of 30-40 assessments. Differences between results and those for serum free control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

both aerobic and microaerophilic conditions. When combined growth factors were inactivated by boiling for 30 minutes, the stimulatory activity was destroyed and the number of attached treponemes was similar to that found for the serum free control. The complex serum requirements of mammalian cells are probably also necessary for the optimum attachment of *T pallidum* to these cells.

Discussion

We have shown previously that metabolically active tissue cells provide a more suitable habitat for *T pallidum* attachment than do resting cells.⁵ At present it is not known what growing cells provide that alters *T pallidum* attachment, but both surface receptors⁸ and extracellular surface glycoproteins, such as fibronectin,⁹ are altered depending on the growth state of the cell. Extracellular matrix components may affect attachment of *T pallidum* to host cells.¹⁰ Fibronectin has been implicated as a potential ligand for *T pallidum* attachment to host cells.⁹ Thus alterations in host cell surface properties may be related to *T pallidum* attachment.

As the metabolic state of the host cell appears to be important in extracellular parasitism by *T pallidum*,

four growth modulatory serum components were tested for their effect on the relation between host and parasite. The most dramatic effect was shown by the iron transport protein, transferrin. Transferrin was as effective as 10% serum in maintaining *T pallidum* motility, and it appreciably increased the degree of attachment to BRGO cells. The reason for the transferrin effect is not known, but may concern its binding of iron. It could either act by donating necessary iron to *T pallidum* or perhaps by chelating iron and removing it from the free state, thus preventing it entering the *T pallidum* cell. This latter effect could be important because *T pallidum* is very sensitive to oxygen reduction products,¹¹ and the hydroxyl radical can be formed intracellularly in iron loaded bacteria.¹² It may also have a direct effect on the BRGO cells, and this effect may secondarily enhance *T pallidum* attachment and viability.

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