EFFECT OF SUGARS AND METABOLIC INTERMEDIATES ON THE ATTACHMENT OF *TREPONEMA PALLIDUM* TO RABBIT CELLS

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SUMMARY. The effect of various energy sources and metabolic intermediates on the attachment of *Treponema pallidum* to baby rabbit genital organ (BRGO) cells in culture was examined. Pyruvate and glucose enhanced the motility of *T. pallidum in vitro*. Pyruvate increased significantly the attachment of treponemes to BRGO cells when compared with the other substrates but all substrates tested stimulated DNA synthesis by cultured BRGO cells. Thus, the effect of pyruvate on attachment may be due to an effect on the treponemes. Prior exposure of the BRGO cells to the glucose analogue 2-deoxyglucose greatly inhibited the attachment of *T. pallidum* whereas three other analogues had no effect. The inhibitory effect of 2-deoxyglucose was partially reversed by the presence of pyruvate in the attachment assay. These results suggest that energy metabolism of both *T. pallidum* and host cells may be required for the initial interaction of *T. pallidum* with its host *in vivo*.

INTRODUCTION

A variety of pathogenic bacteria interact directly with the surface of mammalian cells. For some, this is a necessary step in initiating infection. Interaction with host membranes can result in intracellular penetration (Byrne and Moulder, 1978; Walker and Winkler, 1978) or in attachment and external parasitism of the host cell (Fitzgerald, Miller and Sykes, 1975; Hu, Collier and Baseman, 1977). *Treponema pallidum* readily attaches to a variety of cultured mammalian cells (Fitzgerald *et al.*, 1977) and attachment is believed to be the initial step in the infective process in syphilis (Fitzgerald *et al.*, 1977; Fitzgerald, 1981).

Actively-metabolising host cells appear to be required for attachment by *T. pallidum*. We have shown previously that cultures of resting eukaryotic cells, when stimulated to grow by the addition of serum, were more suitable hosts than unsynchronised cell cultures in which only part of the population was dividing (Wong

Received 16 May 1983; accepted 18 Jul. 1983

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et al., 1983b). Interaction of various bacteria with surfaces is an energy-requiring process (Byrne and Moulder, 1978; Walker and Winkler, 1978; Feldner, Bredt and Razin, 1981); e.g., with *Mycoplasma pneumoniae*, which requires attachment to respiratory epithelium to initiate an infection (Hu *et al.*, 1977), energy metabolism is necessary for adherence to glass surfaces (Feldner *et al.*, 1981).

In the present study, various sugars, metabolic intermediates and sugar analogues were used to determine the effect of energy metabolism on the interaction between *T. pallidum* and mammalian cells.

MATERIALS AND METHODS

Source of T. pallidum. *T. pallidum* (Nichols) was propagated in the testes of adult male rabbits as previously described (Steiner, McLean and Graves, 1981) and the treponemes extracted anaerobically (Wong, Steiner and Graves, 1982). Eagle's minimal essential medium (EMEM) with fetal calf serum (FCS) 10% v/v and 10mM HEPES (Sigma) was used for co-incubation of *T. pallidum* with tissue-culture cells in all experiments, because it had been shown previously to be superior to three other tissue-culture media (Wong *et al.*, 1983a). In the presence or absence of tissue-culture cells, treponemes were incubated at 34°C in microaerophilic (O₂ 3% v/v) conditions as described by Wong *et al.* (1982).

Tissue-culture cells. We had previously examined several cell lines for attachment of *T. pallidum*; the baby rabbit genital organ (BRGO) cell line was superior to others tested (Wong *et al.*, 1983a) and was used for all experiments. This primary cell line was isolated in our laboratory and maintained in EMEM with FCS 10% v/v and 10mM HEPES without antibiotics. The cell line consisted of cells of fibroblast morphology and was isolated from the external genital region of a neonatal New Zealand rabbit. The cells were large, of regular morphology and grew slowly, taking 10–14 days to reach confluence (Wong *et al.*, 1983a). The medium was changed every 3 days and the confluent monolayers were subcultured; phosphate-buffered saline containing trypsin (Sigma) 0.025% w/v and ethylenediamine tetraacetic acid (EDTA) (Sigma) 0.001% w/v was used to remove cells from the tissue-culture flask.

Energy sources, metabolic intermediates and glucose analogues. The interaction between treponemes and BRGO cells was studied in the presence of seven sugars or metabolic intermediates (glucose, mannose, galactose, fructose, pyruvate, phosphoenol pyruvate and glucosamine) and four glucose analogues (1-methyl-D-glucoside, 3-o-methyl-glucose, 2-deoxy-D-glucose and 6-deoxy-D-glucose). All energy sources were tested at a concentration of 1 mg/ml because this was found to be optimal for both glucose and pyruvate. Glucose analogues were tested at a concentration of 5 mg/ml.

Co-incubation of treponemes and tissue-culture cells. Approximately 2×10^4 BRGO cells were co-incubated with 2.1×10^7 treponemes in Leighton tubes in microaerophilic conditions in the presence of different sugars or metabolic intermediates (1 mg/ml). In those experiments where BRGO cells were pre-treated with glucose analogues (5 mg/ml) for 24 h, the cells were washed three times with glucose-containing medium (1 mg/ml) before co-incubation with treponemes (10^7 /ml) in the absence of glucose analogues. The degree of adherence was determined after microaerophilic incubation for 24 h.

Determination of percentage motility of T. pallidum and the number of treponemes attached per BRGO cell. At defined intervals, c. 200 treponemes, randomly selected from triplicate tubes, were examined by dark-field microscopy to determine the percentage of motile treponemes in cell-free medium.

The treponemal adherence to BRGO cells was determined as follows: cover-slips were removed from Leighton tubes, unattached treponemes were washed off with fresh medium and the average number of treponemes attached per cell was determined by counting c. 40 BRGO cells in each of three samples by dark-field microscopy.

Radiolabelling. Freshly-trypsinised BRGO cells (c. 10^3) were dispensed into each well of a 96-well microtitration plate (Sterilin Ltd, 43 Broad St., Teddington TW11 8QZ) and allowed to attach for at least 12 h. The attached cells were washed twice with medium before use. Cells

were treated with glucose analogues (5 mg/ml) for 24 h before 0.5 μ Ci of [3 H]-thymidine (20 Ci/mmol; Amersham International Ltd, Amersham, Bucks) was added to each well. After 24 h, the supernate was discarded, the cells were dissolved in 150 μ l of lysis buffer (0.5M NaOH and sodium dodecyl sulfate 0.05% w/v) and the large mol. wt components were precipitated with trichloroacetic acid (TCA). A multiple cell-culture harvester (Skatron, P.O.B. 283041, Lierbyen, Norway), was used to collect the TCA precipitates on to glass fibre filters (Flow Laboratories, P.O. Box 17, Second Avenue, Industrial Estate, Irvine, Ayrshire KA12 8NB). Acid-insoluble radioactivity was determined by counting the emission from each filter for 5 min in a Packard Tri-carb β scintillation counter, with a scintillation fluid consisting of 2, 5-diphenyloxazole (PPO) 5 g/L and 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene (dimethyl-POPOP) 0.4 g/L in toluene.

RESULTS

Effect of sugars and metabolic intermediates on attachment

T. pallidum metabolises only glucose and pyruvate for energy (Schiller and Cox, 1977; Barbieri and Cox, 1979). Therefore, the effect of different concentrations (0.25–2 mg/ml) of these substrates on the survival of *T. pallidum* was studied. Each enhanced the motility of *T. pallidum* *in vitro* (fig. 1) and the optimal concentration of each metabolite for survival of *T. pallidum* in microaerophilic conditions was 1.0 mg/ml.

The effect of other sugars and metabolic intermediates on the attachment of *T. pallidum* to BRGO cells was then determined. All metabolites were tested at a concentration of 1 mg/ml and all caused some enhancement of attachment (fig. 2). The enormous enhancement of attachment in the presence of pyruvate is noteworthy. This was a statistically significant ($p < 0.001$) increase in attachment over that with all

FIG. 1.—The effect of different concentrations of glucose (—●—) or pyruvate (—○—) on the motility of *T. pallidum* after incubation for 72 h in cell-free medium in microaerophilic conditions. Each point represents the mean of six samples. The bar represents $\pm$ one standard deviation (S.D.).

FIG. 2.—The effect of different energy sources (1 mg/ml) on the attachment of *T. pallidum* to BRGO cells. NONE = no added energy source; GLU = glucose; PEP = phosphoenol pyruvate; PYR = pyruvate; MAN = mannose; GAL = galactose; GLU-NH₂ = glucosamine; FRU = fructose. Each bar represents the mean of 120 determinations $\pm$ S.D.

other metabolites. Pyruvate caused a five-fold or greater increase in attachment over the control tests without sugar or metabolic intermediates and greater than a three-fold increase over tests with any of the other substrates. All the metabolites tested initiated DNA synthesis in the BRGO cells, as measured by [³H]-thymidine incorporation, although no significant differences could be detected between them (data not shown). Because pyruvate did not particularly enhance DNA synthesis in BRGO cells in comparison with the other substrates, the significantly higher level of adherence of *T. pallidum* to BRGO cells may have been due to an effect on treponemal energy metabolism rather than on the growth of the host cells.

Effect of glucose analogues on attachment

Further investigation of the effect of energy metabolism on attachment involved the use of four glucose analogues. In order to separate the effects of analogues on *T. pallidum* from those on the BRGO host cells, the effects on each were studied separately. Only 2-deoxyglucose significantly depressed the growth of the host cells, as measured by DNA synthesis, after incubation for 24 h in the presence of glucose and analogues (fig. 3); 2-deoxyglucose is not metabolised and inhibits glycolysis in eukaryotic cells (Wick *et al.*, 1957). None of the glucose analogues (5 mg/ml) had any effect on the motility of *T. pallidum* during microaerophilic incubation for 24 h.

To determine whether the effect of analogues on BRGO cells would affect treponemal attachment, BRGO cells were pre-treated with the analogues (5 mg/ml) for 24 h and were then washed three times with normal medium before incubation with *T. pallidum* and without the analogues. The percentage motility of treponemes in all

FIG. 3.—The effect of glucose analogues (5 mg/ml) on DNA synthesis in BRGO cells as measured by [³H]-thymidine incorporation. NONE = no added glucose or glucose analogue; GLU = glucose (1 mg/ml); 1-M-G = 1-methyl-D-glucoside; 3-M-G = 3-o-methyl-glucose; 2-D-G = 2-deoxy-D-glucose; 6-D-G = 6-deoxy-D-glucose. Each bar represents the mean of five samples $\pm$ S.D.

FIG. 4.—The attachment of *T. pallidum* to BRGO cells previously exposed for 24 h to glucose analogues (5 mg/ml). The pretreatments were: NONE = no added glucose or glucose analogue; GLU = glucose (1 mg/ml); 1-M-G = 1-methyl-D-glucoside; 3-M-G = 3-o-methyl-glucose; 2-D-G = 2-deoxy-D-glucose; 6-D-G = 6-deoxy-D-glucose. Each bar represents the mean of 120 determinations $\pm$ S.D.

FIG. 5.—The effect of pyruvate (1 mg/ml) on the attachment of *T. pallidum* to BRGO cells previously treated with glucose analogues. Cells were pretreated either with glucose (1 mg/ml) but without glucose analogues (GLU) or with glucose analogues (5 mg/ml) for 24 h: 1-M-G = 1-methyl-D-glucoside; 3-M-G = 3-o-methyl-glucose; 2-D-G = 2-deoxy-D-glucose; 6-D-G = 6-deoxy-D-glucose. Cells were then washed and incubated with *T. pallidum* in the presence (▨) or absence (□) of pyruvate (1 mg/ml). Each bar represents the mean of 120 samples $\pm$ S.D.

samples was *c.* 80–90% at the time of measurement of the number of attached treponemes. BRGO cells which had been incubated previously with 2-deoxyglucose and had had their growth arrested showed little attachment of treponemes (fig. 4). Presumably, energy metabolism in the cells was still inhibited despite the presence of glucose in the attachment assay medium. To test whether pyruvate could reverse this inhibitory effect, BRGO cells were pre-treated with glucose analogues (5 mg/ml) for 24 h and then washed three times in glucose-free medium before co-incubation with treponemes in the presence or absence of pyruvate (1 mg/ml). After microaerophilic co-incubation for 24 h, the extent of treponemal adherence to BRGO cells was determined. The inhibitory effect of 2-deoxyglucose on attachment was partially reversed by the presence of pyruvate in the attachment assay (fig. 5). This did not appear to be due to the ability of pyruvate to reverse the inhibition of DNA synthesis in BRGO cells because the cells were still inhibited when pyruvate was present in the medium containing 2-deoxyglucose (data not shown).

DISCUSSION

Attachment to the host cell appears to be an important step in the pathogenicity of *T. pallidum*. It has been postulated that attachment may be one of the first steps in establishing infection (Fitzgerald *et al.*, 1977), and it would also be important in the penetration of host cells which has been suggested to be a cause of latency in syphilis (Sykes and Miller, 1971).

A metabolically-active parasite and host cell are often required for the successful initiation of infection (Hale and Bonventre, 1979; Hale, Morris and Bonventre, 1979). In order to test this hypothesis with *T. pallidum*, the effect of various energy sources

and glucose analogues on attachment to tissue cells was determined. Pyruvate had by far the greatest enhancing effect. Only glucose and pyruvate have been shown to be used for energy production by *T. pallidum* (Schiller and Cox, 1977). The importance of pyruvate comes from its involvement in the terminal energy yielding steps of catabolism in this organism (Barbieri and Cox, 1979). Further experiments supported the hypothesis that the pyruvate effect was due to its action on *T. pallidum* rather than on the host cells. Pyruvate antagonised the inhibitory effect of the glucose analogue 2-deoxyglucose on the attachment of *T. pallidum* to BRGO cells (fig. 5) but did not reverse the block in the host metabolism caused by 2-deoxyglucose.

There are several possible explanations of the need for an energy source for attachment to host cells. The simplest is that it enhances the survival of *T. pallidum* (fig. 1), thereby increasing the likelihood of a collision between a live bacterium and the host cell. Other possible explanations would involve the synthesis of membrane components and surface proteins, but these seem unlikely in view of the short co-incubation period and the speed of the interaction between host cells and *T. pallidum*. This requirement for an ample energy source for *T. pallidum* is likely to operate in the infectious process. With many bacteria, attachment is a passive step (Byrne and Moulder, 1978; Walker and Winkler, 1978) whereas with *T. pallidum*, active penetration of the host tissue is required. The favoured place of residence of this pathogen is the extracellular matrix surrounding the host cells (Scott and Dammin, 1950). In order to invade host tissue, the treponemes may need to be actively motile (which presumably depends on the availability of glucose or, more especially, pyruvate) and able to penetrate the gel-like ground substance, probably by means of a mucopolysaccharidase (Fitzgerald and Johnson, 1979).

Finally, of what importance is host-cell energy metabolism in the attachment process? We have shown previously that the rate of growth of the tissue is important for the attachment of *T. pallidum* (Wong *et al.*, 1983*a* and *b*). The results obtained with the glucose analogue 2-deoxyglucose indicate that the rate of host energy metabolism also may be important in attachment. Treatment of the tissue cells with glucose analogues for 24 h prior to infection significantly lowered attachment even though the assay was performed in medium without the analogue. One possible explanation is that the surface receptors for *T. pallidum* are lost during energy deprivation, thus fewer sites are available for attachment. Moreover, 2-deoxyglucose blocks glycosylation of proteins as well as energy production, which could provide another possible explanation for the inhibitory effect on treponemal attachment. Another possibility is that a high adenylate energy charge (Atkinson and Walton, 1967) and an increased metabolic rate could cause the secretion of a nutrient such as pyruvate which could attract *T. pallidum* to the tissue cells. Many bacteria show chemotaxis toward a preferred carbon or energy source (Adler, 1979) but nothing is currently known about chemotaxis in *T. pallidum*. Spirochaetes are capable of chemotaxis because it has been demonstrated in at least one other genus (Greenberg and Canale-Parola, 1977). The ability of *T. pallidum* to penetrate the extracellular matrix of the tissues that it invades may be the result of directed motility towards a nutrient source but there are, at present, no data on this intriguing possibility.

The financial support of grants from the Australian National Health and Medical Research Council (NH&MRC), the Utah Foundation, the Heiser Program, the Australian-Britain

Society and Monash University is gratefully acknowledged. We wish to thank Professor S. Faine and Dr S. Graves for use of the facilities of the Monash University Microbiology Department.

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