Surface Properties of *Treponema pallidum* in Relation to Phagocytosis by Human Polymorphonuclear Leucocytes *in vitro*

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Surface charge and hydrophobicity of *Treponema pallidum* have been investigated in relation to phagocytosis by human polymorphonuclear leucocytes (PMNs) *in vitro*. The treponemal surface was relatively hydrophobic and negatively charged but despite these properties, phagocytosis, as assessed by luminol-enhanced chemiluminescence, was minimal in the absence of serum. Pre-opsinization of bacteria with serum reduced surface hydrophobicity but promoted phagocytosis, suggesting that specific immune mechanisms may be more important in controlling phagocytosis of *T. pallidum* *in vitro* than non-specific surface properties. *T. pallidum* evoked a much weaker chemiluminescence response from PMNs than the non-pathogenic treponeme *Treponema phagedenis* biotype Reiterii even though similar numbers of bacteria were phagocytosed, suggesting differences in the reactivity of the surface components of the two organisms toward PMNs. The reactivity of *T. pallidum* towards PMNs could be increased by removal of the bacterial outer membrane by Triton X-100 treatment. These observations reinforce the suggestion that the outer surface of *T. pallidum* is inherently inert.

INTRODUCTION

Syphilis differs from many other bacterial diseases in that early in infection the invading organism, *Treponema pallidum*, evokes little or no acute inflammatory response (Lukehart *et al.*, 1980). Histological examination of tissue early in infection indicates that polymorphonuclear leucocyte (PMN) infiltration is limited and phagocytosis of treponemes is minimal, the vast majority of bacteria remaining extracellular despite a detectable humoral immune response (Penn, 1981; Hanff *et al.*, 1983). It has been suggested that the lack of stimulation of PMN infiltration may be due to masking of treponemal antigens by acquisition of a coat of host-derived tissue components including proteins and mucopolysaccharides (Alderete & Baseman, 1979; Fitzgerald *et al.*, 1979), preventing recognition of antigens by antibody and complement and limiting phagocytosis. Opsonization of bacterial surfaces promotes phagocytosis and a role for anti-*T. pallidum* antibody in promotion of phagocytosis of this organism has been demonstrated *in vitro* (Musher *et al.*, 1983; Lukehart & Miller, 1978); blocking of antibody binding could therefore limit phagocytosis *in vivo*.

Bacterial-phagocyte interactions may also be influenced by non-specific physicochemical properties of the bacterial surface including surface charge and hydrophobicity (Beachey, 1980). These surface properties may be modified by the binding of serum components including complement and antibody (Stendahl *et al.*, 1974; Stjernstrom *et al.*, 1977). Although *T. pallidum* binds host serum components to its surface *in vivo* (Alderete & Baseman, 1979), nothing is known of the influence of bound components on the physicochemical properties of the treponemal surface or their influence on phagocytosis of the organism. A combination of

Abbreviations: HBSS, Hanks' balanced salts solution; PEG, polyethylene glycol; P-PEG, polyethylene glycol palmitate; PMN, polymorphonuclear leucocyte.

treponemal surface properties not conducive to phagocytosis could explain the limited interactions between PMNs and *T. pallidum* *in vivo*.

The influence of serum components on surface properties of *T. pallidum* and phagocytosis of the bacterium has therefore been investigated.

METHODS

Bacteria. The Nichols strain of *T. pallidum* was maintained by intratesticular passage in Californian rabbits and harvested as previously described (Penn & Rhodes, 1982; Penn, 1983). Bacteria were used after storage in extraction medium + 0.1% sodium azide for 48 h at 4 °C. *Treponema phagedenis* biotype Reiterii (*T. phagedenis*) was grown at 37 °C in spirolate broth (Beckton Dickinson) supplemented with 5% (v/v) newborn calf serum (Gibco). Before use bacteria were sedimented by centrifugation for 5 min at 11600 g in an MSE Microcentaur centrifuge and washed twice in the relevant buffer.

Investigation of surface properties of azide-killed *T. pallidum*. Surface properties were investigated by phase partitioning in PEG-Dextran two-phase systems and by electrostatic and hydrophobic interaction chromatography.

Phase partitioning. The methods used were those described by Albertsson *et al.* (1982). Phase systems were prepared in 64 × 10 mm plastic tubes from stock solutions of 40% (w/w) PEG 3350 (Sigma) and 20% (w/w) Dextran T500 (Pharmacia). Surface charge and hydrophobicity were determined in systems containing varying amounts of NaCl (system A) and hydrophobically substituted PEG-palmitate (P-PEG – system B) (Shanbhag & Johanssen, 1974) respectively. Isoelectric points were determined by the method of cross-partition described by Albertsson *et al.* (1982) (system C).

Phase systems were prepared by weighing polymer solutions into the tubes followed by the addition of relevant amounts of buffer, salts and bacterial suspension. The weight of each system was then made up to 1 g with sterile distilled water. The composition of the systems was (A) 6.75% (w/w) dextran, 6.5% (w/w) PEG, 10 mM-sodium phosphate buffer, pH 7.0, and 0 to 20 mM-NaCl; (B) 7.5% (w/w) dextran, 6.5% (w/w) PEG, 3 mM-Tris/HCl buffer, pH 7, and 0 to 0.03% (w/w) P-PEG; (C) as (A) but with 10 mM-sodium citrate buffer, pH 3.0 to 6.0, and either 20 mM-NaCl or 10 mM-Na₂SO₄. Approximately 1×10^8 bacteria, suspended in the relevant buffer, were added per system.

Systems were mixed by inversion 30 to 40 times and 100 µl samples withdrawn for estimation of the total number of bacteria present. Tubes were then left to stand for 45 min at room temperature to allow partition to occur. The upper (PEG) phase was then sampled and, after diluting 1:1 with water, bacterial counts in all samples were determined microscopically in a Thoma counting chamber. The percentage of bacteria in the PEG phase was calculated from these counts and the volumes of the phases.

Electrostatic interaction chromatography. The method used was modified from Pedersen (1980). Packed volumes (200 µl) of ion-exchange resin, pre-swollen and equilibrated in the appropriate buffer, and 0.3 ml buffer containing 8×10^6 bacteria were mixed in 1.5 ml volume capped polycarbonate tubes. Tubes were vortexed for 10 s and the resin beads allowed to settle at room temperature for 10 min. The bacterial counts in the supernates above the beads, and in control tubes containing 0.3 ml bacterial suspension and 0.2 ml buffer, were determined microscopically and reduction of bacterial numbers in the presence of resin taken as a measure of adherence to the beads. Binding to QAE and DEAE Sephadex A50 and SP-Sephadex A25 was tested in 10 mM-sodium phosphate or 100 mM-Tris/HCl buffer, pH 6.8. Binding to DEAE Sephadex A50 at different pH values was tested in 100 mM-sodium citrate buffer + 100 mM-NaCl.

Hydrophobic interaction chromatography. Binding to Octyl-Sepharose CL 4B was tested as above in 100 mM-Tris/HCl buffer, pH 6.8.

Interaction of *T. pallidum* and *T. phagedenis* with PMNs. PMNs were obtained from heparinized human venous blood by the dextran sedimentation method of Bridges *et al.* (1980). PMNs were washed twice in Hanks' balanced salts solution (without phenol red) + 20 mM-HEPES, pH 7.2 (HBSS), and resuspended in HBSS at 1×10^6 ml⁻¹. *T. pallidum* or *T. phagedenis* (1×10^8 ml⁻¹) were pre-opsonized by incubation in 10% (v/v) homologous serum in HBSS at 37 °C for 15 min before mixing with PMNs. Some samples of serum were heated to 56 °C for 30 min to inactivate the C3 component of the complement cascade, before mixing with bacteria. Similar opsonization was done with 10% (v/v) control rabbit serum and a hyperimmune serum raised by repeated injection of *T. pallidum* into New Zealand White rabbits.

Phagocytosis was assayed by the method of luminol-enhanced chemiluminescence (Stevens *et al.*, 1978). The concentration of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in the reaction medium was 10^{-6} M. Volumes (0.25 ml) of bacterial and PMN suspensions were mixed in 1.5 ml capped polycarbonate tubes and incubated at 37 °C on an end-over-end rotator at 30 r.p.m. Control tubes containing similar volumes of PMNs, but lacking bacteria, were also included in each set of assays. Chemiluminescence was measured after 5, 15 and 30 min incubation in a Beckman LS 700 scintillation counter using the 'in coincidence' mode with lower and upper

window settings of 0 and 625 respectively. Results are expressed as counts per minute (c.p.m.). All assays were done in duplicate.

In preliminary experiments phagocytosis was also quantified by microscopy. Bacterial counts in reaction mixtures were determined in a Thoma counting chamber and a reduction in counts in the presence of PMNs compared with control tubes, containing bacteria only, was taken as a measure of phagocytosis.

Electron microscopy. Phagocytosis was confirmed by electron microscopy. After incubation with bacteria, PMNs were sedimented and extracellular bacteria removed by washing in HBSS. PMNs were fixed in 2% (v/v) glutaraldehyde in 200 mosM-phosphate-buffered saline, pH 7.2, and post-fixed in 2% (v/v) osmium tetroxide. The pellet was embedded in Araldite after dehydration through a series of graded alcohols and propylene oxide. Blocks were cured at 60 °C overnight and sections cut on a Reichart Ultracut microtome. Sections were stained with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope.

Triton treatment of *T. pallidum*. Bacteria were exposed to 0.2% (v/v) Triton X-100 in 0.05 M-Tris/HCl buffer, pH 8.0, for 30 min at 37 °C as described by Penn & Rhodes (1982). Bacteria were then sedimented, washed three times and resuspended in HBSS to their original concentration.

Unless otherwise stated, data represent mean values $\pm$ SE from at least three determinations and, where indicated, data were analysed statistically by the Mann-Whitney U test.

RESULTS

Phagocytosis of treponemes by human PMNs

Azide-killed *T. pallidum*, pre-opsonized with normal human serum, were readily phagocytosed by human PMNs as shown both by the reduction of treponemal numbers remaining free in suspension with time and the marked PMN chemiluminescent response (Fig. 1). Microscopic examination of samples of reaction mixtures showed that <5% of PMNs had *T. pallidum* visibly adherent to their outer surface after incubation, suggesting that the majority of bacteria removed from the supernates had been ingested. Also phagocytosis was confirmed by electron microscopy which showed numerous treponemes inside phagocytic vacuoles (Fig. 2). The chemiluminescent response obtained in replicate experiments varied considerably, e.g. after 15 min incubation in normal human serum, mean response = 207 338 c.p.m., SE = 48 900 c.p.m., $n = 19$. The pattern of results obtained in replicate experiments was, however, similar and differences obtained with different serum treatments were reproducible. Chemiluminescence in the presence of normal human serum was measured in each set of assays and was used as a standard against which responses produced by other serum treatments were compared.

The response obtained was influenced by the presence of heat-labile serum components and was markedly reduced in heat-treated serum ($P < 0.05$ at 5 and 15 min) (Fig. 3*b*). The strongest response was observed when bacteria were pre-opsonized with hyperimmune anti-*T. pallidum* rabbit serum ($P < 0.01$ at 5 min) (Fig. 3*a*). Chemiluminescence in the absence of serum was minimal ($P < 0.05$ at all time points) (Fig. 3*c*). Triton-treated *T. pallidum* evoked a much stronger chemiluminescence in the presence of normal human serum than intact bacteria ($P < 0.05$ at 5 min) (Fig. 4).

T. phagedenis was also readily phagocytosed in the presence of normal human serum (Fig. 1). Although similar numbers of both bacteria were phagocytosed the chemiluminescence evoked by *T. phagedenis* was much stronger than that for *T. pallidum* (Fig. 1).

*Surface properties of *T. pallidum**

Phase partitioning. The results of partitioning studies of *T. pallidum* in PEG-Dextran two-phase systems are shown in Fig. 5. Partitioning of bacteria was influenced by increasing concentrations of both NaCl and P-PEG, indicating a negatively charged, relatively hydrophobic cell surface (Fig. 5*a, b*). In preliminary experiments partitioning of bacteria of known surface properties (two *Salmonella typhimurium* isolates designated 395 MS and MR 10, kindly donated by E. Kihlstrom, Linkoping, Sweden) was tested in these systems. *T. pallidum* partitioned in a similar manner to the relatively hydrophobic, negatively charged strain MR 10 (Magnusson *et al.*, 1977) (data not shown).

The negative surface charge was confirmed by the method of cross-partitioning in phase systems containing either NaCl or Na₂SO₄ (Albertsson *et al.*, 1982). The isoelectric point of the

Fig. 1. Correlation of phagocytic uptake and chemiluminescence response of PMNs toward *T. pallidum* (○) and *T. phagedenis* (▼). Bars denote SEMs.

Fig. 2. Electron micrograph of a section through a PMN showing numerous phagocytic vacuoles containing ingested treponemes (arrowed). Magnification $\times 15000$.

Fig. 3. Effect of serum factors on chemiluminescence response of PMNs to *T. pallidum*. Response in normal human serum (○) is compared with that in (a) hyperimmune rabbit serum, (b) heat-treated human serum and (c) no serum (●). Bars denote SEMs.

Fig. 4. Effect of Triton X-100 treatment of *T. pallidum* on PMN chemiluminescence in the presence of normal human serum. □, Control; ■, Triton X-100 treated *T. pallidum*. Bars denote SEMs.

treponemal surface obtained by this method was 3.56 ± 0.3 (Fig. 5c). Some variation was observed in the isoelectric point determined in replicate experiments due to the tendency of bacteria to clump at low pH values.

Electrostatic interaction chromatography. *T. pallidum* bound to both positively charged ion-exchange resins DEAE Sephadex A50 and QAE Sephadex A50 (Fig. 6a). Bound treponemes were readily visible by phase-contrast microscopy, adhering to resin beads. Binding was markedly influenced by the NaCl concentration of the buffer. Minimal binding to Sephadex CL 4B or SP-Sephadex A25 was also detected (data not shown). Binding to DEAE Sephadex A50 was influenced by buffer pH and was lowest at values around pH 3, a value similar to that obtained by cross-partitioning for the tentative isoelectric point of the treponemal surface (Fig. 6b). Standard deviations in these experiments ranged from 0 to 13%.

Hydrophobic interaction chromatography. *T. pallidum* bound to the hydrophobic gel Octyl-Sepharose CL 4B, although bacteria bound in relatively small numbers and only in the presence of high NaCl concentrations (Fig. 6c).

Effect of serum binding on surface properties and phagocytosis of T. pallidum

As pre-opsonization of *T. pallidum* with sera from various sources influenced PMN chemiluminescence, the effect of similar treatments on the surface properties of *T. pallidum* was investigated to try to correlate changes in surface properties with variations in the phagocytic

Fig. 5. Partitioning of *T. pallidum* in PEG-Dextran two-phase systems. (a) With NaCl, (b) with P-PEG, (c) system with 20 mM-NaCl (●) or 10 mM-Na₂SO₄ (○).

Fig. 6. (a) Effect of NaCl concentration on binding of *T. pallidum* to QAE (●) and DEAE (○) Sephadex. (b) Effect of pH on binding of *T. pallidum* to DEAE Sephadex. (c) Effect of NaCl concentration on binding of *T. pallidum* to Octyl-Sepharose CL 4B.

response. Bacteria were pre-opsonized with 10% serum in HBSS for 15 min at 37 °C, sedimented and washed three times in HBSS before resuspension in the relevant buffer and addition to the phase systems. In contrast to *T. pallidum* in the absence of serum, bacteria pre-opsonized with normal human serum or hyperimmune rabbit serum, both previously found to promote phagocytosis, showed a marked reduction in surface hydrophobicity as determined in P-PEG-Dextran two-phase systems. In the absence of serum, $98 \pm 15\%$ (SD) of bacteria partitioned into the P-PEG phase compared with $24 \pm 15\%$ pre-opsonized with normal human serum and 20% (single observation) pre-opsonized with hyperimmune rabbit serum. In contrast, pretreatment of bacteria with heat-treated human serum had no effect on surface hydrophobicity, $87 \pm 19\%$ of bacteria partitioning in the P-PEG phase. No effect of these treatments on surface charge was observed (results not shown).

DISCUSSION

Our observations on the interactions of *T. pallidum* with human PMNs assessed by a chemiluminescence method are in close agreement with those previously reported by Musher *et al.* (1983). Both serum antibody and heat-labile complement components promoted phagocytosis *in vitro*. Promotion of phagocytosis by normal human serum correlates well with previous reports of the presence of neutralizing antibody in normal serum (Hederstedt, 1976) possibly directed against antigens common to both commensal treponemes and *T. pallidum* (Strandberg Pedersen *et al.*, 1981). Paradoxically however, it has proved impossible to detect binding of specific anti-*T. pallidum* antibody to intact treponemes by immunofluorescence (Penn & Rhodes, 1982), even though the observed effects on phagocytosis and surface properties suggest binding has occurred. Further studies with immunogold labelling techniques have also failed to detect antibody binding to azide-killed *T. pallidum* (unpublished observations). It is conceivable that binding of very small quantities of antibody, below levels detectable by these methods, may be sufficient to promote phagocytosis of *T. pallidum*.

We have also observed differences in the chemiluminescence response of PMNs toward *T. pallidum* and a non-pathogenic cultivable treponeme *T. phagedenis*. Although the pattern of responses seen with both bacteria in our studies was similar (Fig. 1) the strength of the chemiluminescent response obtained with *T. phagedenis* was much greater than was observed with *T. pallidum*. The increased response did not appear to be due to greater phagocytosis of *T. phagedenis* since the kinetics of uptake for both bacteria were similar. One possibility is that phagocytosis of *T. phagedenis* stimulates a stronger oxidative metabolic burst than uptake of *T. pallidum*, resulting in increased chemiluminescence. *T. pallidum* and *T. phagedenis* differ in size and the increase in chemiluminescence could be partly attributed to the greater biomass of phagocytosed *T. phagedenis*. However, a second possibility exists that the outer surface of *T. phagedenis* is in some way more reactive towards PMNs than that of *T. pallidum*. The relatively inert nature of the *T. pallidum* surface is supported by previously published reports of the resistance of viable intact treponemes to reaction with antibody (Penn, 1981) and resistance of viable *T. pallidum* to surface iodination (Penn *et al.*, 1985). The inert nature of the *T. pallidum* surface is supported by the observation in this study that Triton-treated *T. pallidum* promoted a much stronger chemiluminescence than intact bacteria (Fig. 4). Triton treatment removes the outer membrane of *T. pallidum* exposing antigens normally inert or cryptic in the intact organism (Penn & Rhodes, 1982). Furthermore, comparison of the surface composition of *T. pallidum* and *T. phagedenis* (Bailey *et al.*, 1985) has shown the presence of a smooth type lipopolysaccharide in *T. phagedenis* but not in *T. pallidum*. The apparent lack of lipopolysaccharide at the *T. pallidum* surface may contribute to the relatively weak chemiluminescence response elicited by *T. pallidum* compared with *T. phagedenis*, the initial lack of reactivity towards PMNs observed *in vivo* and also to treponemal surface hydrophobicity.

The combination of phase partitioning in PEG-Dextran two-phase systems and ionic and hydrophobic interaction chromatography suggests that the native treponemal surface in the absence of serum is negatively charged and relatively hydrophobic. Although all the methods used to assess surface charge gave similar results, some discrepancy was observed between results obtained for surface hydrophobicity by the two methods used. This discrepancy may be explained by the observation that phase partitioning measures overall surface hydrophobicity rather than localized regions of hydrophobic residues involved in binding to Octyl-Sepharose (Miorner *et al.*, 1982).

Negative charge and a relatively hydrophobic surface favour phagocytosis for a number of bacteria (Stendahl *et al.*, 1973; Van Oss, 1978). In contrast, phagocytosis of *T. pallidum* in the absence of serum was minimal, suggesting that non-specific surface properties may be of secondary importance to specific immunological reactions in promoting phagocytosis. The observation that opsonization with normal human serum reduced surface hydrophobicity of *T. pallidum* whilst promoting phagocytosis supports this view, although the mechanism by which hydrophobicity is reduced remains unknown. A similar apparent lack of correlation between hydrophobicity and liability to phagocytosis has been reported for some group A streptococci

where a major surface component, the hydrophobic M protein, appears to confer resistance to phagocytosis (Speert *et al.*, 1981) possibly by masking antigens and preventing binding of antibody and complement. The outer membrane of *T. pallidum* could have a similar role *in vivo*, as evidenced by the lack of reactivity of freshly isolated treponemes with antibody (Penn & Rhodes, 1982) and the increased reactivity towards PMNs of Triton-treated bacteria. The promotion of phagocytosis by antibody and complement observed *in vitro* may be the result of exposure of antigens due to modification or damage of the treponemal surface during the extraction and washing procedures necessary for the preparation of bacteria for these assays. Such modification could probably occur without loss of treponemal motility and could explain our preliminary observations that viable treponemes were phagocytosed as readily as azide-killed bacteria and also behaved similarly in phase-partitioning assays (data not shown). Furthermore, washing may remove loosely-bound host tissue components which *in vivo* could contribute to the lack of surface reactivity by influencing surface properties and the binding of serum factors. In particular, a coat of host proteins or mucopolysaccharides could prevent the interaction between bound complement components and the Fc receptors on the phagocyte surface by simple steric hindrance, as suggested for capsules of some other pathogens (Edebo *et al.*, 1981). Modification of the hydrophobic treponemal surface by binding of hydrophilic mucopolysaccharides (Ofek *et al.*, 1983) could also further reduce the tendency for interaction with PMNs in the absence of serum.

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