Attachment of Mycobacteria to Fibronectin-coated Surfaces

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This report investigates the extent of the expression of fibronectin (FN) binding properties among the mycobacteria and provides preliminary characteristics of the bacterial molecule(s) mediating attachment. Eight BCG substrains, three Mycobacterium tuberculosis strains and four other mycobacterial species all expressed FN-binding capacity. Treatment of organisms with detergent prior to the binding assay destroyed the FN-binding capacity of BCG but not that of Staphylococcus aureus. Trypsin pretreatment eliminated the FN-binding capacity of both BCG and S. aureus. [35S]Methionine-labelled material in supernatants from BCG and M. tuberculosis cultures attached to FN-coated surfaces. These culture supernatants inhibited the attachment of BCG but not S. aureus to FN-coated surfaces. This inhibitory activity of the supernatants was removed by affinity chromatography on FN-Sepharose but was not affected by similar passage over a control column (human serum albumin attached to Sepharose). These results demonstrate that the ability to bind FN is present in all mycobacterial species tested and suggest that attachment is mediated by trypsin-sensitive cell-surface component(s).

INTRODUCTION

Fibronectin (FN) is a glycoprotein found in a soluble form in plasma, cerebrospinal fluid and amniotic fluid and in an insoluble form in extracellular matrix and basement membranes (Mosher, 1980). Fibronectins are characterized by the ability to bind to many different molecules including fibrin, gelatin, collagen, C1q and heparin (Mosher, 1980). Fibronectins also bind to bacteria and phagocytic cells (Hynes & Yamada, 1982; Speziale et al., 1984; Brown, 1986).

The interaction between FN and bacteria has been best characterized for Staphylococcus aureus and some streptococci. S. aureus and the streptococci bind primarily to the FN domain near the amino terminus of the molecule (Speziale et al., 1984; Kuusela et al., 1985), although S. aureus also binds to a second, weaker, interactive site on FN (Kuusela et al., 1985). The staphylococcal surface molecule that binds FN is protein in nature and binds FN in a saturable manner (Proctor et al., 1982; Espersen & Clemmensen, 1982; Ryden et al., 1983). This is contrasted by streptococci, which appear to bind FN via their lipoteichoic acids, although a protein FN-binding component also has been reported (Speziale et al., 1984; Courtney et al., 1983).

The ability of bacteria to attach to FN-coated surfaces may be an important mechanism in the establishment of infections (reviewed by Beachey, 1981). For example, the ability of S. aureus to bind FN has been linked with virulence. The attachment of S. aureus to FN-coated surfaces may be facilitated by the expression of a binding protein, which is trypsin-sensitive and not heat-stable (Proctor et al., 1982; Espersen & Clemmensen, 1982; Ryden et al., 1983). This binding protein may play a role in the establishment of infections, as S. aureus is capable of attaching to FN-coated surfaces before phagocytosis.

Abbreviations: BCG, bacille Calmette–Guérin; FN, fibronectin; HSA, human serum albumin.
attach to endothelial cells bearing cell-surface FN suggests that this may be an early step in invasion and colonization of vascular tissues (Vercellotti et al., 1984). Similarly, streptococci have been observed to attach to buccal epithelial cells bearing FN, suggesting that attachment to FN may be required for tissue colonization (Abraham et al., 1983).

We recently reported that bacille Calmette–Guérin (BCG) attached to FN-coated surfaces (Ratliff et al., 1987). In vitro binding studies demonstrated the specificity of the binding phenomenon in that BCG attached in a dose-dependent manner to surfaces coated with purified FN but not other purified extracellular matrix proteins including laminin, fibrinogen and collagen. Attachment of BCG organisms was inhibited by antibodies to FN but not by antibodies against other extracellular matrix proteins. Similarly, only anti-FN antibodies inhibited the in vivo attachment of BCG or in vitro attachment of these bacteria to surfaces coated with various inflammatory and extracellular proteins. In this report we demonstrate that all the mycobacterial species tested attach to FN-coated surfaces and that the binding appears to be mediated by bacterial surface component(s).

**METHODS**

**Bacteria.** BCG substrains Armand Frappier (Armand Frappier, Quebec, Canada), Tice (Tuberculosis Institute, Chicago, Ill., USA), Glaxo (Glaxo, Inc., Research Triangle, NC, USA) and Connaught (Division of International Marketing, Ontario, Canada) were obtained commercially as lyophilized preparations. Some experiments were done using the reconstituted lyophilized preparations, which consisted of a minimum of 10⁶ c.f.u. mg⁻¹. The remainder of the BCG substrains, Mycobacterium tuberculosis H₃₇R₇, M. avium, M. nonchromogenicum, M. vaccae and M. intracellulare were obtained from the mycobacterial collection of Dr John Stanford. The clinical isolates were obtained from Dr Steven Moser at the Jewish Hospital of St Louis. *Staphylococcus aureus* Woods strain (ATCC 10832) was purchased from the American Type Culture Collection, Bethesda, Md, USA. All experiments were done with viable bacterial preparations.

**Radiolabelling of bacteria.** Two radiolabelling techniques were used. Initially [³²P]orthophosphate was used, but later [³H]uracil was used instead, because the energy emission level is lower for [³H]uracil and also because the [³H]uracil labelling procedure is more rapid. Both labelling procedures measured bacterial attachment to FN equally well. For labelling with [³²P]orthophosphate, mycobacteria were cultured for 7 d at 37 °C in 120 ml Youman's medium under 5% (v/v) CO₂, then washed in PBS (0.15 M-NaCl, 0.1 M-sodium phosphate, pH 7.2) and resuspended in 10 ml of a 1:10 dilution of heart infusion broth (Difco) as previously described (David et al., 1985). [³²P]Orthophosphate (Amersham) was added to a final concentration of 20 μCi ml⁻¹. The bacteria were incubated for 1 h at 37 °C, washed three times in PBS supplemented with 0.1% human serum albumin and resuspended to 10⁸ c.f.u. ml⁻¹ in PBS. Radiolabelling of mycobacteria with [³H]uracil was done as previously described (Altes et al., 1985). Briefly, bacteria were cultured for 3 d in RPMI-1640 supplemented with 0.05% ferric ammonium citrate and 50 μCi [³H]uracil (ARC, Inc.; specific activity 20 Ci mmol⁻¹, 740 GBq mmol⁻¹) ml⁻¹. Labelled bacteria were washed and used in binding assays as described below.

*S. aureus* ATCC 10832 was cultured in RPMI-1640. Exponential-phase cells were washed, resuspended in 10 ml M199 medium containing 100 μCi [³H]thymidine (New England Nuclear; specific activity 6.7 Ci mmol⁻¹, 2479 GBq mmol⁻¹), incubated for 3 h at 37 °C, then were washed and resuspended to 10⁸ c.f.u. ml⁻¹ in PBS.

**Purification of FN.** Human plasma FN was purified as previously described (Pommier et al., 1983). Briefly, the 10% (w/v) polyethylene glycol 3350 precipitate from plasma treated with EDTA, benzamidine and phenylmethylsulphonyl fluoride (PMSF) was resuspended in a buffer of 150 mM-NaCl, 50 mM-KH₂PO₄/K₂HPO₄, and 10 mM-EDTA, pH 7.4. This plasma fraction was then absorbed by passage over Sepharose 4B (Pharmacia), and the FN was purified by elution from gelatin-Sepharose with 1 M-arginine. All buffers used for chromatography and elution contained 5 mM-benzamidine, 1 mM-PMSF, and/or 25 mM-p-nitrophenyl p'-guanidinobenzoate to inhibit residual serine proteases. The purified FN showed a single line on immunoelectrophoresis against anti-whole human serum, and a single major band corresponding to 440 kDa on SDS-PAGE. Upon the reduction of disulphide bonds, SDS-PAGE revealed a closely spaced doublet, as has been reported previously for human plasma FN (Pommier et al., 1984). Antibodies raised against this FN preparation in rabbits and goats gave a monospecific response on immunoelectrophoresis and Ouchterlony double diffusion against whole human plasma.

**Preparation of [³⁵S]methionine-labelled extracellular material.** BCG organisms were first cultured on Lowenstein–Jensen medium at 37 °C, and then subcultured once. All daughter strains were grown in subculture for 5 d, then suspensions were washed three times with Earle's balanced salt solution (Gibco) and resuspended in methionine-free Eagle's minimum essential medium (Flow Laboratories) supplemented with 0.2% glutamic acid, 0.2% asparagine and 0.005% ferric ammonium citrate. Bacterial suspensions containing 2 × 10⁸ organisms and 10 μCi
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[35S]methionine (Amersham; specific activity 40–500 mCi mmol⁻¹, 1.5–18.5 GBq mmol⁻¹) were added to wells of flat-bottomed microtitre trays (Nunc). The trays were incubated for 4–5 d at 37 °C in 5% CO₂. The extent of bacterial proliferation during this period was not monitored. The cultures were then filtered on Millipore filters (Millex-GV, 0.22 μm) and the filtrates, which were free of bacteria, were stored at −70 °C. Nonradiolabelled extracellular material was prepared in an identical manner with unlabelled methionine.

Bacterial adherence assay. Bacterial adherence was determined by quantification of attached radiolabelled bacteria. This method measures FN binding capacity in that bacterial adherence was inhibited by anti-FN antibodies and trypsin pretreatment of bacteria.

Wells in 24-well Costar plates (not treated for tissue culture) were incubated with 125 μg FN ml⁻¹ for a minimum of 1 h at 37 °C. Control wells were coated with human serum albumin (HSA) at 125 μg ml⁻¹. The wells were then washed with normal saline and radiolabelled bacteria (approximately 5 × 10⁶ c.f.u. mycobacteria or 10⁷ c.f.u. S. aureus) were added. The number of c.f.u. was determined by measuring optical density at 520 nm and comparing it with a standard curve relating c.f.u. to OD₅₂₀ for each organism tested. Each set of conditions was assayed in quadruplicate. After addition of bacteria the plates were incubated for 3 h at 37 °C in 5% CO₂, then nonattached bacteria were removed by washing each well three times with PBS. Bound bacteria were detached by addition of 0.5 ml trypsin (500 μg ml⁻¹, plus 0.2 g EDTA 1⁻¹ without Ca²⁺ or Mg²⁺) to each well and incubating for an additional 0.5 h at 37 °C in 5% CO₂ in a humidified chamber. Bacteria thus detached were transferred to counting vials and the radiolabel quantified by liquid scintillation counting. The c.f.u. values reported in Fig. 2 were calculated from standard curves relating c.f.u. to radioactivity.

Binding of 35S-labelled extracellular material to FN. Flat-bottomed microtitre Removawells (0.32 cm² wells, Dynatech) were incubated with FN or HSA as described above. The wells were then washed and 50 μl labelled material (2 × 10⁶ c.p.m.) was added to each well. Assays were done in duplicate. Following incubation at 37 °C for 1 h, the wells were washed with PBS supplemented with 0.1% HSA, then dried, and bound isotope was quantified by liquid scintillation.

Affinity chromatography of mycobacterial supernatants. CNBr-activated Sepharose 4B (Sigma) was placed in 1.0 ml HCl for 20 min. The beads were washed with 100 ml 1.0 mm-HCl followed by 20 ml carbonate buffer (0.5 M, pH 9.0). FN (5 mg ml⁻¹) in carbonate buffer was added to 5 ml beads and incubated with constant mixing for 2 h at room temperature. After coupling, the beads were incubated with 0.2 M-glycine at room temperature for an additional 2 h, then washed alternately with acetate (0.2 M, pH 4.0) and borate (0.2 M, pH 8.5) buffers. The washed beads were placed in a column and equilibrated with 0.1 M-Tris, pH 7.0. HSA was linked to Sepharose 4B in a similar manner.

Samples (2 ml) of bacterial supernatants were applied to 3 ml columns of either FN-Sepharose or HSA-Sepharose beads and incubated for 30 min. The columns were then washed with equilibrating buffer (0.1 M-Tris). The first 2 ml of eluate from each column was collected and tested for inhibitory activity along with bacterial supernatants not exposed to protein-linked Sepharose beads.

Triton X-100 extraction. BCG organisms were labelled with [32P]orthophosphate, and S. aureus with [3H]thymidine, as described above. The organisms were washed three times in PBS, resuspended in 10 ml 2% (v/v) Triton X-100 and incubated for 1 h at room temperature with constant agitation. After incubation the bacteria were washed three times with PBS and assayed for adherence as described above. The bacteria retained most of the radiolabel after Triton X-100 treatment. Triton X-100 extraction of [3H]uracil-labelled BCG gave very similar results.

RESULTS

Characteristics of the bacterial surface component(s) that binds FN

In a previous report we showed that commercial preparations of Glaxo BCG had a lower FN-binding capacity than other BCG strains (Ratli et al., 1988). Since the commercial preparation of Glaxo BCG is grown in the presence of detergents, the possible effect of detergent on FN binding was tested (Fig. 1 a, b). Pretreatment of Armand Frappier BCG with Triton X-100 prior to exposure to FN-coated surfaces abrogated attachment. In contrast, the attachment of S. aureus to FN-coated surfaces was not affected by detergent pretreatment. These results suggested that the low FN binding by commercial Glaxo BCG preparations was due to the effects of detergent. Thus, Glaxo BCG was cultured in the absence of detergent and tested for FN binding (see Fig. 2). The data showed that when grown in the absence of detergent, Glaxo BCG bound to FN-coated surfaces as well as other BCG daughter strains.

Since S. aureus is known to attach to FN via a trypsin-sensitive surface protein (Espersen & Clemmensen, 1982; Ryden et al., 1983), we tested the effects of trypsin pretreatment on the
Fig. 1. Effects of pretreatment of organisms with trypsin or Triton X-100 on the attachment of Armand Frappier BCG and *S. aureus* to FN-coated surfaces. □, Untreated organisms; ☐, organisms treated with Triton X-100 (2%); ☐, organisms treated with trypsin (250 µg ml⁻¹). ■, Negative control (attachment of untreated organisms to HSA). Bars indicate SEM. *, P < 0.025.

Fig. 2. Survey of BCG substrains and other mycobacterial species for binding capacity to FN-coated surfaces. HSA binding ranged from 50 to 875 c.f.u. for all organisms tested and has been subtracted from the values reported.
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Expression of FN-binding properties by mycobacterial species

A survey of the binding capacity of BCG substrains and other mycobacterial species was performed. In this series of experiments all organisms were cultured under identical conditions in Youman's medium in the absence of detergents. All the organisms tested attached to FN-coated surfaces (Fig. 2). The relative binding capacity of the bacteria tested cannot be accurately determined from these data because attachment was assessed in independent assays and because of the variability in the growth characteristics of the organisms.

Secretion of an FN-binding protein by mycobacteria

A previous report showed that supernatants obtained from 4-5-d-old cultures of BCG substrains cultured in the presence of [35S]methionine contained labelled mycobacterial proteins and that the SDS-PAGE profiles of these proteins varied among BCG daughter strains (Abou-Zeid et al., 1986). We tested [35S]methionine-labelled supernatants for their capacity to bind to FN-coated surfaces. All the M. tuberculosis clinical isolates and BCG strains tested released [35S]methionine-labelled material that attached to FN-coated surfaces at levels significantly higher than to HSA-coated surfaces. For example, for M. tuberculosis H37Rv, and Glaxo and Japanese (Tokyo) BCG, about 550-650 c.p.m. (~5% of the added label) bound to FN-coated surfaces, whereas only 80-120 c.p.m. bound to HSA-coated surfaces. To determine whether the secreted FN binding protein(s) may have biological significance, supernatants were tested for their ability to inhibit BCG attachment to FN-coated surfaces (Fig. 3). Supernatants from BCG cultures inhibited the binding of BCG to FN-coated surfaces but did not affect the attachment of S. aureus. The inhibitory activity of the mycobacterial supernatants was removed by passage through an FN-linked Sepharose 4B column but was not affected by passage through an HSA-Sepharose control column (Fig. 4). These data suggest that the inhibitory activity was caused by the presence of an FN-binding component in the bacterial supernatant.

DISCUSSION

Previous reports have shown that most streptococci in groups A, C and G and most S. aureus strains bind to FN (Myhre & Kuusela, 1983; Switalski et al., 1983). Here we show that some fast- and slow-growing, virulent and nonvirulent mycobacterial species attach to FN-coated surfaces. These results suggest that the ability to bind FN is conserved within the genus Mycobacterium.

We previously showed that BCG attached selectively to FN-coated surfaces and that the attachment of BCG to the luminal surface of the bladder during immunotherapy for bladder
cancer was FN-mediated (Ratliff et al., 1987). In this report we tested BCG strains commonly used in immunotherapy for bladder cancer for their FN-binding capacity. Of the commercial preparations tested, only Glaxo BCG expressed minimal ability to bind to FN-coated surfaces. Since Glaxo BCG is cultured in the presence of detergent and the other BCG strains tested were not, we concluded that detergent affected the FN-binding capacity of the bacteria. This hypothesis was supported by the fact that Glaxo BCG cultured in the absence of detergent bound to FN-coated surfaces as effectively as the other BCG strains. Furthermore, pretreatment of Armand Frappier BCG with Triton X-100 eliminated the capacity of the organisms to bind to FN. In contrast, detergent pretreatment of S. aureus did not affect its FN-binding properties. It is not clear whether detergent treatment released the binding moiety or whether the detergent bound to the bacterial surface and blocked attachment.

Surface proteins which bind FN are present on S. aureus (Ryden et al., 1983), but are not affected by non-ionic detergents. The fact that trypsin treatment prevents binding of BCG to FN suggests that a peptide may be involved. The fact that [³⁵S]methionine will label an extracellular FN-binding component similarly suggests a peptide component to the FN-binding molecule of the mycobacteria. However, definitive determination of the binding molecule(s) awaits purification and characterization of these entities.

Soluble protein(s) capable of attaching to FN-coated surfaces are present in the supernatants of cultured mycobacteria. BCG supernatants containing the FN-binding protein(s) also inhibited the attachment of BCG to FN-coated surfaces. Moreover, the inhibitory activity was selectively removed from supernatants by FN-Sepharose affinity chromatography, suggesting that the FN-binding component mediates the inhibitory activity observed in the supernatants. Such soluble inhibitory component(s) have been observed in receptor-containing preparations of both Staphylococcus aureus and Streptococcus pyogenes (Speziale et al., 1984; Ryden et al., 1983). Two separate compounds have been observed to competitively inhibit FN binding by S. pyogenes: lipoteichoic acid (Courtney et al., 1983), and a papain-sensitive protein preparation that did not contain lipoteichoic acid (Speziale et al., 1984). Previous studies on the BCG supernatants have shown them to contain more than 30 distinct radiolabelled proteins on SDS-PAGE with molecular masses ranging from 14 to 200 kDa (Abou-Zeid et al., 1986). Additional studies to determine the characteristics of the FN-binding proteins are in progress.

The physiological significance of the FN-binding properties of the mycobacteria is not known. Studies on S. aureus and the streptococci suggest that attachment to FN is associated with the establishment of an infection (Vercellotti et al., 1984; Abraham et al., 1983). Moreover, FN has been shown to modulate the phagocytic activity of macrophages and polymorphonuclear leucocytes (Brown, 1986; David et al., 1985; Pommier et al., 1983). Whether any of

Fig. 4. Effects of FN-Sepharose affinity chromatography on the ability of supernatant (s/n) from cultured BCG (Armand Frappier) to inhibit binding of BCG to FN. Bars indicate SEM.
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these phenomena affect the mycobacterial/host relationship remains to be established, especially as saprophytic mycobacteria also bind to FN.

In conclusion, we have shown that the ability to bind to FN-coated surfaces is a property of all the mycobacterial species tested. The binding of mycobacteria to FN was inhibited by pretreatment with either trypsin or detergent. A functional FN-binding component, which inhibited the binding of intact mycobacteria to FN, was present in supernatants of cultured BCG, suggesting that the bacteria may secrete or shed their FN binding molecule(s) in vitro. This may aid in the identification and characterization of the mycobacterial binding molecule(s).

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