Characterization of Soluble Fibronectin Binding to Bacille Calmette–Guérin

By JABER ASLANZADEH,¹ ERIC J. BROWN,² SHAWN P. QUILLIN,¹
JULIE K. RITCHEY¹ AND TIMOTHY L. RATLIFF^{1*}

¹Washington University School of Medicine and the Jewish Hospital of St Louis,
Department of Surgery, Division of Urology, 216 South Kingshighway, St Louis,
Missouri 63110, USA

²Washington University School of Medicine, Department of Medicine and Microbiology and
Immunology, St Louis, Missouri 63110, USA

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Fibronectin (FN), a 420 kDa glycoprotein, consists of two similar subunits linked by a disulphide bond near the C-terminal end. FN is present in soluble and matrix forms in various body fluids and tissues and has been shown to bind to a variety of organisms. We characterized the conditions required for ¹²⁵I-FN binding to Bacille Calmette–Guérin (BCG). The binding was dose-dependent, reached saturation within 3 min, and was essentially irreversible for at least 24 h under optimal binding conditions at pH 6.0. In contrast, the binding was reversible (>90% in 24 h) when the pH was increased to 10.0. Scatchard analysis of the dose–response experiments produced a straight line, suggesting the presence of a single class of FN receptor on BCG. ¹²⁵I-FN binding was trypsin-sensitive, suggesting that the BCG-binding molecule is a protein. The number of FN receptors was determined to be 8000–15000 per bacterium. ¹²⁵I-FN binding was pH dependent, with maximal binding at acidic pH. ¹²⁵I-FN binding was sensitive to the presence of NaCl, with 0.08 M-NaCl inhibiting binding by 85%. These data demonstrate that soluble FN binds to a trypsin-sensitive cell-surface component of BCG in an essentially irreversible manner.

INTRODUCTION

Fibronectin (FN), a 420 kDa glycoprotein, consists of two similar subunits linked by a disulphide bond near the C-terminal end. It is present in soluble and matrix forms in various body fluids and tissues (Mosher, 1980). FN binds to specific receptors on eukaryotic cells such as macrophages and polymorphonuclear leucocytes (Wirth & Kierszenbaum, 1984; Proctor *et al.*, 1982*b*). FN interacts with macromolecules such as collagen, fibrin, heparin, heparin sulphate, dextran sulphate, DNA, actin and hyaluronic acid. In addition, FN has been reported to bind to bacteria including *Staphylococcus aureus*, group A, C, and G streptococci, *Treponema pallidum*, *Escherichia coli* and *Salmonella dublin* (Hynes & Yamad, 1982; Speziale *et al.*, 1984; Proctor *et al.*, 1982*a*).

The interaction between FN, both soluble and matrix, and staphylococci has been studied extensively by many investigators. It has been demonstrated that a 29 kDa FN fragment at the NH₂-terminus of the molecule contains the staphylococcal binding site. The binding is mediated through a bacterial surface protein with an apparent molecular mass of 210 kDa (Fröman *et al.*, 1987). The binding is believed to be specific, time-dependent and irreversible, and occurs to

*To whom reprint requests should be addressed at the Department of Surgery, the Jewish Hospital of St Louis, 216 South Kingshighway, St Louis, Missouri 63110, USA.

Abbreviations: BCG, Bacille Calmette–Guérin; FN, fibronectin.

heat-killed as well as to live organisms. Finally, the binding is saturable with as many as 30000 FN molecules per cell (Proctor *et al.*, 1982a).

Bacille Calmette-Guérin (BCG) have been shown to be the treatment of choice for superficial bladder cancer (Mori *et al.*, 1986; Catalona *et al.*, 1987). Ratliff *et al.* (1987) showed that in a mouse model the binding of BCG to the FN exposed on the bladder urothelium was an important initial step for BCG-mediated antitumour activity. *In vitro* experiments examining the interaction between FN and BCG revealed that BCG bound to FN-coated surfaces through a trypsin-sensitive bacterial surface protein. The binding was dose-dependent and could be blocked with antibody specific to FN (Ratliff *et al.*, 1988).

While intravesical BCG appears to be the best available treatment for superficial bladder tumours, the 70% response rate has room for improvement. It was hypothesized that at least some of the failures may have resulted from poor attachment of BCG to the bladder wall. One potential reason for poor BCG attachment may be the presence of soluble FN in the bladder. Animal studies demonstrated that soluble FN prevented the binding of BCG to the matrix FN exposed on the bladder wall (unpublished). In this report we describe the conditions required for the interaction of BCG and soluble FN.

METHODS

Chemicals. All chemicals were purchased from Sigma and were reagent grade unless otherwise stated.

Bacteria. BCG were obtained from Armand Frappier, Quebec, Canada, as a lyophilized preparation containing 10^7 c.f.u. mg^{-1} (manufacturer's specification). Before use, BCG was cultured in Youman's medium for 5 d at 37 °C. The bacteria were harvested by centrifugation and resuspended in buffer to produce approximately 7×10^8 c.f.u. ml^{-1} (determined by standard curves plotting OD₅₂₀ vs c.f.u.).

Preparation of ^{125}I -labelled FN. Human plasma FN was purified as described by Pommier *et al.* (1984). The purified FN, 1.5 mg ml^{-1} , was labelled for 15 min with 1 mCi (37 MBq) ^{125}I Na (Amersham) in a test-tube precoated with 200 μg Iodo-Gen (1,3,4,6-tetrachloro-3,6-diphenylglycouril). The labelled FN was then separated from unbound ^{125}I by chromatography on a 10 ml bed volume Sephadex G25 column. The specific activities of the labelled FN preparations were routinely between 10^6 and 10^7 c.p.m. μg^{-1} .

Binding assay. The binding assay was performed as described by Proctor *et al.* (1982a). Six micrograms of ^{125}I -FN (unless otherwise stated) was added to 1.5 ml volume microcentrifuge tubes (Eppendorf) precoated for 2 h with 1 ml of 1 mg ml^{-1} human serum albumin and containing 7×10^6 BCG suspended in 1.0 ml 0.1 M-Tris buffer, pH 6.0, unless noted otherwise. The ^{125}I -FN was mixed with either 300 μl unlabelled FN (1 mg ml^{-1}) or 300 μl of the appropriate buffer to determine non-specific and total binding, respectively. Specific binding was ascertained by subtracting non-specific from total binding. The reaction mixtures were incubated for 1 h at 22 °C unless noted otherwise. After incubation, the microcentrifuge tubes containing the reaction mixtures were centrifuged at 10000 g for 3 min in a Beckman Microcentrifuge B. The supernatant, containing free radiolabelled FN, was carefully removed. The microcentrifuge tubes were sliced and the pellets containing the radiolabelled FN bound to BCG were analysed for radioactivity. Control experiments using ^{125}I -FN without bacteria produced background counts of <500 c.p.m.

Effect of time on FN binding to BCG. To determine whether FN binding to BCG was saturable, binding assays were carried out as indicated above, except that the BCG/FN mixtures were incubated for 3, 5, 10, 20 and 60 min. After incubation, BCG were harvested, and specific FN binding was determined for each time point.

Effect of temperature on FN binding to BCG. The binding assay was carried out at 4, 22 and 37 °C. The BCG suspensions were preincubated at the appropriate temperature for 1 h to equilibrate the metabolic rate. Samples were taken after 1, 3 or 5 h of incubation.

Effect of pH on FN binding to BCG. The binding assay was conducted as described above. Bacteria were suspended in 1 ml of appropriate buffer, with a pH range of 3–10 (ammonium acetate buffer for pH 3–6 and Tris for pH 7–10). Total and non-specific binding was obtained for each pH by adding 300 μl of either corresponding buffer or unlabelled FN diluted in the appropriate buffer.

Effect of NaCl on FN binding to BCG. Binding assays were carried out as described above, utilizing BCG suspended in buffers supplemented with NaCl concentrations ranging from 0 to 3.0 M.

Reversibility of the BCG/FN interaction. The binding assay was performed at pH 6.0 as described above, except that BCG was irradiated [5000 rad (50 Gy) for 6 min] to prevent growth of bacteria and elaboration of proteases (Proctor *et al.*, 1982a). After centrifugation and removal of unbound radiolabelled FN, half of the samples were resuspended in 1.3 ml of the appropriate Tris buffer and the other half were resuspended in 1.3 ml of the same buffer containing 300 μg unlabelled FN. The bacterial suspensions were then incubated for 0 min, 5 min, 15 min, 1 h, 5 h or 24 h at either pH 6.0 or 10.0, after which specific binding was determined.

Saturability of the binding. BCG were suspended in buffer (pH 6.0). The binding assay was conducted as described above, using an increasing concentration of ^{125}I -FN (0–30 μg). The data obtained were plotted by the method of Scatchard (1949) and the number of fibronectin molecules bound per bacterium and apparent association constant were calculated from the x-axis intercept and slope, respectively.

Effect of trypsin on FN binding to BCG. BCG were incubated for 30 min in a suspension of trypsin (500 $\mu\text{g ml}^{-1}$)/EDTA at 37 °C. Trypsin inhibitor was added and the bacteria were washed in acetate buffer, pH 6.0, three times. These bacteria were subsequently utilized in the FN binding assay as described above.

RESULTS

Fig. 1 illustrates the effect of length of incubation on the specific binding of BCG to FN. Binding reached equilibrium rapidly (<3.0 min). Experiments on the effect of varying temperature on the interaction of BCG and FN indicated that the binding reached equilibrium by 1 h for all temperatures (4, 22 and 37 °C; data not shown). Subsequent binding assays were performed for 1 h at 22 °C for convenience.

Fig. 2 shows the effect of varying the pH on the binding of ^{125}I -FN to BCG. Optimal binding occurred within a pH range of 3.0–6.0. An identical optimal pH range was observed in three separate experiments. Minimal ^{125}I -FN bound at pH 7.0 or higher, suggesting that the ^{125}I -FN/BCG interaction may be reversible at high pH; this would aid in the purification of the BCG receptor molecule. To test this hypothesis we determined the effects of high pH on the reversibility of the ^{125}I -FN/BCG interaction. ^{125}I -FN binding to BCG was carried out at pH 6.0, and the bacterial pellet obtained from centrifugation was resuspended in buffer at pH 10.0 and incubated for various times. Specific ^{125}I -FN binding was reversible at pH 10, with >90% of label released in 24 h (Fig. 3*b*). The supernatant obtained after recentrifugation was also analysed for radioactivity and subjected to SDS-PAGE. The gel was dried and autoradiography was performed. The resultant protein band corresponded to that of FN, indicating that the bound label was indeed FN (data not shown). The reversibility of the ^{125}I -FN/BCG interaction when the pH was maintained within the optimal binding range is shown in Fig. 3(*a*). Less than 20% of the bound FN was released during 24 h incubation at pH 6.0.

To test the effect of salt on the binding of BCG to FN, BCG were suspended in buffer solutions (pH 6.0) supplemented with 0–3.0 M-NaCl (Fig. 4). The specific binding of BCG to

Fig. 1

Fig. 1. Effect of time on the binding of BCG to fibronectin.

Fig. 2

Fig. 2. Effect of pH on the binding of BCG to fibronectin.

Fig. 3. Reversibility of the binding of BCG to fibronectin during 24 h incubation at pH 6.0 (a) and pH 10.0 (b).

Fig. 4. Effect of NaCl in the concentration range 0.15–3.0 M (a) and 0.01–0.08 M (b) on the binding of BCG to fibronectin.

FN was inhibited at NaCl concentrations as low as 0.08 M. Similar results were obtained with urine obtained from volunteers. The salt concentrations of the three urine specimens were: (1) 70 mM-Na; 90 mM-K; (2) 84 mM-Na, 40.5 mM-K; (3) 70 mM-Na, 98.8 mM-K (data not shown).

In order to determine the saturability of BCG/FN binding a dose-response experiment was conducted utilizing 0–30 μg of ${}^{125}\text{I-FN}$ in the presence and absence of excess unlabelled FN. The results (Fig. 5) showed that the binding between BCG and FN is dose-dependent and saturable. The number of binding sites per bacterium was ascertained by extrapolation to the asymptote of the dose-response curve. Calculations by this method indicated that approximately 8000 molecules of FN were bound per bacterium. The data were then plotted by the method of Scatchard (Pommier *et al.*, 1984), and the results suggested that there are a maximum of 15000 molecules of FN bound per bacterium (Fig. 5, inset). Furthermore, the appearance of a straight line indicated a single FN receptor on BCG (Scatchard, 1949).

After trypsin pretreatment of BCG, the specific binding of ${}^{125}\text{I-FN}$ to BCG was inhibited by 70%, from 56.0×10^4 to 17.7×10^4 c.p.m. These data suggest that the FN receptor on BCG may be a trypsin-sensitive protein.

Fig. 5. Saturability of the binding of BCG to fibronectin and (inset) Scatchard analysis of the binding. Bars indicate SEM.

DISCUSSION

Morales *et al.* (1976) demonstrated a significant reduction in the recurrence of superficial bladder tumours following combined intravesical and systemic administration of BCG. Since the original observation, BCG has been used extensively for the treatment of superficial bladder cancer and is now considered by many to be the treatment of choice for this disease (Mori *et al.*, 1986). The exact mechanisms by which BCG mediates antitumour activity are not well understood. However, it has been known for many years that BCG is a potent inducer of delayed hypersensitivity and will activate macrophages and cause them to secrete tumour necrosis factors (Old, 1985).

In vitro studies of the interaction between matrix FN and BCG have been studied extensively in our laboratory because of its possible role in adjuvant BCG immunotherapy for superficial bladder cancer. Our results suggest that BCG attachment to FN within the bladder is a requisite step in the initiation of the antitumour response (Ratliff *et al.*, 1987). FN is observed at the basement membrane and in the submucosa but is absent from the apical surface of the urothelium (Pode *et al.*, 1986). Previous *in vivo* studies have shown that BCG binds specifically to a damaged epithelium where the basement membrane is exposed or a fibrin clot is present (Ratliff *et al.*, 1987). Under such conditions the presence of soluble FN at the site of damage could result in the preferential binding of soluble FN to BCG, which may lower the attachment of BCG to matrix FN in the bladder wall. A potential decrease in attachment could reduce the efficacy of the treatment regimen. Therefore, a better understanding of the interaction between soluble FN and BCG is important for obtaining appropriate clinical results.

The binding of soluble FN to BCG was similar to the interaction previously described for *Staphylococcus aureus* and *Streptococcus pyogenes* (Proctor *et al.*, 1982a) in that the interaction was most probably receptor mediated, rapidly saturable, reaching equilibrium within 3 min, and essentially irreversible. Saturation occurred within 1 h for temperatures ranging from 4 to 37 °C. Furthermore, over 80% of the bound FN remained bound at pH 6.0 for at least 24 h whereas less than 10% remained bound after 24 h when the pH was raised to 10.

S. aureus expresses 7000–30000 receptors per bacterium, with an association constant of 5×10^{-9} M (Proctor *et al.*, 1982a). Scatchard analysis of the FN/BCG interaction demonstrated similar characteristics. Approximately 8000–15000 receptors per bacterium were observed, and the association constant was approximately 9×10^{-9} M. The Scatchard analysis produced a straight line, suggesting that one class of receptor is present.

The FN receptor of *S. aureus* is believed to be a 210 kDa protein which mediates the binding of the organism to a 29 kDa fragment of the molecule at the NH₂-terminus (Fröman *et al.*, 1987). *S. pyogenes* type M5 was clearly shown to bind to a fatty acid binding site on the molecule

through lipoteichoic acid present on the bacterial cell wall (Courtney *et al.*, 1983). However, a different strain of this organism, *S. pyogenes* 1321, was shown to bind to FN through a trypsin-extractable and papain-sensitive surface protein (Speziale *et al.*, 1984). The matrix FN receptor of BCG has been previously shown to be a trypsin-sensitive protein (Ratliff *et al.*, 1988). The loss of the ability of BCG to bind soluble FN after treatment with the protease trypsin indicated that the soluble FN receptor on BCG was also trypsin-sensitive, suggesting that it may be a protein. However, no attempt was made to investigate the structure or composition of this receptor further, or its relationship to the matrix form of the FN receptor.

Binding of FN to BCG differed from that of *S. aureus* in several ways. Proctor *et al.* (1982*a*) showed that NaCl enhanced the interaction between *S. aureus* and FN. In contrast, FN binding to BCG was inhibited by the presence of as little as 0.08 M-NaCl. The inhibitory effects of NaCl were uniformly observed over the optimal pH range of 3–6. In addition, the pH range for FN binding to BCG differed from that observed for *S. aureus*. BCG bound optimally to FN at an acidic pH (pH range 3–6), and a low level of binding occurred at neutral or basic pH. The optimal pH observed for *S. aureus* was 6.0 but binding was absent below pH 5.0 (Proctor *et al.*, 1982*a*). Significant ^{125}I -FN binding was observed for BCG at pH 3.0. The inhibitory effect of pH and salt on the binding of soluble FN to BCG was not observed for the interaction between BCG and matrix FN (data not shown). The reason for the differences is not known.

FN bound to BCG at low pH could be eluted by reincubation of the organism at higher pH. This property of the BCG/FN interaction was utilized to elute radiolabelled FN from BCG. The eluted protein was subjected to gel electrophoresis and autoradiography to demonstrate that the bound label was indeed FN (data not shown).

Since urine contains significant salt levels, the ^{125}I -FN binding assay was performed in the presence of human urine to determine its effect on the binding of soluble FN to BCG. Urine inhibited ^{125}I -FN binding to BCG. The inhibition may have been associated with the salt concentration. Previous work in our laboratory has shown that urine does not interfere with the binding of BCG to matrix FN (data not shown). Moreover, unpublished data show that binding of soluble FN to BCG prior to intravesical instillation inhibits attachment of BCG to the bladder wall. These data suggest that the diluent for BCG should have a neutral or basic pH and should be supplemented with a minimum of 0.08 M-NaCl. Such a diluent would decrease the potential inhibition of BCG attachment to the bladder wall by minimizing the binding of soluble FN.

In conclusion, the binding of ^{125}I -FN to BCG appears to be receptor-mediated. A single class of receptors is present and it is most probably protein in nature. The interaction between ^{125}I -FN and BCG is saturable and essentially irreversible with an association constant of 9×10^{-9} M. Approximately 8000–15000 receptors are present on each bacterium. Both salt and basic pH inhibit binding. Studies are in progress to characterize the BCG receptor more clearly.

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