Intravesical Bacillus Calmette-Guérin Therapy for Murine Bladder Tumors: Initiation of the Response by Fibronectin-mediated Attachment of Bacillus Calmette-Guérin

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ABSTRACT

Intravesical Bacillus Calmette-Guérin (BCG) is considered to be one of the most effective treatments for superficial bladder cancer. Although the mechanisms by which BCG inhibits tumor growth are not known, previous studies have shown that systemic immunization to BCG and the local expression of the immune response in the bladder are associated with a favorable response to BCG therapy. We have investigated the conditions required for the initiation of an immunological response after the intravesical instillation of BCG. Initial histological studies showed that BCG attached to the bladder wall only in areas where the urothelium was damaged by electrocautery and suggested that attachment was associated with the fibrin clot. Quantitative studies verified the histological observations. Minimal BCG attachment (mean <10^2 colony forming units/bladder) was observed in normal bladders in contrast with a mean of 1.42 ± 10^4 colony forming units/bladder in bladders damaged by electrocautery (10 separate experiments). BCG attachment to the bladder wall was durable since organisms were observed in bladders 48 h after instillation. To investigate the proteins to which BCG attached, we tested the binding of BCG to extracellular matrix and inflammatory proteins which comprise a significant portion of the fibrin clot. BCG bound in vivo to coverslips coated in vivo with extracellular matrix proteins but did not bind to control albumin-coated coverslips. BCG also bound to coverslips coated with purified plasma fibronectin but not to coverslips coated with other purified extracellular matrix proteins including laminin, fibrinogen, and type IV collagen. BCG attachment to coverslips coated with either extracellular matrix proteins or purified fibronectin was inhibited by antibodies specific for fibronectin. Moreover, BCG attachment to cauterized bladders in vivo was inhibited by antifibronectin antibodies. These results demonstrate that fibronectin mediates the attachment of BCG to surfaces and suggest that it is the primary component mediating attachment within the bladder. Moreover, the data suggest that the BCG-fibronectin interaction may be a requisite first step for the initiation of the antitumor activity in intravesical BCG for bladder cancer.

INTRODUCTION

Treatment of superficial transitional cell carcinoma of the bladder with intravesical BCG alone has been shown to be an effective treatment for superficial bladder cancer (1–5). In the clinical studies, viable BCG organisms are instilled into the bladder via a catheter once each week for a minimum of 6 consecutive weeks. Such administration eliminates existing tumors and prevents subsequent tumor recurrence. Systemic sensitization to BCG, granulomatous inflammation, and in some cases systemic BCG infections have been observed to occur concomitant with antitumor activity (1, 3, 5). The mechanism(s) by which BCG mediates antitumor activity is not known.

A systemic immune response is often associated with intravesical BCG therapy. A previous report showed that 44% of patients treated with 6 weeks of intravesical BCG converted from PPD skin test negative to positive (5). In addition, granulomatous inflammation in bladder biopsies was observed in 65% of these patients (5). Other investigators also have reported both PPD conversion and granulomatous inflammation in the bladder after intravesical BCG therapy (1, 3). Some investigators (2, 5) have suggested that the development of either a delayed type hypersensitivity to PPD or granulomatous inflammation of the bladder correlate with a favorable antitumor response, although these observations remain controversial (6).

Studies in an animal bladder tumor model also demonstrated that intravesical BCG alone inhibited bladder tumor growth and resulted in the development of delayed type hypersensitivity to PPD (7). Moreover, adoptive transfer studies in athymic mice showed that BCG inhibited bladder tumor growth only in mice receiving splenocytes containing T-lymphocytes, suggesting that T-lymphocytes were required for BCG-mediated antitumor activity (8). Thus, both clinical and animal studies suggest that immunological responsiveness to BCG and the local expressions of immunity within the bladder may be required for antitumor activity.

A requisite first step in the initiation of an immunological response is introduction of the antigen to the immune system. The mechanisms by which BCG gains exposure to the immune system after intravesical instillation are not known. Since a significant correlation between the development of immunological responsiveness to BCG and a therapeutic response has been demonstrated, a clear understanding of the molecular mechanism(s) involved in the presentation of BCG to immunological cells, and the subsequent induction of a local and systemic immune response may provide a basis for enhancing therapeutic results.

We have begun studies to determine the mechanism(s) by which the immunological and antitumor responses to intravesical BCG is initiated. The results reported herein suggest that the first step in the intravesical immunization process and the subsequent antitumor response is FN-mediated attachment of BCG to the bladder wall.

MATERIALS AND METHODS

Bacteria. BCG were obtained from Armand Frappier, Quebec, Canada as a lyophilized preparation containing 10^7 CFU/mg. Before use, BCG were resuspended in 0.1 M PBS, pH 7.4 to the desired concentration.

In Vivo Adherence of BCG. Intravesical BCG instillation was performed as previously described (7). Briefly, mice (C3H/HeN, Charles River) were anesthetized with Nembutal, i.p. (0.05 mg/g animal weight). Anesthetized mice were secured on a grounded plate and an electrode consisting of an Amplatz curved wire guide (Cook, Inc.,...
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Bloomington, IN), insulated by a 24-gauge Teflon sheath (Vicra, Division of Travenol Laboratory, Inc.) was inserted through the urethra into the bladder. The electrode was attached to a Bovie electrocautery unit. While the tip of the electrode was inside the bladder, the cautery into the bladder. The electrode was attached to a Bovie electrocautery insertion of Travenol Laboratory, Inc.) was inserted through the urethra was removed. Controls included cauterized mice with instillation of PBS and noncauterized mice with instillation of BCG. Bladders were surgically removed 30 min after BCG instillation, washed extensively in PBS, and examined histologically or used to quantitate adherent BCG (see below).

BCG Quantitation. Washed bladders were placed in 6 ml of collagenase (1 mg/ml), minced with scissors, and digested in a spinner flask for 40 min at 37°C. BCG CFU were quantitated as described in Section 620.44 of the Federal Register (9). The supernatant was diluted in Youman's medium in serial 1:3 dilutions, and the mixture was solidified with 0.5% agar. Dilutions resulting in 10–50 colonies after 3 weeks of incubation at 37°C were used for quantitating results.

Coating Coverslips in Vivo with ECM and Inflammatory Proteins. Coverslips (0.5 x 1.0 cm) were placed in polypropylene tubes (trimmed to approximately 8 x 15 mm) made porous by punching approximately 50 times with a sharp object. The open end of the tubes was sealed with silicone type A medical adhesive (Stoertz, St. Louis, MO). Tubes containing the coverslips were implanted s.c. in anesthetized mice. The coverslips were harvested 3–4 weeks later (ECM-coated foreign body implants), and the supernatant was cultured on blood agar plates. Only sterile preparations were used for adherence studies.

Adherence of Purified ECM Proteins to Coverslips. Coverslips (0.5 x 1.0 cm) were cleaned with 0.1 N HCl, rinsed, and incubated for 1 h at 37°C in 1.0 ml 0.15 M NaCl (normal saline) containing 120 µg/ml of the respective purified protein. After incubation the coverslips were washed with normal saline and used in adherence assays. Control coverslips were coated with either bovine serum albumin or human serum albumin (Sigma Chemical Co.) in an identical manner.

Radiolabeling of Bacteria. BCG (120 mg of the manufacturer’s lyophilized preparation) were cultured for 7 days in 120 ml Youman’s medium at 37°C in 5% CO2. Bacteria were washed in PBS and resuspended in 10 ml of a 1:10 dilution of heart infusion broth (Difco) as previously described (10). 32P (32P; Amersham) was added to a final concentration of 20 µCi/ml. The bacteria were incubated for 1 h at 37°C, washed 3 times in PBS supplemented with 0.1% human serum albumin, and resuspended to 106 CFU/ml in PBS.

Staphylococcus aureus Woods strain (ATCC 10832) were cultured in RPMI 1640. Log phase cells were washed, resuspended in 10 ml Medium 199 containing 100 µCi [3H]thymidine, and incubated for 3 h at 37°C. Bacteria were washed and resuspended to 106 CFU/ml in PBS.

Coverslip Adherence Assay. Coverslips were submerged in 1.0 ml of bacterial suspension and incubated for 1 h at 37°C. Coverslips were removed, washed extensively in normal saline, and bound bacteria were quantitated by either staining for acid fast bacilli or liquid scintillation counting for radiolabeled bacteria. Radioisotope assays were performed in triplicate while acid fast-staining assays were performed in duplicate. Adherent bacteria stained by the acid fast method were counted in a minimum of 10 separate fields for each coverslip, and adherence is reported as the mean number of bacteria per oil immersion field.

Extracellular Matrix Proteins. Human plasma FN was purified as previously described (11). Briefly, the 10% polyethylene glycol 3350 precipitate from EDTA, benzamide, and phenylmethylsulfonyl fluoride-treated plasma 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 adsorbed by passage over Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and the FN was purified by elution from gelatin-Sepharose with 1 M arginine. Further purification was obtained by adsorption to and elution from, arginine-Sepharose. All buffers used for chromatography and elution contained 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and/or 25 µM p-nitrophenyl p-amidino benzoate to inhibit residual serine proteases. The purified FN showed a single line on immunoelectrophoresis versus anti-whole human serum, and a single major band at M, 440,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Upon the reduction of disulfide bonds, sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a closely spaced doublet, as has been reported previously for human plasma FN (12). Antibodies raised against this FN preparation in rabbits and goats gave a monospecific response on immunoelectrophoresis and Ouchterlony double diffusion against whole human plasma.

Type IV collagen and laminin were purified as previously described (13) and were the kind gift of Dr. Hynda Kleinman (NIIH). Fibrinogen was purchased from Sigma Chemical Co. (Catalog No. F4753).

Inhibition of BCG Binding to Cauterized Bladders by Anti-FN. Mice were anesthetized, catheterized, and the bladder was cauterized as described above. Goat anti-FN antibody and normal goat serum were instilled into respective bladders for 30 min. Serum was removed and BCG was instilled and quantitated as described above. Three mice were included in each experimental group. The results were reproducible in 2 separate experiments, and are reported as a single representative experiment.

Table 1 Effect of bladder urolhelial damage on BCG adherence

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.67</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>7.0</td>
<td>1.7</td>
<td>4.4</td>
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<td>0.42</td>
<td>0.21</td>
<td>0.42</td>
<td>31</td>
<td>82</td>
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</tbody>
</table>

* Bacterial attachment is reported as the number of CFU x 106.

Fig. 1. Time-course analysis of the presence of BCG in cauterized bladders. Each time point included 3 mice and the data were reproducible in 2 separate experiments.

Fig. 2. Effect of trypsin and anti-FN antibody on the attachment of BCG to coverslips coated in vivo with extracellular matrix proteins. * Coverslips coated with bovine serum albumin (BSA) as described in "Materials and Methods." * Coverslips implanted s.c. and processed as described in "Materials and Methods." In this experiment coverslip pretreatment with preimmune goat serum had no effect on BCG attachment while antifibronectin antibodies inhibited attachment (see Figs. 4 and 6).
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Fig. 3. Effect of coating coverslips with varying concentrations of purified FN on BCG binding. The results were reproducible in 3 experiments. *Control coverslip coated with bovine serum albumin as described in "Materials and Methods."*

<table>
<thead>
<tr>
<th>Coating Treatment before BCG Addition</th>
<th>Coverslip Coating</th>
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<tr>
<td>CONTROL 5 SERUM</td>
<td>BSA*</td>
</tr>
<tr>
<td>ANTI-FIBRONECTIN ANTIBODY (1:100)</td>
<td>FN*</td>
</tr>
<tr>
<td>ANTI-FIBRONECTIN ANTIBODY (1:500)</td>
<td>FN</td>
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<tr>
<td>ANTI-FIBRONECTIN ANTIBODY (1:2500)</td>
<td>FN</td>
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Fig. 4. Effect of anti-FN antibody on the binding of BCG to coverslips coated with purified FN. *Coverslips coated with bovine serum albumin (BSA) as described in "Materials and Methods."* *Coverslips incubated with 120 µg/ml purified FN overnight.* Preimmune goat serum.

**RESULTS**

*In Vivo* Adherence of BCG. Histological studies were performed to evaluate BCG retention and attachment after intravesical instillation. The results showed that acid fast bacilli were present only in areas where the urothelium was damaged by electrocautery (data not shown).

Quantitative studies were performed to confirm the subjective histological data. Table 1 shows the effects of urothelial damage on the intravesical attachment of BCG. Electrocautery consistently resulted in elevated BCG colony counts. BCG was detected in only 2 of 10 experiments in which the bladder urothelium lacked intentional damage and no adherent BCG were detected in the other 8 experiments. In all experiments colony counts for bladders damaged by electrocautery exceeded noncauterized bladders. Statistical evaluation of the data showed that the CFU of BCG observed in cauterized bladders was significantly (P < 0.001; Student’s t test) greater than those observed in noncauterized bladders. At 95% confidence limits, 8–132 times more BCG bound to cauterized than to noncauterized bladders.

Because the *in vivo* adherence assays were performed 30 min after BCG instillation, the stability of the attachment of BCG to the bladder was not known. Further studies were performed to determine whether BCG attachment was transient or long lasting. Bladders were removed and CFU were quantitated at various times after instillation (Fig. 1). The results show that BCG were detectable in bladders through 48 h, demonstrating that the attachment of BCG to the bladder was durable.

Attachment of BCG to Coverslips Coated in Vivo with ECM and Inflammatory Proteins. Since the histological data suggested that BCG attachment was associated with the fibrin clot, we initiated *in vitro* studies to evaluate the mechanisms of BCG binding. Foreign body implants (coverslips placed in porous polypropylene tubes and implanted s.c. for 3 weeks) which are known to be coated with ECM proteins were tested for their ability to bind BCG as described for *Staphylococcus aureus* (14). Coverslips coated with bovine serum albumin were used as controls. The data show that BCG attached to ECM-coated foreign body implants but not bovine serum albumin-coated coverslips (Fig. 2). Pretreatment of ECM-coated foreign body implants with trypsin (500 µg/ml for 1 h at 37°C) eliminated BCG binding (Fig. 2). Moreover, pretreatment of ECM-coated foreign body implants with polyclonal antifibronectin antibodies also abrogated BCG binding (Fig. 2). These data suggest that fibronectin is necessary for BCG attachment to ECM-coated foreign body implants and that it could be a mediator of the *in vivo* attachment of BCG to the bladder.

*In Vitro* BCG Attachment to Coverslips Coated with Purified ECM Proteins. Fig. 3 shows that BCG attached to surfaces coated with purified FN in a dose-dependent manner demonstrating that FN alone is sufficient for mediating BCG adherence. As expected, FN-mediated attachment of BCG was inhib-
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itted by anti-FN antibody in a concentration-dependent manner (Fig. 4). The ability of BCG to attach to other purified ECM proteins including laminin, type IV collagen, and fibronogen also was tested (Fig. 5). BCG bound only to FN in contrast to S. aureus which bound to all ECM tested, as has been previously reported (15, 16).

Effect of Anti-FN Antibodies on BCG Attachment to Cauterized Bladders. The in vitro binding studies to the respective purified ECM proteins suggested that FN was both necessary and sufficient for BCG attachment to the coverslips coated in vivo with ECM and inflammatory proteins, and further suggested that FN might mediate the in vivo attachment of BCG to the bladder wall. To test the relevance of these observations for the binding of BCG to cauterized bladders, the effects of anti-FN antibodies on the in vivo attachment of BCG was studied. The results show that pretreatment of cauterized bladders with anti-FN antibodies inhibited the in vivo attachment of BCG but preimmune goat IgG did not (Fig. 6). Taken together these in vivo and in vitro studies suggest that FN mediates binding of BCG to the bladder wall.

DISCUSSION

Fibronectins are a family of glycoproteins found in soluble form in plasma and other body fluids and in an insoluble form in connective tissue and basement membranes (17). Plasma fibronectin has a molecular weight of approximately 440,000 and is composed of two homologous but non-identical disulfide-linked polypeptide chains (17). Fibronectins are major participants in adhesion of both eukaryotic and prokaryotic cells to other cells and substrates, and in the regulation of cell locomotion and morphology (17).

The role of FN in mediating attachment of BCG to the bladder wall is supported by several studies reported herein. In vitro studies demonstrated that FN was important in the attachment of BCG to coverslips coated in vivo with ECM proteins since the attachment was inhibited by anti-FN antibodies. Further studies showed that FN alone was sufficient for the attachment of BCG but other ECM proteins were not. Finally, anti-FN antibodies inhibited the in vivo attachment of BCG to cauterized bladders by >95%, suggesting that FN is the primary mediator of BCG attachment to the bladder wall.

It is well established that FN binds to collagen and fibrin and is a major constituent of healing wounds (18). Pode et al. (19) have analyzed the distribution of FN in normal human bladder mucosa. FN was absent from the apical surface of epithelial cells but was observed at the basement membrane and in the submucosa. The FN distribution suggests that FN-mediated binding of BCG can occur only in the presence of a disrupted urothelium. This hypothesis is supported by our in vivo binding studies in which BCG attached primarily to damaged urothelial surfaces. Either extracellular matrix FN or plasma FN incorporated into the fibrin clot could contribute to the binding of BCG to the damaged urothelium. The relative binding roles of FN from these two sources remain to be determined.

The attachment of BCG to the bladder wall via FN may be important to the antitumor activity of BCG for several reasons. It may function in a passive manner as an attachment matrix that mediates retention of BCG, and thus allows induction of an immune response to the organism as with other foreign invaders. The expression of this anti-BCG immune response within the bladder may then eliminate the tumors. FN also could play a more active role in the induction of the immune response and expression of antitumor activity. Since FN may be opsonic (20), it may direct BCG to macrophages for processing and presentation to the immune system. Furthermore, by entering the macrophages complexed to BCG, FN may become a target of the immune response as an "altered self" antigen. Since FN is important for tumor cell attachment within the bladder, an immunological response to FN could result in the elimination of the tumor cells.

This is the first report of a mycobacterial-FN interaction. Other bacteria including staphylococci and streptococci have been shown to bind to FN while Gram-negative bacteria such as Escherichia coli do not (21-26). The molecular interactions of S. aureus and streptococci with FN have been investigated. While S. aureus binds FN via a cell surface protein, streptococci apparently interact with FN via exposed glycolipids (21-26). Although the nature of the binding molecule on BCG has not been determined, preliminary experiments in our laboratory show that the binding of BCG to FN-coated surfaces is inhibited by pretreatment of BCG with either protease or detergent. The molecular details of the FN-binding molecule on BCG are currently under investigation.

The FN-BCG interaction may have implications beyond its apparent effects on immunotherapy for bladder cancer. Since FN can bind to phagocytes as well as BCG and has been shown to enhance phagocytosis (27), it may modify ingestion of BCG by macrophages by either acting as an opsonin or by direct effects on the macrophage. Such modulation of the BCG-macrophage interaction could alter BCG uptake, intracellular replication, and/or antigen presentation. Thus, the interaction may play a role in the host's defense against the intracellular organisms which in itself may be a subject of potential clinical relevance.

In summary, our studies demonstrate that FN mediates attachment of BCG to surfaces in vitro and suggest an important effector role in BCG attachment to epithelial surfaces in vivo. The data suggest that FN-mediated attachment of BCG to the bladder wall is a requisite first step in the initiation of the antitumor response. Furthermore, this observation may have important implications for mycobacterial colonization and invasion in general.

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Unpublished observation.

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