Fibronectin-mediated Calmette-Guerin Bacillus Attachment to Murine Bladder Mucosa

Requirement for the Expression of an Antitumor Response

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Abstract

Adjuvant intravesical Calmette-Guerin bacillus (BCG) is an effective treatment for superficial bladder cancer. The mechanisms by which BCG mediates antitumor activity are not known. We investigated the initial interaction of BCG with the bladder mucosa to determine whether binding was essential for the development of antitumor activity. Herein, we show that bladder urothelial disruption induced by acrolein, adriamycin, or electrocautery resulted in BCG binding in areas of urothelial damage. Binding induced by each method was inhibited by anti-fibronectin (FN) antibodies but not by antibodies to the basement membrane component laminin. Intravesical BCG binding also was inhibited by pretreating BCG with soluble FN. Inhibition of intravesical FN-mediated BCG attachment prevented immunization via the intravesical route. Moreover, the expression of both delayed hypersensitivity in the bladder of BCG-immunized mice and antitumor activity was inhibited by blocking FN-mediated intravesical BCG attachment.

These data suggest that intralumenal attachment of BCG appears to be mediated by FN. Moreover, these data suggest that intravesical FN mediated attachment of BCG is a requisite step in BCG-mediated antitumor activity in the murine bladder tumor model. (*J. Clin. Invest.* 1990. 85:62–67.) Calmette-Guerin bacillus therapy • bladder therapy • immunotherapy

Introduction

In 1976, Morales and associates (1) first reported the use of intravesical Calmette-Guerin bacillus (BCG) in the treatment of superficial bladder tumors (1). Since their initial report, several investigators have confirmed the efficacy of intravesical BCG. Prospective, randomized studies have shown intravesical BCG to be superior to endoscopic resection alone or intravesical chemotherapy with either thiotepa or adriamycin (2–4).

Although BCG has been extensively used in the treatment of superficial bladder tumors, little is known about the mechanisms by which it mediates antitumor activity. BCG has a significant stimulatory effect on the immune response, specifically the reticuloendothelial system (5–7). This enhancement has been associated with inhibition of tumor growth in several animal models (8). Experimental studies using the mouse bladder tumor, MBT-2, also have suggested that immune mechanisms are important in BCG-induced antitumor activity (9–12). In human trials, intravesical treatments have been associated with the purified protein derivative skin test conversion, granulomatous inflammation in bladder biopsies, and detection of interleukin 2 in urine specimens (13–15). These findings are suggestive of a BCG-induced, cell-mediated immunological reaction associated with the antitumor activity of intravesical BCG.

To understand better the process by which BCG induces an antitumor response, studies were begun to determine the progression of events that lead to immunization and expression of antitumor activity. Initial studies showed that in the murine system BCG attached to areas of the bladder wall damaged by electrocautery but not to intact urothelium (16). Further studies showed that BCG attached to fibronectin (FN)[1] both in vitro and in vivo (17). The functional importance of the BCG–FN interaction was not established.

Herein, we demonstrate that BCG attachment to FN exposed and/or deposited on the lumenal surface of the bladder is necessary for the induction and functional expression of an immune response and for the expression of antitumor activity. In addition, we show that adriamycin and other chemical agents induce conditions that allow intravesical FN-mediated BCG attachment. Such agents in combination with BCG may enhance the efficacy of intravesical bladder tumor therapy.

Methods

Bacteria. Pasteur strain BCG (Armand Frappier, Quebec, Canada) was obtained as a lyophilized preparation containing 10^7 colony forming units (CFU)/mg. One ampule of 120 mg of BCG was resuspended in 100 ml of Youman's medium and cultured for 7 d at 37°C in 5% CO_2.

Radiolabeling of bacteria. After being cultured for 7 d, bacteria were centrifuged at 2,500 rpm for 20 min and resuspended in 4 ml of RPMI 1640 (Kansas City Biologicals, Lenexa, KS) supplemented with 0.2% L-glutamine, 0.2% asparagine, and 0.5% ferric ammonium citrate (Sigma Chemical Co., St. Louis, MO). [^{3}H]uracil, 100 μCi, (American Radiolabeled Chemicals, St. Louis, MO; sp act 20 Ci/m mol) was added to the culture which was divided into two 35-mm petri dishes (Costar, Cambridge, MA) and incubated for 48 h at 37°C in 5% CO_2. Radiolabeled bacteria were washed twice with and resuspended in phosphate-buffered saline (PBS), pH 7.4, to the desired concentration. The number of CFU/ml was determined by measuring the optical density at 595 nm and comparing the absorbance with a standard curve quantitating CFU from optical densities.

Fibronectin. Human plasma FN was purified as previously described (18). Briefly, a 10% polyethylene glycol 3350 precipitate from

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1. *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; FN, fibronectin; UDR, deoxyuridine.

EDTA, benzamidine, and phenylmethylsulfonyl fluoride-treated plasma fraction was then incubated with gelatin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), and FN was eluted with 1 M arginine. All buffers used for chromatography and elution contained 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 25 U/M *p*-nitro-phenyl *p*-amidinobenzoate (Sigma Chemical Co.) 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 kD on SDS-PAGE. Under reduced conditions, SDS-PAGE revealed a closely spaced doublet.

Antibodies. Antibodies to FN were prepared in rabbits and goats and gave a monospecific response on immunoelectrophoresis and Ouchterlony double diffusion against whole human plasma (18). Antilaminin was a gift from Dr. Hynda Kleinman, National Institutes of Health.

In vivo adherence of BCG. Mice (C3H/HEN, Charles River Breeding Laboratories, Inc., Wilmington, MA) were anesthetized with Nembutal, i.p. (0.05 μg/g animal weight). Anesthetized mice underwent electrical or chemical mucosal disruption.

Electrical disruption was accomplished by securing the anesthetized mice on a grounded plate and passing an electrode consisting of an amplatz curved guide wire (Cook, Inc., Bloomington, IL), insulated by a 24-gauge Teflon sheath (Vicra, Division of Travenol Laboratory, Inc., Deerfield, IL) through the urethra into the bladder. A Bovie was attached to the electrode inside the bladder and activated for 4 s at the lowest coagulation setting.

Chemical disruption was accomplished by catheterizing anesthetized mice with a 24-gauge Teflon catheter and instilling either 0.1 ml of adriamycin (Adria Laboratories, Inc., Columbus, OH) weekly for 3 wk or 0.1 ml of acrolein (Eastman Kodak Co., Rochester, NY) 30 min before the binding assay. The concentrations of acrolein utilized ranged from 6.24 to 1.56 μg/ml and doses of adriamycin ranged from 2 to 8 μg/ml. Acrolein at a concentration of 3.12 μg/ml and adriamycin at a dose of 8 μg/ml demonstrated sufficient mucosal disruption and were utilized in BCG attachment assays. Controls consisted of mice that did not undergo mucosal disruption but were catheterized and flushed with 0.1 ml of PBS.

After mucosal disruption 0.1 ml containing 5×10^7 CFU/ml of either radiolabeled or unlabeled BCG were instilled into the bladder and allowed to incubate for 30 min. Bladders were surgically removed and washed extensively by inverting the mucosa and submersion in PBS. In experiments utilizing radiolabeled BCG, bladders were minced in 10 ml of Scinti Verse (Fisher Scientific Co., Pittsburgh, PA) and adherence was determined by measuring ^3H accumulation in the bladder.

Each in vivo binding experiment reported herein consisted of a minimum of nine mice. Results have been reproduced in a minimum of three separate experiments.

Inhibition of BCG binding to disrupted mucosa. Antibodies, described previously to inhibit BCG attachment to cauterized bladders (16), heparin sulfate, and FN were tested for inhibition of BCG binding. Antibodies at varying concentrations were instilled into the bladders of mice in a volume of 0.1 ml. After 30 min the serum was removed, the bladder was rinsed with 0.1 ml of PBS, and radiolabeled BCG were instilled and quantitated as described above.

In inhibition experiments utilizing FN, radiolabeled BCG as prepared above were suspended to 5×10^7 CFU/ml with FN at varying concentrations ranging from 100 to 1 μg/ml for 30 min. The bacteria were then instilled into the bladder as described above.

The effect of topical heparin on BCG adherence after mucosal disruption was studied by instilling 0.1 ml of a 10^{-3} to 1.0 μg/ml sodium heparin solution intravesically for 30 min after injury as described by See and Chapman (19). Bladders were rinsed and radiolabeled bacteria were instilled and processed as described above.

Measurement of delayed-type hypersensitivity (DTH) induced by BCG. Measurement of delayed-type hypersensitivity (DTH) was performed as described by Thomas and Schrader (20) with minor modifications. C3H/HEJ (Charles River Breeding Laboratories, Inc.) mice were immunized to BCG by injecting 5×10^4 CFU BCG in a 1:2 dilution of incomplete Freund's adjuvant at the base of the tail in a total volume of 0.1 ml. 7 d after immunization the mice were anesthetized, catheterized, and cauterized as described above. Mice then received 5×10^7 CFU of BCG or BCG pretreated with FN in a volume of 0.1 ml intravesically, followed immediately by an intraperitoneal injection of 0.1 ml of 10^{-7} M 5-fluoro-2-deoxyuridine (Sigma Chemical Co.). 30 min later 1.5 μCi of [^{125}I]5-iodo-deoxyuridine ([^{125}I]UDR) (Amersham Corp., Arlington Heights, IL; sp act 5 Ci/mg) was injected intraperitoneally. The animals were killed 24 h later and the bladders were harvested. DTH was quantitated by measuring accumulation of [^{125}I]UDR in the bladder. Controls included cauterized mice with instillation of PBS instead of BCG and heat-killed *Staphylococcus aureus* which also bind to FN.

DTH was also assessed by measuring footpad thickness after sensitization with BCG. Mice were treated weekly for 3 wk with 0.1 ml of adriamycin, 8 μg/ml. 1 wk later mice were treated intravesically with 0.1 ml of BCG or FN-pretreated BCG, 5×10^7 CFU/ml for 4 wk. 1 wk after the final BCG treatment, mice were injected in the right hind footpad with purified protein derivative (5 μg in 0.25 μl). The left hind footpad received diluent only. Footpad thickness was measured with a dial gauge caliper 24 h after challenge. An increase in footpad thickness of > 0.20 mm was significant at a level of 1% (21).

Therapy studies. C3H/HEJ mice were anesthetized and catheterized as described above. The MBT-2 tumor (kindly provided by Mark Soloway, University of Tennessee Health Sciences Center, Memphis, TN) was implanted in cauterized bladders and BCG therapy was initiated 24 h later and weekly thereafter as previously described (12). In some experiments FN-pretreated BCG were used to treat tumor bearing mice. In these experiments 10 mice were studied in each group. The results are reported as the mean of three separate experiments.

Results

Previous histological and quantitative studies demonstrated that BCG attached to bladder urothelial surfaces disrupted by electrocautery but not normal urothelial surfaces (Fig. 1 *A*). To determine whether chemical injury could induce BCG binding, bladders underwent mucosal disruption by the intravesical instillation of acrolein or adriamycin. Significant levels of BCG attachment were achieved in the bladders for each treatment (Fig. 1, *B* and *C*). As the concentration of either adriamycin or acrolein increased, the number of bacteria bound to mucosa increased and this attachment correlated with increased mucosal alterations observed on histologic analysis (data not shown).

Previous in vitro studies suggested that BCG attachment to the extracellular matrix could be mediated by FN (16, 17). To evaluate whether FN was the molecule responsible for the BCG attachment observed, inhibition studies were performed. Pretreatment of disrupted urothelial surfaces with anti-FN antibodies reduced BCG attachment to background levels whereas antilaminin antibodies and preimmune serum had no effect on BCG attachment (Fig. 2).

The studies described above suggested that BCG attached to FN exposed within the bladder. Previous studies assessing the binding capacity of tumor cells or *Escherichia coli* within the bladder demonstrated that binding could be inhibited by reconstituting the glycosaminoglycan layer with exogeneous heparin sulfate (19, 22). Similar studies were performed to determine whether pretreatment of disrupted urothelial surfaces with heparin sulfate inhibited BCG attachment. The data show that heparin pretreatment at concentrations of 0.1 μg/ml or greater significantly reduced the intravesical binding of BCG (Fig. 3).

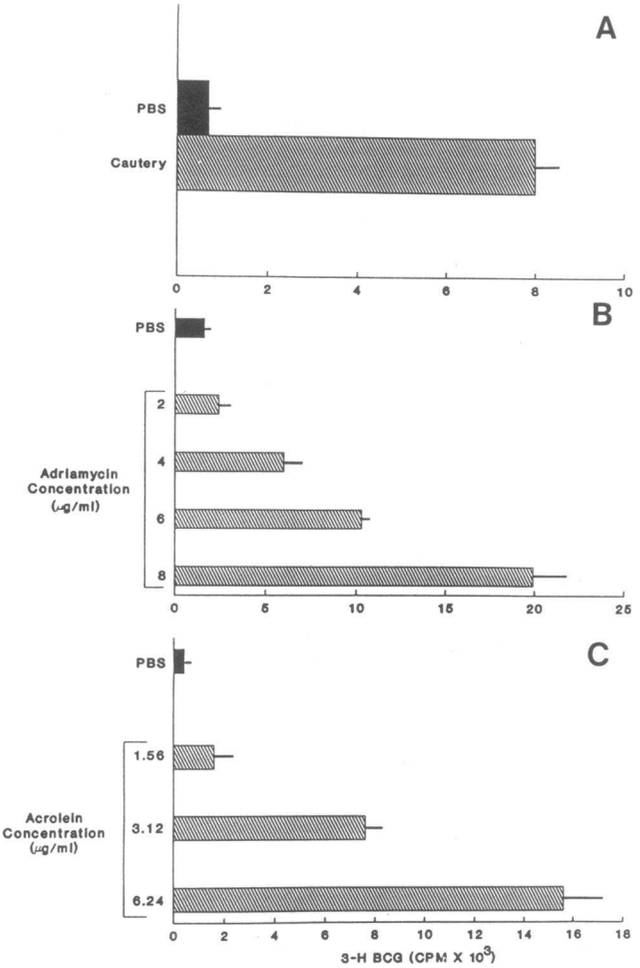

Figure 1. Effect of (*A*) cautery, (*B*) adriamycin, and (*C*) acrolein on the intravesical attachment of BCG. Significant elevation at a minimum of $P < 0.01$ level (Student t test) was observed for cautery; adriamycin 4 μg/ml or higher, acrolein 3.12 μg/ml, and 6.24 μg/ml.

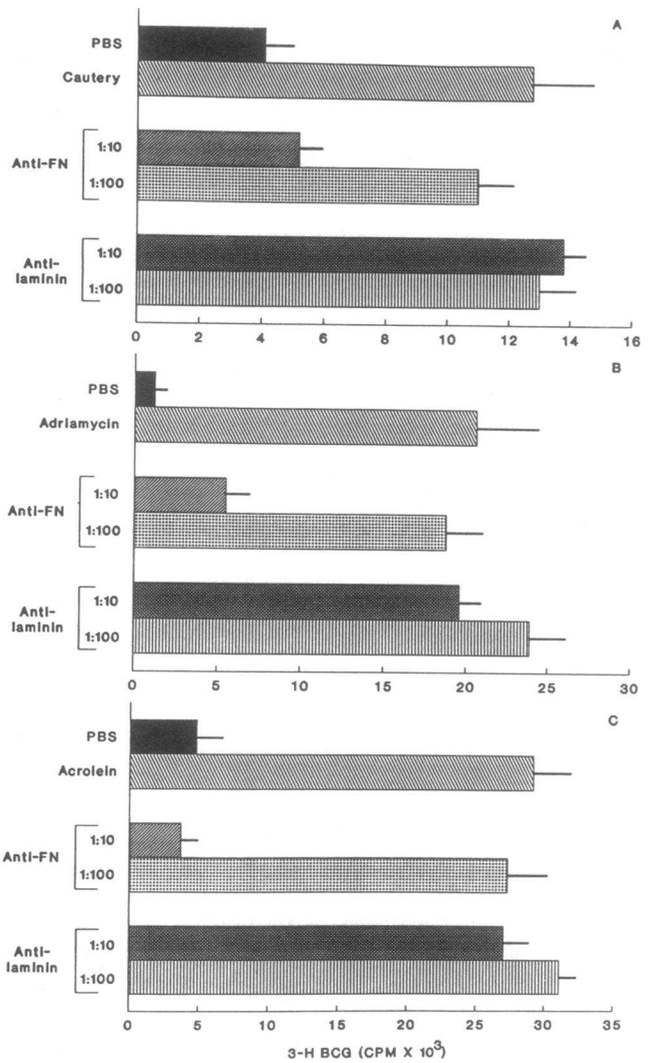

Figure 2. Effect of anti-FN antibodies on the intravesical attachment of BCG to (*A*) cauterized, (*B*) adriamycin-treated, and (*C*) acrolein-treated bladders. Significant ($P < 0.005$, Student t test) inhibition of BCG attachment was observed for all 1:10 dilutions of anti-FN.

Data have shown that BCG receptors for FN bind soluble FN in an essentially irreversible manner at pH below 10 (23). These data suggested that pretreatment of BCG with soluble FN or fragments that retained BCG binding activity at concentrations sufficient to saturate the receptors would block BCG attachment to FN coated surfaces. Studies were performed to test this hypothesis. BCG pretreated with soluble FN were compared with diluent pretreated BCG for attachment to disrupted urothelial surfaces in vivo. As shown in Fig. 4, electrically or chemically treated bladders did not retain BCG pretreated with soluble FN.

Having established conditions for the in vivo inhibition of intravesical BCG attachment, we tested the functional implications of blocking FN-mediated attachment of BCG. Studies were performed comparing the ability of intravesical BCG and FN-pretreated BCG to immunize mice (Fig. 5). The results show that untreated BCG induced a significant DTH footpad response, whereas FN-pretreated BCG did not.

The expression of DTH in previously sensitized mice also was inhibited by blocking intravesical FN-mediated BCG attachment (Fig. 6). Mice were sensitized by intravenous injection of BCG. 3 wk later, mice were challenged intravesically with untreated or FN-pretreated BCG. DTH was measured by

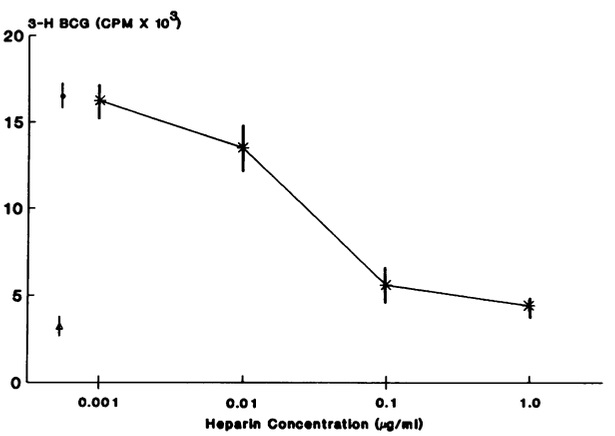

Figure 3. Effect of varying concentrations of heparin on the intravesical attachment of BCG. Heparin significantly ($P < 0.005$, Student t test) reduced BCG attachment at concentrations of 0.1 μg/ml or higher. (●) BCG attachment to FN in the absence of heparin; (△) background BCG attachment to BSA-coated surfaces.

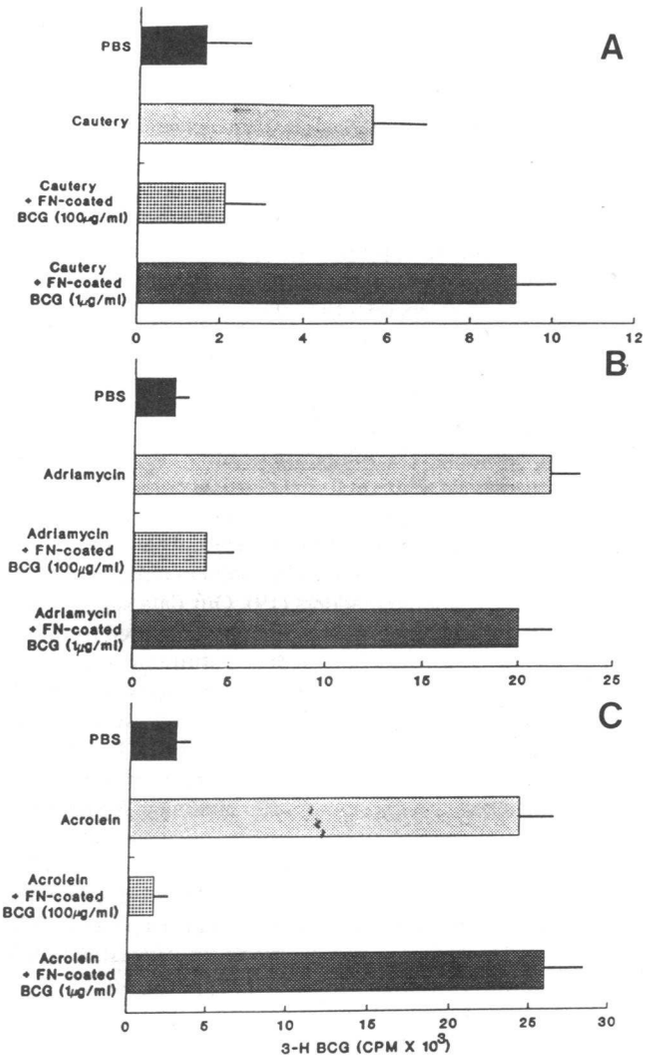

Figure 4. Effect of BCG pretreatment with soluble FN on intravesical binding in (*A*) cauterized, (*B*) adriamycin-treated, and (*C*) acrolein-treated bladders. Significant inhibition ($P < 0.005$, Student t test) was observed for FN-coated BCG in cauterized, adriamycin-treated, and acrolein-treated bladders.

accumulation of ^{125}I-UDR as described by Thomas and Schrader (20). Significant DTH was observed only in mice challenged with untreated BCG. BCG pretreated with FN, which lacked the ability to bind, and *S. aureus,* which bind to FN but were not recognized antigenically, lacked the ability to induce a DTH response in sensitized mice. The accumulation of mononuclear cells within bladders of mice treated with BCG was documented histologically (data not shown).

Finally, the effect of inhibiting FN-mediated attachment of BCG on antitumor activity was determined (Fig. 7). Untreated BCG significantly reduced MBT-2 tumor outgrowth. In contrast, FN-pretreated BCG did not significantly inhibit MBT-2 outgrowth, suggesting that FN-mediated attachment of BCG is required for the expression of antitumor activity.

Discussion

Previous reports have demonstrated that BCG attaches to FN-coated surfaces but not to surfaces coated with other purified

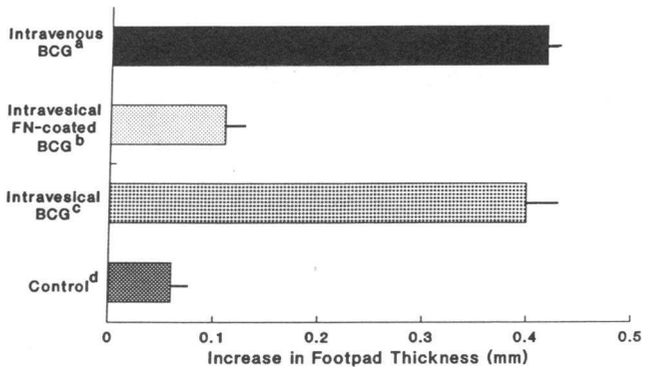

Figure 5. Effect of inhibiting intravesical BCG attachment on immunization. Significant footpad DTH was observed in mice treated intravenously and intravesically with BCG. (*a*) Mice injected intravenously with 5×10^4 CFU BCG on the day of the first intravesical BCG instillation. (*b*) BCG were pretreated with soluble FN and instilled into Adriamycin pretreated bladders once each week for 4 wk. (*c*) Untreated BCG was resuspended in PBS and instilled as in *b*. (*d*) Mice were treated with diluent only as described for *b* and *c*.

extracellular matrix proteins (16). FN-mediated BCG binding was dose dependent and was inhibited by anti-fibronectin antibodies but not antibodies directed against other extracellular matrix proteins. Further studies demonstrated that BCG attached in vivo to urothelial surfaces damaged by electrocautery and the attachment was mediated by FN (17). In this report direct evidence is presented demonstrating that intravesical FN-mediated BCG attachment is necessary for the expression of BCG-mediated antitumor activity in the mouse model.

FN belongs to a family of structurally and immunologically related glycoproteins present on many cell surfaces, in connective tissue, in basement membranes, and in extracellular fluids including plasma (24). Most FN are composed of two homologous but not identical chains linked near the carboxy-terminal end by disulfide bridging (24). The fibronectin molecule consists of globular domains which represent biologically active areas that retain activity even after protease digestion (25).

FN plays a major role in organizing the extracellular matrix and acting as an attachment protein for many different

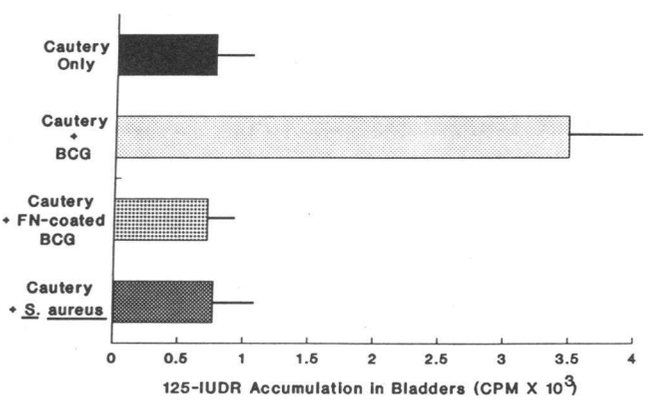

Figure 6. Effect of inhibiting intravesical BCG attachment on the expression of DTH in the bladder. Cautery plus BCG significantly ($P < 0.025$) greater than controls (Student t test).

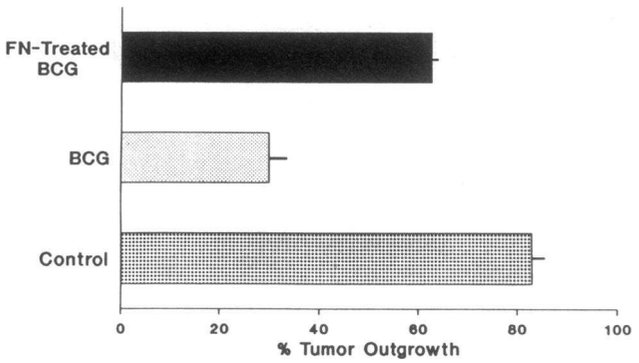

Figure 7. Effect of inhibiting intravesical BCG attachment on the expression of antitumor activity. BCG-treated mice had significantly ($P < 0.0001$) fewer tumors than control. FN-treated BCG mice were not significantly ($P > 0.09$) different than control. FN-treated mice had significantly ($P < 0.01$) more tumors than BCG only mice. Data are reported as a mean of three separate experiments. Statistics: Fisher's exact probability test.

molecules including collagen, fibrinogen, heparin, actin, eukaryotic cells, DNA, hyluronic acid, complement, and itself (26). Recently, fibronectins also have been found to bind to bacteria with the best characterized being *Staphylococcus aureus* and the streptococci (27, 28). The bacteria bind primarily to a FN domain near the amino terminus of the FN molecule via a specific surface protein and/or lipoteichoic acid (28–30). It has been postulated that FN-mediated attachment of the bacteria to the human host may be important in initiating infections such as endocarditis, phlebitis, and foreign body infection (31, 32).

The functional importance of BCG binding to FN in the mouse bladder tumor model was assessed by its effect on both the immune response and antitumor activity. DTH to mycobacterial antigens can be measured after BCG immunization (5–7). In the studies reported herein animals treated with intravesical BCG demonstrated the expected immunological sensitization; however, animals treated intravesically with FN pretreated BCG, which significantly reduced BCG attachment to the damaged urothelium, did not express a significant DTH footpad response. These data demonstrate that intravesical BCG attachment to FN is required for the immunization process. In separate experiments the expression of DTH in previously sensitized mice was abrogated by inhibiting intravesical BCG attachment, further suggesting a link between FN-mediated attachment of BCG and expression of immunity within the bladder.

The dependence of the expression of antitumor activity on the binding of BCG to FN exposed within the bladder was also tested. Inhibition of the binding of BCG to the bladder by pretreating BCG with FN markedly decreased the effectiveness of BCG in preventing tumor outgrowth. These data suggest that antitumor activity also requires FN-mediated BCG attachment. Taken together these studies demonstrate that intravesical BCG attachment, most likely to FN, is necessary for the induction and expression of immunological reactivity and for the expression of antitumor activity in the mouse model.

Further experiments were performed to determine whether chemically induced mucosal disruption also would induce intravesical BCG attachment and whether FN was the functional mediator for BCG attachment. Chemical disruption was investigated because of its potential clinical utility for increasing intravesical BCG binding. In the genitourinary tract, FN is present in the basement membrane and submucosa and not on the apical surface of transitional epithelium (33, 34). If FN is the molecule responsible for attachment, BCG should only attach to bladders in which mucosal disruption has occurred. Our histological and quantitative studies fail to demonstrate BCG binding in bladders in which the mucosa is intact. However, when mucosal disruption was induced by either electrocautery or chemical irritation, BCG bound at significant levels and did so in a dose-dependent manner. BCG attachment was observed only in areas of urothelial damage. Our results also showed that intravesical binding was mediated by FN.

Interestingly, instillation of heparin sulfate into bladders with urothelial damage inhibited BCG binding. FN has heparin-binding domains. The inhibition of BCG binding may result from heparin attachment to FN or possibly replenishment of the glycosaminoglycan layer. The heparin-induced inhibition of BCG binding is of interest since See and Chapman have suggested that topical heparin may be useful in reducing tumor cell adherence in injured bladders (19). Our data suggest that the use of heparin during surgical resection may reduce BCG attachment and thus potentially reduce antitumor activity.

The studies reported herein suggest that intravesical BCG adherence is essential for the development and expression of both an immune response and antitumor activity. The clinical implication of these data is that some reported treatment failures may be related to suboptimal BCG binding to the bladder. Furthermore, the potential exists for enhancing antitumor activity via enhancing attachment. Antitumor activity may be enhanced by augmenting BCG attachment through enhanced exposure to FN. In this regard we have initiated a phase I study testing the effects of intravesical adriamycin pretreatment followed by intravesical BCG. Toxicity associated with BCG instillation after adriamycin are acceptable and similar to those described for BCG alone. The preliminary therapeutic results are encouraging.

In conclusion, BCG attachment to the bladder mucosa appears to be a requisite step for expression of antitumor activity and for the initiation and expression of immunity to BCG. By manipulating this interaction enhanced efficacy of intravesical BCG therapy may be realized. Further laboratory and clinical trials will be required to determine whether increased binding will ultimately result in increased efficacy for bladder therapy.

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