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Interaction Between Fibronectin-bearing Surfaces and Bacillus Calmette-Guérin (BCG) or Gelatin Microparticles

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Abstract

Gelatin, prepared commercially by degradation of animal collagen, was studied to see whether it had an affinity for fibronectin, which has a known affinity for collagen, and whether gelatin-based drugs could be used to target fibronectin-excreting tumours.

Bacillus Calmette-Guérin (BCG) vaccine, an attenuated strain of *Mycobacterium bovis*, is currently the most effective treatment for superficial transitional cell carcinoma of the bladder. The living cells of the BCG vaccine associate with the fibronectin-bearing surfaces of the tumour.

Using a multi-well culture plate technique, gelatin microparticles were shown to be adsorbed onto murine S180 sarcoma cells and this reaction was substantially inhibited by the addition of human plasma fibronectin.

The avidities of various BCG substrains and gelatin microparticles for glass-bound fibronectin were measured and the association constants determined. The gelatin microparticles associated with the fibronectin with equal avidity as the BCG cells.

The results suggest that this model system may allow the investigation of gelatin-based drug delivery devices capable of targeting fibronectin-bearing surfaces associated with some tumours.

Fibronectin is a family of at least 100 spreading-factor glycoproteins with structural homology (Carsons 1989; Goodheart & Silverman 1991; Pagano et al 1991) which attach to or are associated with cellular surfaces. The identified variants are ubiquitous, being found in a variety of non-transformed mammalian and avian cell types. Physiologically, they appear to be involved in a number of cellular phenomena including cell wall adhesion, as a substrate attachment factor and as a spreading factor (Carsons 1989). Because of their involvement with spreading, a number of tumours are thought to express fibronectin at their cell surfaces (Mosher 1989). Antiadhesives have been proposed as cancer treatments based on the fact that fibronectins are involved in the movement of cells in many human diseases such as inflammation, cardiovascular disease and cancer (Humphries 1993). Fibronectins are also believed to be the main cellular proteins reacting with collagen (Engvall & Ruoslahti 1977; Carsons 1989; Hedman & Vaheri 1989; Nakamura et al 1992).

In the case of the rapidly invasive human bladder tumour, Ratliff and his coworkers have demonstrated that Bacillus Calmette-Guérin (BCG) cells are specifically retained on the tumour walls by attachment to fibronectin (Ratliff et al 1987, 1988a, b; Aslanzadeh et al 1989; Ratliff 1989; Hudson et al 1990, 1991; Kavoussi et al 1990). Teppema et al (1992) have discussed current views of this subject in detail. The specific fibronectin receptor in the bacterium has been variously identified as cell-wall proteins of 30–32 kDa (the so-called Antigen 85 complex) (Abou-Zeid et al 1988; Höök et al 1989; Öner et al 1994), of 45 kDa (F. Öner unpublished data) or 55 kDa (Ratliff et al 1993).

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Intravesicular use of BCG vaccine is currently the most effective treatment for superficial transitional cell carcinoma of the bladder, although the exact mode of action is less than certain (Groves 1993). Basement cell fibronectin is believed to be bound by cell-surface receptors expressed by some invasive tumours and, specifically in the case of bladder cancer, is exposed by structural damage to the adjacent urothelium (Coplen et al 1991). Thus, in the case of bladder cancer, fibronectin is either directly on the tumour surface or is indirectly exposed at the edges of the invasive site.

Since fibronectin has a pronounced affinity for collagen, it seemed reasonable to hypothesize that gelatin, a degraded commercially available form of collagen, might also associate with fibronectin-bearing surfaces. The use of microparticles of inert materials such as gelatin as drug delivery systems is well established in the literature (Morris & Cuff 1991; Donbrow 1992). The use of gelatin as a system capable of forming an affinity for fibronectin-bearing surfaces, effectively acting as a targeted drug-delivery system, appeared to be feasible. Carsons (1989) confirmed that fibronectin had a domain capable of reacting with either collagen or gelatin. Gelatin particles interact with macrophages (Tabata & Ikada 1987, 1989) and Nakamura et al (1992) demonstrated that gelatin has a strong affinity for fibronectin. These latter authors suggested that a hydrophilic reaction, involving hydrogen bonding or van der Waals interactions, played a major role in the fibronectin-gelatin association. Here we report some avidities of BCG cells and gelatin microparticles for glass-bound fibronectin and describe the interaction of gelatin particles with a murine S180 sarcoma cell line as an in-vitro model.

Although the S180 murine sarcoma tumour model is irrelevant to bladder cancer, it does excrete fibronectin after in-vitro passage, as confirmed in this present work. In-vitro examination of a passaged MBT-2 murine bladder tumour cell line demonstrated (unpublished) no excretion of fibronectin, no interaction with gelatin and no corresponding change in the cell morphology. Thus, the use of a bladder tumour cell line in-vitro would not necessarily produce data relevant to an in-vivo situation.

Materials and Methods

Glass beads (borosilicate 3 mm diam., Catalogue No. 41-5550-03, PGC Scientific, Gaithersburg, MD) were cleaned in acid dichromate cleaning solution (20 g sodium dichromate plus 400 mL concentrated sulphuric acid) at 100°C for 10 min, followed by copious washing with distilled water and final rinsing with sterile 0·01 M phosphate-buffered saline, pH 7.4 (PBS). Measurement of the bead diameter by microscopy gave a mean diameter of 3·08 mm (s.d. = ± 0·07) (n = 200). The average surface area per bead was calculated to be 0.291 cm^2.

Gelatin microparticles were prepared from bovine skin, lime cured (Type B) gelatin with a Bloom strength of 225 (Sigma Chemical Company, St Louis, MO) by the method described by Öner & Groves (1993). Briefly, 25 mL of a 1% w/v solution of the gelatin was slowly dispersed into 500 mL anhydrous ethanol (ethanol 95%, isopropanol 5%) chilled at −15°C followed by 4% glutaraldehyde to cross-link the gelatin matrix. This system was stirred at −15°C for 1 h followed by an additional 24 h at ~4°C before stopping the reaction with an excess of 5% w/v sodium metabisulphite at 4°C. The microparticles were collected by filtration using a YM10 ultrafiltration membrane (Amicon Division, Beverly, MA) and washed three times with 1/15 M phosphate buffer (pH 7·4) before lyophilization using a Labconco freeze-drier. The final microspheres had a mean volume number diameter of 1·45 μm with a geometric standard deviation (σ_g) of the distribution of 1·66, measured using a Coulter Multisizer II fitted with a 50-μm orifice tube.

S180 murine sarcoma cells were obtained from the National Type Culture Collection (Rockville, MD). The cells were maintained in-vitro at this Institute in CMEM-E (Eagle's minimum essential medium with non-essential amino acids, Earle's basal salts, 5% calf serum, 100 units mL^{-1} penicillin and 100 $\mu g\ mL^{-1}$ streptomycin) in an atmosphere of 5% CO_2, 95% relative humidity at 37°C.

Human fibronectins (from plasma) were obtained from Serva Biochemicals (Westbury, NY), Accurate Chemical and Scientific Corporation (also of Westbury, NY), Alpha Therapeutics (Los Angeles, CA) (the kind gift of Mr Conrad Turner) and Gibco BRL (Grand Island, NY). The materials were reconstituted in PBS as required.

The BCG substrains employed were Tice substrain, manufactured at this Institute, lots 105149, 105B153C and 105178, Connaught substrain, lot 494-11, purchased through the University of Illinois at Chicago hospital pharmacy, Glaxo substrain, lot D1010 (Medeva, Speke, UK) and Pasteur substrain, lot R5590 (provided by Institut Pasteur, Paris, France).

All ampoules were stored at −20°C. The contents of the ampoules were allowed to warm up to ambient temperatures (~25°C) before reconstitution with PBS, and cells were collected and washed once by centrifugation. Only washed cells were employed.

Albumin was a powdered unstabilized human serum albumin, the gift of the Hyland Therapeutic Division of Baxter Healthcare Inc., Deerfield, IL, and was reconstituted in PBS as required.

Urine was from a normal subject, collected over a 24-h period and ultrafiltered through a Pellicon PTGC cassette filter (Millipore, Bedford, MA) to remove cellular and other particulate matter, together with proteins.

Interaction of gelatin microparticles with S180 sarcoma cells

We have previously demonstrated that S180 murine sarcoma cells express fibronectin on their surfaces and, by binding to this fibronectin, BCG cells inhibited S180 adherence to polystyrene substrata (Klegerman et al 1993).

To determine whether gelatin microparticles targeted the tumour cells in a similar fashion, 10^6 viable S180 cells and various concentrations of gelatin microparticles in 1·0 mL CMEM-E without serum were distributed among the centre eight wells of 24-well cluster plates (Costar, Cambridge, MA). After the well plates were incubated for 18 h at 37°C, nonadherent cells were resuspended by gentle aspiration three times with a Pasteur pipette and removed. Numbers of viable cells were determined by counting trypan blue dye-excluding cells in a haemacytometer. Adherent cells removed with 0·25% trypsin in 1 mM EDTA were counted. The fibronectin specificity of the microparticle-cell interaction was determined by adding 10–100 μg human plasma fibronectin to 10^6 viable S180 cells in the presence of 1·5% gelatin microparticles. After incubation overnight, cells were harvested and counted as described.

Adsorption of proteins onto glass

Solutions (50 mL) of either albumin or human plasma fibronectin in PBS at appropriate concentrations were reacted with 19·6 g glass beads (166 cm^2 total surface area) for up to 80 min, using gentle shaking on an Orbit Shaker (Model 3520, Lab-Line Instruments, Melrose Park, IL). The beads and associated solutions were added to a K16/20 chromatographic column (Pharmacia-LKB, Piscataway, NJ), modified by replacing the woven nylon screen at the base of the column with a rigid polyethylene screen glued in place with a rubber-based cement. The beads were washed with PBS, followed by 5 mL of 1·0 M NaOH to desorb the protein, followed by 5-mL distilled water wash. The total protein recovered was estimated using the BCA protein assay (Pierce Chemical Co., Rockford, IL), measuring absorbance at 562 nm with a Beckman DU65 Spectrophotometer (Beckman Instruments, Fullerton, CA).

Adsorption of BCG and gelatin microspheres onto glass beads

About 2×10^9 BCG cells (or 2×10^7 gelatin microparticles) per gram of glass beads (plain or protein-coated) were reacted in a total volume of 5 mL PBS on the Orbit Shaker at 21 ± 1°C for 20 min, by which time the adsorption was generally found to be complete (Fig. 1).

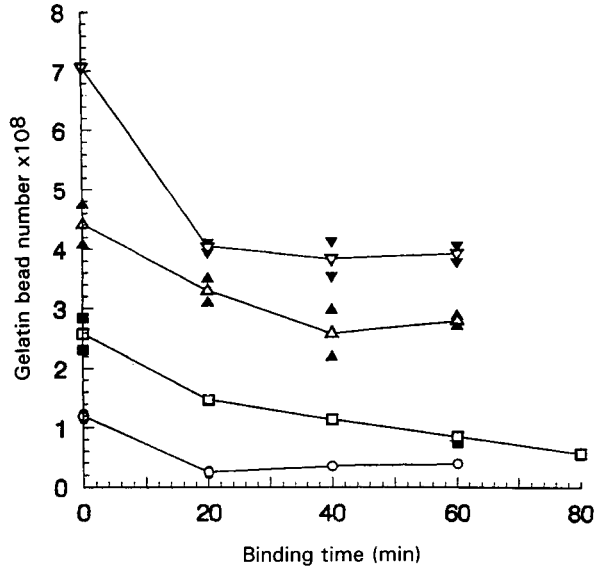

FIG. 1. The rate of gelatin microparticles binding to fibronectin-coated glass beads at 25°C. ○ 1·0, □ 2·0, △ 3·0, ▽ 4·0 mL gelatin microparticle dispersion added. Shaded points are the limits of error for each determination. (Each 1·0 mL of dispersion contains ~1·5–1·7 x 10^8 gelatin microparticles.)

Measurement of adsorption

Gas chromatography. A 80-μL sample was taken from the suspension, placed in a 13 x 100 mm borosilicate screw cap culture tube and processed for total fatty acids (as palmitic acid) by the method described in detail by Olson et al (1990).

Coulter counting. As demonstrated by Groves et al (1991), the primary bacterial particles and associated aggregates are readily determined using a Coulter Multisizer fitted with a 50-μm orifice tube. The method can be used to determine the total number of bacterial cells in a sample. Accordingly, the numbers of bacteria in samples before and after adsorption were again determined, and the numbers adsorbed onto the glass beads were obtained as the difference between the two counts.

Scatchard analysis

The analysis of ligand-macromolecule interactions by Scatchard (1949) was adapted to particulate ligands interacting with surface-adsorbed molecules, assuming that a homogeneous suspension was maintained and the system was evaluated at equilibrium. The Scatchard equation would then take the form:

$$nN_b / [nN_f (n_T - nN_b)] = K_a \quad (1)$$

where N_b is the number of particles bound to surface, N_f is the number of unbound particles, n is the number of ligands per particle capable of binding to surface-adsorbed molecules, n_T is the concentration of surface-adsorbed molecules, and K_a is the monomolecular association constant.

A plot of N_b/N_f vs N_b will yield a straight line of slope $-nK_a$ (the negative avidity constant, K_A) and y-intercept n_TK_a, if only one type of noncovalent interaction occurs. In this case, the particles were BCG cells or gelatin microparticles, the ligands were fibronectin receptors and the surface-adsorbed molecule was human plasma fibronectin adsorbed to glass beads.

Since the beads were saturated with $4{\cdot}54 \times 10^{-13}$ mol fibronectin per square centimeter, $n_T = 1{\cdot}51 \times 10^{-8}$ M. Therefore, K_a could be calculated from the y-intercept and then used to determine n (which should be a fairly consistent function of the total number of ligands per particle) from the slope.

Results and Discussion

The adsorption of albumin or fibronectin onto glass beads was relatively rapid, the albumin saturating at a level of 0·65 μg cm^{-2} and the fibronectin at 0·2 μg cm^{-2}. Assuming molecular weights of 68 and 440 kDa, respectively, this corresponds to approximately $9{\cdot}56 \times 10^{-12}$ and $4{\cdot}54 \times 10^{-13}$ mol cm^{-2} of glass surface, respectively. However, it may be noted that binding of BCG onto uncoated glass beads was at least seven orders of magnitude lower than binding to fibronectin-coated glass (Olson 1992).

The adsorption kinetics of various substrains and lots of BCG onto glass-immobilized human plasma fibronectin, determined by Coulter Multisizer analysis, were remarkably similar (Table 1). The avidities (K_A) monomolecular association constants (K_a) and numbers of binding sites per bacterium varied about twofold, with a minimal affinity exhibited by the Glaxo substrain and a maximal affinity shown by the Pasteur substrain. Parameters determined by gas chromatographic determination of BCG cell mass were broadly similar, but required appreciably more material for the determination. For this reason, Coulter analysis was used for the comparison of the various BCG preparations shown in Table 1.The study of the rate of reaction of gelatin for fibronectin on glass (Fig. 1) showed that the reaction was complete in 20 min. However, as noted by Klegerman et al (1993), the inhibition of this reaction by soluble fibronectin was found to be optimal after overnight incubation.

Surprisingly, the overall avidity of gelatin microparticles for glass-immobilized fibronectin was better than the corresponding measurement for BCG, with the affinity being about the same and the number of binding sites per particle being more than forty times greater (Table 2). The microparticle-fibronectin interaction was evidently not reduced in urine. Addition of soluble fibronectin to the reaction

Table 1. Avidity constants (K_A), association constants (K_a) and number of fibronectin-binding sites per cell (n) of BCG substrains adsorbing to glass-immobilized fibronectin.

Substrain	Method	K_A	K_a (M^{-1})	n
Tice 105149	GC[a]	$2{\cdot}96 \times 10^{-12}$	$4{\cdot}40 \times 10^{7}$	$4{\cdot}86 \times 10^{3}$
	Coulter[b]	$1{\cdot}50 \times 10^{-10}$	$4{\cdot}10 \times 10^{7}$	$1{\cdot}10 \times 10^{4}$
Tice 105178	Coulter	$1{\cdot}40 \times 10^{-10}$	$3{\cdot}94 \times 10^{7}$	$1{\cdot}07 \times 10^{4}$
Tice 105B153c	Coulter	$8{\cdot}43 \times 10^{-11}$	$3{\cdot}34 \times 10^{7}$	$7{\cdot}59 \times 10^{3}$
Connaught	Coulter	$1{\cdot}51 \times 10^{-10}$	$4{\cdot}79 \times 10^{7}$	$9{\cdot}49 \times 10^{3}$
Glaxo	Coulter	—[c]	$2{\cdot}26 \times 10^{7}$	—[c]
Pasteur	Coulter	$1{\cdot}97 \times 10^{-10}$	$5{\cdot}43 \times 10^{7}$	$1{\cdot}09 \times 10^{4}$

[a] BCG quantitation from [16:0] fatty esters by gas chromatography. [b] BCG quantitation by Coulter principle particle analysis. [c] Slope too low for reliable estimate (insufficient number of cells available).

Table 2. Binding parameters of gelatin beads of varying composition absorbed to immobilized fibronectin.

Gelatin type[a]	Solvent[b]	K_A	K_a (M^{-1})	n
A60	PBS	$9{\cdot}17 \times 10^{-9}$	$3{\cdot}49 \times 10^{7}$	$7{\cdot}88 \times 10^{5}$
	Urine	$3{\cdot}42 \times 10^{-9}$	$1{\cdot}52 \times 10^{8}$	$6{\cdot}78 \times 10^{4}$
As above, with fibronectin[c]	PBS	$1{\cdot}53 \times 10^{-4}$	$3{\cdot}33 \times 10^{6}$	—
B60	PBS	$6{\cdot}43 \times 10^{-9}$	$2{\cdot}86 \times 10^{7}$	$6{\cdot}77 \times 10^{5}$
A300	PBS	$4{\cdot}70 \times 10^{-9}$	$2{\cdot}45 \times 10^{7}$	$5{\cdot}78 \times 10^{5}$
B225	PBS	$4{\cdot}79 \times 10^{-9}$	$2{\cdot}33 \times 10^{7}$	$6{\cdot}19 \times 10^{5}$

[a] A = acid-hydrolysed, B = lime-hydrolysed; numbers refer to Bloom number which is proportional to gel strength. [b] PBS = Phosphate-buffered saline; urine = ultrafiltered human urine from a 24-h collection (one source). [c] Number of fibronectin molecules adsorbed onto glass surface $n_T = 3{\cdot}15 \times 10^{-4}$ M.

mixture containing gelatin particles or BCG cells reduced the calculated avidities by five orders of magnitude, verifying the specificity of the interactions (Olson 1992).

The effect of gelatin microparticles on S180 murine sarcoma cells in culture was similar to that of BCG cells, producing a dose-dependent, significant, reciprocal increase of non-adherent cells and decrease of adherent cells (Fig. 2). Like BCG, gelatin microparticles inhibited the adherence of the cells to the plastic support by adsorbing to them (evident by examination of the cultures with an inverted microscope); this adsorption was fibronectin-dependent, since addition of human plasma fibronectin to the medium caused a dose-dependent abrogation of the inhibition (Table 3). Avidities or affinities of either BCG or gelatin microparticles for the S180 cell fibronectin could not be measured directly by the techniques described here because of the inevitable presence of cellular fragments. However, experimentally, 100 μg plasma fibronectin displaced approximately $7{\cdot}5 \times 10^5$ S180 cells from $\sim 9 \times 10^8$ gelatin microparticles. A level of 150 μg fibronectin preincubated with 10^{11} BCG cells prevented $2{\cdot}5 \times 10^5$ S180 cells from binding to $\sim 2 \times 10^{10}$ BCG cells. This suggests that each molecule of soluble fibronectin blocks binding of an equal number of cell fibronectin molecules to about five receptors for both the BCG and gelatin microparticles. The avidity of gelatin and BCG receptors for the bound fibronectin on the S180 cells is therefore approximately the same.

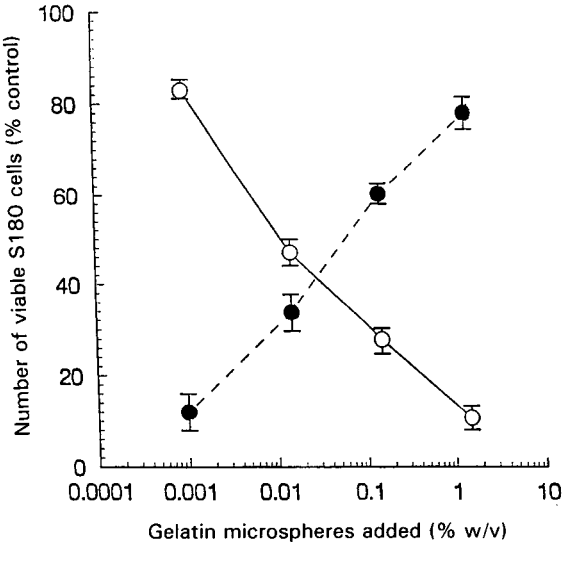

FIG. 2. The effect of increasing the dose of gelatin microparticles at 37°C on the association of murine S180 sarcoma cells for the plastic walls of the wells in the cluster plate. ○ Adherent cells, ● non-adherent cells. As the amount of gelatin is increased, the number of adherent cells decreases and, conversely, the number of non-adherent cells in the well increases. Bar = standard error ($t = 3{\cdot}56$ for 5 degrees of freedom, $P < 0{\cdot}05$).

Effective BCG immunotherapy of superficial bladder cancer apparently depends upon initial adherence of the BCG cells in the immediate vicinity of the tumour cells, mediated by an association of BCG cell-wall fibronectin receptors and basement membrane fibronectin exposed by invasion of the tumour through the urothelium (Ratliff 1989; Teppema et al 1992). Indeed, Ratliff et al (1991) speculated that a purified mycobacterial cell-wall fibronectin receptor could be used to target drugs as an alternative to live BCG for bladder cancer therapy.

Exploitation of fibronectin's gelatin-binding properties would provide a better targeting strategy because gelatin microparticles as drug delivery systems are relatively inexpensive, and easy to produce (Morris & Cuff 1991). Even if mycobacterial cell-wall fibronectin receptors could be produced by recombinant DNA technology, they would be significantly more costly and difficult to prepare and they would be useful for targeting only, requiring coupling to an antitumour drug or to an appropriate drug delivery system. The feasibility and safety of all aspects of such a delivery system would also need to be demonstrated.

The model evaluated in this present work appears to be relevant since association parameters derived for the interaction of the particulate ligands are broadly similar to published values. Aslanzadeh et al (1989) found a K_a

Table 3. The inhibitory effect of fibronectin on the interaction between gelatin microparticles and S180 sarcoma cells in-vitro at 37°C.

Fibronectin (μg/well)	Number of adherent viable cells (% control)
0	19 ± 3
10	46 ± 6*
110	67 ± 10**

* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

value of $1{\cdot}1 \times 10^8$ M^{-1} for association of the Armand Frappier substrain BCG with soluble fibronectin at pH 6·0 and 22°C. Among the BCG substrains that we studied, the Pasteur substrain exhibited the highest K_a, which was about half of this value. The discrepancy may be readily explained by the fact that we studied the interaction at pH 7·4, which Aslanzadeh et al (1989) found to be appreciably less optimal than pH 6·0.

Using an electrophoretic method, Nakamura et al (1992) found a K_a of 5×10^6 M^{-1} for the association of soluble fibronectin and gelatin at pH 6·7 and 21°C. In this present case, insolubilization of the ligands, mimicking in-vivo conditions, may have improved the strength of the interaction. Here we have demonstrated that both gelatin microparticles and whole BCG cells target to fibronectin-coated glass surfaces, as well as a fibronectin-expressing cultured tumour cell, with approximately equal avidity. This strongly suggests that a gelatin-based drug delivery system could be effective in targeting the superficial bladder tumours in-vivo, although whether they would be as efficacious in a clinical situation remains to be demonstrated.

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