The In Vitro Cell Association of Invasin Coated Polylactide-Co-Glycolide Nanoparticles

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Purpose. To determine the effect of particle size and ligand surface density on the cellular association of poly lactide-co-glycolide nanoparticles covalently coated with bacterial invasin.

Methods. Poly lactide-co-glycolide nanoparticles containing a flourescent probe were prepared at four diameters 155nm, 200nm, 375nm and 600nm using standard techniques. Bacterial invasin was covalently coupled to the particles surface at varying surface concentrations using a water soluble carbodiimide. The modified particle's cellular association with HEp2 2B cells in tissue culture was determined using flow cytometry.

Results. Cellular association of modified nanoparticles was time dependent, abolished at low temperature, competitively inhibited by free invasin or the RGD peptide ligand and saturable. Increased cell association was produced by increasing the particle's surface invasin concentration however, this effect was size dependent. Small particles (155 nm and 200 nm) exhibiting a maximal association with increasing invasin concentration whilst the larger particles (375 nm and 600 nm) provide a minimum at low invasin concentrations.

Conclusions. Modified particle cell association provided results commensurate with a receptor dependent uptake mechanism related to the presence of invasin. The size and surface concentration dependency however illustrate that application of these ligands to particulate drug delivery or targeting systems will be controlled by their natural cellular association properties.

KEY WORDS: invasin; PLGA; nanoparticles; phagocytosis; in vitro.

INTRODUCTION

The targeting of particulate systems to cells can be achieved by the attachment of surface ligands, most commonly antibodies (1) for parenteral administration, although other systems for the oral route such as plant lectins (2) are available. A potential pitfall is that although the ligand can provide specific cellular attachment, cellular internalisation may not necessarily follow. One remarkable family of ligands attracting increasing attention are bacterial surface proteins which, have evolved to aid the entry of pathogenic bacteria into mamallian cells. Bacteria such as *Yersinia, Salmonella* and *Shigella* species are able to penetrate epithelia and become internalised by normally non-phagocytic mamallian cells through the surface expression of "enteroinvasive" proteins such as invasin (3). These ligands therefore provide specific attachment coupled with cellular internalisation and may permit the targeting of particulates to any receptor competent mamallian cells. This is of special interest in the field of oral administration of particulate systems (4) where uptake is low (5) and follows specific pathways related to gastro-intestinal physiology (6,7). The coating of particles with enteroinvasive proteins may therefore be a useful mechanism to enhance or modify particle uptake from the gastro-intestinal tract.

Yersinia pseudotuberculosis invasin is a 103kd bacterial outer membrane protein responsible for adhesion to, and penetration into mamallian cells (8). Fusion of invasin gene (*inv*) fragments to the *malE* gene, responsible for *Escherichia coli* maltose bonding protein (MBP), have been constructed and the resulting proteins found to retain invasin activity provided they include the carboxyl terminal 192 amino acids of invasin (9). *E. coli* 71-18 D (*lac proAB*) *thi supE* (F′ *proAB*$^+$ *lac*I^q *lac*Z*DM15*) containing the pJL309 plasmid was formed by fusion of an *inv* 3′ restriction fragment to the 3′ end of *malE* (9) and codes for a fusion protein containing the carboxyl terminal 479 amino acids of invasin (MBP-Inv479). The MBP component of the fusion protein allows purification from bacterial homogenates by affinity chromatography in a single step under physiological conditions. Additionally, since MBP does not contain any cysteine residues, disulphide bridge formation, which is essential for invasin activity, is not disrupted (10).

The utilisation of invasin as a cellular ligand has been tested using latex particles coated with invasin containing membrane preparations (11) which, promoted cellular uptake *in vitro.* Other *in vitro* studies using polystyrene latex covalently linked to a 192 amino acid invasin fragment demonstrated an increased particulate uptake in CACO-2 and MDCK cells (12). *In vivo* a similar system demonstrated that invasin retained biological activity whilst attached to polystyrene particles and was capable of promoting uptake when compared with control systems (12).

In this study the larger invasin fusion protein (MBP-Inv479) has been prepared and crosslinked to the surface of PLGA nanoparticles labelled with a fluorescent dye (DiO), to permit measurement of cell association by flow cytometry. It is proposed that the presence of this protein will facilitate particle entry into Hep2 2B cells. This cell line was chosen because it is known to interact with bacterial invasin and MBP-Inv479 (8,9) thus removing the requirement to determine invasin receptor status. The aim of this study is to examine the cellular association of a range of PLGA nanoparticles sizes coated with various surface densities of MBP-Inv479. These two variables have not been examined previously but will control cell association, additionally this is the first *in vitro* application of invasin linked to biodegradable particulate carriers. The results will be useful to assess whether the system warrants further investigation as a potential delivery route.

MATERIALS AND METHODS

Materials

Sodium Hydroxide (Analar) and Hydrochloric acid (1M Convol) were obtained from B.D.H. Ltd., Lutterworth, Leics., U.K. Acetic Acid (Glacial) was obtained from Fisons Scientific, Loughborough, Leics., U.K. (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), Maltose (Monohydrate ⩾ 98%), Methanol (HPLC), Phosphate Buffered Sa-

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line tablets (pH 7.4), Sodium Dodecyl Sulphate (>99%), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-[N-Morpholino]ethanesulphonic acid >99.5% (MES), 3′3-dioctadecyloxacarbocyanine perchlorate (DiO), Arg-Gly-Asp tripeptide, formaldehyde (37% w/v) and Sephadex G-75 were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Ultima Gold was obtained from Canberra-Packard Ltd., Pangbourne, Berkshire, U.K. Tween 80 from Koch Light Labs., Haverill, UK. Water for Irrigation was obtained from Baxter Healthcare Ltd., Thetford, Norfolk, UK. Poly lactide-co-glycolide (50:50) was prepared in house from lactic and glycolic acids by standard literature techniques.

E. coli 71-18 (pJL309) was a gift from Professor John M. Leong, New England Medical Centre, Boston, Mass., U.S.A. The culture was maintained and MBP-Inv479 prepared using standard culture techniques. The protein employed was judged to be over 80% pure based on SDS-PAGE analysis. Radio-labelled protein was prepared by the addition of ^{3}H glycine to the culture medium and activity determined by liquid scintillation counting (LCS) (Packard 2300 liquid scintillation counter) over 30 minutes, disintegrations with an energy between 0.0-18.6meV counted and disintegrations per minute (DPM) calculated following external standardisation using a ^{60}Co source.

Methods

Nanoparticle Preparation

Poly lactide-co-glycolide was dissolved in dichloromethane (DCM) at a concentration of 1% w/v, containing 0.1 mg/mL of DiO. Varying volumes of the polymer solution were then premixed (on ice) with between 100 to 600 mL of Tween 80 solution at 6,000 rpm (Silverson mixer, Silverson Machines Ltd., Chesham, UK) for 5 minutes. The crude emulsion was Microfluidized for 25 cycles at pressures between 2,000 to 9,000 p.s.i.g. in an ice chilled Microfluidiser (model 110T, Microfluidics Corpn., Boston, USA), DCM was removed by stirring overnight under ambient conditions. Tween 80 was removed by gel permeation chromatography through a 2.3 × 30 cm column of Sepharose CL4B, with elution followed by UV absorbance (250 nm) and the fraction coincident with the first peak collected. Nanoparticle size was determined by photon correlation spectroscopy using a Malvern Zetasizer 4 instrument. The varying of phase volumes and microfluidsation pressures permitted the preparation of nanoparticles with diameters between 130 to 600 nm. Nanoparticle fluorescence (excitation 484 nm, emission 570 nm) was linear with concentration and unlabelled nanoparticles did not fluoresce.

Nanoparticle Surface Modification

EDC (0.284 g) and NHS (11.4 mg) (15:1 molar ratio) were dissolved in MES buffer (300 mM, pH 6.5, volume equivalent to 1/6th of nanoparticle suspension volume). Nanoparticle suspension (equivalent to 20 mg PLGA) was added, the volume adjusted to 1.5 mL with WFI then stirred at room temperature for twenty four hours. Nanoparticles were separated by gel permeation chromatography (Sephadex G75, 5 × 1.6 cm) with MES buffer (50 mM pH 6.5), the eluate's absorbance monitored (250 nm), and the 4 mL fraction coincident with the first peak collected. The required volume of MBP-Inv479 solution was added to the isolated nanoparticles along with MES buffer (300 mM pH 6.5, 83 μL) and the volume adjusted to 4.5 mL using WFI. The resulting suspension was stirred at 4°C for five hours. Surface modified colloid was separated by chromatography using Sepharose CL 4B (14 × 1.6 cm), eluted with WFI (unless otherwise stated). The eluate's absorbance (250 nm) was monitored, and the fraction coincident with the first peak collected. This turbid fraction was adjusted to 10 mL and stored at 4°C. MBP-Inv479 concentration was determined by standard techniques [13] using bovine serum albumin as a reference or by Liquid Scintillation Counting of labelled protein.

Incubation of HEp2 2B Cells with Particulates

Cells were maintained in RPMI1640 medium supplemented with FCS 10% v/v, L-glutamine, Fungizone, gentamicin and sodium bicarbonate adjusted to pH 7.4 and incubated at 37°C in a 5% CO_2 atmosphere. Cells were harvested by trypsinisation, the cell density adjusted to 1×10^5 cells/mL then 1 mL seeded into multiwell plates and adjusted to 5 mL with RPMI 1640. The medium was replaced each day, and the cells grown to a density of 5×10^5 cells per well. Medium was removed and 1 mL of treatment suspension containing 100 μg of nanoparticles in RPMI 1640 added. Final nanoparticle concentration was then adjusted with RPMI 1640 and plates incubated at 37°C in a 5% CO_2 atmosphere for 2 hours unless otherwise stated. Following incubation medium was removed, cells rinsed with ice cold PBS (3 × 1 mL), trypsinised and resuspended in 0.5 mL of ice cold PBS. This suspension was transferred to a vial containing 0.25 mL of 3% w/v formaldehyde in PBS, vortexed and stored at 4°C until analysed.

Flow Cytometry

Cell association in DiO labelled nanoparticles was determined by flow cytometry using a Coulter EPICS instrument. A 100 μL volume of cell suspension was flushed through the analysis zone at 20 μL/minute, both transmitted and scattered light was analysed. A linear relationship between polymer concentration and total fluorescence by flow cytometry was demonstrated for each batch of particles (data not shown). A control plot of side scattered against forward scattered light intensity was created for non-treated HEp2 2B cells, this profile was employed to distinguish cell associated fluorescence from that of free particles and to subtract cell autofluorescence. A histogram of fluorescence intensity against cell number was constructed by analysing 5,000 cells and total cell associated fluorescence was calculated for each suspension by integrating the area under this graph. Different instrument settings were required to detect cells and isolated nanoparticles therefore it was not possible to calibrate the instrument using nanoparticle suspensions. Percentage cell association, defined as the percentage of maximal observed fluorescence in an experiment, was therefore calculated and employed to define particle cell association for treatments involving the same batch of particles.

Since the specific fluorescence of different particle batches was found to vary results for treatments involving different particles could not be directly compared. However, particle fluorescence as determined by flow cytometry is di-

rectly related to that determined by fluorimetry, then the relationship observed between fluorescence and nanoparticle concentration can be used to normalise the flow cytometer data. Normalisation was achieved by multiplying total fluorescence, determined by flow cytometry, by the gradient of the relevant fluorimeter calibration series. This allowed a normalised cell associated fluorescence to be calculated.

RESULTS AND DISCUSSION

The kinetics of nanoparticle association with HEp2 2B cells demonstrates that cellular association increases during the first two hours with no significant increase occurring on longer incubation periods of up to six hours (Figure 1). Cellular association of uncoated particles is less than 10% of coated. A declining rate of cell association with time is a common feature of studies involving particulates, the profile's shape depending on experimental conditions [14]. According to Silverstein and Steinman (15) such slowing indicates adsorptive endocytosis, fluid phase uptake resulting in a linear association versus time profile. The present results can be compared to those of Valberg *et al.,* (16) who studied colloidal gold uptake into pulmonary macrophages, demonstrating a linear cell association versus time profile.

HEp2 2B nanoparticle uptake was assessed at 4°C and 37°C, and a 16 fold increase in modified nanoparticles association was observed at the higher temperature (Figure 2). Since cell metabolism is effectively halted at 4°C the result demonstrates that cell association is energy dependent and therefore related to endocytosis (15). Figure 2 also demonstrates a six fold increase in the cell association of surface modified with respect to unmodified nanoparticles at 4°C and over ten fold at 37°C. Cell internalisation is negligable at 4°C indicating that cell binding to the external surface is mediated principally by surface attached MBP-Inv479, and that attachment of unmodified particles is minimal in comparison. The results demonstrate that at 37°C internalised nanoparticles represent over 90% of the total cell association measured by this technique.

It has been demonstrated that the presence of denatured protein on a particle's surface can enhance its endocytotic capture (17). Since the coupling method employed to facilitate MBP-Inv479 attachment will produce a degree of denaturation it is conceivable that any increase in cell association is due to a general effect rather than to MBP-Inv479 *per se.* To investigate this possibility, bovine serum albumin (BSA) was crosslinked to nanoparticles and their cell association determined. Successful crosslinking was evidenced by a change in particle zeta potential (results not shown) although bound protein density was not determined. Figure 3, illustrates that BSA modified particle association is not significantly greater than unmodified, whilst the association of MBP-Inv479 modified particles is greatly increased. Suggesting that MBP-Inv479 modified nanoparticle cell association is due to a specific particle cell interaction. These results agree with those of Tabata and Ikada (18) who found non-opsonic compounds such as BSA do not enhance macrophage phagocytosis of poly(lactide) microspheres.

To determine if cellular association of MBP-Inv479 modified nanoparticles was receptor mediated experiments were conducted in the presence of free MBP-Inv479 as a competitive binding ligand. The results (Figure 4) demonstrate the ability of free MBP-Inv479 to reduce modified nanoparticle cell association to levels not significantly different to unmodified, indicating cellular association via the same receptor as free MBP-Inv479. However, this does not conclusively prove interaction with the invasin receptor, since MBP-Inv479 also possesses a maltose/maltodextrin binding component. Modified nanoparticles were therefore incubated with the arginine-glycine-aspartic acid tripeptide (RGD), which competes for the invasin binding site (19). RGD similarily inhibits modified nanoparticle cell association in a concentration dependant fashion (Figure 5) indicating a competitive

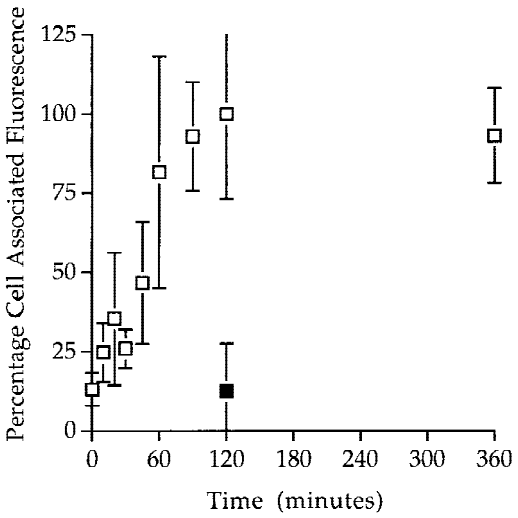

Fig. 1. Time course of cellular association of MBP-Inv479 coated PLGA nanoparticles. □ 100 mg of 196 nm diameter PLGA particles with a MBP-Inv479 concentration of 791 ng/mg incubated under standard conditions with HEp2 2B cells. ■ un-coated PLGA nanoparticle incubated as before. Mean association, n = 3 ± standard deviation. No significant difference between coated nanoparticle treatments at two and six hours, $p < 0.05$.

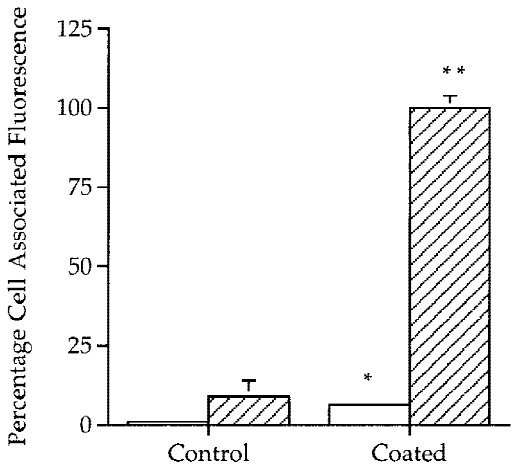

Fig. 2. Cellular association of nanoparticles as a function of temperature. 100 mg of 375 nm diameter PLGA particles incubated under standard conditions except for temperature. Control, unmodified PLGA nanoparticles; modified nanoparticles with a MBP-Inv479 concentration of 2,640 ng/mg. □ 4°C and ▨37°C. Mean association, n = 3 ± standard deviation. *Association significantly greater than control $p < 0.005$ at 4°C; **association significantly greater than control $p < 0.0025$ at 37°C.

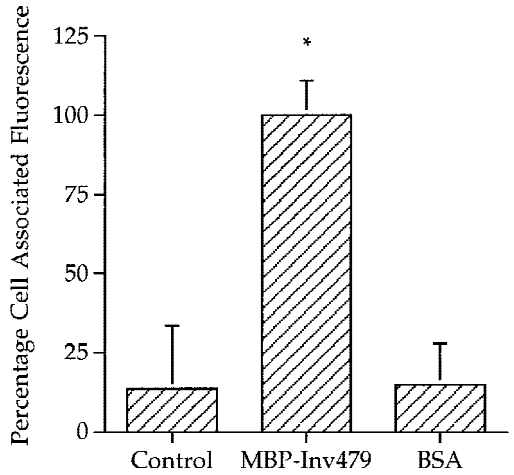

Fig. 3. Cellular association of nanoparticles cross linked with protein. Control, 100 mg of unmodified 196 nm diameter PLGA nanoparticles; MBP-Inv479, 100 mg of 196 nm diameter modified nanoparticles with MBP-Inv479 concentration of 791 ng/mg; BSA, 100 mg of 196 nm diameter nanoparticles crosslinked with BSA. Mean association n = 3 ± standard deviation. *Significantly greater than control $p < 0.0025$.

inhibition (20). Association falls to 17% of maximum at 2.5×10^{-6} M, with 50% inhibition occurring at 9×10^{-7} M. This suggests that surface modified particles and RGD bind to closely related if not identical receptor sites, and confirms the interaction of modified nanoparticles with the invasin receptor. This provides evidence for a specific and competitive interaction concurrent with a receptor mediated cell association process.

HEp2 2B cells were incubated with increasing concentrations of modified labelled nanoparticles, in the presence of excess blank (modified but non-fluorescent) nanoparticles and cell association determined. The results (Figure 6) demonstrate saturability of cellular association indicative of adsorptive endocytosis (15,21). The non-specific cell association component was linear and extremely small with the specific component deviating only slightly from total association at particle concentrations greater than 20 μg/mL. This indicates a saturable receptor/ligand interaction present in systems such as low density lipoprotein cell association (22).

The above indicates that MBP-Inv479 surface modified nanoparticle cell association is facilitated by MBP-Inv479 interaction with the invasin receptor present on the surface of Hep2-2B cells. The "zipper" hypothesis used to explain the internalisation of invasin bearing microorganisms, requires a continual series of receptor/ligand interactions around the "particle" to facilitate cellular entry (15). To determine if MBP-Inv479 surface density could influence internalisation, experiments were conducted utilising nanoparticles with varying coverage and different diameters (Figure 7). The results demonstrate that increased MBP-Inv479 surface coverage, irrespective of particle size, produces an increased cell association, reflecting literature findings for IgG and fibronectin coated particles (18). Two types of response were observed, small particles (155 nm and 200 nm) where cell association plateaus at high MBP-Inv479 coverage whilst larger particles (375 nm and 600 nm) exhibit a minimum at low coverage. These two forms of cell association profile may be attributable to a different mode of uptake for small and large particles.

Small diameter nanoparticles (155 nm and 200 nm) cannot stimulate their own phagocytosis therefore pinocytosis represents their only route for cellular uptake. Pinocytosis decreases as a function of diameter and is known to be restricted to particles less than approximately 150 nm (15) but without a sharp demarcation (23). The "zipper" hypothesis excludes small particles from phagocytosis due to the existence of a diameter below which the adjacent ligand molecule angle is too acute to provide the continual receptor/ligand interactions required to stimulate internalisation (21). Increasing ligand density will increase the number of potential

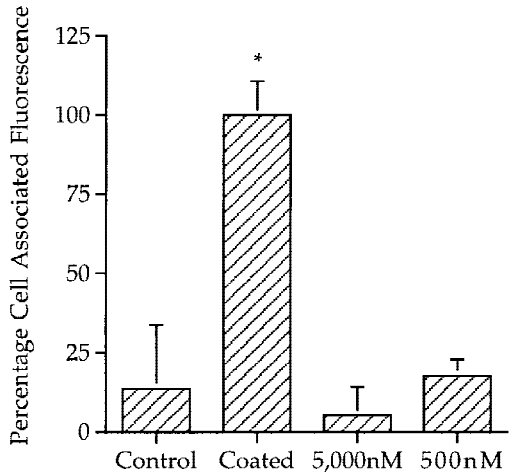

Fig. 4. Cellular association in presence of free MBP-Inv479. Control, 100 mg of unmodified 196 nm diameter nanoparticles; Coated, 100 mg of 196 nm diameter modified nanoparticles with MBP-Inv479 791 ng/mg; 5,000 nM, as "Coated" except nanoparticles co-incubated with 5,000 nM free MBP-Inv479; 500 nM, as "Coated" except nanoparticles co-incubated with 500 nM free MBP-Inv479. Mean association n = 3 ± standard deviation. *Significantly greater than control $p < 0.0025$.

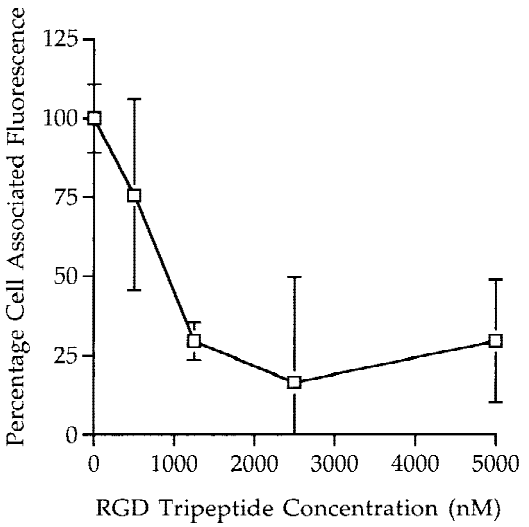

Fig. 5. Cellular association in the presence of RGD tripeptide. 100 mg of 196 nm diameter PLGA nanoparticles modified with 791 ng/mg MBP-Inv479 and incubated in the presence of increasing concentrations of RGD. Mean association n = 3 ± standard deviation.

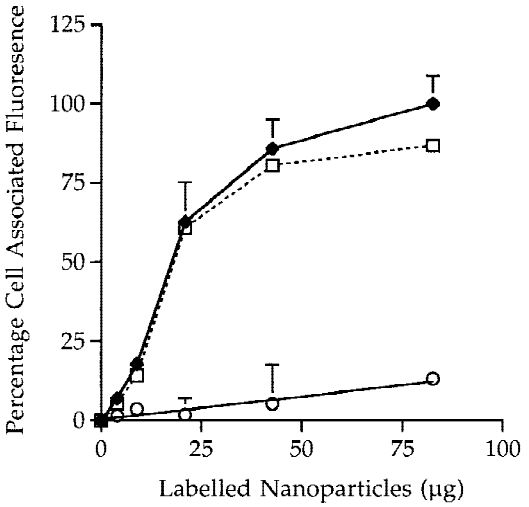

Fig. 6. Saturability of modified nanoparticle cell association. Modified nanoparticles incubated under standard conditions in the presence and absence of 1 mg unlabelled modified nanoparticles. Labelled nanoparticles 156 nm diameter PLGA nanoparticles modified with 1.31 ng/mg MBP-Inv479. Unlabelled nanoparticles 140 nm diameter PLGA nanoparticles modified with 1.52 ng/mg MBP-Inv479. ♦ total cell association (in the absence of unlabelled colloid); o nonspecific cell association (in the presence of unlabelled colloid); □ specific cell association (difference between total and nonspecific association). Mean association n = 3 ± standard deviation.

receptor interactions until a point at which the maximum number of interactions will occur and the rate of particle pinocytosis will approach the maximum allowed by the process's constitutive rate. Larger particles (375 nm and 600 nm) are taken up more readily and do not exhibit a plateau but an initial minimum followed by an increase as ligand density

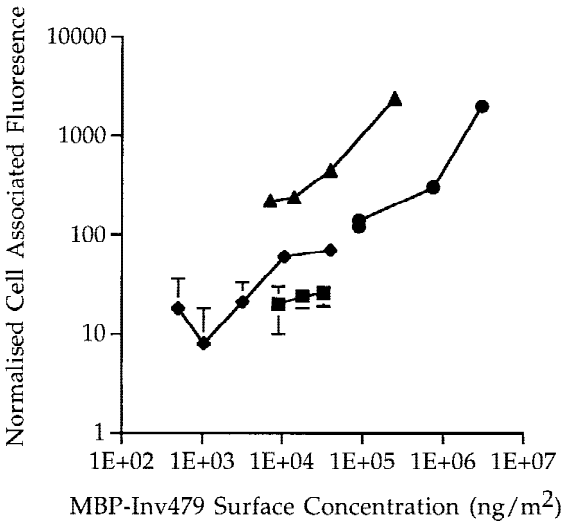

Fig. 7. Effect of MBP-Inv479 surface density and nanoparticle size upon cellular association. Cell incubation performed using standard conditions; ♦ 155 nm diameter PLGA nanoparticles; ■ 200 nm; ▲ 375 nm; ● 600 nm. Mean association n = 3 ± standard deviation. PLGA has a density of 1.39 g/cm^3, therefore the total surface area for a given nanoparticle mass can be calculated based on the measured hydrodynamic diameter. The amount of bound protein can be measured and the degree of protein coverage expressed as a quantity bound per unit surface area (ng/m^2).

increases. This is concurrent with the zipper hypothesis that larger particles stimulate phagocytosis by promoting continual receptor/ligand interactions around their surface. Internalisation is thus dependent only upon receptor ligand interactions and is not limited by the rate of pinocytotic vesicle production. This is in agreement with the observation (24) that the internalisation of MBP-Inv479 expressing *Staphylococcus aureus* increases in a linear fashion as ligand density increases. These results indicate that ligand density and particle size are critical parameters controlling cellular association.

CONCLUSIONS

MBP-Inv479 surface modified particles are associated with HEp2 2B cells in an energy dependent fashion which, is highly specific, saturable, and inhibited by both free MBP-Inv479 and RGD. This is concurrent with receptor mediated endocytosis involving integrins of the β1 subfamily which are responsible for the internalisation of invasin bearing bacteria. Increasing MBP-Inv479 surface density produces an increased cell association with two types of profile resulting depending on particle diameter. Small particles only capable of pinocytosis exhibit a plateau indicating that as ligand surface density increases it produces a maximal cellular association probably equivalent to the rate of pinocytosis. Larger particles do not exhibit a maximum in their ligand surface density cell association profiles probably due to an ability to stimulate phagocytosis via the zipper mechanism. This illustrates that the utility of invasin type ligands to promote the cellular interaction of particulates will be controlled by the particle's diameter, the ligand's surface density and ultimately the ligand's natural biological cellular association properties.

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