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Bacterial internalization mediated by β_1 chain integrins is determined by ligand affinity and receptor density

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Binding of bacteria to β_1 chain integrin receptors results in either bacterial adherence or uptake by cultured cells (Isberg, 1991). In this report we show that *Staphylococcus aureus* coated with high affinity ligands for the β_1 chain integrin family can be internalized efficiently, whereas bacteria coated with low affinity ligands are poorly internalized. Overproduction of the $\alpha_5\beta_1$ integrin increased the efficiency of bacterial internalization, indicating that the uptake efficiency is directly related to the level of expression of the receptor. By using latex beads or *S.aureus* coated with mAbs directed against the $\alpha_5\beta_1$ integrin, a roughly semi-logarithmic correlation was observed between the affinity of the receptor–ligand interaction and the rate of bacterial internalization. Evidence is presented that high affinity binding of the bacterium allows the microorganism to compete efficiently with receptor–ligand interactions at the basolateral surface of the cell.

Key words: bacterial uptake/integrins/invasin/ligand affinity/receptor density

Introduction

The ability of a microbe to establish a niche in the host is an important determinant of its pathogenesis. Some virulent bacteria are able to adhere to the external surface of host cells and this property allows them to colonize specific tissues (Abraham *et al.*, 1983; Van de Water *et al.*, 1983; Flock *et al.*, 1987). In contrast, other pathogens can enter normally non-phagocytic host cells (Moulder, 1985; Finlay and Falkow, 1988; Maurelli and Sansonetti, 1989; Isberg, 1990). The ability to enter into host cells may be a requirement for multiplication in the host, as is the case with the obligate intracellular bacterium *Chlamydia trachomatis* (Moulder, 1991), or it may be an important step in gaining access to deeper tissues, as with enteroinvasive bacteria such as salmonellae and yersiniae (Une, 1977; Finlay and Falkow, 1988).

After ingestion by the host, the enteroinvasive bacterium *Yersinia pseudotuberculosis* enters cells exposed at the surface of the lumen of the intestine and reaches the lamina propria where it initiates spread to other organs via the lymphatic system (Une, 1977). Internalization of *Y.pseudotuberculosis* by normally non-phagocytic cells occurs by at least three distinct routes (Isberg, 1990). The most efficient of these pathways is mediated by a single bacterial surface protein called invasin, which binds to several heterodimeric β_1 chain integrins (Isberg and Falkow, 1985; Isberg *et al.*, 1987; Isberg and Leong, 1990). The fact that invasin promotes bacterial internalization by binding to integrin receptors raises an interesting problem: the β_1 chain integrins are usually associated with cellular attachment to the extracellular matrix (Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992) and are not typically involved in phagocytosis.

Previous studies of the internalization of particles by β_1 chain integrins have given conflicting results. For instance, beads coated with fibronectin, which binds the $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins, were shown to be phagocytosed by BHK cells, although internalization was predominantly observed in round cells and was less obvious in spread cells (McAbee and Grinnell, 1985; Grinnell and Geiger, 1986). On the other hand, bacteria such as *Streptococci* spp., *Staphylococcus aureus* and *Pseudomonas aeruginosa* can be coated by a variety of integrin ligands such as collagen and fibronectin (Flock *et al.*, 1977; Abraham *et al.*, 1983), but these organisms usually adhere to host cells without subsequent internalization (Van de Water *et al.*, 1983). A similarly paradoxical situation has been observed *in vitro* using cultured HEp-2 cells (Rankin *et al.*, 1992). Invasin-coated bacteria are efficiently internalized whereas fibronectin-coated bacteria are not, even though both bind to the $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins (Isberg and Leong, 1990).

The difference in the ability of invasin and fibronectin to mediate internalization is probably not due to a difference in the binding sites on the receptor (Tran Van Nhieu and Isberg, 1991). Also, the presence of a particular domain of invasin responsible for internalization is unlikely since the minimal portion of invasin that binds integrins (Leong *et al.*, 1990) is also sufficient to promote uptake (Rankin *et al.*, 1992). One distinct difference between these two ligands is that invasin binds to the $\alpha_5\beta_1$ integrin with a much higher affinity than does fibronectin (Tran Van Nhieu and Isberg, 1991). In this report we show that high affinity binding to β_1 chain integrins is sufficient to promote bacterial internalization under conditions in which integrin receptor concentration is limiting.

Results

S.aureus coated with anti-integrin mAbs are efficiently internalized by cultured HEp-2 cells

We have found that invasin-coated bacteria are much more efficiently internalized by mammalian cells than are fibronectin-coated bacteria, even though both ligands bind identical receptors, probably at the same site (Isberg and Leong, 1990; Tran Van Nhieu and Isberg, 1991; Rankin *et al.*, 1992). To determine if mere binding to β_1 chain integrins is sufficient by itself to promote bacterial internalization, we decided to use anti-integrin mAbs as

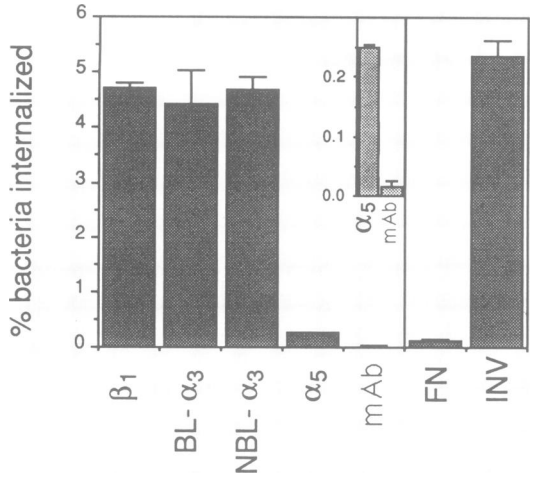

Fig. 1. Anti-β_1 chain integrin antibodies promote efficient internalization of *S.aureus* in cultured HEp-2 cells. *S.aureus* was coated with anti-integrin antibody (left panel), MBP-inv479 hybrid protein or fibronectin (right panel) as described (Materials and methods). The coated bacteria were then used to infect monolayers of HEp-2 cells and internalized bacteria were quantified by the gentamicin protection assay (Isberg and Falkow, 1985). Anti-β_1 and anti-α_3 antibodies promoted bacterial internalization at levels comparable to invasin, whereas anti-α_5 antibody or fibronectin were about 20-fold less efficient. β_1: *S.aureus* coated with anti-rat IgG followed by rat mAb AIIBII; BL-α_3: *S.aureus* coated with anti-mouse IgG followed by mouse mAb P1B5; NBL-α_3: *S.aureus* coated with anti-mouse IgG followed by mouse mAb J143; α_5: *S.aureus* coated with anti-mouse IgG followed by mouse mAb P2E6; mAb: *S.aureus* coated with anti-mouse IgG followed by mouse mAb 9G11, an irrelevant mAb; Fn: *S.aureus* coated with anti-fibronectin mAb clone III (Telios) followed by human fibronectin (Sigma); Inv: *S.aureus* coated with anti-MBP antiserum followed by MBP-Inv479. Each value is the mean of 3–10 independent determinations.

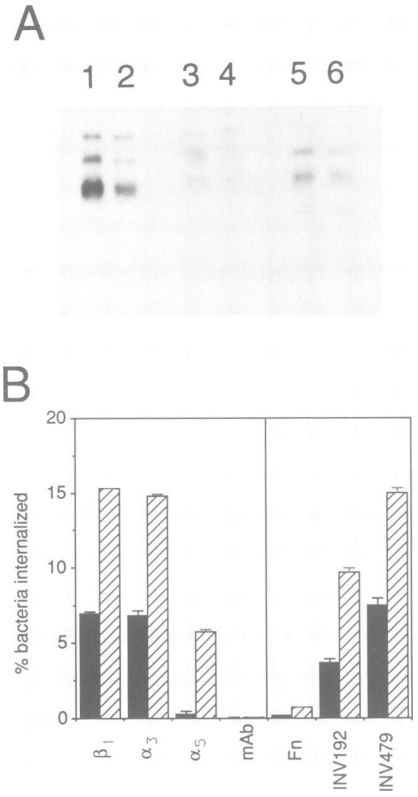

Fig. 2. (**A**) Immunoprecipitation analysis of β_1 chain integrins expressed by the Hα-1 transfectant that overproduces the $\alpha_5\beta_1$ integrin. Approximately 10^7 cells/lane of HEp-2 cells (HEp-2) or of the cellular clone transfected with the pSVneo-α_5 plasmid (Hα-1) were labeled as described (Materials and methods) and detergent solubilized extracts were precipitated with anti-integrin monoclonal antibodies, followed by incubation with anti-mouse beads or anti-rat beads. The immunoprecipitates were analyzed by SDS–PAGE on a 7.5% polyacrylamide gel under non-reducing conditions, followed by transfer to an Immobilon filter, probing with streptavidin–horseradish peroxidase and chemiluminescent detection (Materials and methods). Results were quantified by scanning densitometry. Lanes 2, 4, 6: immunoprecipitates from HEp-2 cells. Lanes 1, 3, 5: immunoprecipitates from Hα-1 cells. Lanes 1, 2: immunoprecipitation with anti-β_1 mAb AIIBII and anti-rat beads. Lanes 3, 4: immunoprecipitation with anti-α_5 mAb B2G2 and anti-rat beads. Lanes 5, 6: immunoprecipitation with anti-α_3 mAb P1B5 and anti-mouse beads. The Hα-1 clone expresses ~10-fold more $\alpha_5\beta_1$ integrin and about two times more $\alpha_3\beta_1$ and β_1 chain integrins than parental HEp-2 cells, as estimated by scanning densitometry (Materials and methods). (**B**) The efficiency of bacterial internalization via the $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins is dependent on the receptor expression level in HEp-2 cells. *S.aureus* was coated with anti-integrin antibodies, with MBP-Inv479 or with fibronectin (as in Figure 1) and used to infect monolayers of Hα-1 cells (hatched bars) or HEp-2 cells (black bars). The percentage of internalized bacteria was determined by the gentamicin protection assay (Isberg and Falkow, 1985). The efficiency of bacterial internalization is dependent on the level of expression of the integrin. β_1: *S.aureus* coated with anti-rat IgG and rat mAb AIIBII, α_3: *S.aureus* coated with anti-mouse IgG and mouse mAb P1B5, α_5: *S.aureus* coated with anti-mouse IgG and mouse mAb P2E6, mAb: *S.aureus* coated with anti-mouse IgG and mouse mAb 9G11, Fn: *S.aureus* coated with anti-fibronectin mAb clone III (Telios) and human fibronectin (Sigma). Inv192: *S.aureus* coated with anti-MBP antiserum and MBP-Inv192. Inv479: *S.aureus* coated with anti-MBP antiserum and MBP-Inv479. Each value is the mean of 3–10 determinations.

bacterial coating ligands and to test the ability of these coated bacteria to be internalized by cultured mammalian cells.

S.aureus cells were coated first with rabbit anti-mouse IgG antiserum and then with anti-integrin antibodies (Materials and methods). The coated bacteria were then tested for their ability to be internalized by cultured HEp-2 cells. Bacteria coated with anti-$\alpha_3\beta_1$ or anti-β_1 mAbs were internalized by cells with an efficiency comparable to invasin coated bacteria (Figure 1; bars BL-α_3, NBL-α_3 and β_1), whereas anti-$\alpha_5\beta_1$ coated bacteria were internalized ~20-fold less efficiently (Figure 1; bar α_5), but ~10-fold above the control *S.aureus* coated with an irrelevant mAb (Figure 1; bar mAb). Both the anti-$\alpha_3\beta_1$ integrin mAb that blocks cell attachment to fibronectin (Figure 1; P1B5, BL-α_3) and the one that does not block cell attachment (Figure 1; J143, NBL-α_3) allowed similar efficiencies of bacterial internalization, suggesting that the specific site bound on the integrin receptor had little effect on the efficiency of integrin-mediated bacterial internalization. The internalized bacteria coated with anti-$\alpha_3\beta_1$ or anti-$\alpha_5\beta_1$ integrin mAb were found within endocytic vacuoles inside the cell in a fashion similar to that seen with invasin coated bacteria (not shown). Thus, internalization of the coated bacteria appeared indistinguishable from invasin-mediated uptake.

The $\alpha_5\beta_1$ integrin can efficiently promote bacterial internalization when over-expressed in HEp-2 cells

From the previous experiments it appeared that invasin-mediated bacterial internalization can be mimicked by mAb binding to the same β_1 chain integrin receptors. *S.aureus* coated with anti-α_5 mAb, however, were internalized by cells about 20-times less efficiently than an invasin derivative

Table I. Characterization of anti-$\alpha_5\beta_1$ integrin monoclonal antibodies

Mab	Specificity[a]	ED_{50} M[b]	Binding inhibition[c] %								Group[d]
			IIA1	VD1	IVF4	IVG5	IIIB8	VC5	VD10	6F4	
IIA1	$\alpha_5\beta_1$ bl.	5.0×10^{-10}	100	50	0	0	0	0	0	0	A
VD1	$\alpha_5\beta_1$ bl.	8.0×10^{-9}	67	100	0	0	0	0	0	0	A
IVF4	$\alpha_5\beta_1$ bl.	2.5×10^{-8}	0	0	100	32	50	0	0	65	B2
IVG5	$\alpha_5\beta_1$ bl.	5.8×10^{-8}	44	13	81	100	85	0	0	85	B3
IIIB8	$\alpha_5\beta_1$ nbl.	2.0×10^{-8}	0	0	93	81	100	0	0	87	B1
VC5	$\alpha_5\beta_1$ nbl.	8.9×10^{-10}	0	0	0	0	0	100	0	0	C
VD10	β_1 nbl.	2.7×10^{-7}	0	0	0	0	0	0	100	0	D
6F4	α_{β_1} nbl.	8.3×10^{-8}	0	0	60	25	40	0	0	100	B1

[a]The specificity of the mAb was determined from Figure 3. bl: mAb blocking cell binding to invasin or fibronectin coated surfaces. nbl: mAb which does not interfere with cell binding to invasin or fibronectin coated surfaces (Materials and methods).
[b]The ED_{50} of each mAb corresponds to the concentration of unlabeled mAb required to give 50% maximal inhibition of binding to monolayers of HEp-2 cells of trace amounts of the same mAb labeled with ^{125}I (Materials and methods).
[c]The % inhibition represents the relative ability of the individual mAbs, indicated on top, to inhibit cell-binding of the labeled mAb, indicated on the left column, when pre-incubated with cells at a 100-fold excess (Materials and methods).
[d]Groups are deduced from the relationship between the pattern of percentage of inhibition by the different mAbs. The mAbs IVF4, IVG5, IIIB8 and 6F4 present related patterns of inhibition and define the subgroups B1–B3.

(MBP-Inv479), anti-β_1 or anti-α_3 coated *S.aureus*. This difference observed in the efficiency of internalization could be explained if the $\alpha_5\beta_1$ integrin is poorly expressed in HEp-2 cells. Immunoprecipitation analysis of an extract of surface-labeled cells using anti-α_3, anti-α_5 and anti-β_1 integrin mAbs followed by scanning densitometry showed that the parental HEp-2 cells expressed about 7% as much as $\alpha_5\beta_1$ as $\alpha_3\beta_1$ integrin (Figure 2A; lanes 4 and 6). To determine whether the $\alpha_5\beta_1$ integrin was competent to internalize bacteria, the expression of the $\alpha_5\beta_1$ was increased by transfecting HEp-2 cells with the pSVneo-α_5 plasmid (Materials and methods) containing the full length cDNA clone coding for the human α_5 subunit (Argraves *et al.*, 1986). A HEp-2 cell clone, Hα-1, expressing higher amounts of the $\alpha_5\beta_1$ was selected (Materials and methods). The transfected clone Hα-1 was shown to have expression levels of the $\alpha_5\beta_1$ integrin that were about 10-times higher than with the parental HEp-2 cells (Figure 2A; lanes 2 and 4). In addition, the surface expression level of the entire family of β_1 chain integrin receptors was increased by ~50% relative to the parental line (Figure 2A; lanes 1 and 2).

S.aureus was coated with anti-integrin mAbs and monolayers of the Hα-1 clone were infected with the coated bacteria. As shown in Figure 2B, the efficiency of internalization of anti-α_5 mAb coated *S.aureus* by Hα-1 was increased 10-fold relative to the parental HEp-2 cells (Figure 2B; α_5). The efficiency of internalization by the Hα-1 cellular clone of *S.aureus* coated with anti-β_1 mAb (β_1), anti-α_3 mAb (α_3) or the invasin derivative (MBP-Inv479) increased only 2- to 3-fold compared with the parental HEp-2 cells, in agreement with the relative increase of β_1 integrin levels found in this clone. Interestingly, internalization of fibronectin coated *S.aureus* was also increased approximately 3-fold in the Hα-1 clone, although the levels of internalization were still far below those found with invasin (Figure 2B; bar Fn). These results show that the $\alpha_5\beta_1$ integrin can promote bacterial internalization with an efficiency comparable to the $\alpha_3\beta_1$ integrin when over-expressed in HEp-2 cells (Figure 2B; compare α_3 solid bar to α_5 hatched bar) and that the efficiency of bacterial internalization mediated by these β_1 chain integrin receptors is dependent on their level of expression in HEp-2 cells.

Identification of anti-$\alpha_5\beta_1$ integrin monoclonal antibodies that vary in their efficiency of promoting bacterial internalization

The above results indicated that the efficiency of bacterial uptake depended on the number of β_1 chain integrin receptors recognized by the ligand coating the bacterium. However, invasin and fibronectin bind identical receptors on HEp-2 cells (Isberg and Leong, 1990) and yet fibronectin usually mediates bacterial adherence rather than internalization (Van de Water *et al.*, 1983; Rankin *et al.*, 1992; Cheng,S.Y., unpublished results). The difference between these two ligands must reflect a difference in the nature of the interaction between these ligands and their integrin receptors. We decided to use mAbs to investigate further the interaction of bacteria and integrin receptors by testing if mAbs recognizing different sites on the $\alpha_5\beta_1$ integrin receptor could promote different levels of bacterial uptake by cultured HEp-2 cells.

Monoclonal antibody-producing hybridoma clones were isolated using purified placental $\alpha_5\beta_1$ integrin as an antigen (Materials and methods). Seven clones were isolated that specifically recognized the $\alpha_5\beta_1$ integrin as shown by immunoprecipitation of surface labeled cells without or with pre-incubation with a previously characterized anti-$\alpha_5\beta_1$ mAb (Materials and methods; Tran Van Nhieu and Isberg, 1991). One clone (VD10) recognized an epitope present in the β_1 subunit (Table I).

S.aureus was coated with these different anti-α_5 mAbs and then assayed for uptake by monolayers of HEp-2 cells (Figure 3A) or the Hα-1 overproducing clone (Figure 3B). In the case of the parental HEp-2 cells, the mAbs promoted levels of bacterial internalization ranging from 0.02% (Figure 3A; IVG5) to 2.5% (Figure 3A; IIA1). In the case of the Hα-1 clone the bacteria were internalized 0.2–5.5% (Figure 3B; IIA1), consistent with the specificity of these mAbs for the $\alpha_5\beta_1$ integrin. The fact that blocking mAbs, defined as mAbs able to inhibit cell attachment to invasin or fibronectin (Figures 3A and 4B; IIA1, VD1 and IVF4) or non-blocking mAbs (Figures 3A and 4B; IIIB8 and VC5) promoted comparable efficiencies of uptake indicated that the binding site on the $\alpha_5\beta_1$ integrin recognized by the mAb was not important for bacterial internalization, but rather some other physical property caused this difference.

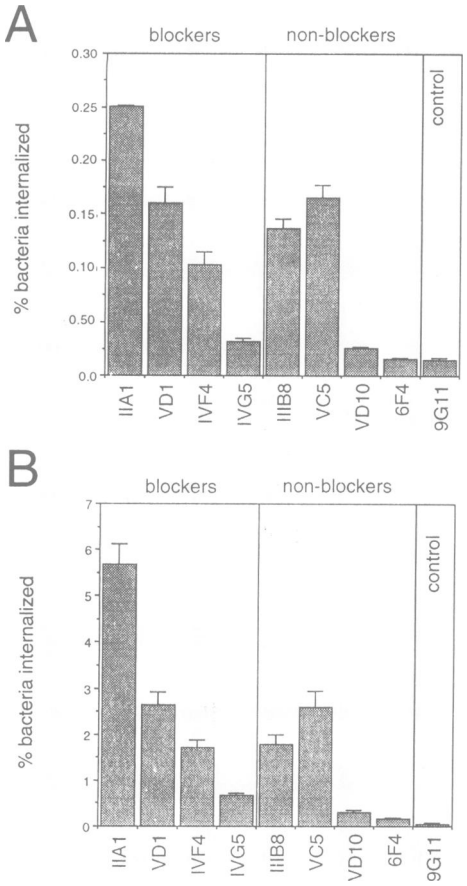

Fig. 3. Various anti-$\alpha_5\beta_1$ antibodies promote different levels of bacterial internalization. (**A**) HEp-2 cells. (**B**) Hα-1 transfectant. *S.aureus* was coated with the various anti-α_5 mAbs and used to infect monolayers of HEp-2 cells (HEp-2) or Hα-1 cells (Hα-1). Internalized bacteria were quantified using the gentamicin protection assay (Isberg and Falkow, 1985). Each bar is the mean of 5–10 determinations. Individual mAbs used to coat *S.aureus* are denoted on the x-axis.

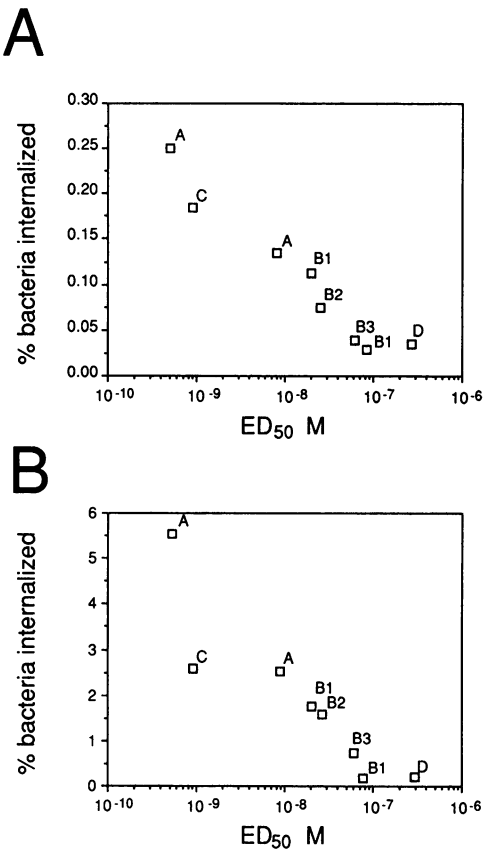

Fig. 4. Relationship between the antibody affinity and the efficiency of bacterial internalization. The ED_{50} of the various mAbs for HEp-2 cells monolayers was determined (Materials and methods). The ED_{50} is plotted as a function of the bacterial internalization promoted by the mAb for HEp-2 cells (panel **A**, □) or Hα-1 cells (panel **B**, □). In each case mAbs having an ED_{50} >100 nM are relatively inefficient at promoting bacterial internalization. Higher affinity mAbs show a rough semi-logarithmic relationship between their ED_{50} and the percentage of bacterial internalization that they promote. Letters refer to group of mAbs, as defined by cross-inhibition experiments (Table I).

To determine if mAbs that promoted uptake bound to related sites the mAbs were labeled with rhodamine (Materials and methods) and used in cross-inhibition experiments. These mAbs could be classified into four different groups in this fashion (Table I; Groups A–D). Group B mAbs consisted of mAbs that were able to inhibit partially each others binding and could be subdivided in three subgroups (Table I; Groups B1–B3). The existence of these four distinct mAb groups was confirmed by independently demonstrating that two mAbs belonging to different groups could bind a single $\alpha_5\beta_1$ integrin receptor simultaneously (Materials and methods; Tran Van Nhieu,G. and Isberg,R., unpublished results). The fact that mAbs IIA1 (Group A), IVF4 (Group B2) and VC5 (Group C) defined three distinct binding groups and were able to promote significant levels of bacterial internalization (Figures 3A and 4B; IIA1, IVF4 and VC5) indicated that binding a particular site on the $\alpha_5\beta_1$ integrin cannot be critical for bacterial internalization.

The affinity of the ligand for the $\alpha_5\beta_1$ integrin receptor determines the efficiency of bacterial internalization

In order to test if the affinity of the ligand was the critical determinant of bacterial internalization, the affinity of the eight different anti-$\alpha_5\beta_1$ mAbs was determined based on their ED_{50}, as previously described (Tran Van Nhieu and Isberg, 1991; Materials and methods; Table I, ED_{50}). In contrast to the binding site results, a good correlation was found between the ED_{50} of each antibody and its capacity to promote bacterial internalization. High affinity antibodies having an ED_{50} of the order of 10^{-9}–10^{-10} M promoted consistently higher cellular uptake (Figures 3A and 4; mAbs IIA1, VD1 and VC5) than lower affinity mAbs. mAbs having an affinity for the receptor with an ED_{50} of the order of 10^{-7} M, in contrast, were at least 10 times less efficient at promoting uptake (Figure 3A and 4; mAbs VD10 and 6F4) than the high affinity mAbs. For comparison, invasin having a high affinity for the $\alpha_5\beta_1$ receptor (ED_{50} invasin $= 6.2 \times 10^{-9}$ M) (Tran Van Nhieu and Isberg, 1991) is ~20-fold more efficient than fibronectin (ED_{50} fibronectin $= 4.1 \times 10^{-7}$ M) at promoting bacterial uptake (Figure 1).

The relationship between affinity and uptake was maintained in both HEp-2 cells and the Hα-1 overproducer (Figures 4A and 5B). For the low affinity mAbs having an ED_{50} of the order of 10^{-7} M the efficiency of bacterial uptake remained relatively low, approximately 0.02% for HEp-2 cells (Figure 4A) and 0.2% for the Hα-1 transfectant (Figure 4B). The efficiency of bacterial uptake decreased as a rough semi-logarithmic function of the ED_{50}

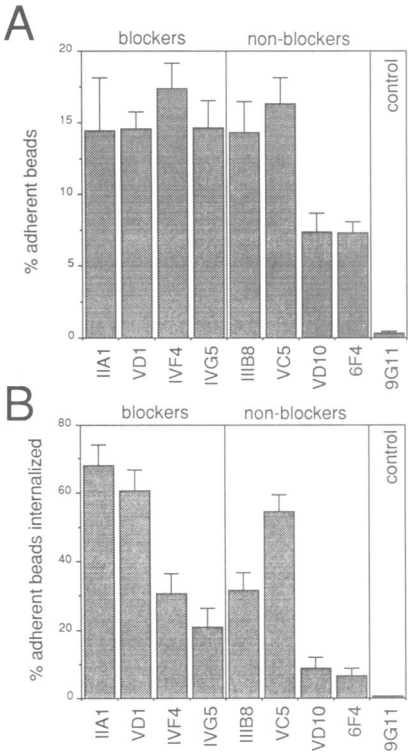

Fig. 5. Adherence and internalization of anti-$\alpha_5\beta_1$ mAb coated beads by HEp-2 cell monolayers. Latex beads (1.1 μm) were coated with the various mAbs and incubated with monolayers of HEp-2 cells at a multiplicity of 5 beads/cell. After incubation for 2 h at 22°C unbound beads were washed with PBS. (**A**) For determination of cell-associated beads the cells were fixed with paraformaldehyde and adherent beads were counted microscopically (Materials and methods). The percentage of cell-associated beads is calculated by dividing the number of cell-associated beads by the number of input beads. (**B**) For determination of internalized beads, after removal of the unbound beads by washing with PBS, prewarmed medium was added to the cell monolayers and adherent beads were allowed to be internalized for 45 min at 37°C. The cells were subsequently fixed and probed with anti-mouse IgG antibody–FITC. The percentage of beads internalized was calculated by subtracting the number of beads labeled with FITC from the total number of beads bound determined under phase contrast and dividing by the total number of beads (Materials and methods). Each bar represents an average of 10 microscopic field determinations including 40–60 HEp-2 cells. Individual mAbs used to coat latex beads are denoted on the x-axis. Control: beads coated with an irrelevant mAb. Low affinity antibodies promote adherence of latex beads to HEp-2 cells, but a low efficiency of subsequent internalization. Each value represents the mean of determinations on three independent coverslips.

(r =0.917), although the slopes were somewhat different for the two cell lines. The one exception was the high affinity non-blocking mAb VC5 (Group C), which deviated significantly for the semi-logarithmic correlation especially in the case of the Hα-1 transfectant. It seems likely that the site recognized by this mAb must have some influence on the level of bacterial internalization, perhaps due to steric constraints.

These results indicate that the affinity of the ligand for the integrin receptor is the critical determinant of bacterial internalization and that the importance of ligand affinity is more acute when the integrin receptor is over-expressed. To gain further support for the importance of affinity a derivative of invasin was analyzed that promotes less efficient bacterial internalization than MBP-Inv479 (Rankin *et al.*, 1992). This particular derivative (MBP-Inv192) was purified and the affinity of the purified protein for whole HEp-2 cells was

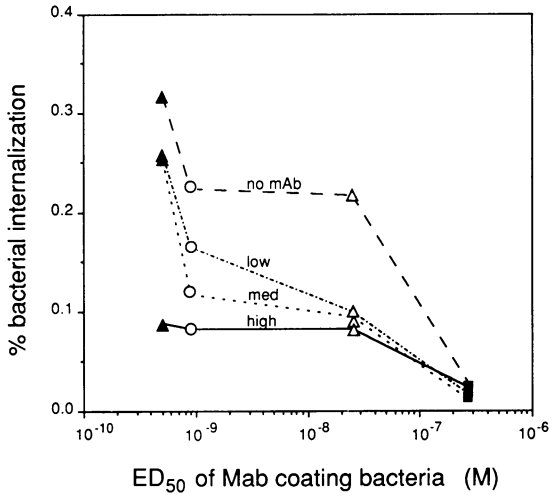

Fig. 6. Effect of $\alpha_5\beta_1$ receptor–ligand interactions at the cell ventral surface on bacterial internalization. *S.aureus* coated with anti-$\alpha_5\beta_1$ mAbs was used to infect HEp-2 cells plated on high (IIA1), intermediate (VC5) or low (VD10) affinity mAbs (Table I, Materials and methods). The percentage of internalized bacteria was determined by the gentamicin protection assay (Isberg and Falkow, 1985). The percentage of internalization is plotted as a function of the relative ED_{50} of the anti-$\alpha_5\beta_1$ mAbs. *S.aureus* coated with: IIA1 (▲); VC5 (○); IVF (△); VD10 (■). HEp-2 cells plated on: the high affinity mAb IIA1 (_____); the intermediate affinity mAb IVF4 (- - - -); the low affinity mAb VD10 (-·-·-·-); no mAb (- - - - -).

determined as previously described (Tran Van Nhieu and Isberg, 1991; data not shown). The two hybrids differ in their K_d by ~12-fold [K_d (MBP-Inv479) = 5 nM versus K_d (MBP-Inv192) = 70 nM]. As expected, the lower affinity ligand is less efficient at promoting bacterial internalization than the higher affinity ligand (Figure 2B; compare MBP-Inv192 and MBP-Inv479).

Low affinity receptor–ligand interaction promotes extracellular attachment whereas high affinity interaction results in internalization

The less efficient internalization observed with lower affinity ligands could be the result of two distinct phenomena. A lower affinity could result in a smaller number of bacteria binding to the cell, effectively decreasing the number of bacteria being sampled by the cell. Alternatively, a lower affinity could result in surface adhesion without subsequent internalization. In order to distinguish between these two possibilities, the efficiency of surface adhesion of particles coated by integrin ligands was quantified. To this end latex beads coated with the various mAbs were allowed to bind to cells and the bound beads were subsequently counted microscopically. In parallel experiments bound beads were allowed to be internalized for 30 min at 37°C and the number of internalized beads was determined using fluorescence microscopy (Grinnell and Geiger, 1986; Materials and methods).

As shown in Figure 5A, for most of the mAbs analyzed ~15–17% of the initial input beads bound the cells, although the two lowest affinity mAbs, VD10 and 6F4, promoted cell binding at approximately one-half this efficiency. The binding was specific, as beads coated with an irrelevant mAb did not significantly bind to cells (Figure 5A; bar 9G11).

In contrast to the binding results, a pattern very similar to the one observed with *S.aureus* was obtained when

internalization was measured (Figure 5B). High affinity mAb (IIA1, VD1 and VC5) promoted internalization of ~60–75% of the bound beads, intermediate affinity mAb (IIIB8, IVF4, IVG5) promoted internalization of ~20–30% of the bound beads, whereas low affinity mAbs (VD10 and 6F4) allowed internalization of only 6–8% of the bound beads (Figure 5B). Similar results were obtained when determining the relative percentage of internalization of mAb-coated beads bound to the Hα-1 transfectant using the same experimental conditions. In this case, however, more beads (up to 50% of the initial input beads) were bound to the cells (data not shown). It should be noted that the percentages of internalization were higher in the case of coated beads than in the case of coated *S.aureus*. This difference probably results from a variety of different parameters in these two protocols (i.e. multiplicity of infection, difference in sedimentation rate of latex beads, gentamicin protection versus antibody accessibility). In spite of these differences the overall results from these two protocols were identical.

These results demonstrate that the efficiency of adhesion of the particles to the cell surface is not the limiting factor, but rather that the bound particles are inefficiently internalized if the receptor–ligand interaction is of low affinity.

High affinity ligands can compete with receptor–ligand interactions at the ventral surface of the cell to promote bacterial internalization

The facts that low affinity ligands can promote stable adherence of the bacterium to the cell without internalization and that overproduction of the $\alpha_5\beta_1$ integrin in HEp-2 cells results in a significant increase in internalized bacteria suggests that the number of available receptors is an important determinant in discriminating between surface adhesion and bacterial uptake. β_1 chain integrin receptors promote cell attachment to the extracellular matrix (Hynes, 1987) and it is likely that interactions between these receptors and the extracellular matrix proteins at the basolateral surface of the cell limit the number of receptors available for invasive bacteria. This binding could represent an important source of competition for available receptors. To test this possibility we decided to determine the efficiency of bacterial internalization by cells plated on $\alpha_5\beta_1$ integrin ligands of varying affinities.

In order to determine if binding of the mammalian cells to a matrix of high affinity affects the availability of receptor for uptake, HEp-2 cells were plated on plastic surfaces previously coated with either the high affinity mAb IIA1 (Figure 6; high), the intermediate affinity mAb IVF4 (Figure 6; med), the low affinity mAb VD10 (Figure 6; low) or no $\alpha_5\beta_1$ ligand (Figure 6; no mAb). Cells plated on these different ligands were then challenged with *S.aureus* coated with either low (Figure 6; solid squares), intermediate (Figure 6; open triangles) or high (Figure 6; open circles and solid triangles) affinity anti-α_5 mAbs. As shown in Figure 6, bacteria coated with the low affinity mAb VD10 were poorly internalized, independent of the affinity of the substrate on which the HEp-2 cells were plated (Figure 6; solid squares). In contrast, bacteria coated with high affinity ligands IIA1 or VC5 were generally more efficiently internalized by HEp-2 cells plated on no ligand or a low affinity ligand than if the cells were plated on a high affinity ligand (Figure 6; solid triangles and open circles, compare no mAb, low, med and high). This result indicates that integrin interactions at the ventral surface of the cell can modulate the ability of the cell to internalize bacteria and that bacteria coated with high affinity ligands compete more efficiently to allow bacterial internalization.

Discussion

In this report we showed that *S.aureus* coated with anti-integrin mAbs could be internalized by cultured cells at an efficiency similar to that observed with invasin-coated bacteria. The results presented here are consistent with the notion that no domain other than the cell binding domain of invasin (Leong *et al.*, 1991) is required for internalization (Rankin *et al.*, 1992) and that invasin promotes bacterial uptake simply by binding to its β_1 chain integrin receptors with high affinity. These results are in distinct contrast to studies on internalization of *Salmonella typhimurium*, in which binding and signaling for internalization are clearly encoded by different components (Galan *et al.*, 1992). This emphasizes the point that bacteria utilize multiple strategies to get internalized by host cells.

In this study we have found that both the $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins could efficiently mediate bacterial internalization, even though it has been previously suggested that in contrast to the $\alpha_5\beta_1$ receptor the $\alpha_3\beta_1$ integrin does not participate in the endocytotic cycle (Bretscher, 1992). These results can be explained either by supposing that cycling properties of the $\alpha_3\beta_1$ integrin receptor in HEp-2 cells differ from that in the previous study or that there is an increase in the efficiency of internalization of $\alpha_3\beta_1$ integrins after cross-linking by ligands immobilized on the bacterial surface. Support for this latter explanation exists, since soluble antibodies bound to $\alpha_3\beta_1$ are poorly endocytosed unless the receptor-bound antibodies are cross-linked by a secondary antibody (DeStrooper *et al.*, 1991). The mechanism that causes this transition to an internalization-competent state has not been investigated, although it may involve tyrosine phosphorylation since clustering of β_1 chain integrins by binding to polyvalent ligands results in an increase of phosphorylation of the proteins pp125, at least one of which is a tyrosine kinase (Guan *et al.*, 1991; Kornberg *et al.*, 1991; Schaller *et al.*, 1992). Furthermore, inhibitors of tyrosine protein kinases also block bacterial uptake mediated by invasin (Rosenshine *et al.*, 1992).

Anti-$\alpha_5\beta_1$ mAbs that promoted different levels of bacterial uptake were isolated, generating ligands of contrasting affinities, in order to mimic the differences between invasin and fibronectin. Anti-$\alpha_5\beta_1$ mAbs that recognized a site different from that of invasin or fibronectin could still promote high levels of bacterial internalization, indicating that internalization did not require binding to a particular site on the integrin. It appears, however, that simple bacterial adherence to the cell surface is the result of low affinity ($ED_{50} \approx 10^{-7}$ M) binding to integrin receptors, whereas high affinity ligands (ED_{50} up to 8×10^{-8}–5×10^{-10} M) are more competent at promoting internalization. In addition, increasing the integrin receptor density on the surface of the cell resulted in an overall increase in the levels of internalization promoted by the different integrin ligands. It should be emphasized that this increase in internalization was mainly the result of an increase of the number of particles adhering to the over-

producer clone. Once bound, the internalization efficiency of a particle was primarily determined by the relative affinity of the ligand for the $\alpha_5\beta_1$ integrin. Perhaps this is because a larger number of receptor−ligand interactions is required for uptake of a bacterium-sized particle than is required for simple surface adherence. Consistent with this point are results indicating that high affinity ligands can compete with integrin receptor−ligand interactions at the ventral surface of the cell (Figure 6). After attachment of the bacterium to the cell sequential receptor−ligand interactions must occur between the bacterial ligand and the integrin receptors. If the affinity of the bacterial ligand is high enough to displace the initial interaction between integrins and the extracellular matrix, or if there is sufficient receptor available on the surface, successful zippering (Griffin *et al.*, 1975; McAbee and Grinnell, 1985) can occur and the internalization process can be completed.

Also, integrin receptors have been proposed to link the extracellular matrix to the cytoskeleton via binding of the cytoplasmic domain of the β_1 subunit to various actin-binding proteins, such as α-actinin (Otey *et al.*, 1990) and talin (Buck and Horwitz, 1987). Although actin polymerization is required for bacterial internalization (Finlay and Falkow, 1988), association of integrins with cytoskeletal structures in focal plaques (Hynes, 1987) may limit the ability of integrin receptors to mediate bacterial internalization. Therefore, a high affinity of bacterial ligand binding to the integrin may allow an efficient sequestering of receptor away from a particular cytoskeletal structure.

Time-lapse videomicroscopy indicates that most bacterial internalization mediated by invasin occurs near the mammalian cell−ECM interface (Francis *et al.*, 1992). Therefore, the pool of receptors available for bacterial internalization is in a region of the cell where competition for binding to the ECM or anchoring of the integrins to the cytoskeleton is predicted to be important. Further studies on the interaction of integrin receptors with both the ECM and cytoplasmically localized factors should give insight into how this competition occurs, as well as identify additional host components involved in the bacterial internalization process.

Materials and methods

Cell lines, bacterial strains and growth conditions

Human cultured HEp-2 cells were grown in RPMI 1640 medium (Irvine) containing 5% newborn calf serum. JAR cells, derived from a human choriocarcinoma, and K562 cells, an erythroleukemia cell line, were grown in RPMI 1640 containing 10% fetal calf serum. For mAb production the NY Fox myeloma cells and hybridomas were grown in RPMI 1640 containing 20% fetal calf serum and 20 mM HEPES, pH 7.0. The plasmid pECE-FNRα containing the full-length cDNA clone coding for the α_5 subunit (Giancotti and Ruoslahti, 1990) was kindly provided by Drs W.A.Argraves and E.Ruoslahti. In order to increase the efficiency of transfection by the electroporation of HEp-2 cells, the plasmid pSVneo-α_5 was constructed by cloning the 4.3 kb *XbaI*−*SalI* fragment of pECE-FNRα containing the α_5 subunit encoding cDNA into the *AccI* site of pSV2-neo (Southern and Berg, 1982) after filling in with Klenow polymerase. This allowed transcription of the α_5 subunit gene under the control of the SV40 late promoter. *S.aureus* 377 McCowan was grown as described by Rankin *et al.* (1992) and was inoculated into Penassay broth from a fresh plate the day prior to the cellular internalization assay.

Antibodies and reagents

The rat monoclonal antibody AIIBII against β_1 chain integrins was kindly provided by Dr Caroline Damsky (University of California at San Francisco, San Francisco, CA). The anti-invasin mAb 9G11 used as a negative control was provided by John Leong (Tufts University, Boston, MA). The mouse mAb P1B5 against the $\alpha_3\beta_1$ integrin was obtained from Telios Pharmaceuticals. Rabbit antiserum against the maltose binding protein was a gift from Dr Carol Kumamoto (Tufts University, Boston, MA). The rabbit anti-mouse IgG, rabbit anti-mouse IgG linked to horseradish peroxidase and streptavidin linked to horseradish peroxidase were from Zymed Laboratories. Protein A beads were from Bio-Rad (Richmond, CA), goat anti-mouse beads and goat anti-rat beads were purchased from Sigma (St Louis, MO). NHS-LC-Biotin was purchased from Pierce Pharmaceuticals (Rockford, IL), and 5 (and-6)-carboxytetramethylrhodamine succinimidyl ester was from Molecular Probes, Inc. (Eugene, OR). The hybrid proteins MBP-Inv479 and MBP-Inv192, respectively containing the 479 and 192 carboxy-terminal amino acids of invasin fused to the maltose binding protein, were purified as described by Leong *et al.* (1990).

Isolation and characterization of monoclonal antibodies against the $\alpha_5\beta_1$ receptor

RBF-DNJ mice (Jackson Laboratories, Bar Harbor, ME) were immunized with three intraperitoneal injections of ~50 μg of $\alpha_5\beta_1$ integrin, purified from human placenta by invasin-affinity chromatography (Tran Van Nhieu and Isberg, 1991), at two-week intervals. Six months after the last injection and 3 days before fusion the mice were injected intravenously with the receptor preparation, the animals were sacrificed and their spleens fused with NYFox myeloma cells as described (Galfre and Milstein, 1981).

MAbs against the $\alpha_5\beta_1$ integrin receptor were screened using the $\alpha_5\beta_1$ receptor immobilized in an ELISA based assay. About 50 ng of receptor aliquots were spotted on an Immobilon-P filter (Millipore) using a 96-well dot-blot apparatus (Bio-Rad). The filters were then removed from the apparatus and allowed to block in PBS containing 1% BSA for 2 h. Droplets of hybridoma supernatants were transferred from the fusion plates to the receptor-containing dots on the filter using a Transtar 96 cartridge (Costar). The filters were incubated for 60 min at 37°C in a humid chamber, washed three times with PBS, incubated with anti-mouse IgG antibody linked to horseradish peroxidase and positive reactions were visualized by standard colorimetric detection methods (Portsmann *et al.*, 1981). The mAbs were then screened for the ability to block binding of K562 cells to invasin or fibronectin as described (Tran Van Nhieu and Isberg, 1991). For this study eight positive clones were further characterized by immunoprecipitation analysis of surface-labeled cells (see below). Immunodepletion of the cell extract using the anti-α_5 mAb B2G2 (C.Damsky, UCSF, CA) strongly diminished the amount of material precipitated by the mAbs IIA1, VD1, IIIB8, IVF4, IVG5, VC5 and 6F4, consistent with the specificity of these seven mAbs for the $\alpha_5\beta_1$ integrin. In contrast, the mAb VD10 precipitates were mostly depleted by the anti-β_1 mAb AIIBII (C.Damsky, UCSF, CA) (data not shown). Also, the VD10 mAb recognized the β_1 subunit in a standard Western blot assay (data not shown), consistent with the specificity of this mAb for the β_1 subunit.

Transfection of HEp-2 cells and analysis of a clone overexpressing the $\alpha_5\beta_1$ integrin

HEp-2 cells grown to 65% confluency in 175 mm tissue culture flasks were trypsinized, washed once with 100% fetal calf serum, twice with PBS and resuspended in PBS at a density of 5×10^6 cells/ml. Cells were transferred to a 0.4 cm electroporation cuvette (Bio-Rad) and incubated on ice for 10 min. Plasmid DNA (50 μg) was added and the cells were electroporated at 450 V, 500 μF. The cells were returned to ice for 10 min before plating. After 3 days G418 was added to the medium at a final concentration of 400 μg/ml. After 10 days individual G418-resistant clones were transferred to 96-well plate using a cloning ring, and clones expressing a higher level of the $\alpha_5\beta_1$ integrin were identified by an ELISA procedure as follows. Cells grown in a 96-well plate were fixed in PBS containing 2% paraformaldehyde, blocked with RPMI 1640 containing 5% newborn calf serum and probed with the anti-$\alpha_5\beta_1$ mAb VD1 (Table I), followed by detection using anti-mouse IgG linked to horseradish peroxidase. The amount of bound mAb was quantified using ABTS (Zymed) followed by absorbance measurement at 405 nm in an EIA reader (Bio-Rad, model no. 2550). The amount of receptor expressed was determined by normalizing the absorbance at 405 nm by the number of cells according to the crystal violet staining method (Brasaemle and Attie, 1988). Further quantification of receptor expression was performed by surface labeling and immunoprecipitation with anti-integrin mAbs as described below.

Immunoprecipitation analysis

Cells were surface-labeled with the impermeant reagent NHS-LC-biotin (Pierce) as described (Isberg and Leong, 1990). After labeling the cells were lysed in PBS containing 1% Triton X100, 1 mM PMSF, 0.5 mM $CaCl_2$,

1 mM $MgCl_2$ for 60 min on ice. Cell debris was pelleted by centrifugation for 30 min at 4°C and anti-mouse IgG beads (Sigma) were added to the soluble extract. After 30 min at 4°C, the beads were pelleted, the anti-integrin mAbs (usually 200 μl of hybridoma supernatant for the equivalent of 5×10^6 cells) were added to the precleared extract and the mAbs were allowed to bind for 60 min on ice. Immune complexes were pelleted by subsequent incubation with rabbit anti-mouse or rabbit anti-rat IgG beads (Sigma) and analyzed by SDS–PAGE on a 7.5% polyacrylamide gel, followed by Western blot analysis probing with streptavidin-linked to HRP. Bands were visualized by the ECL chemiluminescent system (Amersham) and their intensity was quantified by scanning densitometry (Molecular Dynamics, model no. 300B).

For immunodepletion experiments the soluble cell extract was incubated with anti-α_5 (B2G2) or anti-β_1 mAb (AIIBII) for 60 min at 4°C, followed by incubation with anti-rat IgG beads (Sigma). After 60 min at 4°C the beads were pelleted. The supernatant was transferred to a fresh tube. The second anti-integrin mAb was then added and immunoprecipitation was performed as described above.

ED_{50} determination of anti-$\alpha_5\beta_1$ mAbs to HEp-2 cells

mAbs (5–20) mg were purified from 1 l of hybridoma culture on a protein A column using the MAPS buffer system (Bio-Rad). In order to iodinate the purified mAbs VD1, IVF4, IIIB8 and VD10 50 μg of mAb were labeled with [^{125}I]NaI using Iodobeads following the manufacturer's instructions (Pierce). The binding activity of mAbs IIA1, IVG5, VC5 and 6F4 was sensitive to the Iodobead labeling technique, so these mAbs were labeled using the Bolton–Hunter reagent (Bolton and Hunter, 1973) for 15 min on ice as described by Tran Van Nhieu and Isberg (1991). Binding was unaffected by this procedure. To quantify the binding affinities, $\sim 5 \times 10^5$ HEp-2 cells were seeded in a 24-well plate. The unfixed HEp-2 cells were washed and incubated with labeled mAb for 18 h at 4°C in RPMI 1640 containing 20 mM HEPES, pH 7.0 and 0.4% BSA. After incubation the cells were washed three times with PBS, lifted from the plastic surface in 2 mM EDTA, PBS and the radioactivity in the suspension was quantified. In preliminary experiments saturation curves were constructed by incubating unfixed HEp-2 cells with increasing concentrations of labeled mAbs for 18 h at 4°C. The lowest concentration of mAb giving quantifiable counts, corresponding to concentrations ranging from the half-maximal binding concentration down to 10% of this value depending on the mAb, was then used as a labeled tracer in displacement experiments using increasing concentrations of the corresponding purified mAb as an inhibitor. The concentration of non-labeled mAb required to give half-maximum inhibition (ED_{50}) was used as an estimation of mAb affinity for HEp-2 cells. Each experiment was performed 3–4 times and the ED_{50} is the mean of these experiments.

Cross-inhibition experiments of mAbs binding to K562 cells

Purified mAbs (500 μg) were labeled with succinimidyl-rhodamine (Molecular Probes) for 15 min on ice as described (Rankin *et al.*, 1992). K562 cells growing in RPMI 1640 containing 10% FCS were washed once with PBS containing 2 mM EDTA and resuspended in RPMI 1640 containing 20 mM HEPES, pH 7.0 and 0.4% BSA at a density of 10^8 cells/ml. Aliquots of cell suspension (50 μl) were placed in V-bottomed microtiter wells and pre-incubated with unlabeled purified mAbs at a final concentration of 200 μg/ml, corresponding to 500 times the concentration of tracer mAb. After 60 min at 22°C rhodamine-labeled mAbs were added at a concentration of 2 μg/ml. Binding was then allowed to occur for 2 h at 22°C, the cells were washed twice with 200 μl of PBS and resuspended in 100 μl PBS. Samples were transferred to a Titertek Fluoroplate (Flow Laboratories) and rhodamine fluorescence emission was quantified using a microfluorometer (Fluoroskan II, Perkin Elmer). Percentages of inhibition were determined taking binding of the tracer mAb in the presence of excess identical unlabeled mAb as 100% inhibition, while 0% inhibition was that determined in the absence of competitor mAb. Each value is the mean of three independent determinations.

Cellular internalization assay of S.aureus coated with anti-$\alpha_5\beta_1$ mAbs

S.aureus was coated with MBP-invasin hybrid proteins as described, using rabbit anti-MBP in a primary coating step (Rankin *et al.*, 1992). To coat *S.aureus* with anti-integrin mAbs rabbit anti-mouse IgG (Zymed) was used as a primary antibody and the purified anti-integrin mAbs as secondary antibody. Briefly, a fresh culture of *S.aureus* was inoculated into Penassay broth from an overnight culture at a dilution of 1:10 and allowed to grow for 120 min at 37°C. Aliquots (500 μl) were transferred to microfuge tubes, washed once in PBS after centrifugation for 30 s in a microfuge at 10 000 r.p.m. and resuspended in 500 μl PBS containing 10 μg of rabbit anti-mouse IgG mAb. The tubes were placed on a small benchtop roller (Labindustries, Inc., model no L1237) and rotated for 45 min at 4°C. The bacteria were then washed three times with PBS after successive 10 000 r.p.m. centrifugations and incubated with 10 μg of the anti-integrin mAbs in 500 μl PBS for another 45 min. Under these conditions the concentration of mAb corresponds to saturation of *S.aureus* protein A binding (data not shown). The coated bacteria were then washed twice with PBS and were used to infect cells at an m.o.i. of 1.0 (typically ~10 μl of the bacterial suspension/5×10^5 cells). The number of internalized bacteria was determined by the gentamicin protection assay as described by Isberg and Falkow (1985).

Quantification of mAb-coated bead attachment and internalization by HEp-2 cells

Although the quantification of internalized *S.aureus* was sensitive and reproducible, the quantification of *S.aureus* specifically attached to cells proved to be difficult due to clumping of bacteria on the surface of the cell. Therefore, particle attachment promoted by the different anti-$\alpha_5\beta_1$ integrin mAbs was determined using latex beads.

The procedure for coating latex beads is derived from Grinnell *et al.* (1986) (Chen,S.Y. and Skvorak,A., unpublished results). Latex beads (1.1 μm, LB-11, Sigma) were coated with rhodamine-labeled mAbs at a concentration of 10 μg/ml in 500 μl of PBS for 90 min at 30°C. PBS (50 μl) containing 5% BSA was then added and the incubation was allowed to continue for an additional 1 h. The beads were then washed three times in PBS containing 0.5% BSA and the amount of mAb coating per bead was determined in a fluorometer (Fluoroskan II, Perkin Elmer). The estimated efficiency of coating did not show any significant variations for the different mAbs and corresponded to ~10^6 molecules/bead (data not shown). Coated beads were then incubated with HEp-2 cells plated on cover slips at a multiplicity of 5 beads per cell in RPMI 1640 containing 20 mM HEPES, 1% BSA for 2 h at 22°C. Following incubation the monolayers were washed 5 times with PBS, fixed in PBS containing 2% paraformaldehyde and the number of adherent beads was determined microscopically using rhodamine fluorescence. For quantification of uptake, medium pre-warmed at 37°C was added to the monolayers, after the PBS washes, and the cells were incubated for 1 h at 37°C to allow internalization of adherent beads. The cells were fixed and non-internalized beads were detected by probing with anti-mouse IgG antibody linked to FITC. The ratio of internalized beads was determined by subtracting the number of FITC-labeled beads from the total number of bound beads and dividing by the total number of bound beads. To test the efficiency of FITC labeling of the beads, monolayers of HEp-2 cells incubated with coated beads were treated as above except that the beads were permeabilized for 4 min with 0.1% Triton X100 after fixation. In these control experiments the number of FITC-labeled beads in permeabilized cells did not differ significantly from the total number of beads (data not shown). Each determination represents a count of 500–1000 beads on an average of 20 fields on three independent cover slips.

Infection of HEp-2 cells plated on anti-$\alpha_5\beta_1$ mAbs

Twenty-four-well plates were coated with anti-α_5 mAbs in PBS at a concentration of 10 μg/ml for 2 h at 22°C. To facilitate cell attachment to the plastic surface, rat tail collagen type I was added at a concentration of 100 μg/ml and the plates were incubated for another 1 h at 22°C. The wells were then washed three times with PBS and blocked in PBS containing 1% BSA overnight at 4°C. HEp-2 cells were then seeded on the coated wells at a density of 5×10^5 cells/well and allowed to bind at 37°C for 2 h prior to infection with *S.aureus* coated with anti-α_5 mAbs. The number of internalized bacteria was determined by the gentamicin protection assay (Isberg and Falkow, 1985).

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References

Abraham,S.N., Beachey,E.H. and Simpson,W.A. (1983) *Infect. Immun.*, **41**, 1261–1268.

Albelda,S.M. and Buck,C.A. (1990) *FASEB J.*, **4**, 2868–2880.

Argraves,W.S., Pytela,R., Suzuki,S., Millan,J.L., Pierschbacher,M.D. and Ruoslahti,E. (1986) *J. Biol. Chem.*, **261**, 12922–12924.

Bolton,A.E. and Hunter,W.M. (1973) *Biochem. J.*, **133**, 529–539.

Brasaemle,D.L. and Attie,A.D. (1988) *Biotechniques*, **6**, 418–419.

Bretscher,M. (1992) *EMBO J.*, **11**, 405–410.

Buck,C.A. and Horwitz,A.F. (1987) *J. Cell. Sci. Suppl.*, **8**, 231–250.

DeStrooper,B., Van Leuven,F., Carmeliet,G., Van Den Berghe,H. and Cassiman,J.-J. (1991) *Eur. J. Biochem.*, **199**, 25–33.

Finlay,B.B. and Falkow,S. (1988) *Biochimie*, **70**, 1089–1099.

Flock,J.I., Froman,G., Jonsson,K., Guss,B., Signas,C., Nilsson,B., Raucci,G., Hook,M., Wadstrom,T. and Lindberg,M. (1987) *EMBO J.*, **6**, 2351–2357.

Francis,C.L., Starnbach,M.N. and Falkow,S. (1992) *Mol. Microbiol.*, **6**, 3077–3087.

Galan,J.E., Pace,J. and Hayman,M.J. (1992) *Nature*, **357**, 588–589.

Galfre,G. and Milstein,C. (1981) *Methods Enzymol.*, **73**, 3–46.

Giancotti,F.G. and Ruoslahti,E. (1990) *Cell*, **60**, 849–859.

Griffin,F.M.J., Griffin,J.A., Leider,J.E. and Silverstein,S.C. (1975) *J. Exp. Med.*, **142**, 1268–1282.

Grinnell,F. and Geiger,B. (1986) *Exp. Cell. Res.*, **162**, 449–461.

Guan,J.L., Trevithick,J.E. and Hynes,R.O. (1991) *Cell. Regul.*, **2**, 951–964.

Hemler,M.E. (1990) *Annu. Rev. Immunol.*, **8**, 365–400.

Hynes,R.O. (1987) *Cell*, **48**, 549–554.

Hynes,R.O. (1992) *Cell*, **69**, 11–25.

Isberg,R.R. (1990) *Mol. Biol. Med.*, **7**, 73–82.

Isberg,R.R. (1991) *Science*, **252**, 934–938.

Isberg,R.R. and Falkow,S. (1985) *Nature*, **317**, 262–264.

Isberg,R.R. and Leong,J.M. (1990) *Cell*, **60**, 861–871.

Isberg,R.R., Voohris,D.L. and Falkow,S. (1987) *Cell*, **50**, 769–778.

Kornberg,L.J., Earp,H.S., Turner,C.E., Prockop,C. and Juliano,R.L. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8392–8396.

Leong,J.M., Fournier,R.S. and Isberg,R.R. (1990) *EMBO J.*, **9**, 1979–1989.

Maurelli,A.T. and Sansonetti,P.J. (1989) *Annu. Rev. Microbiol.*, **42**, 127–150.

McAbee,D.D. and Grinnell,F. (1985) *J. Cell Physiol.*, **124**, 240–246.

Moulder,J.W. (1985) *Microbiol. Rev.*, **49**, 298–337.

Moulder,J.W. (1991) *Microbiol. Rev.*, **55**, 143–190.

Otey,C.A., Pavalko,F.M. and Burridge,K. (1990) *J. Cell Biol.*, **111**, 721–729.

Portsmann,B., Portsmann,T. and Nugel,E. (1981) *J. Clin. Chem. Clin. Biochem.*, **19**, 435–439.

Rankin,S., Isberg,R.R. and Leong,J.M. (1992) *Infect. Immun.*, **60**, 3909–3912.

Rosenshine,I., Duronio,V. and Finlay,B.B. (1992) *Infect. Immun.*, **60**, 2211–2217.

Schaller,M.D., Borgman,C.A., Cobb,B.S., Vines,R.R., Reynolds,A.B. and Parsons,J.T. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 5192–5196.

Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.

Tran Van Nhieu,G. and Isberg,R.R. (1991) *J. Biol. Chem.*, **266**, 24367–24375.

Une,T. (1977) *Microbiol. Immunol.*, **21**, 505–506.

Van de Water,L., Destree,A.T. and Hynes,R.O. (1983) *Science*, **220**, 201–204.

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