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Micromechanics of filopodia mediated capture of pathogens by macrophages

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Abstract The biological function of filopodia has been extensively studied while only little work has been done on their mechanical properties. In the present study, we apply magnetic microbeads to explore the capturing and initial step of phagocytosis of pathogens by macrophages through filopodia. Microbeads were covered by the bacterial coat protein invasin which is known to trigger the invasion of the intestine by the bacteria *Yersinia enterocolitica*. These mimetics of bacteria were placed in the vicinity of J774 mouse macrophages exhibiting long filopodia. The specific adhesion of beads to the tip of a filopodium induced the retraction of the protrusion resulting in the dragging of the bead towards the cell body. The dynamics of the retraction process was analyzed by following the in-plane motion of the bead. We estimated the minimal force developed by filopodia and compared the results with previous magnetic tweezer studies of mechanical force induced growth of protrusions (Vonna et al. 2003). We show that very thin filopodia can generate astonishingly large retraction forces over large distances (>10 μm) and can act as an efficient mechanical tool to detach pathogens adhering on surfaces.

Keywords Colloidal probes · Phagocytosis · Yersinia · Invasin · Macrophage · J774 · Filopodium

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Introduction

Depending on the biological processes, cells can generate a variety of protrusions that can be classified according to their shape. The two major types include first, tube-like protrusion exhibiting lengths between a few hundred nanometers (called microspikes) or several micrometers (called filopodia), and second, several micrometer broad (veil-like) lobes called lamellipodia. Filopodia are multifunctional protrusions. They have been reported to act as path finding probes during the development of neuronal networks where they can determine the direction of growth cones (Davenport et al. 1993; Gomez et al. 2001; Mallavarapu and Mitchisona 1999). More recently, evidence was provided that filopodia contribute to the communication between cells through receptor–ligand interaction. Thus, long filopodia formed during the development of wing imaginal discs (cell assembly providing the adult wing during the morphogenesis of drosophilia) appear to mediate contact with cells of tissue by receptor ligand interaction (De Joussineau et al. 2003; Ramirez-Weber and Kornberg 1999). A similar role for filopodia was

proposed to occur during the sea urchin gastrulation (Miller et al. 1995) or phagocytosis (Miller et al. 2001).

While numerous studies of the biological function of filopodia have been published, little attention has been given to their mechanical properties despite the fact that similar structures, the lamellipodia, are known to play a key mechanical role in cell motility. Thus, forces developed by this type of protrusions were shown to induce the reorganization of networks of the extracellular matrix proteins or to change the behavior of adjacent cells (Stopak et al. 1985). They can even act as a mechanical probe sensing the softness of substrates (Lo et al. 2000).

In the particular case of phagocytosis, electron microscopic studies of phagocytosis show that the initial process of the capture of the bacteria *Yersinia enterocolitica* is mediated by filopodia and provide evidence that the attraction to the cell body is mediated by bundles of actin which penetrate deeply into the cytoplasm (Svitkina et al. 2003; Young et al. 1992). This initial step of the attachment between a macrophage filopodium and a pathogenic body preceding the final engulfment was described earlier in the literature for similar systems (Koerten et al. 1980; Wago 1984). A dominant role for the filopodium is often postulated but no real mechanical mechanism of this process is proposed, despite the fact that forces are necessary to capture of pathogens sticking to surfaces. In most studies of phagocytosis the capture and binding of bacteria is mediated by centrifugation of cellular suspensions. They concentrate on the later stages of the process while the retrieval process is seldom considered in these experiments.

In the present work, we studied the mechanical and dynamic aspects of the capture of mimetics of pathogens by specific adhesion and retraction of macrophages filopodia. Superparamagnetic beads are functionalized by adsorption of the coat protein invasin of bacteria of the *Y. enterocolitica* family. To capture the ends of filopodia, the magnetic beads are positioned close to isolated cells by manipulation with magnetic field gradients and the binding of the bead is monitored by analyzing its brownian motion. The binding process between the filopodium and the bead is indicated by a sudden impediment of the motion. Then the motion of the bead dragged by the retracting filopodium is followed by particle tracking until it forms contact with the cell. A minimal force that can be generated by the specifically induced retraction of the filopodium is estimated by comparison with mechanical studies performed on macrophages with the magnetic tweezers technique and published previously (Vonna et al. 2003).

Materials and methods

Macrophages were obtained from the mouse macrophage cell line J774 (European Collection of Animal Cell Cultures, Salisbury, UK). Cells were cultivated in an incubator at 37°C and 5% CO_2. The cell culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies GmbH, Eggenstein, Germany). Before each experiment, the cells were detached from the culture dish and a part of the suspended cells transferred onto a glass substrate for an incubation of 24 h in the same culture medium. The cell density was chosen in such a way that adhering cells were well separated or formed little groups with less than five cells.

The expression and purification of the invasin protein is described in a previous paper (Wiedemann et al. 2001). The protein is covalently bound to tosyl groups activated on superparamagnetic Dynabeads (Dynal, Hamburg, Germany) according to the procedure provided by the supplier. The 4.5 μm diameter polystyrenes are filled with nanoscopic ferromagnetic particles and thus behave in a magnetic field similar to paramagnetic bodies. In order to verify the binding of invasin, the beads were incubated at 4°C with rabbit anti-invasin antibody, diluted by 1:1,000. Beads were then washed three times with 1% PBS to remove unbound antibodies and then incubated with Alexa 488-labeled goat anti-rabbit antibodies, diluted by 1:200 (Molecular Probes). Beads that are effectively covered by invasin exhibit a fluorescent rim under a confocal fluorescent microscope.

The glass substrate covered with cells was placed on the stage of an AXIOVERT 100 phase contrast microscope (Zeiss, Oberkochen, Germany) and the beads were gently added to the adhering cells adjusting their number to one bead per cell. Some beads could be dragged in the vicinity of the cell with a permanent magnet. Immediately after this step, the motion of the beads was followed by visual inspection. Occasionally, randomly distributed beads on the surface of the substrate were contacted by filopodia extending several micrometers into the medium and are attracted towards the cell body. Actin filaments within the filopodia were visualized by incubation with rhodamin phalloidin. The fluorescent label purchased from Molecular Probes was diluted by a factor of 20. After fixation with acetone (at −20°C) the cells were observed with a fluorescent microscope. An example of the actin distributions is shown in Fig. 1.

Since we were interested in the capturing and retrieval process of the beads, it was necessary to start the experiments at low optical resolution at which the

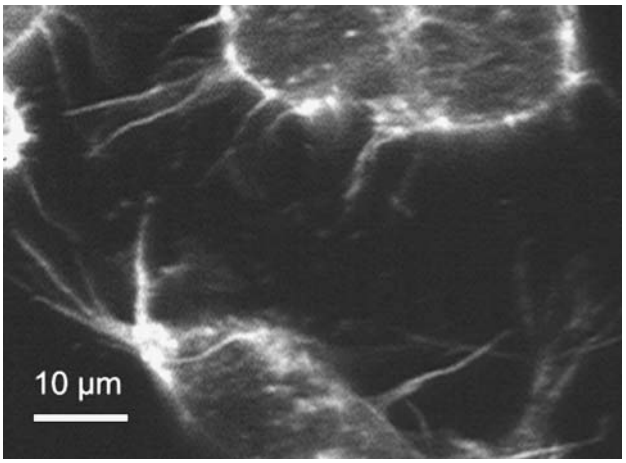

Fig. 1 Fluorescent micrograph of fixed J774 macrophages on which actin was stained with rhodamin phalloidin. Note that fiolopodia are stabilized by actin bundles and emanate as nearly straight hairs from the cell surface

filopodia exhibiting submicrometer diameters are not easily observable. To overcome this problem we observed the Brownian motion of beads in the neighborhood of the cell. Their binding to filopodia was indicated by a drastic reduction of the initially vivid random walk. Once such an event occurred, the subsequent motion of the bead was analyzed by particle tracking techniques.

Results

Snapshots of a typical capture process are shown in Fig. 2 at different stages of the capturing and retraction process: (1) situation of free bead, (2) and (3) stages of the bead bound and pulled by the filopodium and (4) the bead in contact with the cell body. The trajectory of the bead in the image plane (defined as a x–y plane) during the process is presented in the graph of Fig. 2. The time difference between two circles of the trajectory is 0.44 s. The different stages corresponding to the snapshots are indicated on the graph by arrows. The total engulfment occurs with a delay to the order of 20 min after the initial contact between the filopodium and the bead. Closer inspection of the randomness of the motions and the distance between the two bead

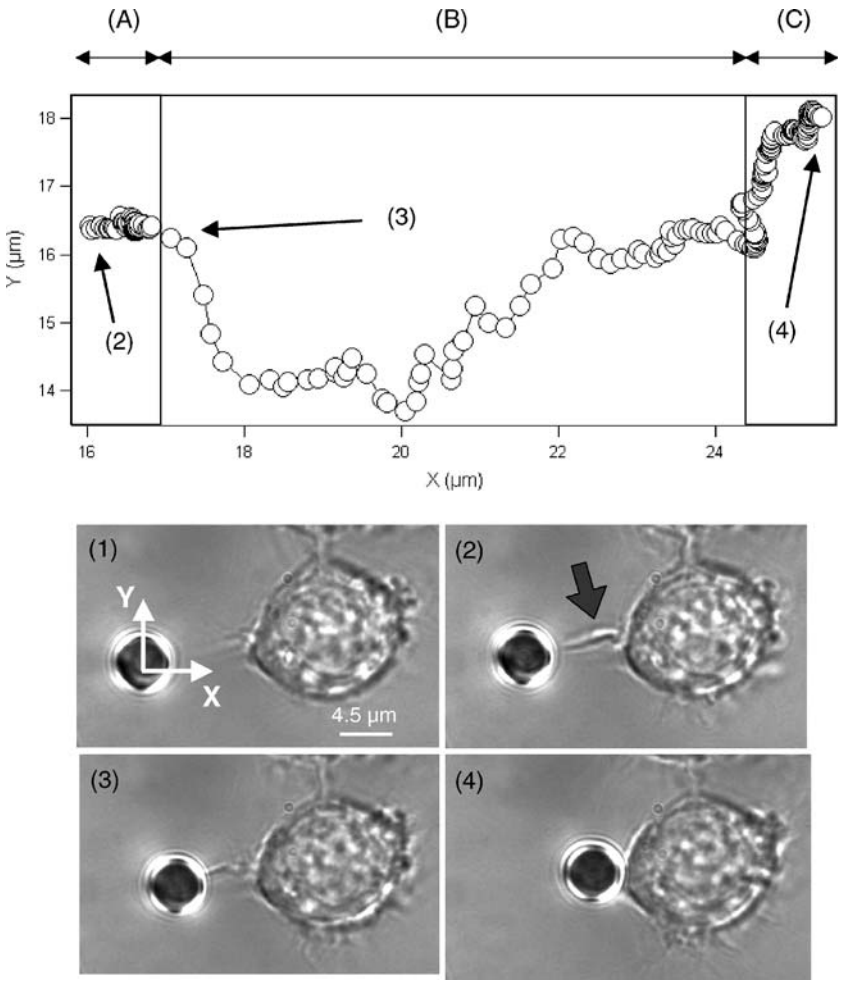

Fig. 2 Consecutive snapshots of a filopodium mediated capture process showing the situation *1* before, *2* and *3* during the retraction of the filopodium, and *4* after attachment of the bead to the cell surface. The *black arrow* on snapshot *2* shows the thickening of the filopodium near the cell. The graph on the top shows the trajectory of the bead in the image plane (defined as x–y plane). The three regimes denoted by *A*, *B* and *C* correspond to the three steps of the capture process. The *arrows* indicate the different positions of the bead for three of the snapshots. Time interval between points is 0.44 s

positions (circles) measured at fixed time intervals (0.44 s) show three distinct motional phases: first, a slow motion towards the cell (A), second, a rapid movement towards the cell (B), and finally a slow motion ending in the fixation of the bead on the cell surface (C). One can clearly distinguish the phase of rapid motion which occurs about 180 s after the initial slow motion. It is followed by a slowing down after about 200 s. In this particular case, the retraction of the filopodium towards the cell in phase (B) occurs with velocities reaching 155 ± 5 nm/s. In some cases it was possible to observe before the beginning of the phase (A), the rapid Brownian motion of the free bead turning into a restricted quasi-random motion after being attached to a filopodium.

About 23 capture events were analyzed to measure the velocities of the rapid retraction phase. Figure 3 shows a histogram of the velocities measured. The fast velocities range between 30 and 160 nm/s. The average velocity measured for this set of experiments is 85 nm/s. The velocities in the incubation phase (i.e., before the fast retraction) and phase (C) are of the order of 10 nm/s.

In order to test whether the three-phase retraction process characterized by a faster middle phase (B) is induced by the pathogenic surface of the bead we also studied the retraction process for polystyrene beads coupled to the cell surface in nonspecific way by covering the bead with basic functional groups (NH_2). In this case we never observed a remarkable retraction of a filopodium after contact with the bead. Nevertheless, growing protrusions were able in some cases to reach the bead. In contrast to the behavior of invasin coated beads, the nonspecifically coupled bead was dragged and transported along the cell surface. The motion of the bead followed the random movements of the cell but didn't show a clear directed motion inward the cell as observed with invasin-coated beads.

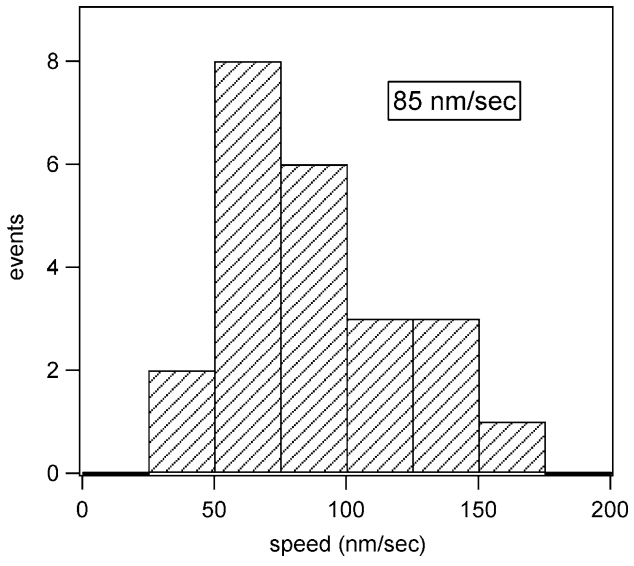

Fig. 3 Histogram showing the distribution of the pulling speeds observed for 23 different cells. The velocities range from 30 to 160 nm/s, and the average velocity measured for this set of experiments is 85 nm/s

As will be shown below, the net force necessary to pull a free microbead in an aqueous medium is of the order of tens of femtonewtons. Much stronger forces can be generated during the attraction phase. In order to estimate these forces and to gain some insight into the process leading to this remarkable retraction of the filopodium, we studied some particular cases more closely. In one case, the filopodium pulled on an invasin-coated bead adhering on the substrate as shown in Fig. 4. In the first step, the retraction of the filopodium displaces the cell toward the immobile bead. The forces acting in this process lead finally to the detachment of the bead. In another case the bead was initially bound on the surface of another cell as shown in Fig. 5. Astonishingly, the forces generated by the filopodium of the cell on the right are large enough to deform the cell on which the bead was attached. The retraction speed of the filopodium shown in Fig. 5 (and consequently the deformation of the cell) is around 10 nm/s . By comparing this deformation with the deflections generated by magnetic tweezers we can estimate the minimum force generated by the filipodium. It is important to note that the retraction process was accompanied by a thickening of the filopodium near the cell surface.

Discussion

Our present study strongly suggests that the capture of pathogenic particles by filopodia is a triphasic process. The first is an induction phase after invasin mediated binding of the bead. It is followed by phase (A) of the capture process. The characteristic incubation time can vary from one to another experiment. This phase is followed by the attraction process with speeds varying from 30 to 160 nm/s. In the third phase, the speed of attraction is reduced and the process of engulfment starts. Inspection of a large number of such processes suggests that during the third phase, the diameter of the filopodium expands in the region near the cell surface (shown by a black arrow on the snapshot (2) of Fig. 2). It also appears that this thickening process is necessary to build-up large retraction forces. The remarkable differences between the capture process for invasin and nonspecifically coated (positively

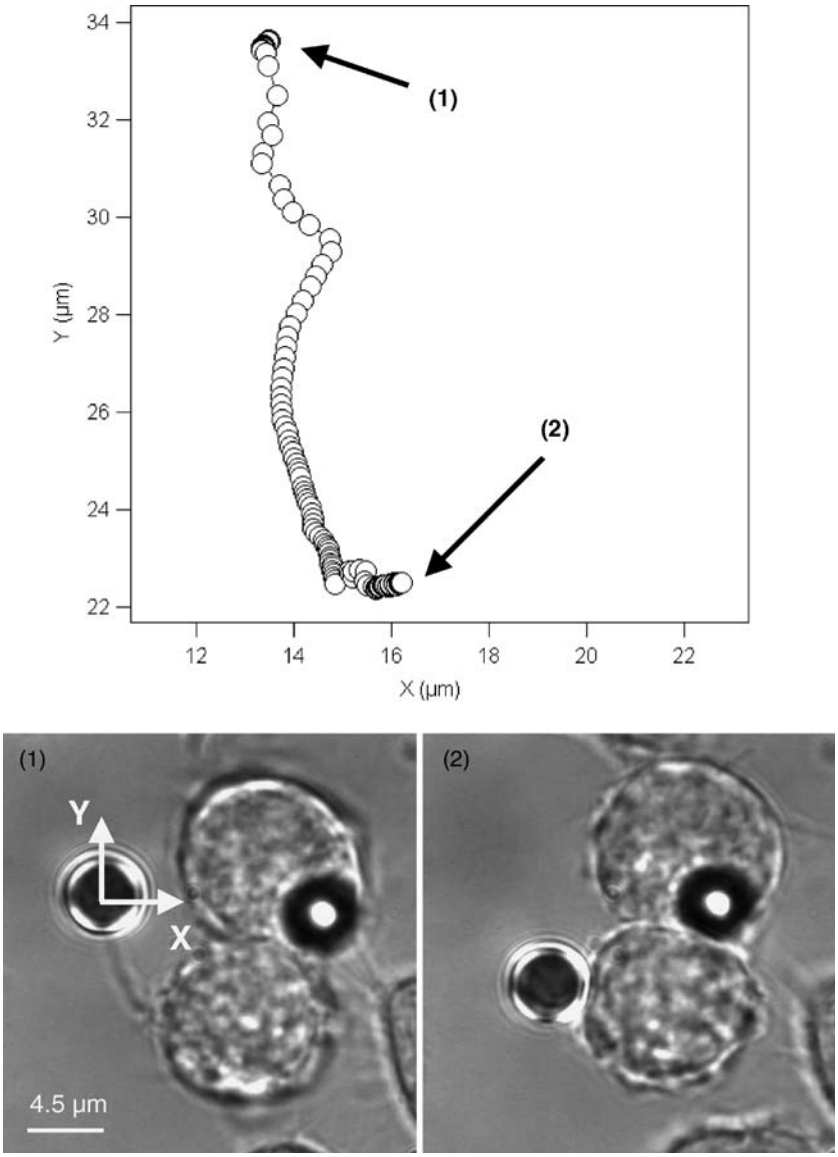

Fig. 4 Capture of an invasin-coated bead sticking to the glass surface. The graph shows the trajectory of the bead in image plane (defined as *x*–*y* plane). The *arrows* on the graph indicate the positions of the bead corresponding to the two snapshots. Time interval between points is 0.44 s

charged) beads shows that the fast attraction process is determined by activation of cell signaling pathways involving the reorganization of the actin cortex. In fact, invasin has been shown to activate actin polymerization via CDC42Hs after binding with β1 integrins (Wiedemann et al. 2001; Young et al. 1992). In contrast, this pathway is not activated when positively charged beads bind electrostatically to the negatively charged glycocalix of the macrophage (Chenevier et al. 2000). This specific action of invasin is actually known to serve the Yersinia bacteria for invading and penetrating with high efficiency the epithelial intestinal barrier (Pepe and Miller 1993). Our results show that phagocytosis can be initiated through filopodia which generate the capture forces that attract the pathogenic body until it contacts the cell and the uptake process by the engulfment of the body starts.

The net forces necessary to move a bead in an aqueous medium are very small. It must be just sufficient to pull the bead against the frictional force F_f. To a first approximation we can assume that the frictional force is similar to that of a bead moving freely in water and is thus given by the Stokes law: $F_f = 6\pi\eta r v$, where r is the radius of the bead and v the average speed. In our case, the average speed of the 4 µm bead is 85 nm/s and we thus expect a frictional force of $F_f \sim 0.01$ pN. Extreme frictional force of 0.85 nN could arise if the bead moves over surfaces exposing polymer brushes although this situation is not given in our case (Sengupta et al. 2003). Cells are able to develop forces in the range between 0.01 pN and 0.85 nN through processes that are not powered by actin-myosin motors. One possible mechanism is the decomposition of actin bundles at the tip of pseudopods by reversal of the growth process which is generally

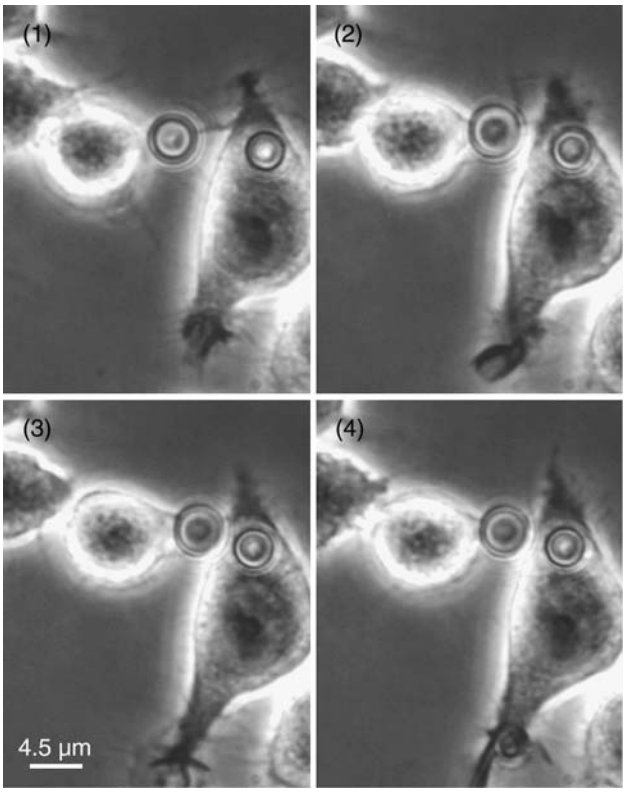

Fig. 5 Consecutive snapshots of two macrophages pulling on the same bead. The filopodium of the cell at the right is pulling on a bead already attached to the surface of the cell at the left. It is clearly seen that the cell on the left is drastically deformed by the right cell. The retraction speed of the filopodium (and consequently the deformation of the cell) is in this case around 10 nm/s

attributed to the continuous insertion of actin monomers at the tip of the protrusion (Loisel et al. 1999). If we assume that the forces generated by the actin decomposition are similar to that of the growth process we can expect with some confidence that the membrane tension of the lipid protein bilayer is sufficient to drag the bead as the actin bundles are depolymerizing. Thus, forces up to 0.03 nN are required to pull out tube-like protrusions (e.g. by optical tweezer) (Dai and Sheetz 1999). If the bead interacts weakly with the substrate, this process could generate forces large enough to pull the bead bound to the tip of the filopodium. However, in view of these weak forces it is remarkable that the protrusions are able to detach beads sticking on surfaces (Fig. 4) or to deform other cells (Fig. 5).

Below we attempt to estimate the minimal forces filopodia would have to generate for the deformation process. For this purpose we consider the deformation amplitude of the cell body shown in Fig. 5 and compare it with data of our previous study where we show that local forces applied to J774 macrophages with magnetic tweezers generate conical protrusions (Vonna et al. 2003). We found first, that a minimal local force of 0.5 nN is necessary to generate the protrusions and second, that the speed is independent of the applied force. We thus postulated that the deformation (in the range of 0.5 and 10 nN) is controlled by the actin polymerization. By assuming that the mechanism of the deformation induced by a filopodium pulling on a invasin-coated bead (as in Fig. 5) is the same as that induced with magnetic tweezers, we expect that the forces generated by filopodia are larger than 0.5 nN. Such forces can be generated by the growth and shrinkage of actin bundles or actin-myosin assemblies. It is commonly assumed that this process is activated during phagocytosis which follows the recognition of ligands by specific receptors on the surface of the phagocytic cells (Niedergang and Chavrier 2005). In our case, the pagocytic uptake of the bead is specifically induced by the binding of the protein invasin covering the bead to integrins containing β_1 chains (Wiedemann et al. 2001). Micromechanical studies of phagocytic processes performed by the micropipette aspiration technique showed that a build-up of cortical tension is responsible for the internalization of pathogens (Evans et al. 1993; Herant et al. 2006; Swanson et al. 1999; Zhelev and Hochmuth 1995). Our results suggest that a similar process associated with the decomposition of the actin filaments within filopodia could be responsible for the retraction force. A major message of our study is that mechanical forces can be mediated over large distances (>10 µm) through filopodia showing that these cellular extensions can play a key role as capturing tools in immunological processes.

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