

Interaction with *Borrelia burgdorferi* Causes Increased Expression of the CR3 Integrin and Increased Binding Affinity to Fibronectin via CR3

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Abstract

We have previously demonstrated that the $\alpha_M\beta_2$ integrin (known as CR3 or Mac-1) expressed on neutrophils (PMNs) and/or on CHO Mac-1 transfected cells, in the presence of serum complement binds *B. burgdorferi* and promotes an increased non-opsonic adhesion, in the presence of serum complement. In this study we demonstrate that: 1) living motile *B. burgdorferi* and recombinant lipidated OspA and OspC, up-regulate CR3 expression on PMNs; 2) in the absence of serum, *B. burgdorferi* induces increased adhesion of CHO cells expressing CR3 to fibronectin, an extracellular matrix protein. Both the I-domain and the lectin-like domain of CR3 are involved in the binding recognition and activation because mAb anti I-domain and N-acetyl-glucosamine inhibit cell adhesion to fibronectin. These data indicate that *B. burgdorferi* whole cells, but not Osps, activate CR3 integrin; since this receptor plays a key role in priming neutrophils to important inflammatory events, the interaction of *B. burgdorferi* with neutrophils via the CR3 may enhance their role both in defence and in disease.

Introduction

One of the pathogenic factors which could contribute to *Borrelia burgdorferi* (*B. burgdorferi*), the ethiological agent of Lyme borreliosis, dissemination and persistent infection is its capacity to attach itself to several cellular targets such as endothelial cells (Comstock and Thomas, 1989), neuroglia (Garcia-Monco *et al.*, 1989), fibroblasts (Hechemy *et al.* 1992; Klempner *et al.* 1989), lymphocytes (Dorward *et al.*, 1997), platelets (Coburn *et al.*, 1993), Schwann cells (Garcia-Monco *et al.*, 1992) and to proteoglycans of the extracellular matrix (Isaacs *et al.*, 1994), mainly decorin (Guo *et al.*, 1995). Studies carried out by J. Leong and J. Coburn since 1993 have demonstrated that *B. burgdorferi* also binds to some cellular

receptors such as the $\alpha_{IIb}\beta_3$ platelet glycoprotein, the $\alpha_V\beta_3$ vitronectin receptor and the $\alpha_5\beta_1$ fibronectin receptor (Coburn *et al.*, 1993; Coburn *et al.*, 1995). These receptors belong to a family of surface expressed transmembrane proteins, integrins, that are heterodimeric transmembrane proteins consisting of an α chain associated to a β chain, giving rise to more than 20 different receptors (Virjio *et al.*, 1996) which play a role in cell to cell and cell to extracellular matrix adhesion. Some of these integrins act also as receptors for pathogenic agents, beside their role as adhesion receptors.

In a previous study (Cinco, *et al.*, 1997) we demonstrated that *B. burgdorferi* are recognised by polymorphonuclear leukocytes (PMNs), in the absence of specific antibodies, mainly via the CR3 receptor (known also as Mac-1 or CD11b/CD18 or the $\alpha_M\beta_2$ integrin) and that binding of borreliae to CR3 involves both the I-domain and the lectin-like domain of the integrin α chain. It has been demonstrated that the lectin-like domain is involved in the activation of CR3 by interactions with surface receptors belonging to the family of the GPI-anchored proteins, such as CD16, CD14 and CD87 (Todd *et al.*, 1996). The co-operation of these molecules with CR3, induces activation of the integrin, leads to higher affinity binding to the ligand, cytoskeleton arrangements and signalling for cellular responses. The neutrophil functions triggered by CR3 activation are: the adhesion to stimulated endothelium or to the extracellular matrix, an enhanced phagocytosis of iC3b-coated particles and an enhancement of cytotoxicity.

It has been reported that *B. burgdorferi* outer surface proteins (Osps) such as OspA, besides a role as modulators of inflammation (Radolf *et al.*, 1991; Sellati, *et al.*, 1996; Ma and Weis, 1993; Morrison *et al.*, 1993; Wooten *et al.*, 1996; Ebnet *et al.*, 1997; Sellati *et al.*, 1998), are capable of exerting activation and priming of human PMNs. Since the proinflammatory effects of Osps appear to develop through molecular mechanisms which resemble those triggered by the stimulated CD14 receptor, and the cellular response to CD14 recognition involves CR3 activation, it should be interesting to investigate whether the stimulation of PMN adhesion to extracellular matrix induced by Osps proceeds via CR3 activation or not, and the role played in this phenomenon by the lectin-like domain of the integrin.

To this purpose, experiments were carried out employing a whole cell suspension of *B. burgdorferi* and recombinant proteins OspA and OspC and, as CR3 expressing cells, human PMNs and CR3 transfected CHO cells (CHO Mac-1). We demonstrated that *B. burgdorferi*, and lipidated OspA and OspC in the absence of serum, were able to induce up-regulation of the CR3 on human neutrophils; *B. burgdorferi* living cells, but not the Osps,

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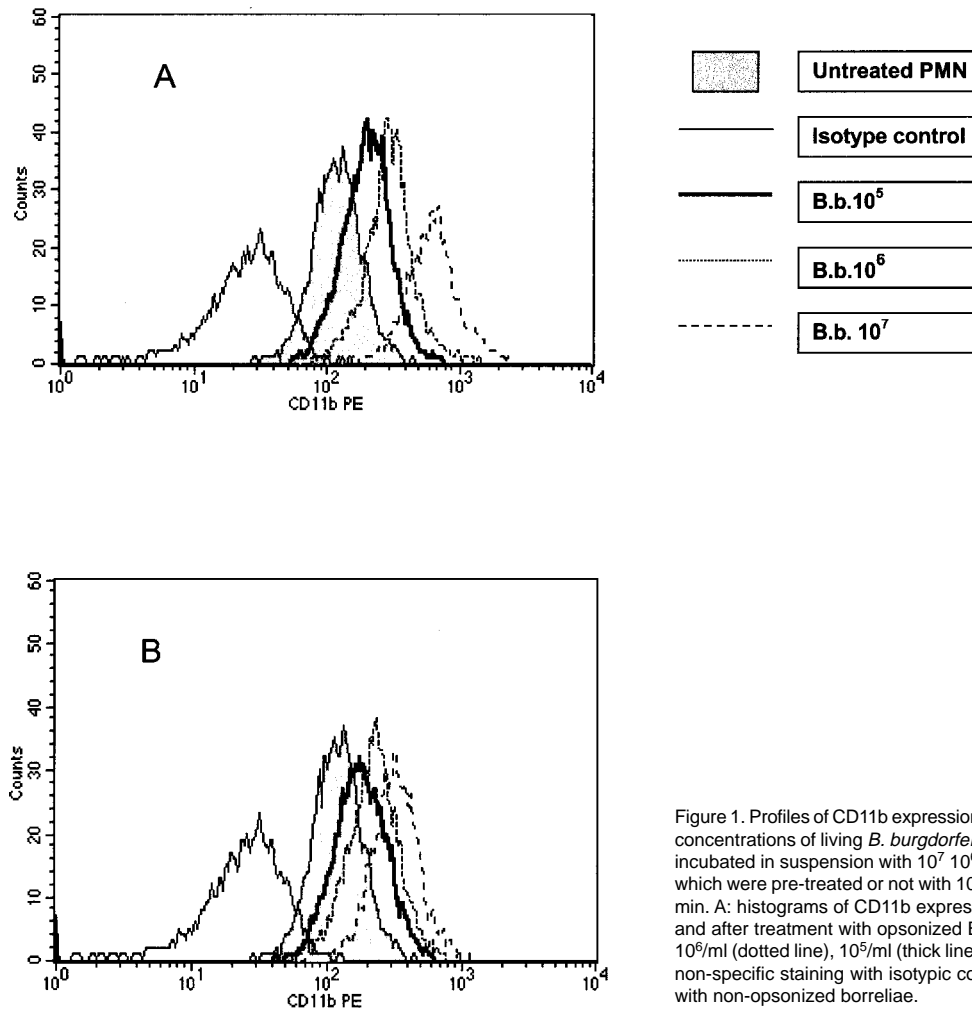


Figure 1. Profiles of CD11b expression of PMNs after treatment with different concentrations of living *B. burgdorferi*, strain BITS. PMNs ($5 \times 10^6/\text{ml}$) were incubated in suspension with 10^7 , 10^6 and 10^5 Borreliae for 15 min at 37°C which were pre-treated or not with 10% normal human serum (NHS) for 15 min. A: histograms of CD11b expression of PMNs baseline (shaped area) and after treatment with opsonized *B. burgdorferi* at $10^7/\text{ml}$ (dashed line), $10^6/\text{ml}$ (dotted line), $10^5/\text{ml}$ (thick line). Histogram in thin line represents the non-specific staining with isotypic control Mab. B. The same experiments with non-opsonized borreliae.

stimulate higher adhesion to fibronectin by CHO Mac-1. The cellular adhesion induced by *Borrelia* beside involving the I- domain of the CR3, was also influenced by the lectin-like domain of the integrin.

Results

PMNs Respond to *B. burgdorferi* and OspA and OspC With CR3 Up-Regulation

One of the consequence of PMN activation by an agonist is up-regulation of CR3, which occurs by the quick recruitment of CR3 from the granules where is stored. Another consequence is an increased avidity for the ligand, *i.e.* extracellular matrix. To verify the first statement, we looked for enhanced surface expression of the CR3 by cytofluorimetric study of PMNs treated with whole cell *B. burgdorferi* or recombinant OspA and OspC. Different concentrations of live *B. burgdorferi* cells were found to induce surface expression of CR3 on PMNs (Figure 1, 2), in a dose dependent fashion, both in the absence and presence of serum. The kinetics of surface CR3 expression indicated that the stimulation develops in an average of 15 min and as few as 10^6 live Borreliae, corresponding to one Borrelia/PMN was required to detect a significant level of

the CD11b subunit expression. Similar kinetics of expression has been reported for classical agonists of inflammation such as LPS and FMLP (Kishimoto *et al.*, 1989). Upon exposure to 0.5 to 5 μg lipidated OspA and OspC, PMNs increased the expression of CD11b (Figure 3, A); no stimulation of the integrin was observed upon exposure to the unlipidated lipoproteins. These results agree with the data from the literature (Radolf *et al.*, 1995; Wooten *et al.*, 1998) reporting that the lipid moiety of lipoproteins is crucial for cell activation.

Since it has been reported (Morrison *et al.*, 1997) that CR3 up-regulation is one of the consequence of PMNs priming by OspA proceeding via the CD14 recognition, to ascertain the contribution of this receptor to CR3 overexpression, we performed the up-regulation experiments in the presence of mAb 60 bca CD14 blocking antibody and the non blocking isotype mAb 21: as reported in Figure 3,B, up-regulation of CR3 decreased when PMNs were pre-treated with mAb 60 bca antibody, but still remained at significant levels of stimulation, indicating that the CD14 receptor only facilitates the activation, but other molecules transfer the signal, or the stimulation is due to a direct interaction of OspA with the CR3 domains.

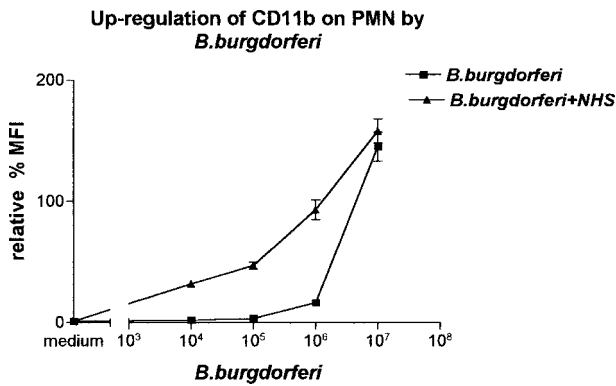


Figure 2. Graph of dose-dependence up-regulation of CR3 on PMNs surface. Experiments as in Fig.1. Fluorescence was measured by flow cytometry: each data point represents the % variations of mean fluorescence intensity compared to the baseline fluorescence value observed with medium alone. Data were means \pm standard deviations of three independent determinations. * $p \leq 0.005$.

B. burgdorferi, But Not Osps Induces CHO-Mac-1 Adhesion to Fibronectin (FN)

In order to verify whether *B. burgdorferi* not only induces overexpression of the integrin, but also a conformational change in the molecule, leading to higher binding affinity and adhesion to the ligand, we performed the adhesion experiments on fibronectin (FN), employing CHO cells transfected and not with CR3, instead of PMNs, to avoid the interference with other co-receptors; in fact CHO cells constitutively express neither CD14, nor the Toll-2 receptors, which have been recently found to mediate signalling for cellular responses induced by bacterial lipoproteins and LPS (Kirshing *et al.*, 1998)

In our experiments *B. burgdorferi* living cells stimulated, in dose dependent fashion, the adhesion of CHO Mac-1 cells on surfaces coated with protein of the extracellular matrix. As illustrated in Fig.4 (A) treatment of CHO Mac-1 cells with 7×10^5 *Borreliae* doubled adherence of the cells to FN-coated plastic wells, and the adhesion was not lowered by polymyxin B. The highest adherence was found with *E. coli* LPS. *B. burgdorferi* treated CHO Mac-1 cells appeared in the typical "spread" pattern, when observed by light microscopy (data not shown). The direct interaction and activation of the integrin by *B. burgdorferi* was proven by the absence of any adhesion of the CHO not transfected cells.

In order to verify the contribution of the CR3 I-domain and the lectin-like domain in the *B. burgdorferi* dependent adhesion of CHO Mac-1 cells to FN, the cells were treated with 10^8 *B. burgdorferi* in the presence of mAb M1/70, recognising the I-domain, or 100 mM NADG, which is known to interact with the lectin-like site of CR3. As reported in Fig.4 (B), the adherence of CHO Mac-1 cells induced by *B. burgdorferi* dropped to the control value with both antagonists. Taken as a whole these results indicate that living *B. burgdorferi* activates the integrin $\alpha_M\beta_2$ to a higher affinity binding of the ligand, and that the lectin-like domain of the integrin contributes to this phenomenon. When we came to examine the capacity of Osps to induce adhesion of CHO Mac-1 cells to FN, variable and not significant results were observed: 0.5 μg OspCL and OspAL induced a significant FN adhesion of CHO cells (data not shown),

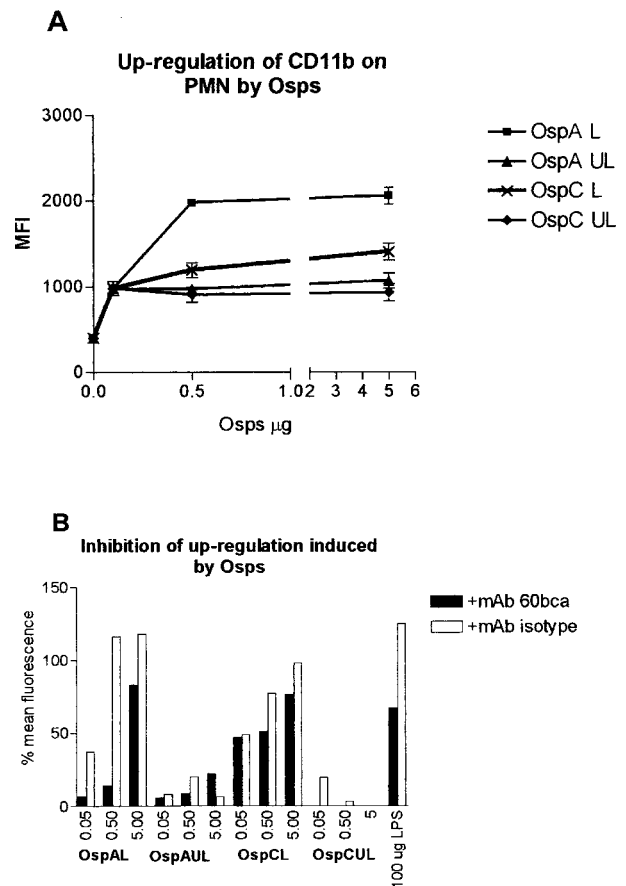


Figure 3 Up-regulation of CR3 on PMNs after treatment with OspA and OspC lipidated and unlipidated. (A) 100 μl of PMNs ($5 \times 10^6/\text{ml}$) were incubated in suspension with 0.05, 0.5, and 5 $\mu\text{g}/\text{ml}$ of OspA and OspC lipidated and not lipidated for 15 min at 37°C, in the presence or not of serum complement (NHS). Data represent the mean fluorescence intensity of each condition. (B) The same experiments were performed in the presence of 5 $\mu\text{g}/\text{ml}$ of either CD14 blocking mAb (60bca) or its isotype control (mopc 21), and 100 $\mu\text{g}/\text{ml}$ *E. coli* LPS as control of stimulation. Fluorescence measurements as in Figure 2.

but this observation was not confirmed by dose response experiments. Therefore we concluded that the *Borrelia* lipoproteins we used, did not directly interact with CR3.

Discussion

We have previously demonstrated that *B. burgdorferi* strain BITS binds to the CR3 receptor and attaches to integrin expressing cells, *i.e.* PMNs and Mac-1 CHO transfected cells. In this study we investigated further this interaction and the possible consequence of such binding, employing living *B. burgdorferi* and recombinant OspA and OspC lipidated and not. We used CHO Mac-1 transfected cells in some experiments, to avoid interference from other receptors present on PMNs surface such as CD14, which was found to bind to OspA and *Borrelia* lipoproteins (Wooten *et al.*, 1998; Sellati *et al.*, 1998) and trigger leukocyte activation. The results presented here indicate that living *Borreliae* activate the integrin in a dose dependent manner and the activation is serum independent. The evidence of such activation includes up-regulation of CR3 on PMNs, and increased binding affinity

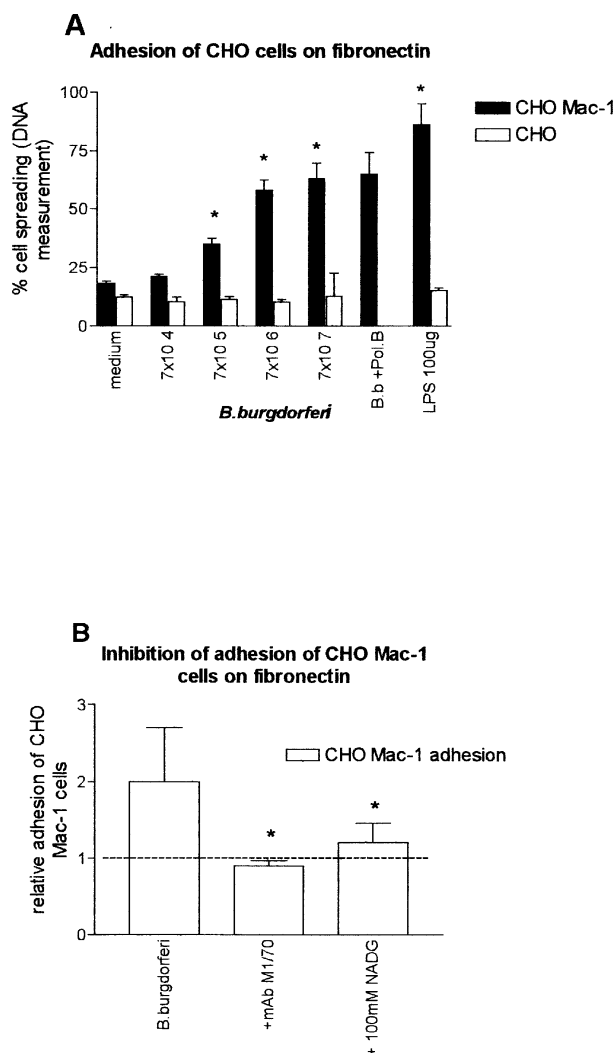


Figure 4. Spreading of CHO cells, transfected (Mac-1) and not transfected with CR3, on fibronectin coated plates, induced by living *Borreliae*. (A) Cells (2×10^5 in 100 μ l final volume) were treated for 45 min at 37°C, with different amounts (10^4 to 10^9) of living *Borreliae*, 100 μ g/ml *E. coli* LPS and 5 μ g/ml Polymyxin B, in FN coated 96 well. Measurement of adhesion was calculated from DNA content released after lysis of adherent cells. * $p \leq 0.005$ (B) Effect of reagents that bind to CR3 domain and to Lectin-like domain on *B. burgdorferi* induced adhesion of CHO Mac-1 cells to FN. CHO (2×10^5 in 100 μ l final volume) were treated, for 45 min at 37°C with 10^8 living *B. burgdorferi*, in the presence of medium or 2 μ g mAb M1/70, or 100 mM NADG. Measurement of adhesion as in (A). Dotted line indicates unstimulated adhesion, *i.e.* in the presence of incubation medium. * $p \leq 0.005$.

by CHO cells for heterologous ligand, *i.e.* fibronectin. Further evidence of specific integrin activation by *B. burgdorferi* is that the adherence of CHO Mac-1 cells on fibronectin is inhibited by both mAb M1/70 and NADG. This latter finding gives a further evidence that the lectin-like domain of CR3 contributes to recognition and binding of *B. burgdorferi*, a datum which confirms previous adhesion studies (Cinco *et al.*, 1997): in fact adhesion of spirochetes to CR3 expressing cells was found to be inhibited by mannose and NADG, sugars known to bind to the COOH terminal lectin-site of the integrin CR3 (Cinco *et al.*, 1997; Cinco *et al.*, 1998). The lectin-like domain of CR3 was recently recognised as influencing the CD11b/CD18 binding

activity of *Candida albicans* (Forsyth *et al.*, 1998), by modulating the function of the I-domain and thereby mediating the adhesion of this pathogen to leukocytes. We cannot exclude that glycoconjugates participate in CR3 activation also in *B. burgdorferi*, on the basis of our results and of the data obtained by several authors (Hulinska *et al.*, 1992; Sambri *et al.*, 1992), even if intrinsic expression of glycosylated proteins on the *Borrelia* surface has not been proven yet. The recombinant lipidated OspA and OspC do not directly interact with CR3, since they do not induce the spreading of CHO Mac-1 cells, but stimulate CD11b/CD18 overexpression probably via CD14 and other unidentified co-receptors; therefore the *Borrelia* counter receptor (adhesin) responsible for CR3 recognition and activation, remains still unknown.

The meaning of our results shows that *Borrelia* components induce a priming state on neutrophils through CR3 up-regulation and activation: as a possible consequence, PMNs become adhesive not only for the stimulating borreliae but also for other ligands, such as the extracellular matrix and possibly, the endothelium. It has been widely demonstrated that the outer surface lipoproteins of *B. burgdorferi* up-regulate the expression of adhesion molecules ICAM-1, E-Selectin and VCAM on vascular endothelium (Sellati *et al.*, 1996; Wooten *et al.*, 1996; Ebnet *et al.*, 1997). Our results point out that *B. burgdorferi* Osps, possibly the same that stimulate adhesion molecules on endothelium, stimulate also the counter part, the CD11b/CD18 receptor on PMNs: therefore, *in vivo*, the whole process of neutrophil binding to endothelium, as consequence of *B. burgdorferi* infection, is amplified on both PMNs and endothelium and represents the first step of perivascular inflammation.

Furthermore, since the family of β_2 integrins dependent adhesion to certain biological surface such as fibronectin, is a prerequisite for granulocytes to fully respond to stimuli that usually give a transient response, or do not give any response at all with the cells in suspension (Nathan, 1997, Dri *et al.*, 1991) and *B. burgdorferi* induces an increased adhesion to the matrix, we may speculate that another consequence of the CR3 activation by *Borrelia* might be the priming of granulocytes to degranulation and massive H_2O_2 release in time, thus causing tissue damage.

Experimental Procedures

Bacterial Strains, Culture Conditions and Labelling

The borreliae used were from our laboratory: high passage strain BITS isolated from *I. ricinus*, belonging to *Borrelia garinii*. Culture conditions were done as previously reported (Cinco *et al.*, 1997). Recombinant OspA and OspC lipidated and unlipidated were kindly provided by Dr. Huebner (Aventis, Swiftwater Pasteur, PA, USA)

Human Neutrophils (PMNs) and CHO Cell Preparations

PMNs were isolated from heparinized peripheral blood of healthy donors by dextran sedimentation followed by Fycoll-Hypaque density gradient centrifugation and hypotonic lysis, as previously described (Cinco *et al.*, 1997). CHO Mac-1 cells, which had been transfected with stable wild-type human Mac-1 (clone CHO Mac-1 1-135) were cultured and checked for receptor expression as previously described (Cinco *et al.*, 1997). The not transfected CHO cells (clone CHO-F185.1) were used as controls. Adhesion experiments were performed in MEM (Minimal Essential Medium) solution.

Monoclonal Antibodies and Reagents

The following monoclonal antibodies (mAbs) were used: phycoerythrin-conjugated (PE) clone 2LPM19C, recognising the CD11b subunit of the integrin CR3 and isotype-matched control IgG2a (Dako SpA Milano); mAb M1/70 rat clone, recognising and blocking CD11b I-domain (ATCC), mAb 60bca blocking the CD14 receptor, and its isotype mopc 21 non blocking

mAb (kindly sent by Mario Philipp, Tulane, University, New Orleans, USA). Fibronectin (FN) was prepared from human plasma according to Ruoslahti (Ruoslahti *et al.*, 1982). N-acetyl-D-glucosamine (NADG) and LPS from *E. coli* were from Sigma, Chem. Every solution was prepared in pyrogen free water and the possible contamination by endotoxin was checked, in the experiments of cell adhesion, by the *Limulus* amoebocyte lysate pyrogen (BioWhittaker, Italia Srl) and by the addition of 5 µg Polymyxin B.

Up-Regulation of CR3 Integrin on PMNs

PMNs suspensions (5x10⁶/ml), performed as reported above, were incubated in Hank's balanced salt solution (HBSS) for 15 min at 37°C, with different concentrations of *B. burgdorferi* whole cells, or recombinant OspA and OspC lipidated and unlipidated. Suspensions were directly stained with 1 µg of PE-labelled anti CD11b, the subunit of the integrin CR3, mAb 2LPM19c, or isotype PE conjugated control. Reaction with antibody was performed at 4°C for 30'; then the cells were washed twice and fixed with 1% paraformaldehyde. Inhibition of up-regulation was performed by pre-treatment of PMNs with 5 µg mAb 60bca CD14 receptor blocking antibody or its isotype control mopc 21 non blocking mAb.

Cellular Adhesion on Fibronectin-Coated Surfaces

The assay was performed on polystyrene 96-well plates (NUNC, Inc. Naperville, IL) coated with 20 µg/ml FN. The FN-coated plates were washed with PBS prior the addition of 10⁵ CHO Mac-1 cells and different amounts of strain BITS or Osps or the corresponding amount of medium buffer in a final volume of 100 µl. When indicated, 2 µg mAb M1/70 or 200 mM NADG were added. After 45 min incubation at 37°C, the plates were filled with PBS, sealed with caps and centrifuged upside down at 200 x g for 5 min. After centrifugation the wells were flicked empty, filled with 100 µl distilled water and frozen. Quantification of adherent cells was achieved on the basis of DNA fluorescence titration measurements, using Hoechst 33258, and an automated 96-well plate cell reader, according to Rago (Rago *et al.*, 1990). The percentage of cell adhesion was calculated on the basis of a calibration curve drawn with known amounts of cells.

Flow Cytometry

Analysis of PMNs expression of CD11b was performed by a FACScan flow cytometer (Becton Dickinson Immunocytometry system, San Jose, CA). Cells were gated for forward and right angle light scatter and then analysed for fluorescence emission at 585 nm. For each analysis 10.000 events were accumulated in list mode and data were analyzed with LYSYS II^o software. Results were expressed as mean fluorescence intensity (MFI) subtracting the non-specific staining obtained with isotype PE labelled mAb.

Statistics

Data from repeated experiments were analysed by the Mann-Whitney nonparametric test (P≤95%) using GraphPad Prism version 2.0 for windows, GraphPad software, San Diego, California, USA.

Acknowledgements

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