

Integrin CR3 Mediates the Binding of Nonspecifically Opsonized *Borrelia burgdorferi* to Human Phagocytes and Mammalian Cells

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Like other pathogens, the spirochete *Borrelia burgdorferi*, the agent of Lyme disease, possesses multiple pathways for cell binding; adhesion to phagocytic cells is of particular interest since it reportedly occurs even in the absence of specific antibodies. This study sets out to investigate how *B. burgdorferi* binds to human polymorphonuclear leukocytes (PMNs) when an exogenous complement is added and how the CR3 complement receptor, known as Mac-1 or $\alpha_m\beta_2$ integrin, is involved in the binding process. Experiments performed on PMNs and CHO Mac-1-expressing cells demonstrate that binding is inhibited by monoclonal anti-iC3b site antibodies, fibrinogen, and *N*-acetyl-D-glucosamine. These findings, which are not present with non-Mac-transfected CHO cells, indicate that the integrin $\alpha_m\beta_2$ acts as a receptor for spirochetes in nonimmune phagocytosis; furthermore, binding occurs on different domains of the CD11b subunit, involving the iC3b site and the lectin domain. The interaction of *B. burgdorferi* with $\alpha_m\beta_2$ integrin adds a novel pathway to *Borrelia*-phagocyte binding; not only does this binding affect the early stages of phagocytosis, but also it can influence the effector intracellular mechanisms which are activated by the β_2 integrin, as are the cytotoxic mechanisms.

Lyme disease is a multisystemic illness, characterized by a chronic course during which spirochetes may be found in affected organs (1). This suggests that *Borrelia burgdorferi* is able to survive for a long time and that although many spirochetes are removed by phagocytic cells, clearance is incomplete. Phagocytosis of *B. burgdorferi* by professional phagocytes, monocytes, and neutrophils proceeds without opsonization (3–5, 20); it is more effective in the presence of whole serum (3); and there is evidence of both killing and/or survival (15, 20).

Many authors report that the uptake of antibody-opsonized *B. burgdorferi* (4, 16) proceeds via the Fc receptors and leads to lysosome degradation (15, 17); however, on the other hand, recent studies by Montgomery et al. have demonstrated that when unopsonized borreliae are ingested they do not colocalize with Fc receptors, showing that this receptor is not apparently involved in the uptake (16). It therefore remains unclear how borreliae enter phagocytes in the absence of specific opsonization.

Since it has been found that phagocytosis and adhesion of unopsonized borreliae increase in the presence of serum (5) and, as Kochi and Johnson (13) reported, that borreliae are covered by the iC3b fraction following activation of the human complement, this study aims to investigate whether the integrin CR3 (known as Mac-1) is involved in the adhesion of unopsonized spirochetes. It is already widely accepted that this receptor for complement protein iC3b promotes the attachment and ingestion of some intracellular parasites such as *Leishmania major* (18), *Rhodococcus equi* (10), *Legionella pneumophila* (19), *Listeria monocytogenes* (9), and *Bordetella pertussis* (21).

Mac-1, a $\alpha_m\beta_2$ member of the β family of integrins expressed on the plasma membrane of mammalian polymorpho-

nuclear leukocytes (PMNs), mononuclear phagocytes, and natural killer cells, consists of a unique α (CD11b) chain covalently associated with the β (CD18) subunit. CR3, previously considered to be the main iC3b receptor, is known to have multiple adhesion domains, which are localized at different points on the CD11b subunit. The primary domain is responsible for iC3b, ICAM-1, fibrinogen, and factor X recognition, and a second domain, COOH-terminal to the primary domain, has a lectin-like activity and binds sugars such as β -glucans, mannose, *N*-acetyl-D-glucosamine, glucose, and lipopolysaccharide (7, 8, 27, 28).

MATERIALS AND METHODS

Bacterial strains, culture conditions, and labelling. The borreliae used were *Borrelia garinii* BITS isolated from *Ixodes ricinus* and the low-passage *B. burgdorferi* sensu stricto human isolate Tirelli. The spirochetes were cultured at 34°C in Barbour-Stoenner-Kelly (BSK) medium as previously reported (5). When required, borreliae were fluorescein labelled as previously described (5). In brief, a culture of approximately 10^8 borreliae ml⁻¹ was separated from the medium by centrifugation at $900 \times g$ for 40 min and then washed in Hanks balanced salt solution (HBSS) plus 0.1% gelatin. The sediment was resuspended in 1 ml of HBSS (pH 7.4) containing 0.2 mg of fluorescein isothiocyanate (FITC) (Sigma, St. Louis, Mo.) and, after a 30-min incubation at room temperature, washed twice in HBSS. For experiments on cell adhesion, borreliae were radiolabelled by being grown for 3 days in BSK medium supplemented with [³H]adenine (Amersham Life Science) at 2.5 μ Ci/ml and then washed thoroughly in Eagle minimal essential medium (MEM; Sigma Chemical Co.). This gave optimal labelling of spirochetes, with approximately 10,000 cpm incorporated per 10^7 spirochetes. Both fluorescein-labelled and [³H]adenine-labelled borreliae were centrifuged and resuspended in BSK medium plus 20% glycerol at 5×10^8 cells/ml, and aliquots were frozen at -80°C.

MAbs and reagents. As potential inhibitors of CR3-mediated adhesion, the following monoclonal antibodies (MAbs) were used: monoclonal anti-human CD11b clone 2LPM19c (Dako), MAbs M1/70 and OKM1, and MAb MY904 directed against the α -chain of CR3 (donated by R. Cramer, University of Trieste). In CHO cell adhesion, MAbs were added at 2 to 5 μ g/ml in MEM. Human fibrinogen (Sigma) at 0.5 mg/ml and *N*-acetyl-D-glucosamine (GluNAc; Sigma) at different concentrations were tested to investigate potential inhibition of CR3 binding. Anti-human CD11b or isotype-matched control immunoglobulin G2a phycoerythrin-conjugated MAb (clone 2LPM19c) was used to stain CR3 receptors on CHO cells: the cells were stained for 30 min on ice, washed twice with phosphate-buffered saline (PBS; pH 7.4), and resuspended in PBS-0.5%

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paraformaldehyde for cytometric reading. Dextran sulfate (Sigma) at 10 $\mu\text{g/ml}$ was also used to check whether the proteoglycans were involved in adhesion.

Preparation of human PMNs and cell cultures. PMNs were isolated from heparinized peripheral blood of healthy donors by dextran sedimentation followed by hypotonic lysis and Ficoll-Hypaque density gradient centrifugation as previously described (5).

CHO Mac-1 cells, which had been transfected with stable wild-type human Mac-1 (clone CHO-Mac-1-1.35) (7), and clone CHO-F185.1 cells, which did not express CR3, were used in our study of *Borrelia* adhesion to Mac-1. The cells were generously donated by David Mosser (Temple University, Philadelphia, Pa.). They were cultured as described previously (26) in alpha minimal essential medium (α -MEM; GIBCO-BRL)-10% dialyzed fetal calf serum-16 μM thymidine-0.1 μM methotrexate-2 mM glutamine-penicillin G plus streptomycin (100 U/ml and 100 mg/ml, respectively). Under these conditions, clone CHO-Mac-1-1.35 expressed a high level of Mac-1 receptor compared to the Mac-1-negative clone CHO-F185.1. The two clones were periodically checked for receptor density by flow cytometry as described above (Fig. 1).

Binding of [^3H]adenine-labelled *B. burgdorferi* to cultured cells. CHO cells were seeded at 2×10^5 cells per 2-cm 2 well in 24-well plates and allowed to form a confluent monolayer. Radiolabelled spirochetes were thawed, washed twice in MEM, and resuspended in the same medium. During the experiments, 2×10^7 borreliae in 400 μl of MEM supplemented with 1% bovine serum albumin were added to each well with or without 5 to 10% fresh normal human serum (NHS) and, as required, the other reagents as described below.

Cells and spirochetes were rocked and incubated for 60 min at 34°C and then fixed by the addition of 0.5% paraformaldehyde. The monolayers were gently washed twice with MEM to remove unbound organisms. Cell-bound borreliae were released by solubilization with 0.5 Triton X-100-0.1 M NaOH, and the lysates were loaded in ISTAMED scintillation liquid. Radioactivity was counted with a Beckman LS6000TA counter. Suspensions of the *Borrelia* inoculum containing the same amount of bacteria used in the experiment were also counted. Four wells were prepared for each sample.

Binding of fluorescein-labelled *B. burgdorferi* to PMNs and CHO cells. Purified PMNs or CHO cells were suspended in HBSS with or without 5 to 10% NHS. As required, monoclonal antibodies or GluNAc was added as specified in the figures. A 100- μl volume of cell suspension (1×10^6 to 3×10^6 cells/ml) was mixed with 20 μl of fluorescein-labelled borreliae ($3 \times 10^8/\text{ml}$) and incubated at 37°C for 60 min with shaking. Finally 1 ml of PBS-1% EDTA was added to each vial, and the contents were analyzed by cytometry.

Flow cytometry. A FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with an air-cooled argon ion laser and filter setting for FITC (530 nm) and phycoerythrin (585 nm) was used. A total of 10,000 cells were computed in list mode and analyzed with LYSYS II software (Becton Dickinson). Forward-scatter and side-scatter gates were used to define the viable cell fraction and to avoid aggregates and debris. Single green fluorescence emission of FITC-labelled borreliae bound to CHO cells or PMNs was expressed as a percentage. To select the binding of spirochetes to PMNs, the specific background fluorescence of PMNs alone was used as the threshold level and a marker was set. PMNs with a fluorescence intensity higher than the threshold level were considered to represent significant adhesion. The percent binding was calculated as the number of positive cells with respect to the total events counted (10,000). The expression of Mac-1 antigen on CHO cells was quantified as a red fluorescent signal. For each experiment, unstained CHO cells or PMNs or cells stained with isotype-matched MAbs were used as negative controls to set the threshold for positivity.

Statistics. Data from repeated experiments were analyzed by the Mann-Whitney nonparametric test ($P \leq 95\%$) of GraphPad software (ISTAMED).

RESULTS

Binding of *B. burgdorferi* to PMNs. Fluorescent *B. burgdorferi* cells were added to freshly isolated human PMN suspensions at a ratio of 20:1, which had previously proved optimal for adhesion studies (4). Different reagents were added to the bacterium-PMN mixture, and binding of spirochetes to phagocytes was quantified by flow cytometer. Figure 1 shows that binding of spirochetes to PMNs was low (18.3% of the cells) in the absence of NHS but dramatically increased (47.31%) when 10% fresh NHS was added, suggesting that this adhesion might be serum dependent. When heat-inactivated NHS was used, adhesion remained at the baseline level of 15% (data not shown). Addition of MAb anti-CD11b 2LPM19c (2 $\mu\text{g/ml}$), directed against the iC3b domain of the CR3 integrin, led to an inhibition of serum-dependent adhesion. Although the use of whole-antibody 2LPM19c can lead to a potential cross-linking and capping of receptors, this finding suggests that the CR3 receptor is involved in the binding. Figure 1D to F shows the

inhibitory effect of GluNAc on serum-dependent adhesion: *B. burgdorferi* adhesion values fell when GluNAc concentrations increased, from an initial 47.31% in the absence of GluNAc to 35.60% in the presence of 100 mM GluNAc, 28.4% in the presence of 200 mM GluNAc, and 10.1% in the presence of 400 mM GluNAc. Thus, it would appear that GluNAc acts as a dose-related inhibitor of *B. burgdorferi*-PMN adhesion: this finding indicates that interactions between *B. burgdorferi* and the Mac-1 receptor may involve a number of binding sites, other than C3b1, such as the lectin domain, which is able to bind certain complex polysaccharides, including β -glucan, mannose, and GluNAc (27).

Binding of *B. burgdorferi* to CHO cells transfected with cloned complement receptor Mac-1. Adhesion assays with nontransfected CHO cells and cells transfected with human CD11b/CD18 (9) were carried out to investigate in greater detail the CR3-mediated serum-dependent *Borrelia* binding. Both clones of cells were used, the nontransfected clone CHO-F185 (Mac-1-) and clone CHO-Mac-1-1.35 expressing Mac-1 (Mac-1+). Surface expression of Mac-1 was monitored by using phycoerythrin-labelled MAb anti-CD11b, clone 2LPM19c, as shown in Fig. 2. The levels of fluorescence for CHO Mac-1- and CHO Mac-1+ were <1 and 95 to 99% in all the experiments.

Parallel adhesion experiments were carried out on CHO cells attached on plates and inoculated with radiolabelled spirochetes and on CHO cells in suspension inoculated with fluorescein-labelled *B. burgdorferi* in the absence or presence of different concentrations of NHS. As shown in Fig. 3, spirochetes bound to a very small extent to the Mac-1- cell type both in the presence and in the absence of NHS. In contrast, addition of increasing amounts of NHS to Mac-1-transfected CHO cells led to a dose-dependent increase in cell binding. This increase was demonstrated by a rise in the fluorescent CHO Mac-1+ fraction (Fig. 3B) from 18 to 37 and 54% (in the presence of 0.5 and 10% NHS, respectively) as a consequence of the adhesion of fluorescein-labelled spirochetes. Considering the experiments with [^3H]adenine-labelled *B. burgdorferi* and the level of labelling, the increase was from 8×10^5 to about 1.4×10^6 spirochetes/ml under the different conditions specified in Fig. 2. Heat treatment of NHS at 56°C for 30 min, to inactivate complement, reduced binding to the cells to baseline values. These findings were obtained with CHO Mac-1+ cells and not with CHO Mac-1- cells, further indicating that serum-dependent binding of *B. burgdorferi* to mammalian cells implies that the Mac-1 receptor (integrin CR3) is involved.

Effect of reagents on *B. burgdorferi* binding to Mac-1+ transfected CHO cells. A number of reagents that inhibit receptor-ligand interactions were tested to further investigate the domains of CR3 integrin involvement in *B. burgdorferi* binding, as suggested by the first series of experiments performed with PMNs. Potential inhibitors of CR3 binding at the α_m subunit with different domain specificities were used: fibrinogen and the CD11b, 2LPM19c, and M1/70 MAbs (27) (2 to 5 mg/ml) were chosen as being representative of substances blocking the I domain of the α_m chain, which also recognizes the iC3b protein, ICAM-1 receptor, and Factor X (7, 8, 29). GluNAc and MAbs CD11b, OKM1, and MY904, which interact close to the lectin site and lipopolysaccharide site, recently mapped on the C-terminal region of the α_μ subunit, where lectin activity and sugar recognition occur (27, 29), were added in the inhibition experiments. As observed in the previous experiments performed with PMNs, GluNAc led to a progressively lower, dose-dependent adhesion of *B. burgdorferi* to CHO Mac-1+ cells compared to the control: 200 and 400 mM GluNAc lowered the binding to 71 and 47.7%, respectively, with

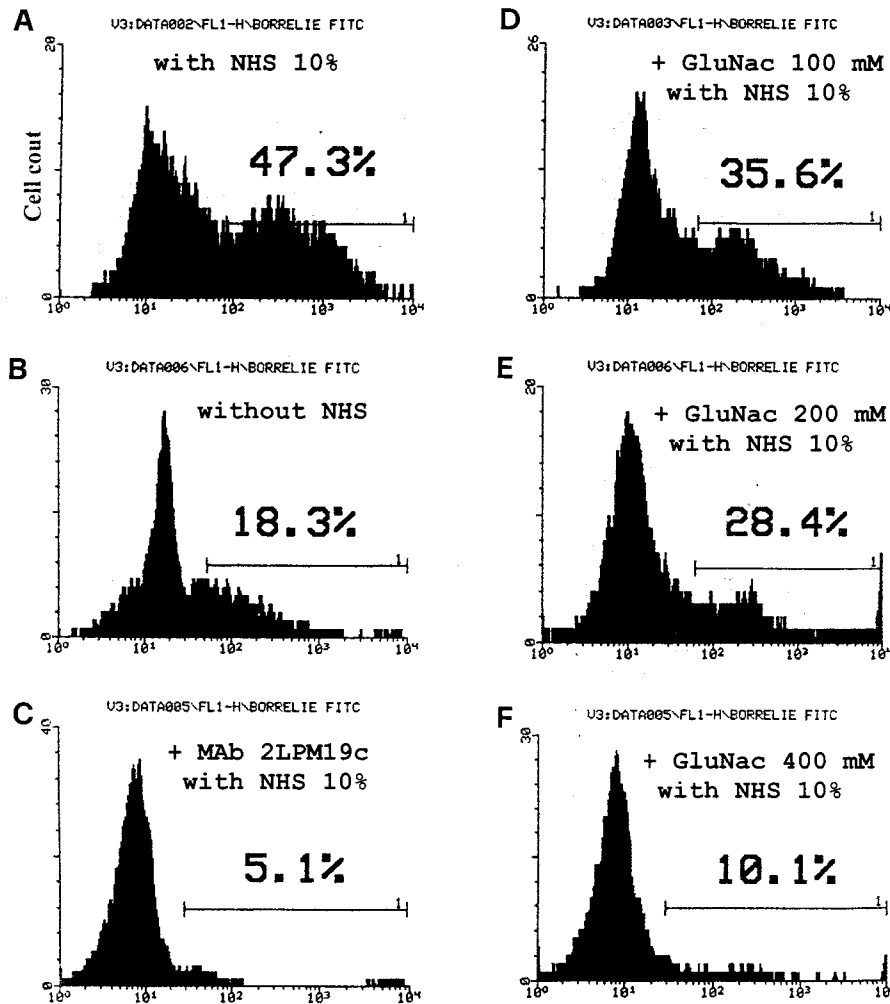


FIG. 1. Flow cytometry profiles of the PMN fraction that was fluorescent after adhesion to FITC-labelled *B. burgdorferi*. Fluorescence intensity is expressed in arbitrary units. (A) PMNs plus FITC-labelled spirochetes (1/20) with 10% NHS; (B) without NHS; (C) with 10% NHS plus anti-Mac-1 CD11b, clone 2LPM19c. (D to F) PMNs plus FITC-labelled borreliae with 10% NHS, plus increasing concentrations of GluNac: (D) 100 mM GluNac; (E) 200 mM GluNac; (F) 400 mM GluNac. PMN vitality was not affected by 400 mM GluNac. Data from one representative experiment of three performed are shown. In each panel the marker 1 indicates the number of fluorescent cells analyzed.

radiolabelled spirochetes (Fig. 4A) and to 55.5 and 40.0%, respectively, with FITC-labelled borreliae (Fig. 4B). Other binding inhibitors considered were fibrinogen, giving only 47.2% adhesion (Fig. 4A), and MAbs 2LPM19c and M1/70, which lowered binding to 52.7 and 46.6% (A) and 34.1 and 42.2% (B). MAbs OKM1 and MY904 had no inhibitory effect on binding; indeed, OKM1 seems to stimulate adhesion (Fig. 4A).

Adhesion experiments performed with 10 μ g of dextran sulfate per ml, in the absence of NHS, excluded the involvement of proteoglycans present on CHO cells (12) in binding (data not shown).

CHO binding to different high-passage strains of *B. burgdorferi*. Experiments of adhesion on CHO were also carried out with a low-passage human isolate of *B. burgdorferi* sensu stricto Tirelli. Preliminary data obtained indicated that the rate of serum-dependant adhesion was not significantly different from that of strain BITS, which is a low-passage spirochete. Research into this topic, using other strains and genospecies of *B. burgdorferi*, is in progress.

DISCUSSION

We have examined the adhesion rate of *B. burgdorferi* BITS to PMNs in the absence of specific opsonins and found that binding to neutrophils increases significantly when fresh, not heat-inactivated, NHS is present. Binding is inhibited by the addition of MAbs directed toward the iC3b site located on the CD11b subunit of the integrin CR3. These findings, together with the knowledge that *B. burgdorferi* activates complement and fixes iC3b protein on the surface (13), suggest the involvement of CR3 complement receptor (integrin $\alpha_m\beta_2$, CD11b/CD18, or Mac-1) in adhesion and confirm our previous findings of a greater phagocytic activity of PMNs on nonopsonized borreliae within whole blood (3). Further evidence of Mac-1 binding of *B. burgdorferi* comes from our experiments on CHO cells, transfected with the whole human Mac-1 integrin, which were carried out by two different methods. Parallel results obtained by the two procedures demonstrate that *B. burgdorferi* attachment to CHO Mac-1-expressing cells is serum dependent and is blocked by MAbs which recognize the iC3b binding site of the integrin. Similarly, the iC3b binding site is inhibited

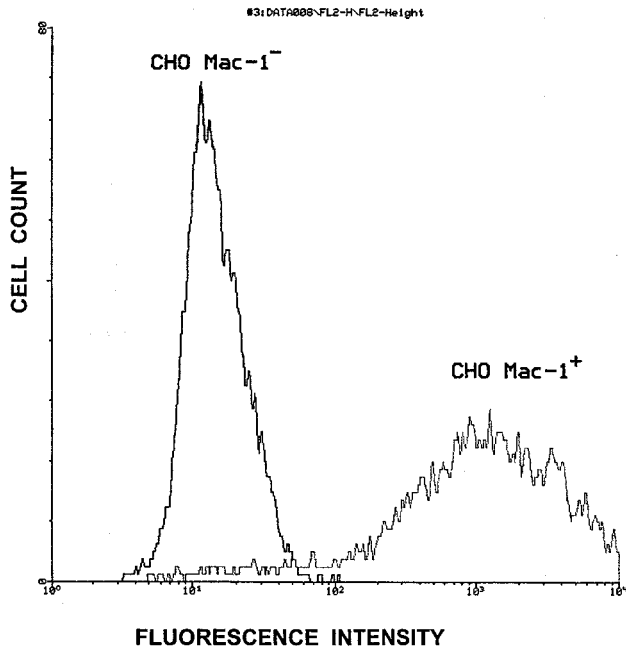


FIG. 2. Fluorescence emission of CHO-F185 (Mac-1⁻) and CHO-Ma-1.35 (Mac-1⁺) cells after reaction with MAb CD11b clone 2LPM19c, conjugated with phycoerythrin.

by fibrinogen. On the other hand, MAbs OKM1 and MY904, which bind to different domains of the CD11b subunit, failed to block serum-dependent adhesion: no serum-dependent binding was observed with control transfected CHO Mac-1.

Our data on binding inhibition with GluNAc in both PMNs and CHO cells strongly suggest that *B. burgdorferi* binds to the CR3 integrin not only via the iC3b site but also via the lectin site, which is known to possess binding specificity for many sugars, including GluNAc (26). This implies that the spirochetes bring glycoproteins or glycolipids with sugar specificity on the outer membrane. This statement, and the interaction of *B. burgdorferi* with the CR3 lectin site, is supported by (i) the detection of glycoconjugates associated with the spirochete outer envelope containing at least four carbohydrates including GluNAc (11); (ii) incorporation of GluNAc into carbohydrate residues on OspA and OspB (24); (iii) extensive literature on the role of this recognition site in phagocytosis of unopsonized yeasts (23) and, furthermore, observations derived from studies on “coiling phagocytosis” commonly occurring in *B. burgdorferi*, suggesting that this phenomenon is correlated with the presence of carbohydrate moieties on the spirochete and to lectin-type receptors on the phagocyte (22). It is therefore expected that CR3 integrin plays a twofold role, promoting opsonphagocytosis via iC3b attached to *B. burgdorferi* and promoting lectinphagocytosis, via the lectin-like site binding a *B. burgdorferi* sugar residue.

The ability of *B. burgdorferi* to bind to a wide variety of mammalian cells has been shown to occur through diverse cell receptors. Indeed, these bacteria bind glycosphingolipids (2) and proteoglycans (12, 14), binding activities which are not related to integrins. Coburn et al. have clearly shown that an important integrin of type $\alpha_{IIb}\beta_3$ acts as a receptor for *B. burgdorferi* on platelets (6). Our findings propose a novel integrin-mediated binding for *B. burgdorferi* involving the integrin of the $\alpha_m\beta_2$ type. This binding is more complex and entails multiple site-specific ligand interactions, which have hitherto never

been described for this bacterium. As a consequence of the CR3 integrin on PMNs and monocytes, it is expected that CR3-mediated binding significantly influences the rate of phagocytosis of spirochetes in the absence of specific anti-*B. burgdorferi* opsonins. It may also affect secondary events after the initial spirochete-phagocyte recognition, of primary importance in promoting intracellular killing of *B. burgdorferi*. As a β_2 integrin, CR3 bound to ligands mediates transmembrane chemical signalling and stress to the cytoskeleton; it has been demonstrated that uptake into phagocyte via Mac-1 may enable the microorganism to bypass a critical killing pathway such as O₂-dependent killing; this is true for some intracellular microorganisms such as *Leishmania* (18) and *Histoplasma* (25)

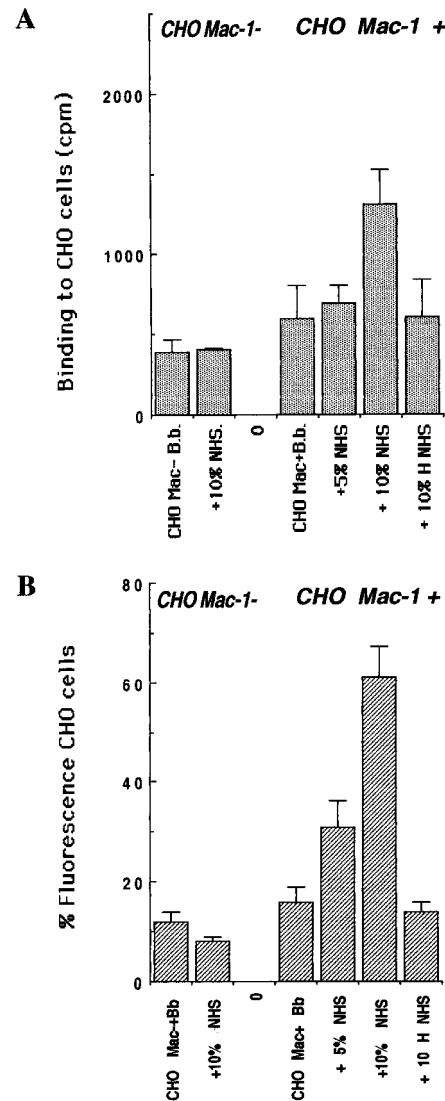


FIG. 3. Serum-dependent attachment of *B. burgdorferi* to CHO Mac-1⁻ (clone CHO-F185) and CHO Mac-1⁺ (clone CHO-Mac-1-1.35). Experiments were performed in the presence of 0, 5, or 10% NHS or 10% heat-treated NHS (H NHS). (A) Binding of radiolabelled *B. burgdorferi* to CHO cells adsorbed to plastic. Background binding to bovine serum albumin-coated plastics was 84 ± 6.7 cpm. Labelling of the spirochetes was specified in the text. CHO cell vitality in the presence of 400 mM GluNAc was tested by trypan blue exclusion. (B) Adhesion measured as the percentage of cells becoming fluorescent after the binding of FITC-labelled *B. burgdorferi*. Bars express the means \pm standard deviations of three or four independent determinations.

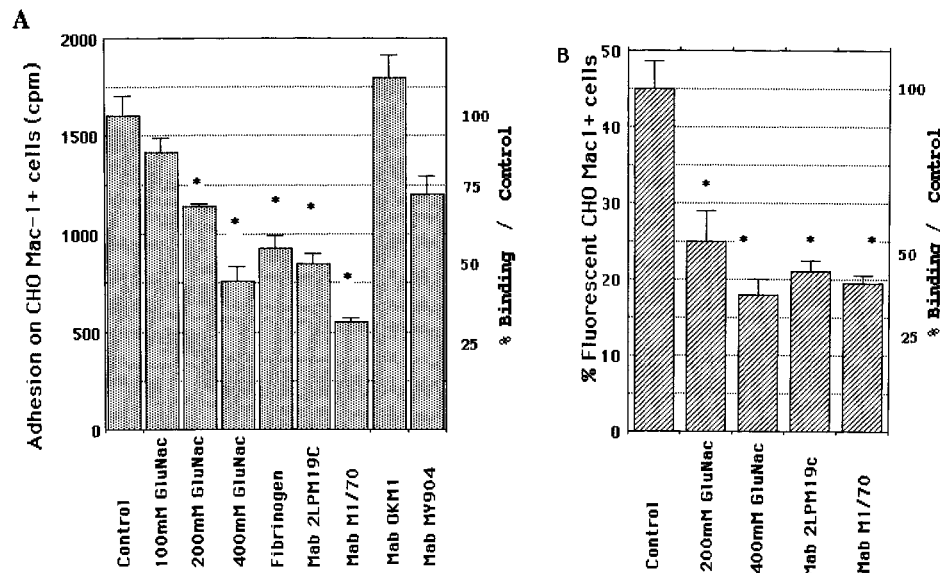


FIG. 4. Inhibition of *B. burgdorferi* binding on CHO Mac-1+ cells, measured with radiolabelled (A) and FITC-conjugated (B) *B. burgdorferi* as described in the legend to Fig. 3. All assays were performed in the presence of 10% NHS without (control) or with the addition of reagents as specified in the figure or in the text. Fibrinogen at a final concentration of 0.5 mg/ml and GluNac at 100, 200, and 400 mM were added to the mixture. MAbs were incubated with the CHO cells seeded on plastic or in suspension, prior to the addition of the spirochetes. Bars express the means \pm standard deviations of three or four independent determinations. *, $P < 95\%$.

and other intracellular parasites, which are internalized through this receptor. However, according to recent reports (28), polysaccharides bound to CR3 via the lectin site determine a novel priming state which leads to CR3 activation of cytotoxicity of target cells. Nevertheless, with iC3b-opsonized bacteria which lack the appropriate saccharide, Mac-1 does not trigger cytotoxicity. Therefore if the adhesion of *B. burgdorferi* to phagocytes in the absence of specific antibodies occurs both via the iC3b site and via the lectin domain, this would trigger intracellular killing mechanisms. Studies are in progress to investigate the correlations between CR3-mediated adhesion to leukocytes and PMN activation.

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