CR3 (CD11b/CD18) EXPRESSES ONE BINDING SITE FOR Arg-Gly-Asp-CONTAINING PEPTIDES AND A SECOND SITE FOR BACTERIAL LIPOPOLYSACCHARIDE

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Human phagocytes express a family of cell surface glycoproteins known as LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), and p150,95 (CD11c/CD18) (1). Each of these receptors is a dimer composed of a β chain (CD18) that is identical in all three members and a noncovalently associated α chain (CD11) that is unique to each molecule. CR3 was first recognized as a receptor for the surface-bound complement protein C3bi (2), but was later shown to function in the recognition of several other particles, including zymosan (3), *Escherichia coli* (4), and *Histoplasma capsulatum* (5) and in the binding of polymorphonuclear leukocytes (PMN)[1] to protein-coated surfaces (6). Ross et al. (3) first suggested that CR3 has two distinct binding sites because certain anti-CR3 mAbs that blocked the binding of C3bi did not block the binding of zymosan and vice versa. Dana et al. (6) later showed that the anti-CR3 mAb 904 blocked the binding of PMN to protein-coated plastic, but not the binding of C3bi. We have extended these studies by using ligands of defined structure to define and map the binding sites of CR3.

Previous work from this laboratory has characterized two structurally distinct classes of ligand that bind CR3. The first class of ligand is exemplified by the proteins C3bi, fibrinogen, and Leishmania gp63. CR3 binds these proteins by recognizing a region that contains the amino acid sequence Arg-Gly-Asp in C3bi and gp63 (7, 8), or the structurally similar Lys-x-x-Gly-Asp in fibrinogen (9). The second class of ligand is exemplified by bacterial LPS. CR3 (as well as LFA-1 and p150,95) recognizes rough LPS, lipid A, and the biosynthetic precursor *O*-(2-amino-2-deoxy-β-D-glycopyranosyl)-(1-6)-2-amino-2-deoxy-2-D-glucose acetylated at positions 2, 3, 2′, and 3′ with β-hydroxymyristoyl moieties and bearing phosphate groups at positions 1 and 4′ (lipid IVa) (4).

Since peptides containing Arg-Gly-Asp are structurally different from LPS, we

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[1] *Abbreviations used in this paper:* EC3b, EC3bi, EIgG, ELPS, and EIVa, sheep erythrocytes coated with C3b, C3bi, IgG, LPS, or lipid IVa, respectively; Fg, fibrinogen; LAD, leukocyte adhesion deficiency; lipid IVa, *O*-(2-amino-2-deoxy-β-D-glycopyranosyl)-(1-6)-2-amino-2-deoxy-2-D-glucose acetylated at positions 2, 3, 2′, and 3′ with β-hydroxymyristoyl moieties and bearing phosphate groups at positions 1 and 4′; PDB, phorbol dibutyrate; PMN, polymorphonuclear leukocyte.

sought to determine if these two ligands interact with the same or different binding sites on CR3. Here we show that an mAb against CR3, which blocks the binding of C3bi, does not block the binding of LPS, and another monoclonal anti-CR3, which blocks binding of lipid IVa-coated erythrocytes, does not block binding of C3bi-coated erythrocytes. Further, synthetic peptides that competitively inhibit the binding of C3bi do not inhibit, but rather enhance, the binding of IVa. Thus, separate binding sites are used to recognize the two classes of ligand. Here we also confirm that both binding sites are on the same molecule since cells from patients with a genetic deficiency in CD18 fail to bind both C3bi and lipid IVa.

Materials and Methods

Subjects. Three patients of French Canadian origin with CD18 deficiency (also known as leukocyte adhesion deficiency [LAD]) have been recently described[2]. All were 2–4 yr of age at the time of this study, and all had a history of recurrent infections and extreme leukocytosis.

Peptides. Peptide L10 (LGGAKQAGDV), based on residues 402–411 of the γ chain of human fibrinogen (Fg), was synthesized by Peninsula Laboratories, Inc. (Belmont, CA). An inactive analogue of L10, AcL10, was prepared by acetylating lysine 406 as described (9). Both L10 and AcL10 were repurified by HPLC on a reverse phase column. Peptides C3-12 (TRYRGDQDATMS), based on residues 1390–1401 of human C3, was a generous gift of Dr. M. Ginsberg (La Jolla, CA).

Antibodies. mAbs OKM1 (IgG2b) and OKM10 (IgG2b), directed against the α chain (CD11b) of CR3 (2) were a gift of Dr. G. Goldstein, (Ortho Pharmaceutical, Raritan, NJ); mAb 904 (IgG1), also directed against the α chain (CD11b) of CR3 (10) was a gift of Dr. J. Griffin (Dana-Farber Cancer Center, Boston, MA); mAb IB4 (IgG2a), directed against the β chain (CD18) of CR3, LFA-1, and p150,95, was as described (2); and mAb 3G8 (IgG1), directed against the low avidity Fc receptor of neutrophils (FcRIII, CD16) was as described (11).

mAbs IB4, 904, OKM10, and $F(ab)_2$ antimurine IgG (Boehringer Biochemicals, Indianapolis, IN) were labeled with ^{125}I by the iodogen procedure (12) to sp act of $0.5–5 \times 10^6$ cpm/μg.

Cells. PMN were purified from fresh human blood on Ficoll-Hypaque gradients (13). As observed by other investigators, PMN from the CD18-deficient patients bound very poorly to protein-coated surfaces (data not shown), but we could obtain normal high plating efficiency (>90%) by adding the cells to uncoated Terasaki microtest wells as follows. 10,000 PMN in HAP buffer (Dulbecco's PBS containing 0.5 mg/ml human serum albumin, 3 mM glucose, and 0.3 μ/ml aprotinin) were added to uncoated wells of Terasaki tissue culture trays and incubated at 37°C for 30 min. The resulting monolayers were stable to repeated washes.

Sheep erythrocytes were coated with C3b (EC3b), C3bi (EC3bi), or IgG (EIgG) as previously described (14). Sheep erythrocytes were coated with rough (Re) LPS (ELPS) or lipid IVa (EIVa) as described (4). *E. coli* K12 (strain MC1061) was grown and labeled with fluorescein as previously described (4).

Attachment of Ligand-coated Particles to PMN. Monolayers of PMN were produced as described above. Where indicated, cells were incubated for an additional 10 min at 37°C with PMA (30 ng/ml) or phorbol dibutyrate (PDB) (300 ng/ml). The PDB was then washed away by extensive rinsing of the plate in PBS followed by a 20-min incubation at 20°C in a large volume of HAP. In some experiments, monolayers of PMN were then incubated with mAbs for 15 min at 0°C. Ligand-coated erythrocytes and the indicated peptides were added to the monolayers, erythrocytes were allowed to settle for 15 min at 0°C, and the plate was warmed to 37°C for 15 min. Unattached erythrocytes were removed by washing, and binding of erythrocytes to PMN was scored by phase contrast microscopy as described (14). Binding of *E. coli*

[2] Detmers, P. A., S. D. Wright, E. Olsen-Egbert, R. Adamowski, Z. Chad, L. G. Kabbash, and Z. A. Cohn, submitted for publication.

was assessed by a similar method as previously described (4). Results are reported as attachment index, the number of erythrocytes or *E. coli* per 100 PMN.

Expression of Cell Surface CR3. Monolayers of PMN were established, pulsed with PDB as described above, then treated with antibodies as described in the text. To measure the amount of cell surface CR3, cells were cooled to 0°C and incubated for 50 min with 50 μg/ml anti-CR3 (OKM10 or 904). The presence of anti-receptor antibody bound to the cell surface was then determined by measuring the binding of ^{125}I anti-mouse F(ab)$_2$ as described (15). Background binding was determined in parallel wells from which anti-CR3 was omitted. Parallel experiments used fluoresceinated F(ab)$_2$ in place of the iodinated F(ab)$_2$ anti-mouse IgG to visualize the cell surface CR3.

Results

Phagocytes Deficient in the CD18 Complex Fail to Bind C3bi, E. coli, and LPS. We have identified three LAD patients whose leukocytes are nearly devoid of all members of the CD18 complex. Less than 4% of normal levels of LFA-1, CR3, and p150,95 are expressed on the PMN[2]. These cells exhibited normal binding of EC3b and EIgG (Table I) as expected by their normal expression of CR1 and FcR[2] and failed to bind EC3bi as expected from their lack of CR3. The capacity of CR3 to bind EC3bi is dramatically enhanced by treatment of normal PMN with phorbol esters (15). Treatment of PMN from the LAD patients, however, failed to promote binding of EC3bi.

To confirm that CD18 is necessary for the recognition of LPS, we measured the binding of ELPS and unopsonized *E. coli*, both of which bind to phagocytes via LPS. PMN from the CD18-deficient patients failed to bind *E. coli*, ELPS, or EIVa (Table

TABLE I

Attachment of Ligand-coated Particles to PMN from Patients Deficient in CD18

		Attachment index				
Exp.	Subject	EC3b	EC3bi	EC3bi + PMA	EIVa	*E. coli*
1	Patient 1	406	1.5	3.8	5.8	16
	Control 1	273	86	832	205	223
	Control 2	370	90	770	201	380
2		EC3b	EC3bi + PMA	ELPS		
	Patient 1	395	1.9	16		
	Patient 2	508	2.0	2		
	Control 1	364	182	63		
	Control 2	371	258	55		
3		EIgG	EC3bi	EC3bi + PMA	EIVa	ELPS
	Patient 2	552	5.0	9	1.3	7
	Patient 3	579	5.6	6.3	3.7	4.1
	Control 1	552	116	760	168	44
	Control 2	800	163	888	370	102

Monolayers of PMN were established on uncoated tissue plastic surfaces. Where indicated, PMA (30 ng/ml) was added for 10 min at 37°C. The indicated particles were added and binding to PMN was determined after a 30-min incubation at 37°C. Data are reported as the attachment index, the number of erythrocytes bound per 100 PMN. These results are confirmed in two additional experiments (not shown).

I). These data confirm the hypothesis that members of the CD18 family function to bind LPS.

Separate Epitopes on CR3 Mediate Binding of C3bi and LPS. We asked whether LPS binds to CR3 at a site distinct from the site that recognizes C3bi. Monolayers of PMN were stimulated with PDB to maximally enhance the binding activity of CR3 for C3bi (15). Cells were cooled, incubated with a panel of mAbs against the α chain of CR3, and ligand-coated erythrocytes were added. mAb 904 against the α chain of CR3 effectively blocked the binding of EIVa (Table II). These data confirm the role of the CD18 family in the recognition of LPS. They also suggest that CR3 is the predominant receptor for LPS on PMN and that p150,95 and LFA-1 make a minor contribution. This is consistent with the observation that CR3 is far more abundant on phorbol-stimulated PMN than either LFA-1 or p150,95 (1).

Though mAb 904 inhibited binding of EIVa, it did not inhibit the binding of EC3bi. In contrast, a second mAb against the α chain of CR3, OKM10, effectively inhibited the binding of EC3bi but did not inhibit binding of EIVa (Table II). The inhibition observed was unlikely to be due to masking of the cell surface since mAb 3G8, directed against the FcRIII of PMN, had no effect on adhesion, and Fc receptors are more abundant on the cell surface than CR3 (15). More importantly, a third mAb against the α chain of CR3, OKM1, did not inhibit binding of EC3bi or EIVa. These data suggest that CR3 expresses two different epitopes, one that binds LPS and is recognized by mAb 904, and a second that recognizes C3bi and other proteinaceous ligands and is recognized by mAb OKM10.

904 and OKM10 Bind Separate Epitopes. We confirmed that 904 and OKM10 bind separate epitopes by measuring their ability to compete for binding to PMN. A 50-fold excess of unlabeled 904 had no effect on the binding of radioiodinated OKM10 to PMN (Table III). Similarly, a 50-fold excess of OKM10 did not affect the binding of radioiodinated 904 to PMN.

We also determined whether incubation of PMN at 37°C with either OKM10

TABLE II

The Two Binding Specificities of CR3 Are Inhibited by Different Monoclonal Antireceptor Antibodies

	Attachment index		
mAb	EC3bi	EIVa	EC3b
Control	993	206	733
3G8	938	302	888
OKM1	848	263	915
OKM10	215	234	815
904	730	28	817

Monolayers of PMN were established by plating cells for 35 min at 37°C then pulsing for 10 min at 37°C with PDB. To prevent the decline in receptor activity observed during prolonged stimulation with phorbol esters, the PDB was washed from the cells with a 20-min incubation at 20°C in a large excess of HSA-containing buffer as previously described (15). Cells were incubated for 15 min at 0°C with mAbs, ligand-coated erythrocytes were added, and the attachment index was scored as described in Materials and Methods. All antibodies were added as ascites fluids diluted to yield a final concentration of ~100 μg/ml of IgG. This experiment is representative of seven studies. Similar results were obtained with purified Igs used at 25 μg/ml.

TABLE III

mAb OKM10 and 904 Do not Compete for the Same Binding Site on CR3

Competitor	Binding of ^{125}I-OKM10	Binding of ^{125}I-904
-	3,829	5,772
904	3,867	600
OKM10	159	4,631

PMA-stimulated PMN were incubated with 1 μg/ml ^{125}I-OKM10 or ^{125}I-904 in the presence of 50 μg/ml of the indicated mAbs. After 60 min at 0°C, cells were washed and cell-associated radioactivity was counted.

or 904 caused redistribution of CR3. PMN were incubated with or without these antibodies for 30 min at 37°C exactly as described in Table II, then expression of CR3 was measured as described in Materials and Methods. Incubation of PMN at 37°C with either OKM10 or 904 caused no change in the amount of cell surface CR3 (not shown). The mAbs, thus, were unlikely to have caused internalization or shedding of CR3. Parallel experiments monitored the distribution of cell surface CR3 by immunofluorescence. In all cases, CR3 was observed to be uniformly distributed on the cell surface. Thus, at the resolution of fluorescence microscopy, neither antibody caused changes in the distribution of CR3.

Synthetic Peptides that Inhibit Binding of EC3bi Do not Inhibit Binding of EIVa. To confirm that C3bi and LPS bind to CR3 at distinct sites, a synthetic 12 amino acid peptide corresponding to residues 1390–1401 of human C3 (previously implicated as the recognition sequence in C3bi [7, 9]) was used to competitively inhibit binding of EC3bi to stimulated PMN. This peptide, C3-12, strongly inhibited binding of EC3bi with half-maximal inhibition at concentrations of ~0.4 mg/ml (Fig. 1). In parallel assays, C3-12 did not inhibit the binding of EC3b to CR1 on PMN. Most importantly, C3-12 did not inhibit binding of EIVa. Rather, this peptide caused a strong enhancement of the binding of EIVa with a dose response that corresponded to that for inhibition of binding of EC3bi. Thus, ligation of the C3bi binding site with a soluble ligand, C3-12, enhances the binding efficiency of the LPS binding site.

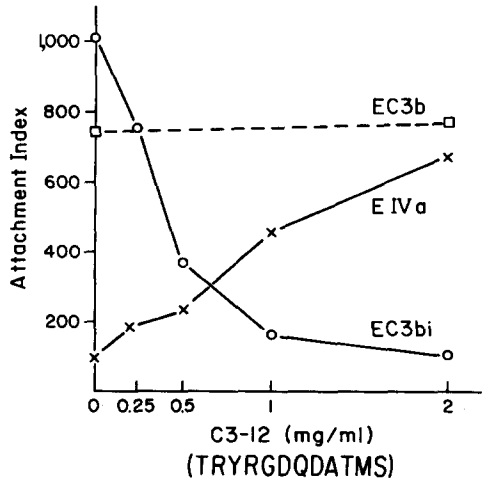

FIGURE 1. A synthetic peptide from the recognition site of C3bi inhibits the binding of EC3bi to PMN but enhances the binding of EIVa. Monolayers of PMN were established, pulsed with PDB, and washed. The binding of EC3bi (O), EIVa (×), and EC3b (□) was measured in the presence of the indicated concentrations of peptide C3-12. This experiment is representative of three separate studies.

To confirm the specificity of the results with C3-12, additional inhibitory peptides were used. We have recently shown that CR3 recognizes not only C3bi but also fibrinogen, and that peptides based on the sequence of Fg recognized by CR3 inhibit binding of both Fg and C3bi to CR3 (9). The recognition sequence in Fg does not contain Arg-Gly-Asp, but an alternative sequence in which the positive charge of Arg is supplied by a nearby Lys group. Chemical acetylation of that Lys group abolishes the activity of the peptides based on the Fg sequence. A decamer based on the recognition sequence of Fg, L10, effectively inhibited binding of EC3bi to PMN and enhanced the binding of EIVa with a similar dose dependence (Fig. 2). The specificity of these effects is confirmed by the observation that an analogous peptide, AcL10, in which the Lys group is acetylated, neither blocked binding of EC3bi nor enhanced binding of EIVa. These data confirm that occupation of the C3bi and Fg binding site of CR3 does not block, but instead enhances binding of EIVa.

Discussion

We confirm here that members of the CD18 family of receptors on PMN are required for recognition of bacterial LPS. A genetic deficiency in CD18 renders cells incapable of binding both EC3bi and ELPS (Table I). The binding site used to recognize LPS, however, is distinct from that for C3bi. mAbs against CR3 that inhibit the binding of ELPS do not inhibit the binding of EC3bi and vice versa (Table II). Further, synthetic peptides that competitively inhibit the binding of EC3bi do not inhibit binding of ELPS. Rather, these peptides enhance the binding of LPS (Figs. 1 and 2). Thus, although the binding sites for C3bi and LPS are separate, they are functionally linked.

The binding site for C3bi functions in the recognition of not only C3bi but also Leishmania gp63 (8), Fg (9), and an undefined structure on endothelial cells (16). Binding of each of these ligands is inhibited by mAb OKM10 but not by mAb 904. Further, binding of all of these ligands can be inhibited by a similar set of synthetic peptides. For example, peptides based on the recognition sequence of Fg competi-

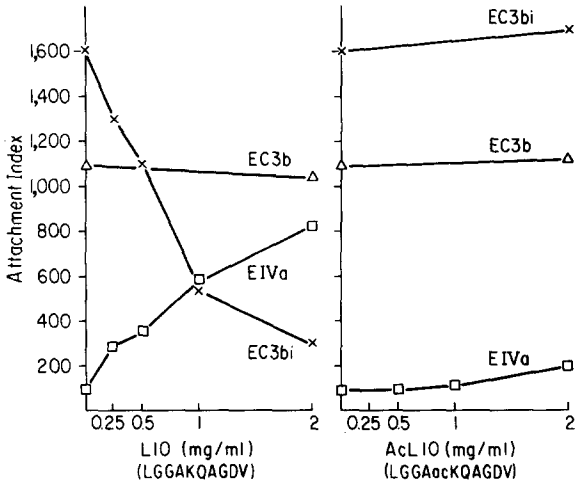

FIGURE 2. Effect of peptides based on the recognition sequence of fibrinogen on the binding of EC3bi and EIVa to PMN. The binding of EC3bi (×), EIVa (□), and EC3b (Δ) to PDB-stimulated PMN was measured in the presence of the indicated concentrations of synthetic peptides. L10, based on the COOH terminus of the γ chain of Fg, inhibited binding of EC3bi and enhanced the binding of EIVa. Acetylation of the lysine group of L10 yields a peptide, AcL10, with no inhibitory activity. This experiment is representative of three separate studies.

tively inhibit the binding of Fg, C3bi (9), gp63 (8), and binding of PMN to endothelial cells (16). It thus appears that this binding site serves for several structurally similar peptide ligands. The α chain of CR3 contributes strongly to the specificity of this binding site since C3bi and gp63 are not bound by LFA-1 and p150,95, receptors that differ from CR3 in the identity of the α chain.

In contrast, the binding site for LPS on the CD18 family appears affected little by the identity of the α chain since LFA-1, CR3, and p150,95 exhibit equivalent ability to bind LPS (4). The precise structural features recognized by the LPS binding site are not known, and this site may function in recognition of other molecules in addition to LPS. For example, *H. capsulatum* is recognized by all three members of the CD18 family (5), and a component of the cell wall of this yeast may thus also be recognized by the "LPS" binding site.

The presence of two binding sites, one for peptide and a second for lipids, may be a common property of receptors related to the CD18 family. CD18 is a member of a structurally related superfamily of adhesion-promoting receptors termed Integrins (17). Members of this superfamily recognize ligands that contain the sequences Arg-Gly-Asp or Lys-x-x-Gly-Asp. For example, the vitronectin receptor recognizes the triplet Arg-Gly-Asp in vitronectin (18), and gpIIb/IIIa of platelets recognizes both a sequence in fibronectin containing Arg-Gly-Asp and a sequence in fibrinogen containing the sequence Lys-Gln-Ala-Gly-Asp (19). The vitronectin receptor exhibits an additional binding site that recognizes ganglioside GD2 (20). Gangliosides are structurally similar to LPS in that both species are amphipathic with a strongly anionic hydrophilic group. The binding site on CD18 for LPS may thus be the evolutionary homologue of the binding site on the vitronectin receptor for GD2. It is not known whether the vitronectin receptor can bind bacterial LPS.

The ability of a single receptor to recognize two distinct ligands is not unique to the integrins. The insulin-like growth factor II receptor binds both insulin-like growth factor II and mannose-6-phosphate (21), and the bacterial membrane protein Tar mediates independent chemotactic responses to aspartate and maltose via distinct binding sites (22). In the case of insulin-like growth factor II, binding of the two ligands is cooperative. While the precise biological significance of multifaceted receptors remains unclear, this property may allow the sensitivity of cells to an individual ligand to be controlled by a second or regulatory ligand.

Summary

Polymorphonuclear leukocytes (PMN) from three patients deficient in the CD18 family of receptors (LFA-1, CR3, and p150,95) exhibited an inability to bind erythrocytes coated with C3bi or bacterial LPS. These observations confirm that the CD18 family, and CR3 in particular, can bind the structurally dissimilar molecules C3bi and LPS. Further studies showed that LPS and C3bi bind to CR3 at distinct sites. mAb OKM10 against CR3 blocked binding of C3bi to PMN but did not block the binding of LPS. In contrast, mAb 904, directed against a different epitope on CR3, blocked binding of LPS to PMN but not binding of C3bi, thus suggesting that different regions of CR3 were involved in binding these two ligands. In addition, synthetic peptides based on the sequence in C3bi recognized by CR3 competitively blocked the binding of C3bi to CR3 but did not block the binding of LPS. Rather, occupation of the peptide binding site on CR3 by the synthetic peptides enhanced binding

of LPS. These results indicate that CR3 has two distinct binding sites, one that recognizes ligands composed of protein and a second that recognizes LPS.

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