12. Seed, B., and Aruffo, A. *Proc. nat. Acad. Sci., USA* 84, 3365 (1987).
13. Kishimoto, T. K., Hollander, N., Roberts, T. M., Anderson, D. C., and Springer, T. A. *Cell* 50, 193 (1987).
14. Hogarth, P. M., Walker, I. D., McKenzie, I. F. C., and Springer, T. A. *Proc. nat. Acad. Sci., USA* 82, 526 (1985).
15. Pischel, K. D., Marlin, S. D., Springer, T. A., Woods, V. L. Jr, and Bluestein, H. G. *J. clin. Invest.* 79, 1607 (1987).
16. Dahms, N. M., and Hart, G. W. *J. Immunol.* 134, 3978 (1985).

N1.9 Differential effects on leucocyte functions of CD11a, CD11b, and CD18 mAb

MICHAEL S. DIAMOND, S. CLAIBORNE JOHNSTON, MICHAEL L. DUSTIN, PETER MCCAFFERY, and TIMOTHY A. SPRINGER

Mac-1 (CD11b/CD18), LFA-1 (CD11a/CD18), and p150,95 (CD11c/CD18) constitute a family of functionally important leucocyte integrins that share a common β-subunit (M_r, 95 000) and participate in cellular adhesion [1, 2]. Mac-1 (α-subunit, M_r 165 000) is expressed basally on granulocytes, monocytes, and NK-cells. A second intracellular, vesicular pool is mobilized to the surface in granulocytes and monocytes by inflammatory mediators (fMLP, C5a, phorbol esters). Mac-1 is known to bind soluble ligands (iC3b, clotting factor X, fibrinogen) [1, 3–5] and to promote a series of neutrophil functions including adherence to endothelial cells, margination through high endothelial venules, homotypic aggregation, and chemotaxis [6]. LFA-1 (α-subunit, M_r 170 000) is expressed on B- and T-lymphocytes, NK-cells, monocytes, and granulocytes. mAb to LFA-1 block cytolytic T-cell-mediated killing and NK-mediated killing by inhibiting adhesion to target cells, and block T helper cell responses by preventing cell–cell contact. Ligands for LFA-1 have recently been characterized and cloned and include ICAM-1 (CD54) [7, 8] and ICAM-2 [9]. p150,95 (α-subunit, M_r 150 000) is expressed on phagocytic cells, NK-cells, certain cytolytic T-cells, and $CD5^-$ B-cells [6]. The role of p150,95 is less clearly defined, although mAb inhibit conjugate formation between cytotoxic T-cells and target cells as well as some neutrophil and monocyte adherence functions [10–12]. Recently, both rosetting experiments [13] and affinity chromatography [14] have suggested iC3b as a ligand for p150,95.

In this study, we have characterized functionally the Workshop antibodies directed to the CD11a, CD11b, and CD18 antigens for their ability to inhibit homotypic cell aggregation, to block the binding of T-lymphoma or B-lymphoblastoid cells to immunoaffinity-purified, solid-phase Mac-1 and LFA-1, and to decrease the binding of ^{125}I-labelled Mac-1 to iC3b-coated sheep erythrocytes (iC3b-E). The specificity of Workshop antibodies for CD11a, CD11b, and CD18 was defined elsewhere by their reactivity with COS cells transfected with CD11a/CD18, CD11b/CD18, and CD11c/CD18 cDNA clones [Larson *et al.*, N1.8].

Since Mac-1 is known to bind to a multiplicity of soluble and cellular ligands, we hypothesized that, by testing the Workshop CD11b and CD18 panels, we might find particular antibodies that block restricted functions; such a trend might suggest the presence of multiple functional domains on Mac-1 that are critical for ligand specificity. A similar technique has been utilized with five other CD11b antibodies to distinguish two functional domains, one responsible for iC3b-E binding and a second for neutrophil spreading on plastic and neutrophil chemotaxis [15, 16]. For our studies three separate assays were selected: a binding assay between purified ^{125}I-Mac-1 and iC3b-E, a Mac-1-dependent neutrophil homotypic cell–cell aggregation assay, and a ^{51}Cr-SKW3 T-lymphoma cell binding assay to solid-phase purified Mac-1. In the latter two assays, Mac-1 interacts with undefined ligands on neutrophils and T-cells.

In the homotypic neutrophil aggregation assay (Table 1), the data demonstrate that, in general, the CD18 β-chain Workshop antibodies are better blockers of phorbol-ester-induced aggregation than the CD11b panel. Six of the eight CD18 antibodies tested inhibit neutrophil aggregation by at least 50 per cent and four of the eight inhibit it by more than 75 per cent; furthermore, other non-Workshop β-chain antibodies (TS1/18, 60.3) have been described as inhibitors of neutrophil aggregation. In contrast, only one of six α-chain antibodies, N301

Table 1. Summary of the blocking of Mac-1 dependent functional assays with Workshop antibodies

Workshop no.	mAb name	SKW3 T lymphoma cell binding to Mac-1 (%±SD)	Neutrophil aggregation (%±SD)	^{125}I-Mac-1 binding to iC3b-E (%±SD)
CD11b				
N301	LPM19C	0.2±1.7	3.7 (ND)	21.9±0.7
N302	1±B6.E2	3.7±1.1	70.8±7.0	8.8±0.7
N303	5A4.C5	0.3±0.1	89.6±2.0	12.4±1.2
N305	MN41	1.4±1.0	91.1±1.1	10.9±0.7
N307	TMG6-5	1.3±1.1	77.8±1.0	10.4±0.2
N308	VIM12	37.9±2.4	94.8±2.0	46.6±2.7
CD18				
N207	M232	18.3±1.1	9.7±1.9	11.1±1.5
N214	IC11	61.6 (ND)	63.9 (ND)	91.8 (ND)
N216	MHM23	33.3±10.6	42.9±28.0	12.8±0.7
N218	CLB-54	17.5±3.8	7.0±0.4	83.7±8.6
N219	YFC51.1	12.2±1.2	10.7±2.4	68.9±3.5
N221	YFC118.3	30.8±3.8	24.7 (ND)	105.5±4.5
N229	GRF1	27.9±13.2	52.6±36.5	24.1±0.7
N306	MJ5/1	7.6±3.2	90.8±1.7	19.5±2.6
Control mAb				
N304	MO1 (CD14)	47.7±19.3	89.6±1.2	118.7±4.8
	OKM1 (CD11b)	36.7 (ND)	—	76.9 (ND)
	OKM9 (CD11b)	35.1±7.0	—	—
	OKM10 (CD11b)	30.3 (ND)	—	—
	LM2/1 (CD11b)	34.0 (ND)	95.2±0.6	100.0±8.9
	60.3 (CD18)	—	—	−1.6±0.4
	TS1/18 (CD18)	29.7±8.1	46.4±16.8	—
	TS2/18 (CD2)	35.2±9.4	—	136.9±20.7

Mac-1 is purified from granulocyte lysates by immunoaffinity chromatography [18; Diamond and Springer, in preparation]. Purified Mac-1 is plated on to 96-well microtitre plates (Linbro-Titertek) for 60 min at room temperature. Plates are washed thrice and non-specific sites are blocked (HBSS, 10 mM Hepes pH 7.3, 0.5 per cent human serum albumin (HSA), 2 mM $MgCl_2$, 0.5 mM $MnCl_2$) for 2 h at room temperature, and then pre-incubated with a 1/400 final mAb concentration for 30 min at 4°C. The methods for performing and quantitating the cell binding assay have been described [19]. Results are expressed as the percentage of ^{51}Cr–SKW3 T-lymphoma cells bound relative to the input. Background cell binding to plates (HSA alone) routinely averages about 5 per cent. For the T lymphoma binding assay, SD represents the standard deviation of two separate experiments performed in triplicate.

The quantitative neutrophil homotypic aggregation assay is performed as described previously [20] using 40 ng/ml phorbol dibutyrate (PDB). The percentage aggregation is defined as:

$$\left(1-\frac{\text{no. of free cells}}{\text{total no. of cells}}\right)\times 100.$$

Sheep erythrocytes sensitized with iC3b (iC3b-E) are prepared as before [21] except that human C5-deficient serum is substituted for murine A.J C5-deficient serum. ^{125}I-Mac-1 is prepared by labelling immunoaffinity-purified Mac-1 by the Iodogen method. The assay is performed in V-bottom 96-well microtitre plates that are pre-blocked with HBSS, 10 mM Hepes pH 7.3, 0.5 per cent HSA (HHHSA). For each experiment ^{125}I-Mac-1 is pre-incubated with a 1/400 final dilution of each mAb in the HHHSA, 1 mM Mn^{2+} for 30 min at 4°C. Subsequently, 50 μl of 2×10^8 iC3b-E is added (in HHHSA), and the mixture is shaken gently at 37°C for 20 min. The mixture is pelleted and washed four times in HHHSA, 1 mM Mn^{2+}; the cells are lysed with 100 μl of 100 mM NaOH; the solutions are collected and counted for gamma emission. Quantitation is expressed as the percentage of ^{125}I-Mac-1 bound relative to a non-blocking binding control (LM2/1). Background is determined by binding to IgM-E (M1/87) and subtracted; it averages between 5 and 10 per cent of the signal. For the iC3b-E assay, SD represents the standard deviation of three separate experiments performed in triplicate. ND in all experiments represents points that were determined in only one series of experiments performed in triplicate.

(LPM19C), completely blocks aggregation, while two other antibodies, N302 (14B6.E2) and N307 (TMG6-5), consistently give partial inhibition in our assay.

The SKW3 T lymphoma cell binding assay provides an antibody inhibition profile that is quite distinct from the neutrophil aggregation assay (Table 1). CD11b α-chain antibodies are significantly better blockers of cell binding to solid-phase purified Mac-1. In this assay, five of the six CD11b antibodies blocked binding by more than 90 per cent. Surprisingly, all non-Workshop CD11b antibodies tested were non-blocking; LM2/1, OKM1, OKM9, and OKM10 did not decrease SKW3 cell binding. In addition, no CD18 β-chain antibody blocked as strongly as any of the α-chain antibodies, although three of seven antibodies tested lowered binding by more than 50 per cent.

The iC3b-E binding assay (Table 1) shows an antibody inhibition pattern that is similar to T lymphoma binding for CD11b antibodies, as five of six antibodies block strongly. The similarity of CD18 antibody inhibition between SKW3 and iC3b-E is significantly weaker; only four of eight antibodies show qualitatively similar blocking potential.

Because the data is derived exclusively from antibody inhibition assays it is not possible to speculate on the specific location of functional domains on Mac-1 for each of its independent ligand-binding activities; such information awaits the evaluation of genetic chimera and deletion mutants in functional mAb binding assays. However, certain trends are apparent from the antibody blocking data.

1. Only one antibody, N301 (LPM19C) is able to block all three functions significantly; this antibody must bind either to a critical site for ligand binding activity or attach in such a way that it thoroughly disrupts the tertiary structure of Mac-1 and prevents multiple ligands from binding.

2. CD18 β-chain antibodies appear to inhibit cell–cell aggregation more consistently while CD11b α-chain antibodies are more effective for inhibiting T-lymphoma cell binding to solid-phase Mac-1. This disparity suggests the presence of a unique Mac-1 ligand on T-cells although, as yet, no cell–cell functional studies have demonstrated a role for a Mac-1-dependent T-cell–monocyte or T-cell–granulocyte adhesion. Previous studies [3] have suggested that Mac-1 possesses distinct ligand binding sites for iC3b or fibrinogen and the neutrophil aggregation ligand because iC3b and fibrinogen binding to phorbol-ester-stimulated neutrophils is inhibited by arginine–glycine–aspartic acid (RGD) peptides while neutrophil aggregation is not. Our antibody blocking data support this since iC3b-E binding and neutrophil aggregation are inhibited by a different pattern of CD11b antibodies. However, the antibody-blocking patterns of iC3b-E binding or SKW3 binding show similarities as the same five CD11b antibodies inhibit by more than 75 per cent; we hypothesize that the putative T-cell ligand binds to a region on Mac-1 that is either adjacent or overlapping with the iC3b or fibrinogen-binding domain, and is distinct from the binding site of the neutrophil aggregation ligand.

Alternatively, the common feature between binding of SKW3 and iC3b-E may be the use of purified Mac-1; mechanisms of mAb blockade, such as effects on conformation or cell-surface distribution, may differ for purified and cell-surface molecules.

3. Antibody N308 (VIM12) binds to a distinct epitope on Mac-1 that is removed from the site of most functional ligand interactions. This antibody is unable to inhibit SKW3 or neutrophil-dependent Mac-1 binding and only weakly inhibits iC3b-E binding.

The Workshop CD11a and CD18 antibodies were evaluated for their ability to inhibit LFA-1-dependent adhesive functions. Phorbol ester treatment prompts JY cells to aggregate homotypically by an LFA-1 and ICAM-1 dependent mechanism [17] and promotes SKW3 cells to aggregate by an LFA-1-dependent, ICAM-1-independent mechanism. Recently, this laboratory has cloned and expressed a novel adhesion protein, ICAM-2, which is highly homologous to ICAM-1 and may be responsible for LFA-1-mediated SKW3 aggregation [9].

The blocking studies of LFA-1 dependent aggregation and cell binding to solid-phase LFA-1 provide some interesting results (Table 2).

1. CD18 β-chain antibodies block cell–cell adhesion more strongly than the CD11a antibodies. Five of seven CD18 antibodies strongly inhibit SKW3 aggregation and four of seven antibodies potently block JY aggregation. In contrast, only five of 23 CD11a antibodies strongly block SKW3 or JY aggregation. This situation parallels the case for Mac-1 dependent cell aggregation where CD18 antibodies blocked to a greater degree than CD11b antibodies.

2. Antibodies that are able to block LFA-1-mediated JY aggregation, in general, are also able to block LFA-1-mediated SKW3 aggregation. There are seven cases where either a CD11a or CD18 antibody blocks both JY and SKW3 aggregation strongly, five cases where an antibody which strongly blocks one type cell aggregation inhibits the other partially, and no cases where strongly inhibiting antibodies of JY aggregation do not correlate with inhibition of SKW3 aggregation. These observations are not that surprising if we hypothesize that SKW3 aggregation depends on ICAM-2; we know from sequence data [9] that ICAM-1 and ICAM-2 are 35 per cent homologous and so it is possible that ICAM-1 and ICAM-2 bind LFA-1 in a single or spatially overlapping domain.

Table 2. LFA-1 functional blocking studies

Workshop no.	mAb name	Homotypic aggregation		JY cell binding to LFA-1 (%±SD)
		SKW3	JY	
—	No antibody	+	++	51.0±2.7
—	W6/32 (HLA-A)	+	++	42.2±7.4
N205	BU-49 (non-CD11a)	+	++	42.5±13.1
N206	GRT22 (non-CD11a)	+	++	69.9±5.3
CD11a				
N201	11H6	+	++	52.3±2.3
N202	CRIS-3	+	++	49.5±0.6
N203	122-2A5	−/+	++	43.2±15.6
N204	BU-17	+	++	59.3±0.4
N208	0501	+	++	60.3±1.7
N209	MEM-25	−	−/+	14.4±10.4
N210	MEM-30	−/+	++	49.4±12.2
N211	MEM-83	+	++	44.6±15.7
N212	MEM-95	−	−	2.8±1.0
N213	25.3.1	−/+	−/+	28.3±19.8
N215	459	+	++	33.6±0.4
N217	MHM24	−/+	−	33.1±10.3
N220	YTH 81.5	+	++	39.0±8.9
N222	1524	−	−/+	14.1±3.8
N223	2F12	−/+	−	4.9±2.7
N224	F110.22	−/+	++	34.3±2.3
N225	TMD-1	−	−	21.0±18.5
N226	ITM3-2	+	−/+	65.8±1.0
N227	CC5 1D7	−	−	6.3±3.4
N228	VIPIIIB1	+	++	75.6±22.2
N230	GRS3	−/+	++	33.7±22.1
N231	HI111	−/+	++	5.7±2.7
N232	M10	+	++	41.5±8.8
CD18				
N207	M232	−	−	19.9±12.3
N214	IC11	−	−	24.6±9.1
N216	MHM23	−	−/+	9.0±3.0
N218	CLB-54	−	−	29.0±10.6
N219	YFC51.1	−	−	24.3±9.2
N221	YFC118.3	−/+	++	39.5±16.1
N229	GRF1	+	++	32.2±3.5

The qualitative aggregation assay is performed as previously described [7]. SKW3 T-lymphoma and JY B-lymphoblastoid cells are stimulated with 50 ng/ml of phorbol-12-myristate acetate. The assay is scored as follows. SKW3 (after 5 h): −, no aggregates; −/+, few aggregates; +, many aggregates. JY (after 1 h): −, no aggregates; −/+, few aggregates; + +, large clumping aggregates. The cell binding assay will be described in greater detail [Dustin and Springer, in preparation]. Briefly, immunoaffinity-purified LFA-1 is plated on to plastic microtitre plates. The plates are non-specifically blocked with 1 per cent BSA and then pre-incubated with a 1/400 final dilution of the Workshop antibodies. ^{51}Cr-labelled JY cells are centrifuged (10 g for 5 min) on to LFA-1 coated plates and incubated for 10 min at 37°C. The cells are washed four times, removed with EDTA, and quantitated by gamma emission. Results are expressed as the percentage of ^{51}Cr-JY cells bound relative to the input. The background binding to BSA is 3 per cent. Binding experiments were performed twice in quadruplicate.

3. The antibody blocking of JY binding to purified LFA-1 does not correlate completely with the inhibition of aggregation. Only five of 33 antibodies inhibited binding by more than 75 per cent. A noticeable change in the blocking character of the CD18 β-chain antibodies is observed; while four of seven β-chain antibodies strongly block homotypic cell aggregation, only two of seven are able to decrease JY binding to solid-phase LFA-1 by more than 50 per cent. It is possible that certain CD18 β-chain antibodies, by binding, induce a conformational change on the membrane of cells but are unable to alter solid-phase protein; this may explain why these antibodies block LFA-1 dependent cell–cell interactions better than binding to solid-phase LFA-1.

References

1. Sanchez-Madrid, F., Nagy, J., Robbins, E., Simon, P., and Springer T. A. *J. exp. Med.* 158, 1785 (1983).
2. Hynes R. O., *Cell* 48, 549 (1987).
3. Wright, S. D., Weitz, J. I., Huang, A. J., Levin, S. M., Silverstein, S. C., and Loike, J. D. *Proc. nat. Acad. Sci., USA* 85, 7734 (1988).
4. Altieri, D. C., and Edgington, T. S. *J. Biol. Chem.* 263, 7007 (1988).
5. Altieri, D. C., Bader, R., Mannucci, P. M., and Edgington, T. S. *J. cell. Biol.* 107, 1893 (1988).
6. Kishimoto, T. K., Larson, R. S., Corbi, A. L., Dustin, M. L., Staunton, D. E., and Springer, T. A. *Adv. Immunol.* 46, 149 (1989).
7. Staunton, D. E., Marlin, D. S., Stratowa, C., Dustin, M. L., and Springer, T. A. *Cell* 52, 925 (1988).
8. Simmons, D., Makgoba, M. W., and Seed, B. *Nature* 331, 624 (1988).
9. Staunton, D. E., Dustin, M. L., and Springer, T. A. *Nature* 339, 61 (1989).
10. Pholman, T. H., Stanness, K. A., Beatty, P. G., Ochs, H. D., and Harlan, H. M. *J. Immunol.* 136, 4548 (1986).
11. Miller, L. J., Bainton, D. F., Borregaard, N., and Springer, T. A. *J. clin. Invest.* 80, 535 (1987).
12. Keizer, G. D., Borst, J., Visser, W., Schwarting, R., de Vries, J. E., and Figdor, C. G., *J. Immunol.* 138, 3130 (1987).
13. Myones, B. L., Dalzell, J. G., Hogg, N., and Ross, G. D. *J. clin. Invest.* 82, 640 (1988).
14. Micklem, K. J. and Sim, R. B. *Biochem. J.* 231, 233 (1985).
15. Dana, N., Styrt, B., Griffin, J., Todd, R. F. III, Klempner, M., and Aranout, M. A. *J. Immunol.* 137, 3259 (1986).
16. Anderson, D. C., Miller, L. J., Schmalstieg, F. C., Rothlein, R., and Springer, T. A. *J. Immunol.* 137, 15 (1986).
17. Rothlein, R., Dustin, M. L., Marlin, S. D., and Springer, T. A. *J. Immunol.* 137, 1270 (1986).
18. Miller, L. J., Weibe, M., and Springer T. A. *J. Immunol.* 138, 2381 (1987).
19. Haskard, D., Cavender, D., Beatty, P., Springer, T., and Ziff, M. *J. Immunol.* 137, 2901 (1986).
20. Patarroyo, M., Beatty, P. G., Serhan, C. N., and Gahmberg, C. G. *Scand. J. Immunol.* 22, 619 (1985).
21. Rothlein, R. and Springer, T. A. *J. Immunol.* 135, 2668 (1985).

N2 Cluster report: CD16

REINHOLD E. SCHMIDT and BICE PERUSSIA

The CD16 molecule has been described as the low-affinity Fc receptor (FcγRIII) for complexed IgG [1]. It is expressed on NK-cells, granulocytes (PMN), and macrophages. Biochemically, CD16 previously was detected on all cells as a 50–70 kDa band in SDS–gel electrophoresis. More recently Lanier has described structural differences in the CD16 glycoprotein from granulocytes and NK-cells [2]. Endo-F and N-glycanase treatment resulted in a 29–30 kDa polypeptide from granulocytes. In contrast, after identical treatment, a complex pattern of peptides with two predominant bands at 36 and 38 kDa was precipitated from NK-cells. Before this Workshop polymorphic CD16 mAb had been described only as directed against the NA1 molecule on granulocytes.

Simmons and Seed have reported a cDNA clone encoding CD16 determinants [3]. They demonstrated that the CD16 gene encoded for a phospholipid-anchored protein. Messenger RNA transcripts of CD16 gene were detected in granulocytes and NK-cells as well. The gene was confirmed by Peltz *et al.* and mapped to chromosome 1 by spot-blot analysis of sorted chromosomes [4]. Other groups have described a defect in CD16 expression on granulocytes of patients with paroxysmal nocturnal haemoglobinuria (PNH) [5, 6].

The CD16 molecule represents the functional receptor structure for performing antibody-dependent cellular cytotoxicity (ADCC). A number of recent studies, therefore, have focused on functional aspects of this molecule in cell activation. Several groups have described the induction of cytotoxicity by activating NK-cells, granulocytes,