Differential function of LFA-1 family molecules (CD11 and CD18) in adhesion of human monocytes to melanoma and endothelial cells

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SUMMARY

Human peripheral blood monocytes from normal, healthy donors express the leucocyte function-associated antigen (LFA)-1, CR3 and p150,95. These heterodimeric antigens are members of a glycoprotein family sharing a common beta subunit but endowed with distinct alpha chains. They have been shown to play an important role in cell–cell interactions. In the present study we have investigated the role of these molecules in the interaction of monocytes with endothelial cells and melanoma (tumour) cells. Heterotypic cell–cell interactions were studied in single cell conjugate assays and by adhesion of monocytes to monolayers of cells. The results demonstrate that monoclonal antibodies directed against LFA-1 alpha, CR3 alpha, p150,95 alpha and the common beta chain strongly reduce the number of conjugates (71, 50, 60 and 89% inhibition, respectively), formed between monocytes and melanoma or endothelial cells in a single cell assay. In contrast, adhesion of monocytes to monolayers of the same cells seems only to depend on p150,95, since only antibodies directed to the alpha chain of this molecule and to the common beta chain inhibited adhesion. Interestingly, the number of conjugates formed with melanoma cells in single cell assays was at least twice the number of conjugates formed between monocytes and endothelial cells, whereas no differences were observed in the adhesion of monocytes to monolayers of these cells. However, the basis for this phenomenon is not yet clear. These results indicate that not only LFA-1 but also CR3 and p150,95 can mediate adhesion to target cells in suspension, but that monocyte adhesion to monolayers is caused by a different mechanism in which the p150,95 molecule seems to play a prominent role.

INTRODUCTION

One of the molecules involved in the non-specific adhesion of target cells to lymphocytic cytotoxic effector cells (Springer *et al.*, 1982; Krensky *et al.*, 1984; Spits *et al.*, 1986) is the leucocyte function-associated antigen (LFA)-1. The LFA-1 molecule is crucial for lytic interaction between cytotoxic T cells (CTL) or natural killer cells and their respective targets (Krensky *et al.*, 1984; Schmidt *et al.*, 1985). It is widely distributed and expressed by T and B lymphocytes, null cells, monocytes and granulocytes, and is composed of two non-covalently associated molecules, an alpha chain of 170,000 MW and a beta chain of 95,000 MW. LFA-1 is structurally related to the antigens CR3

Abbreviations: ACD, acid citrate dextrose; BSA, bovine serum albumin; CTL, cytotoxic T lymphocyte; DMEM, Dulbecco's modification of Eagles minimal essential medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAM, goat anti-mouse; LFA-1, leucocyte function-associated antigen-1; PBS, phosphate-buffered saline.

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and p150,95 cell surface molecules, since they share the same beta chain (95,000 MW, CD18) but have distinct alpha chains (170,000, 165,000 and 150,000 MW, respectively for LFA-1, CR3 and p150,95 (CD11)) (Sanchez-Madrid *et al.*, 1983; Keizer *et al.*, 1985). Both CR3 and p150,95 are expressed by monocytes and granulocytes but are generally absent on T and B lymphocytes (Todd *et al.*, 1982; Lainer *et al.*, 1985).

The role of LFA-1 in CTL interaction is well documented but much less is known about the function of the LFA-1 family molecules in monocyte cell–cell interactions. This prompted us to study the role of the LFA-1 family molecules in the interaction of monocytes to two cell types that are involved in important adhesion phenomena: melanoma cells (monocytes as potential cytotoxic effector cells) and endothelial cells (physiological blood cell adhesion). Recent findings indicate that the LFA-1 family molecules mediate adhesion and migration of human monocytes (G. D. Keizer *et al.*, submitted for publication). In the present study we demonstrate that on human monocytes not only LFA-1 but also CR3 and p150,95 play an important role in the adhesion to melanoma cells and endothelial cells.

MATERIALS AND METHODS

Monocyte isolation

Highly purified peripheral blood monocytes were isolated from blood of normal human donors as described previously (Figdor *et al.*, 1982, 1984). Briefly, the mononuclear cells of a buffy coat, prepared from 500 ml blood containing acid citrate dextrose (ACD), obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) were isolated by density centrifugation with a blood component separator and subsequently fractionated to monocytes and lymphocytes by centrifugal elutriation. The monocyte preparation was over 95% pure (non-specific esterase and May Grunwald Giemsa staining) and the viability of the cells was always more than 95% as judged by means of trypan blue exclusion.

Cultured cells

Melanoma cell lines established in our laboratory as described previously (De Vries, Rümke & Bernheim, 1972) were grown in Dulbecco's modification of Eagles minimal essential medium (DMEM) supplemented with streptomycin (50 µg/ml), penicillin (100 IU/ml), NaH-CO$_3$ (3·7 g/l), glutamine (2 mM) and 10% fetal calf serum (FCS; Gibco, Paisley, Renfrewshire, U.K.). Human endothelial cells, isolated from human umbilical cord veins, were obtained from Dr J. H. Reinders from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). These cells were cultured in RPMI-1640 medium (Flow Laboratories, Irvine, Ayrshire, U.K.), supplemented with 20% human serum, penicillin, streptomycin, fungizone and 2 mM glutamine.

Monoclonal antibodies

The following monoclonal antibodies were used. SPV-L7 recognizes the alpha chain of LFA-1. It precipitates a 170,000/95,000 MW complex and was obtained as described previously (Spits *et al.*, 1983). Bear-1 was found to be similar to anti-CR3 and OKM-1 (Todd *et al.*, 1982; Keizer *et al.*, 1985) and precipitated a 165,000/95,000 MW complex that is found to be associated with C3bi receptor function (Beller, Springer & Schreiber, 1982). S-HCl 3, kindly provided by Dr R. Schwarting (Schwarting, Stein & Wang, 1985) precipitated a 150,000/95,000 molecule. CLB54 is directed against the beta chain of LFA-1, CR3 and p150,95 (Miedema *et al.*, 1984). Other monoclonal antibodies used were Bear-2, which reacts with a monocyte-specific differentiation antigen with a MW of 55,000 and is similar to Mo2 (Todd *et al.*, 1982) (data not shown). This antibody was used as a control for positive antibody binding. CEM III 2.1.1 specific for keyhole limpet haemocyanin was used as a control antibody for negative antibody binding. In addition, two melanoma-specific antibodies were used, AMF-6 and AMF-7 (De Vries *et al.*, 1986). These antibodies were selected for their capacity to inhibit the reaction of cytotoxic T-lymphocyte clones that preferentially lysed human melanoma cells. AMF-6 (225,000/ > 450,000 MW) reacts with a high MW melanoma-associated proteoglycan. AMF-7 (150,000/90,000) detects a melanoma-associated surface membrane antigen that is involved in the adhesion and spreading of melanoma cells. This antigen was termed 'melanoma-associated cellular adhesion molecule-1' (MACAM-1) (De Vries *et al.*, 1986). The isotype of the test and control monoclonal antibodies are of the

IgG1 subclass, except S-HCl 3 antibody, which is of the IgG2b isotype. The reaction pattern of a control antibody of the IgG2b isotype did not differ from that of the IgG1 control antibodies (results not shown).

Membrane immunofluorescence

Adherent cells were detached with EDTA (5 mM), resuspended in DMEM supplemented with 10% FCS and washed with PBS containing BSA (0·2%) and azide (0·02%). Cells (1–3 × 10^5) were incubated for 30 min at 0° with the various monoclonal antibodies (1:1000 ascites dilution). After this incubation period the cells were washed twice with PBS (+ BSA + azide) and incubated with FITC-labelled GAM F(ab)$_2$ (1:30 diluted, Nordic, Tilburg, The Netherlands) for 30 min at 0°. Finally, 0·5 ml PBS (+ BSA + azide) was added and the relative fluorescence intensity of the cells was analysed with a FACS (FACS IV, Becton-Dickinson, Mountain View, CA).

Monocyte conjugate assay in suspension

A modification of the method of Bonavida, Bradley & Grimm (1983) was used to determine conjugate formation on a single cell level. Freshly isolated human peripheral blood monoytes were used as effector cells in binding to melanoma cell lines, human endothelial cells, lymphocytes and erythrocytes. After washing twice with PBS the monocytes were labelled with carboxy fluorescein diacetate (CFDA) to distinguish them from the target cells under the fluorescence microscope. Monocytes and target cells were incubated for 30 min with or without antibodies in a 1:1 ratio in medium with or without 10% human serum at 30°; subsequently the cell suspension was centrifuged for 5 min at 200 *g* to establish conjugate formation. The pellet was resuspended in 1% molten agarose (1:5 ratio of type I and VII agarose, Sigma, St Louis, MO) and plated onto slides. After fixation in 0·04% formaldehyde in PBS the percentage conjugates was determined. The percentage of conjugates was determined by:

$$\frac{\text{number of bound monocytes to target}}{\text{total number of monocytes}} \times 100.$$

Adhesion of monocytes to monolayers

The capacity of monocytes to adhere to various substrates was determined by means of an ELISA (Keizer, Figdor & De Vries, 1986). Monocytes were labelled with anti-monocyte antibodies coupled to peroxidase by incubation at 4° for 45 min. It was demonstrated that these anti-monocyte antibodies did not affect the adherent proportion of the monocytes. Subsequently the cells were washed twice in PBS and resuspended in serum-free medium, Linolea (Yssel *et al.*, 1984). Cells (5 × 10^4) were then incubated with or without antibodies for 30 min at 37°, 5% CO$_2$ and 100% humidity in a 96-well tissue culture plate (Costar 3596, Cambridge, U.K.) that contained a monolayer of target cells. After the incubation period the non-adhering monocytes were removed by washing with PBS. The number of attached peroxidase-labelled cells was determined by adding substrate and photospectrometric determination of the staining in a one-step ELISA. The staining correlated directly with the number of adherent cells as was determined by a standard calibration curve or by counting the number of non-adherent cells (not shown). The values obtained with cells incubated in the absence of antibodies was set at 100%.

RESULTS

Expression of LFA-1, CR3 and p150,95 and melanoma-associated antigens by monocytes, melanoma and endothelial cells

In order to show that LFA-1, CR3 and p150,95 are only expressed on monocytes and not on melanoma and endothelial cells we incubated human monocytes, melanoma cells (BK-mel) and endothelial cells with various antibodies. The results in Table 1 show that LFA-1, CR3 and p150,95 are present on the monocytes, and that these molecules are not expressed by these melanoma cells and endothelial cells. In accordance with previous data (De Vries *et al.*, 1986) the antibodies AMF-6 and AMF-7 recognized melanoma-associated antigens, which are not expressed on monocytes and endothelial cells, except AMF-7 which showed cross-reactivity with the cultured endothelial cells.

Requirements for conjugate formation in suspension

In order to determine the conditions necessary for conjugate formation between monocytes and other cells we investigated the temperature dependence and the requirement for calcium and/or magnesium ions in monocyte conjugate formation. In Table 2 it is demonstrated that only minimal binding occurs at temperatures of 0 compared to 30, indicating that the conjugate formation between monocytes and melanoma cells or endothelial cells is temperature dependent. Furthermore, conjugate formation was found to be Mg^{2+} dependent, whereas Ca^{2+} was not required.

Time–course of conjugate formation in suspension

When monocytes are incubated with melanoma or endothelial cells striking differences in the degree of binding can be

Table 1. Expression of LFA-1, CR3 and p150,95 and melanoma-associated antigens on monocytes, melanoma cells and endothelial cells*

Monoclonal antibody	Antigen recognized	Monocytes	BK melanoma cells	Endothelial cells
Neg. control	KLH	(0)†	1 (0)	1 (0)
SPV-L7	LFA-1 (α chain)	13 (95)	1 (2)	1 (5)
Bear-1	Mol (α chain)	21 (94)	1 (4)	1 (6)
S-HCl 3	p150,95 (α chain)	9 (90)	1 (3)	1 (2)
CLB54	LFA-1, Mol, p150,95 (β chain)	24 (94)	1 (4)	1 (6)
Bear-2	Mo2	8 (87)	1 (3)	1 (4)
AMF-6	HMW antigen	1 (1)	5 (84)	1 (8)
AMF-7	MACAM-1	1 (1)	8 (93)	3 (49)

* Cells were labelled with monoclonal antibody and GAM F(ab)₂ FITC directly after separation (monocytes) or after culture for 1 week (endothelial cells) or prolonged culture (melanoma cells).

† Data are expressed as mean relative fluorescence index and mean percentage positive cells (in parentheses). Similar results were obtained in two other experiments.

Table 2. Requirements for conjugate formation of monocytes and melanoma or endothelial cells

		% conjugates between monocytes and:			
		BK melanoma cells		Endothelial cells	
Temperature of conjugate formation	Human serum, Mg^{2+} or Ca^{2+} added	Donor 1	Donor 2	Donor 1	Donor 2
30	Human serum	41	25	27	13
0	Human serum	5	4	3	2
30	—	9	3	4	2
30	Mg^{2+} and Ca^{2+}	39	20	22	13
30	Mg^{2+}	41	20	25	14
30	Ca^{2+}	10	6	6	4

Conjugate formation was performed as described in the Materials and Methods at 30 and 0. Mg^{2+} and Ca^{2+} were added in a concentration of 0·0025% w/v and 0·005% w/v, respectively. Data shown are the percentage fluorescent monocytes in conjugates. Three-hundred monocytes were counted to determine percentage conjugate formation.

Table 3. Inhibition of conjugate formation between monocytes and melanoma or endothelial cells*

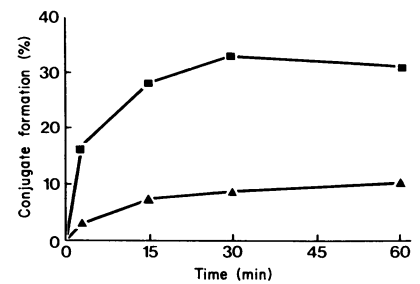| Antibodies added | % inhibition of conjugate formation† between monocytes and: | |
	Melanoma cells	Endothelial cells
None	0	0
Neg. control (anti-KLH)	10 ± 11	2 ± 2
SPV-L7	71 ± 14	64 ± 2
Bear-1	50 ± 16	52 ± 3
S-HC1 3	60 ± 11	57 ± 4
CLB54	86 ± 10	89 ± 3
Bear-2	8 ± 11	7 ± 4
AMF-6	−11 ± 20	7 ± 2
AMF-7	−26 ± 26	9 ± 5

* Conjugate formation was performed as described in the Materials and Methods. Antibodies were added in a concentration of 5–10 μg/ml.

† Data shown are the mean percentage inhibition ± SE calculated by the formula:

$$1 - \frac{\text{conjugate formation} + \text{antibodies}}{\text{conjugate formation control} \, (-\text{antibodies})} \times 100.$$

Three-hundred monocytes were counted to determine percentage conjugate formation. Five different inhibition experiments were carried out. The number of conjugates formed between monocytes and melanoma cells ranged from 25 to 41% with different monocyte preparations. With endothelial cells the maximum number of conjugates was 16%.

Figure 1. Time–course of binding of human monocytes to melanoma cells (BK-mel) (■) and endothelial cells (▲) in suspension. Assay was performed as described in the Materials and Methods. Data of one representative experiment.

tion between monocytes and melanoma cells or endothelial cells required different incubation times, a time–course of conjugate formation in suspension was performed. Figure 1 shows that cell–cell adhesion reaches equilibrium within 30 min at 30°, irrespective of the cell type to which the monocyte is bound.

Role of LFA-1, CR3 and p150,95 in conjugate formation in suspension

In order to determine which antigens of the LFA family mediate conjugate formation, experiments were carried out in which the capacity of various monoclonal antibodies to inhibit conjugate formation was studied. Antibodies that recognize LFA-1, CR3 and p150,95 inhibited monocyte–melanoma cell and monocyte–endothelial cell conjugate formation, while control antibodies did not affect this interaction (Table 3). Conjugate formation is almost completely blocked by antibodies that recognize the common beta chain of LFA-1, CR3 and p150,95. Furthermore, it is demonstrated that antibodies recognizing melanoma-associated surface antigens did not inhibit conjugate formation between monocytes and melanoma cells. In several experiments an enhanced binding of monocytes to melanoma cells was observed when the anti-melanoma antibodies were present

observed. As shown in Tables 2 and 3 the number of conjugates formed between monocytes and melanoma cells was significantly higher than that between monocytes and endothelial cells. In order to investigate whether optimal conjugate forma-

Table 4. Inhibition of conjugate formation of monocytes to melanoma or endothelial cell monolayers*

| Antibodies added | % inhibition of number of monocytes that adhere to a monolayer† | |
	Melanoma cells	Endothelial cells
None	0	0
Neg. control (anti-KLH)	1 ± 2	1 ± 1
SPV-L7	2 ± 2	1 ± 2
Bear-1	7 ± 4	7 ± 4
S-HC1 3	30 ± 4	30 ± 6
CLB54	28 ± 5	29 ± 6
Bear-2	1 ± 1	2 ± 1
AMF-6	2 ± 1	3 ± 1
AMF-7	3 ± 1	3 ± 2

* Conjugate formation was performed as described in the Materials and Methods. Antibodies were added in a concentration of 5–10 μg/ml.

† Data shown are the mean percentage inhibition ± SE calculated by the same formula as described in Table 3. Five different inhibition experiments were carried out.

during conjugate formation. This phenomenon is most probably caused by binding of the monocyte via its Fc gamma receptor to the antibody-coated melanoma cells, since monocytes of 70% of all donors have a receptor for mouse IgG1 (Tax *et al.*, 1984).

Conjugate formation between monocytes and target cells grown in monolayer

In order to quantify the proportion of monoytes that adhere to a monolayer of cells, an adhesion assay was used in which, prior to adhesion, the monocytes were labelled with an antibody conjugated to peroxidase. Such labelling does not affect the adhesive properties of the cells (Keizer *et al.*, 1986). The number of monocytes that binds to a plastic surface is almost 100%, whereas approximately 70–80% of the monocytes adhere to monolayers of melanoma or endothelial cells. In contrast to binding of monocytes and these cells in suspension, binding of monocytes to monolayers of melanoma and endothelial cells does not depend on the type of target cells used. No differences were observed between the number of monocytes that bind to a monolayer of melanoma cells compared to the number of monocytes that adhere to an endothelial cell monolayer. Inhibition studies performed with anti-LFA-1 family antibodies demonstrate that only p150,95, but not LFA-1 or CR3, is involved in the adhesion of monocytes to a monolayer of target cells (Table 4). Monoclonal antibodies that recognize the common beta chain of LFA-1, CR3 and p150,95 also markedly reduce the number of monocytes that adhere to a target cell monolayer.

Anti-melanoma antibodies AMF-6 and AMF-7 had no effect on the number of monocytes that adhere to a monolayer of melanoma cells.

In conclusion, these data indicate that antibodies against the alpha chains of LFA-1 and CR3 do not contribute to the adhesion of monocytes to a monolayer of cells, whereas the same antibodies effectively inhibit conjugate formation in a single cell assay. Apparently, different interaction mechanisms are involved in the formation of conjugates of monocytes with single cells and with monolayers of cells.

DISCUSSION

LFA-1, CR3 and p150,95 expressed on monocytes are involved in the formation of conjugates between monocytes and single melanoma or endothelial cells. In contrast, adhesion of monocytes to monolayers of cells seems only to be mediated by the alpha chain of p150,95, whereas LFA-1 and CR3 play no significant role. Furthermore, the results presented here demonstrate for the first time that different members of this family of structurally related antigens contribute to the binding of monocytes to melanoma or endothelial cells in two different assays.

Conjugate formation is shown to be a temperature-sensitive and Mg^{2+}-dependent process, which is in agreement with recent findings of Pawlowski *et al.* (1985) who studied monocyte–endothelial cell interactions. These findings with monocytes are in line with those obtained with T cells, since Spits *et al.* (1986) and others (Springer *et al.*, 1982) described that binding of CTL to specific targets also was dependent on both temperature and Mg^{2+} concentration.

LFA-1 is known to be involved in the adhesion strengthening between effector and target cells (Springer *et al.*, 1982; Krensky *et al.*, 1984; Spits *et al.*, 1986). It is most likely that LFA-1 expressed on monocytes has the same function in conjugate formation between monocytes and target cells in suspension. These results correspond with the recent findings of Strassmann *et al.* (1986) who describe participation of LFA-1 in the interaction between murine activated macrophages and neoplastic cells. Our results show that not only LFA-1, but also CR3, mediates conjugate formation between monocytes and target cells. The latter finding is in line with previous studies that already indicated that CR3 has lectin-like properties and promotes adherence between phagocytic cells and certain surfaces (Ross, Cain & Lachmann, 1985; Arnaout *et al.*, 1983). From these data it can be concluded that CR3, which is mainly expressed on monocytes and granulocytes, seems to be responsible for adhesion strengthening on phagocytic cells, similar to LFA-1. Interestingly, LFA-1 and CR3 do not participate in the adhesion of monocytes to melanoma/endothelial cells when these cells are growing in monolayers. Under these conditions, p150,95 is the only antigen involved in this interaction. These data suggest that the structures to which LFA-1 and CR3 can bind are absent or less abundant on the available surface. This notion is supported by the finding that cells growing in a monolayer show a reorganization of cellular structures when they adhere to a substrate. Molecules required for adhesion move to the interface substrate/cell surface (Chen & Singer, 1982). Our data are in accordance with the results reported by Harlan *et al.* (1985) and others (Wallis *et al.*, 1985) who suggest that a p150,95-like antigen is responsible for adherence of neutrophils to plastic surfaces and monolayers of cells. Recently, Anderson *et al.* (1986) described that CR3 mediates adherence of granulocytes to a serum-coated surface. Since we do not use the same antibodies directed to CR3, it is possible that our antibody recognizes a different epitope that is not involved in monocyte adhesion to a plastic or cell surface, but plays a role in monocyte cell–cell interaction. In addition, it is evident that molecules other than LFA-1 family glycoproteins are involved in this process since the addition of anti-p150,95 antibodies to monocytes that adhere to a substrate results in a blocking percentage of only 30%. Furthermore, these observations indicate that LFA-1 and CR3 might function as receptors that recognize counterstructures on target cells in suspension, whereas only p150,95 appears to be involved in adhesion of monocytes to monolayers of cells.

Up until now it has been unclear whether the common beta chain of LFA-1, CR3 and p150,95 itself contributes to cell–cell interactions. A known function of the beta chain is that it is required for the surface expression of the alpha–beta heterodimer complexes of these molecules (Sanchez-Madrid *et al.*, 1983; Springer *et al.*, 1984).

An interesting finding is the significantly higher number of monocytes that bind in suspension to melanoma (tumour) cells in comparison to endothelial cells (Fig. 1). A possible explanation could be that monocytes have receptors recognizing determinants more strongly expressed on malignant cells. This preferential adhesion is supported by recent data indicating that the number of conjugates formed between monocytes and melanoma cells is higher than the number of conjugates formed between monocytes and normal human fibroblasts (te Velde *et al.*, 1986). The regulation of the mechanism underlying a

differential binding of monocytes to malignant versus non-malignant cells in suspension deserves further investigation.

The binding of monocytes to melanoma cells and endothelial cells does not result in lysis in a 3-hr single cell cytotoxic assay (results not shown). This is in line with data of others (Koren & Herberman, 1985), who show that monocytes isolated by centrifugal elutriation do not spontaneously lyse target cells, not even in 24-hr assays. Monocytes probably need to be activated for tumour cell killing, which is currently under investigation.

Taken together, these data indicate that the LFA-1 family molecules are involved in the binding of monocytes to tumour cells and to endothelial cells suggesting that these molecules are involved in monoyte tumour cytotoxicity and that they may be associated with extravasation of monocytes through vessel walls.

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