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Role of CD18-dependent and CD18-independent mechanisms in the increased leukocyte adhesiveness and in the variations of circulating white blood cell populations induced by anti-CD3 monoclonal antibodies

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Abstract Anti-CD3 antibodies induce a quick and profound depletion of peripheral blood mononuclear cells (PBMCs) that is not well understood. We studied the effect of OKT3, a mouse monoclonal antibody against the human CD3 complex, on the in vitro adhesion of human PBMCs to monolayers of fresh and fixed human umbilical vein endothelial cells (HUVECs). OKT3 induced an increased adhesiveness of PBMCs. This phenomenon was blocked with anti-CD18 antibodies, indicating the participation of $\beta 2$ integrins. As this increased adhesiveness could explain the lymphopenia by adhesion of PBMCs to endothelial cells and their sequestration in some peripheral vascular beds, we studied the effect of anti-CD18 antibodies in vivo on mice injected with 145/2C11, a hamster monoclonal antibody against murine CD3. Mice treated with 145/2C11 presented with a transient granulocytopenia and a sustained reduction in PBMCs. A monoclonal anti-CD18 antibody prevented the granulocytopenia but had no effect on the drop in PBMCs. Consequently, the in vivo depletion of PBMCs after administration of an anti-CD3 monoclonal antibody involves CD18-independent mechanisms, while the transient drop in polymorphonuclear cells appears to be CD18-dependent.

Key words Monoclonal antibodies, endothelial cells · Endothelial cells, monoclonal antibodies · Leukocyte adherence, monoclonal antibodies

Introduction

Monoclonal antibodies raised against the CD3 complex of the lymphocytic cell membrane are potent immunosuppressors [2, 4, 19]. They induce a rapid and sustained reduction in the number of peripheral blood mononuclear cells (PBMC) that is not clearly understood. Various mechanisms have been proposed to explain this occurrence, such as opsonization followed by trapping in the reticuloendothelial system, cell lysis, and apoptosis [10, 14, 23]. Some time ago, Dustin and Springer observed that CD3 crosslinking with OKT3 transiently stimulates the adhesiveness of T cells to polystyrene microtiter wells coated with intercellular adhesion molecule 1 (ICAM-1) [7]. They demonstrated that this phenomenon resulted from a higher avidity of CD11 a, a $\beta 2$ integrin for the ligand ICAM-1. An increased adherence of lymphocytes to endothelial cells could explain the quick OKT3-induced lymphopenia by a shift of the mononuclear cells from the leukocyte circulating pool to the marginal pool where they cannot be detected by normal blood sampling.

As the adhesion of leukocytes to endothelial cells is a multistep phenomenon involving not only CD11 a and its ligands ICAM-1 and ICAM-2 but also many other sets of adhesion molecules [6, 16], it seemed interesting to study the adherence of human PBMCs treated with OKT3 to monolayers of human umbilical vein endothe-

lial cells (HUVECs). Our results demonstrated that it is enhanced. The adhesion of activated blood cells to the vascular endothelium was shown to be detrimental to the host in other experimental settings [11], and this could be related to some of the side effects of the anti-CD3 antibodies. Therefore, we also tested in vivo, in mice, the efficacy of anti-CD18 antibodies directed against $\beta 2$ integrins in preventing alterations of white blood cell counts induced by 145/2C11, a hamster anti-mouse CD3 monoclonal antibody.

Materials and methods

Antisera

OKT3, the mouse monoclonal antibody (mAb) directed against the human CD3 complex, was kindly provided by Ortho-Cilag (Brussels, Belgium); the mouse IgG 2a was obtained from Immunotech (Fleurus, Belgium). Two antisera against human CD18 were used: one (SW) was a gift from Professor Samuel Wright (New York), the other (BB) was purchased from British Biotechnology (Abingdon Oxon, UK). Two mAb raised against human CD11a were also tested: one (PM) was given to us by Pasteur-Merieux (Lyon) and the other (BB) came from British Biotechnology.

145/2C11, a hamster mAb directed against the murine CD3 complex, was kindly provided by O. Leo (Brussels). The antibody against mouse CD18 was an ascitis fluid recovered from nude mice injected i.p. with the hamster hybridoma ATCC HB226 (American Type Culture Collection, Rockville, Md., USA). The IgG1 was an anti-DNP antibody produced in our own laboratory.

Preparation of endothelial cells monolayers

Human umbilical vein endothelial cells (HUVECs) were harvested from human umbilical cords treated with collagenase and cultured as previously described [9, 13, 17]. In short, the cells were cultured in M199 medium supplemented with 20% fetal calf serum, essential amino acids, heparin, endothelial cell growth factor, penicillin, and streptomycin. Cells from the second passage were transferred into polystyrene Lab-Tek Chamber slides (Life Technologies, Gibco, Merelbeke, Belgium). Each chamber received 0.5 ml of a cell suspension containing 2.10^5 HUVECs/ml. After overnight incubation, when the cells were at confluence, the monolayers were used immediately in an adhesion assay or treated with a periodate/lysine/paraformaldehyde fixative that stabilizes antigens in situ without altering their antigenicity [18]. They were then stored in the ice box.

Blood cell preparations

Heparinized blood was drawn from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density gradient centrifugation and resuspended in autologous plasma or serum at a concentration of 5.10^6 cells/ml.

Adhesion of PBMCs to HUVECs

Each chamber of the chamber slide containing the HUVEC monolayer received 1 ml of the PBMC suspension. Two micrograms of OKT3 was added to half of the chambers; the others served as a control to determine the basal level of adhesion. The slides were incubated for 1 or 2 h at 37 °C and at room air. The supernatant was removed and the chamber was rinsed twice with saline at 37 °C. The walls of the chambers were removed and the slides were stained with May-Grünwald-Giemsa stains. Residual PBMCs adhering to HUVECs were counted manually and expressed as numbers of adhering cells/100 HUVECs. Values of five replicates were averaged. An adhesion index was calculated using the formula: number of adhering cells per 100 HUVECs in the OKT3 well/number of adhering cells per 100 HUVECs in the control well. Thus, a value of 1 or close to 1 indicated no increase in the basal level of adhesion. The validity of the method was assessed by two independent observations with eight PBMC suspensions. The variation in the mean adhesion index was less than 10%. In the experiments designed to study the role of adhesion molecules, mAbs against CD18 or CD11a were added to the PBMC suspension immediately before OKT3 or, in some experiments, 30 min before OKT3. The various dosages are indicated in Tables 2 and 3.

Mice experiments

Balb/c mice were purchased from IFFA Credo (Brussels). Thirty animals aged 10–12 weeks were divided into five groups of six animals. The first group was simply anesthetized with ether and bled by retro-orbital puncture. The other four groups were bled at various times after an i.v. injection of 25 µg of 145/2C11. White blood cell (WBC) counts and differential counts were performed on all of the blood samples. This technique was chosen instead of repeatedly bleeding the same mice to avoid multiple stress episodes which, in rodents, are accompanied by high circulating corticosteroid levels known to influence WBC counts. In a second experiment, mice were injected i.p. either with an anti-CD18 antibody (100 µl ascitis fluid containing 3.5 mg protein) or with a similar amount of IgG1; 2 h later, the animals in each group were divided into two subgroups receiving an i.v. injection of either saline or 25 µg of 145/2C11. After half an hour they were bled, and WBC and differential counts were made. Results were expressed for each group as means ± SEM of the total number of circulating polymorphonuclear cells (PMN) and PBMCs. All of the animal experiments were performed according to the "Principles of laboratory animal care" (NIH publication no. 86–23, revised 1985).

Statistics

Data are expressed as means ± SEM. Significance of differences was assessed by a one-way analysis of variance.

Results

Incubation of PBMCs with OKT3 significantly increased their adhesiveness to HUVECs (Fig 1). This is an active phenomenon that is completely inhibited at 4 °C. Fixation of HUVECs with periodate/lysine/paraformaldehyde did not significantly change the adhesion index of OKT3-treated PBMCs (Table 1). There-

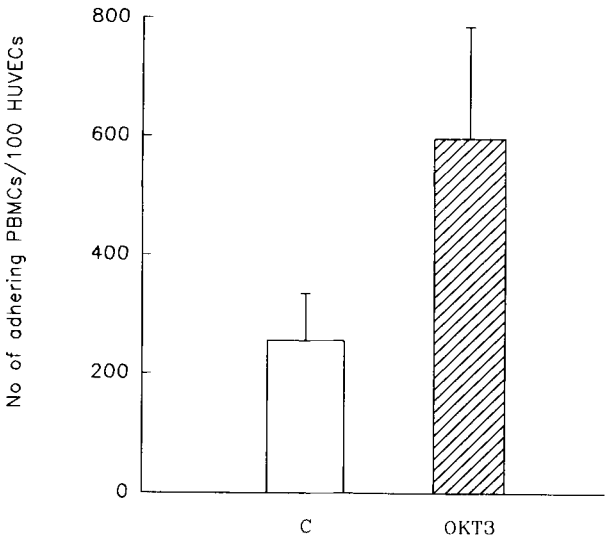

Fig. 1 Effect of OKT3 on adhesion of PBMCs to HUVECs. Results are expressed as means ± SEM of eight different cell suspensions (*C* control suspension)

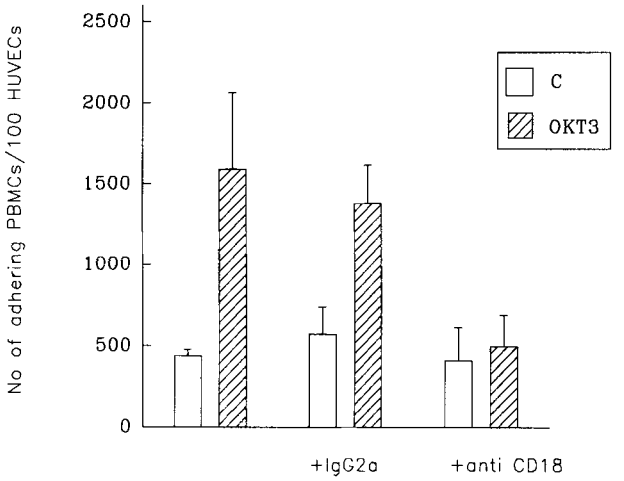

Fig. 2 Inhibition by the anti-CD18 antibody SW of the increased adhesion of PBMCs to HUVEC induced by OKT3. Results are expressed as means ± SEM (*C* control cell suspension)

fore, all of the subsequent experiments were performed with fixed cells. The increased adhesiveness of PBMCs to HUVEC monolayers persisted for at least 2 h: the mean adhesion index of three different PBMC suspensions was 2.14 ± 0.59 after 1 h of incubation with OKT3 and 2.12 ± 0.54 after 2 h of incubation with the antibody. Longer incubation periods could not be tested because the control PBMCs unhooked themselves from the monolayers and results were impossible to interpret after 2 h. The effect of OKT3 depended on the presence of heat labile factors. Cells incubated for 1 h with OKT3 had an adhesion index of 2.56 ± 0.37 when they

Table 1 Effect of temperature and fixation of HUVECs on the adhesion index of PBMCs incubated with OKT3. The incubation time was 1 h. Figures in parentheses correspond to the numbers of tested cell suspension. Adhesion indices are expressed as means ± SEM (N.D. not determined)

	37 °C	4 °C
Fresh HUVECs (4)	2.19 ± 0,68	N.D.
Fixed HUVECs (4)	2.37 ± 0.13	0,94 ± 0.05*

* $P < 0.005$

Table 2 Effect of monoclonal anti-CD18 and anti-CD11a antibodies on the adhesion index of PBMCs incubated with OKT3. The antibodies were added to the cell suspension a few minutes before OKT3, except for the experiment performed with mAb PM at the dose of 100 μg/ml, where the antibody was added 30 min before OKT3. Adhesion indices are expressed as means ± SEM. Incubation time with OKT3 was 1 h

mAb	Isotype	Dosage (μg/ml)	Number of tests	Treatment of the PBMC suspension	
				OKT3 alone	OKT3 + mAb
Anti-CD18 (SW)	IgG2a	10	3	3.53 ± 0,73	1.34 ± 0.13*
Anti-CD11a (BB)	IgG2a	5	4	2.49 ± 0,18	2.10 ± 0.18
Anti-CD18 (BB)	IgG1	5	7	2.45 ± 0.20	1.92 ± 0.26
		20	4	2.56 ± 0.30	1.70 ± 0.18*
Anti-CD11a (PM)	IgG1	10	3		2.52 ± 0.19
		100	3	2.66 ± 0.47	2.68 ± 0.11

* $P < 0.05$ for OKT3 + mAb vs OKT3 alone

were suspended in normal autologous serum, but when they were suspended in heat-inactivated serum their adhesion index fell to 1.20 ± 0.06. Two different anti-CD18 antibodies significantly reduced the adhesion index of PBMCs incubated with OKT3 (Table 2) without affecting the adhesiveness of control cells (Fig. 2, Table 3). Two other mAbs with the same isotypes but raised against CD11a had no effect on the increased adhesiveness of PBMC induced by OKT3, indicating that the effect of anti-CD18 antibodies was not due to some general, nonspecific mechanism (Table 2). One of these nonblocking antibodies (BB) was tested on control PBMCs; it did not alter their basal adhesiveness (Table 3).

The mAb 145/2C11 induced a profound and longlasting depletion of PBMCs in mice. There was also a transient drop in the number of PMNs, but at 3 h, the values were back to normal (Table 4). This phenomenon was observed repeatedly, although its extent was highly variable, depending on the various batches of mice. The administration of an anti-CD18 antibody 2 h before the challenge with 145/2C11 had no effect on the reduction

Table 3 Absence of effect of various mAbs binding CD11a or CD18 on basal PBMC adhesiveness. Number of adhering cells are expressed as means ± SEM

mAb	Isotype	Blocking activity	Dosage (μg/ml)	Number of tests	Number of adhering PBM/100 endothelial cells	
					Untreated PBMCs	Treated PBMCs
Anti-CD11a (BB)	IgG2a	0	5	4	647 ± 151	870
Anti-CD18 (SW)	IgG2a	+	10	3	440 ± 38	410
Anti-CD18 (BB)	IgG1	+	20	4	619 ± 75	659

Table 4 Effect of 145/2C11 on white blood cell counts in mice. Number of cells are expressed as means ± SEM at various times after injection of the mAB

Time (hours)	Number of mice	PMNs (cells/mm^3)	PBMCs (cells/min^3)
0	6	2706 ± 378	6329 ± 481
1	6	786 ± 255*	475 ± 87*
3	6	2334 ± 252	251 ± 42*
6	5	2306 ± 287	333 ± 81*
24	6	2962 ± 185	304 ± 41*

* $P < 0.001$ vs value at time 0

Table 5 Effect of an anti-CD18 mAb on the alterations of white blood cell counts induced by 145/2C11. The animals were bled half an hour after the injection of 145/2C11. Number of cells are expressed as means ± SEM

Group	Pretreatment		Number of mice	PMNs (cells/mm^3)	PBMCs (cells/mm^3)
	−2 h	Time 0			
A	IgG1	Saline	6	4554 ± 837	4329 ± 322
B	IgG1	145/2C11	6	3661 ± 411	1893 ± 442
C	anti-CD 18 mAb	Saline	6	6437 ± 170	3945 ± 370
D	anti-CD 18 mAb	145/2C11	5	6234 ± 556	1572 ± 155

P = NS for PMNs, A vs C or D; $P < 0.01$ for PMNs, B vs all other groups; $P < 0.001$ for PBMCs, A vs B and C vs D

in PBMCs. However, no granulocytopenia was observed in these animals, which actually had a slightly, but not significantly, higher number of circulating PMNs than the normal controls (Table 5).

Discussion

In our in vitro experimental setting, CD3-positive cells are activated by cross-linking OKT3 through the FcIgG receptors of the monocytes in the PBMC suspension and also, possibly, through Fc receptors present on the HUVECs. This activation is accompanied by an increased adhesiveness of these PBMCs to HUVEC monolayers, which could theoretically result from the binding of OKT3-coated cells to the endothelial Fc receptors. However, it is quite unlikely that this simple mechanism plays a significant role in this phenomenon. Indeed, coating control PBMCs (not treated with OKT3) with anti-CD18 mAbs or a nonblocking anti-CD11a antibody did not alter their binding capacity to HUVECs (Fig. 2, Table 3). Moreover, the increased adhesiveness of the PBMCs is the result of an active process that is abrogated at 4 °C and that requires the presence of heat labile factors in the serum. It is also inhibited by anti-CD18 antibodies, which indicates the involvement of β2 integrins. Previous studies have shown that homotypic lymphocyte adhesion and binding of lymphocytes to purified ICAM-1 molecules adsorbed on polystyrene plates are stimulated by anti-CD3 antibodies [7, 15]. This enhanced adhesiveness results from an increased avidity of the CD11a/CD18 molecule expressed on the lymphocyte surface for its ligands. It is a transient phenomenon that does not completely account for our observations that the enhanced binding of PBMCs to HUVECs lasts for at least 2 h in our experimental setting. Taken together with the findings from previous studies, our results suggest that β2 integrins play an essential role in the initial steps of the increased adhesion of PBMCs, but later other mechanisms must play a role in its maintenance. Indeed, adhesion of PBMCs to HUVECs is a multistep phenomenon involving many sets of adhesion molecules other than the β2 integrins and their ligands [6, 16].

In addition to causing a profound depletion of circulating mononuclear cells, the injection of 145/2C11 into mice also causes a transient decrease in PMNs that is always present, although its magnitude varies widely from one experiment to another. Raasveld et al. recently demonstrated a transient peripheral blood granulocytopenia with pulmonary sequestration of granulocytes in patients receiving OKT3 [21]. At the same time, these authors measured high circulating levels of C3a desarg, and they suggested that complement activation by the monoclonal antibody could be responsible for the decrease in circulating PMNs. Our present data indicate that β2 integrins are also involved in this phenomenon. Indeed, the reduction in circulating PMN levels was

not observed after injection of 145/2C11 into mice pretreated with an anti-CD18 mAb. Actually, C5a increases the surface expression of CD11b/CD18 on normal granulocytes [3, 17], and it seems reasonable to postulate that the drop in circulating PMNs after injection of anti-CD3 antibodies results from complement activation, which in turn induces an upregulation with activation of CD11b/CD18 and an enhanced adhesion of these cells to the endothelial lining of the blood vessels. These activated PMNs, trapped in the pulmonary circulation, release degranulation products that have been incriminated in the acute respiratory side effects of the first dose of OKT3 [21]. The blocking effect of anti-CD18 antibodies might be beneficial in preventing this complication.

Pretreatment of mice with anti-CD18 antibodies did not prevent the decrease in PBMCs after 145/2C11 administration. This could have been due to the lack of efficacy of our anti-CD18 preparation, but this explanation seems unlikely in view of its effect on PMN levels in these animals. Thus, in vivo PBMC depletion is a CD18-independent phenomenon. Yet, the absence of effect of an anti-CD18 antibody on the drop in PBMCs in mice is not in contradiction with the blocking effect of two similar sera on the increased adhesiveness of PBMCs to HUVEC monolayers induced by OKT3. These in vitro studies were conducted with HUVEC monolayers treated with a periodate/lysine/paraformaldehyde fixative that does not alter the antigenicity of the cell surface but stabilizes the expression of the various molecules [18]. Consequently, in our model, we detected only the effect of OKT3 on the adhesiveness of the mononuclear cells without testing the consequences of alterations of the endothelial cell surface induced by the monoclonal antibody. The fixed HUVECs express ICAM-2 and low levels of ICAM-1, which are the ligands for the $\beta 2$ integrins present on leukocytes. The interaction of these molecules is blocked by anti-CD18 antibodies. In contrast, in vitro adhesion of T lymphocytes to endothelial cells activated by Il1 or LPS is only marginally inhibited by an anti-CD18 antibody [12]. In mice experiments, PBMCs are activated by anti-CD3 antibodies and release various cytokines, such as interferon γ and TNF [1, 8] which are known to induce an upregulation of ICAM-1 and VCAM-1 on the endothelial cells [5]. The lack of effect of anti-CD18 antibodies on the lymphopenia caused by 145/2C11 could result, at least partially, from induction at the surface of endothelial cells of VCAM-1, which would interact with the $\alpha 4\beta 1$ integrin present on mononuclear cells and induce their binding and trapping in some vascular beds or parenchyma by a CD18-independent mechanism [20]. Granulocytes do not bear $\alpha 4\beta 1$; their adhesion to endothelial cells depends mainly on the interaction between $\beta 2$ integrins and their ligands, ICAM-1 and ICAM-2, which explains the efficacy of anti-CD18 antibodies in preventing 145/2C11-induced granulocytopenia. The possible role of other, as yet unidentified, molecules in the OKT3-induced lymphopenia and in vivo increased adhesion of PBMCs to endothelial cells should, however, not be overlooked. Indeed, Thornhill et al. have recently demonstrated that the increased adhesiveness for T lymphocytes of endothelial cells activated with a combination of TNFα and interferon γ was only partially inhibited by anti-VCAM-1 and/or anti-CD18 antibodies, suggesting the participation of other adhesion molecules [22].

In conclusion, our results indicate that CD18 dependent as well as CD18-independent mechanisms are involved in the alterations of the numbers of circulating WBCs induced by anti-CD3 antibodies. The profound reduction in PBMCs seems to be a multifactorial phenomenon.

In addition to the role of adhesion molecules, one must also consider other explanations, such as sequestration of opsonized lymphocytes and/or various mechanisms of cell lysis or apoptosis [10, 14, 23].

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Note added in proof While our manuscript was in press, the following article reaching similar conclusions was published in Blood. Buysmanns, Bemelman FJ, Schellekens PTA, van Kooyk Y, Figdor CG, ten Berge I (1996) Activation and increased expression of adhesion molecules on peripheral blood lymphocytes is a mechanism for the immediate lymphocytopenia after administration of OKT 3. Blood 87: 404–411

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