Monocyte Procoagulant Activity Induced by In Vivo Administration of the OKT3 Monoclonal Antibody

By O. Pradier, M. Surquin, P. Stordeur, L. De Pauw, P. Kinnaert, P. Vereestraeten, P. Capel, M. Goldman, and D. Abramowicz

The first injection of OKT3 in kidney transplant recipients activates the common pathway of coagulation. This may result in early thrombosis of graft vessels. To this day, the cells involved in this phenomenon have not been identified. The aim of this study was to investigate whether circulating monocytes participated in this OKT3-induced coagulopathy. The procoagulant activity (PCA) of circulating monocytes rose from (mean ± SEM) 0.15 ± 0.02 μM/mL to 0.40 ± 0.05 μM/mL at 3 hours (P = .002) and 0.56 ± 0.21 at 5 hours (P = .045) after the initial OKT3 injection. These monocytes displayed increased tissue factor expression at the same moments (mean fluorescence intensity: 14 ± 2 before OKT3 injection versus 54 ± 14 at 3 hours, P = .008 and 34 ± 7 at 5 hours, P = .01). Tissue factor mRNA was detected in blood by reverse transcriptase-polymerase chain reaction as early as 2 hours after OKT3 administration. The circulating monocytes also displayed a steady increase in membrane expression upregulation of ICAM-1, CD29, CD11b, and CD11c. In vitro experiments showed that OKT3 as well as 2 mitogenic, humanized anti-CD3 antibodies potently induced monocytic PCA whereas the 4 nonmitogenic anti-CD3 antibodies tested were over 1,000-fold less potent than OKT3. We conclude that (1) OKT3 induces in vivo tissue factor gene upregulation and membrane expression resulting in increased PCA of circulating monocytes; and (2) nonmitogenic anti-CD3 antibodies seem devoid of significant procoagulant properties.

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From the Departments of Hematology-Immunology and Nephrology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium.

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Address reprint requests to O. Pradier, MD, Department of Hematology-Immunology, ULB-Hôpital Erasme, 808, route de Lennik B1070, Belgium.

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OKT3 IS A MURINE IgG2a monoclonal antibody (MoAb) that recognizes the ε chain of the CD3-T-cell receptor complex present on mature T lymphocytes. In vivo injection of OKT3 is known to prevent and treat effectively acute rejection episodes. However, the first administration of OKT3 triggers a transient, polyclonal activation of T cells. This results in a systemic release of cytokines responsible for flulike symptoms and occasional more serious side effects. In addition, we and others have observed that the first OKT3 injection results in a systemic activation of blood coagulation, as indicated by increased plasma levels of prothrombin fragment 1.2. This procoagulant effect is of clinical importance, as we observed an increased incidence of irreversible intragraft thrombosis after injection of OKT3 in kidney transplant recipients.

Previous in vitro experiments have shown that OKT3 was able to induce membrane expression of the tissue factor (TF) molecule on both monocytes and endothelial cells. Endothelial TF expression appears to be mainly triggered by tumor necrosis factor-α (TNF-α), a cytokine produced in large amounts after OKT3 injection. The mechanisms leading to increased monocyte TF expression after OKT3 challenge in vitro are not as well defined. Although T-cell-derived soluble factors are probably involved, signals mediated by adhesion molecules could also contribute to increased TF expression. Indeed, cross-linking of CD11b/CD18 on monocytes results in marked enhancement of TF activity. Once expressed, TF will bind factor VII/VIIa and initiate coagulation through the extrinsic pathway.

Although these in vitro experiments indicate that both monocytes and endothelial cells are able to express tissue factor in response to OKT3, the role of these cells in the systemic activation of coagulation triggered by OKT3 in vivo remains unknown. The first aim of the present study was to investigate whether the initial injection of OKT3 triggered TF expression and activity on circulating monocytes in vivo. Secondly, we measured the expression of several adhesion molecules on the monocyte membrane. Finally, we investigated whether nonmitogenic anti-CD3 antibodies induced monocytic procoagulant activity in vitro, despite their inability to trigger cytokine release.

MATERIALS AND METHODS

Blood collection and cell isolation in kidney transplant recipients. All patients were kidney transplant recipients who were studied on the day of surgery. OKT3 (5 mg intravenous [IV]) was administered after induction of narcosis. The moment of OKT3 injection was defined as T = 0. Associated medications were azathioprine (2 mg/kg) and methylprednisolone (8 mg/kg IV) administered 2 to 4 hours before surgery. Control patients received cyclosporine A (6 mg/kg before surgery) instead of OKT3. In these patients, T = 0 was defined as the time after induction of narcosis.

Blood samples were collected through a central venous catheter immediately before (T = 0) and 3, 5, 8, 16, and 24 hours after the first injection of OKT3. In control patients, blood sampling was performed with the same timing. After discarding the first 10 mL, 24 mL of blood was collected into vacutainer tubes containing EDTA as the anticoagulant (Venoject; Terumo Europe, Leuven, Belgium). A differential count was made with an automatic cell counter (Cell Dyn 3500; Abbott, Santa Clara, CA) and the red blood cells were then removed with lipopolysaccharide (LPS)-free buffered NH4Cl solution (10 minutes at 0°C). The white blood cells (WBC) were then pelleted by centrifugation at 90g to remove platelets. The mononuclear cells (PBMC) were further isolated from WBC by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density gradient centrifugation followed by two washes in phosphate-buffered saline.
OKT3 AND MONOCYTE PROCOAGULANT ACTIVITY

Table 1. Description of the Anti-CD3 MoAbs Used in This Study

<table>
<thead>
<tr>
<th>Name</th>
<th>Mouse Isotype</th>
<th>Human (OKT3 CDR grafted)</th>
<th>Description</th>
</tr>
</thead>
</table>
| OKT3         | IgG2a         | IgG4 mutated             | Anti-CD3 MoAb used in vivo; 209-IgG1 and 209-IgG4, two human antibodies of the IgG1 and IgG4 isotype, respectively, grafted with the complementary determining region (CDR) of OKT3. The nonmitogenic anti-CD3 MoAbs assayed were Table 1: 209-IgG1 a/a and 209-IgG4 a/a, obtained by mutations in the CH2 domain of the Fe portion of 209-IgG1 and 209-IgG4, respectively. These changes resulted in the loss of FcyRI and FcyRII binding and, hence, in the loss of mitogenic properties. In addition, OKT3 D and OKT3 E, two nonmitogenic murine IgG2b MoAbs, were also assayed. PBMC were challenged with increasing doses of soluble anti-CD3 MoAbs. After culture, the cells were frozen/thawed once and procoagulant activity was determined. Statistical analysis. Results are shown as mean ± SEM. Differences between patient groups were evaluated by Student’s t-test. For the in vitro evaluation of MoAbs, repeated ANOVA followed if significance (P < .05) by Bonferroni multiple comparisons posttest was performed.

RESULTS

PBMC from kidney transplant recipients display increased tissue factor type PCA after the first injection of OKT3. As shown in Fig 1, no significant change in procoagulant activity was observed in circulating PBMC of control patients. By contrast, the first injection of OKT3 resulted in a large increase in PBMC’s procoagulant activity, which was already manifest at 3 hours, reached a maximum around 5 hours and then slowly decreased. Procoagulant activity was still slightly above control values at 24 hours, but no further increase was seen after the second OKT3 injection (data not shown). The increased procoagulant activity was caused by TF, as indicated by the inhibitory effect of the anti-factor VII MoAb added to the normal plasma used in the clotting assay (Table 2).

Increased expression of membrane tissue factor on circulating monocytes after the first injection of OKT3. Figure 2 shows a fluorocytometric analysis of TF expression on monocytes identified by physical characteristics as well as CD14 staining. Only background staining was observed before OKT3 injection. A slight increase in fluorescence was observed when monocytes obtained 3 hours after OKT3 injection were incubated with the control MoAbs, but the mean

(PBS). A second differential count was obtained and the number of monocytes was then adjusted to 1 × 10⁷/ml before determination of procoagulant activity and immunophenotyping. The endotoxin level of the reagents was constantly <10 pg/mL as measured by a Limulus Amoebocyte Lysate assay (Chromogenix Nodia, Möln达尔, Sweden).

Ex vivo procoagulant activity of blood mononuclear cells. PBMC were frozen/thawed twice before determination of their procoagulant activity in a one-stage clotting assay as previously described. Clotting time was recorded with a KClO fibrometer (Aem-lung, Lemgo, Germany) and interpolated into milliunits (mU) by reference to a standard curve. One unit corresponds to the amount of thromboplastin (Simplastine; Organon Teknica, Durham, NC) yielding a normal clotting time of 12.4 seconds. In some experiments, an anti-factor VII MoAb (12D10; Centocor, Malvern, CA) was added to the normal pooled human plasma to assess the extrinsic pathway’s involvement in this clotting assay.

Ex vivo cytofluorometer analysis of TF and adhesion molecule expression on monocytes. PBMC (50 μL of the cell suspension adjusted to 10⁶ monocytes/ml) were cocultivated for 30 minutes at 37°C with anti-CD14-PE (Leu M3-PE; Becton Dickinson, San Jose, CA) and fluoresceinated MoAbs specific for either: (1) TF (4508 CJ murine IgGl MoAb; American Diagnostica, Greenwich, CT); (2) CD29, the β1 subunit of the VLA integrin family (BD-15, murine IgG1; Serotec, Oxford, UK); (3) CD11b, the α2 subunit of the β2 integrin family (44, murine IgG1; Serotec); (4) CD11c, the α3 subunit of the β2 integrin family (3.9, murine IgG1; Serotec), or (5) CD54-ICAM-1, a member of the Ig superfamily (84H10, murine IgG1; Immunotech, Marseille, France). Control staining for TF and adhesion molecules was performed with a pool of nonreducing fluorescein isothiocyanate (FITC)-labeled murine IgG1 (Serotec and Immunotech). After washing with PBS supplemented with 0.02 mol/L HEPES, 1% glutamine, and 10% LPS-free fetal calf serum (FCS; Myocline, Gibco, Paisley, Scotland) at 37°C in air atmosphere supplemented with 5% CO₂, FCS was decomplemented by heating 30 minutes at 56°C. The following mitogenic MoAbs were assayed (Table 1): OKT3, the murine IgG2a MoAb used in vivo; 209-IgG1 and 209-IgG4, two human antibodies of the IgG1 and IgG4 isotype, respectively, grafted with the complementary determining region (CDR) of OKT3. The nonmitogenic anti-CD3 MoAbs assayed were Table 1: 209-IgG1 a/a and 209-IgG4 a/a, obtained by mutations in the CH2 domain of the Fe portion of 209-IgG1 and 209-IgG4, respectively. These changes resulted in the loss of FcyRI and FcyRII binding and, hence, in the loss of mitogenic properties. In addition, OKT3 D and OKT3 E, two nonmitogenic murine IgG2b MoAbs, were also assayed. PBMC were challenged with increasing doses of soluble anti-CD3 MoAbs. After culture, the cells were frozen/thawed once and procoagulant activity was determined. Statistical analysis. Results are shown as mean ± SEM. Differences between patient groups were evaluated by Student’s t-test. For the in vitro evaluation of MoAbs, repeated ANOVA followed if significance (P < .05) by Bonferroni multiple comparisons posttest was performed.

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Procoagulant activity of PBMC. Procoagulant activity of PBMC from kidney transplant recipients after the first injection of OKT3 (N = 9) and from controls (N = 4). PBMC were isolated by density gradient centrifugation, adjusted to 1 x 10^6 monocytes/mL, and stored at -70°C until determination of procoagulant activity. The background procoagulant activity of this assay is 0.12 mU/mL. Results are shown as mean (±SEM) of procoagulant activity in mU/mL. * P < .05; ** P = .003 for comparisons between OKT3 group (●, N = 9) and controls (□, N = 4).

Fluorescence intensity remained far below the one recorded after staining with the anti-TF MoAb (Fig 2). Figure 3 shows the mean fluorescence intensity for TF on monocytes from OKT3 and control kidney transplant recipients. The expression of TF was markedly increased in OKT3 patients (Fig 3). The maximum staining was observed at 3 hours, with increased levels still present at 16 hours before returning to near basal values at 24 hours. No increased monocyte TF expression was found after the second OKT3 injection (data not shown).

Detection of tissue factor mRNA in whole blood after OKT3 injection. Reverse PCR experiments indicated that the blood from patients treated with OKT3 contained increased amounts of tissue factor mRNA (Fig 4). TF mRNA appeared sometime between 1 and 2 hours after OKT3 injection, was maximal at 2 hours, and slowly returned to basal levels at 24 hours.

Increased expression of adhesion molecules on circulating monocytes after the first injection of OKT3. The expression of ICAM-1, CD11b, CD11c, and CD29 on monocytes is

Table 2. Inhibition of the Procoagulant Activity Present on Circulating Monocytes Three Hours After OKT3 Administration by the 12D10 Anti-factor VII MoAb

<table>
<thead>
<tr>
<th>Monocytes Procoagulant Activity 3 h After OKT3 Injection</th>
<th>PCA in mU (one-stage clotting assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Plasma</td>
<td>12D10-Treated Plasma</td>
</tr>
<tr>
<td>Patient 1</td>
<td>0.54</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.44</td>
</tr>
</tbody>
</table>
shown in Fig 5. Except for CD29 that slowly increased after surgery in control patients, no significant changes were observed on circulating monocytes isolated from patients treated with cyclosporin A. However, OKT3 led to marked enhancement of these four adhesion molecules. Near-maximal levels of ICAM-1, CD11b, and CD11c were already present at 3 hours. CD11c expression decreased rapidly thereafter, whereas ICAM-1 and CD11b showed sustained expression for at least 8 hours. Different kinetics occurred for CD29 expression, which steadily increased during 16 hours. The background staining of monocytes observed after incubation with control MoAbs increased moderately after OKT3 injection but remained far below the adhesion molecule staining.

**Induction of procoagulant activity on PBMC by mitogenic and nonmitogenic soluble anti-CD3 MoAbs.** The observation that monocytes displayed strong tissue factor activity in vivo as they did in vitro after OKT3 stimulation suggested that in vitro assays of monocyte procoagulant activity could help predict the in vivo procoagulant properties of other anti-CD3 antibodies. As previously shown,11 incubation of PBMC with soluble OKT3 resulted in a dose-dependent increase in procoagulant activity (Fig 6). Significant effects were already visible with 0.1 ng/mL of OKT3, and plateau levels were reached with 10 ng/mL. The two mitogenic, OKT3-CDR-grafted, humanized MoAbs (209 IgG1 and 209 IgG4) also triggered large increases in procoagulant activity. Although they seemed somewhat less potent on a molar basis than OKT3, the maximum levels seen at 1,000 ng/mL were equivalent to those observed with OKT3. On the opposite, the nonmitogenic anti-CD3 MoAbs, whether of human or murine origin, induced only modest increases in procoagulant activity, and were at least 1,000-fold less potent than OKT3 on a molar basis.
our study does not exclude a possible role for endothelial cells, it should be pointed out that the kinetics of blood coagulation activation closely parallel the expression of tissue factor mRNA came from monocytes as they seem to be the only circulating cell able to express this gene. Kinetic experiments showed that TF mRNA appeared first with a maximum at 2 hours, followed by the expression of monocytic membrane tissue factor 1 hour later, at the same time as the maximal procoagulant activity of PBMC. Thrombin generation rapidly followed, as indicated by increased levels of prothrombin fragment 1.2, which reached a maximum 4 hours after OKT3 injection.

Induction of tissue factor after OKT3 injection occurred in the context of generalized monocyte activation, as shown by the increased expression density of several adhesion molecules on circulating monocytes. Along this line, injection of a mitogenic anti-CD3 MoAb in mice resulted in activation of splenic macrophages, as these cells displayed increased amounts of messenger RNA for both interleukin-1 and interleukin-6. Increased expression of monocytic adhesion molecules might have two important consequences regarding OKT3 coagulopathy. First, it could explain the increased susceptibility of graft vessels to thrombotic events. Indeed, the cold ischemia associated with kidney graft preservation also results in upregulation of adhesion molecules on endothelial cells, so that activated monocytes are likely to localize preferentially to the graft endothelium leading to in situ thrombosis. Second, signaling through adhesion molecules is known to further enhance monocyte tissue factor activity (see below).

It is now admitted that the acute events triggered by OKT3 in vivo are similar to those elicited by endotoxin. Indeed, proinflammatory cytokines are released in comparable amounts and kinetics, Furthermore, injection of LPS in human volunteers also results in the activation of the coagulation's extrinsic pathway, again with kinetics comparable to those observed after OKT3 injection. Although some of the molecular mechanisms underlying LPS- and OKT3-induced monocyte activation probably differ, our findings in OKT3 patients suggest that the study of monocytes in septic patients is worth investigating.

Although the present study was not designed to investigate the mechanisms responsible for tissue factor expression on monocytes after OKT3 injection, data from other experimental systems indicate that T-cell-derived cytokines like TNF-α, interferon-γ, or other mediators such as macrophage-procoagulant inducing factor, could be involved. In addition, cross-linking of CD11b/CD18, a process that also upregulates TF activity, could take place after OKT3 injection. Indeed, the complement system is activated and C3bi is produced. This molecule binds to CD11b/CD18, so that opsonization by C3bi will result in monocytic CD11b/CD18 cross-linking.

The prominent in vivo monocyte activation triggered by OKT3 led us to examine several anti-CD3 MoAbs that could be considered for clinical use for their ability to induce in vitro TF activity on monocytes. Only the MoAbs able to stimulate cytokine release and cell proliferation as a consequence of monocyte Fc receptor binding induced high procoagulant activity of monocytes in vitro. Interestingly, an IgA switch variant of OKT3 that is not mitogenic in vitro, because human monocytes lack Fc receptors for IgA, has recently been injected to patients, with no evidence of coagulation activation. Thus, our in vitro assay does indeed appear...
to correlate with in vivo findings, rendering it a potentially interesting screening tool for possible procoagulant activity of new MoAbs. Whether the nonactivating MoAbs studied in the present work or others25,32 as are effective immunosuppressive agents as OKT3 is not fully established yet. Preliminary evidence indicates that T10B9, a nonmitogenic IgM anti- \( \gamma \)T-cell receptor antibody, reverses acute renal allograft rejection in the same proportion of patients as OKT3.32 With regard to the prophylactic use of anti-CD3/\( \gamma \)T-cell receptor MoAbs, recent studies showed that an anti-CD3 IgA MoAb48 and an anti-\( \gamma \)T-cell receptor MoAb41 both effectively deplete or modulate circulating CD3+ cells. However, comparative trials will be needed to see if they allow for the same improvement of long-term kidney graft survival as does OKT3.32

In conclusion, the present work shows that in vivo activation of circulating mononuclear cells during the self-limited septic-like syndrome that follows OKT3 injection results in coordinate expression of tissue factor gene and protein. This phenomenon is likely to have contributed to the thrombosis observed after OKT3 therapy. Nonmitogenic anti-CD3 antibodies appear devoid of significant procoagulant properties, and could become useful therapeutic alternatives to OKT3.

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