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Anti-ICAM-1 Monoclonal Antibody R6.5 (Enlimomab) Promotes Activation of Neutrophils in Whole Blood

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R6.5 (BIRR-1, Enlimomab), a murine IgG2a mAb to the human ICAM-1, inhibits leukocyte adhesion to the vascular endothelium, thereby decreasing leukocyte extravasation and inflammatory tissue injury. In initial clinical trials, R6.5 proved to be beneficial in reducing both disease activity in refractory rheumatoid arthritis and the incidence of acute rejection after kidney and liver allograft transplants. However, adverse effects such as fever, leukopenia, or cutaneous reactions were not infrequent. We studied the effects of R6.5 on neutrophil function in whole blood samples ex vivo. Surprisingly, at the concentrations achieved in clinical trials, R6.5 activated neutrophilic granulocytes, as indicated by a significant increase in expression of the adhesion molecule $\beta_2$-integrin CD11b, a concurrent decrease in L-selectin expression, and an enhancement of the oxidative burst activity. Neutrophil activation was not exerted by an anti-ICAM-1 mAb of the IgG1 isotype, by isotype-matched, irrelevant anti-2-phenyloxazolone mAb, or by F(ab')$_2$ fragments of R6.5. Neutrophil activation was completely inhibited by soluble complement receptor type 1. We conclude that in whole blood, R6.5 activates resting neutrophils in a complement-dependent manner. This finding can explain, at least in part, the side effects associated with R6.5 therapy. The Journal of Immunology, 1999, 162: 2353–2357.

Materials and Methods

Reagents

The reagents used were: PBS (Life Technologies, Paisley, U.K.), ammonium chloride (Riedel-de Haën AG, Seelze, Germany), potassium bicarbonate (Riedel-de Haën AG), tetrasodium EDTA (Sigma, St. Louis, MO), etc.
acid citrate dextrose (ACD) (Baxter Healthcare, Norfolk, U.K.), dextran (m.w. 70,000, Sigma), dichlorofluorescin diacetate (DCFH-DA, Eastman Kodak, Rochester, NY), phycoerythrin (PE) conjugates of anti-CD11b mAb (IgG2a, clone D12) and anti-CD62L mAb (IgG2a) (both from Becton Dickinson, San Jose, CA), murine anti-human ICAM-1 mAbs R6.5 (IgG2a) and RR1/1 (IgG1) as well as F(ab')2 fragments of R6.5 (all provided by Boehringer Ingelheim Pharmaceuticals Inc.); anti-2-phenyloxazolone (anti-Ox) mAb (IgG2a, clone DKE-18) and anti-CD3 mAb OKT3 (IgG2a, clone T3G2a) (both provided by Dr. M. Kaartinen, Haartman Institute, University of Helsinki), FMLP and PMA (Sigma); and soluble complement receptor type 1 (sCR1) (a gift of Dr. U. S. Ryan, T Cell Sciences, Inc., Needham, MA).

**Experimental design**

The DCFH oxidation method (27), as modified for whole blood samples (28), was used to evaluate neutrophil intracellular hydrogen peroxide formation in response to anti-ICAM-1 mAbs (R6.5, F(ab')2 fragments of R6.5, and RR1/1), irrelevant anti-Ox mAb, or OKT3. A peripheral blood sample was obtained and placed in a tube containing anticoagulant, dextran, DCFH-DA, and the appropriate mAb. While the sample was incubated with the mAb, DCFH-DA diffused into leukocytes. The leukocyte-rich plasma layer yielded by dextran-facilitated sedimentation was subsequently collected, and neutrophils were surface-labeled with PE-conjugated anti-CD11b and anti-CD62L mAbs. This experimental design made possible the flow-cytometric dual-color quantification of neutrophil oxidative burst activity and adhesion molecule expression. In some experiments, activation of the complement system was inhibited by adding sCR1 to the tube before blood sampling. Finally, in specific experiments, R6.5 was prefiltered (Minisart 0.2 µ, Sartorius AG, Goettingen, Germany) to remove Ig aggregates.

**Blood samples**

Healthy volunteers were recruited from the laboratory staff. The anticoagulant ACD (final concentration was 56 mM in blood) was added to polystyrene tubes (Falcon no. 2058, Becton Dickinson Labware, NJ). Each tube was further supplemented with dextran (6 mg/ml) and DCFH-DA (100 µM) as well as R6.5 (0–100 µg/ml), F(ab')2 fragments of R6.5 (100 µg/ml), RR1/1 (100 µg/ml), anti-Ox mAb (100 µg/ml), or OKT3 (100 µg/ml). The final concentration of sCR1 was 20 µg/ml blood. After prewarming the tubes at 37°C in the dark, a venous blood sample was obtained using an 18-gauge open needle (Terumo Europe, Leuven, Belgium). The tubes were capped, reversed, three times, and immediately placed back into the water bath at 37°C. After incubation for 5 min, the leukocyte-rich plasma layers were collected and further incubated for 10 min at 37°C. The leukocyte-rich plasma suspensions were then divided into 50-µl aliquots, cooled, and retained in an ice-cold water bath for subsequent labeling of the cells with fluorescent mAbs.

**Processing of leukocyte-rich plasma**

The aliquots of leukocyte-rich ACD-plasma at 0°C were labeled with saturating concentrations of PE-conjugated anti-CD11b mAb (clone D12) or PE-conjugated anti-CD62L mAb (29–31). After incubation for 30 min at 0°C, contaminating E were lysed with an ice-cold lysing solution containing ammonium chloride (8.26 g/l), potassium bicarbonate (1.0 g/l), and tetrasodium EDTA (0.037 g/l). Cells were washed twice with PBS, resuspended in 500-µl aliquots of PBS, retained on ice, and analyzed immediately. The time that elapsed from withdrawal of the blood sample until data acquisition was always <3 h.

**Flow cytometry**

A FACScan flow cytometer (Becton Dickinson) and Lysys II software were used for acquisition and dual-color analysis of the data, as described previously in detail (28–30). After appropriate color compensations, the instrument settings were not changed. The amplifier linearity and the stability of instrument settings were regularly checked by fluorescent beads (Calibrite, Becton Dickinson). Neutrophils were identified on the basis of their light scattering properties. Red (PE) and green (DCF) fluorescence were used for acquisition and dual-color analysis of the data, as described previously. Two aliquots of leukocyte-rich plasma devoid of R6.5 (one supplemented with PMA (1 µg/ml, final concentration) and the other left without supplements) were incubated at 37°C for a total of 10 min. A third aliquot was similarly incubated for 5 min, supplemented with FMLP (10−6 M), and further incubated for 5 min. Labeling of leukocytes was performed according to standard procedures.

**Results**

R6.5 activates neutrophils in whole blood

To examine the effects of the IgG2a anti-ICAM-1 mAb R6.5 on resting neutrophils, samples of peripheral blood from five healthy volunteers were incubated with increasing concentrations of R6.5 (0, 10, 50, and 100 µg/ml blood). R6.5 accounted for marked and consistent increases in the neutrophil CD11b fluorescence intensity. Compared with the R6.5-negative samples, the three concentrations of R6.5 resulted in median fold increases (ranges) of 11.8 (7.5–16.0), 15.5 (12.8–19.8), and 16.3 (13.4–21.1), respectively. Concomitantly, the CD62L fluorescence intensity decreased by 30.3% (20.1–36.7%), 37.1% (33.9–47.8%), and 47.5% (43.1–57.1%), respectively. Finally, the median fold increases observed in the DCF fluorescence intensity were 2.0 (1.4–3.5), 2.2 (1.8–4.1), and 2.8 (1.9–4.3), respectively (Fig. 1).

To evaluate the magnitude of neutrophil activation by R6.5, an additional set of experiments was performed with FMLP, PMA, and R6.5 as the activating agents. R6.5 stimulation was performed as described previously. Two aliquots of leukocyte-rich plasma devoid of R6.5 (one supplemented with PMA (1 µg/ml, final concentration) and the other left without supplements) were incubated at 37°C for a total of 10 min. A third aliquot was similarly incubated for 5 min, supplemented with FMLP (10−6 M), and further incubated for 5 min. Labeling of leukocytes was performed according to standard procedures.
In three independent experiments, the ranges of fold increases in CD11b fluorescence intensity were as follows: 14.1–18.1 (induced by R6.5), 13.6–18.1 (induced by FMLP), and 31.1–39.9 (induced by PMA). The ranges of decreases in CD62L fluorescence intensity were 53–64, 99–100, and 100%, respectively. Finally, the ranges of fold increases in DCF fluorescence intensity were 2.1–2.6, 0.9–1.1, and 10.3–15.0, respectively.

The irrelevant anti-Ox mAb did not affect CD11b or DCF fluorescence intensity. Furthermore, the neutrophil responses induced by the intact R6.5 were not observed with F(ab\(^9\))\(_2\) fragments of R6.5 or with the IgG1 anti-ICAM-1 mAb RR1/1 (Fig. 2). Taken together, the results indicate that neutrophil activation was specific for R6.5 and dependent upon the IgG2a Fc fragment of the mAb. In an attempt to rule out the action of a possibly precipitated fraction of R6.5, we repeated the experiment presented in Fig. 1 with three volunteers, using a microfiltered solution of the mAb (100 \(\mu\)g/ml). This procedure did not alter the neutrophil-activating effect of R6.5 (data not shown).

Role of the complement system

As the observed neutrophil activation was dependent upon the Fc fragment of R6.5, we proceeded to study whether the complement system was involved: sCR1, a potent inhibitor of both the classical and alternative complement pathways, was used (32). In ACD-anticoagulated blood, sCR1 at 20 \(\mu\)g/ml completely abolished the increases in both the neutrophil CD11b and DCF fluorescence intensities induced by R6.5 (100 \(\mu\)g/ml) (Fig. 3A).

Finally, to confirm that the assay applied in the present study is sensitive enough to detect complement-dependent increases in neutrophil CD11b and DCF fluorescence intensities, we examined the effects of the murine IgG2a anti-CD3 mAb OKT3. OKT3 has cytolytic activity against T cells by virtue of its ability to induce Fc-triggered activation of the classical complement pathway (33) with widespread complement-mediated leukocyte responses, including neutrophil activation (34). In the whole blood assay, OKT3 (100 \(\mu\)g/ml blood) increased the neutrophil CD11b fluorescence intensity to 16.5 times the baseline level and increased the DCF fluorescence intensity to 2.3 times the baseline level (median results in three volunteers, Fig. 3B).

Discussion

The present results indicate that the intact IgG2a anti-ICAM-1 mAb R6.5, at clinically relevant concentrations, activates neutrophils in whole blood specimens, as shown by a markedly increased expression of CD11b, by decreased L-selectin expression, and by increased oxidative burst activity. The neutrophil response was entirely dependent upon the activated complement system. It is worth noting that murine mAbs of the IgG2a isotype may fix C1q of human complement (35). Indeed, we found that an anti-ICAM-1 mAb of the IgG1 isotype did not activate neutrophils.

The presence of activated circulating neutrophils (i.e., cells showing increased surface expression of CD11b/CD18) is well...
documented in patients with systemic inflammation (36–38), for which the level of CD11b expression relates to the occurrence of organ failure (39). Considering these facts, we did not extend our study to in vivo experiments on healthy volunteers.

Frequent adverse effects are reported after the i.v. administration of R6.5 to humans. In general, fever and leukopenia are typical of complement-mediated neutrophil activation in the circulation (36). In a clinical trial on 18 renal allograft recipients receiving R6.5, 3 patients (17%) complained of chills after the initial infusion (26). In the phase I/II study on 32 refractory rheumatoid arthritis patients receiving R6.5, 16 (50%) had fever, 7 (22%) complained of pruritus, and 3 (9%) developed urticaria (i.e., symptoms and signs common in generalized anaphylaxis). After the initial dose of R6.5, the number of circulating neutrophils decreased, but the difference between treatment and control groups was not significant (24). This may be due to the nominal function of R6.5 attenuating neutrophil emigration. In keeping with this possibility, ICAM-1-deficient mice present with moderate leukocytosis (19). Interestingly, in three studies on patients with severe rheumatoid arthritis treated with anti-CD4 mAbs, chills and fever were less frequent regardless of the isotype of the mAb used (40–42).

R6.5 has also been used to reduce ischemia-reperfusion injury in the setting of acute ischemic stroke. An international, multicenter, randomized, placebo-controlled, double-blind trial recruited 625 stroke patients. The results failed to suggest a beneficial effect on the clinical outcome. Mortality and infant sizes were slightly larger in the treatment group. Moreover, six cases of aseptic meningitis occurred in the group receiving R6.5, whereas no such cases were seen in the placebo group (43). Increased expression of endothelial ICAM-1 has been found in humans to be associated with neutrophil emigration into ischemic brain tissue (44). It is presently not known whether R6.5 can prevent neutrophil infiltration in human cerebral infarction.

In conclusion, our findings show that R6.5 activates neutrophil granulocytes in a complement-dependent manner. This could well explain the multiple adverse effects reported in patients treated with R6.5. The results presented will favor the use of a preparation of R6.5 that is devoid of complement-fixing capacity but able to mediate the valuable therapeutic function of specific binding to ICAM-1.

Acknowledgments

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References