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Different CD3/T cell receptor monoclonal antibodies have distinct capacities to induce adhesion of T lymphocytes to endothelium

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Murine CD3/T cell receptor (TCR) monoclonal antibodies (mAbs) induce immediate peripheral lymphocytopenias of different degree and duration. Lymphocytopenia is of short duration after the administration of immunoglobulin A CD3 mAb, but it persists much longer after the administration of immunoglobulin G2a CD3 mAb. Peripheral lymphocytopenia after the administration of WT31, a murine immunoglobulin G1 TCR mAb, appears to be dependent on the polymorphism of Fc γ RIIa. In high responders, lymphocytopenia is comparable to that observed after immunoglobulin G2a CD3 mAb; in low responders, no lymphocytopenia occurs. In vitro, both immunoglobulin A and immunoglobulin G2a CD3 mAbs induce immediate activation of CD11a/CD18, with concomitant up-regulation of CD11b/CD18 on T cells, each of which is shown to be involved in the concurrent adhesion of T cells to endothelium. WT31 induces an immediate activation of CD11a/CD18 as well as T cell adhesion to endothelium in Fc γ RIIa high responders only, interestingly without changes in the level of expression of CD11b/CD18. We conclude that the immediate occurrence of peripheral lymphocytopenia after the administration of CD3/TCR mAb is mediated by changes in the level of expression or avidity (or both) of adhesion molecules on T cells, whereas the persistence of this lymphocytopenia depends on the isotype of the CD3/TCR mAb and on the presence of suitable Fc receptors. (J Lab Clin Med 1997;130:91-101)

Abbreviations: CLB = Central Laboratory of the Netherlands Red Cross Blood Transfusion Service; FACS = fluorescence-activated cell sorter; Fc γ R = Fc gamma receptor; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; ICAM-1 = intercellular adhesion molecule-1; IgA = immunoglobulin A; IgG2a = immunoglobulin G2a; mAb = monoclonal antibody; MFI = mean fluorescence intensity; MHC = major histocompatibility complex; PBMC = peripheral blood mononuclear cell; PBS = phosphate-buffered saline solution; PE = phycoerythrin; TCR = T cell receptor; VCAM-1 = vascular cell adhesion molecule-1

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Supported by the Dutch Kidney Foundation (Grant C91.1158).

Submitted for publication Dec. 18, 1996; accepted Feb. 5, 1997.

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0022-2143/97 \$5.00 + 0 5/1/81594

Murine mAbs directed against the CD3/TCR complex induce peripheral lymphocytopenias of different severity and duration.¹⁻⁴ Previously we investigated the kinetics of lymphocytopenia after treatment with murine CD3/TCR mAbs of the IgG2a (i.e., 1X2a and OKT3) and IgA class (i.e., 1XA).¹⁻³ 1X2a and 1XA are switch variant mAbs of the same idiootype and react with the same epitope on the CD3 molecule, whereas OKT3 recognizes a different epitope. Two different patterns of peripheral lymphocytopenia were discerned after administration of these CD3/TCR

mAbs, depending on their isotype. Both CD3 mAbs of the *IgG2a* and *IgA* class induce a rapid depletion of lymphocytes from the peripheral blood compartment, but lymphocytopenia after *IgG2a* CD3 mAb is persistent, whereas lymphocytopenia after *IgA* CD3 mAb is quickly reversed.¹⁻³ Because both *IgG2a* CD3 mAb and *IgA* CD3 mAb induce an immediate transient activation and up-regulation of adhesion molecules *in vitro*, paralleled by transient adhesion of T cells to a monolayer of vascular endothelial cells, we postulated that the mechanism of disappearance of T cells from the peripheral blood compartment might be the binding of these cells to the endothelium of the peripheral lymphoid organs.⁴ The persistence of peripheral lymphocytopenia, then, might be mediated by at least two other, possibly cooperating, mechanisms: (1) opsonisation of T cells by CD3 mAb and complement, with sequestration of these cells in the mononuclear phagocyte system by means of binding to Fc and complement receptors; (2) up-regulation of the adhesion molecules VCAM-1 and ICAM-1 on the endothelium induced by cytokines such as tumor necrosis factor- α and interferon γ , which are known to be released after the administration of CD3 mAb of the *IgG2a* class.^{5,6} The latter leads to increased adhesiveness of endothelium to the counterstructures present on peripheral blood lymphocytes.⁷⁻¹⁰ The murine *IgA* isotype switch variant CD3 mAb does not bind to human Fc receptors, does not activate lymphocytes, and hardly results in the release of cytokines. Moreover, monomeric murine *IgA* is generally assumed to have poor activating capacity for human complement.^{2,11} This may explain why administration of this mAb *in vivo* induces a rapid lymphocytopenia that is transient.

Recently it was shown that a murine CD3/TCR mAb of the *IgG1* isotype (i.e., WT31) induces a long-lasting T lymphocytopenia comparable to that observed after CD3 mAb of the *IgG2a* class only in 70% of the patient population.^{12,13} This may be explained by polymorphism of Fc γ RIIa, the human Fc receptor for murine *IgG1*, of which two allelic forms exist. They have been classified as Fc γ RIIa-H131 and Fc γ RIIa-R131, according to their binding capacity to murine *IgG1*.¹⁴ Seventy percent of the human population carries the Fc γ RIIa-R131 form (high responders), whereas the other 30% of the population bears the Fc γ RIIa-H131 form (low responders).¹⁵ The administration of WT31 to patients of the high-responder phenotype induces T cell proliferation, cytokine release, and the occurrence of adverse effects. In contrast, in low-responder patients, WT31 does not induce the release

of cytokines, does not lead to adverse effects *in vivo*, and does not stimulate lymphocytes *in vitro*.^{13,15,16} However, it is not clear whether WT31 can activate or up-regulate the expression of adhesion molecules on lymphocytes and endothelium, as can CD3 mAbs of the *IgG2a* or *IgA* class, nor whether it can fix complement, as can *IgG2a* CD3 mAb.

To elucidate the role of CD11a/CD18 and CD11b/CD18 and Fc receptor binding in the pathogenesis of lymphocytopenia, CD3/TCR mAbs of three different isotypes were tested on their capacity to activate or up-regulate expression of adhesion molecules on T lymphocytes *in vitro* and their capacity to induce adhesion of these T cells to endothelial cell monolayers. Blocking experiments were performed to study which adhesion molecule(s) are involved. In addition, clinical findings were correlated to our *in vitro* results.

METHODS

Patients. In 20 kidney transplant recipients who were treated prophylactically with either the *IgG2a* (1X2a) or the *IgA* (1XA) switch variant CD3 mAb—which was administered twice daily, 0.5 mg intravenously for 10 days—peripheral lymphocyte counts were determined immediately before the first administration of CD3 mAb and at 15 minutes, 30 minutes, and 1, 3, and 6 hours thereafter.² The first dose was preceded by 500 mg methylprednisolone and 25 mg promethazine, administered 1 hour before the CD3 mAb.

In addition, the number of lymphocytes in time was measured after the first administration of WT31 in 6 renal transplant recipients with acute rejection.¹³ The antibody had been purified at the Dutch National Institute of Public Health and Environmental Protection.¹⁷ Three of these patients were high responders and 3 were low responders, as determined by *in vitro* T cell proliferation induced by WT31.¹⁵ Each of the 3 low responders and the first treated high responder received an initial intravenous dose of 6 mg WT31, whereas in the other two high responders an initial dose of 1 mg was given. In 1 out of 3 low-responder and 2 out of 3 high-responder patients, the first WT31 dose was preceded by 50 mg prednisone, whereas the other patients did not receive any premedication.

Healthy control individuals. In 20 healthy control individuals the phenotype of Fc γ RIIa was determined on granulocytes and monocytes by indirect immunofluorescence.¹⁸ The mAbs used to discriminate Fc γ RIIa-H131 and Fc γ RIIa-R131 donors were IV.3 (Medarex, Lebanon, N.H.), which bind both allotypic forms of Fc γ RIIa, and 41H16 (provided by Dr. B. M. Longenecker, Edmonton, Alberta, Canada), which selectively recognizes the Fc γ RIIa-R131 allotype.^{19,20} FITC-labeled F(ab')₂ fragments of goat anti-mouse immunoglobulin (G26M17F, CLB, Amsterdam, the Netherlands) were used as secondary anti-

body. All incubations for phenotypic analysis were performed in the presence of 20% (vol/vol) heat-inactivated (30 minutes at 56° C) human serum to avoid cytophilic binding of mouse mAbs. Fluorescence was quantitated with the flow cytometer (FACScan; Becton Dickinson, Mountain View, Calif.). To confirm FcγRIIIa-H131 and FcγRIIIa-R131 phenotypes, mononuclear cells were also tested in a T cell proliferation assay, with murine IgG1 and human IgG2 anti-CD3 mAbs used as stimulants.^{15,18} From these healthy control individuals 6 high responders and 6 low responders were selected, and peripheral blood was taken for in vitro studies.

Lymphocyte counts. Total leukocyte and differential counts were determined by flow cytometry (Technicon H1 system; Bayer/Technicon, Tarrytown, N.Y.) in blood anticoagulated with ethylenediaminetetraacetic acid.

In vitro studies: Adhesion molecule expression. Heparinized peripheral blood samples from 12 healthy individuals, 6 high responders and 6 low responders, were put in a waterbath of 37° C. A 5 ml sample was kept apart, and the remaining blood was divided into multiple aliquots, which were each incubated with one of the following mAbs in different concentrations: the murine isotype switch variant CD3 mAbs 1X2a (IgG2a isotype) and 1XA (IgA isotype), the murine TCR mAb WT31 (IgG1 isotype), and the following irrelevant murine isotype control antibodies: F23-49 (mouse IgG2a, directed against *Mycobacterium tuberculosis*),²¹ TEPC15 (mouse IgAκ myeloma protein; Sigma, St. Louis, Mo.), or CLB-M1451 (mouse IgG1, directed against plant allergens; CLB). To study whether the effects on expression of adhesion molecules were specific for antibodies directed to the CD3/TCR complex, the same experiments were also performed with murine IgG2a mAbs directed against either the CD4 molecule (CLB-T4) or against a MHC class I antigen (W6/32). At 3, 30, and 60 minutes after addition of the mAbs, whole blood samples were taken. PBMCs were obtained by centrifugation of heparinized blood on Ficoll-Paque (density 1.076; Pharmacia, Uppsala, Sweden). Contaminating erythrocytes were lysed by incubation with NH₄Cl for 10 minutes on ice, followed by 2× washing of the PBMCs in PBS containing 0.5% wt/vol bovine serum albumin and 0.01% wt/vol sodium azide. The PBMCs were incubated for 30 minutes at 0° C in PBS containing 0.5% wt/vol bovine serum albumin and 0.01% wt/vol sodium azide with appropriate dilutions of the mAb CD11a-FITC (Dako; clone MHM24,IgG1k), [NKI-L16]-FITC, which recognizes a Ca²⁺-dependent epitope on the CD11a/CD18 complex that is expressed only on activation of the complex^{22,23} or CD11b-PE (Dako; clone 2LPM19c,IgG1k). In addition, part of the cells was incubated with the biotinylated mAb LECAM-1 (directed against L selectin, clone SK11, IgG2a; Becton Dickinson), followed by incubation with PE-labeled streptavidin (Becton Dickinson). Next, we performed two-color staining of these samples with a CD3 mAb to determine the total number of T cells. Because two-color staining with a FITC or PE-labeled detecting CD3 mAb might be hampered by

the in vitro bound CD3/TCR mAb, before these experiments we studied which detecting CD3 mAb could be used for optimal detection of T cells without causing interference with the already bound CD3/TCR mAb. In the case of WT31, at any concentration tested, two-color staining was possible with CD3-PE or CD3-FITC (both from Becton Dickinson; Leu-4; clone SK7, IgG1). In experiments with 1X2a and 1XA, two-color staining was optimal when using a mixture of directly labeled CD4 and CD8 mAb. In control experiments, the mixture of these CD4 and CD8 mAbs (Becton Dickinson; Leu-3a; clone SK3, IgG1 and Leu-2a; clone SK1, IgG1, respectively) was shown to detect numbers of T cells equal to the number detected by the Leu-4 mAb. Determination of the total number of CD3⁺ cells was performed by a direct labeling procedure with Leu-4-FITC (Becton Dickinson).

The same experiments were performed on PBMCs incubated with CD3/TCR mAbs after their isolation from whole blood by Ficoll density gradient centrifugation.

Analysis of cell suspensions was done on FACScan (Becton Dickinson). Lymphocytes in the PBMC population were gated on forward and sideward scatter.

Functional adhesion studies. Functional adhesion studies were performed in parallel with the above-described assays. PBMCs from the same 12 healthy control individuals were washed 3 times in Tris-buffered Earle's balanced salt solution containing 5% FCS. CD3⁺ cells were obtained as follows: B lymphocytes, monocytes, and natural killer cells were removed by incubation with murine mAb anti-CD19 (CLB, clone 11G1), anti-CD14 (CLB, clone 8G3), and anti-CD16 (CLB, gran-1) followed by incubation with sheep-anti-mouse IgG-coated Dynabeads (N-0212; Dynal A.S., Oslo, Norway). This procedure resulted in >95% pure CD3⁺ cell populations, as was confirmed by FACS analysis after staining with FITC-labeled CD3- and PE-labeled CD16 and CD56 mAbs (NK-Simulset; Becton Dickinson). CD3⁺ cells were resuspended and adjusted to a concentration of 2 × 10⁶ cells/ml and incubated on ice for 30 minutes with either 1X2a, 1XA, WT31, CLB-T4, W6/32, or the irrelevant isotype control antibodies F23-49, TEPC15, and CLB-M1451 in the indicated concentrations. Phorbol myristate acetate PMA, 50 ng/ml (Sigma), was used as a positive control. Next, the cells were washed 2 times at 4° C with Iscove's modified Dulbecco's medium containing 5% FCS. Because cross-linking is assumed to occur in vivo after the administration of CD3 mAb of the IgG2a class and after administration of WT31 to patients with the high-responder phenotype, adhesion studies were also performed after cross-linking of the mAb-coated CD3/TCR complex with goat-anti-mouse Ig. Therefore, after incubation with the T cell mAb, one half of the cell suspension was cross-linked by 2 μg/ml goat anti-mouse Ig (GM17D, CLB), immediately followed by 3 minutes of 10 g centrifugation at 37° C; no goat-anti-mouse Ig was added to the other half.²⁴ Then 25 μl of these cell suspensions was added to a monolayer of an immortalized endothelial cell line, which is derived from human umbilical vein endothelial cells,²⁵ and was

cultured in 96-well plates (Nunc Micro Well F96; Nunc A.S., Roskilde, Denmark) on 1% gelatin (G1890; Sigma) in PBS. The remaining part of the cell suspensions was put in a shaking waterbath at 37° C, from which samples were added to endothelial cell monolayers after 30 and 60 minutes. The 96-well plates were incubated for 15 minutes at 37° C (5% CO₂). Then the nonadherent fraction was removed by washing three times with 200 µl of 37° C Earle's balanced salt solution containing 5% FCS. The adherent fraction was stained with Diff-Quick (i.e., a three-step staining set consisting of [1] fixation in methanol with fast green, 0.002 gm/L; [2] eosin G in phosphate buffer, pH 6.6; and [3] thiazine dye in phosphate buffer, pH 6.6 [Baxter Dade A.G, Düringen, Switzerland]). All experiments were performed in triplicate.

Blocking studies were performed with mAbs directed against either the common β_2 integrin chain (CD18: TS 1/18,²⁶ against CD11a [NKI-L7]²⁷ or against CD11b [Bear-1]²⁸) in a final concentration of 10 µg/ml, which was added to the endothelial cell monolayer simultaneously with the T cells that had been incubated with the different CD3/TCR mAbs for 3 minutes at 37° C. This concentration of 10 µg/ml had shown in previous studies to result in a maximal inhibition of adhesion.

The adhesion was scored blindly by 3 technicians independently in a semiquantitative way (score ranging from 0 to 5; 0 = no T cells adherent to endothelium; 1 = <50 T cells/field; 2 = 50 to 100 T cells/field; 3 = 100 to 250 T cells/field; 4 = 250 to 350 T cells/field; 5 = >350 T cells/field; scored at a magnification of $\times 200$).

With therapeutic plasma levels considered to be about 1 µg/ml, the studies on expression of lymphocyte adhesion molecules as well as the adhesion studies were performed with different concentrations of the mAb, as follows: for WT31, 0.01, 0.1, 1 and 10 µg/ml; for 1X2a and 1XA, 0.01, 0.1, 1 and 5 µg/ml.

T cell binding studies. Differences between the three T cell mAbs in their binding to T cells might influence the degree of activation or up-regulation of adhesion molecules. Therefore, the T cell binding of the three mAbs was analyzed as follows: PBMCs from 4 healthy control individuals were isolated by Ficoll-density gradient centrifugation and incubated for 30 minutes on ice with 1X2a, 1XA, WT31, or the irrelevant murine isotype control antibodies in a concentration ranging from 0 to 4 µg/ml. The cells were washed, and subsequently PE-labeled rabbit F(ab')₂ anti-mouse Ig was added (code R 0439; Dako, Glostrup, Denmark) at multiple dilutions ranging from 1:5 to 1:160. After 30 minutes of incubation on ice, the cells were washed and the MFI was measured by FACS analysis with a gate to identify the lymphocyte fraction. Because binding of the detecting rabbit F(ab')₂ anti-mouse Ig may be different for the diverse murine isotype mAbs, the actual measured MFI values cannot be compared. Therefore, the concentration of T cell mAb that was needed to reach a 50% value of the maximal level in MFI was used as a measure for T cell binding.

Complement studies. The ability of each of the three T cell mAbs to induce binding of C3 activation products on T cells was determined by FACS analysis. Heparinized whole blood samples from 6 healthy high-responder and 6 healthy low-responder control individuals were incubated for 15 minutes at 37° C with 1 µg/ml 1X2a, 1XA, WT31, or the irrelevant murine isotype control antibodies. Mononuclear cells were isolated by centrifugation on Ficoll-paque. Deposition of C3 activation products on T cells was detected with a biotinylated mAb directed against a neo-epitope on C3b, C3bi, and C3c (C3-28, CLB²⁹), followed by a second incubation step with PE-labeled streptavidin and a third incubation with a FITC-labeled CD3 mAb (Leu-4; Becton Dickinson).

Calculations and statistical analysis

Absolute number of double-positive cells. The percentage of double-positive cells was calculated by means of PCLYSYS software from Becton Dickinson and was multiplied times the absolute number of lymphocytes at the same time point, resulting in the absolute number of double-positive cells. All values are expressed as mean \pm SEM.

MFI measurements. At all time points the MFI was calculated with PCLYSYS software. All values are expressed as mean \pm SEM.

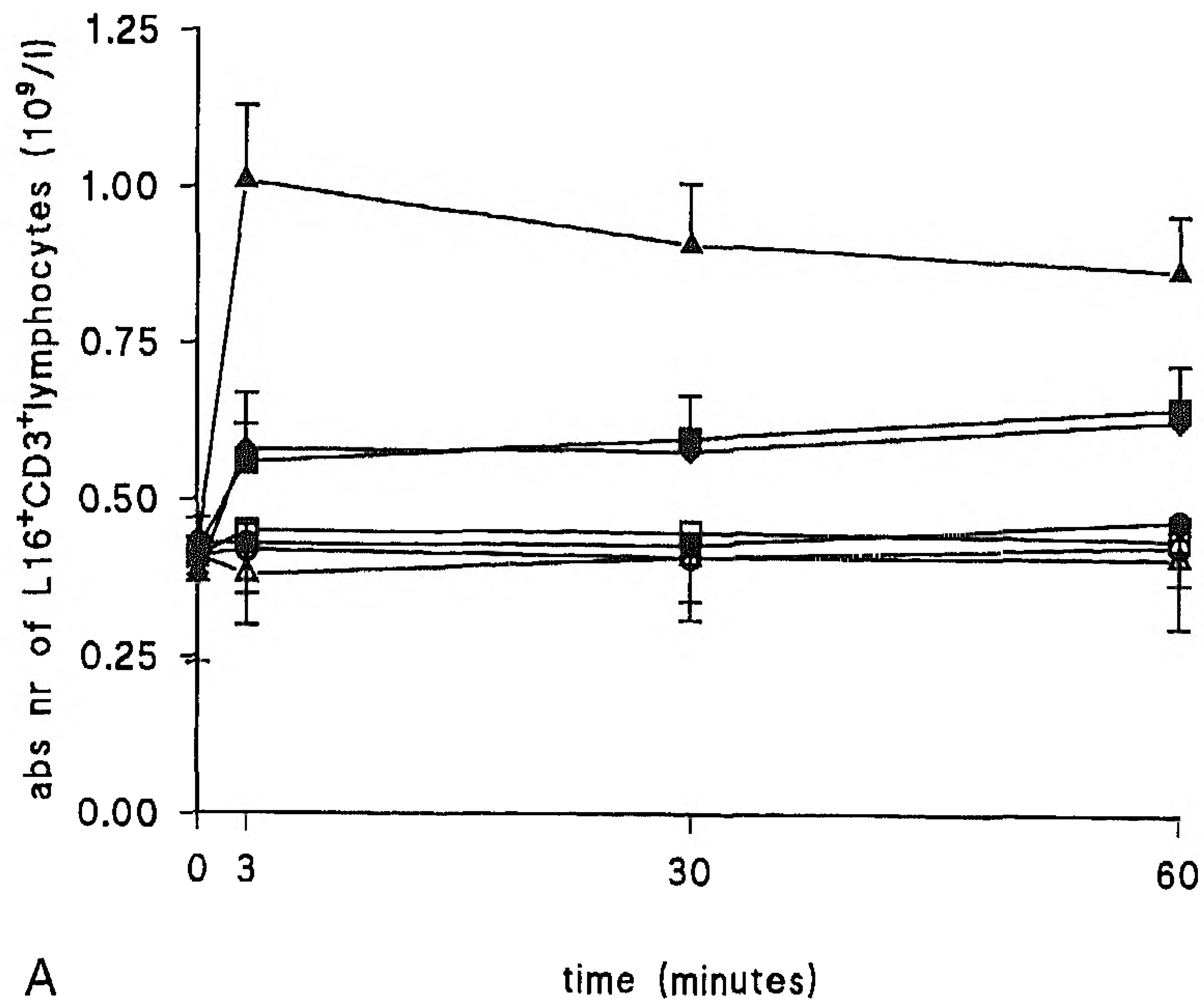
RESULTS

Lymphocytopenia after in vivo administration of different CD3/T cell mAbs. Administration of either 1X2a or 1XA induced a disappearance of virtually all T lymphocytes from the peripheral blood compartment within 15 minutes. However, CD3 depletion after 1XA was of shorter duration than that after 1X2a administration; whereas at least during the first 2 days of IgG2a treatment the number of CD3⁺ cells remained low as compared with pretreatment values, 1XA-induced T cell depletion lasted for several hours only.^{1,2} After administration of the IgG1 TCR mAb WT31, the occurrence of a peripheral lymphocytopenia appeared to be dependent on the Fc γ receptor IIa status of the patient receiving WT31. Also, in high-responder patients an immediate and long-lasting disappearance of lymphocytes was observed after the administration of WT31, whereas in low-responder patients the number of peripheral blood lymphocytes remained unchanged (data not shown). On the basis of these results, two phases with different underlying pathogenetic mechanisms can be distinguished in the CD3/TCR mAb-induced peripheral T lymphocytopenia: (1) an immediate decline in the number of peripheral T lymphocytes and (2) persistence of the peripheral lymphocytopenia.

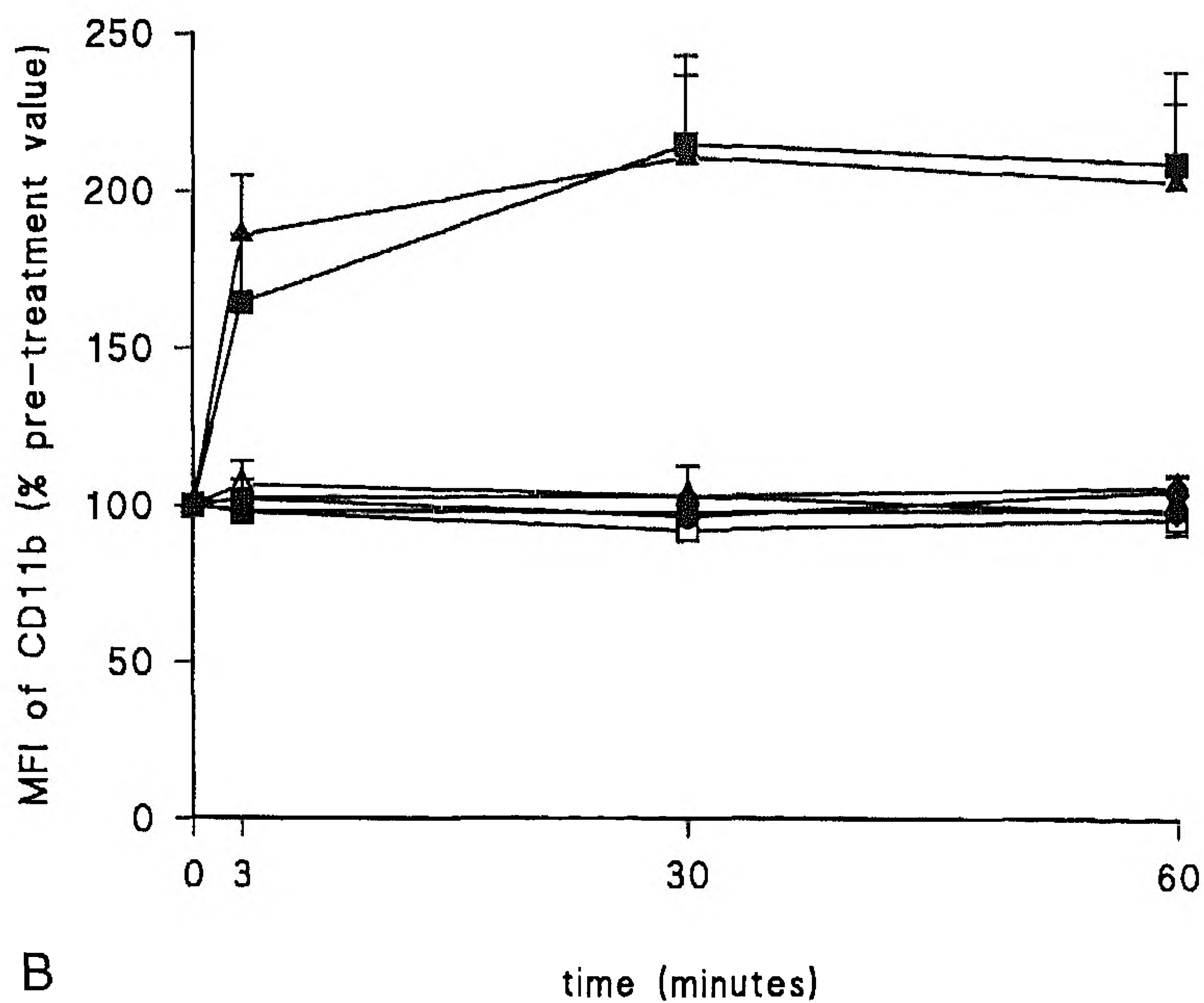
In vitro effects of CD3/TCR mAbs

Expression of adhesion molecules in vitro

MABS DIRECTED AGAINST CD3. In each of the 12 healthy control individuals, the addition of 1 µg/ml



A



B

Fig. 1. Comparison of the effect of 1X2a, 1XA, and WT31 in high responders or WT31 in low responders on the expression of NKI-L16 (directed against the activated form of CD11a/CD18) and CD11b on peripheral blood T lymphocytes from healthy control individuals. Depicted are the number of NKI-L16⁺CD3⁺ lymphocytes (A) and the MFI of CD11b on CD3⁺ T cells, presented as a percentage of the preaddition value (B) after the administration of 1 μg/ml of either 1X2a (▲, n = 12), 1XA (■, n = 12), or WT31 to whole blood samples of high responders (◆, n = 6) or low responders (●, n = 6). *Open symbols* show the effect of 1 μg/ml of the irrelevant murine control monoclonal antibodies of the IgG2a (Δ), IgA (□), or IgG1 (○) isotype. Results are represented as mean ± SEM.

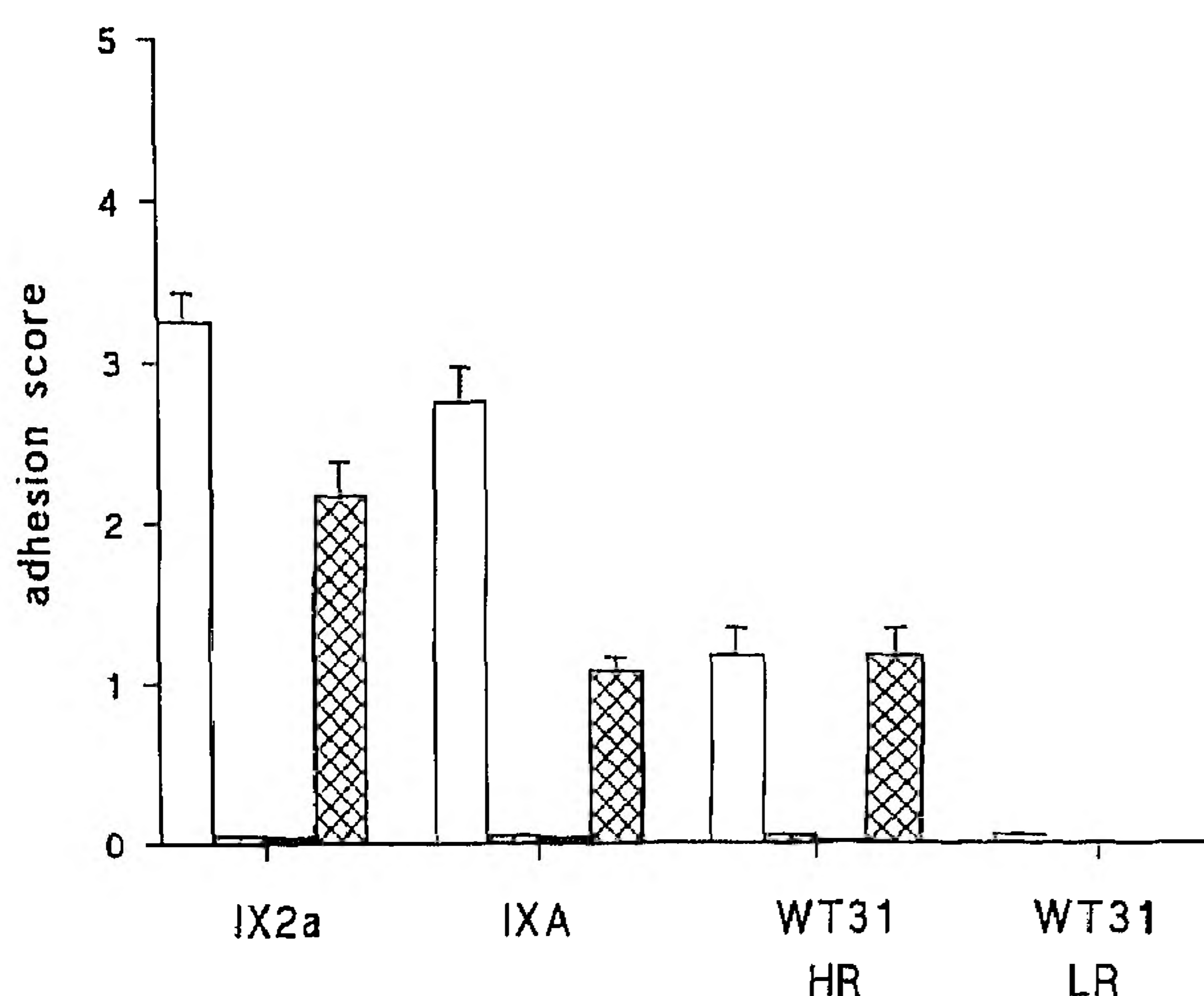


Fig. 2. Comparison of the effect of 1 $\mu\text{g/ml}$ 1X2a, 1XA, or WT31 in high responders or WT31 in low responders on the capacity to induce adhesion of purified T cells to monolayers of endothelial cells (open bars) and blocking of this adhesion by mAbs against CD11a (hatched bars) against CD18 (solid bars) or against CD11b (cross-hatched bars). These results were obtained after 3 minutes of incubation with the respective CD3/TCR mAbs, without cross-linking by goat-anti-mouse Ig. Results are represented as mean \pm SEM for 6 healthy high responders and 6 healthy low responders for WT31 and for all 12 individuals for 1X2a and 1XA.

1X2a or 1XA to whole blood induced an immediate increase in the number of T cells bearing the activated form of CD11a/CD18, as detected by NKI-L16 expression, and an up-regulation of the CD11b/CD18 molecule (Fig. 1).

MABS DIRECTED AGAINST THE TCR. Incubation of whole blood from 6 healthy high responders with 1 $\mu\text{g/ml}$ WT31 induced an immediate increase in the number of NKI-L16-positive T cells. In contrast, no change was observed after the addition of WT31 to whole blood from 6 healthy low responders (Fig. 1, A). No changes in CD11b expression were observed, either in the high responders or in the low responders, while the addition of 1 $\mu\text{g/ml}$ 1X2a or 1XA in the same experiments did induce an increase in CD11b expression (Fig. 1, B). No effects on L-selectin expression, either in percentage of positive cells or in mean fluorescence intensity, were observed on CD3⁺ T cells after the addition of either 1XA, 1X2a, or WT31. Incubation of whole blood with 1 $\mu\text{g/ml}$ of the irrelevant murine mAbs of the IgG2a, IgA, or IgG1 isotype did not alter the expression of NKI-L16 or CD11b (Fig. 1, A and B), nor did incubation with the CD4 or the MHC class I mAb (data not shown). Dose-response studies showed a maximal effect on NKI-L16 and CD11b expression at a concentration of 1 $\mu\text{g/ml}$ for 1X2a or

1XA. Regarding WT31, increasing the concentration to 10 $\mu\text{g/ml}$ had only a small additional effect on NKI-L16 expression in high responders but did not affect the NKI-L16 expression in low responders. Even at this high concentration of WT31, no changes in CD11b expression were observed in high responders or low responders (data not shown). When similar experiments were performed on PBMCs instead of whole blood, the same results were obtained (data not shown).

Functional adhesion studies. Purified T cells from each of the 12 healthy control individuals showed an immediate adhesion to endothelial cell monolayers after incubation for 3 minutes with 1 $\mu\text{g/ml}$ 1X2a or 1XA (Fig. 2). For WT31, a discrimination could be made between high responders and low responders: in high responders, incubation with 1 $\mu\text{g/ml}$ WT31 also induced adhesion of T cells to endothelium, whereas no T cell adhesion could be detected in the low-responder group, when adhesion was studied without cross-linking (Fig. 2). The irrelevant murine isotype control antibodies, the CD4, or the MHC class I mAbs also did not affect adhesion of T cells to endothelial cell monolayers (not shown). Incubation with 50 ng/ml phorbol myristate acetate, used as a positive control, resulted in an adhesion score of 3.75 ± 0.22 after 3 minutes. Dose-response curves showed that the T cell mAb-induced adhesion was dose dependent. A maximal adhesion was observed with 1 $\mu\text{g/ml}$ of each of the three mAbs tested. For WT31, increasing the dose to 10 $\mu\text{g/ml}$ still did not result in adhesion of T cells to endothelial cell monolayers in the low-responder group. Adhesion blocking studies showed that coincubation with either a CD11a or a CD18 mAb completely inhibited the adhesion observed after 3 minutes of incubation with either 1X2a, 1XA, or WT31. However, coincubation with a CD11b mAb resulted in partial inhibition of the CD3 mAb-induced adhesion but did not suppress the WT31-induced adhesion (Fig. 2). Studies in which incubation with the T cell mAb was followed by cross-linking with goat-anti-mouse Ig showed that with each of the three mAbs, subsequent cross-linking resulted in higher adhesion scores as compared with preincubation with the same mAb without cross-linking. After cross-linking, adhesion was also induced by WT31 in the low responders (data not shown). For the CD3 mAbs 1X2a and 1XA, adhesion was maximal after 3 minutes of cross-linking at 37° C, followed by a decrease. For WT31, maximal adhesion was observed after 30 minutes of cross-linking, both in high responders and in low responders (data not shown).

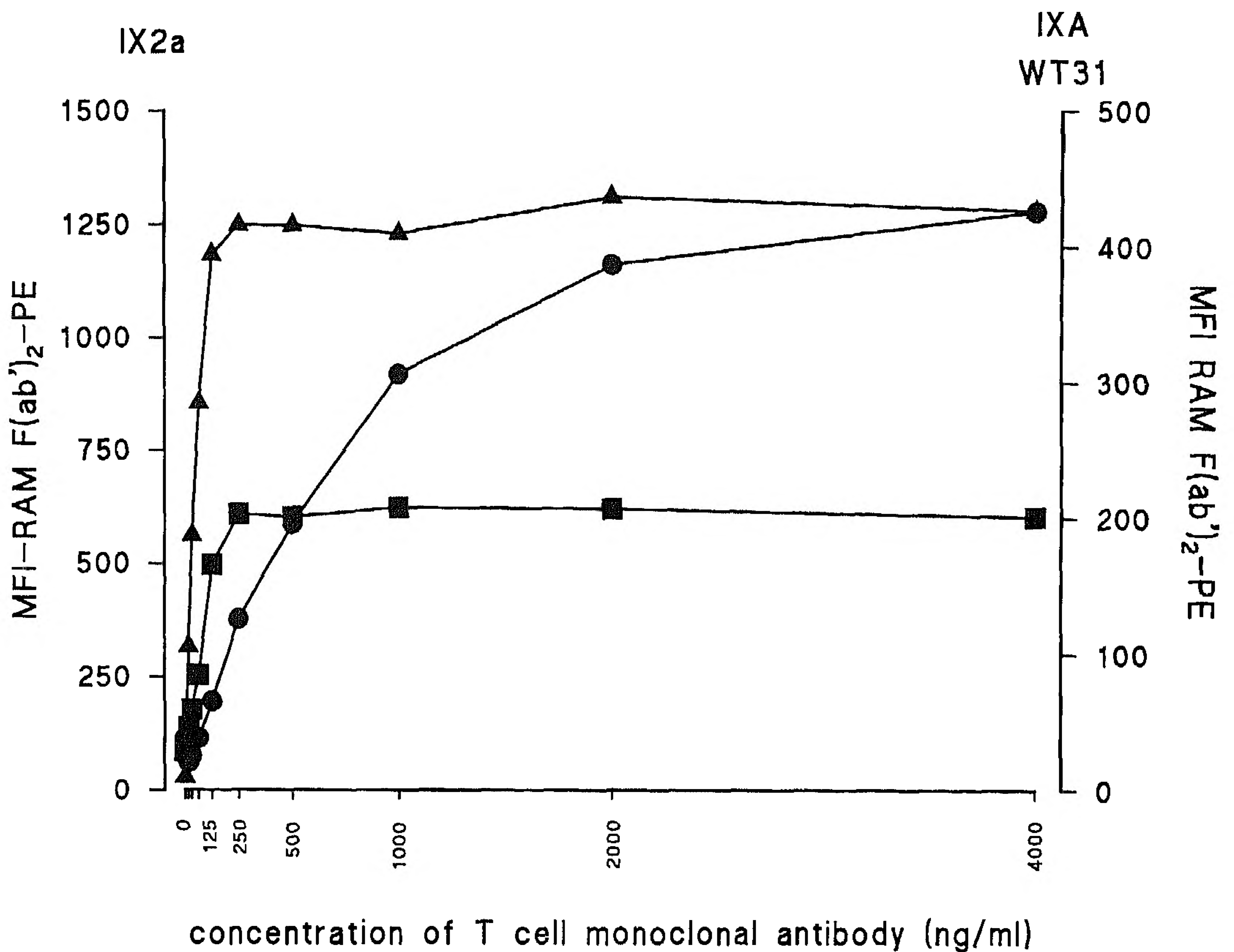


Fig. 3. Affinity of 1X2a (▲), 1XA (■), and WT31 (●) for T cell binding, as detected by FACS analysis. Depicted is the MFI of PE-labeled rabbit F(ab')₂ fragments-anti-mouse Ig. The data of one representative out of four experiments is shown.

T cell binding studies. Fig. 3 shows a difference in T cell binding between the CD3 mAbs 1X2a and 1XA on the one hand and WT31 on the other hand. When an optimal dilution (1:10) of the detecting PE-labeled rabbit F(ab')₂ anti-mouse Ig conjugate was used, for the CD3 mAbs 1X2a and 1XA a 50% value of the maximal MFI could already be reached at concentrations of 41.5 and 76.5 ng/ml, respectively, whereas for WT31 a higher concentration (585 ng/ml) was needed. This suggests that WT31 has a lower affinity for binding to the CD3/TCR complex than do the switch variant CD3 mAbs 1X2a and 1XA.

Complement activation. Fixation of C3 activation products on T cells could be observed only after incubation of whole blood with the IgG2a mAb 1X2a, not after incubation with the IgA switch variant 1XA or the IgG1 TCR mAb WT31 (Fig. 4). Increasing the 1XA or WT31 dose to 10 μg/ml still could not induce any fixation of C3 activation products on T cells. No differences were observed between high responders and low responders concern-

ing the ability to induce deposition of C3 activation products on T cells (not shown).

DISCUSSION

To interpret the CD3/TCR mAb-induced lymphocytopenia in our different patient groups, the preadministered corticosteroid dose has to be taken into account. To diminish first-dose side effects, the first administration of CD3/TCR mAb is generally preceded by the administration of a single dose of corticosteroids, which are known to induce a reversible peripheral lymphocytopenia by themselves.^{30,31} This lymphocytopenia starts 30 minutes after corticosteroid administration and reaches its maximum effect in about 6 hours.^{30,31} For this reason it is unlikely that corticosteroids have something to do with the immediate decline in peripheral T lymphocytes after CD3/TCR mAb administration. However, persistence of the lymphocytopenia might be modified by corticosteroids. In chimpanzees, administration of the IgA switch variant CD3 mAb without corticosteroid premedication induced a clear saw-

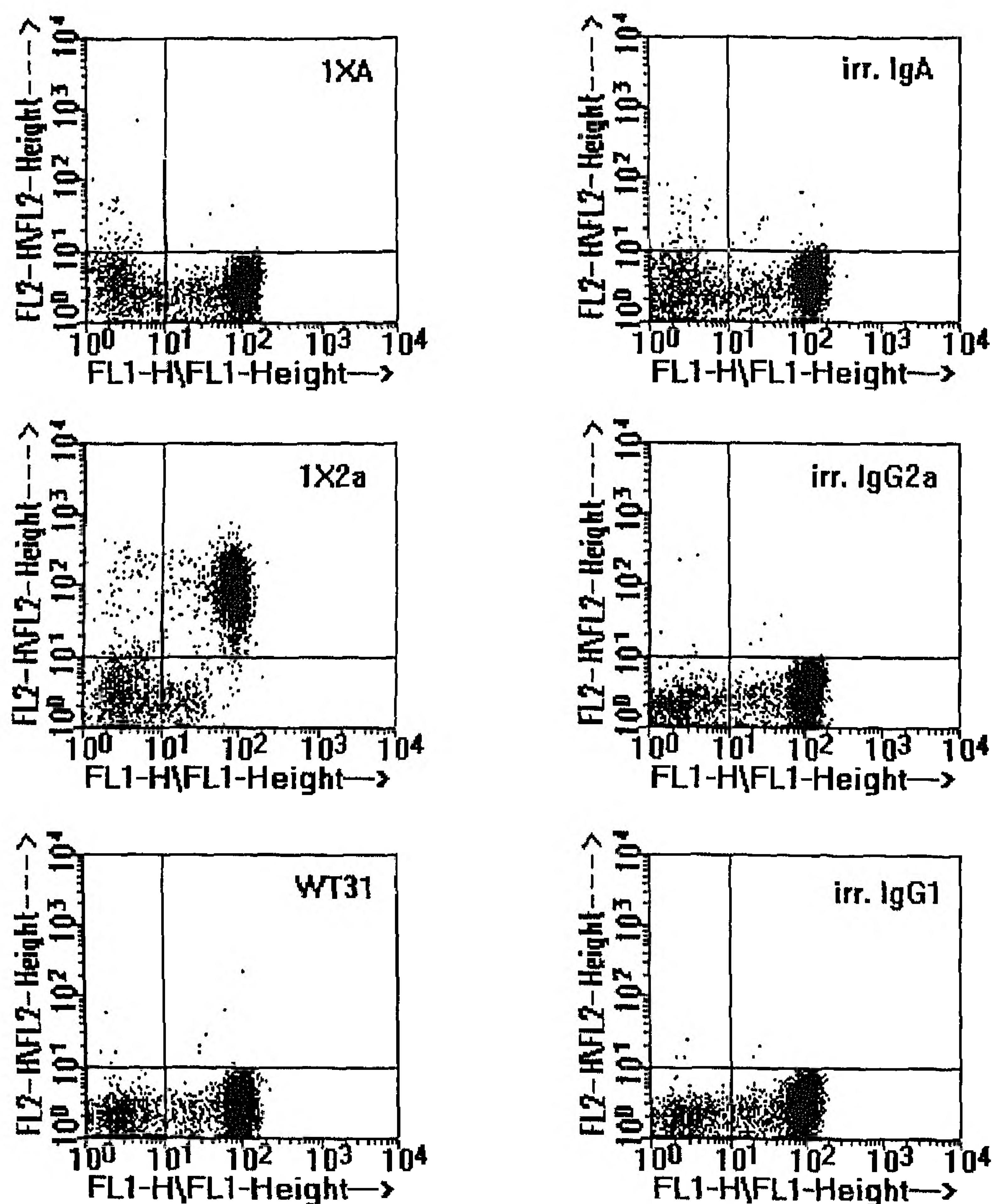


Fig. 4. Fixation of C3 activation products on T cells after incubation of heparinized whole blood from a healthy high responder for 15 minutes at 37° C with 1 μ g/ml 1X2a, 1XA, WT31, or the irrelevant isotype control antibodies. FL-1 depicted on the x axis represents binding of a FITC-labeled CD3 mAbs; FL-2 depicted on the y axis represents binding of the biotinylated C3-28 mAb (which recognizes C3 activation products) as detected with PE-labeled streptavidin.

tooth curve with respect to the number of T cells: a very fast decline and an almost complete recovery within 24 hours.¹ This effect is also visible in human subjects on day 8 of the treatment with the IgA switch variant mAb CD3. On day 1 the recovery is partial but clearly present, and is apparently modified by pretreatment with high-dose corticosteroids.² For the reasons mentioned we feel it justified to discuss patterns of CD3 lymphocytopenia even after corticosteroid administration.

Theoretically, three mechanisms may be involved in the CD3/TCR mAb-induced disappearance of T lymphocytes from the peripheral blood compartment: (1) activation up-regulation (or both) of adhesion molecules on lymphocytes resulting in adhesion to vascular endothelium; (2) Fc-Fc receptor interaction with activation of mononuclear cells re-

sulting in systemic cytokine release and leading to up-regulation of adhesion molecules on vascular endothelium; (3) interaction of the Fc part of the mAb with an appropriate Fc receptor or coating of T cells by complement activation products (or both interaction and coating), giving rise to sequestration of the cells in the mononuclear phagocyte system, eventually followed by lysis of these cells. However, in a previous article we presented arguments that make it unlikely that cell lysis is a major mechanism in the observed lymphocytopenia.³²

On account of the observations in vivo, two phases can be discerned in the peripheral lymphocytopenia after CD3/TCR mAb, and these may have different underlying pathogenetic mechanisms (summarized in Table I). For antibodies that induce an immediate decline in the number of T lympho-

Table 1. Summary of in vitro effects of CD3/TCR mAb

mAb	Peripheral T lymphocytopenia	Expression of adhesion molecules		Adhesion to endothelium without cross-linking	Effect of inhibitory mAb			Suitable Fc receptors available	Complement fixation on T cells
		Number of (NK1-L16) ⁺ T cells	MFI of CD11b on T cells		CD11a mAb	CD18 mAb	CD11b mAb		
1X2a	Immediate, long lasting	↑ ↑ ↑	↑ ↑	+++	Total	Total	Partial	Yes	Yes
1XA	Immediate, short lasting	↑ ↑	↑ ↑	++	Total	Total	Partial	No	No
WT31 HR	Immediate, long lasting	↑ ↑	—	++	Total	Total	No	Yes	No
WT31 LR	No lymphocytopenia	—	—	—	ND	ND	ND	No	No

ND, Not determined.

cytes, the common denominator is that they induce both activation and an increase in expression of adhesion molecules in vitro. CD3 mAbs of either the IgG2a or the IgA isotype were able to induce a transient adhesion of T cells to endothelial cells, and this could completely be blocked by either a CD11a or CD18 mAb and partially by a CD11b mAb. Therefore, activation of the CD11a/CD18 molecule seems to be a prerequisite for the CD3 mAb-induced T cell adhesion to endothelium, but increased expression of CD11b may be needed for optimal CD3 mAb-induced adhesion. WT31 could induce adhesion of T cells from high responders to endothelium without apparent involvement of CD11b. Because administration of WT31 in vivo to high responders is able to induce an immediate peripheral T cell lymphocytopenia, we conclude that up-regulation of the CD11b/CD18 molecule is not essential for the induction of such a peripheral T lymphocytopenia. In low responders, the WT31 mAb appeared to induce neither activation nor up-regulation of adhesion molecules on T cells. In agreement with this observation, no immediate peripheral lymphocytopenia was observed after WT31 administration to low responder patients.

The difference in ability to induce an up-regulation of CD11b between CD3 mAbs and the TCR mAb WT31 is difficult to explain. Because each of the studied mAbs binds to the CD3/TCR complex, a difference in intracellular signal transduction is unlikely to be responsible. The lower affinity of WT31 as compared with that of the CD3 mAb for their respective ligands may play a role in the observed differences, although other yet-unknown factors may be involved as well. In vivo up-regulation of CD11b/CD18 probably results from a direct signaling pathway that is initiated by binding of CD3/TCR mAbs to the CD3/TCR molecule and leads to stimulation of the inositol phospholipid metabolism, thereby giving rise to activation of protein kinase C and Ca²⁺ fluxes, which in turn may lead to up-

regulation of CD11b/CD18 expression on T cells.^{33,34} Although 1XA is a nonactivating mAb, it is able to induce Ca²⁺ mobilization in peripheral blood T lymphocytes,³⁵ which may be involved in the observed activation of CD11a/CD18 and increased expression of CD11b/CD18.

The common denominator for antibodies that induce persistence of peripheral lymphocytopenia is interaction of the Fc part of the mAb with an appropriate Fc receptor. Indeed, persistence is not observed after administration of 1XA to all patients or WT31 to low responders, corresponding with the absence of an appropriate Fc receptor for these mAbs. Various mechanisms may be responsible for the Fc receptor-mediated decline in peripheral T lymphocytes. Binding of the Fc part of the mAb to its Fc receptor can lead to sequestration of lymphocytes in the mononuclear phagocyte system. In addition, interaction of the Fc part of the CD3/TCR mAb on T cells with its Fc receptor on mononuclear cells induces activation of the latter, resulting in the release of cytokines such as tumor necrosis factor- α and interferon- γ . Indeed, a systemic cytokine release has been observed after the administration of IgG2a CD3 mAb or WT31 to high responders, whereas only a minimal amount of tumor necrosis factor- α was observed after WT31 administration to low responders or after the administration of 1XA.^{2,5,6,13} These cytokines have been shown to induce an up-regulation of ICAM-1 and VCAM-1 on the vascular endothelium in vitro, resulting in increased adherence of T cells to endothelium.^{7,8,36} Because WT31 does not induce any complement activation and persistence of the lymphocytopenia is still observed after the administration of this mAb to high-responder patients, it seems justified to conclude that complement activation is certainly not an essential mechanism for this effect to occur. Whether activation of the complement system only would be sufficient for persistence of lymphocytopenia cannot be concluded from the current experiments.

In conclusion, two different phases can be distinguished in the CD3/TCR-induced T lymphocytopenia. The first phase of immediate disappearance can be explained by activation or increased expression (or both) of adhesion molecules. This seems to be sufficient to induce an immediate decline in peripheral T cells in vivo. The second phase of persistence of the peripheral lymphocytopenia is directly dependent on the heavy chain of the mAb and the presence of an appropriate Fc receptor for this mAb.

We thank F. N. J. van Diepen and S. L. Yong for technical assistance and Professor Dr. C. G. Figdor and Dr. Y. van Kooyk for providing us with the FITC-labeled NKI-L16 monoclonal antibody. We also thank Dr. F. J. Bemelman for critically reading the manuscript.

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