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Efficiency of T cell triggering by anti-CD3 monoclonal antibodies (mAb) with potential usefulness in bispecific mAb generation

Abstract T cell triggering can be achieved by monoclonal antibodies (mAbs) specific for the CD3/TcR complex. In the presence of appropriate costimulation and/or progression factors, such triggering permits the generation of effector cells for immunotherapy protocols involving the redirection of T cell lysis against tumor cells by mAbs bispecific for anti-CD3/anti-tumor cells (bs-mAbs). Focusing our analysis on the clinically relevant bs-mAb OC/TR, we found that bs-mAbs generated with the same anti tumor specificity, but two other anti-CD3 mAbs, TR66 and OKT3, have the same and a significantly lower lytic potential, respectively, compared with that of OC/TR. To evaluate the relevance of the anti-CD3 component, we examined several anti-CD3 mAbs with respect to binding parameters and the ability to trigger T lymphocytes. Competitive binding assays suggested that all anti-CD3 mAbs recognized the same or overlapping epitopes, although mAbs BMA030 and OC/TR bound with lower avidity than did αCD3 (the bivalent anti-CD3 mAb produced by the hybrid hybridoma OC/TR), TR66 and OKT3, as determined by measurement of the affinity constants. In all lymphocyte populations examined, which included resting peripheral blood mononuclear cells (PBMC), activated PBMC and T cell clones, OKT3, BMA033 and OC/TR failed to mobilize Ca^{2+} without cross-linking, whereas αCD3, in both murine and murine-human chimeric versions, TR66 and BMA030, did not require cross-linking. The ability to induce CD3 modulation was associated in part with the induction of Ca^{2+} fluxes. Despite the differences in the behavior of these mAbs in triggering the events that precede proliferation, all of them ultimately led to expression of the IL-2 receptor and to proliferation in T cells in the presence of accessory cells. Our data suggest that anti-CD3 mAbs that bind more rapidly (strong Ca^{2+} mobilizers) and more tightly under physiological conditions are good candidates for retargeting T cells in the bs-mAb clinical application.

Key words T cell activation · Ca^{2+} flux · CD3 modulation · T cell proliferation · Anti-tumor bs-mAb Retargeting

Abbreviations bs-mAb, bispecific monoclonal antibody; chi: chimeric; m: murine; IC_{50}: dose of competitor required to inhibit mAb binding by 50%; PBMC, peripheral blood mononuclear cells; r, recombinant; TAA, tumor-associated antigen.

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Introduction

TcR heterodimers provide T cells with the specificity for cognate recognition of antigen within the MHC, but the cell surface expression and function of the TcR are both dependent on the associated CD3 complex [41]. The physiological TcR-MHC/antigen interaction, together with the cooperative interaction of accessory molecules, leads to clonotypic T cell activation through several related steps, beginning with second messenger generation and ending with the development of effector functions. The same biochemical events can be engaged in polyclonal T cell populations by antibodies specific for framework epitopes of the TcR or the invariant chains of the CD3 complex [3, 32].

Triggering of any T cell, irrespective of its fine specificity, by anti-CD3 mAbs has been therapeutically exploited by construction of bispecific mAbs (bs-mAb) in conjunction with a number of different anti-tumor-associated antigen (TAA) mAbs. Engagement and cross-linking of the TcR-CD3 complex alone may lead to a state of T cell unresponsiveness (anergy) or even to cell death by apoptosis [16]. Thus, adoptive immunotherapy based on the use of anti-CD3/anti-TAA bs-mAbs requires the ex vivo activation

of T cells for effective anti-tumor reactions. Properly activated T lymphocytes that are specifically retargeted to tumor cells by bs-mAbs can lyse tumor cells and inhibit their in vitro growth [25, 31]. This approach has resulted in eradication of human tumors in xenotransplanted mice [14, 26]. Numerous anti-CD3 mAbs have been used to study the activation of T cells [1, 11, 17, 20, 28–30], but only a few have been used to generate anti-tumor bs-mAbs [26, 31]. Several phase I trials have suggested the clinical potential of anti-tumor/anti-CD3 bs-mAb [2, 4, 8, 9, 40], and at least one phase II trial in ovarian carcinoma patients has demonstrated the benefit of these reagents [6]. Using hybrid hybridomas derived from the fusion of the same anti-ovarian carcinoma hybridoma with two different anti-CD3 hybridomas (OKT3 and TR66) [10, 38] or immunized spleen cells [10], we evaluated the relevance of the fine binding specificity of the anti-CD3 component of the bs-mAb in redirecting T cell-induced lysis. Since the three bs-mAbs differed in cytolytic potential, the parental anti-CD3 mAbs and some others were examined for their ability to trigger T lymphocytes activation. Our results show that these mAbs behave differently in triggering the events that precede proliferation, such as Ca^{2+} mobilization and CD3 modulation, but all of the mAbs tested ultimately lead to expression of the IL-2 receptor and to proliferation of T cells in the presence of progression factors.

Material and methods

Anti-CD3 antibodies

BMA030 (mIgG$_{2a}$) and BMA033 (mIgG$_3$) were kindly provided in purified form by Dr. Kurrle (Behringwerke, Marburg, Germany). OKT3 (mIgG$_{2a}$) and TR66 (mIgG$_1$) hybridomas were obtained from the ATCC (Rockville, Md.) and kindly provided by Dr. A. Lanzavecchia (Basel Institute), respectively. Three anti-ovarian carcinoma/anti-CD3 hybrid hybridomas, the trioma OC/TR (mIgG$_1$) and the quadromas T3/OC1 (mIgG1/G2a) and T3/OC2 (mIgG1), derived from the fusion of hybridoma MOv18 [15] with spleen cells of a mouse immunized with human T cells and hybridomas OKT3 and TR66, respectively, were also used [10]. OC/TR has been fully characterized and used in preclinical and clinical studies [3, 6, 13, 23]. The mAbs were purified from mouse ascitic fluid or cell culture supernatant by affinity chromatography on insolubilized protein A according to their isotype. The bs-mAbs and, in the case of the mAb produced by the trioma OC/TR, the anti-CD3 bivalent component (referred to below as αCD3) were fractionated on a hydroxylapatite HPLC column as described elsewhere [23]. Their purity and integrity was confirmed by SDS-PAGE and isoelectrofocusing using the automated microprocessor-driven Phastsystem (Pharmacia LKB Biotechnology, Uppsala, Sweden), by analytical gel filtration, and by FACScan (Becton Dickinson, Erembodegem, Belgium) analysis of the target cells. Chi-αCD3, obtained by chimerizing the variable regions of the αCD3 with the human L- and IgG1-constant regions [18], was kindly provided in purified form by Dr. L. Coney (Centocor, Malvern, Pa.) All mAbs were kept at 4°C in sterile saline and were used as entire IgG molecules.

Preparation and culture of lymphocytes

PBMC were obtained from heparinized peripheral blood of healthy adult donors by centrifugation on Lymphoprep (Nycomed, Oslo, Norway), washed three times, and counted. For evaluation of mAb-binding characteristics, PBMC were activated with PHA (Wellcome, Dartford,

UK) (1µg/ml) and stimulated with 100 U/ml human recombinant (r) IL-2 (kindly provided by Glaxo Institute for Molecular Biology, Geneva, Switzerland) as already described [22]. For evaluation of Ca^{2+} fluxes, PBMC were stimulated for 4 days with 50 U/ml rIL-2 plus 10 ng/ml mAb BMA030. Two alloreactive T-cell clones (DM3 and DM13) were used in Ca^{2+} flux analysis and for evaluation of bs-mAb cytotoxic activity. Both clones were restimulated every 2 weeks with 1µg/ml PHA and irradiated (3,000 R) allogeneic PBMC and stimulated with rIL-2 (25 U/ml). The culture medium consisted of RPMI 1640 (Gibco, Ghent, Belgium) supplemented with 1% non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 30 U/ml penicillin-streptomycin (Gibco) and 5% pooled heat-inactivated human AB serum.

Assay of bs-mAb cytotoxic activity

Cytotoxicity was determined in a standard 4-h ^{51}Cr-release assay. Ovarian carcinoma IGROV1 cells were incubated for 1 h at 37°C with 100 µCi Na$_2$^{51}CrO$_4$/10^6 cells (ICN Biomedicals, Milan, Italy). After washing, cells (5000/well) were seeded in triplicate in U-shaped 96-well plates with DM13 alloreactive T cell clone at an effector-to-target cell ratio of 10:1 and the indicated amount of bs-mAb in a final volume of 0.2 ml/well. After 4 h of incubation, 0.1 ml/well of supernatant was withdrawn and radioactivity measured in a β-counter. Spontaneous release of ^{51}Cr, determined in cells incubated with medium alone, was always < 20%. Maximum ^{51}Cr release was measured in cells lysed in 1% NP-40. SEM of triplicates never exceeded 5–10%.
Percent specific lysis was calculated as

$$100 \times \frac{\text{Experimental release cpm } - \text{ spontaneous release cpm}}{\text{Maximum release cpm } - \text{ spontaneous release cpm}}$$

Anti-CD3 mAb binding assays

Based on their sensitivity to labeling procedures, mAbs αCD3, BMA030, TR66 and OC/TR were labeled with ^{125}I (Amersham, Aylesbury, UK) by lactoperoxidase-catalyzed iodination [12] to a mean specific activity of 8.4 µCi/g. OKT3 was labeled by the Bolton-Hunter method to a final specific activity of 4.4 µCi/g.
The K$_{aff}$ and number of sites recognized by the anti-CD3 mAbs were evaluated by Scatchard analysis. Activated PBMC (2×10^5/well in 0.05 ml RPMI-1640 plus 1% FCS) were incubated for 3 h at 0°C with serial dilutions of ^{125}I-mAb. After three washes with cold buffer (PBS plus BSA, 0.03% w/v), cell-bound radioactivity was measured directly in a counter. Background was determined in the presence of a 100-fold excess of cold mAb. For competition assays, activated PBMC were seeded at 2×10^5 cells per well in 0.05 ml of RPMI-1640,1% FCS, in the presence of a fixed amount of ^{125}I-mAb (5×10^5 cpm/well) and different concentrations of competitor. After incubation for 3 h at 0°C or 37°C, cells were washed three times and assessed directly for radioactivity in a γ-counter. The dose of cold competitor required to inhibit radiolabeled mAb binding by 50% (IC$_{50}$) was extrapolated from dose-response curves.

Assays of cytoplasmic free Ca^{2+}

After addition of 3 µl of 20% (v/v) Plutronic F-127 (Molecular Probes, Eugene, Ore.), lymphocytes (10^7 in 1 ml of Hank's solution, Gibco) were loaded with 3 M fluo-3/AM (Molecular Probes) and incubated for 20 min in the dark at 37°C. After dilution (1/5) in HBSS containing 1% FCS and a further 45 min incubation at 37°C, cells were washed three times and resuspended at a concentration of 1–2×10^6 cells/ml in Hepes-buffered saline (Sigma, St. Louis, Mo.). Before flow cytometry, cells were incubated for 10 min at 37°C in a water bath. Cells were stimulated by anti-CD3 mAbs (2 µg/ml) and analyzed on a Facscan. In all experiments, 8 µg/ml of rabbit anti-mouse Ig (Chemicon, Temecula, Calif.) was added to crosslink the prebound anti-CD3 mAb before re-analysis on a FACScan. Events were acquired and analyzed with Lysis II software.

Proliferation assays

Responder donors PBMC able to bind and to proliferate equally well in the presence of anti-CD3 IgG_1 and IgG_{2a} mAbs [20] were selected. PBMC (1.25×10^6 cells/ml) were cultured for 4 days in the presence of 50 U/ml of human rIL-2. On days 2 and 4 of culture, cells were plated in triplicate in 96-well U-bottomed plates at 10^4 cells/well in 200 μl of medium. ^{3}H-Thymidine (25 μl, 0.4 mCi, Dupont NEN, Dreieich, Germany) was added to each well, and after 4 h cells were harvested with a cell harvester (Skatron, Lier, Norway) on glassfiber filters, whereupon ^{3}H-thymidine incorporation was determined by scintillation counting (1500 TriCarb Packard).

Immunofluorescence and flow cytometry

PBMC stimulated for 1–4 days with anti-CD3 mAb and rIL-2 (0.5×10^6 cells) were incubated on ice with 25 μl of goat anti-mouse Ig-FITC (Immunotech, Marseille, France), anti-CD3-FITC (B-B11, IQP, Groningen, The Netherlands), or anti-CD25-PE (Dako, Denmark). After washing, cells were analyzed for fluorescence intensity on a Facstar+ (Becton Dickinson) with Lysis II software.

Statistical analysis

Data were analyzed using the Student's t-test, Mann-Whitney or an ANOVA test (Instat, GraphPad software).

Results

Anti-ovarian carcinoma/anti-CD3 bs-mAb induce different cytotoxicity

We showed previously that T3/OC1 (MOv18×OKT3) mediates a lytic activity lower than that induced by OC/TR (MOv18×αCD3) [13]. Using a different T cell clone, we confirmed that the T3/OC1 was less efficient than OC/TR and T3/OC2 (MOV18×TR66) bs-mAbs, which are raised with different anti-CD3 specificities, in retargeting (Fig. 1). Indeed, in each test and at both bs-mAb concentrations (100 and 1 ng/ml), the lysis mediated by the bs-mAb with OKT3 was significantly lower.

Anti-CD3 mAbs have different binding properties

To determine whether the affinity and avidity of different anti-CD3 mAbs might determine the different cytotoxic potential of bs-mAb, binding properties of the three parental anti-CD3 mAbs (αCD3, OKT3, TR66), another anti-CD3 mAb (BMA030) and bs-mAb OC/TR were assessed by Scatchard analysis and competition assays. The experiments were initially performed at 0°C to ensure a high binding level. Scatchard analysis of the binding data revealed comparable K_{aff} values for αCD3 (7.4×10^8 M^{-1}), OKT3 (7×10^8 M^{-1}) and TR66 (3.6×10^{-8} M), and 4–10 times lower values for BMA030 (0.8×10^8 M^{-1}) (Fig. 2) and OC/TR (1×10^8 M) (not shown). Competitive binding assays at 0 °C suggested that all anti-CD3 mAbs recognized the same or overlapping epitopes (see Fig. 3 for a representative experiment of αCD3 competition), although OC/TR and BMA030 bound with lower avidity, than αCD3, TR66 and OKT3.
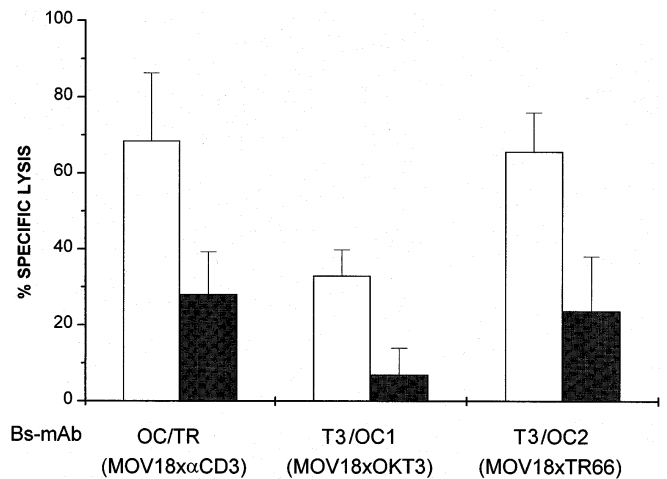

Fig. 1 Anti-ovarian carcinoma/anti-CD3 bs-mAb redirected lysis of IGROV1 cells by a CTL clone in a 4-h ^{51}Cr-release assay. DM13 CTL clone cells were used at an effector to-target cell ratio of 10:1 in the presence of 100 ng/ml □ or 1 ng/ml ■ of whole bs-mAb. Lysis in the absence of bs-mAb or in the absence of CTL was less than 5%. Data are reported as mean±SEM of 3–5 independent experiments

Competition experiments performed at 37°C to evaluate binding properties in physiological conditions (Fig. 3) revealed an inhibition induced by each anti-CD3 that resembled the pattern observed at 0°C, despite an increase in the IC_{50} values, which was particularly evident for BMA030 (IC_{50}=12.9 nM at 0°C and 264 nM at 37°C) and OKT3 (IC_{50}=8.5 at 0°C and 794 nM at 37°C).

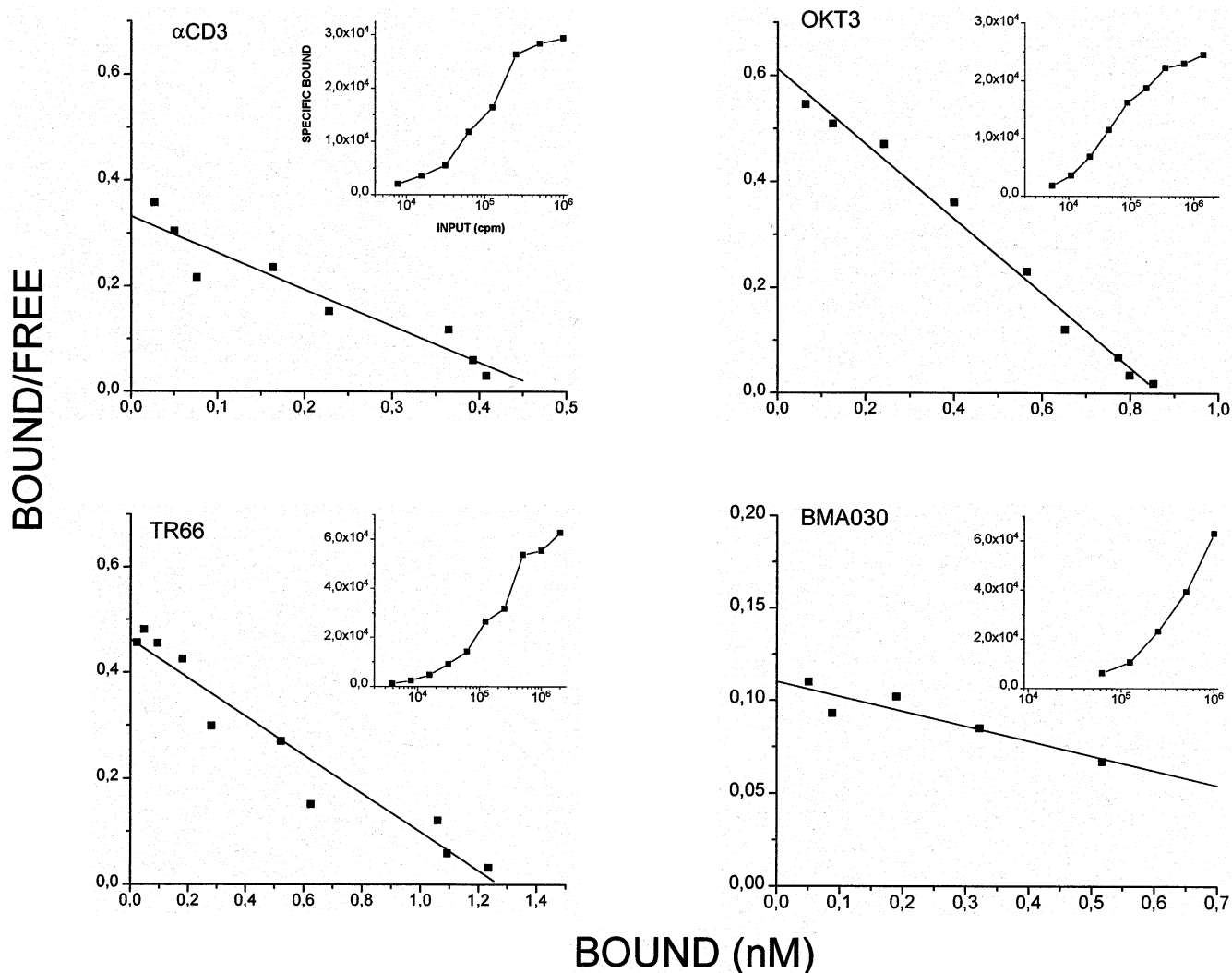## Stimulation by different anti-CD3 mAb induces different calcium fluxes

The calcium response after stimulation with seven different anti-CD3 mAb without further cross-linking was studied on different cell populations: resting PBMC, BMA030-activated PBMC and $CD8^+$ or $CD4^+$ T cell clones (Table 1). In all experiments, BMA030, TR66, αCD3 and chi-αCD3

Table 1 Calcium response in different lymphocyte preparations after stimulation with different anti-CD3 mAb

Anti-CD3 mAb	Calcium response[a] in			
	PBMC		T cell clones	
	Resting	BMA030-activated	DM13 CD8+	DM3 CD4+
BMA030	193±21 (5)[b]	171±24 (5)	387±83 (2)	148±23 (4)
TR66	186±13 (6)	172±17 (4)	384±51 (3)	147±19 (4)
OKT3	122± 6 (5)	99± 4 (3)	127±22 (2)	90± 4 (3)
OC/TR	125± 9 (4)	102± 4 (4)	171±12 (2)	99± 6 (4)
αCD3	218±11 (2)	278 (1)	455 (1)	163±17 (2)
chi-αCD3	198±11 (3)	–	355 (1)	137±22 (2)
BMA033	118 (1)	98 (1)	96 (1)	96 (1)

[a] Evaluated as free cytoplasmic Ca^{2+} levels in fluo-3-loaded cells. Data are reported as: (mean fluorescence after addition of anti-CD3/ mean fluorescence of baseline) × 100

[b] Mean ± SD (number of experiments)

Fig. 2 Scatchard plots of ^{125}I-anti-CD3 mAb binding on human activated PBMC. *Inset* binding plots. Statistical analysis of the curves: αCD3, $R^2=0.95195$ ($P<0.0001$); OKT3 $R^2=0.99451$ ($P<0.0001$); TR66, $R^2=0.97066$ ($P<0.0001$); BMA030 $R^2=0.9188$ ($P<0.05$)

mAbs induced calcium mobilization, whereas OKT3, BMA033 and OC/TR generated only low or no detectable calcium fluxes. Figure 4A shows representative FACS profiles on resting PBMC. A higher (6 µg/ml) or lower (10 ng/ml) concentration of OKT3 did not generate a Ca^{2+} response at the levels obtained with TR66 or BMA030 (data not shown). The extent of Ca^{2+} increase was dependent on the T cell type and activation status, and the responses were highest in the $CD8^+$ T cell clone, but the various anti-CD3 mAb gave a similar cline of response with all the cell types used. After cross-linking with rabbit anti-mouse or anti-human IgG, the Ca^{2+} response became comparable for all anti-CD3 mAbs (Fig. 4B). By comparing monovalent OC/TR with the murine and the chimeric versions of the bivalent parental αCD3 and by cross-linking with anti-mouse or anti-human Ig, we confirmed that binding valency plays a role in inducing Ca^{2+} mobilization. However, bridging to monocytes present in the PBMC population by mAb or bs-mAb Fc portion did not induce Ca^{2+} mobilization, perhaps because bridging occurs later or because the number of monocytes is insufficient.

OC/TR and BMA033 do not induce CD3 modulation

Modulation of the CD3 molecule was evaluated on resting PBMC of responder donors (see "Materials and methods"). Cells were cultured in the presence of rIL-2 and soluble anti-CD3 mAb (10 ng/ml) and analyzed for CD3 expression and the residual presence of the anti-CD3 mAb on the cell surface from day 1 to day 4 (Table 2). At the concentrations tested, there was no competition between the anti-CD3 used for detection and the anti-CD3 mAb used for stimulation (data not shown). Down-regulation of CD3 was observed after 24 h stimulation with TR66, BMA030, αCD3 and chi-αCD3, but not with OC/TR or BMA033; OKT3 induced intermediate down-regulation (Fig. 5A). The modulation pattern by the mAbs was highly reproducible, except for BMA030, with PBMC from different donors (Table 2). CD3 expression returned to initial values on day 4, despite the continuous presence of the anti-CD3 mAbs during the

Table 2 CD3 modulation induced by different anti-CD3 mAbs on PBMC

| Culture condition | CD3 expression at day[a] | | | |
	1	2	3	4
Control	1.00	1.00	1.00	1.00
IL-2	1.01±0.08	1.07±0.09	1.25±0.28	1.06±0.09
IL-2 + BMA030	0.52±0.33	0.71±0.53	1.03±0.65	1.15±0.54
IL-2 + TR66	0.30±0.06	0.45±0.28	1.07±0.17	0.91±0.31
IL-2 + OKT3	0.58±0.16	0.74±0.18	1.15±0.23	1.16±0.30
IL-2 + OC/TR	0.91±0.10	1.06±0.11	1.39±0.33	1.25±0.19
IL-2 + αCD3	0.22±0.12	0.74±0.59	1.11±0.83	1.19±0.54
IL-2 + chi α-CD3	0.29±0.07	0.46±0.127	1.08±0.18	1.27±0.22
IL-2 + BMA033	0.94±0.05	0.92±0.06	1.20±0.30	1.28±0.22

[a] Data are mean ± SEM of three independent experiments and are expressed as: mean fluorescence in presence of anti-CD3/mean fluorescence in control cells

induction period. The anti-CD3 mAbs were detectable on the cell surface by staining with an anti-mouse or anti-human Ig-FITC on day 1 (Fig. 5B).

All anti-CD3 mAbs induce cell proliferation and IL-2 receptor expression

Under the culture conditions described above, the levels of cell activation induced by the soluble anti-CD3 mAb were compared by measuring cell proliferation and the expression of the IL-2 receptor (CD25) in three independent experiments. On day 2, a proliferative response was observed with all anti-CD3 mAbs, but the bs-mAb OC/TR was significantly less potent in inducing proliferation than was its parental αCD3 (Fig. 6A). After 4 days of culture, a similar proliferative response was observed for all anti-CD3 mAbs (Fig. 6B). Results were similar for the expression of the IL-2 receptor. A significantly lower percentage of CD25+ cells was observed in culture with OC/TR on day 2 (Fig. 6A), but this percentage increased to near that with other anti-CD3 mAbs on day 4 (Fig. 6B).

Discussion

The significant clinical potential of bs-mAbs containing anti-CD3 in retargeting T cell cytotoxicity against tumor cells led us to evaluate the possible correlation between their cytotoxic efficiency and the binding and activating potential of the parental anti-CD3 mAbs. We focused our analysis on the bs-mAb OC/TR, which is now being used in clinical trials [4–6]. Comparison of the parental mAb αCD3 with several other anti-CD3 mAbs showed that all the tested anti-CD3 mAbs ultimately led to full T cell activation in a system in which progression factors are provided. This was true despite an apparent inability of OC/TR, OKT3 and BMA033 to trigger Ca^{2+} mobilization. Recently, Zhu and Carter [42] demonstrated a direct correlation between antigen-binding efficiency and late T cell activation events in a panel of humanized variants of the anti-CD3 mAb UCHT1. We analyzed the influence of anti-
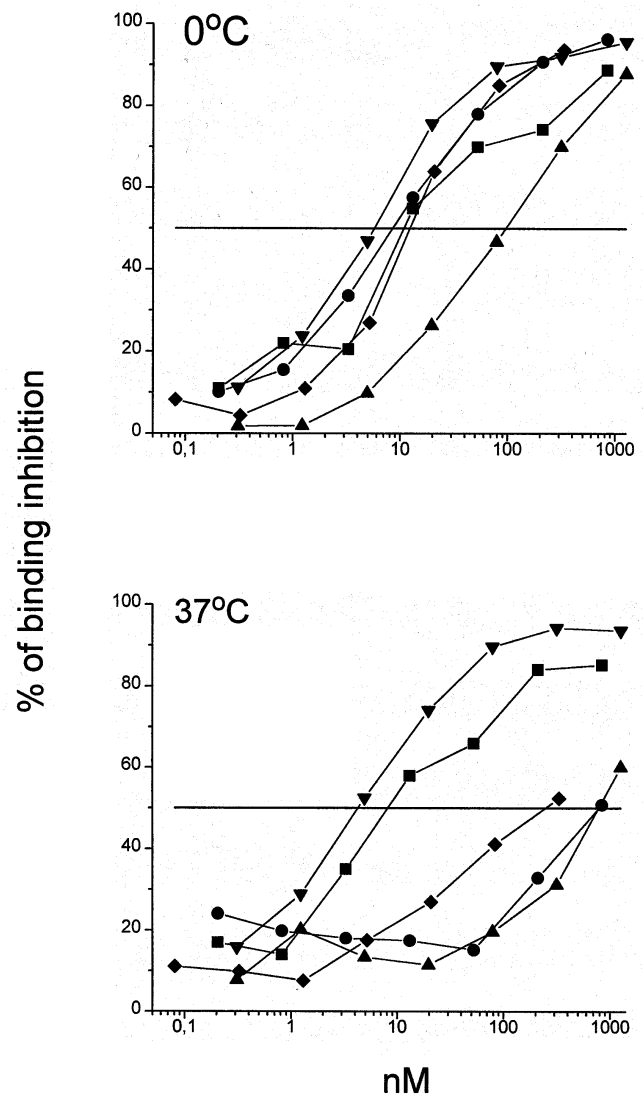

Fig. 3 Binding properties of different anti-CD3 mAb. ^{125}I-αCD3 mAb (10 nM) binding to human activated PBMC competed with titrated doses of unlabeled homologous or heterologous mAb, as described in Materials and methods for each radiolabeled mAb at 0°C or at 37°C. Competing mAb: TR66 (■); OKT3 (●); αCD3 (▼); BMA030 (◆); OC/TR (▲). Data are representative of the experiments performed with radiolabeled TR66, OKT3, BMA030 and OC/TR with similar results

CD3 mAb-binding parameters on early (Ca^{2+} mobilization) and intermediate-late (CD3 modulation, CD25 expression and proliferation) events.

Our use of the calcium probe fluo-3 in flow cytometry to measure the increase in cytoplasmic Ca^{2+} [37] permitted a semi-quantitative evaluation with clear-cut advantages over spectrofluorimetry. Previous studies, conducted mainly with probes that require ultraviolet light [1, 7, 11, 17, 20, 24, 27–30], have led to conflicting conclusions about a requirement for cross-linking and/or bridging of the TcR-CD3 complex in increasing the cytosolic Ca^{2+} concentration. Several variables, such as the type of T cell population studied or the concentration, fine binding specificity, binding affinity or valency of the mAb, might account for the discrepant results.
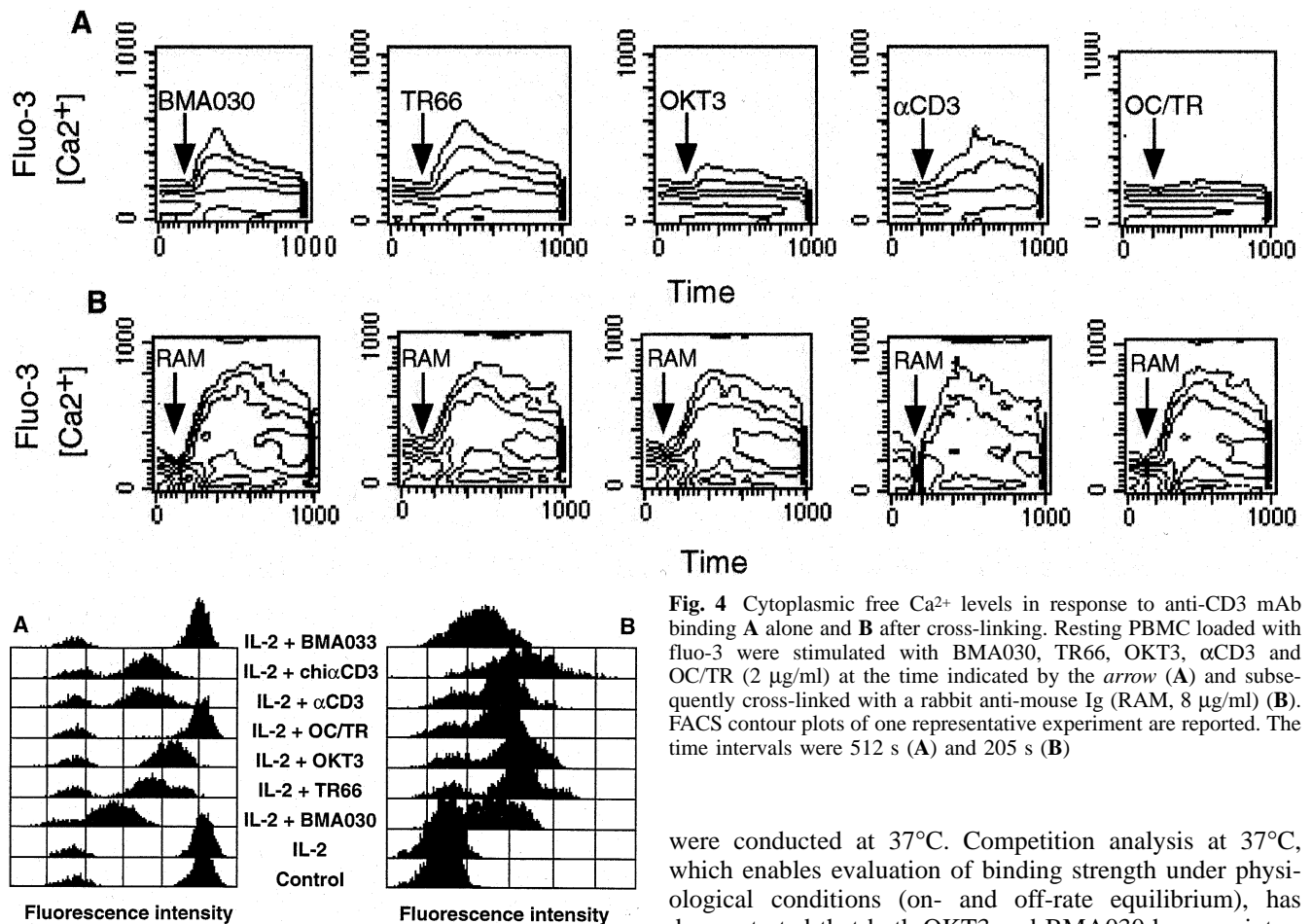
Fig. 4 Cytoplasmic free Ca^{2+} levels in response to anti-CD3 mAb binding **A** alone and **B** after cross-linking. Resting PBMC loaded with fluo-3 were stimulated with BMA030, TR66, OKT3, αCD3 and OC/TR (2 µg/ml) at the time indicated by the *arrow* (**A**) and subsequently cross-linked with a rabbit anti-mouse Ig (RAM, 8 µg/ml) (**B**). FACS contour plots of one representative experiment are reported. The time intervals were 512 s (**A**) and 205 s (**B**)

Fig. 5A, B Degree of CD3 modulation by different anti-CD3 mAbs. **A** After 1 day of culture in medium alone (control), or in the presence of rIL-2 or rIL-2 + anti-CD3 mAbs (10 ng/ml), CD3 expression was measured using an anti-CD3-FITC mAb. **B** Staining cells with FITC-goat anti-mouse Ig or anti-human Ig in the case of chi-αCD3 showed residual anti-CD3 mAb used for the stimulation. Data are from a single experiment representative of three performed with PBMC from different donors (see Table 2)

All of our anti-CD3 mAbs competed with each other in binding assays, and the amounts required at 0°C to obtain the same inhibition were clearly related to their binding affinity. These data imply that the mAbs tested are directed to the same or spatially close epitopes. Previous analysis [34] identified at least three epitope groups on human CD3 through which T cell activation by anti-CD3 mAb can proceed. OKT3, TR66 and BMA030, which belong to the same CD3 recognition group but bind with a different avidities, differ in their ability to promote Ca^{2+} mobilization. Our data suggest that the fine binding specificity of anti-CD3 mAb per se plays only a minor part in triggering early activation events, although any firm conclusions await further structure-function analyses.

No clear correlation between the ability to trigger Ca^{2+} mobilization and K_{aff} values was observed, at least in the cases of OKT3 and BMA030. However, our K_{aff} determinations, like most of those reported in the literature, were performed at 0–4°C, whereas Ca^{2+} mobilization studies

were conducted at 37°C. Competition analysis at 37°C, which enables evaluation of binding strength under physiological conditions (on- and off-rate equilibrium), has demonstrated that both OKT3 and BMA030 have an intermediate ability to compete with the ^{125}I-αCD3 binding, which was less than the competition exerted by TR66 and homologous mAb.

OKT3 and BMA033 mAbs and OC/TR bs-mAb that were virtually unable to promote Ca^{2+} mobilization during the first minutes of incubation were also less able or at least slower to promote the modulation of CD3 molecules and expression of CD25. However, all of the mAbs and the bs-mAb OC/TR ultimately induced proliferation, although with a slower kinetics. These data indicate that early Ca^{2+} mobilization is not a sufficient predictor of final activating ability and that full T cell activation can occur even in the absence of a detectable Ca^{2+} increase, due possibly to persistent but undetectable Ca^{2+} mobilization, or to the activation of another signal transduction pathway. The requirement for TcR/CD3 cross-linking in T cell activation is difficult to extrapolate to the physiological interaction between the TcR and the MHC-peptide complex displayed on the surface of antigen-presenting cells. Indeed, this interaction takes place with as few as 100 complexes and has low affinity and a high off-rate [35]. A model of TcR serial triggering was recently proposed [36] according to which the high off-rate of the TcR is instrumental because it allows a single peptide-MHC complex to engage many TcRs in successive rounds of ligation, triggering and dissociation. According to this model, a higher affinity could result in less stimulation because the lower off-rate may
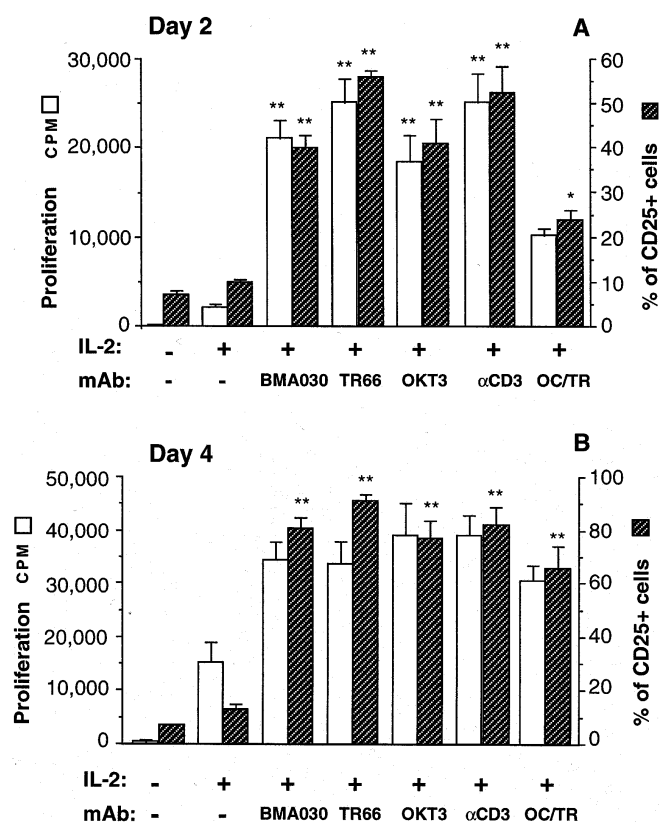
Fig. 6A, B T cell proliferation and CD25 expression after stimulation with different anti-CD3 mAb. On days 2 (**A**) and 4 (**B**) of culture, proliferation was measured by 4-h ³H-thymidine incorporation (*open bars*), and the percentage of CD25+ cells (*hatched bars*) was measured by flow cytometry. Data are the mean ± SEM of three independent experiments

prevent TcR reusage. In the case of surrogate antigenic stimulation through anti-CD3 mAbs, the number of concurrent interactions is 1000 times that between the TcR and the MHC-peptide complex. Moreover, TcR signaling characteristically requires that Ca^{2+} mobilization be sustained for at least 30 min to induce proliferation and cytokine production [21, 39]. Thus, high binding affinity of anti-CD3 mAbs might not be detrimental, provided that their off-rates are low enough to sustain prolonged Ca^{2+} mobilization.

In conclusion, we suggest that the best candidates for anti-tumor/anti-CD3 bs-mAb generation are anti-CD3 mAbs, such as αCD3 and TR66, which bind rapidly (strong Ca^{2+} mobilizers) and tightly (good competitors at 37°C) under physiological conditions. These mAbs, even in monovalent form, as in the bs-mAb configuration, can be expected to promote and sustain full activation in vivo. Indeed, the higher in vitro cytolytic potential of bs-mAb generated with CD3 and TR66 than of anti-TAA/OKT3 bs-mAb [13] (present report) supports this prediction. On the other hand, the in vivo use of bs-mAb with high promoting ability must be considered with caution in light of the finding that F(ab')₂ of the OC/TR bs-mAb, when injected i.v. even at dose of 0.2 mg, induced a generalized in vivo activation with severe side effects [2, 33] similar to those induced by 5 mg of OKT3 [19]. Thus, treatment protocols

with autologous ex vivo-activated lymphocytes coated with OC/TR must be appropriately designed [5] to avoid toxicity while retaining maximum anti-tumor bs-mAb retargeting potential.

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