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Research Article

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Cell Retargeting by Bispecific Monoclonal Antibodies

Evidence of Bypass of Intratumor Susceptibility to Cell Lysis in Human Melanoma

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Abstract

Intratumor heterogeneity for susceptibility to cytotoxic T lymphocytes (CTL)-mediated lysis represents a major obstacle to cancer adoptive immunotherapy. To overcome the heterogeneity observed in terms of susceptibility of target cells to cell-mediated lysis, in this study we used two purified bispecific monoclonal antibodies (bsmAbs) that recognize molecules expressed by cytotoxic effector cells (CD3 and IgG Fc receptorial molecules), as well as one high molecular weight melanoma-associated antigen (HMW-MAA). The ability of these reagents to enhance or induce a relevant in vitro cytotoxic activity by a CTL clone (CTL 49) isolated from PBL of a melanoma patient was tested on a large panel of autologous and allogeneic melanoma cell lines and clones. Functional studies revealed that the CTL 49 clone lysed all the HMW-MAA$^+$ tumor lines in the presence of bsmAbs and that these reagents affected the target lysis in a cooperative fashion. The effectiveness of bsmAbs in overcoming the heterogeneous susceptibility of human melanoma cells to cell-mediated lysis may find practical implications in cancer adoptive immunotherapy. (*J. Clin. Invest.* 1992. 90:1093–1099.) Key words: tumor heterogeneity • melanoma-associated antigen • autologous tumor • antibody bridging • cytotoxic activity

Introduction

Autologous tumor-specific cytolytic T cells have recently been used in cancer adoptive immunotherapy as an alternative to IL-2-activated effectors (1–4). These attempts stem from experimental evidence that tumor-infiltrating-specific T cells (TIL)[1] are more efficient than lymphokine-activated killer cells in mediating the regression of established neoplasia displaying significant intratumor heterogeneity for cell-mediated lysis (5, 6). However, tumor cells may escape recognition by specific PBL- or tumor-infiltrating-specific-derived cytotoxic T lymphocytes (CTL) because of the heterogeneity in the expression on the cell surface of MHC products and tumor-associated antigens acting as restriction elements. Thus, protocols of adoptive immunotherapy using injection of cytolytic T lymphocytes should take into account lysis-resistant neoplastic cells (2–7). These observations prompted us to develop alternate approaches aimed at increasing the effectiveness of autologous effector cells in killing tumor cells. One of the most promising strategies toward this goal, which has found several applications both in vitro and in vivo, is represented by the development of bispecific monoclonal antibodies (bsmAbs) able to focus CTL to tumor targets (8).

We devised a high efficiency biological protocol for producing hybrid hybridomas. Our approach includes a conventional somatic fusion between hybridoma lines, each carrying a stable and de novo inserted gene conferring resistance to a selected drug. Gene transfer in the parental hybridomas has been successfully achieved by using replication-deficient retrovirus-derived shuttle vectors conferring selectable features to the fusion partners. Simple genetic complementation produces vector-containing particles, which then are released in the culture medium and infect the hybridoma cells. Such procedures allow the recovery of 30–50% resistant cells within 10–14 d after the beginning of the selection pressure. The hybrid immunoglobulins obtained by following this procedure are a mixture of several combinatorial structures derived from heavy and light chains assembly. The experimental evidence gathered in the last years indicates that there are from three to five dominant assembly families, corresponding to the bispecific immunoglobulins and other molecular combinations. These observations might not be fully representative of all possible combinations, as they are influenced by evidence that the stringent selection pressure and the selection by binding analysis favor the desired combinations.

This study reports the results obtained using an in vitro model to focus effector cells to melanoma cells using two bsmAbs obtained by following the above technique (9). At variance from data of other groups, we have used autologous lymphocytes as effector cells. The bsmAbs react with two molecules expressed by effector cells (CD3 and Fc receptor [FcR]), which, upon being bound by agonistic antibodies, can activate the cytolytic machinery on T and natural killer cells, respectively. The antibodies recognize a differentiation antigen of the melanocyte lineage, expressed by the majority of melanomas (10). The results of the present study clearly show that bsmAbs can significantly increase the lysis of tumor cell lines and clones exerted by autologous lymphoid cells via a T cell receptor (TCR)-independent pathway, thus overcoming intratumor heterogeneity of susceptibility to CTL lysis.

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1. *Abbreviations used in this paper:* bsmAbs, bispecific monoclonal antibodies; CTL, cytotoxic T lymphocytes; E/T ratio, effector to target ratio; FcR, Fc receptor; HMW-MAA, high molecular weight melanoma-associated antigen; HPHT, high performance hydroxylapatite; IEF, isoelectric focusing; IIF, indirect immunofluorescence; SNK, Student-Newman-Keuls; TCR, T cell receptor; TIL, tumor-infiltrating-specific T cell.

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Methods

Tumor cells and clones. Me 665/1, Me 665/2, and 665/R melanoma cell lines, obtained from two metastases and from a tumor recurrence of the same patient (patient 665), have been elsewhere described (7). Tumor clones were isolated at early in vitro passages from Me 665 cell line using a two-layer soft agarose technique (7). Other melanoma cell lines used in this study were established from primary lesions (Me 1007) and from lymph node metastases (Me AQB, Me MRN, and Me CRL) (11, 12). Me 9229/4 is a clone derived from a cell line obtained from a lymph node metastasis. VSKB LCL is an EBV-transformed B cell line (gift of Dr. F. H. Bach, Immunobiology Research Center, Minneapolis, MN). All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine. Mycoplasma contamination was monitored by electron microscopy and by the GIBCO Mycotest Kit (Gibco Laboratories, Grand Island, NY).

Effector cells CTL 49 is an alloreactive T cell clone directed to HLA-A2 related determinants not expressed on the autologous melanoma cells (13). The clone was obtained as follows from PBL of a melanoma patient (patient 665), from whom tumor cells lines and clones were originated (see *Tumor cells and clones*): 5×10^6 PBL of the patient were stimulated with 5×10^6 irradiated (8,000 rad) VSKB-LCL cells (HLA-A2,3; B44,35; CW4; DR1,4; DP4,DP6) in 10 ml of 20% heat-inactivated human serum (HS) in RPMI 1640 medium. On day 10, a secondary mixed lymphocyte culture was implemented by restimulating the residual PBL with irradiated VSKB cells. 4 d later, the PBL were cloned by limiting dilution in round-bottom 96-well plates (Costar Corp., Cambridge, MA) in 0.2 ml of HS-RPMI 1640 supplemented with 40 U/ml of rIL-2 (kindly provided by Glaxo IMB, Geneva, Switzerland) (cloning medium) (7). Irradiated VSKB cells (5×10^4/well) were used as feeders. The CTL 49 clone was selected on the basis of the lytic ability to the allogeneic line VSKB and to the autologous melanoma cells.

mAbs and bsmAbs. The mAbs used included mAb CBT3 (an IgG_{2a} anti-CD3, kindly provided by Dr. A. Lanzavecchia, Basel, Switzerland), AB8.28 (an IgG_{2a} specific for a molecule displaying FcR activity, mostly expressed on natural killer cells) (14), and Ep2 (an IgG_{2a} anti-HMW-MAA) (15). These mAbs were used to generate hybrid hybridomas secreting bsmAbs CBT3 × Ep2 and AB8.28 × Ep2 by following a high efficiency biological method recently described (9).

bsmAbs purification and characterization. The bsmAbs were purified according to the procedure indicated by the European Economic Community guidelines for immunopharmaceuticals derived from biotechnological processes (16). IgGs were precipitated from ascitic fluid by the $(NH_4)_2SO_4$ technique and loaded on a protein A monoclonal antibody purification system (MAPS) preparative column (100 × 25 mm) (Affi-Prep; Bio-Rad Laboratories, Richmond, CA). The retained IgGs were then eluted with 0.1 M glycine-HCl buffer (pH 3.0) and immediately neutralized with 1 M Tris buffer (pH 8.5). The eluted IgG fraction was then dialyzed against a 60 mM NaH_2PO_4 solution (pH 6.8) containing 0.05% NaN_3, and loaded on a 100 × 7.8 mm hydroxylapatite column (MAPS high performance hydroxylapatite [HPHT]). The retained material was eluted by running a 60-min gradient from 60 to 180 mM NaH_2PO_4 at pH 6.8. The eluted peaks containing different IgG idiotypes were collected and extensively dialyzed against PBS at pH 7.4. The peak shown to contain the true bsmAb has undergone a second HPHT step to eliminate any residual peak-to-peak contamination. The degree of purification calculated by densitometric scanning of isoelectric focusing (IEF) gels resulted > 98% (not shown). A sample of the IgG fraction was digested with pepsin to obtain a $F(ab')_2$ preparation (17). Discrete peaks of purified parental and hybrid IgG molecules were subjected to IEF on a 5% acrylamide/3% ampholytes (range 3.5–10; Pharmacia Fine Chemicals, Piscataway, NJ) gel in a vertical apparatus under nonreducing conditions (anion = 0.02 M acetic acid; cation = 0.02 M sodium hydroxide).

Indirect immunofluorescence (IIF) and cytofluorography analysis. IIF was performed incubating 2×10^5 cells with the optimal dilution of each purified mAb and bsmAb in 5% FCS–RPMI 1640, staining with FITC-labeled $F(ab')_2$ fraction of a goat anti-mouse Ig serum (Technogenetics, Milan, Italy). Cytofluorimetric analysis of cell surface antigens was performed on an EPICS C (Coulter Electronics, Inc., Hialeah, FL). Maintaining constant main operative settings of the EPICS C throughout the whole study allowed a dependable comparison of the serological reactivity of mAbs from parental hybridomas and hybrid hybridomas. The analysis of each sample included the evaluation of the percentage of positive cells obtained after background subtraction. The mean channel fluorescence intensity on a logarithmic scale was also recorded for each sample tested.

Cytotoxicity assay. Cytotoxic activity was determined using a conventional ^{51}Cr-release assay. Target cells were labeled for 90 min at 37°C with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Int'l., Amersham, Bucks., UK). After three washings with medium, the cells were seeded in round-bottom 96-well plates (1×10^3 cells/well in 100 μl HS–RPMI 1640). Effector cells were added to each well at an effector to target (E/T) ratio ranging from 50:1 to 5:1. In some experiments, the CTL 49 clone was incubated before use for 24 h at 37°C with a purified preparation of the individual mAbs or bsmAbs, at a final concentration of 300 ng/ml. The clone was then extensively washed and used as effector in the assay. The final step was the addition of 10 μl of the mAbs or bsmAbs either in intact or in $F(ab')_2$ form to the cell mixture. 300 ng/ml antibody concentration was chosen since it represents a plateau for dose–response killing and was kept constant to make the results obtained from different targets comparable to each other. The plates were subsequently centrifuged at 60 *g* for 5 min and incubated at 37°C for 4 h. Then, 100 μl of supernatants were harvested from each well, mixed with 0.7 μl of Ready-Solve HP cocktail (Beckman Instruments, Geneva, Switzerland) in Micronic PPN tubes (Flow Laboratories, Settimo Milanese, Italy), and counted in triplicate in a β-counter. Maximum ^{51}Cr release was measured by incubating cells with 1% NP-40 detergent, whereas the spontaneous release was determined by incubating the target cells in medium alone. Percent lysis was calculated as follows: % cytotoxicity = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Statistical analysis. Lysis was performed in the presence or absence of various combinations of bsmAbs and monospecific Abs using CTL 49 on the same target. The significance of the differences among different combinations was evaluated through the analysis of variance. Briefly, an *F* test value was scored for each E/T ratio using the percent cytotoxicity from each of the replicate wells. The significance of the differences between any of the combinations was then evaluated by the Student–Newman-Keuls (SNK) multiple range test using the "Tadpole" software. Statistical analysis by the SNK test was performed at the $P = 0.01$ level for all experiments.

Results

Characterization of the hybrid molecules. The bsmAbs used were grown in vivo as peritoneal tumor in Balb/c mice. The ascites obtained contained ~ 5 mg of IgG/ml. Fig. 1 *A* shows the chromatographic profile pattern of the purification AB8.28 × Ep2 bsmAb, which presented five major peaks. When analyzed by IEF under nonreducing conditions, the five peaks were resolved into multiple bands with partially overlapping pI ranges (Fig. 1 *B*). Peak 1 (lane *C*, Fig. 1 *B*) displayed a banding pattern indistinguishable from parental AB8.28, and peak 5 (lane *G*, Fig. 1 *B*) showed a banding pattern overlapping with the parental Ep2 mAb. The bands of Peak 3 (lane *E*, Fig. 1 *B*) were instead in a pI range intermediate between that of the parental mAbs. Contamination of hybrid mAbs by parental mAbs have been ruled out by means of adsorption experiments done on the Ig preparation corresponding to the individual peaks (data not shown).

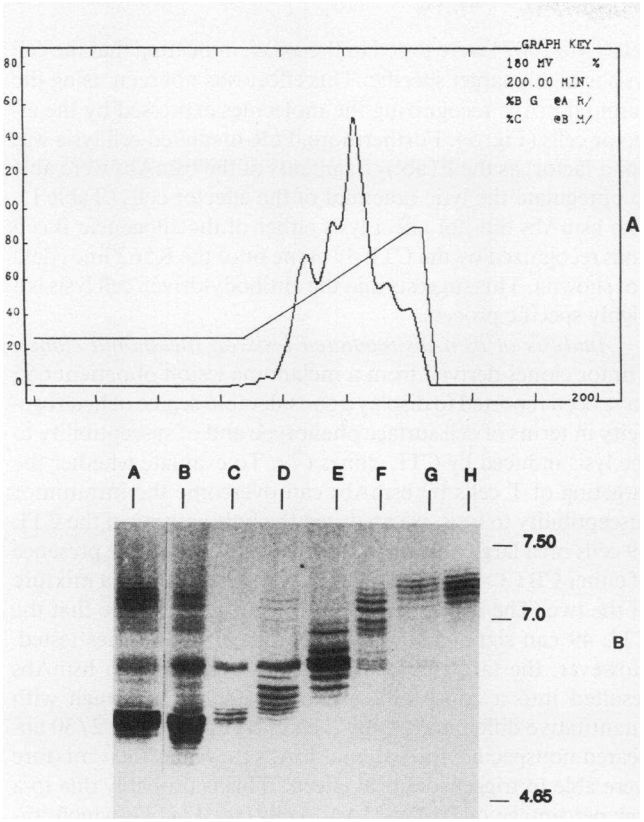

Figure 1. (*A*) HPLC purification of AB8.28 × Ep2 bsmAb on an HPHT column and analysis of the discrete peaks. *y*-axis = OD absorbance (280 nm) (*left side*) and phosphate gradient (mM) (*right side*). *x*-axis= elution time in minutes. See Methods for details. (*B*) IEF characterization of the discrete peaks obtained after HPLC of the AB8.28 × Ep2 bsmAb (peaks 1–5, corresponding to lanes *C–G*). Lane *A*, mixture of parental AB8.28 and Ep2 mAbs. Lane *B*, parental AB8.28 mAb. Lane *H*, parental Ep2 mAb.

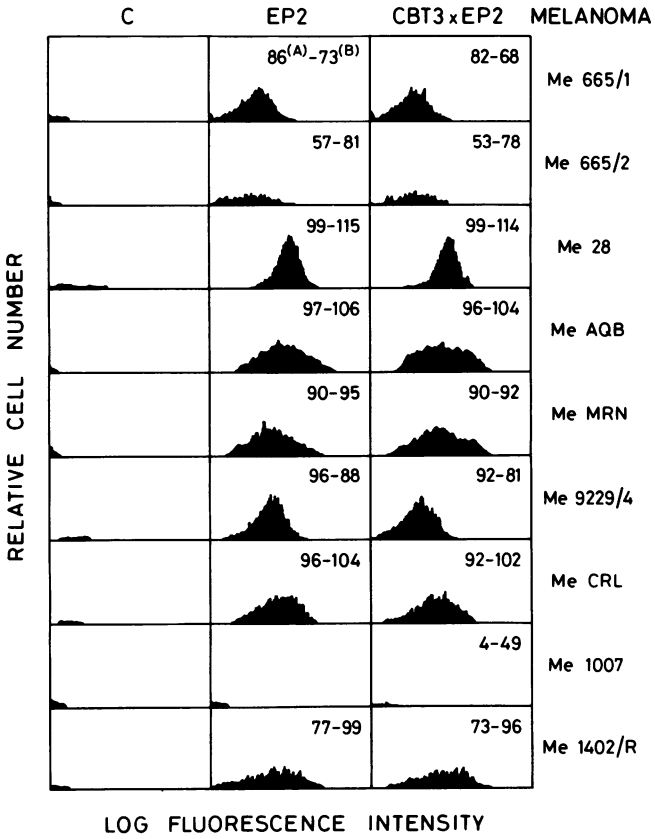

Figure 2. EPICS C analysis of the antigenic phenotype of two autologous (665/1 and 665/2) and seven allogeneic (Me 28, AQB, MRN, 9229/4, CRL, 1007, and 1402/R) melanoma cell lines. Cells were reacted and stained by IIF with Ep2 mAb and with purified bsmAb CBT3 × Ep2. C represents the control performed using a FITC-labeled F(ab')$_2$ preparation of goat anti-mouse Ig serum. The fluorescence intensity was recorded on a logarithmic scale. (A) Percentage of positive cells after background subtraction; (B) mean fluorescence intensity (arbitrary units).

Serological reactivity of CBT3 × Ep2 and AB8.28 × Ep2 bsmAbs on melanoma cell lines, tumor clones, and the effector clone CTL 49. To investigate the extent of expression of the high molecular weight melanoma-associated antigen (HMW-MAA) on melanoma cell lines and clones, cytofluorimetric analysis using bsmAb CBT3 × Ep2 and the parental Ep2 mAb was performed. As shown in Fig. 2, all melanoma cell lines tested showed high levels of expression of the HMW-MAA, with the exception of Me 1007, a cell line derived from a primary tumor.

The panel of nine tumor clones isolated from a single metastatic lesion (665/2) displayed a variable expression of the Ep2 antigen (Fig. 3). Most of the tumor clones featured between 20 and 80% of HMW-MAA$^+$ cells, with the exception of the clone 2/30, in which only 10% of the cells were HMW-MAA$^+$. These observations clearly indicate that HMW-MAA, which is recognized by Ep2 mAb, represents an ideal target, being expressed by the majority of melanoma cells with an epitope density able to mediate antibody binding and cell killing. The CTL 49 cells (CD3$^+$, WT31$^+$, CD8$^+$) were selected as the effector clone because of their ability to lyse the autologous melanoma cell lines in a TCR-independent fashion (13). CTL 49 cells were tested for recognition by all the parental and the bsmAbs used in the study. Fig. 4 shows that CBT3 and AB8.28 mAbs and their bispecific counterparts react with the CTL 49 clone.

bsmAbs-mediated lysis of melanoma cell lines. To determine whether bsmAbs are capable of inducing or modulating the cytotoxic activity of the alloreactive clone CTL 49, a panel of autologous and allogeneic melanoma cell lines were tested in a ^{51}Cr-release assay upon the addition of either CBT3 × Ep2 or AB8.28 × Ep2 or a mixture of the two. From the data reported in Fig. 5, it can be inferred that CTL 49 lyses the autologous lines 665/1 and 665/2 (27 and 30% lysis, respectively). The killing activity increased markedly after the addition of 300 ng/ml of either CBT3 × Ep2 or AB8.28 × Ep2 bsmAbs. This activity has been observed both on autologous cells and on the allogeneic lines (AQB and MRN). From the data of Fig. 5, it can also be seen that the activity of the two bsmAbs seems to have an enhancing effect: the CTL 49 clone, constitutively unable to lyse the allogeneic target Me CRL even in the presence of bsmAb AB8.28 × Ep2, became cytotoxic upon the addition of the CBT3 × Ep2 bsmAb. The percent lysis was further increased when the incubation mixture contained both bsmAbs. Furthermore, the CBT3 × Ep2 bsmAb appeared to be more efficient in the ^{51}Cr-release assay, since it induced a 60% lysis

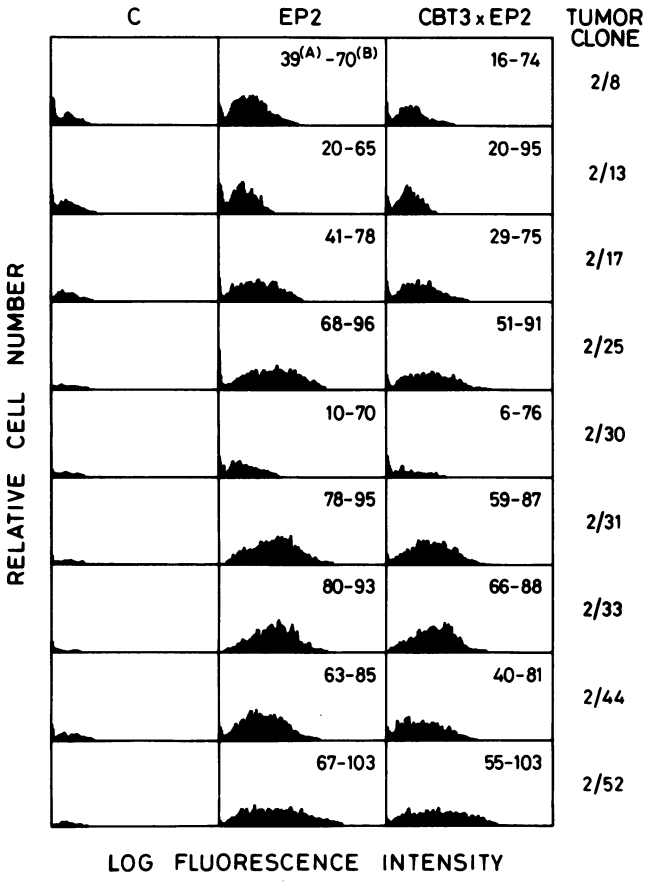

Figure 3. EPICS C analysis of the antigenic phenotype of a tumor clone from the 665/2 melanoma lesion. Cells were reacted and stained by IIF with Ep2 mAb and purified bsmAb CBT3 × Ep2. C represents the control performed using an FITC-labeled $F(ab')_2$ preparation of goat anti-mouse Ig serum. The fluorescence intensity was recorded on a logarithmic scale. (A) Percentage of positive cells after background subtraction; (B) mean fluorescence intensity (arbitrary units).

on the cell line AQB. The improvement in lysis efficiency was also present at a low E/T ratio, i.e., 5:1 (see the MRN and AQB cell lines in Fig. 5). No cell lysis was detected when the MAA^- cells (Me 1007) were tested in the assay, indicating that the cell lysis is highly target specific. This effect was not seen using the parental mAbs recognizing the molecules expressed by the effector cells (Fig. 5). Furthermore, FcR-mediated cell lysis was not a factor, as the $F(ab')_2$ fragments of the bsmAbs were able to upregulate the lytic potential of the effector cells (Table I). The bsmAbs did not affect lysis either of the allogeneic B cell lines recognized by the CTL 49 clone or of the K562 line (data not shown). This suggests that the antibody-driven cell lysis is a highly specific process.

Analysis of bsmAbs-mediated lysis on melanoma clones. Tumor clones derived from a melanoma lesion of patient 665 have been reported to display a considerable degree of heterogeneity in terms of cell surface phenotype and of susceptibility to the lysis induced by CTL clones (7). To evaluate whether the targeting of T cells by bsmAbs can overcome the intratumor susceptibility to lysis, we analyzed the lytic activity of the CTL 49 cells on a large number of tumor cell clones in the presence of either CBT3 × Ep2 and AB8.28 × Ep2 bsmAbs or a mixture of the two. The results summarized in Fig. 6 indicate that the CTL 49 can significantly lyse four out of nine clones tested. However, the targeting of the effector cells through bsmAbs resulted into a consistant lysis of all targets, although with quantitative differences. Only the killing of the clone 2/30 appeared nonspecific: the parental mAbs as well as their mixture were able to trigger identical effects. This is probably due to a low percentage of $HMW\text{-}MAA^+$ cells (see Fig. 3), which impairs the bsmAbs targeting of CTL 49 cells. The significance of the differences observed in the experiments reported in Figs. 5 and 6 have been statistically analyzed using the SNK multiple range test. The bars of Figs. 5 and 6 displaying significant differences are marked by an asterisk.

Lytic activity of CTL 49 after overnight incubation with bsmAbs. Owing to the E/T ratios, cell interactions mediated by bsmAbs are expected to be delayed in vivo to variable extents. We have analyzed whether prolonged incubation (24 h) at 37°C of CTL 49 clone with the bsmAbs can influence the cytotoxic potential on different cell targets. These included both autologous as well as allogeneic tumor cells. As can be seen from the data of Table II, CTL 49 clone can lyse the relevant targets independent of the exposure time of the bsmAbs in the reaction mixture. This indicates that the effector cells retain their cytolytic activity unaltered for ≥ 24 h.

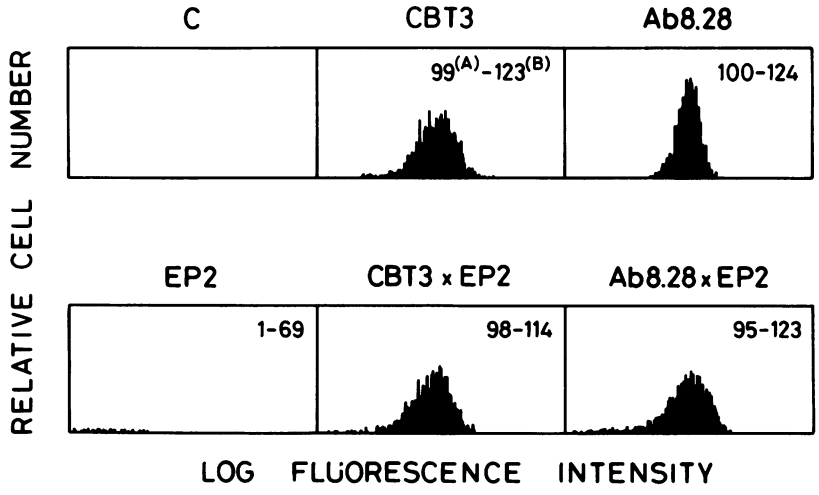

Figure 4. EPICS C analysis of the antigenic phenotype of the CTL 49 clone. Cells were reacted and stained by IIF with mAbs CBT3, AB8.28, Ep2, and with purified bsmAbs CBT3 × Ep2 and AB8.28 × Ep2. C represents the control performed using a FITC-labeled $F(ab')_2$ preparation of goat anti-mouse Ig serum. The fluorescence intensity was recorded on a logarithmic scale. (A) Percentage of positive cells after background subtraction; (B) mean fluorescence intensity (arbitrary units).

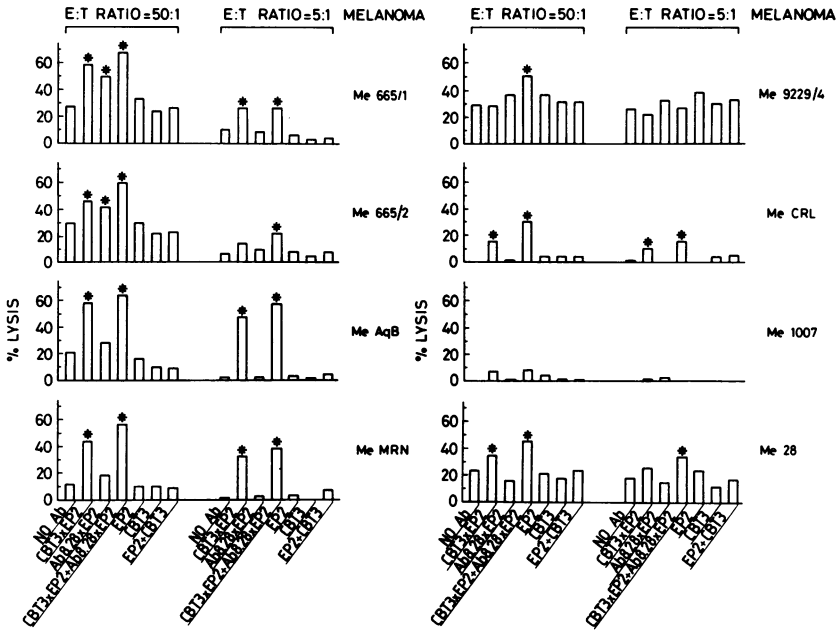

Figure 5. Evaluation of cytotoxic activity of clone CTL 49 mediated by CBT3 × Ep2 and AB8.28 × Ep2 bsmAbs against autologous cells (*Me 665/1, Me 665/2*), and allogeneic melanoma cell lines Me AQB, Me MRN, Me 9229/4, Me CRL, Me 1007, and Me 28. The CTL 49 clone was tested in a 4-h ^{51}Cr-release assay at two different E/T ratios (*50:1* and *5:1*) against each target in absence (*No Ab*) or presence of purified bsmAbs (*CBT3 × Ep2* and *AB8.28 × Ep2*) or a mixture of the two bsmAbs (*CBT3 × Ep2 + AB8.28 × Ep2*), as indicated on the *x*-axis. The bars Ep2, CBT3 and Ep2 + CBT3 indicate the percent lysis obtained adding the parental Ep2 mAb and CBT3 mAb, or a combination of the two mAbs. Lysis of Me 665/1, Me 665/2, Me AQB, Me MRN, Me CRL, and Me 28 (*E:T ratio = 50:1*) and lysis of Me 665/1, Me AQB, Me MRN, and Me CRL (*E:T ratio = 5:1*) were significantly different ($P = 0.01$; SNK test) in the presence of CBT3 × Ep2 or the combination of CBT3 × Ep2 and AB8.28 × Ep2 when compared with the lysis of the same targets in the absence of the bsmAbs. At E:T ratio = 50:1, lysis of Me 665/1 and Me 665/2 in the presence of AB8.28 × Ep2 is significantly enhanced over lysis in the absence of antibodies ($P = 0.01$, SNK test). An asterisk marks the differences reaching statistical significance.

Discussion

Tumor cell heterogeneity can hamper the efficacy of therapeutic strategies, including adoptive immunotherapy. Intratumor heterogeneity has mainly been studied in human melanoma, where experimental evidence indicates the coexistence, within a discrete neoplastic population, of various phenotypic and functional features, such as distinct patterns of oncogene expression (18), in vitro (19) and in vivo (10) antigenic phenotype, and susceptibility to lymphokine-mediated antigenic modulation (12, 20). Melanoma heterogeneity also includes a varying susceptibility to cell-mediated lysis (7) and to cytotoxic/cytostatic activity of individual cytokines (11). This study reports the results of an ongoing effort to overcome the intratumor heterogeneity in susceptibility to lymphocyte-mediated lysis by the use of a combination of bsmAbs obtained by biological protocols. Such protocols, and the characterizations of bsmAbs so produced, have been previously reported (9).

Table I. Comparison of Lytic Activity of CTL 49 Clone in Presence of Intact or F(ab')$_2$ CBT3 × Ep2 bsmAb

Targets	No Ab	CBT3 × Ep2*	CBT3 × Ep2 F(ab')$_2$*
665/1	27	59‡	55
665/2	30	48	45
Me AQB	20	59	62
Me MRN	11	43	40
Me CRL	0	18	16
Me 1007	0	8	2
Me 28	23	37	35

* Both intact and digested bsmAbs were used at 300 ng/ml (final concentration). ‡ Percent lysis at E/T ratio of 50:1 in a 4-h ^{51}Cr-release assay.

The structure identified by the Ep2 mAb has been shown to be identical to that recognized by the 225.28 mAb. The latter mAb reacts to a carbohydrate determinant carried by the HMW-MAA, which consists of a condroitinsulphate proteoglycan and has been shown to be endowed with serological features of clinical relevance such as homogeneous expression in melanoma cells and among metastatic foci, thus making this target ideal for immunodiagnostic and therapeutic intervention (21).

Using bmsAbs purified at homogeneity by HPLC on HPHT, we have shown that (*a*) the tumor reactivity of the CBT3 × Ep2 and AB8.28 × Ep2 bsmAbs is maintained in most of the primary and metastatic melanoma cell lines and clones; (*b*) TCR-independent lysis of the HMW-MAA$^+$ tumor cells by an autologous CTL clone is significantly increased by the addition of CBT3 × Ep2 and AB8.28 × Ep2 bsmAbs; (*c*) the improved performance of the lytic machinery induced by the bsmAb CBT3 × Ep2 clearly indicates that the two reagents act through distinct transmembrane signaling pathways endowed with distinct efficiency. The ability of agonistic antibodies to activate effectors cells is maintained in bsmAbs obtained according to the reported procedures. Finally, and of major relevance for its clinical fallout, (*d*) the triggering of distinct activation pathways may increase the lytic power.

Unlike the effector cells used in other studies (22–27), the CTL 49 clone used in the present study is per se capable of recognizing and lysing the autologous melanoma cells in a TCR-independent fashion. As previously reported (13), the killing activity of CTL 49 cells takes place through multiple lytic mechanisms mediated by both direct cell–cell contacts and by the release of soluble cytotoxic factors.

The results obtained with the cell line CRL provided further information about the effects of bsmAbs on cell cytotoxicity. CTL 49 was unable to lyse the allogeneic melanoma cell line CRL, either when used alone or upon the addition of

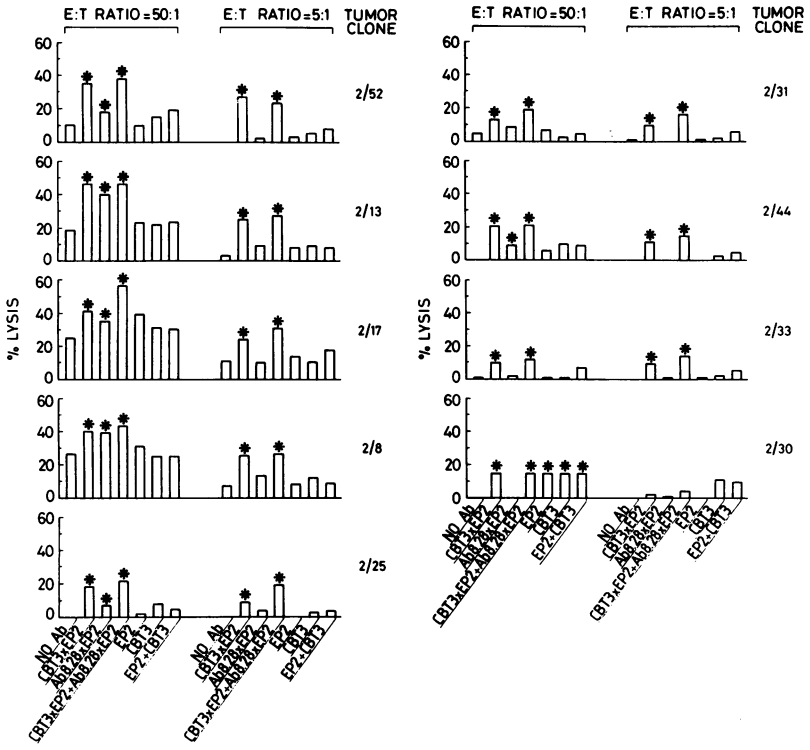

Figure 6. Evaluation of the cytotoxic activity of clone CTL 49 mediated by CBT3 × Ep2 and AB8.28 × Ep2 bsmAbs against nine tumor clones (*2/52, 2/13, 2/17, 2/8, 2/25, 2/31, 2/44, 2/33,* and *2/30*) isolated from the autologous lesion Me 665/2. The CTL 49 clone was tested in a 4-h ^{51}Cr-release assay at two different E/T ratios (*50:1* and *5:1*) against each target in absence (*No Ab*) or presence of purified bsmAbs (*CBT3 × Ep2* and *AB8.28 × Ep2*) or of a mixture of the two bsmAbs (*CBT3 × Ep2 + AB8.28 × Ep2*), as indicated on the *x*-axis. The bars Ep2, CBT3, and Ep2 + CBT3 indicate the percent lysis obtained by adding the parental Ep2 mAb and CBT3 mAb, or a combination of the two mAbs. Lysis of all tumor clones at E:T ratio = 50:1 and 5:1 (except tumor clone 2/30 at E:T ratio = 5:1) in the presence of CBT3 × Ep2 or CBT3 × Ep2 + AB8.28 × Ep2 differed significantly ($P = 0.01$, SNK test) from lysis in absence of antibodies. Similarly, lysis of all tumor clones (except 2/31, 2/33 and 2/30) at E:T ratio = 50:1 was significantly different in the presence of AB8.28 × Ep2, in comparison with lysis in the absence of antibodies ($P = 0.01$, SNK test). An asterisk marks the differences reaching statistical significance.

AB8.28 × Ep2. However, CBT3 × Ep2 retargeting was followed by significant lysis in the same system. The presence of both bsmAbs yelded a higher percent lysis of the CRL line than that produced by CTL 49 in the presence of CBT3 × Ep2.

The observed cooperative effects of the two bsmAbs suggest that other lytic pathways are involved in the multistep targeting process, and that such activation might be inefficient until a threshold is reached. A second signal of the same receptor (or other receptors) driving discrete pathways may be needed to implement the machinery of the cell or, alternatively, to increase its efficiency. When the functional studies were performed using nine tumor clones isolated from the patient from whom the autologous CTL 49 cells were obtained, the addition of bsmAbs overcame heterogeneity in susceptibility to cell lysis. Whereas the use of the CTL 49 clone alone was followed by efficient lysis in only four out of nine tumor clones, all HMW-MAA$^+$ cells were specifically lysed in the presence of bsmAbs, in a fashion similar to that observed with the autologous and allogeneic melanoma cell lines. These data suggest that physical juxtaposition is necessary to increase lysis efficiency and they also confirm the specificity of the bsmAbs-driven killing effects.

The lack of lysis of Me 1007 (Fig. 5) and of the tumor clone 2/30 (both HMW-MAA$^-$) suggests that the bridging between effectors cells and melanoma targets occurs only via HMW-MAA. Moreover, the higher efficiency of CBT3 × Ep2 compared with AB8.28 × Ep2 indicates a preferential CD3-mediated lysis, as confirmed on a large panel of tumor cell lines and clones, where a cross-linking or bridging is required for triggering T cells by a monovalent α-CD3 mAb (as it is for the purified CBT3 × Ep2). In all the functional studies, the bsmAbs appeared to cooperate in functional terms, even when AB8.28 × Ep2 alone is unable to induce CTL 49 cells to lyse an allogeneic melanoma cell line. Two features of bsmAbs-in-

Table II. Lytic Activity of CTL 49 Clone after Preincubation with mAbs or bsmAbs

		Targets				
CBT3 × Ep2*	Preincubation‡	Me665/1	Me665/2	Me665/R	AQB	MRN
−	—	25§	13	21	0	18
−	CBT3	30	15	37	6	30
−	CBT3 × Ep2 (1)	60	38	45	31	52
−	AB8.28	ND	ND	7	7	24
−	AB8.28 × Ep2 (2)	ND	ND	0	0	23
−	(1) + (2)	ND	ND	30	30	56
+	—	63	32	51	ND	ND
+	CBT3	64	29	53	ND	ND
+	CBT3 × Ep2	63	40	49	ND	ND

ND, not done. * bsmAb CBT3 × Ep2 was used at 300 ng/ml. ‡ CTL 49 cells were incubated for 24 h at 37°C in the presence of 300 ng/ml of each antibody. § Percent lysis at E/T of 50:1 in a 4-h ^{51}Cr release assay.

duced lytic activity should be emphasized in view of possible in vivo application, namely that the killing activity induced by the reagents selected during this study was retained by the $F(ab')_2$ fragments of the bsmAbs (Table I), and that their ability to arm the CTL 49 clone against tumor cells was maintained for up to 24 h. The present results have been confirmed by data obtained with the use of antibodies generated by other groups (28, 29).

The conclusion of the present work is twofold: (*a*) bsmAbs may find a place in adoptive immunotherapy for their ability to overcome intratumor heterogeneity and (*b*) selected combinations of new bsmAbs may lead to an increased antitumor activity by CTL. The last issue is gaining a wider relevance in light of an increasing number of surface molecules reported to transduce cell activation signals (30–33). Some of these molecules (e.g., CD16) resulted in sharing with CD3 the use of a common ξ chain for the transmembrane signaling (34). Retargeting and cytotoxicity triggering using different sets of mAbs specific for conventional and alternative activation pathways are presently underway (35).

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