

A MONOCLONAL ANTIBODY SPECIFIC FOR A COMMON  
DETERMINANT OF THE HUMAN T CELL RECEPTOR  $\gamma/\delta$   
DIRECTLY ACTIVATES CD3<sup>+</sup>WT31<sup>-</sup> LYMPHOCYTES TO  
EXPRESS THEIR FUNCTIONAL PROGRAM(S)

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Human T lymphocytes express receptors for antigen that are associated in a molecular complex to CD3 surface proteins. In most CD3<sup>+</sup> cells these receptor molecules are represented by disulphide-linked heterodimers formed by an  $\alpha$  and a  $\beta$  chain that recognize antigen(s) in an MHC-restricted fashion (1, 2). However, another small subset has recently been identified that does not express  $\alpha/\beta$  molecules in association with CD3 (3, 4). These cells do not react with the WT31 mAb (specific for a framework determinant of the TCR- $\alpha/\beta$ ) and do not express CD4 and CD8, the molecules associated with class II or class I MHC antigen recognition (4). In these cells, CD3-associated molecules are represented by the surface product of the TCR- $\gamma$  and  $-\delta$  genes (5-8).

Previous studies have indicated that TCR- $\gamma/\delta$ <sup>+</sup> cells, similarly to cells expressing TCR- $\alpha/\beta$ , can be activated by antibodies directed to surface CD3 or CD2 antigens (9, 10). However, no direct evidence exists that stimuli acting via the surface receptor itself would result in activation of the functional program of the cells. We have now isolated a mAb reacting with the surface receptor expressed on CD3<sup>+</sup>WT31<sup>-</sup> cells by immunizing with a cell line derived from human peripheral T cells expressing the CD3<sup>+</sup>WT31<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> surface phenotype. This antibody, termed BB3, reacts with a large subset (>60%) of CD3<sup>+</sup>WT31<sup>-</sup> lymphocytes and immunoprecipitates from the surface of these cells a disulphide-linked dimer associated to CD3 proteins. Treatment of CD3<sup>+</sup>WT31<sup>-</sup> cells with BB3 mAb led to a rapid intracellular Ca<sup>2+</sup> increment and resulted in the induction of the functional program(s) of the cells such as triggering of the lytic machinery and lymphokine production.

This work was supported in part by grants awarded by the Consiglio Nazionale delle Ricerche (CNR) "Piano Finalizzato Oncologia" to L. Moretta and A. Moretta and by Associazione Italiana per la Ricerca sul Cancro (AIRC). O. Viale and I. Prigione are recipients of a fellowship awarded by AIRC.

## Materials and Methods

*Isolation and Culture of CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> Lymphocytes.* Peripheral blood lymphocytes from normal volunteers were isolated by Ficoll-Hypaque gradients and then separated into E-rosetting-positive and -negative populations. E-rosetting cells were then stained with a mixture of anti-CD4 and anti-CD8 mAbs followed by treatment with rabbit complement for 1 h at 37°C. Viable cells were cultured either at 10<sup>5</sup> cells/ml (for MV1 cell population) or under limiting dilution conditions (for T cell clones) in the presence of irradiated autologous PBL, as a source of feeder, 1% PHA (vol/vol) and 50 U/ml of rIL-2 in 96-well microtiter plates. Cell lines and clones were screened directly by FACS analysis, for the presence of surface CD3 and the simultaneous lack of CD4 or CD8 antigens. The mAbs used in these experiments were Leu-4 (anti-CD3) (Becton Dickinson and Co., Basel, Switzerland), MAR 206 (directed to CD2), B9.4 (anti-CD8), CK.79 (anti-CD4), and WT31, specific for a framework determinant of the TCR- $\alpha/\beta$ . A polyclonal cell line, termed MV1, expressing the CD2<sup>+</sup>3<sup>+</sup>4<sup>-</sup>8<sup>-</sup>WT31<sup>-</sup> surface phenotype (>95% pure) was selected for mice immunization.

*Production of the BB3 mAb.* 6-wk-old male BALB/c mice were immunized with the MV1 cell line as previously described (11). The immunization schedule consisted of three weekly intravenous injection of 10<sup>7</sup> MV1 cells. After 10 d, the mice received a booster injection of 1.5  $\times$  10<sup>7</sup> cells followed by splenectomy 3 d later. Immune splenocytes were fused with P3-U1 myeloma cells (11). The initial screening of hybridoma supernatants (SN) was carried out by indirect immunofluorescence against the immunizing MV1 cell line or against CD3<sup>+</sup>WT31<sup>+</sup> cell lines derived from the same donor. To this end cells were stained with hybridoma supernatants (50  $\mu$ l/10<sup>5</sup> cells) followed by fluoresceinated goat F(ab)<sub>2</sub> anti-mouse Ig as previously described (12). According to this screening a hybridoma, termed BB3, that reacted with MV1 cells but not with CD3<sup>+</sup>WT31<sup>+</sup> cell populations, was isolated and further subcloned by limiting dilution.

*Two-Color Flow Cytofluorometric Analysis.* Analysis of total PBMC for the distribution of CD3, BB3, and WT31 antigens was performed using two-color fluorescence cytofluorometric analysis, as previously described (13). Cells were stained with phycoerythrin (PE)-conjugated OKT3 (IgG2a) mAb (Ortho Pharmaceuticals, Raritan, NJ) and with either WT31 (IgG1) (Sanbio Biological Products, Uden, The Netherlands) or BB3 (IgG1) mAb followed by FITC-conjugated goat anti-mouse IgG1 antibodies (Southern Biotechnology Associates Inc., Birmingham, AL). After incubation at 4°C, cells were extensively washed in cold RPMI-1640 medium and then resuspended in the same medium supplemented with 5% FCS. Lymphocytes expressing CD3 but not WT31 or BB3 exhibited a red (PE) fluorescence, but no green (FITC) fluorescence.

*Characterization of the BB3 Antigen.* MV1 or CD3<sup>+</sup>WT31<sup>-</sup> cloned cells were washed five times in cold RPMI-1640, twice in PBS and then surface-labeled with <sup>125</sup>I using the lactoperoxidase/glucose oxidase-catalyzed iodination (14). After labeling, cells were washed once in PBS and resuspended at 0°C for 30 min in lysis buffer. The lysis buffer was as follows: 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN<sub>3</sub> and contained 1% NP-40 or digitonin. Digitonin was added at 1 g/100 ml at the above solution, boiled, stirred for 2 min, cooled, allowed to stand at room temperature for at least 4 d, and then filtered. After spinning, the supernatants were filtered and dialyzed with PBS and precleared three times with 20  $\mu$ l of packed protein A-Sepharose beads for 2 h under rotation. Lysates were then incubated for 2 h with 200  $\mu$ l of BB3 culture supernatant or 50  $\mu$ l of a 1:10 dilution of anti-Leu-4 mAb. 20  $\mu$ l of packed protein A-Sepharose beads were then added and samples were incubated overnight at 4°C under rotation. The immunoprecipitate was eluted from protein A-Sepharose by boiling for 5 min in buffer containing 1% SDS in the presence or absence of 5% 2-ME and analyzed on 11% discontinuous SDS-polyacrylamide gels (15). The nonequilibrium pH gradient electrophoresis (NEPHGE) was carried out using pH 3.5-10 ampholines followed by 11% SDS-PAGE gels for size separation as described (16). The iodinated sample was applied at the acidic end.

*Functional Analysis of CD3<sup>+</sup>WT31<sup>-</sup> Cell Populations and Clones.* Production of IL-2 from clones and cell lines was assessed by culturing 10<sup>5</sup> cells/well in the presence of 2 ng/ml PMA and either anti-CD3 mAb (10  $\mu$ l of culture supernatant), or BB3 mAb (10  $\mu$ l of culture supernatant). After 24 h, culture supernatants were removed and each tested in an indicator

cell system consisting of the IL-2-responsive murine CTLL cell line as described previously (17). Data were expressed as arbitrary units of IL-2 activity. One unit was defined as the amount of IL-2 required for the induction of a half-maximal proliferative response by CTLL cells.

The cytolytic activity of the clones was tested in a 4-h  $^{51}\text{Cr}$ -release assay in which  $\text{CD3}^+\text{WT31}^+$  or  $\text{CD3}^+\text{WT31}^-$  cells were used as effector cells against the hybridomas producing anti-CD3, anti-CD2, and BB3 mAbs, respectively. All these target cells were used at  $5 \times 10^3$  cells/well for a final E/T cell ratio of 10:1. A second type of cytolytic assay was performed using the murine P815 line as target cell (18). In this assay  $5 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells were added to each well containing  $5 \times 10^3$  effector cells in the presence of either one of the following mAbs: anti-CD3 (20  $\mu\text{l}$  of culture supernatant), BB3 (20  $\mu\text{l}$  of culture supernatant) and WT31 (20  $\mu\text{l}$  of a 1:20 dilution of the stock solution). The final culture volume was 0.2 ml for each well. After a 4-h culture period, 0.1 ml was removed from each well and counted in a gamma counter for the assessment of  $^{51}\text{Cr}$  release. Percent specific  $^{51}\text{Cr}$  release was determined as previously described (19).

*Determination of Free Cytoplasmic  $\text{Ca}^{2+}$  Concentration.* Determination of  $[\text{Ca}^{2+}]_i$  was performed as previously described (20). Briefly,  $\text{CD3}^+\text{WT31}^-$  cells were loaded with acetoximethyl ester of Fura-2 (1  $\mu\text{M}$  final concentration) and the fluorescence of the cellular suspension ( $10^6/2$  ml) was monitored with an LS-5 spectrofluorimeter (Perkin-Elmer Corp., Pomona, CA) using 2 ml quartz cuvette. The cell suspension was excited at 340–380 nm and fluorescence measured at 510 nm. Five slit widths were used for both excitation and emission. All measurements were performed at 37°C using a thermostatically controlled cuvette holder and stirring apparatus.  $[\text{Ca}^{2+}]_i$  were calculated by the method of Grynkiewicz et al. (21). In the  $\text{Ca}^{2+}$  depletion experiments, EGTA (final concentration 10 mM) was added to the cellular suspension from a 500-mM stock (pH 7.4) 1–10 min before the addition of the stimulatory mAb.

## Results and Discussion

*Cell Distribution of BB3 Antigenic Determinant.* A polyclonal  $\text{CD3}^+4^-8^- \text{WT31}^-$  cell line termed MV1 was used for mice immunization. This cell line was derived from peripheral blood lymphocytes depleted of  $\text{CD8}^+$  and  $\text{CD4}^+$  cells by specific mAbs and complement and cultured in the presence of rIL-2. We have previously shown that the CD3-associated molecules expressed in this cell line are likely to be represented by the TCR- $\gamma/\delta$  gene products (22). Among the various hybridoma supernatants tested, one, termed BB3, reacted with the immunizing MV1 line but not with a large panel of TCR- $\alpha/\beta$ -bearing lymphocyte populations or clones. On the other hand, large proportions of  $\text{CD3}^+\text{WT31}^-$  clones, derived from six different donors, reacted with BB3 mAb. For example, 12 of 16  $\text{CD3}^+\text{WT31}^-$  clones derived from one donor and 9 of 12 derived from another donor were  $\text{BB3}^+$ . In addition, BB3 mAb reacted with variable, but usually small, percentages of peripheral blood lymphocytes. These percentages ranged between 0.5 and 8% in 12 different individuals tested and paralleled the percentages of  $\text{CD3}^+\text{WT31}^-$  cells, as indicated by two-color immunofluorescence experiments. A representative experiment is shown in Fig. 1: 7% of PBMC were stained by anti-CD3 mAb but were unreactive with WT31 mAb. When the same lymphocyte suspension was analyzed using anti-CD3 in combination with BB3 mAb, 6.6% of the cells were simultaneously stained by the two mAbs. Moreover,  $\text{BB3}^+$  cells were restricted to  $\text{CD3}^+$  cell populations, since no  $\text{BB3}^+\text{CD3}^-$  cells could be detected. Taken together, these results indicate that most, but not all,  $\text{CD3}^+\text{WT31}^-$  peripheral blood lymphocytes are recognized by BB3 mAb. We next analyzed whether after interaction with the BB3 mAb, BB3 surface molecules would undergo modulation since such phenomenon has been shown to

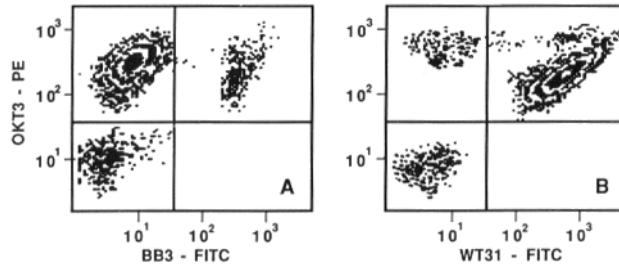


FIGURE 1. Distribution of BB3<sup>+</sup> cells in peripheral blood lymphocytes. Peripheral blood lymphocytes were stained with PE-OKT3 (anti-CD3) and FITC-BB3 (A) or with PE-OKT3 and FITC-WT31 (anti-TCR- $\alpha/\beta$ ) (B), the contour plot was divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with red and green fluorescence (*upper right*), and cells with only green fluorescence (*lower right*). 6.6% of lymphocytes were simultaneously stained with OKT3 and BB3 (A) and 7% of cells were OKT3<sup>+</sup> and WT31<sup>-</sup> (B).

occur after interaction of TCR- $\alpha/\beta$  or CD3 (23, 24) with the respective mAbs. We found that BB3 antigenic determinant was susceptible to mAb-induced surface modulation in a similar manner as CD3 molecules. Previous studies have shown that TCR- $\alpha/\beta$  molecules undergo comodulation with CD3 after cell treatment with either anti-TCR or anti-CD3 mAbs (24). Similar studies performed in both MV1 and several CD3<sup>+</sup> WT31<sup>-</sup> clones indicated that antigen modulation induced by BB3 mAb caused a simultaneous downregulation of surface CD3 molecules. Similarly, mAb-induced CD3 modulation led to the disappearance of BB3-reactive surface antigen (data not shown). These data strongly suggest that the surface molecules recognized by BB3 mAb in CD3<sup>+</sup> WT31<sup>-</sup> cells are physically linked to the CD3 molecular complex.

**Biochemical Characterization of Proteins Recognized by BB3 mAb.** The BB3 mAb immunoprecipitated from <sup>125</sup>I surface-labeled MV1 cells an 80-kD surface protein under nonreducing conditions and three bands of ~44, 42, and 38 kD under reducing conditions (in the presence of 1% NP-40) (Fig. 2). Similar figures were obtained from all of the eight additional CD3<sup>+</sup> WT31<sup>-</sup> BB3<sup>+</sup> clones analyzed: in all instances, only disulphide-linked molecules could be precipitated (data not shown). Under ex-

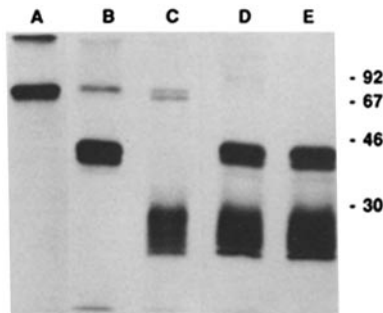


FIGURE 2. SDS-PAGE analysis of surface molecules immunoprecipitated from the CD3<sup>+</sup> WT31<sup>-</sup> BB3<sup>+</sup> cell line MV1 by BB3 and anti-CD3.  $\sim 15 \times 10^6$  MV1 cells were surface labeled with <sup>125</sup>I using the lactoperoxidase technique. One-third of the labeled cells were lysed in buffer containing 1 g/100 ml digitonin and the remaining cells were lysed in buffer containing 1% NP-40. Lysates were pre-cleared and then immunoprecipitation was performed with either anti-Leu-4 (anti-CD3) or BB3 mAbs coupled to protein A-Sepharose beads. MV1 cells were lysed in NP-40 and immunoprecipitated by BB3 mAb under nonreducing conditions (lane a) or under reducing conditions (lane b). The same cells were also lysed in digitonin-containing buffer and immunoprecipitated by anti-Leu-4 mAb under nonreducing conditions (lane c) or under reducing conditions (lane d). In lane e, cells were lysed in digitonin and immunoprecipitated with BB3 mAb under reducing conditions.

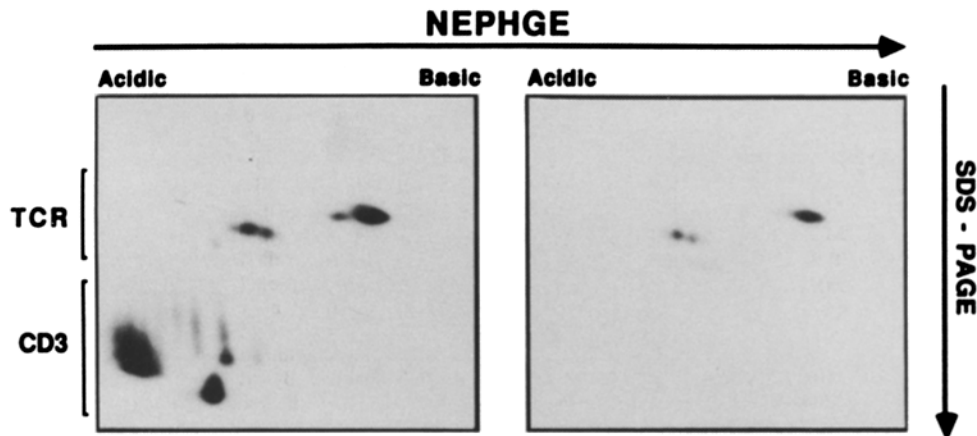


FIGURE 3. NEPHGE analysis of the TCR molecular complex present on the  $CD3^+ 4^- 8^- WT31^-$  clone 6.6.1. In the left panel,  $^{125}I$ -labeled 6.6.1 cells were lysed under conditions that preserve the CD3-TCR complex association (1% digitonin) and were immunoprecipitated with anti-Leu-4 (anti-CD3 mAb). In the right panel, the CD3-TCR molecular complex was dissociated by lysing the same cells in buffer containing 1% NP-40. The lysate was then immunoprecipitated by using the BB3 mAb. Samples were analyzed by NEPHGE. In the second dimension, 11% acrylamide gels were used for SDS-PAGE analysis.

perimental conditions that preserve the TCR/CD3 association (lysis in the presence of 1% digitonin), the BB3 mAb coprecipitated the three bands above together with CD3 molecules. Similar figures were obtained when the same cell lysate was immunoprecipitated with anti-CD3 mAb. The identity between the 44-, 42-, and 38-kD molecules precipitated by BB3 mAb and those precipitated by anti-CD3 mAb was confirmed in two-dimensional electrophoresis experiments (using pH-dependent separation in the first dimension followed by size separation in the second dimension, NEPHGE) (17). As shown in Fig. 3, in a BB3<sup>+</sup> clone, the CD3-associated molecules immunoprecipitated by anti-CD3 mAb displayed the same electrophoretic mobility as the molecules immunoprecipitated by BB3 mAb. It is evident that, in both instances, immunoprecipitated molecules were represented by a 44-kD molecule displaying a basic pI ( $\sim 7.5$ ) and by two more acidic proteins (pI  $\sim 6$ ) with a mol mass of  $\sim 42$  and 38 kD. Similar data have been reported by Lanier et al. (8) by immunoprecipitation experiments using an anti-CD3 mAb on a  $CD3^+ WT31^-$  cell population. The finding that in immunoprecipitation experiments performed under conditions that do not preserve TCR-CD3 interactions (1% NP40) BB3 mAb only precipitated TCR proteins and not CD3 molecules, strongly suggests that the antigenic determinant recognized by BB3 is present on TCR- $\gamma/\delta$  and not on CD3 molecules.

*BB3 mAb Induces  $CD3^+ WT31^-$  Lymphocytes to Express their Functional Program.* Previous studies have indicated that  $CD3^+ WT31^-$  cells, similarly to conventional  $CD3^+ WT31^+$  (TCR- $\alpha/\beta^+$ ) lymphocytes respond to a series of stimuli including PHA, anti-CD3 mAb, and appropriate combinations of anti-CD2 mAbs (9, 10). T cell responses resulted in activation of the cellular functional program including lymphokine production and triggering of the lytic machinery. Although the

TABLE I  
*IL-2 Production Induced by Anti-CD3 or Anti-TCR- $\gamma/\delta$  Antibodies*

Cells	Surface phenotype	IL-2 production upon stimulation with mAbs*		
		Anti-CD3	BB3	None
MV1 <sup>†</sup>	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	11	10	0
M 4.3	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	12	10	0
M 2.1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	24	22	0
T 2.1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	16	8	0
MV113	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	0	0	0
T 12	CD3 <sup>+</sup> 4 <sup>+</sup> 8 <sup>-</sup> WT31 <sup>+</sup> BB3 <sup>-</sup>	11	0	0
T 1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>+</sup> WT31 <sup>+</sup> BB3 <sup>-</sup>	36	0	0

\* 10<sup>6</sup>/ml cells were cultured in the presence of a final concentration of 1:20 hybridoma supernatant and 2 ng/ml of PMA. After 24 h supernatants were collected and tested for IL-2 activity using the CTLL indicator system. Data are expressed as arbitrary units of IL-2 activity as described in Materials and Methods.

<sup>†</sup> Cells were represented by MV1 polyclonal cell line, used for mice immunization, or by cloned cells.

induction of T cell responses by anti-CD3 mAb suggested the existence of a functional activation pathway initiated by CD3/TCR complex, no direct proof existed that the TCR- $\gamma/\delta$  itself was capable of delivering activation signals to the cell. Thus, we investigated whether TCR- $\gamma/\delta$ <sup>+</sup> clones, as well as the immunizing MV1 cell line, could be triggered by the BB3 mAb to release IL-2. As shown in Table I, three CD3<sup>+</sup> WT31<sup>-</sup> BB3<sup>+</sup> clones and the MV1 cell line, which produced IL-2 after stimulation with anti-CD3 mAb, released similar amounts of IL-2 also after stimulation with BB3 mAb. On the other hand, the remaining clone did not release IL-2 with neither anti-CD3 nor BB3 mAb. Moreover, BB3 mAb failed to activate two

TABLE II  
*Lysis of mAb secreting hybridomas, by CD3<sup>+</sup> WT31<sup>-</sup> or CD3<sup>+</sup> WT31<sup>+</sup> effector cells*

Effector cells	Surface phenotype	Hybridoma target cells		
		Anti-CD3	BB3	Anti-CD2 <sup>§</sup>
MV1*	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	56 <sup>†</sup>	96	0
M 4.3	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	81	91	7
M 2.1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	43	89	0
T 2.1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	84	91	1
MV113	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	65	83	7
T 12	CD3 <sup>+</sup> 4 <sup>+</sup> 8 <sup>-</sup> WT31 <sup>+</sup> BB3 <sup>-</sup>	36	0	0
T 1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>+</sup> WT31 <sup>+</sup> BB3 <sup>-</sup>	42	0	0

\* Effector cells were represented by MV1 polyclonal cell line or by cloned cells.

<sup>†</sup> Data are expressed as percent <sup>51</sup>Cr release from labeled hybridoma target cells at a 10:1 E/T cell ratio.

<sup>§</sup> Note that in this experiment only a single anti-CD2 mAb (MAR 206) was used. We have previously shown that CD3<sup>+</sup> WT31<sup>-</sup> cells can be activated via surface CD2 molecules only in the presence of two appropriate anti-CD2 mAb-producing hybridomas.

TABLE III  
*mAb-induced Lysis of P815 Target Cells by CD3<sup>+</sup> WT31<sup>-</sup> or CD3<sup>+</sup> WT31<sup>+</sup> Effector Cells*

Effector cells	Surface phenotype	mAb added to the cytolytic test*			
		Anti-CD3	WT31	BB3	None
6.6.1 <sup>†</sup>	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	82 <sup>§</sup>	2	81	3
6.25.4	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	77	4	64	4
5.25.10	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	89	1	78	1
MV1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>-</sup> BB3 <sup>+</sup>	83	5	84	6
5.50.1	CD3 <sup>+</sup> 4 <sup>-</sup> 8 <sup>-</sup> WT31 <sup>+</sup> BB3 <sup>-</sup>	64	51	3	3

The cytolytic test against <sup>51</sup>Cr-labeled P815 murine mastocytoma cells was performed as described in Materials and Methods.

\* The various antibodies were added at the onset of the cytolytic test.

<sup>†</sup> Effector cells were represented by MV1 polyclonal cell line or by cloned cells.

<sup>§</sup> Data are expressed as a percent specific <sup>51</sup>Cr release at 1:1 E/T ratio.

CD3<sup>+</sup> WT31<sup>+</sup> (TCR- $\alpha/\beta$ <sup>+</sup>) clones that produced IL-2 after stimulation with anti-CD3.

Given the ability of CD3<sup>+</sup> WT31<sup>-</sup> clones and populations to lyse target cells after stimulation with mAbs directed to the CD3/TCR molecular complex (9), we investigated whether activation of the lytic machinery could also be induced by BB3 mAb. To this end, we used as triggering target the hybridoma producing BB3 mAb. As shown in Table II, all the CD3<sup>+</sup> WT31<sup>-</sup> BB3<sup>+</sup> cells analyzed efficiently lysed the BB3-producing hybridoma as well as an anti-CD3 hybridoma. On the contrary, no lysis occurred when a single anti-CD2 mAb-producing hybridoma (mAb 206) was used. In fact, as previously shown, the activation of the CD2 pathway requires the combined use of suitable anti-CD2 mAbs and does not occur when the signal is given by a single anti-CD2 mAb (25).

To further define the ability of BB3 mAb to trigger the lytic machinery of TCR- $\gamma/\delta$ <sup>+</sup> cells, we used a cytolytic assay in which P815 target cells (which express surface Fc- $\gamma$  receptors) are lysed by CD3<sup>+</sup> cytolytic cells provided that soluble anti-CD3 mAb is present (18). As shown in Table III, BB3 mAb was as efficient as anti-CD3 mAb in inducing lysis of P815 cells by TCR- $\gamma/\delta$ <sup>+</sup> clones and MV1 cell line. The CD3<sup>+</sup> WT31<sup>+</sup> cytolytic clone used as control did not lyse P815 in the presence of BB3 but did so in the presence of WT31 mAb.

We have also investigated the role of free cytoplasmic Ca<sup>2+</sup> levels in the transduction of signals induced after cell stimulation with BB3 mAb. In previous studies (10) we demonstrated that signaling via either CD3 or CD2 surface molecules resulted in a rapid increase of free intracytoplasmic Ca<sup>2+</sup> levels in both CD3<sup>+</sup> WT31<sup>-</sup> and CD3<sup>+</sup> WT31<sup>+</sup> lymphocytes. In addition, experiments performed in the presence of the Ca<sup>2+</sup> chelator EGTA indicated that the [Ca<sup>2+</sup>]<sub>i</sub> raise was consequent to an early release from the internal stores followed by a more sustained Ca<sup>2+</sup> influx from the extracellular compartment (10). Here we show that triggering of surface TCR molecules of CD3<sup>+</sup> WT31<sup>-</sup> lymphocytes by BB3 mAb induces a rapid increase of [Ca<sup>2+</sup>]<sub>i</sub> levels, followed by a plateau phase (Fig. 4 B). Similar events occurred when cells were stimulated by anti-CD3 antibodies (Fig. 4 A). Although not shown, experiments of Ca<sup>2+</sup> mobilization performed in the presence of EGTA confirmed that BB3

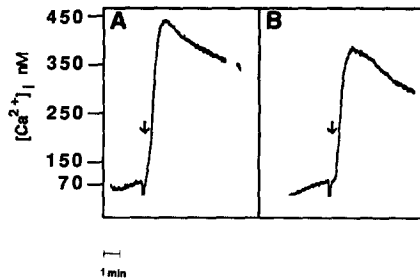


FIGURE 4. Comparative analysis of  $[Ca^{2+}]_i$  mobilization in 6.6.1 cloned CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> cells in response to anti-CD3 (A) or BB3 (B) mAb. Cloned cells were loaded with acetoxymethyl ester Fura-2 as described in Materials and Methods. Stimuli were added in the presence of 1 mM extracellular  $Ca^{2+}$ . The arrows indicate the time of the addition of the stimuli.

mAb induced both a rapid  $Ca^{2+}$  release from the internal stores followed by an influx from the extracellular compartment.

Our present data provide evidence that stimuli that act directly on the TCR- $\gamma/\delta$  molecules are capable of inducing TCR- $\gamma/\delta^+$  cell activation. Similarly to TCR- $\alpha/\beta$ , the early events that follow ligand-receptor interaction are represented by a rapid  $[Ca^{2+}]_i$  increase that reflects sequential  $Ca^{2+}$  mobilization from intracellular and extracellular compartments. In addition, functional analyses revealed that BB3 mAb induced, in CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> cells, both triggering of the cytolytic machinery and production of IL-2. Therefore, it is conceivable that upon binding to TCR- $\gamma/\delta$ , a natural ligand (antigen?) may similarly trigger CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> cells to express their functional program. Along the same line, an antibody described by Moingeon et al. (5), reactive with a clonotypic determinant of a fetal TCR- $\gamma/\delta^+$  clone, inhibited the cytolytic activity of this clone against the NK-sensitive K562 target cells and induced proliferation of the same clone in the absence of exogenous IL-2 (5). More recently, the same group described a second antibody (Ti- $\gamma$ A) directed to the TCR- $\gamma/\delta$  of adult T lymphocytes (26). Although no functional data have been reported, Ti- $\gamma$ A mAb appears to be similar to BB3 since it recognized TCR- $\gamma/\delta$  molecules expressed by the majority of CD3<sup>+</sup>WT31<sup>-</sup> peripheral lymphocytes and cell clones. However, a remarkable difference between the two antibodies is represented by the reactivity of the Ti- $\gamma$ A antibody with the TCR- $\gamma/\delta$  proteins, independently upon the C $\gamma$  gene segment used, whereas BB3 reactivity appears to be restricted to cells that express the disulphide-linked form of the TCR- $\gamma/\delta$ . It is noteworthy that the majority of CD3<sup>+</sup>WT31<sup>-</sup> clones obtained under our experimental conditions displayed the disulphide-linked form of the TCR molecules, in which the  $\gamma$  chain is represented by the molecular product of C $\gamma$ 1 gene segment (5, 8, 27), and reacted with BB3 mAb. In contrast, less than one-third expressed the non-disulphide-linked form of the TCR and were not BB3 reactive. In this context, the PEER leukemic cell line, known to express the molecular product of the C $\gamma$ 2 gene (that is non-disulphide-linked to  $\delta$  chain) (27) did not react with BB3 mAb. Therefore, the BB3 mAb allows us to further dissect the CD3<sup>+</sup>WT31<sup>-</sup> cell subset and the two populations so defined may correspond to cells that express different TCR- $\gamma/\delta$  types.

### Summary

In an attempt to select mAbs specific for human TCR- $\gamma/\delta$ , a polyclonal CD3<sup>+</sup>4<sup>-</sup>8<sup>-</sup>WT31<sup>-</sup> (TCR- $\gamma/\delta^+$ ) cell line (MV1) was used for mice immunization.



An mAb, termed BB3, reacted with MV1 cells but not with a large panel of CD3<sup>+</sup>WT31<sup>+</sup> (TCR- $\alpha/\beta$ <sup>+</sup>) cell populations or clones. In addition, BB3 mAb reacted with the majority of CD3<sup>+</sup>WT31<sup>-</sup> clones derived from six different donors. Double-color fluorescence experiments and FACS analysis showed that BB3<sup>+</sup> cells were restricted to the CD3<sup>+</sup> fraction of peripheral blood lymphocytes; in addition, in several donors the percentages (0.5–8% of total PBL) of BB3<sup>+</sup> cells paralleled those of CD3<sup>+</sup>WT31<sup>-</sup> cells. Surface molecules recognized by BB3 were susceptible to antibody-induced modulation; in addition, cell treatment with either BB3 or anti-CD3 mAb caused the simultaneous downregulation of the two molecules. That BB3 molecules are physically linked to CD3 antigen was further supported by immunoprecipitation experiments. Thus, under conditions that preserve the TCR-CD3 association, both BB3 and anti-CD3 mAb precipitated from <sup>125</sup>I-labeled MV1 cells the same set of molecules. These consisted in the 18–28-kD CD3 molecules and in three bands of ~44, 42, and 38 kD under reducing conditions. When cell lysis was performed in 1% NP-40, the molecules immunoprecipitated by BB3 mAb were represented by an 80-kD band under nonreducing conditions, which resolved, under reducing conditions, in the three 44-, 42-, and 38-kD bands. Similar disulphide-linked forms of the TCR molecules were revealed in all of the other eight CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> clones analyzed. Analysis of TCR molecules by electrophoresis (NEPHGE) showed that BB3 or anti-CD3 precipitated a 44-kD molecule displaying a basic PI (~7.5) and two more acidic proteins (PI ~6) with a mol mass of 42 and 38 kD. Studies aimed to define whether stimuli directly acting on TCR- $\gamma/\delta$  could induce CD3<sup>+</sup>WT31<sup>-</sup> cell activation revealed that (a) In the presence of PMA, soluble BB3 mAb induced IL-2 production by MV1 cell line and by three other CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> clones analyzed. (b) BB3 mAb-producing hybridoma used as triggering target, was efficiently lysed by CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> effector cells (but not by CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>-</sup> conventional CTL). (c) Soluble BB3 mAb induced CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> effector cells to lyse the Fc receptor-positive P815 target cells. (d) BB3-TCR- $\gamma/\delta$  interaction on CD3<sup>+</sup>WT31<sup>-</sup>BB3<sup>+</sup> cells induced a rapid increase of [Ca<sup>2+</sup>]<sub>i</sub> levels, similar to that observed in response to anti-CD3 mAbs.

We thank Miss Rosanna Bellomo for technical assistance and Miss Cinzia Miriello for typing the manuscript.

*Received for publication 19 January 1988.*

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