TWO SUBSETS OF HUMAN T LYMPHOCYTES EXPRESSING γ/δ ANTIGEN RECEPTOR ARE IDENTIFIABLE BY MONOCLONAL ANTIBODIES DIRECTED TO TWO DISTINCT MOLECULAR FORMS OF THE RECEPTOR

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The majority of human peripheral blood T lymphocytes express surface receptor for antigen (TCR) consisting of a disulphide-linked heterodimer with a M_r of ~90 kD, which is noncovalently linked to CD3, a set of proteins displaying molecular weight ranging between 20 and 28 kD (1, 2).

While both TCR chains, an acidic α chain (mol wt, 45–52) and a more basic β chain (mol wt, 37–45), contain variable and constant region domains, the CD3 proteins are invariant (3–5). Cell surface expression of TCR-α/β can be revealed by the use of mAbs such as WT31, which react with all T lymphocytes expressing TCR-α/β (6). Several lines of evidence now exist that CD3 molecules can be expressed in association with TCR proteins different from the conventional α and β chains (7–11). Indeed, both $CD3^+WT31^-$ T cell leukemias and T cell clones (12) derived from immunodeficient patients have been shown to express TCR molecules encoded by two different sets of rearranging genes termed γ and δ (13–21). Further experiments performed on $CD3^+WT31^-$ populations or clones derived from normal donors provided evidence that expression of these CD3-associated proteins characterize a small T lymphocyte subset displaying unique phenotypic and functional characteristics (9, 11, 22, 23). Among TCR-γ/δ, at least two distinct forms have been identified (12). While the Cγ1 gene segment usage correlates with the expression of a disulphide-linked form of receptor, the usage of the Cγ2 gene segment results in a non-disulphide-linked form (24, 25). The recent availability of mAbs directed against TCR-γ/δ offers the possibility to directly analyze these surface proteins. In this context, we recently selected an mAb (termed BB3) that reacts with TCR molecules expressed on approximately two thirds of peripheral $CD3^+WT31^-$ lymphocytes (26). Another mAb termed δ-TCS-1 has recently been selected that reacts with the δ chain of the TCR complex.

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By using these two mAbs, we analyzed a large panel of $CD3^+WT31^-$ clones. We show that ~100% of such clones were reactive with one or another mAb. More importantly, $BB3^+$ clones failed to react with δ-TCS-1 mAb, and, conversely, those stained by δ-TCS-1 did not react with BB3 mAb. The mutually exclusive expression of the antigenic determinants recognized by the two antibodies was further confirmed by functional experiments in which triggering of the lytic machinery of $BB3^+$ or δ-$TCS\text{-}1^+$ clones only occurred in the presence of the appropriate mAb. Biochemical analyses revealed important differences in the TCR molecules immunoprecipitated from the two types of clones. BB3-reactive $CD3^+WT31^-$ clones only expressed the disulphide-linked form of TCR, whereas δ-$TCS\text{-}1^+$ clones expressed the non-disulphide-linked form of TCR. In addition, two-dimensional (2D)[1]-PAGE analysis revealed major differences in the charge mobility of TCR chains immunoprecipitated from the two types of clones.

Materials and Methods

Cloning of $CD3^+$ $WT31^-$ PBL. PBL were isolated from seven different normal volunteers. $CD3^+WT31^-$ lymphocytes were then isolated and cloned under limiting dilution as previously described (9, 27). Briefly, cells were cultured at various cell concentrations per well in round-bottomed wells (Greiner Labor Technik, Nurtinger, Federal Republic of Germany) in the presence of irradiated feeder cells (5,000 rad) in RPMI 1640 medium containing 10% FCS (Gibco Laboratories, Grand Island, NY), 0.5% vol/vol PHA, and an exogenous source of IL-2 derived from PHA-stimulated human spleen cell suspensions (27). Microcultures were considered as clones on the basis of the frequency of proliferating microculture in each experiment (28). Clones were screened directly by FACS analysis for the presence of surface CD3 and the simultaneous lack of reactivity with WT31 mAb. Clones were then expanded in the presence of rIL-2 (Cetus Corp., Emeryville, CA) and in the absence of feeder cells always in the same type of microtiter plates.

Isolation of Thymocytes. Single cell suspensions were obtained from normal thymus fragments that had been removed from children (<5 yr old) during corrective cardiac surgery. Thymocytes were isolated by teasing the thymus fragments in RPMI 1640 supplemented with 10% FCS. Viable mononuclear cells were obtained by Ficoll/Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. For the isolation of cell subpopulations, thymocytes were then incubated with saturating amounts of anti-CD4 plus anti-CD8 antibodies for 30 min at 4°C, followed by an additional 45 min at 37°C with 1/3 dilution of noncytotoxic rabbit complement (Rabbit complement-MA; Cederlane Laboratories, Ontario, Canada).

mAbs. The mAbs used in these studies were represented by JT3A (29) and anti-Leu-4 (anti-CD3) (Beckton Dickinson and Co., Basel, Switzerland), B9.4 (anti-CD8) (30), CK7.9 (anti-CD4) (9), WT31 (directed against the TCR complex expressed by TCR-α/β-bearing cells) (6), BB3 directed to ~60–70% of $CD3^+WT31^-$ cells (26), δ-TCS-1 directed to the δ chain of $CD3^+WT31^-$ lymphocytes (T cell Sciences Inc., Cambridge, MA), MAR 21 (anti-CD7) (29) MAR 199 (anti-HLA-DR), CD2-1, and CD2-9, which recognize distinct epitopes of the CD2 molecule and, when used in combination, are able to induce T cell activation (29, 31).

Flow Cytofluorometric Analysis. The techniques used have been described in detail elsewhere (5, 29). Briefly, aliquots of 10^5 cloned cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig. Control aliquots were stained with the fluorescent reagent clone. All samples were then analyzed on a flow cytometer (FACS II; Becton Dick-

[1] *Abbreviations used in this paper:* NEPHGE, nonequilibrium pH gradient electrophoresis; 2D, two dimensional.

inson Immunocytometry Systems, Mountain View, CA) gated to exclude nonviable cells. Results are expressed as arbitrary normalized fluorescence histograms, i.e., number of cells vs. fluorescence intensity.

Cytolytic Assays. The ability of various mAbs to trigger the cytolytic activity of clonal T lymphocytes was assessed in a 4-h ^{51}Cr-release assay in which target cells were represented by the murine mastocytoma cell line termed P815 (28, 32). In this assay, 5×10^3 ^{51}Cr-labeled P815 cells were added to each well containing 5×10^3 effector cells (for a final E/T ratio of 1:1) in the presence of either one of the following mAbs: anti-CD3 (JT3A) (10 μl of culture supernatant), BB3 (10 μl of culture supernatant), δ-TCS-1 (10 μl of 1:200 dilution of the stock solution) WT31 (10 μl of a 1:50 dilution of the stock solution), and CD2-1 + CD2-9 (50 μl of a 1:1 mixture of the two culture supernatants). The final culture volume was 200 μl for each well. After a 4-h culture period, 100 μl was collected from each well and counted in a gamma counter for ^{51}Cr release. Percent-specific ^{51}Cr-release was calculated as previously described (27, 28).

Characterization of Radioiodinated Cell Surface Proteins. Cloned cells (~1.5×10^6) were washed five times in cold RPMI 1640, twice in PBS, and then surface-labeled with ^{125}I using the lactoperoxidase/glucose oxidase-catalyzed iodination (33). 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% NaN3 and contained 1% NP40 or digitonin. Digitonin was added at 1 g/100 ml to the above solution, boiled, stirred for 20 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 then precleared three times with 20 μl of packed protein A–sepharose beads for 2 h under rotation. Lysates were then incubated for 2 h with 200 μl of BB3 culture supernatant or 50 μl of a 1:10 dilution of anti-Leu-4 mAb or δ-TCS-1 mAb. 20 μl 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 (34). The nonequilbrium pH gradient electrophoresis (NEPHGE) was carried out using pH 3.5–10 ampholines followed by 11% SDS-PAGE gels for size separation as described (12).

Results

Distribution of BB3$^+$ and δ-TCS-1$^+$ Cells in Peripheral Blood and Thymus. To study the expression of the TCR-γ/δ surface determinants recognized by BB3 or δ-TCS-1 mAbs, we analyzed unfractionated, as well as CD4$^-$8$^-$ T cell populations isolated from peripheral blood or thymus. In E-rosetting cells isolated from normal peripheral blood, the percentages of BB3$^+$ cells ranged from 0.5–12%, whereas δ-TCS-1$^+$ cells were 0.2–2.3% (in 10 different donors). In addition, in all donors, the percent of BB3$^+$ cells exceeded that of δ-TCS-1$^+$ cells. TCR-γ/δ-enriched populations were obtained by treating E-rosetting cells with anti-CD4 plus anti-CD8 mAbs and complement. The resulting populations contained variable proportions (40–90%) of CD3$^+$ cells (most of which did not react with WT31 mAb). Consistently, the sum of the percentages of BB3$^+$ and δ-TCS-1$^+$ cells approximated those of CD3$^+$(WT31$^-$) cells in seven different donors tested. Data from two representative donors are shown in Table I. Moreover, in polyclonal cell lines obtained by in vitro expansion (in rIL-2) of FACS-purified CD3$^+$WT31$^-$ lymphocyte populations, the sum of the percentages of BB3$^+$ and δ-TCS-1$^+$ cells was ~100% (data not shown). In freshly prepared, unfractionated, thymocyte cell suspensions, δ-TCS-1$^+$ cells were 1–5.5%, whereas BB3$^+$ cells could not be detected in four thymuses analyzed. Purified CD4$^-$8$^-$ thymocytes contained variable proportions of CD3$^+$ cells and few WT31$^+$ cells. In these TCR-γ/δ-enriched populations, δ-TCS-1$^+$ cells approximated the per-

TABLE I

Reactivity of BB3 and δ-TCS-1 mAbs in Unfractionated or CD4⁻8⁻ Peripheral Blood (PB) T Lymphocytes or Thymocytes

Lymphocyte population	Exp.	CD3	WT31	BB3	δ-TCS-1
Unfractionated PB E^+ cells	1	96*	82	12	1
	2	90	85	3.5	0.5
$CD4^-8^-$ PB E^+ cells	1	89	3	75	8
	2	68	10	45	10
Unfractionated thymocytes	1	42	40	0	3
	2	46	ND	0	5.5
$CD4^-8^-$ thymocytes	1	60	8	0	52
	2	50	0	0	48

* Cells were analyzed by flow cytofluorometry; data are presented as percent-positive cells (after subtraction of staining with the fluorescent conjugate alone).

centages of $CD3^+WT31^-$ cells, while $BB3^+$ cells could not be detected. Two representative experiments are shown in Table I.

Phenotypic Analysis of T Cell Clones Derived from Peripheral Blood $CD3^+WT31^-$ Lymphocytes. Taken together, the above data suggested that BB3 and δ-TCS-1 mAbs recognized different subsets of TCR-γ/$δ^+$ cells. In addition, it appeared that $BB3^+$ and δ-$TCS\text{-}1^+$ cells could account for most, if not all, peripheral blood $CD3^+WT31^-$ cells. To define whether indeed BB3 and δ-TCS-1 mAbs reacted with determinants of TCR-γ/δ expressed by different peripheral blood T cells we analyzed a large number of $CD3^+WT31^-$ cell clones. Clones were derived by limiting dilution from $CD3^+WT31^-$ peripheral blood populations of healthy donors, as previously described (9, 27). Clones so obtained were screened for the expression of WT31 and CD3 surface antigen and the contaminating $WT31^+$ clones were discarded. 5 out of 72 $CD3^+WT31^-$ clones analyzed expressed low amounts of CD8 surface antigen, whereas the remaining were $CD4^-8^-$. Clones were analyzed for their reactivity with BB3 or δ-TCS-1 mAbs. Approximately 70% of the $CD3^+WT31^-$ clones analyzed were stained by BB3 mAb; more importantly, none of such $BB3^+$ clones reacted with δ-TCS-1 mAb. δ-TCS-1-reactive clones were ~30%, and none of them reacted with BB3 mAb (Fig. 1). These experiments sug-

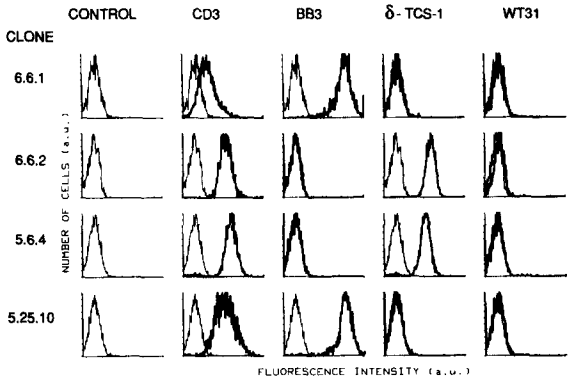

FIGURE 1. Flow cytofluorometric analysis of surface antigens expressed by $CD3^+WT31^-$ human T cell clones. Cells were stained with either one of the following mAbs: anti-CD3, WT31, BB3, or δ-TCS-1 as described in Materials and Methods. Fluorescein-conjugated goat anti-mouse Ig was used as second reagent. Note that clones 6.6.1 and 5.25.10 were stained by BB3 but not by δ-TCS-1 mAb; on the contrary, clones 6.6.2 and 5.6.4 were stained by δ-TCS-1 but not by BB3 mAb.

TABLE II

Appropriate Anti-receptor mAbs Trigger the Lytic Machinery of both BB3⁺ and δ-TCS-1⁺ Clones

Clone‡	Phenotype	mAb added to the cytolytic test*					
		None	Anti-CD3	WT31	BB3	δ-TCS-1	CD2-1⁺ CD2-9
6.6.2	δ-TCS-1⁺	2§	64	0	1	63	38
6.12.4	δ-TCS-1⁺	0	41	0	0	36	31
6.12.3	δ-TCS-1⁺	0	62	1	2	63	37
5.25.3	δ-TCS-1⁺	2	58	2	1	47	42
5.25.11	δ-TCS-1⁺	1	73	ND	2	74	35
5.6.4	δ-TCS-1⁺	5	57	4	5	55	40
6.25.4	BB3⁺	1	92	0	83	1	27
6.6.1	BB3⁺	0	90	0	72	0	25
6.12.8	BB3⁺	0	82	ND	81	2	ND
5.50.17	BB3⁺	3	71	2	67	4	53
5.25.10	BB3⁺	3	69	0	68	3	30
5.12.4	BB3⁺	2	68	ND	65	2	ND
5.25.A	WT31⁺	3	62	42	2	3	38

* The various antibodies were added at the onset of the 4-h ^{51}Cr-release assay containing the Fc receptor-positive P815 target cells.

‡ Representative CD3⁺4⁻8⁻WT31⁻ clones reacting with δ-TCS-1 mAb or with BB3 mAb were tested against ^{51}Cr-labeled murine target cells P815 at an E/T ratio of ~1:1.

§ Data are expressed as a percent-specific ^{51}Cr-release.

gest that TCR surface determinants recognized by BB3 or δ-TCS-1 mAb are expressed by two distinct, non-overlapping CD3⁺WT31⁻ lymphocyte populations. Interestingly, all the five CD8⁺ clones (derived from different donors) expressed the δ-TCS-1 determinant.

Both Types of the CD3-associated Receptors Expressed on CD3⁺ WT31⁻ T Lymphocytes Are Functionally Active. It has been shown that mAbs directed to CD3 or TCR complex molecules can trigger the lytic machinery of cytolytic T cells carrying the typical α/β heterodimer (35). In addition, recent studies indicated that anti-CD3 mAbs can also induce the cytolytic activity of CD3⁺WT31⁻ clones (36). Moreover, we showed that all the CD3⁺WT31⁻ clones derived from normal peripheral blood display cytolytic activity (37). In an attempt to define whether BB3 and δ-TCS-1 mAbs could trigger the lytic machinery of clones expressing the corresponding antigenic determinant, the same set of clones analyzed above was assessed for the ability to kill appropriate target cells in the presence of BB3 or δ-TCS-1 mAbs. Target cells were represented by the Fcγ receptor-positive murine mastocytoma cell line P815 (32). In Table II, we show that P815 cells were not lysed by either BB3⁺ or δ-TCS-1⁺ clones alone (that is in the absence of stimuli added). However, both types of clones displayed a strong cytolytic activity in the presence of PHA or anti-CD3 mAbs. On the other hand, antibodies to other surface molecules expressed on the effector cells such as HLA-DR or CD7 were uneffective (not shown). Moreover, no lysis was detected upon addition of WT31 mAb (this mAb triggered conventional CD3⁺WT31⁺ cytolytic clones used as control). In this cytolytic assay, BB3 mAb induced cytolytic activity of CD3⁺WT31⁻ cells; however this effect was restricted to BB3⁺ clones (Table II). Conversely, δ-TSC-1⁺ clones were induced to kill by δ-TCS-1 but not

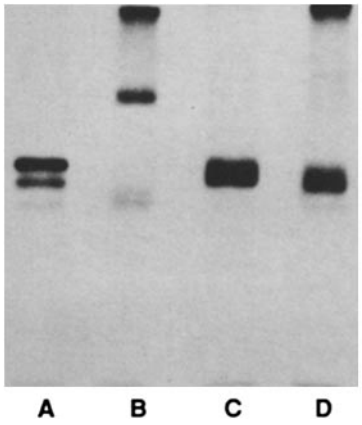

FIGURE 2. SDS-PAGE analysis of surface molecules immunoprecipitated by BB3 or δ-TCS-1 mAbs from two representative $CD3^+WT31^-$ clones. 5×10^6 cloned cells were surface labeled with ^{125}I using the lactoperoxidase technique as described in Materials and Methods. After labeling, cells were lysed in a buffer containing 1% Nonidet P40 (NP40). Cell lysate from clone 5.50.17 ($CD3^+WT31^-BB3^+$ δ-TCS-1^-) was immunoprecipitated with BB3 mAb under reducing (*A*) or nonreducing (*B*) conditions, whereas cell lysate from clone 5.25.3 ($CD3^+WT31^-BB3^-$ δ-TCS-1^+) was immunoprecipitated with δ-TCS-1 mAb under reducing (*C*) or nonreducing (*D*) conditions.

by BB3 mAb. These experiments further confirm that BB3 and δ-TCS-1 mAbs recognize antigenic determinants expressed on different subsets of $CD3^+WT31^-$ cells. In addition, they provide direct evidence that TCR molecules recognized by the two mAbs can transduce activation signals leading to triggering of the lytic machinery. This suggests that both types of receptors are functional and may therefore participate in the complex process of antigen recognition/transduction of the signal, which ultimately results in the ability of a cell to express its functional program.

Reactivity with BB3 or δ-TCS-1 mAb Correlates with Different Forms of CD3-associated TCR Structures. We next analyzed the biochemical characteristics of TCR molecules expressed in peripheral blood-derived $CD3^+WT31^-$ clones reacting with either BB3 or δ-TCS-1 mAb. To this end, cells of $BB3^+$ or δ-TCS-1^+ clones were surface iodinated, and molecules reactive with the corresponding anti-TCR mAb were immunoprecipitated and analyzed by SDS-PAGE. Under nonreducing conditions, the BB3 mAb immunoprecipitated from $BB3^+$ clones a major 80-kD band (and, in some cases, a minor 38-kD band). This band, under reducing conditions,

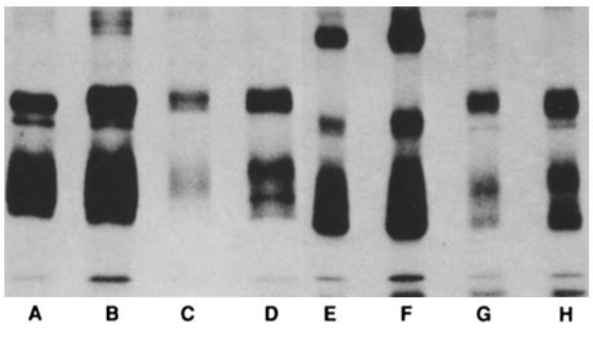

FIGURE 3. SDS-PAGE analysis of CD3-associated molecules immunoprecipitated from four distinct $CD3^+WT31^-$ clones. 10^7 cloned cells were surface labeled and then lysed in a buffer containing digitonin as indicated in Materials and Methods. Cell lysates were then immunoprecipitated by using the anti-Leu-4 (anti-CD3) mAb. The clones analyzed in this experiment were clones 6.6.1 and 5.25.10 ($CD3^+WT31^-BB3^+$ δ-TCS-1^-) and clones 6.5.4 and 5.25.3 ($CD3^+WT31^-$ BB3 δ-TCS-1^+). (*A–D*) CD3-associated molecules were analyzed under reducing conditions. (*E–H*) CD3-associated molecules were analyzed under nonreducing conditions. Clone 6.6.1 ($BB3^+$) (*A* and *E*); clone 5.25.10 ($BB3^+$) (*B* and *F*); clone 6.5.4 (δ-TCS-1^+) (*C* and *G*); clone 5.25.3 (δ-TCS-1^+) (*D* and *H*).

resolved in two major components of 44 and 41 kD, respectively (and a minor component of 38 kD [Fig. 2]). This molecular pattern has been observed in all 13 $BB3^+$ clones analyzed, as well as in a $BB3^+$ polyclonal population, thus indicating that $BB3^+$ cells express the disulphide-linked form of the TCR-γ/δ. In δ-TCS-1-reactive T cell clones, immunoprecipitated molecules appeared differently from those immunoprecipitated by BB3 mAb in $BB3^+$ cells. Thus, both under reducing and nonreducing conditions, a predominant diffuse band of 41–44 kD was detected, while no high molecular weight band was present.

That cells reactive with either BB3 or δ-TCS-1 mAb express different CD3-associated TCR molecules was further confirmed by immunoprecipitation experiments using anti-CD3 mAb. Under conditions that preserve the association between CD3 and TCR molecules (in digitonin-containing buffer) anti-CD3 mAb coprecipitated from $BB3^+$ clones a disulphide-linked form of the TCR, whereas the nondisulphide-linked form was detected in immunoprecipitates from δ-$TCS\text{-}1^+$ clones (Fig. 3). Similar data have been obtained in all 11 δ-$TCS\text{-}1^+$ clones analyzed, thus indicating that δ-$TCS\text{-}1^+$ cells express a nondisulphide-linked form of TCR-γ/δ. It should also be noted that, under nonreducing conditions, in addition to the predominant diffuse band at 41–44 kD, a minor 38-kD band (similar to that found in $BB3^+$ cells) was detected (Fig. 3, lanes *G* and *H*). In most clones analyzed, however, this band could not be detected under reducing conditions, possibly because it integrated into the major 41–44-kD band. It has been suggested that this 38-kD band may represent the molecular product of the δ genes (20).

To better define the molecular differences of TCR molecules expressed by $BB3^+$ or δ-$TCS\text{-}1^+$ cells, we further performed immunoprecipitation experiments followed by 2D gel electrophoresis. In these experiments, immunoprecipitated molecules were first separated according to their pI by NEPHGE, followed, in the second dimension, by SDS-PAGE. Fig. 4 shows the mobility pattern of CD3-associated TCR molecules immunoprecipitated by anti-CD3 mAb from the $BB3^+$ clone termed 5.25.10. Under reducing conditions (Fig. 4 *B*), two components of ~38 and 41 kD displaying a similar charge, and a third more basic 44-kD subunit could be detected. A single

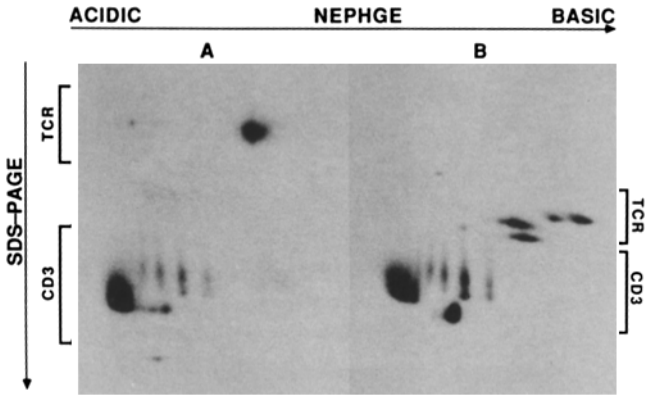

FIGURE 4. 2D-PAGE analysis of the CD3/TCR molecular complex immunoprecipitated from the $CD3^+WT31^-BB3^+$ δ-$TCS\text{-}1^-$ clone 5.25.10. Cells were lysed under conditions that preserve the CD3/TCR complex association (buffer-containing digitonin) and immunoprecipitated with anti-Leu-4 (anti-CD3) mAb. The CD3/TCR complex was analyzed under nonreducing conditions (*A*) and under reducing conditions (*B*). Samples were analyzed by NEPGHE and, in the second dimension, 11% acrylamide gels were used for SDS-PAGE analysis.

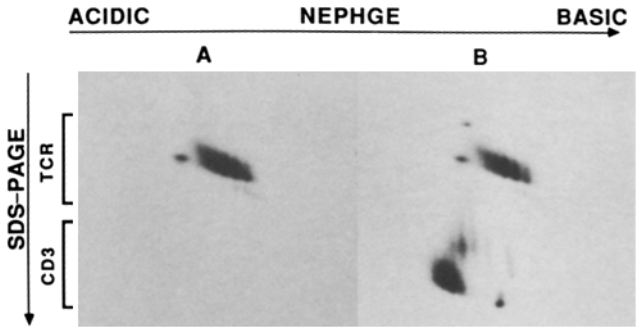

FIGURE 5. 2D-PAGE analysis of the CD3/TCR molecular complex expressed by the $CD3^+WT31^-BB3^-$ δ-TCS-1^+ clone 6.6.2. Cells were labeled with ^{125}I and then lysed either in the presence of 1% NP40 (*A*) or in the presence of digitonin (*B*) and then immunoprecipitated by using the δ-TCS-1 mAb. Samples were run under reducing conditions.

80-kD spot displaying a charge mobility intermediate with respect to those observed for separated chains was observed under nonreducing conditions (Fig. 4 *A*).

Fig. 5 shows the 2D-PAGE analysis of CD3-associated molecules immunoprecipitated from the δ-TCS-1^+ clone termed 6.6.2. In Fig. 5 *A*, immunoprecipitation was performed with δ-TCS-1 mAb and in *B* with anti-Leu-4 mAb (under reducing conditions). In both cases, TCR molecules could be resolved in at least two series of spots migrating with an apparent mol wt of 42–44 and of 41–43 kD, which displayed a similar heterogeneity in charge. In addition to these two series of spots (corresponding to the diffuse 41–44-kD band observed in one-dimensional SDS-PAGE), a third series of poorly labeled spots similar in charge heterogeneity to the most basic spots of the previous two sets was observed. This third family of spots is likely to correspond to the 38-kD band observed in SDS-PAGE. When compared with the TCR molecules immunoprecipitated from the $BB3^+$ clone (Fig. 4), it was clear that TCR molecules precipitated from the δ-TCS-1^+ clone (Fig. 5) were more heterogeneous in charge, relatively more acidic and, in addition, they did not include the very basic 44-kD spots.

To define whether the pattern displayed by the TCR molecules precipitated from either the 5.25.10 ($BB3^+$) clone or the 6.6.2 (δ-TCS-1^+) clone was unique (for those two clones), or rather, it was common to all $BB3^+$ or δ-TCS-1^+ clones, respectively, we analyzed a large panel of clones belonging to the two phenotypic groups.

As many as 13 $BB3^+$ and 11 δ-TCS-1^+ clones were analyzed. Fig. 6 shows the 2D-PAGE analysis (under reducing conditions) of anti-CD3 precipitated molecules of six representative clones (3 $BB3^+$, Fig. 6, *A-C* and three δ-TCS-1^+, *D-F*). It is evident that $BB3^+$ clones displayed very similar TCR mobility patterns as the one shown in Fig. 4, although some differences in labeling of the 38-kD subunit was observed. Of three δ-TCS-1^+ clones, two (Fig. 6 *D* and *E*) displayed a TCR heterogeneity pattern similar to that shown in Fig. 5. The third clone (*F*) showed a relatively reduced number of spots that correspond to the more acidic spots of the previous two clones.

Discussion

In the present study, on the basis of the differential reactivity with two anti-TCR-γ mAbs, we identified two distinct, nonoverlapping subsets within human peripheral blood $CD3^+WT31^-$ T lymphocytes. Perhaps more importantly, these subsets express different molecular forms of CD3-associated TCR structures. Studies per-

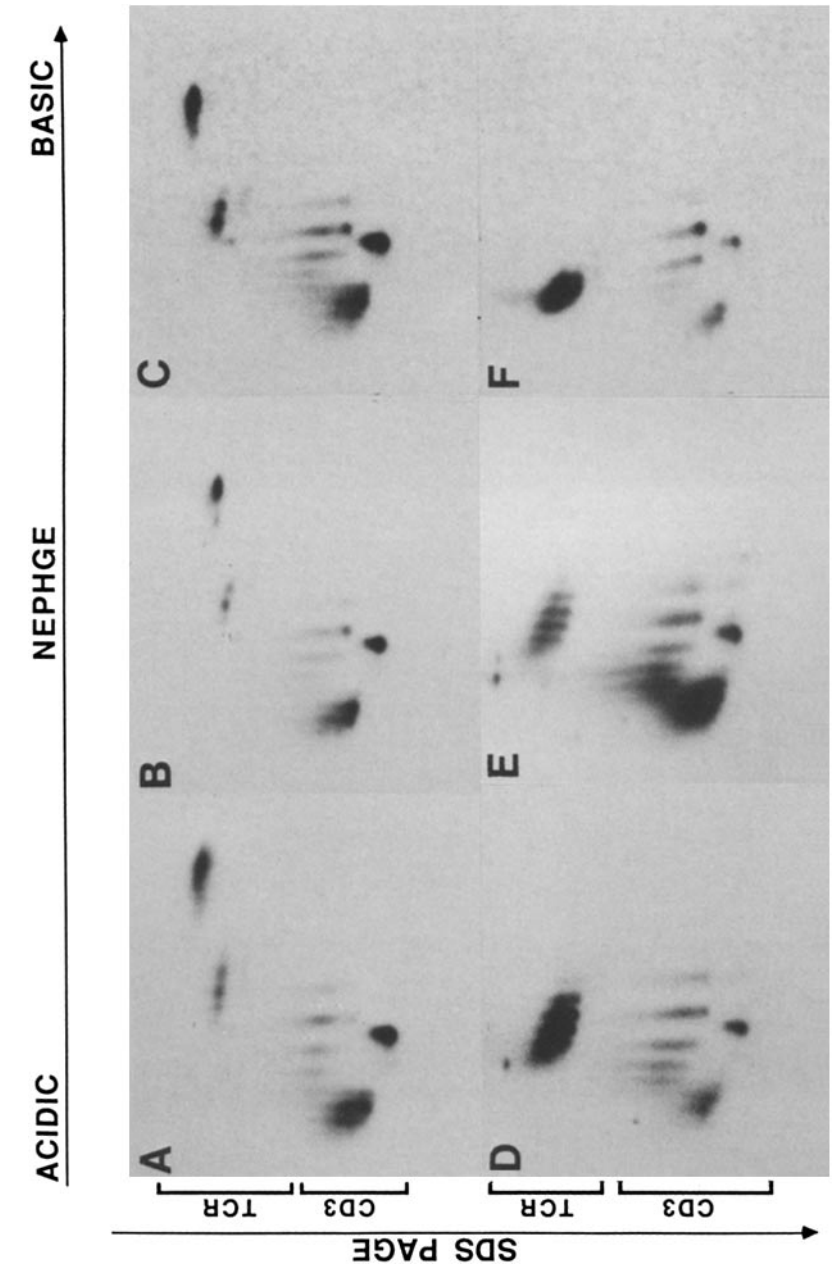

FIGURE 6. Comparison between the CD3-associated molecules expressed by $BB3^+$ or by δ-TCS-1^+ clones. 10^7 cells were surface labeled with ^{125}I and lysed in buffer containing digitonin under conditions that preserve the CD3/TCR association. Cell lysates were then immunoprecipitated using the anti-Leu-4 (anti-CD3) mAb. (*A*) Clone 6.25.4 ($BB3^+$); (*B*) clone 6.12.8 ($BB3^+$); (*C*) clone 5.12.4 ($BB3^+$); (*D*) clone 5.25.3 (δ-TCS-1^+); (*E*) clone 5.15.11 (δ-TCS-1^+); (*F*) clone 5.6.4 (δ-TCS-1^+). All the samples were analyzed by NEPHGE and the second dimension was performed using 11% acrylamide gels for SDS-PAGE analysis under reducing conditions.

formed both at the population and at the clonal level revealed that reactivity with BB3 or δ-TCS-1 was mutually exclusive, as no cells that were simultaneously stained by the two mAbs could be identified. Since this sharp phenotypic distinction was evident both in "resting" PBLs and in long term cultured T cell clones, it is possible to conclude that the differential expression of the antigenic determinants recognized by the two mAbs is a stable phenotypic property of two peripheral $CD3^+WT31^-$ subsets. In addition, it is important to stress that all $CD3^+WT31^-$ clones analyzed reacted with either anti-TCR mAb. Therefore, BB3- or δ-TCS-1-reactive cells are likely to account for most, if not all, of TCR-γ^+ cells present in human peripheral blood. On the other hand, only δ-TCS-1-reactive cells could be detected within the thymus. The lack of $BB3^+$ cells within the thymus could suggest that these cells may represent an independent subset characterized by extra thymic maturation. An alternative explanation could be that $BB3^+$ cells may represent a minor subset originating from $CD3^-4^-8^-$ thymocytes, which rapidly leaves the thymus upon surface expression of CD3/TCR.

The mutually exclusive expression of the antigenic determinants recognized by the two antibodies was further confirmed by experiments in which BB3 and δ-TCS-1 mAb were shown to selectively trigger the lytic machinery of peripheral blood-derived $BB3^+$ and δ-$TCS\text{-}1^+$ clones, respectively. These experiments further substantiated the notion that all $CD3^+WT31^-$ lymphocytes express cytolytic function, and they also provided direct evidence that the cytolytic effector function can be induced, in both types of clones, by stimuli acting via surface TCR molecules. One important corollary of this finding is that both types of TCR-γ/δ^+ lymphocytes express a functionally active TCR that can transduce activation signals upon binding to the natural ligand (38). In this context, it has recently been shown that human (39), as well as murine (40), TCR-γ^+ cells can recognize and lyse allogenic cells in a specific manner. This finding, together with the fact that the TCR-γ and -δ chains are formed by multiple rearranging genes that code for variable regions strongly supports the concept that antigen(s) may be the natural ligand also for TCR-γ^+ cells.

Phenotypic analysis performed at the clonal level confirmed the finding (at the population level) that peripheral T cells reactive with BB3 are more frequent than δ-$TCS\text{-}1^+$ cells, since $BB3^+$ clones represented ~2/3 of all $CD3^+WT31^-$ clones analyzed. In addition, all such clones expressed both CD7 and CD2 differentiation antigens. With regard to the expression of CD7, it should be mentioned that, in some clones, the fluorescence intensity was highly reduced as compared with that detected in conventional TCR-α/β^+ clones (not shown). Functional studies indicated that CD2 surface molecules expressed by $BB3^+$ or δ-$TCS\text{-}1^+$ clones transduced activation signals leading to triggering at the lytic machinery of the cell (Table II). Therefore the "alternative" pathway of T cell activation inititiated via surface CD2 molecules appears to be expressed (and to function) in both types of TCR-γ^+ cells. However, since both Peer- and Molt-13 leukemic cell lines, which express a non-disulphide-linked form of TCR and react with δ-TCS-1 mAb (not shown), do not express surface CD2 molecules, one may expect that $CD2^-$ δ-$TCS\text{-}1^+$ cells may exist. Although analysis of a greater number of clones may be required, it is noteworthy that all of the five $CD3^+WT31^-$ clones expressing CD8 antigens belonged to the δ-$TCS\text{-}1^+$ subset. Another recently described TCR-γ-specific mAb (Ti-γA) (41) reacted with both $CD8^-$ and $CD8^+$ ($CD3^+WT31^-$) cells. According to Jitsukawa

et al. (41), this mAb recognizes a fraction of lymphocytes expressing the disulphide-linked form of TCR-γ. However, if indeed CD8 is expressed only in cells bearing the non disulphide-linked form of the TCR-γ, it is likely that (different from BB3) Ti-γA mAb recognizes an antigenic determinant that is expressed also by the non disulphide-linked form of the TCR-γ.

In addition to defining two cell subsets within human TCR-γ/δ$^+$ cells, BB3 and δ-TCS-1 mAbs identify two forms of receptors that display major molecular differences. Thus, SDS-PAGE analysis performed under both reducing and nonreducing conditions showed that BB3-reactive molecules are represented by disulphide-linked heterodimers, whereas molecules immunoprecipitated by δ-TCS-1 mAbs were always represented by the nondisulphide-linked form of the TCR-γ. Previous studies provided evidence that two distinct Cγ gene segments may be used by T lymphocytes expressing the two molecular forms of TCR-γ (25). Based on the amino acid sequences, predicted from nucleotide sequence of constant gene segments, it has been proposed that Cγ1 molecular product, but not Cγ2, can form interchain disulphide bond (24, 25, 42). In fact, while Cγ1 encodes a cysteine residue that is found on the NH_2-terminal side of the presumed membrane-spanning region, in Cγ2 this cysteine is absent. If indeed this cysteine is responsible for interchain disulphide linkage, only heterodimers formed by Cγ1-encoded γ chains would form disulphide-linked heterodimers. In this context, preliminary experiments performed on two δ-TCS-1$^+$ clones (which expressed non-disulphide-linked TCR) showed the rearrangement of Cγ2 gene in both chromosomes. Studies are presently in progress to analyze Cγ1 and Cγ2 gene rearrangement in a panel of BB3$^+$ or δ-TCS-1$^+$ clones.

Further evidence for the molecular differences existing between the TCR expressed by BB3$^+$ or δ-TCS-1$^+$ lymphocytes was provided by 2D-PAGE analysis. Thus, CD3-associated TCR molecules immunoprecipitated from BB3$^+$ clones (under reducing conditions) were represented by two proteins of 41 and 38 kD displaying the same pI (of ~6) and by a more basic (pI ~7.5) 44-kD protein. A similar molecular pattern in 2D-PAGE analysis has been described by Lanier et al. (11). By using an antiserum raised against γ peptides, these authors identified the two more acidic (38 and 41 kD) proteins as products of the TCR-γ genes. If this is the case, the more basic 44-kD protein may represent the delta chain in the (BB3$^+$) disulphide-linked form of TCR-γ. In δ-TCS-1-reactive cells, immunoprecipitated molecules were represented by two major bands (mol wt ~41–44) displaying a similar mean pI (~5.5), both characterized by different degrees of glycosilation and by a noticeable charge heterogeneity. The major difference between the 2D-PAGE pattern of BB3 or δ-TCS-1-reactive TCR molecules was represented by the absence, in the latter, of the more basic (pI 7.5) 44-kD band. In immunoprecipitates from δ-TCS-1$^+$ cells, under non-reducing conditions, a 38-kD band could be detected that was no more observed in SDS-PAGE under reducing conditions (possibly because it was integrated in the 41–44-kD band as the consequence of a shift in the apparent molecular weight). On the other hand, this 38-kD band could be detected in clones 6.6.2 and 5.6.4 (δ-TCS-1$^+$) also under reducing conditions in 2D-PAGE analysis. It is noteworthy that Hata et al. (20) showed that a similar 38-kD molecule immunoprecipitated from IDP2 cell line reacted with a mAb specific for the δ chain. It should be noted, however, that in the IDP2 cell line (derived from an immunodeficient patient) the 38-kD molecule was noncovalently associated to a γ chain of higher molecular weight (55 kD).

Since the leukemic cell line termed PEER, which express a similar type of TCR-γ/δ, reacts with δ-TCS-1 mAb, we expected to detect at least some clones expressing the 55-kD γ chain, however none of the 11 δ-TCS-1$^+$ clones (derived from seven normal individuals analyzed so far) expressed this molecular form of the receptor. It would be of interest to define whether also the 38–55-kD TCR heterodimer is expressed in all normal individuals.

In conclusion, the availability of BB3 and δ-TCS-1 mAbs allows the direct assessment of two subsets of TCR-γ$^+$ T lymphocytes characterized by the expression of distinct molecular forms of TCR. This may be of help for defining the proportions of these subsets in different tissues in normal and disease states and perhaps for a better understanding of the T cell development.

Summary

Two mAbs directed to the TCR-γ/δ were analyzed for their pattern of reactivity with CD3$^+$WT31$^-$ cell populations or clones. In normal individuals, the BB3 mAb reacted with ~2/3 of peripheral blood CD3$^+$WT31$^-$ lymphocytes, whereas δ-TCS-1 stained ~1/3 of such cells. In addition, the sum of the percentages of BB3$^+$ and δ-TCS-1$^+$ cells approximated the percentages of peripheral blood CD3$^+$WT31$^-$ lymphocytes in seven normal donors tested. Also, in peripheral blood–derived polyclonal CD3$^+$WT31$^-$ populations, cultured in IL-2, cells reacting with one or another mAb accounted for the whole cell population. On the other hand, only δ-TCS-1-reactive cells, but not BB3$^+$ cells, could be detected in unfractionated as well as in CD4$^-$8$^-$ thymocyte populations. Analysis of peripheral blood–derived CD3$^+$WT31$^-$ clones showed that 70% of 72 clones analyzed reacted with BB3 mAb, but not with δ-TCS-1 mAb. On the other hand, δ-TCS-1 mAb stained the remaining BB3$^-$ clones. Five clones expressing medium-low amounts of CD8 antigen were BB3$^-$ δ-TCS-1$^+$. Both types of clones lysed the Fcγ receptor–bearing P815 target cell in the presence of anti-CD3 mAb (but not of mAb directed against HLA-DR, CD7 molecules, or TCR-α/β). In this cytolytic assay, BB3 mAb induced target cell lysis only by BB3$^+$ clones, whereas δ-TCS-1 mAb was effective only with δ-TCS-1$^+$ clones. The CD3-associated surface molecules expressed by BB3$^+$ or δ-TCS-1$^+$ clones were analyzed after cell surface iodination and immunoprecipitation with the corresponding anti-TCR mAb or with anti-CD3 mAb (in digitonin-containing buffer). In SDS-PAGE, molecules immunoprecipitated from 13 BB3$^+$ clones displayed, under nonreducing conditions, a molecular weight of 80 kD (in some cases, a minor 38-kD band could be detected). Under reducing conditions, two major components of 44 and 41 kD (and a minor component of 38 kD) were detected. On the other hand, TCR molecules immunoprecipitated from 11 different δ-TCS-1$^+$ clones appeared as a diffuse band of 41–44 kD, both under reducing and nonreducing conditions (under nonreducing condition, an additional 38-kD band was present). Therefore, BB3$^+$ cells express a disulphide-linked form of TCR-γ/δ whereas δ-TCS-1$^+$ cells express a non-disulphide-linked form. TCR molecules immunoprecipitated from BB3$^+$ clones and analyzed by two-dimensional electrophoresis were represented, under reducing conditions, by two bands of ~38 and 41 kD displaying a similar pI and a third more basic 44 kD band. Under nonreducing conditions, BB3-reactive molecules were represented by a single 80-kD band (displaying an intermediate pI with

respect to that of the reduced chains). TCR molecules immunoprecipitated from δ-TCS-1$^+$ clones were represented, in both reducing and nonreducing conditions, by a diffuse 41–44-kD band (in which, however, two distinct components could be detected). In addition, a poorly labeled band of lower molecular weight was present. All these molecules displayed a similar pI and a high charge heterogeneity. Since similar molecular patterns were consistently detected among all BB3$^+$ as well as among all δ-TCS-1$^+$ clones analyzed, we conclude that BB3 and δ-TCS-1 mAbs identify two distinct molecular forms of TCR-γ/δ expressed by two different peripheral blood T cell subsets.

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