Brief Definitive Report

HUMAN PERIPHERAL BLOOD LYMPHOCYTES BEARING T CELL RECEPTOR γ/δ

Expression of CD8 Differentiation Antigen Correlates with the Expression of the 55-kD, Cγ2-encoded γ Chain

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A small subset of human peripheral T lymphocytes express CD3-associated receptor for antigen (TCR) composed of γ and δ chains (1). A remarkable phenotypic feature of most of these cells is the lack of surface expression of both CD4 and CD8 differentiation antigens, which are known to define the two major subsets of TCR α/β^+ cells (1). Recently, by the use of two anti-TCR-γ/δ mAbs (BB3 and δ-TCS-1), we identified two distinct, nonoverlapping subsets of $CD4^-8^-$ T cells (2, 3). Clonal analysis showed that cells reacting with BB3 mAb expressed disulphide-linked TCR, whereas expression of the non-disulphide-linked form of TCR was always associated with δ-TCS-1 reactivity. In both types of TCR-γ/δ, the γ gene product displayed a molecular mass ranging between 41 and 44 kD. In no instance (>25 $CD4^-8^-$ clones analyzed), could we detect the high molecular form (55 kD) of the γ chain that had been previously revealed in a T cell leukemia (4) or in polyclonal cell lines derived from normal thymocytes (5) or from an immunodeficiency patient (1).

In the present study, we analyzed a series of TCR-γ/δ^+ clones characterized by the surface expression of CD8 antigen. We show that these cells express a non-disulphide-linked, δ-TCS-1-reactive, Cγ2-encoded form of TCR. More importantly, TCR molecules contained the heavy (55–60 kD) form of the γ chain.

Materials and Methods

Cloning of $CD3^+$ $WT31^-$ Peripheral Blood Lymphocytes or Thymocytes. Peripheral blood lymphocytes or thymocytes were isolated as previously described. $CD3^+WT31^-$ cells were then isolated by sorting at the FACS and cloned under limiting dilution (3, 6, 7). Microcultures were considered as clones on the basis of the frequency of proliferating microculture in each experiment. Clones were screened directly by FACS analysis for the presence of surface CD3

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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.

mAbs and Flow Cytofluorometric Analysis. mAbs used in these studies were represented by JT3A and anti-Leu-4 (anti-CD3) (Beckton Dickinson & Co., Basel, Switzerland) B9.4 (anti-CD8), WT31 (anti TCR-α/β), BB3 directed to ~60–70% of $CD3^+WT31^-$ cells, δ-TCS-1 directed to the δ chain of $CD3^+WT31^-$ lymphocytes (T Cell Sciences Inc., Cambridge, MA) (3) and MAR 21 (anti-CD7). Cytofluorometric analysis was performed as previously described (2, 7).

Characterization of Radioiodinated Cell Surface Proteins. Cloned cells (~5×10^6) were surface labeled with ^{125}I using the lactoperoxidase/glucose oxidase-catalyzed iodination and were lysed in a buffer containing digitonin as previously described (2, 3). Lysates were then incubated for 2 h with 200 μl of BB3 culture supernatant or with 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. The non equilibrium pH gradient electrophoresis (NEPHGE) was carried out using pH 3.5–10 ampholines followed by 11% SDS-PAGE gels for size separation as described (3).

DNA Analysis. The genomic configurations of the three J segments (JP1, JP, and J1) upstream to Cγ1 and the two J segments (JP2 and J2) upstream to Cγ2 were tested by Southern blotting through different combinations of J-specific probes and restriction endonucleases (Eco RI, Bam HI). Due to sequence homology, the J1 probe (0.7-kb Hind III–Eco RI segment, a kind gift of T. Rabbitts, MRC, Cambridge, UK) (8) detects the J2 region as well. The JP1 and JP probes (1.0-kb Hind III–Sac I and 0.1-kb Hind III–Eco RI, respectively) were subcloned from a recombinant phage isolated from a human placental DNA library. The JP2 probe (0.26-kb Hind III–Eco RI) and a 0.24-kb Xba I–Pst I genomic segment containing the Cγ2 second exon were kindly provided by P.-G. Pelicci (9).

Results and Discussion

In previous studies we derived TCR-γ/δ^+ cells from $CD4^-8^-$ peripheral blood T cells (2, 3, 6, 7). Although this type of selection resulted in highly enriched TCR-γ/δ^+ populations, it excluded most of the TCR-γ/δ^+ cells expressing CD8. To analyze TCR-γ/δ^+ $CD8^+$ cells, we purified the $WT31^-$ fraction of peripheral blood E-rosetting cells (under conventional experimental conditions WT31 mAb only recognizes TCR-α/β^+ cells). The resulting cell populations contained variable proportions (ranging between 40 and 80%) of $CD3^+$ cells. Cells were seeded under limiting dilution conditions and the derived clones were analyzed by indirect immunofluorescence and FACS analysis for expression of CD3, WT31, BB3, δ-TCS-1 and CD8 surface antigens. Of >300 $CD3^+WT31^-$ clones analyzed, ~70% reacted with BB3 mAb, whereas most of the remaining clones were δ-TCS-1^+. Eight clones were $CD8^+$ and their reactivity with δ-TCS-1 mAb suggested that they expressed a non-disulphide-linked form of TCR-γ/δ (3). Three $CD8^+$ clones (derived from two different individuals) could be expanded extensively and were therefore analyzed in more detail.

Cell surface iodination, followed by immunoprecipitation with anti-CD3 mAb, under conditions that preserve the CD3/TCR association (1% digitonin), showed that indeed $CD8^+$ clones expressed a non-disulphide-linked form of TCR-γ/δ (Fig. 1 *A*, lanes *a* and *b*). However, the molecular mass of the CD3-associated molecules was considerably higher (55–60 kD) than that of $CD8^-$ TCR-γ/δ^+ clones (40–44 kD) isolated from the same donor (a representative clone is shown in lanes *c* and *d*).

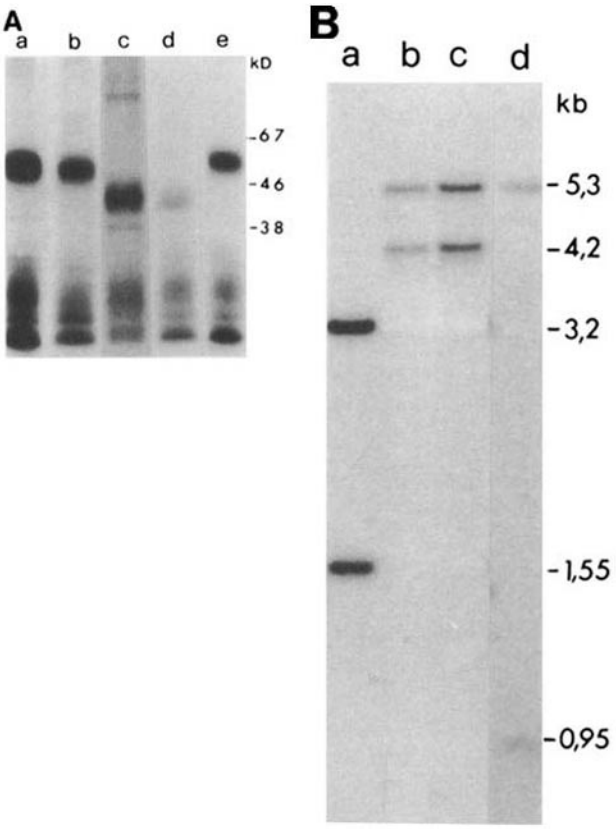

FIGURE 1. (*A*) SDS-PAGE analysis of CD3-associated TCR molecules immunoprecipitated from $CD8^+$ and $CD8^-$ TCR-γ/δ^+ clones. 10^7 cloned cells were surface labeled and then lysed in buffer containing digitonin as indicated in Materials and Methods. Cell lysates were immunoprecipitated by using anti-Leu-4 (anti-CD3) mAb. Molecules immunoprecipitated from clone D1.12 ($CD8^+$) under nonreducing (lane *a*) and reducing (lane *b*) conditions and from clone D5.100 ($CD8^-$) under nonreducing (lane *c*) and reducing (lane *d*) conditions are shown. The CD3-associated molecules immunoprecipitated from clone T65 (derived from thymocytes and expressing the δ-TCS-1^+CD8^+ surface phenotype) run under reducing conditions are also shown (lane *c*). (*B*) Southern blot analysis of Eco RI–digested DNA from clone MV28 (lane *b*), clone MV120 (lane *c*), and clone D1.12 (lane *d*) hybridized to a J1 probe. The germline J-1 and J-2-containing fragments (1.55- and 3.2-kb, respectively) are shown in lane *a* (fibroblast DNA). The V segment rearranged genes, according to reference 11, were as follows: the fragment of 5.3-kb corresponded to V3; the 4.2-kb to V8, and the 0.95 kb to V4.

The reactivity with a polyclonal anti-γ chain rabbit antiserum indicated that the 55–60-kD and the 40–44-kD bands expressed by the two types ($CD8^+$ or $CD8^-$) of δ-TCS-1^+ clones represented the molecular product of the γ chain genes (data not shown). Interestingly, a similar 55-kD band was precipitated also from a δ-TCS-1^+ $CD8^+$ clone derived from $WT31^-$ thymocytes (Fig. 1 *A*, lane *e*). Although, only 3 (peripheral blood–derived) $CD8^+$ δ-TCS-1^+ clones could be analyzed (due to their low frequency and also to major difficulties in their in vitro expansion), it should be stressed that all the 25 $CD8^-$ δ-TCS-1^+ clones analyzed so far expressed the low molecular mass (40–44 kD), non-disulphide-linked form of TCR-γ/δ (see also reference 3). Therefore, in the panel of clones analyzed, expression, or lack thereof, of CD8 surface antigens appears to correlate with two different molecular sizes of non-disulphide-linked TCR-γ/δ. It is noteworthy that Brenner et al. (1) described a similar type of TCR-γ/δ molecules in immunoprecipitates from the IDP2 polyclonal cell line containing ~50% $CD8^+$ cells.

To better characterize the TCR-γ/δ molecules expressed by $CD8^+$ clones, we further performed two-dimensional PAGE of CD3-associated molecules immunoprecipitated after lysis of surface iodinated cells in digitonin-containing buffer. The TCR molecular complexes of the three $CD8^+$ clones isolated from peripheral blood are

shown in Fig. 2, (*A–C*). It is evident that no substantial differences in charge mobility could be detected between different clones. The TCR molecular complex immunoprecipitated from one of these $CD8^+$ clones (panel *E*) is also compared with those expressed by a $BB3^+$ (panel *D*) or a $CD8^-$ δ-TCS-1^+ (panel *F*) clone derived from the same individual. It can be seen that the spots corresponding to the 55-kD γ chain expressed in $CD8^+$ clones are slightly more acidic than those corresponding to the 40–44-kD γ chains expressed in either $BB3^+$ or $CD8^-$ delta-TCS-1^+ clones. The charge mobility of the delta-chain expressed in $CD8^+$ clones could not be compared with that of the other two forms of TCR because of the poor labeling of this chain under the experimental conditions used.

The analysis of the DNA configuration at the TCR-γ loci by means of a probe that can detect both the J1 and J2 regions showed that the three clones had rearranged both chromosomes (Fig. 1 *B*). Thus, the absence of the germline J1- and

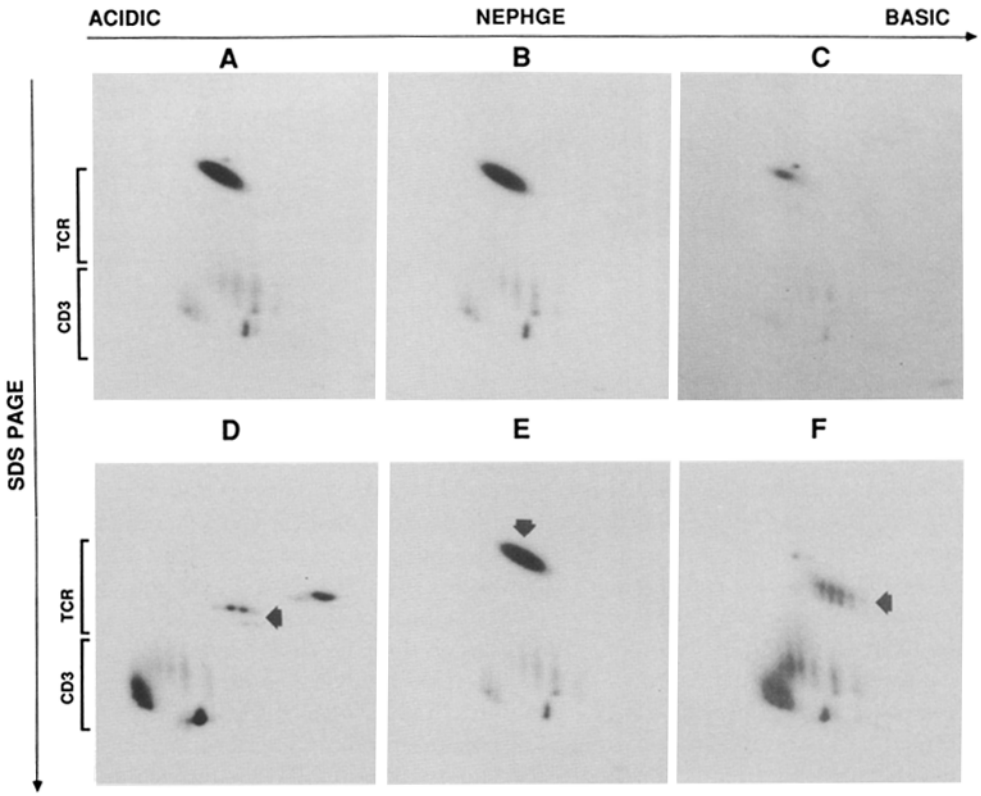

FIGURE 2. CD3-associated TCR molecules in $CD8^+$ δ-TCS-1^+ clones MV120 (*A*) D1.12 (*B*) and MV28 (*C*). (*D–F*) A comparison of the three different forms of TCR-γ/δ complex is shown. (*D*) the $BB3^+$ clone GA17, (*E*) the $CD8^+$ δ-TCS-1^+ clone D1.12, and (*F*) the $CD8^-$ δ-TCS-1^+ clone D5.100. In these experiments $\sim 10^7$ cloned cells were labeled with ^{125}I and lysed in digitonin-containing buffer. Cell lysates were then immunoprecipitated using the anti-Leu-4 mAb. All the samples were analyzed by NEPHGE and the second dimension was performed using 11% acrylamide gels for SDS-PAGE analysis under reducing conditions. The arrows indicate the position of the molecules immunoprecipitated by a polyclonal rabbit antiserum specific for γ chain in a separated experiment (not shown).

J2-containing segments (lanes *b, c,* and *d*), together with the lack of hybridization to JP1- and JP2-specific probes (data not shown), clearly indicated that both the productive and nonproductive V-J recombination had involved the most 3′ J segment, i.e., J2. It follows that the constant portion of the expressed γ chains in the three clones should correspond to the Cγ2 gene segments, since the Cγ1 gene segments, mapping upstream to J2, have been deleted. In each clone, the type of the rearranged Vγ genes could be inferred from the size of the Eco RI (Fig. 1 *B*) and Bam HI (not shown) novel bands (10) as follows: the 5.3-kb fragment corresponded to V3, the 4.2-kb fragment to V8, and the 0.95-kb fragment to V4.

Unlike the Cγ1 locus that codes for a constant region made up of three exons, the Cγ2 locus is polymorphic in size. At least two major alleles have been identified by gene cloning; one contains a duplication, the other a triplication of the second exon (9, 11). The 55-kD γ chain expressed by the PEER leukemia cell line has been shown to be coded by a Cγ2 triplicated second exon (11). Southern analysis by means of an exon 2-specific probe indicated that at least one copy of the triplication allele is present in all three clones (not shown). Based on these data, it is likely that the 55-kD γ chain found in $CD8^+$ clones, similarly to that present in PEER leukemia, may represent the surface product of a Cγ2 gene segment containing a triplication of the second exon. Although no direct evidence is provided, it is conceivable that differences in size and acidity between the γ chains expressed by $CD8^+$ or $CD8^-$ clones may reflect the different number of amino acid residues, as well as the number of sites available for *N*-linked glycosylation, as proposed by two different groups (9, 11).

Summary

We analyzed the CD3-associated molecules present on peripheral blood-derived TCR-γ/$δ^+$ clones that express CD8 surface antigens. Clones were derived by limiting dilution from $CD3^+WT31^-$ FACS-purified populations derived from several donors. Eight of >300 TCR-γ/$δ^+$ clones analyzed expressed CD8 and reacted with δ-TCS-1 mAb. Cell numbers suitable for more detailed analyses could be obtained from four clones, including one derived from thymus. Analysis of CD3-associated TCR molecules immunoprecipitated by anti-Leu-4 (anti-CD3) mAb under conditions that preserve the CD3/TCR association (1% digitonin) showed a predominant 55–60-kD molecule both under reducing and nonreducing conditions. On the other hand, the δ-TCS-1-reactive molecules immunoprecipitated from 25 $CD3^+$ δ-$TCS\text{-}1^+$ $CD8^-$ clones, in all instances, displayed a 40–44-kD mol mass. In two-dimensional PAGE, TCR-γ molecules precipitated from δ-$TCS\text{-}1^+$ $CD8^+$ clones appeared more acidic than those of $BB3^+$ or δ-$TCS\text{-}1^+$ $CD8^+$ clones. Southern analysis confirmed that this type of non-disulphide-linked TCR-γ/δ is also coded for by the Cγ2 gene segment.

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References

1. Brenner, M. B., J. McLean, D. P. B. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel. 1986. Identification of a putative second T cell receptor. *Nature (Lond.).* 322:145.
2. Ciccone, E., S. Ferrini, C. Bottino, O. Viale, I. Prigione, G. Pantaleo, G. Tambussi,

A. Moretta, and L. Moretta. 1988. A monoclonal antibody specific for a common determinant of the human T cell receptor γ/δ directly activates $CD3^+WT31^-$ lymphocytes to express their functional program(s). *J. Exp. Med.* 168:1.

3. Bottino, C., G. Tambussi, S. Ferrini, E. Ciccone, P. Varese, M. C. Mingari, L. Moretta, and A. Moretta. 1988. 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. *J. Exp. Med.* 168:491.
4. Weiss, A., M. Newton, and D. Gommie. 1986. Expression of T3 association with a molecule distinct from the T-cell antigen receptor heterodimer. *Proc. Natl. Acad. Sci. USA.* 83:6998.
5. Bank, I., R. A. De Pinho, M. B. Brenner, J. Cassimeris, F. W. Alt, and L. Chess. 1986. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. *Nature (Lond.).* 322:179.
6. Ciccone, E., O. Viale, C. Bottino, D. Pende, N. Migone, G. Casorati, A. Moretta, and L. Moretta. 1988. Antigen recognition by human T cell receptor γ-positive lymphocytes. *J. Exp. Med.* 167:1517.
7. Ferrini, S., C. Bottino, R. Biassoni, A. Poggi, R. P. Sekaly, L. Moretta, and A. Moretta. 1987. Characterization of $CD3^+CD4^-CD8^-$ clones expressing the putative T cell receptor gamma gene product. *J. Exp. Med.* 166:277.
8. Le Franc, M. -P., and T. H. Rabbitts. 1985. Two tandemly organized human genes encoding the T-cell gamma constant-region sequences show multiple rearrangement in different T-cell type. *Nature (Lond.).* 316:464.
9. Pellicci, P.-G., M. Subar, A. Weiss, R. Dalla-Favera, and D. R. Littman. 1987. Molecular diversity of the human T-gamma constant region genes. *Science (Wash. DC).* 237:1051.
10. Forster, A., S. Huck, N. Ghanem, M.-P. Lefranc, and T. H. Rabbitts. 1987. New subgroups in the human T cell rearranging V gene locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1945.
11. Littman, D. R., M. Newton, D. Crommie, S. L. Ang, J. G. Seidman, S. N. Gettner, and A. Weiss. 1987. Characterization of an expressed CD3-associated Ti γ-chain reveals C-γ domain polymorphism. *Nature (Lond.).* 326:85.