TCT.1, a Target Molecule for γ/δ T Cells, Is Encoded by an Immunoglobulin Superfamily Gene (Blast-1) Located in the CD1 Region of Human Chromosome 1

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Summary

We have recently generated a series of γ/δ T cell clones able to kill, after in vitro immunization, an Epstein-Barr Virus–transformed B cell line (designated E418) in a non–major histocompatibility complex–requiring fashion. A monoclonal antibody, termed anti-10H3, produced against E418 was selected by its ability to block these cytotoxic interactions. Further analysis indicated that the inhibitory effects of anti-10H3 were highly selective (i.e., no blocking activity with multiple control clones used as effector cells; no alteration of the natural killer–like function mediated by the relevant γ/δ clones against $10H3^+$ tumor cells such as Rex). The molecule immunoprecipitated by anti-10H3, termed TCT.1, was characterized as a 43-kD protein broadly distributed in the hematopoietic system. The TCT.1 molecule has been further studied here by protein microsequencing. Results show that the TCT.1-derived peptide sequences are virtually identical to corresponding regions of Blast-1, a previously described surface protein with unknown function. The likely identity of the two molecules has been strengthened by analyzing the susceptibility of TCT.1 to phosphatidylinositol-specific phospholipase C digestion in light of the known anchorage of Blast-1 to the cell membrane through a glycosyl-phosphatidylinositol-containing lipid. The TCT.1/Blast-1–encoding gene is well characterized; it belongs to the immunoglobulin gene superfamily and it is located in the same band of chromosome 1 as the CD1 gene cluster. Together, these data further support the view that proteins distinct from the conventional class I/II histocompatibility molecules are involved in specific T cell recognition.

Non–MHC-restricted cytotoxic interactions mediated by T lymphocytes are likely to include two distinct phenomena: (*a*) the TCR-independent recognition on target cells of monomorphic molecules corresponding to the so-called NK-like activity (1–7), which may well reflect the conservation by T lymphocytes of the more primitive cytotoxic functions of NK cells (7, 8); and (*b*) the TCR-mediated specific recognition of target molecules that are distinct from the conventional MHC class I/II gene products (9–12). Although suggested in early studies with α/β^+ T lymphocytes (12), it is only with the characterization of the γ/δ receptor that the existence of the latter phenomenon has been generally accepted. Indeed, because class I/II molecules appear to restrict TCR-γ/δ-dependent antigen-specific T cell responses infrequently, the search for a novel T cell recognition mechanism has led several authors to propose either additional MHC-encoded proteins (e.g., the TL [13, 14] and the Qa antigens [15]; reviewed in reference 16) or structurally close but MHC-independent molecules (e.g., the CD1 proteins [17, 18]) as potential TCR target structures. In this regard, we have recently developed several γ/δ alloreactive clones derived from PBL of a healthy individual (9). Two of them, E102 and E117, generated against the E418 EBV-transformed B cell line, were found to display a non–MHC-restricted cytotoxicity against the immunizing E418 cells as well as a substantial lytic activity against irrelevant NK target cell lines such as K562 and Rex. The molecular characterization of the γ and δ chain–encoding genes indicated that both clones express an infrequent TCR-γ/δ, associating Vγ3-JP2-Cγ2/Vδ1-(D)-Jδ1-Cδ chains. To identify a putative target molecule recognized by E102 and E117 clones, we selected, after mice immunization with E418 cells, a mAb, anti-10H3, able to block the cytotoxic activity of both clones against E418, while not altering the E102-E117/K562-Rex cytotoxic interactions (9). Anti-10H3 was found to precipitate a 43-kD molecule, designated TCT.1, with a broad distribution on hema-

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topoietic cells. In the present study, we have strengthened these functional findings through the identification of five additional γ/δ T cell clones (E31, E38, E66, E69, and E116), and characterized by microsequencing the TCT.1 protein. Our data indicate that TCT.1 is most likely identical to Blast-1, a molecule of the Ig superfamily encoded in the CD1 region of human chromosome 1 (19).

Materials and Methods

Generation of Cloned Cell Lines. E31, E38, E66, E69, and E116 γ/δ CTL were generated from PBL of the same donor as E102 and E117 in a MLC using E418 EBV-transformed B cell line as described previously (9).

Monoclonal Antibodies. An anti-10H3 mAb directed against the TCT.1 molecule was generated as described previously (9). W6/32 mAb recognizes a monomorphic determinant of the HLA class I gene product (20). Anti-NKTa mAb recognizes an α/β clonotypic determinant (21). Anti-TiγA mAb (22) directed against a Vγ9-encoded epitope recognizes approximately two-thirds of human γ/δ PBL. Anti-TCR-δ1, kindly provided by M. B. Brenner (Dana-Farber Cancer Institute, Boston, MA), reacts with a constant determinant of the TCR δ chain (23). δTCS1 mAb (24) reacts specifically with a structure encoded by Vδ1-Jδ1 gene products (25, 26). A13 and TiVδ2 (27) react with Vδ1 and Vδ2 gene products, respectively. BMA031, kindly provided by Dr. R. Kurrle (Behring Company, Marburg, Germany), reacts with a monomorphic determinant of the TCR-α/β receptor. OKT3, OKT4, and OKT8 (Ortho Diagnostics, Raritan, NJ) react with CD3, CD4, and CD8 proteins, respectively.

Cytotoxic Assays. The cytotoxic activity of the cloned cell lines was measured by a conventional 3-h ^{51}Cr release assay using triplicate cultures in V-bottomed plates. The E/T ratio was 10:1 on 5,000 target cells/well. Percent specific cytotoxicity was calculated conventionally; standard deviations were <5%. REX (α/β T cell leukemia) and E418 1324 (EBV-transformed B cell line, kindly provided by Dr. J. Colombani, St. Louis Hospital, Paris) cell lines were used as targets in cytotoxicity assays. W6/32 (anti-class I) and 10H3 (anti-TCT.1) mAbs were used in functional assays. Target cells were preincubated for 2 h at 37°C with saturating concentration of each antibody before addition of effector cells.

Purification and Amino Acid Sequence Determination of TCT.1 Molecule. 2×10^{10} E418 cells were lysed at 10^7 cells/ml in 200 ml of X-100 Ripa buffer containing 1% Triton X-100, 0.15 M NaCl, 0.5% sodium deoxycholate, 1 mM PMSF, 20 mM iodoacetamide, and 2 μg/ml trypsin inhibitor. Solubilized material was recovered in the supernatant after centrifugation for 20 min at 2×10^4 *g*, and each 50-ml sample was applied to three distinct irrelevant mAb chromatography columns (anti-neomycin coupled to protein G) at a flow rate of 0.1 ml/min. The recovered material was then loaded on an anti-10H3/protein G 1-ml column at the same flow rate. The columns were washed sequentially with 10 ml of 20 mM Tris, pH 8, 10% ethylene glycol, 0.1% *N*-octylglucopyranoside, and with 10 ml of the same buffer supplemented with 0.25 M NaCl. Bound material was eluted sequentially with 7.5 ml of 50 mM glycine-HCl (pH 2.5) and 7.5 ml of 50 mM triethylamine (pH 11) at the flow rate of 1 ml/min. 2.5-ml fractions of each buffer were collected and pooled. Samples were desalted, concentrated, and subjected to electrophoresis on a 10% acrylamide preparative SDS gel. The fragment containing the putative 43-kD molecule was cut and electroeluted in 1 mM *N*-ethyl morpholyne (pH 9) using an electroelution apparatus (ISCO, Inc., Lincoln, NE) (2 h at 10 mA). 0.2 nmol was subjected to gas phase NH_2-terminal sequencing (28), while 2 nmol of electroeluted material was digested with 2.5 μg of endoproteinase Asp-N (Boehringer Mannheim Biochemicals) in 100 mM Tris (pH 8.2) for 2 h at 37°C. Digested material was either separated by SDS-PAGE on a 16.5% acrylamide gel, electroblotted onto polyvinylidene difluoride (29) membranes and cut (S3), or loaded onto a 2 × 100-mm RP-300 reverse-phase column (Brownlee Labs, Inc., Santa Clara, CA) installed in an 130-A high pressure liquid chromatograph (Applied Biosystems, Inc., Foster City, CA). The column was eluted at a flow rate of 0.15 ml/min. The eluate was monitored at 214 nm and fractions were collected (S14, S18, S19, S23, S24, and S116). The NH_2 terminal and the different peptides amino acid sequences were determined by sequential degradation on 470-A protein microsequencer (Applied Biosystems, Inc.) using modified Edman chemistry (30).

Phospholipase C Treatment of Cells. 10^6 cells (E418, Rex, and PHA blasts) were washed once with PLC buffer (RPMI containing 2 mg/ml BSA, 10 mM Hepes, pH 7.4 and 5×10^{-5} M 2-ME), resuspended in 1 ml of PLC, and incubated at 37°C for 60 min with 1 U of PI-specific PLC (Immunotech S.A., Luminy Marseille, France) (31) or with no enzyme, as described previously (19). The cells were washed once with PLC buffer followed by two washes with PBS containing 0.5% FCS and then subjected to immunofluorescence analysis. Indirect immunofluorescence studies were performed at 4°C with saturating concentrations of each mAb and FITC-conjugated goat anti-mouse Ig serum (1).

Results and Discussion

Generation and Characterization of Cloned Cell Lines. E31, E38, E66, E69, and E116 were generated from PBL of the same donor as the previously described E102 and E117 γ/δ T cell clones (9). As E102 and E117, all these clones appeared to be $CD3^+$, BMA031$^-$ (TCRα/β^-), TCR-δ1$^+$, δTCS1$^+$, A13$^+$, TiVδ2$^-$, TiγA$^-$, CD4$^-$, CD8$^-$, and NKH1$^-$ (data not shown). They were also found to express a Vδ1(D)-Jδ1-Cδ/Vγ3-JP2-Cγ2 heterodimer, which is infrequent in human peripheral blood (data not shown).

E31, E38, E66, E69, and E116 were assayed for cytotoxic activity against E418-immunizing cells as well as the Rex

Table 1. *Cytotoxic Activity of E31, E38, E66, E69, and E116 γ/δ T Cell Clones towards E418 and Rex Target Cells*

		E31	E38	E66	E69	E116
E418	Media	44	54	49	54	44
	10H3	1	3	0	5	4
	W6/32	41	47	44	54	46
Rex	Media	61	46	26	71	54
	10H3	59	46	20	69	56
	W6/32	61	51	26	69	60

Cytolytic experiments were performed either in media or in the presence of anti-10H3 or anti-W6/32 mAbs. E418- and Rex ^{51}Cr-labeled cells (5×10^3) were preincubated for 2 h with saturating concentration of each antibody before the addition of effector cells (5×10^4). The indicated values correspond to percent of specific lysis calculated conventionally.

NK target cell line. As shown in Table 1, all these clones display a strong cytotoxic activity against both the E418-immunizing cell line and the irrelevant Rex tumor cells. The lysis of the two target cell types was not altered by the anti-W6/32 mAb (class I specific), while anti-10H3 blocked the cytotoxic interactions with E418 exclusively (i.e., no effect on the TCT.1$^+$ Rex lysis). These data further support the view that such effector T cell clones have two distinct pathways of target cell recognition: non–MHC-dependent specific recognition (of TCT.1 in the present case) and NK-like activity. The involvement of the γ/δ receptor in TCT.1 recognition is not directly established; it is, however, supported by the observation that: (*a*) the additional five clones described here have the same unusual γ and δ rearrangements as E102 and E117 (data not shown); and (*b*) the cytotoxicity of series of randomly selected clones (CD3$^-$/NK, α/β^+, γ/δ^+ cells) against a variety of TCT.1$^+$ target cells is not altered by anti-10H3 mAb (9). Note also that the interactions of the TCT.1-specific γ/δ clones and E418 cells are inhibited by anti-TCR antibodies (this point has to be mentioned although it is not conclusive because such results may reflect the transduction of a negative signal in effector cells even when the TCR is not involved in target cell recognition [32]).

Amino Acid Sequence Identity of TCT.1 and Blast-1. Partial amino acid sequencing was performed to further characterize the TCT.1 molecule. A crude cell lysate from 20 × 10^9 E418 EBV-transformed B cells was subjected to affinity chromatography with anti-10H3 mAb. After preparative gel electrophoresis, the 43-kD specific material was electroeluted, and ~2.2 nmol was obtained. A fraction of the purified protein (0.2 nmol) was used in gas phase NH_2-terminal sequencing (28), while the remaining was digested by the endoproteinase Asp-N, known to specifically cleave peptide bonds NH_2-terminally at aspartic acid. The degradation peptides were either resolved by C8 reverse-phase HPLC (S14, S18, S19, S23, S24, and S26) or separated in SDS-PAGE preparative gel before electroblotting (29) onto polyvinylidene difluoride membranes (out of the six peptides obtained by the latter approach, only one, S3, could be sequenced unambiguously). The NH_2-terminal sequence of the whole protein (S0) and that of seven different peptides were thus determined after Edman degradation (30). A computer search performed with the PC gene program (33) revealed a high sequence homology between S0 and the corresponding region of a previously described surface molecule, termed Blast-1 (19, 34). The whole series of sequences (S0, S3, S14, S18, S19, S23, S24, and S26) have thus been aligned with that of Blast-1 (19, 34). As shown in Fig. 1, the initial NH_2-terminal amino acids of TCT.1 could not be assigned with sufficient confidence. The following sequence (S0) is identical to Blast-1 in 20 out of the 23 characterized amino acids. Among the three differences, two amino acid determinations were ambiguous (shown between parentheses in Fig. 1). Concerning the S3, S19, S23, and S26 peptides, 17/21, 13/16, 15/18, and 11/12 identities were observed, respectively, with Blast-1 peptides. In the S3 and S19 peptides, three amino acids could not be identified (represented by an X). The sequences of S14 (six amino acids), S18 (six amino acids), and S24 (10 amino acids) peptides were identical to the corresponding Blast-1 fragments.

Membrane Anchorage of TCT.1 through Glycosyl Phosphatidylinositol–containing Lipid. It has been previously reported (19) that Blast-1 is anchored to the cell surface through glycosyl-phosphatidylinositol (GPI)[1]-containing lipid. Phospholipase C (PLC) treatment was demonstrated to specifically release the Blast-1 protein from cell membrane with a very unique pattern, namely total disappearance of the Blast-1 an-

[1] *Abbreviations used in this paper:* GPI, glycosyl-phosphatidylinositol; PLC, phospholipase C.

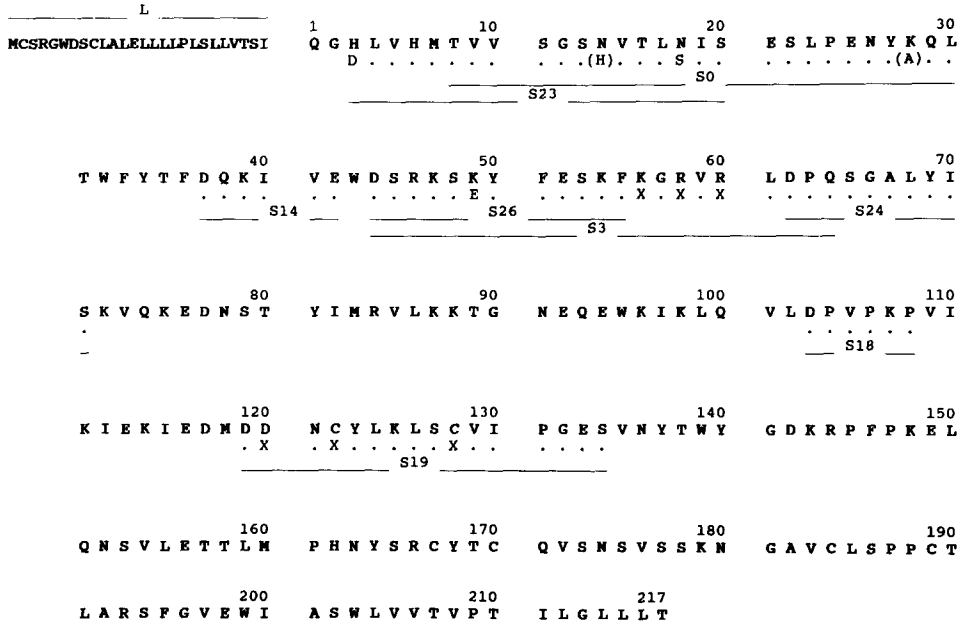

Figure 1. NH_2-terminal (S0) and Asp-N–derived peptide (S3, S14, S18, S19, S23, S24, and S26) amino acid sequences of TCT.1 aligned with the corresponding sequences of human Blast-1 protein (34). Ambiguous residues are presented between parentheses. An X indicates the residues where no assignment could be made. Dots correspond to amino acid sequence identities.

tibody reactivity on PHA-activated T cells, while a degree of expression is maintained on B lymphocytes (19). Experiments were therefore performed here with TCT.1 under the same experimental conditions reported for Blast-1 (19). As shown in Fig. 2, treatment of E418 cells with PI-PLC resulted in a marked but incomplete decrease (even with increasing enzyme concentrations) in binding of the anti-10H3 mAb assessed by immunofluorescence analysis. This decrease was specific for TCT.1 and was not observed with the control W6/32 mAb directed against the integral transmembrane MHC class I gene product. In contrast and consistent with the results published by Staunton et al. (19), the treatment of PHA-activated T cells resulted in the abrogation of anti-10H3 binding. Similar results were also obtained with the Rex T cell line used in the cytotoxicity assay (Fig. 2). Together, the high degree of similarity throughout the protein sequences and the PI-PLC digestion profile strongly support the view that the TCT.1 and Blast-1 molecules are encoded by the same gene. Note that it has been recently suggested, on the basis of cDNA sequence similarity, that Blast-1 is a member of the CD48 cluster group (35). The significance of the partial TCT.1/Blast-1 release from certain cell types after PI-PLC digestion will have to be assessed for both structural and functional aspects in further studies.

Because TCT.1/Blast-1 is a GPI-anchored molecule, we have tested the effect of PI-PLC treatment of E418 target cells with respect to cytotoxicity by clone E69. It was found that such treatment leads to a very strong decrease of target cell lysis (data not shown). These results are in line with a potential role of TCT.1 as a ligand on target cells. However they are not conclusive because other GPI-linked molecules, which may be important in E/T cell interaction, such as for example LFA-3, are removed from cell membranes after PI-PLC treatment.

Structural and Functional Characteristics of Blast-1. Previous analysis of the Blast-1 molecule showed that it belongs to the Ig gene superfamily (19, 34). The strongest sequence similarity (81% considering conservative amino acid substitutions) was observed with the protein OX45 (36), which has been proposed as Blast-1 rat homologue. Blast-1 was also found to display a high degree of homology with the LFA-3 molecule, particularly in the NH_2-terminal domain (61% considering conservative amino acid substitutions). In addition to primary sequence similarities, the three molecules (TCT.1/Blast-1, OX45, and LFA-3) share major structural characteristics, including: (*a*) polypeptide length (217, 218, and 210 amino acids, respectively); (*b*) organization in two Ig-related domains, a distal domain of a V-SET subtype without cysteine residues and a proximal domain of a C2 set; (*c*) N-linked glycosylation sites (five, five, and six, respectively); and (*d*) GPI anchorage (19, 36, 37). In light of these findings, it has been suggested that Blast-1 may be involved in nonspecific cell-cell adhesion, perhaps as an additional CD2 ligand (19). Our data, which provide the first observations on TCT.1/Blast-1 functional activity, do not favor this hypothesis, because its recognition appears to be limited to unique effector cells that share a common TCR.

Cytogenetic studies have shown that the Blast-1 gene is located in a position indistinguishable from that of the CD1 cluster at chromosome 1 q22-q23 (19). This point is of particular interest in light of the recent results obtained with anti-CD1c antibodies (17, 18). Indeed, inhibition of $CD1c^+$ target cell lysis by γ/δ T cell clones using anti-CD1c reagents has been reported with identical characteristics as those described here (i.e., no blocking activity of anti-CD1c antibodies with a series of randomly selected clones used as effector cells; no blocking of the NK-like function). While gene location and functional properties of the specific antibodies tend to suggest a related evolution of the CD1c and Blast-1/TCT.1 genes, comparing the structure of the two proteins does not provide additional elements to support this view. Indeed, the overall degree of sequence similarity is low and the distal domain of CD1 is not of the Ig type (38). In any case, our findings strongly suggest that TCT.1/Blast-1 is an additional

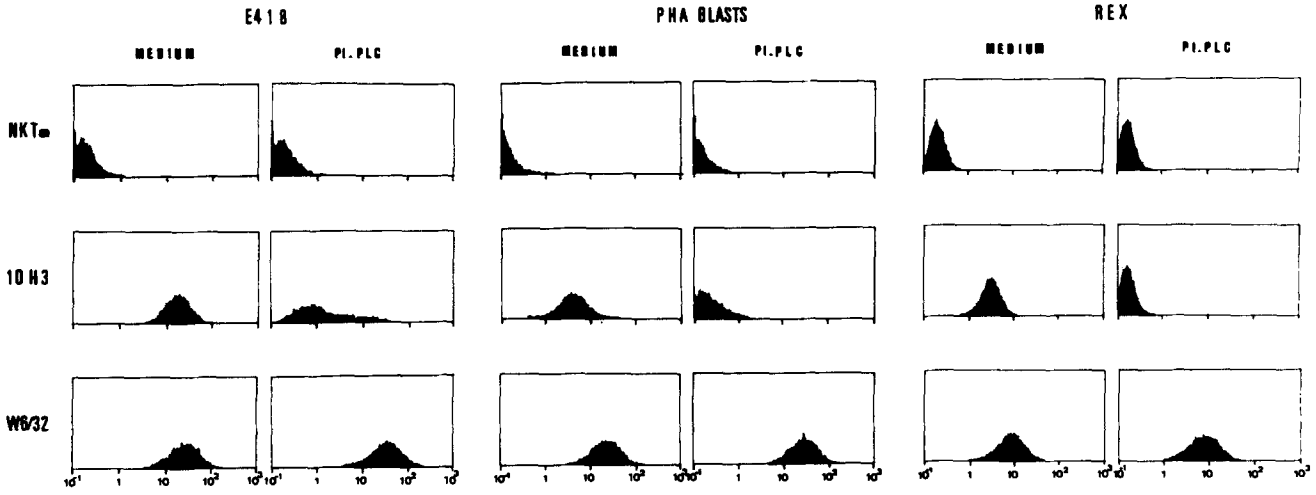

Figure 2. Immunofluorescence analysis of PI-PLC-treated or untreated E418, PHA-activated T cells, and Rex T cell line with NKTa (as negative control) anti-10H3 and anti-W6/32 mAbs.

molecule to be added on the increasing list (i.e., TNKtar [12]; Qa [15]; TL [13, 14, 16]; CD1 [17, 18]; bacterial toxins [39]; heat shock proteins [11]; and Igs [10]) of potential TCR ligands susceptible to direct specific T cell responses in a non-MHC class I/II–requiring fashion. Further studies will have to assess whether TCT.1 physically interacts with the TCR and to determine which TCR regions (e.g., complementary determining regions) may be involved.

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