Expression and Function of a Variant T Cell Receptor Complex Lacking CD3-γ

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Summary

A T cell line termed DIL2 has been derived from an infant with a polyclonal T cell receptor (TCR)/CD3 cell surface expression defect. Indirect immunofluorescence showed that the expression of certain TCR/CD3 epitopes (like those detected by WT31 and BMA031 monoclonals) was strongly reduced (around five-fold) on DIL2, whereas other epitopes (like those detected by SP34 and Leu4) were only around two-fold lower than in normal T cell lines. Specific immunoprecipitates of surface-radioiodinated DIL2 cells contained TCRα, TCRγ, CD3-δ, CD3-ε, TCR-γ, and TCR-δ chains, but lacked CD3-γ. This structural TCR/CD3 variant was, however, capable of transducing certain activation signals, since normal proliferation and a low but significant calcium flux was observed in DIL2 cells after engagement with specific antibodies. Our data suggest that a functional TCR/CD3 complex can be expressed on the surface of T cells in the absence of CD3-γ.

Materials and Methods

Cell Lines. Cell lines were derived from PBLs cultured in RPMI 1640 medium containing 10% FCS (Flow Laboratories, Rockville, MD).
MD) and 1% PHA (Difco Laboratories, Inc., Detroit, MI). After 3 d, cells were re-stimulated weekly with 50 IU/ml of rIL-2 (kindly provided by Hoffmann-La Roche, Nutley, NJ), and, in some cases, also with an inactivated mixture (ratio 1/4) of allogeneic lymphocytes and EBV-transformed lymphoblastoid cells (GUS-1). T cells from a mild case of TCR/CD3 immunodeficiency (D, reference 11) as well as from different normal individuals (genetically named C) were cultured in parallel during 1-2 mo, and the latter were used as controls in all experiments.

**Antigens.** Several mAb were used for cytofluorometric analysis. Their names, specificities, Ig isotypes and sources are listed in Table 1. For specific immunoprecipitations, the following reagents were used: mAb SP34 is a murine IgG3 which recognizes CD3-ε (12), mAb APA 1/2 recognizes CD3-δ (Fig. 2 D) and both were obtained by immunization of Balb/c mice with purified human CD3 proteins; mAb HMT3.2 recognizes CD3-γ (Fig. 2 D), was obtained by immunization of armenian hamsters with purified human CD3 proteins and was generously donated by Dr. Ralph Kubo (National Jewish Center, Denver, CO); mAb OKT3 was a generous gift from Dr. G. Goldstein (Ortho Pharmaceuticals, Raritan, NJ); TG5 is a rabbit anti-CD3 antibody raised against the CD3-γ C-terminal peptide GLIQGNQLRRN and was kindly donated by Dr. Denis Alexander (Cambridge Research Station, UK); J31 is a rabbit anti-CD3 antibody raised against the last 13 C-terminal amines of human CD3-δ (Alarcon, B., S. C. Ley, F. Sanchez-Madrid, R. S. Blumberg, S. T. Ju, M. Fresno, and C. Terhorst, unpublished results); N40 is a rabbit anti-CD3 antibody raised against a human TCR-γ peptide (Sancho, J., and C. Terhorst, unpublished results); C41 is a rabbit antisera that reacts with the constant region of HLA class I heavy chains (13).

**Cytofluorometry.** Cells (10⁵-5 × 10⁵) were incubated at 4°C for 30 min with appropriate amounts of either fluorescein-conjugated or purified mAbs (Table 1) in 100 µl of PBS containing 1% FCS. After two washings, F(ab')₂ goat anti-mouse IgG (Ortho Pharmaceuticals, Raritan, NJ) was used as a second step reagent before cytofluorographic analysis of 10⁵ cells in an Epics C cell sorter (Coulter Electronics, Hialeah, FL). Quantitative comparisons of fluorescence intensities were performed with an appropriate program which calculated the mean fluorescence in linear scale for each sample (Epics cytologic software 2.01; Coulter).

**Antibody-Binding Radioimmunoassays.** The reactivity of different mAbs with T cell lines was tested by RIA as described (14). Briefly, 5 × 10⁵ cells were labeled with an excess of mAb, washed three times and developed with 28 µl of 0.25 M phosphate buffer (pH 8.6) containing 10 mM phenanthroline (Sigma Chemical Co.) and mixed with 28 µl of 0.25 M phosphate buffer (pH 8.6) containing 10 mM phenanthroline (Sigma Chemical Co.). Half of each sample was incubated overnight at 30°C in the presence of 0.25 IU of N-glycosidase F (Genzyme, Boston, MA) and the other half was left untreated.

**Southern and Northern Blotting Analysis.** Total DNA or RNA was prepared from cell lines, digested with endonucleases in the case of DNA, subjected to electrophoresis, blotted, hybridized, washed and autoradiographed as described previously (17). The following DNA probes were obtained and labeled as described (18): TCR-α, TCR-β, CD3-δ, CD3-γ, CD3-ε, TCR-γ and β-actin.

**Functional Assays.** Proliferation assays were set up in 96-well round-bottomed microtiter plates (8 × 10⁵ cells per well in 0.2 ml; Costar, Cambridge, MA). Increasing dilutions of mAb in Tris 50 mM, pH 8, were incubated in plates overnight at 4°C. The plates were then washed three times with 199 medium (Flow Laboratories) to eliminate unbound mAb. Cells in RPMI with 10% FCS were incubated in the plates for 3 d in a controlled humidity chamber at 5% CO₂ and 37°C. In some cases, inhibition of T cell proliferation was assayed by adding increasing amounts of anti-CD3 (Leu4, 5-70 ng/ml), anti-CD5 (0.3-10 µg/ml; Coulter Electronics, Hialeah, FL) or anti-IL-2 mAb (3-50 µg/ml; Genzyme), at the beginning of the cultures. Proliferation was measured by 3H-thymidine uptake after 72 h as previously described (18).

For IL-2 production assays, 16 × 10⁵ cells were incubated in the presence of plastic-bound mAb or inactivated allogeneic cells in a medium containing anti-CD25 (10 µg/ml) to avoid autologous use of secreted IL-2. Maximum synthesis of IL-2 was induced with 1 µM Ionomycin (Sigma Chemical Co.) plus 12 ng/ml phorbol myristate acetate (Sigma Chemical Co.) plus a 1/60,000 dilution of an anti-CD28 mAb (Kolt2, generously donated by Dr. K. Sagawa, Kurume University, Japan). Supernatants of triplicate experiments were collected at day 3 for an IL-2 bioassay using CTLL2 (19).

**Calcium Mobilization during Stimulation with 10 µg/ml of anti-CD3 mAb (SP34) and crosslinking with 30 µg of anti-mouse Ig antisera (Cappel Laboratories, Malvern, PA) was analyzed using cells pre-loaded with the calcium-sensitive fluorescent dye Indo-1 (3 µM; Molecular Probes, Junction City, OR). Maximum calcium influx was induced with 1 µM Ionomycin. Quantitative comparisons of cytofluorometric results were performed as stated above (under Cytofluorometry).

**Results**

**Expression of the T Cell Receptor Complex.** Analysis by cytofluorometry of several T cell lines derived from a mild case of TCR/CD3 immunodeficiency (showing low levels of sur-
The reactivity of each mAb against DIL2 was analyzed by immunofluorescence and flow cytometry as described in Materials and Methods. The mean linear fluorescence fraction reflects the quantitative binding of each mAb to DIL2, relative to a normal T cell line. The symbols (+++ + + +), (+++ + +), (+ + + + +), (+ + +), (+ +), and (-) denote reactivity patterns with DIL2 comparable to OKT11, Leu4, OKT3, WT31, and MsIgG, respectively (Fig. 1). ND not determined.

Surface TCR/CD3 in peripheral blood T lymphocytes (11) revealed that their expression of certain TCR/CD3 epitopes became progressively higher upon culturing in IL2-containing media. This may reflect the selection imposed by our particular culture conditions. By 1 to 2 mo of culture, the expression of several T cell markers in one of these CD4 positive T cell lines (DIL2) was as summarized in Table 1, and remained stable thereafter. Quantitative analysis of the cytofluorometric data (shown as mean linear fluorescence fraction in Table 1) indicated that DIL2 had two- to five-fold less expression of the tested TCR/CD3 epitopes as compared to controls. Antibody-binding radioimmunoassays using the same mAb (Table 1) essentially confirmed these findings (DIL2 expressed around 50% less TCR/CD3 complexes than controls, data not shown). Also, striking differences were observed in the reactivity patterns of different TCR/CD3-specific mAbs with DIL2. Thus, most DIL2 cells were positive (>75%) when stained with CD3-e-specific mAb (Leu4, SP34, Table 1, Fig. 1). In contrast, other epitopes like those recognized by OKT3 and WT31 were detectable in only a fraction of the cells (<70% and <55%, respectively, Fig. 1). Based on these two criteria (fluorescence intensity and reactivity pattern), the following mAb hierarchy from higher to lower reactivity was observed: OKT11, Leu4, OKT3, WT31, MsIgG, and OKT8, respectively (Fig. 1).

Table 1. Phenotypic Characterization of DIL2 Cells

| Name     | Specificity | Isotype | Mean linear fluorescence fraction | Reactivity pattern with DIL2 | Source                |
|----------|-------------|---------|-----------------------------------|-----------------------------|-----------------------|
| OKT11    | CD2         | IgG2a   | 0.90                              | +++ + + +                    | Ortho, NJ             |
| OKT4     | CD4         | IgG2b   | 1.20                              | +++ + + +                    | Ortho, NJ             |
| DR       | HLA class II| IgG2a   | 1.30                              | +++ + + +                    | B. Dickinson, CA      |
| RIL-2    | CD25        | IgG2a   | 1.00                              | +++ + + +                    | Coulter, FL           |
| Leu4     | CD3-e       | IgG1    | 0.55                              | +++ +                         | B. Dickinson, CA      |
| SP34     | CD3-e       | IgG3    | 0.60                              | +++ +                         | C. Terhorst, MA       |
| T3b      | CD3-e?      | IgG2a   | 0.40                              | ++ +                          | J. E. de Vries, HOLLAND |
| OKT3     | CD3-eγ+δ    | IgG2a   | 0.30                              | ++ +                          | Ortho, NJ             |
| RIL28C8  | CD3-e?      | IgG2a   | 0.30                              | ++ +                          | E. L. Reinherz, MA    |
| RIL24B6  | CD3-e?      | IgG2a   | 0.30                              | ++ +                          | E. L. Reinherz, MA    |
| CRIS7    | CD3-e?      | IgG2a   | 0.25                              | ++ +                          | R. Vilella, SPAIN     |
| WT31     | TCR-α+β     | IgG1    | 0.27                              | +                             | Sanbio, HOLLAND       |
| BMA031   | TCR-α+β     | IgG2b   | 0.19                              | +                             | R. Kurle, FRG         |
| KOLT2    | CD28        | IgG1    | 1.17                              | +                             | K. Sagawa, JAPAN      |
| TiyA     | TCR-Vγ9     | IgG2a   | ND*                               | -                             | T. Hercend, FRANCE    |
| TCRδ1    | TCR-CD1     | IgG1    | ND                                | -                             | T Cell Sciences, MA   |
| OKT8     | CD8         | IgG2a   | ND                                | -                             | Ortho, NJ             |
| MsIgG    | Neg. control| IgG     | ND                                | -                             | Coulter, FL           |
| X63      | Neg. control| -       | ND                                | -                             | F. Sanchez-Madrid, SPAIN |

The reactivity of each mAb against DIL2 was analyzed by immunofluorescence and flow cytometry as described in Materials and Methods. The mean linear fluorescence fraction reflects the quantitative binding of each mAb to DIL2, relative to a normal T cell line. The symbols (+++ + + +), (+++ + +), (+ + + + +), (+ + +), (+ +), and (-) denote reactivity patterns with DIL2 comparable to OKT11, Leu4, OKT3, WT31, and MsIgG, respectively (Fig. 1). ND not determined.

Figure 1. Representative reactivity patterns of anti-TCR/CD3 mAbs with DIL2 (solid line) as compared with a control T cell line (dotted). Leu4 (anti-CD3-e), OKT3 (anti-CD3-εγ+δ) and WT31 (anti-TCR-α+β) profiles are shown as logarithm of relative fluorescence vs cell number. The reactivity profile with OKT11 (anti-CD2) is shown for comparison. The vertical line in each panel indicates the upper limit of the negative control staining (MsIgG).
Calcium mobilization by DIL2 (left panels) as compared to a control T cell line (right panels). Both samples were incubated with anti-CD3, 3 min later with anti-mouse Ig, and finally with ionomycin (ion), and analyzed for calcium content changes in an EPICS cytometer (Coulter Electronics, Hialeah, FL). The basal calcium levels are shown in the upper panels. The numbers in each panel indicate the percentage of positive cells above an arbitrary upper limit defined on the basal profiles by a vertical line. All panels represent the highest percentage for each treatment.

Figure 3.

Figure 4. Impaired IL-2 synthesis by DIL2 cells (filled symbols), as compared to a control (open symbols) T cell line, after stimulation with increasing amounts of plastic-bound anti-ε (Leu4) or anti-εγεδ (OKT3) mAb. Production of IL-2 was always <0.5 IU/ml when using only medium or irrelevant mAbs (ORM1, IgG2b; ORM5, IgG1; Leu5b, IgG2a). Addition of ionomycin plus phorbol myristate acetate plus an anti-CD28 mAb restored normal synthesis levels of IL-2 by DIL2 cells (>100 IU/ml; normal control >100 IU/ml).

lower binding of DIL2 may be deduced: Leu4/SP34 > OKT3/T3b/RW28C8/RW24B6/CRIS7 > WT31/BMA031 (Table 1). Taken together, these results suggested that most DIL2 cells expressed a relatively high level of the TCR/CD3 complex in their surface.

Absence of CD3-γ. DIL2 cells were next labeled with 125I to allow a preliminary biochemical characterization of the abnormal TCR/CD3 on their cell surface. The results using an anti-CD3-ε (ε hereafter) mAb demonstrated the presence of mature αβ heterodimers associated to ε on DIL2 cells (Fig. 2 A, left panel). However, N-glycanase digestion of anti-ε precipitates, which allowed a clear distinction between γ and δ chains, revealed that γ was undetectable in DIL2 precipitates (Fig. 2 A, middle panel). To confirm these results and to rule out the presence of free γ chains on the surface of DIL2, a direct precipitation was performed with an anti-γ mAb (HMT3.2, characterized in Fig. 2 D). No γ chains could be detected in these conditions either (Fig. 2 A, right panel).

In contrast, control precipitates revealed normal levels of HLA class I proteins in DIL2 lysates (Fig. 2 A, right panel). Analysis by two-dimensional electrophoresis of the radioiodinated material showed that β chains were associated to the surface TCR/CD3 of DIL2. It also confirmed that the surface α and β chains of DIL2 carried mature-type oligosaccharides (not shown).

Although no γ could be detected on the surface of DIL2, it was still possible that some was present in the cytoplasm but could not associate to the rest of the TCR/CD3 complex for exportation. To rule out this possibility, DIL2 cells were metabolically labeled and precipitated with antibodies specific for the different CD3 polypeptide chains (Fig. 2 B). The results demonstrated a complete lack of γ product also in the cytoplasm of DIL2, whereas δ and ε were readily detectable. Similar results were obtained using an anti-γ chain antiserum (TG5, Fig. 2 C). Thus, both a monoclonal (HMT3.2) and an antiserum (TG5) which were specific for γ showed that this chain was not present in DIL2 cells. In conclusion, the biochemical defect in DIL2 involved primarily a profound γ protein deficiency. Surprisingly, detectable levels of mature TCR/CD3 complexes containing α, β, δ, ε, and γ, but not γ chains, reached the cell surface. It was therefore of interest to analyze the genes and transcripts coding for those TCR/CD3 components in DIL2.

The existence of large deletions in the γ and δ chain genes of DIL2 was ruled out by Southern blot analysis of genomic DNA with several restriction enzymes, since no differences were found between normal and DIL2 cells in the number and size of restriction fragments corresponding to those and
all other TCR/CD3 components (not shown). Interestingly, an \( \epsilon \)-specific probe revealed that DIL2 was homozygous for a previously described 8.1 kb TaqI restriction fragment length polymorphism (20). This rare genotype (<10% frequency in normal Spaniards) was shared within the family only by the other affected sib (V, reference 21) but it is unlikely that it was directly related to the TCR expression defect, because it was also found in normal individuals.

To assess the expression of mRNA transcripts encoding the different TCR/CD3 components, total RNA isolated from DIL2 and a normal control T cell line was analyzed by Northern hybridization techniques. The results revealed that some crosshybridizing transcripts for all TCR/CD3 complex chains were present in DIL2 (data not shown). This ruled out a complete transcriptional defect for the observed lack of \( \gamma \) in DIL2.

**Functional Analysis.** To determine whether the \( \gamma^- \) TCR/CD3 complex expressed by DIL2 was functional with regard to antigen recognition and signal transduction, three sequential T cell activation parameters (calcium mobilization, IL-2 synthesis, and cell proliferation) were independently tested after TCR/CD3 crosslinking using several anti-CD3 or anti-TCRcig reagents (and, in some cases, appropriate inactivated allogeneic feeder cells).

First, calcium flux was low but detectable in DIL2 (Fig. 3). The impairment of calcium mobilization in DIL2 was a TCR/CD3-specific phenomenon, because addition of a calcium ionophore (which by-passes membrane signals) completely reverted the defect (Fig. 3, ion). In contrast, CD3 crosslinking in saturating conditions (10 \( \mu \)g/ml) using an anti-mouse Ig reagent showed that only a few DIL2 cells (around 30% compared to more than 70% in controls) significantly increased their calcium content above basal levels. In addition, the average increase of calcium concentration in DIL2 cells was around three-fold lower than in controls (see Materials and Methods). Therefore, calcium influx after CD3 crosslinking was impaired, but not absent, in DIL2.

Second, IL-2 production by DIL2 cells after stimulation with a wide range of plastic-bound anti-CD3 antibody concentrations was found to be markedly impaired as compared to a normal T cell line (four- to five-fold lower, Fig. 4). Interestingly, the observed impairment of IL-2 synthesis by DIL2 occurred irrespective of the binding ability of the stimulatory mAb (both low, like OKT3 and high, like Leu4). In addition, the synthesis of IL-2 in response to allogeneic antigens was analyzed in T cell lines derived from the donor of DIL2 or from normal individuals, cultured in the presence of an allogeneic lymphoblastoid B cell line (GUS-1) as described in Materials and Methods. The T cell lines thus obtained were challenged with either GUS-1 or an irrelevant (complete HLA mismatch) B cell line, and assayed for IL-2 synthesis. Irrelevant B cells consistently induced less than 0.5 IU/ml of IL-2 in both control and \( \gamma^- \) cells, and GUS-1 induced around 50 IU/ml of IL-2 in controls, but less than 5 IU/ml in \( \gamma^- \) cells. The impairment in IL-2 synthesis by DIL2 was also shown to be a TCR/CD3-specific feature, as it could be reverted by using transmembrane signals (calcium ionophore plus phorbol ester) together with mAb against other surface molecules (CD28, see footnote to Fig. 4). In conclusion, IL-2 synthesis by DIL2 cells after CD3 crosslinking (and by other \( \gamma^- \) T cells after allogeneic stimulation) was markedly impaired in vitro.

Third, the proliferation of DIL2 cells induced by an identical concentration range of plastic-bound anti-CD3 mAb was, in contrast, found to be dose-dependent and comparable to control T cell lines (Fig. 5). Similar results were obtained with all tested anti-CD3 (Leu4, OKT3, T3b, SP34) and anti-TCR-\( \alpha \beta \) (WT31) mAb, irrespective of their binding ability to DIL2 cells (not shown). The observed proliferation of DIL2 to plastic-bound Leu4 could be completely abolished by addition to the cultures of increasing amounts of soluble Leu4 (data not shown), demonstrating the specificity of the response. Also, the normal proliferation of DIL2 cells in response to both Leu4 and OKT3 was shown to be dependent on IL-2/IL-2 receptor interactions, because addition to the cultures of increasing amounts of anti-IL2 or anti-CD25 mAb effectively abolished their proliferative response in a dose-dependent fashion (data not shown).

To rule out that the normal proliferation of DIL2 to anti-CD3 challenges was due to an unusually high responsiveness
of this T cell line to the low levels of IL-2 detected in the assays (<5 IU/ml, Fig. 4), its proliferative response to exogenous rIL-2 was titrated out (Fig. 5). It was found to be similar to that of normal controls.

Taken together, our results suggest that this particular T cell line expressing a variant form of surface TCR/CD3 complex which lacked γ was nevertheless in part functional, since it was capable of fluxing some calcium and undergoing normal TCR/CD3-mediated proliferation responses. Also, proliferation took place through the IL-2/IL-2 receptor pathway, despite the fact that IL-2 synthesis was markedly impaired in similar experimental conditions. The observed low levels of secreted IL-2 by DIL2 must therefore have been sufficient for its autocrine requirements.

Discussion

A Natural CD3-γ Expression Defect. The surface expression of partial TCR/CD3 complexes lacking γ chains was shown to be possible in DIL2. The lack of γ, however, strongly reduced the expression of certain TCR/CD3 epitopes (namely those recognized by T3b, OKT3, RW28C8, RW24B6, CR157, WT31, and BMA031), suggesting that this group of mAbs require γ expression to bind the complex efficiently. These data correlate with those obtained by others in murine cell line expressing a variant form of surface TCR/CD3 complex which lacked γ was nevertheless in part functional, since it was capable of fluxing some calcium and undergoing normal TCR/CD3-mediated proliferation responses. Also, proliferation took place through the IL-2/IL-2 receptor pathway, despite the fact that IL-2 synthesis was markedly impaired in similar experimental conditions. The observed low levels of secreted IL-2 by DIL2 must therefore have been sufficient for its autocrine requirements.

The virtual absence of γ chains in DIL2 cells by immunoprecipitation is difficult to explain at present, because some γ-specific mRNA is detectable by Northern analysis. It could be proposed that the biochemical basis of the defective phenotype may involve a small natural mutation in the gene for CD3-γ. This defect would prevent its normal association to the rest of the TCR/CD3 complex and also its recognition by γ-specific mAb, but not the exportation of a partial TCR/CD3 lacking γ chains to the cell surface. However, since allelic exclusion of the TCR does not operate on CD3 genes, this hypothesis would require the segregation of two independent small mutations (in coding and/or regulatory regions) into this particular donor (his parents were unrelated, reference 10). Interestingly, an ε gene polymorphism detected in the family suggests that the two affected siblings may share their γεε haplotypes (see above). We are presently sequencing DIL2 γ cDNA and regulatory genomic DNA segments to address this issue (see note added in proof).

Function of a CD3-γ− T Cell Receptor (TCR)/CD3 Complex. Previous studies with T cell mutants have demonstrated that the normal expression and function of the TCR/CD3 complex requires the presence of all its components (4-9). However, some of these variants did retain certain functional features, allowing to establish structure/function relationships (4, 5, 24). Similarly, DIL2 allows to propose that CD3-γ is dispensable for TCR-induced autocrine proliferation. With regard to other functional features, it cannot be ruled out that the impaired calcium flux and IL-2 secretion observed in stimulated DIL2 cells may have been due to their two-to five-fold lower antibody-binding ability as compared to controls. Also, clonal variation and/or the intrinsic heterogeneity of a T cell line like DIL2 cannot be ruled out as a cause of its observed impaired activation features.

Pathological Implications. It is surprising that the donor of DIL2 may himself be viable, unless alternative or redundant pathways of T cell activation are operative (18). Indeed, redundant and overlapping functions among proteins of the immune system allow the existence of healthy immunodeficient individuals, the most common being congenital C2-, IgA-, HLA class I- and adenosine deaminase-deficiencies (reviewed in reference 25). Recently, a healthy infant with a similar TCR/CD3 defect has been reported (26). The biochemical basis of this deficiency was, however, different from that of DIL2, and may involve a δ defect (27). It is therefore tempting to speculate that δ-containing TCR/CD3 and γ-containing TCR/CD3 are in fact redundant recognition structures. The close evolutionary relationship of δ and γ genes would support this concept (28).

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