SURFACE EXPRESSION OF ONLY γδ AND/OR αβ T CELL RECEPTOR HETERODIMERS BY CELLS WITH FOUR (α, β, γ, δ) FUNCTIONAL RECEPTOR CHAINS

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The specificity of T lymphocytes derives from the properties of clonally distributed membrane receptors. For most peripheral T cells ($CD4^+CD8^-$ and $CD4^-CD8^+$), antigen is corecognized with molecules of the MHC by a receptor consisting of clonally diverse αβ heterodimers associated with the multichain CD3 complex (1–3). Recently, a subpopulation of T cells was described, usually lacking expression of the CD4 and CD8 cell surface molecules, that uses as its receptor a distinct heterodimer (γδ) associated with CD3 (4–7). The specificity of this new TCR is not well characterized, although one report of reactivity with a polymorphic MHC-linked molecule has appeared (8).

The genes encoding each of the chains of the two receptor heterodimers have been cloned and analyzed (9–15). Each chain is encoded by a set of gene segments that undergoes rearrangement during differentiation of the T cell, although the number of such segments, their diversity in the germline, and the pattern of rearrangement varies for each locus. Ontogenetic studies have revealed that γ and δ are rearranged before β and α (14, 16–20), and T cells expressing γδ-CD3 complexes on their cell surfaces appear in the murine thymus several days before αβ-CD3-expressing cells (21, 22).

Investigation of TCR gene expression in various cloned cell lines has shown that most αβ-expressing cells lack an in-frame γ gene rearrangement (23) and that most γδ-expressing cells lack mRNA capable of encoding functional α or β chains (4, 7). In addition, the δ locus is contained within the α locus, such that Vα-Jα rearrangement will delete the δ locus on that chromosome (14). These findings, together with biochemical studies showing mutually exclusive expression of one or the other of the types of TCR (αβ or γδ) on individual T cell clones and the ontogenetic pattern of receptor expression noted above (21), have led to the hypothesis that γδ- and αβ-expressing T cells represent distinct lineages derived from a common precursor. Successful expression of a γδ receptor is thought to preclude further receptor gene rearrangement, and hence, αβ expression. A failure to successfully rearrange either γ or δ permits continued rearrangement and the eventual expression of an αβ receptor.

This model explains the rare occurrence of in-frame γ transcripts in $\alpha\beta^+$ T cells and the absence of functional α or β transcripts in $\gamma\delta^+$ cells.

Although the absence of in-frame γ rearrangements in most αβ T cells makes it unlikely that many $\gamma\delta^+$ cells differentiate further into $\alpha\beta^+$ cells, it is very difficult from in vivo studies to rule out the existence of a small population of T cells in transition from expression of one type of receptor to another. Furthermore, some mature T cells have been found to contain at least three potentially functional receptor chains (11, 24). Because all these chains are expected to share the general structural features of prototypic Ig domains, it is of interest to know whether pairing and expression of chains typically associated with distinct TCR dimers could occur and give rise to unusual types of receptors, perhaps with unique specificities. In the present report, we analyze these issues by intentionally introducing functional α and/or β genes into $\gamma\delta^+$ cells and analyzing the resultant cell surface expression of CD3-associated heterodimers. Our results demonstrate the potential for simultaneous expression of both receptor types by a single cell and also reveal a clear absence of dimers containing mixtures of chains other than the typical αβ or γδ combinations. In one case, creation of a functional αβ dimer led to the loss of γδ expression on the membrane, consistent with an induced switch from one receptor type to another. However, further study indicated that this change most likely reflected intracellular competition for CD3 components, rather than a genetic event. Overall, the data support the view that cells express the two types of receptor dimers in a mutually exclusive manner and that mixtures of receptor chains other than of the αβ or γδ types do not occur to a significant extent nor contribute to receptor diversity.

Materials and Methods

Cell Lines and Antibodies. The human T cell tumor line Jurkat (clone E6.1), expressing clonotypic TCR (Ti)[1] αβ heterodimers (25), and the tumor line PEER, expressing γδ heterodimers (6), have been described. 2B4 is a cytochrome *c*-specific, I-E^k-restricted murine T hybridoma, and its characteristics have been described in detail (26). DN7.1 is a γδ-expressing murine hybridoma produced by cell fusion between C57BL/6 dull $Ly1^+$ double-negative thymocytes and $Ti\beta^-$-BW5147, as described (27).

mAbs used in this study were as follows: A2B4.2, a mouse anti-clonotypic antibody recognizing the Tiα chain of 2B4 (2, 28); 2C11, a hamster anti-murine CD3 chain antibody (29); OKT3 (30) and anti-Leu-4 (Becton Dickinson & Co., Sunnyvale, CA), mouse anti-human CD3 antibodies; WT31, a mouse anti-human Tiαβ heterodimer antibody (31); βF1, a mouse anti-human Tiβ chain framework antibody (32); anti-TCRδ1, a mouse anti-human Tiδ chain framework antibody (33); and anti-Cγm1, a mouse anti-human γ chain constant region antibody (Hochstenbach and Brenner, submitted for publication). Myeloma protein P3 was used as a control antibody. An antiserum directed against a COOH-terminal peptide of murine γ chains and described previously in detail (7) was kindly provided by J. Coligan (National Institute of Allergy and Infectious Diseases, National Institutes of Health). The anti-γ antibody used was purified by affinity chromatography on an appropriate peptide-bearing column.

Transfection. The constructs of expressible forms of the 2B4α and β chain genes were described previously (2). Briefly, for the α chain, a cDNA clone corresponding to the 2B4α chain was cloned into a vector containing the Friend spleen focus-forming virus LTR and neo^r gene (p2B4αFneo). For the β chain, a rearranged genomic clone was subcloned in

[1] *Abbreviations used in this paper:* CHAPS, 3-[(3-Cholamidopropyl)dimethyl-ammonio]-propanesulfonate; Ti, clonotypic TCR.

pSV2gpt (p2B4βSV2gpt), providing a distinct selective marker usuable in conjunction with the α gene construct. All transfections were performed by spheroplast fusion (2, 34). Transfected cells were cultured at 10^5 cells per well in 96-well microtiter plates, and were selected 48 h later in DMEM containing 10% FCS and appropriate concentrations of selective drugs. For 2B4α transfection, 1 and 2 mg/ml of G418 were used for PEER and DN7.1, respectively, and for 2B4β transfection, DN7.1 transfectants were selected in the presence of 1 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine. To obtain 2B4α- and β-transfected DN7.1, one 2B4β-transfected DN7.1 clone (DN1-β-1.35) was super-transfected with p2B4αFneo. Growing colonies were visualized 2–4 wk after transfection and were expanded and maintained in selective medium. Positive clones were screened by both surface staining with A2B4 and RNA dot blot analysis using Vα2B4 or Vβ2B4 probes.

Flow Microfluorimetry. Cell surface phenotype was determined by flow cytometry on a FACS analyzer (Becton Dickinson & Co.). 5×10^5 cells were stained with culture supernatants of A2B4, OKT3, or 2C11, or a 1:500 dilution of ascites of WT31, followed by fluoresceinated $F(ab')^2$ goat anti-mouse Ig (Cappel Laboratories, Cochranville, PA), as described (2).

Northern Blot Analysis. Cytoplasmic RNA was prepared (35) from E6.1, PEER, PE-α-1.3, 2B4, DN7.1, and 2B4α- and/or β-transfected DN7.1. 10 μg of total RNA from each cell line was subjected to electrophoresis on a 1% agarose gel containing 0.22 M formaldehyde for 600 V-h, transferred to nitrocellulose (36), and hybridized to various cDNA probes labeled by the hexamer-priming method (37). The hybridized blots were washed with 0.1 × SSC and 0.1% SDS at 60°C. The probes used were a 1.4-kb Eco RI fragment corresponding to the V, J, and C regions of the human α chain clone Py1.4 (13); a 750-bp Bgl II-Apa I fragment corresponding to the human Cβ region from pΓβFneo (38); a 1.6-kb Eco RI fragment corresponding to the V, J, and Cγ1 region of human γ chains (kindly provided by Dr. T. W. Mak); a 1.5-kb Eco RI fragment corresponding to human Cδ (15); a 340-bp PstI-Pvu II fragment corresponding to the V region of the 2B4α chain (Vα11.2, 39) from pGEM-2B4α (2); a 310-bp Eco RI-Sca I fragment corresponding to the V region of the 2B4β chain (12; now called Vβ3, [40]) from p2B4βSV2gpt (2); and a 600-bp Eco RI fragment corresponding to the murine Cγ2 region (M. Avigan and R. N. Germain, unpublished results).

Cell Labeling and Immunoprecipitation. For cell surface labeling, 4×10^7 T cells were purified on Ficoll density gradients, washed with PBS, and labeled using lactoperoxidase in the presence of 2 mCi sodium ^{125}I, as described (4). After 3 h of lysis in 0.3% 3-[(3-Cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS)/Tris-buffering saline containing 7.5 mM iodoacetamide (Sigma Chemical Co., St. Louis, MO) and 1 mM PMSF (Sigma Chemical Co.), the lysates were precleared, as described (4). For βF1 and anti-TCRδ1 immunoprecipitation, 1% Triton X-100 was added before addition of specific mAb to augment the dissociation of the TCR-CD3 complex. The specific immunoprecipitations were performed using 100 μl P3 (negative control), A2B4, 2C11 and anti-TCRδ1 hybridoma supernatants, 0.5 μl βF1 ascites, or 1 μg anti-Leu-4 (Becton Dickinson & Co.) in the presence of 100 μl rat anti-mouse κ chain-specific mAb 187.1 to facilitate binding of the immune complexes to 100 μl 10% (vol/vol) protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The beads were washed in 0.1% Triton X-100 containing buffer. Before loading the samples on a 10% acrylamide gel, half of each immunoprecipate was reduced in DTT as described. For diagonal gels, samples were run under nonreducing conditions for 12 h in 7.5% SDS-PAGE tube gels at 0.25 mA per tube. Tube gels were then incubated in reducing sample buffer for 2 h at room temperature, layered on top of a 10% SDS-PAGE slab gel, and run 12 h at 6 mA per gel. Gels were dried, and labeled proteins were visualized by autoradiography.

For metabolic labeling, 2×10^7 exponentially growing cells were resuspended in 4 ml of methionine and cysteine-free RPMI 1640 (Select-Amine kit; Gibco Laboratories, Grand Island, NY) supplemented with 10% dialysed FCS and 20 mM Hepes. After a 30-min starvation at 37°C, 0.5 mCi of ^{35}S-methionine and 0.5 mCi of ^{35}S-cysteine was added, followed by a 3-h labeling period. Cells were harvested and lysed for 1 h in 1% (wt/vol) digitonin-(Aldrich Chemical Co., Milwaukee, WI) supplemented Tris-buffered saline (TBS) (50 mM Tris, pH 7.6, 140 mM NaCl) containing freshly added 1 mM PMSF and 8 mM IAA. The lysates were precleared by incubating with normal rabbit serum and 187.1 hybridoma culture supernatant, followed by addition of 200 μl of a 10% (wt/vol) cell suspension of fixed *Staphylo-*

coccus aureus Cowan I (Pansorbin; Calbiochem-Behring Corp., San Diego, CA). Specific precipitations were carried out by adding 0.25 μl βF1 ascites, 0.5 μl anti-Cγm1 ascites, 0.2 μl anti-TCRδ1 ascites, 1 μl 1 mg/ml anti-Leu-4 or 0.5 ml NMS together with 200 μl of 187.1 supernatant to each sample. After a 1-h incubation, 100 μl of 10% (vol/vol) protein A-Sepharose (Pharmacia Fine Chemicals) was added, and the mixture was rocked for 1 h. The immunoprecipitates were washed four times in 0.1% Triton X-100, TBS and three times in 1% digitonin, TBS. Half of each sample was reduced with DTT, the other half was analyzed under nonreducing conditions. The samples were resolved on a 10% acrylamide gel, and fluorography was performed as described (41).

Results

Conversion of $\gamma\delta^+$ PEER Cells into $\alpha\beta^+$ cells After the Expression of a Transfected Tiα Gene. PEER, a human $\gamma\delta^+$ leukemia cell line, has been extensively analyzed (6, 42). This cell has the unusual property of also containing an in-frame β mRNA that gives rise to β chains that assemble intracellularly with CD3 components, but are not transported to the cell membrane (43, 44). The receptor gene phenotype of this cell ($\gamma^+\delta^+\beta^+\alpha^0$) made it a candidate for a rare transitional cell differentiating from a $\gamma\delta^+$ to an $\alpha\beta^+$ lymphocyte. To determine if this tumor cell might be capable of demonstrating feedback inactivation of the γδ loci upon expression of a functional αβ-CD3 complex, we introduced an α chain-encoding cDNA clone into PEER cells.

We have previously shown that both mouse α–human β and human α–mouse β heterodimers can be expressed in association with the human CD3 complex on the cell surface after transfection of mouse Ti genes into human T cells (2, 45). Therefore, the human PEER cell line was transfected with an expressible form of a functional full length cDNA clone of the Tiα gene of the murine I-E^k-restricted, cytochrome *c*-specific T cell hybridoma 2B4 (2, 26). Transfectants were screened by both surface staining with the A2B4 mAb, which is known to react with the 2B4α chain in isolation or associated with a variety of partner chains (2), and by RNA dot blot analysis using a Vα2B4 probe. More than 30 2B4α-transfected PEER clones were obtained in this manner. Six independent clones have been analyzed in detail by staining and immunoprecipitation, and all six showed almost identical patterns of TCR expression. We describe here a representative clone, PE-α-1.3.

Fig. 1 shows the staining profiles of the parental PEER line and of the Tiα-transfected clone PE-α-1.3. PEER shows a broad, somewhat dull pattern of surface CD3 expression. As expected, staining with A2B4 is absent, as is staining with WT31, known to react with a common epitope on human αβ dimers. In contrast, PE-α-1.3 shows bright staining with anti-CD3 as well as WT31 and A2B4 antibodies. The most likely explanation for this result is the expression of human β-mouse α heterodimers on the cell surface, reactive with both the A2B4 and the WT31 antibodies. However, because Van de Griend et al. (46) have recently found that WT31 reacts with some $\gamma\delta^+$ T cells, the positive staining of PE-α-1.3 with WT31 does not conclusively indicate Ti αβ expression.

To more directly determine the nature of the receptor chains expressed on the surface of the transfected PEER cells, immunoprecipitation and SDS-PAGE were performed. Both PEER and PE-α-1.3 cells were surface iodinated, followed by lysis with 5 mM CHAPS to preserve the Ti/CD3 complex (6). Labeled proteins were immunoprecipitated from the cell lysates with various mAbs (Fig. 2). No specific bands were observed after treatment of the PEER cell lysate with either A2B4 or

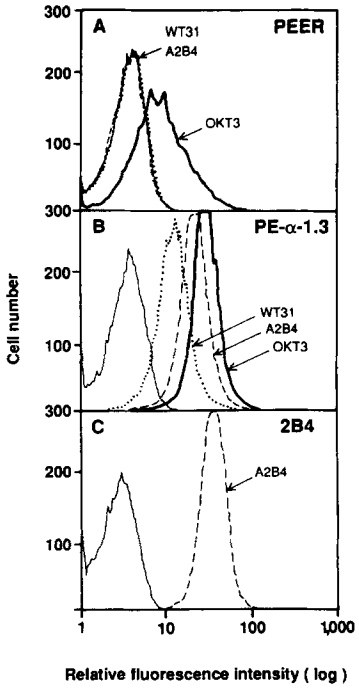

FIGURE 1. Cell surface expression of Ti/CD3 by 2B4α-transfected PEER cells. 5×10^5 PEER (*A*), PE-α-1.3 (*B*), or 2B4 (*C*) were stained with culture supernatants of A2B4.2 (- - - -), OKT3 (——), or a 1:500 dilution of ascites of WT31 (· · ·), or medium alone (——), followed by fluoresceinated $(Fab')_2$ goat anti-mouse Ig and then analyzed using a FACS analyzer. The results represent analysis of 10^4 cells.

with an mAb reactive with a framework determinant of human β chains (βF1, 32). As expected, anti-Leu-4 (anti-CD3) precipitated a 55-kd γ chain together with a 40-kd δ chain in association with CD3 proteins. On PEER cells, the TCR γ and δ chains are noncovalently associated. Use of a recently described mAb directed at human δ chains (anti-TCRδ1, 33) also led to precipitation of the 55-kd γ chain and the CD3 chains, but very little visible δ chain. This difficulty in visualizing the PEER δ chain after surface iodination has been noted previously (6, 42).

In contrast to the results with the parental cell line, precipitation of proteins from the PE-α-1.3 lysate with anti-Leu-4 revealed the presence of an 80-kd species that was recognized both by A2B4 and βF1 and, therefore, represents a Ti αβ heterodimer. This Ti αβ species yielded a broad 39–43-kd band upon reduction. These data are consistent with the staining results and indicate that 2B4α and PEER β chains are associated with the CD3 complex on the surface of PE-α-1.3. Surprisingly, anti-Leu-4 precipitation did not show any detectable 55-kd γ chain. This apparent loss of γ chain expression was confirmed by the failure to detect γ chain protein after immunoprecipitation with the anti-TCRδ1 antibody. Six independent transfectant clones all showed this same loss of surface γδ expression concomitant with the appearance of surface αβ dimers.

These data made several points. First, the absence of a βF1-reactive species on the PEER cell surface before transfection implied that the PEER β chain cannot be transported to the cell surface in association with either the γ or δ chain. Nevertheless, these findings also indicated that the PEER β chain is functional and can be expressed with an α chain on the cell surface. Second, the unexpected loss of γδ sur-

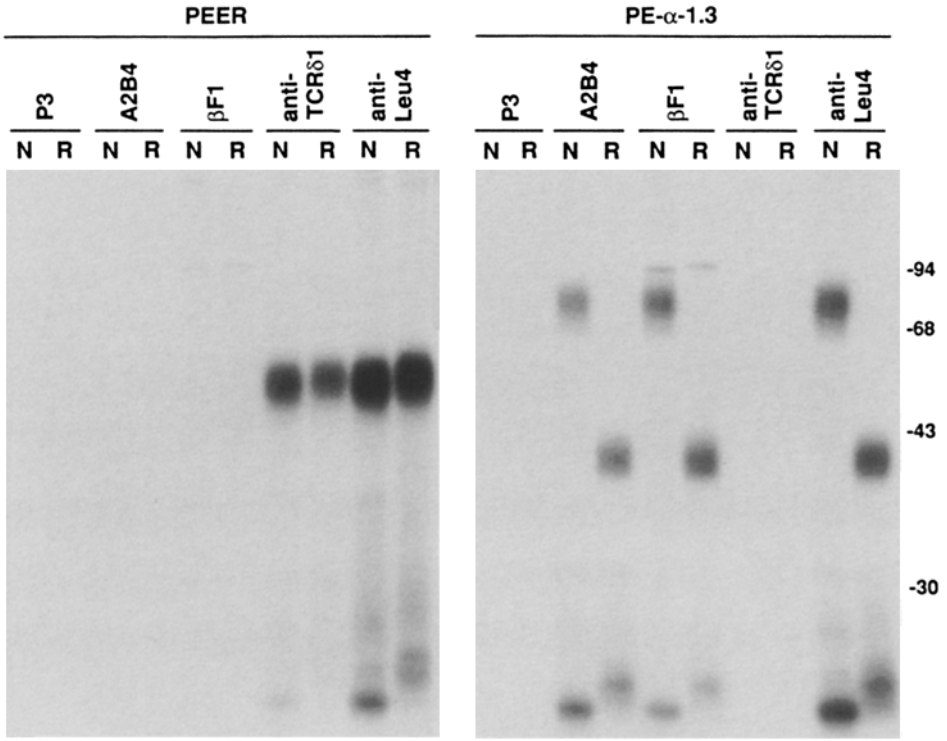

FIGURE 2. Immunoprecipitation of proteins from lysates of surface-labeled PEER and PE-α-1.3 cells. PEER and PE-α-1.3 cells were surface iodinated, lysed, and the lysates were used for immunoprecipitation with the indicated mAbs. Immunoprecipitated samples were analyzed by SDS-PAGE using 10% gels under nonreducing (*N*) and reducing (*R*) conditions. Molecular size markers are indicated as kD in the margin.

face expression at the time of acquisition of the αβ phenotype suggested a possible feedback regulation of receptor expression in this cell line.

Introduction of a Functional α Gene into PEER Does not Lead to a Change in γδ mRNA Expression. To determine if the loss of surface γδ dimer expression after α gene transfection into PEER was due to negative regulation of γδ mRNA levels, the steady-state amounts of Ti mRNAs in the parent and transfectant cell lines were examined. Fig. 3 shows the results of Northern blot analysis using cDNA probes corresponding to the constant regions of human Tiα, β, and δ genes and to the V region of the mouse 2B4α gene. Cα and Cβ blots show that both PEER and PE-α-1.3 express no human α mRNA, but they do have approximately equal and high levels of full-length 1.3-kb β mRNA. Cγ and Cδ blots revealed only slightly reduced levels of mature 1.6-kb γ chain and 2.2- and 1.3-kb mature δ mRNA in the transfected cells. 1.7- and 0.8-kb δ mRNAs, which do not contain V region sequences (15), were also detected in both cells. Fig. 3 also shows the abundant expression of 2B4α mRNA in PE-α-1.3. These findings suggest that the switch in Ti phenotype after α gene transfection is not the result of a direct feedback effect of successful αβ dimer formation on the transcription of γδ genes or the rate of degradation of the respective mRNAs.

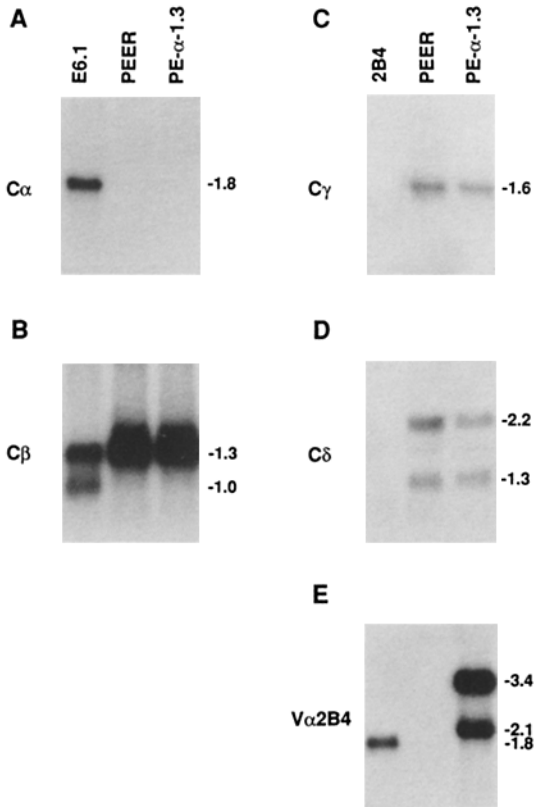

FIGURE 3. Northern blot analysis of Tiα-transfected PEER cells. 10 μg of cytoplasmic RNA from E6.1, PEER, and PE-α-1.3 were separated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and the blots hybridized to human α (*A*), human β (*B*), human γ (*C*), human δ (*D*), or Vα2B4 (*E*) probes. Molecular sizes in kb are indicated in the margin.

The Failure to Detect γδ Expression on PE-α-1.3 May Be Due to Competition for CD3 by αβ Dimers. We next labeled PEER and PE-α-1.3 cells biosynthetically in order to test whether γ and δ protein can be detected intracellularly. Fig. 4 shows the immunoprecipitation results using lysates prepared from cells labeled for 3 h. βF1 precipitation of protein from the PEER lysate revealed that a large amount of β protein is synthesized in PEER cells. Only a fraction of the β chains is precipitated with anti-Leu-4, indicating that both free β chains and CD3-associated β chains exist intracellularly, as reported previously by Koning et al. (43). The 45- and 48-kD bands that are associated with CD3 correspond to the TCR γ chain precursors, because they were recognized by anti-Cγm1, a monoclonal anti-γ chain antibody (53). Immunoprecipitation by anti-TCRδ1 antibody showed a typical single δ chain band that migrated more rapidly under nonreducing conditions. Under nonreducing conditions, δ could be unequivocally identified after anti-CD3 precipitation despite the presence of the more heavily labeled β species.

The pattern of Ti chains precipitated from lysates of labeled PE-α-1.3 was strikingly different from that obtained with PEER. βF1 precipitation again showed abundant intracytoplasmic β chain. However, the anti-Leu-4 antibody coprecipitated only β, but no detectable γ chains along with CD3. Precipitation with anti-Cγm1 antibody did reveal a small amount of γ chain precursor in the PE-α-1.3 lysates, but

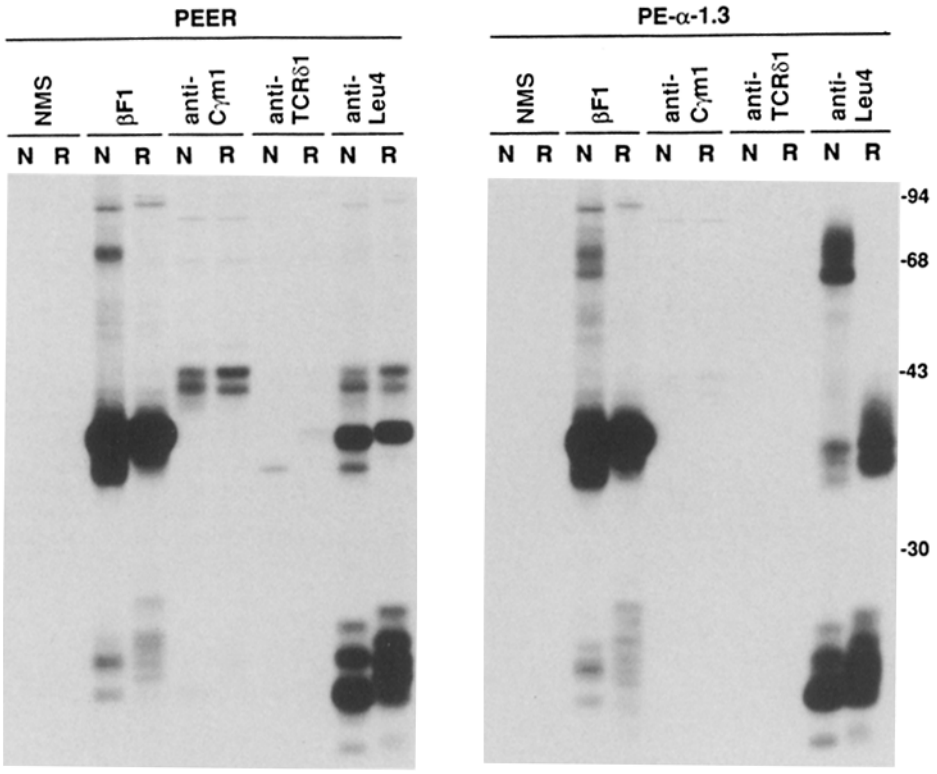

FIGURE 4. Immunoprecipitation of proteins from lysates of metabolically-labeled PEER and PE-α-1.3 cells. PEER and PE-α-1.3 cells were metabolically labeled for 5 h with ^{35}S-methionine and ^{35}S-cysteine, lysed, and the lysates used for immunoprecipitation with indicated mAbs. Immunoprecipitated samples were analyzed by SDS-PAGE using 10% gels under nonreducing (*N*) and reducing (*R*) conditions. Molecular size markers in kD are indicated in the margin.

these chains were not detectably associated with CD3 components. Furthermore, we could only observe δ chain after prolonged exposure of the gels (data not shown). Thus, after the introduction of a functional α chain–encoding gene into PEER, TCR δ chain polypeptides are virtually undetectable, and γ chain precursors are present in only minor amounts. Because δ and γ mRNA are only modestly reduced under these conditions, these findings are most consistent with a post-translational decrease in intracellular γ and δ levels. This may be the result of abundant αβ dimers in the transfectant outcompeting γδ for limiting CD3 components, leading to degradative loss of unassembled δ and γ chains.

Transfection to Ti α and β Genes into a Murine γδ$^+$ Hybridoma. Because of the asymmetry in chain synthesis in the transfected PEER cell, leading apparently to the competitive loss of γδ assembly with CD3, we could not use this model to extend our analysis of the possible pairings of Ti chains of various types. In an attempt to overcome this experimental limitation, and also to determine if there is a special bias in αβ vs. γδ association with CD3 proteins, we have examined a second independent system involving the introduction of Ti α and/or β genes into a murine γδ$^+$ hybridoma.

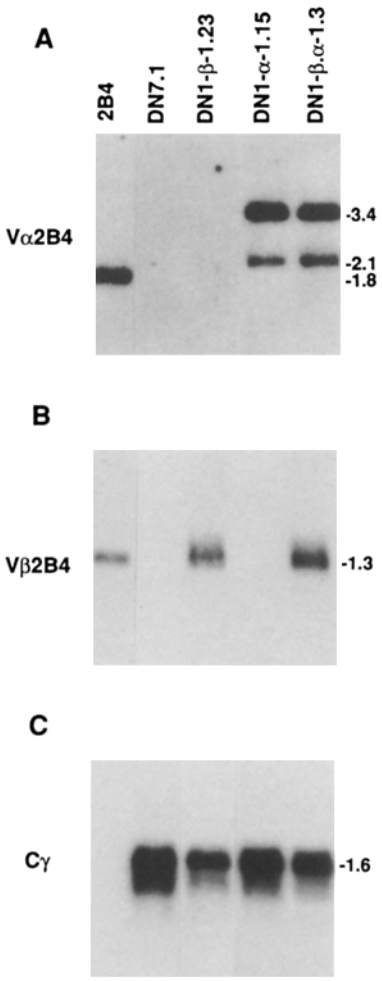

FIGURE 5. Northern blot analysis of 2B4α- and/or β-transfected DN7.1 hybridomas. 10 μg of cytoplasmic RNA from 2B4, DN7.1, DN1-β-1.23, DN1-α-1.15, and DN1-β.α-1.3 were separated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and the blots hybridized to Vα2B4 (*A*), Vβ2B4 (*B*), or Cγ (*C*) probes. Molecular sizes in kb are indicated in the margin.

The DN7.1 $\gamma\delta^+$ murine hybridoma, previously described in detail (27), was chosen for these experiments. Briefly, DN7.1 was produced by fusion of C57BL/6 dull $Ly1^+$ $CD4^-CD8^-$ thymocytes with a BW5147 lymphoma variant that has lost the expression of its functional Ti β gene. DN7.1 therefore expresses BW5147α chain mRNA but not mature β chain mRNA. On the cell surface, DN7.1 expresses disulfide-linked 47-kb δ and 34-kD γ chains but no αβ dimers. Fig. 5 shows the results of Northern blot analysis of representative clones of 2B4 α and/or 2B4β gene-transfected DN7.1. The parent DN7.1 does not express any mRNA hybridizing with V region probes for the 2Bα or β genes. The 2B4β single-gene tranfectant (DN1-β-1.23) has the expected 2B4β gene transcript and, similarly, the 2B4α single-transfectant (DN1-α-1.15) expresses specific 2B4α mRNA. The two Vα11-containing transcripts of 2.1 and 3.4 kb are larger than the authentic 2B4α transcript and are due to the nature of the cDNA expression vector used to express this cDNA. Finally, a 2B4α and 2B4β gene-transfected DN7.1 (DN1-β.α-1.3) expresses both specific α and β chain transcripts.

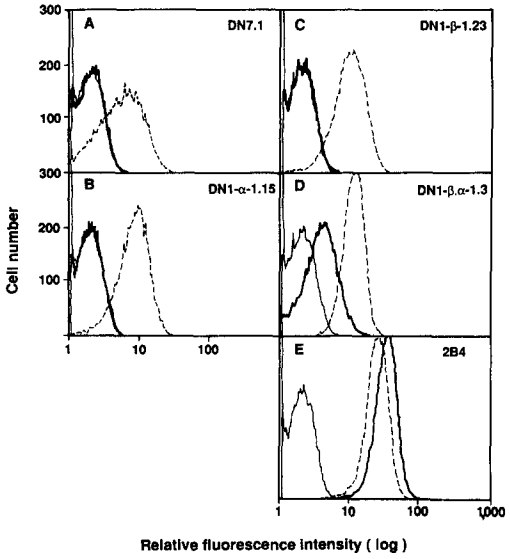

FIGURE 6. Cell surface expression of 2B4α chain by 2B4α- and/or β-transfected DN7.1 cells. 5 × 10^5 DN7.1 (*A*), 2B4 (*E*), or Ti-transfected DN7.1 cells (*B–D*) were stained with culture supernatant of 2C11 (- - - -), A2B4.2 (——), or medium alone (——), followed by fluoresceinated $(Fab')_2$ goat anti-mouse Ig, and then analyzed by a FACS analyzer. The results represent analysis of 10^4 cells.

Fig. 6 depicts immunofluorescence profiles of the DN7.1 cell and of 2B4 α and/or β gene transfectants stained with A2B4 and/or the anti-mouse CD3ε antibody 2C11. All cells showed expression of the CD3ε epitope, consistent with the continued expression of some type of Ti-CD3 complexes on the cell surface. The parental DN7.1 cells, the β gene single transfectant, and the 2B4α gene single transfectant did not express 2B4α chain on the surface. This result indicates that 2B4α chain does not pair with either the γ or δ chain to yield heterodimers able to associate with CD3

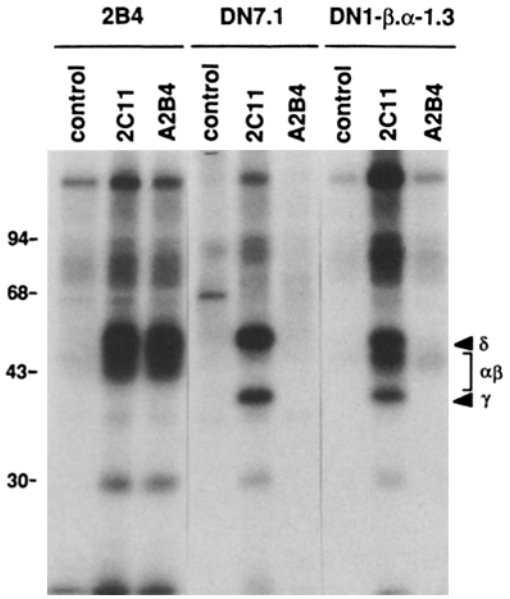

FIGURE 7. Immunoprecipitation of proteins from lysates of surface-labeled 2B4αβ-transfected DN7.1 cells. 2B4, DN7.1, or DN1-β.α-1.3 were surface iodinated, lysated, and the lysates used for immunoprecipitation with 2C11, A2B4.2, or normal mouse serum as control. Immunoprecipitated samples were analyzed by SDS-PAGE using 10% gels under reducing conditions. Molecular size markers in kD are indicated in the margin. The band of ~60 kd in the control lane of DN7.1 was not observed on repeat gels.

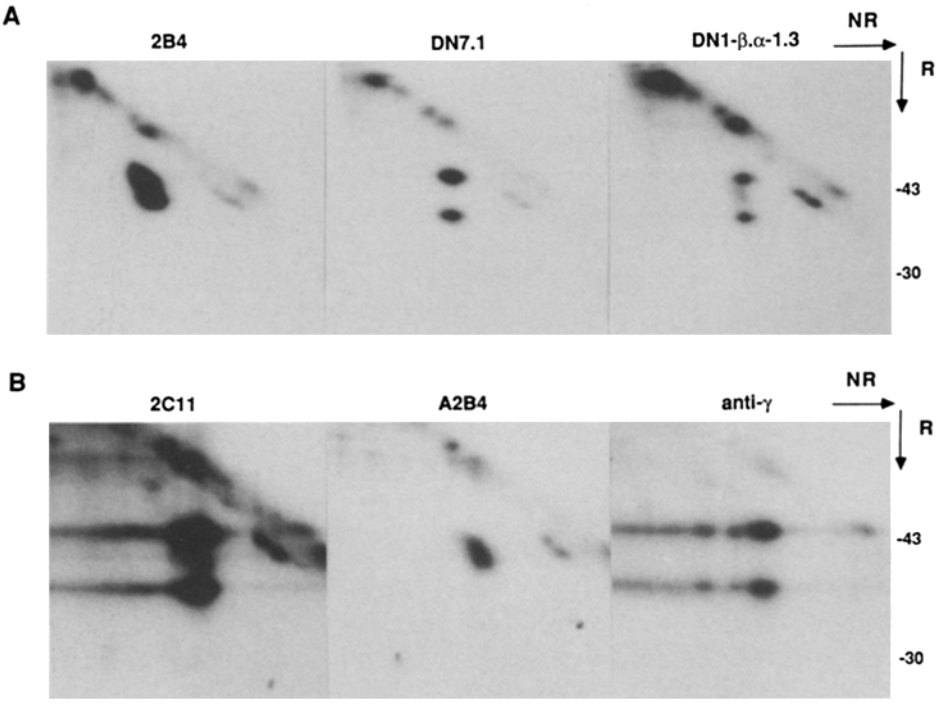

FIGURE 8. Two-dimensional gel analysis of Ti/CD3 complexes on Tiαβ-transfected DN7.1 cells. 2B4, DN7.1, and DN1-β.α-1.3 cells were surface iodinated, lysed, and the lysates used for immunoprecipitation. Immunoprecipitated samples were analyzed in diagonal gels (7.5% nonreducing (*NR*) gel for the first dimension and 10% reducing (*R*) gel for the second dimension). (*A*) Proteins from each lysate of 2B4, DN7.1, or DN1-β.α-1.3 were precipitated with 2C11. (*B*) Proteins from the lysate of DN1-β.α-1.3 were precipitated with 2C11, A2B4, or anti-γ heteroantibodies, respectively. Molecular size markers in kD are indicated in the margin.

and be transported effectively to the membrane. The control 2B4α and β gene double transfectants express 2B4α chain on the surface as expected (Fig. 6 *D*), although the level of the expression is only 20% that of 2B4.

Absence of Unusual Receptor Dimers on Cells Expressing all Four Ti Chains. To analyze all the Ti/CD3 complexes on Tiαβ-transfected $\gamma\delta^+$ hybridomas, 2B4 ($\alpha\beta^+$), DN7.1 ($\gamma\delta^+$), DN1-β-1.23 (Tiβ transfectant), and DN1-β.α-1.3 (Tiαβ transfectant) cells were surface iodinated, cell lysates prepared, and the labeled proteins immunoprecipitated with either 2C11 or A2B4 (Fig. 7). Both 2C11 and A2B4 precipitated αβ dimers from the control 2B4 lysate. The uncommonly high molecular weight receptor band in this precipitate has been recently shown to be BW5147β chain (T. Saito and R. Germain, unpublished observation). Precipitation of DN7.1 with 2C11 showed the two expected bands, corresponding to the 47-kd δ chain and the 34-kd γ chain. No proteins were specifically immunoprecipitated from the DN7.1 lysate by A2B4. In contrast, when 2B4αβ-transfectant DN1-β.α-1.3 proteins were precipitated with 2C11, a new broad band was observed between the γ and δ chain bands at ~39–43 kd, indicating the presence of αβ dimers (2B4α-2B4β and BW5147α-2B4β) expressed in association with CD3. Confirming this, A2B4 precipitation results in visualiza-

tion of an αβ band migrating in between the positions of the γ and δ chains. No associated γ or δ chains are seen in A2B4 precipitates from these transfectant lysates. These results suggest that αβ dimers exist independently from γδ dimers on the same cell membrane. Similar findings about simultaneous expression of all four Ti chains on the surface of a single cell were obtained using the β gene single-transfectant DN1-β-1.23 (data not shown). However, the lack of the 2B4 α chain precluded direct demonstration of the preferential expression of αβ and γδ dimers seen with the double (αβ) transfectant.

To provide a more detailed picture of the various combinations of Ti chains expressed on the cell membrane of these transfectants, the unreduced-reduced "diagonal" gel technique was used. In Fig. 8 *A*, surface-labeled cell lysates from 2B4 ($\alpha\beta^+$), DN7.1 ($\gamma\delta^+$), and DN1-β.α-1.3 ($\alpha\beta^+\gamma\delta^+$) were immunoprecipitated with a single antibody, 2C11. Consistant with the results from one-dimensional gels, DN7.1 yielded two off-diagonal spots representing the γ and δ chains, whereas, the $\alpha\beta^+$ transfectant also showed a typical αβ dimer in between these γ and δ chain spots that comigrated with the control αβ precipitate from 2B4. In Fig. 8 *B*, the surface proteins of DN1-β.α-1.3 were precipitated using three different antibodies; 2C11 (anti-CD3), A2B4 (anti-2B4α), and an anti-γ heteroantiserum. 2C11 again precipitated all four Ti chains together with CD3. In contrast, precipitation with A2B4 brought down only αβ chains, without any coprecipitating γ or δ. Finally, precipitation with an anti-γ antiserum revealed only γ and δ chains, without any α or β. These results indicate that when all four Ti chains were expressed on the cell surface of single clone, Ti molecules showed preferential chain association, that is, only αβ and/or γδ, but no αγ, αδ, βγ, or βδ combinations were observed.

Discussion

We have investigated the effects of simultaneously expressing functional chains of both TCR types (αβ and γδ) in a single T cell. In one model system involving the human cell PEER, creation of an expressible αβ dimer after gene transfer led to the extinction of surface γδ expression and good αβ expression. This phenotypic conversion did not appear to be the result of gene regulation, but rather intracellular competition for CD3 components necessary for membrane receptor expression. This was consistent with data from a murine model, in which simultaneous surface expression of both γδ and αβ dimers was observed. In neither case were mixed dimers found on the cell surface, indicating a stringent chain specificity to heterodimer formation and/or transport.

Based on initial ontogenic studies demonstrating the sequential expression of Tiγ, β, and then α genes, it was proposed that some immature thymocytes might express γβ receptors with self-MHC reactivity (16, 17). More recent results have indicated that surface γ- and β-expressing thymocytes are distinct, with no evidence for dual-expressing cells (21). However, the existence of a transitional population could not be ruled out by these studies. Our results provide direct evidence against the notion of such a transitional γβ-expressing population, as even when all four subunits (α, β, γ, and δ) are present in a single cell, surface membrane Ti molecules consist solely of αβ and γδ heterodimers.

In this context, PEER is a very unusual cell that, unlike other $\gamma\delta^+$ T cells, pos-

sesses a functional β protein. This is the phenotype one might expect for a cell differentiating from $\gamma\delta^+$ to $\alpha\beta^+$, a process that might occur even though a direct relationship between the receptors in the two differentiation states (via γβ dimers) is ruled out. Indeed, $\gamma\delta^+$ PEER cells showed just such a change in surface receptor expression after transfection of an intact Tiα gene.

Examination of the mechanism by which this switch occurred made it unlikely to represent a normal physiologic mechanism. Thus, the failure to detect CD3-associated γ and δ chains in Tiα-transfected PEER was not to transcriptional down-regulation of γ or δ genes after the expression of complete αβ dimers, as the Tiα-transfectant possessed apparently full-length γ and δ mRNA in amounts similar to the parental PEER line. Rather, it appears that the Tiα-transfectant produces so much αβ dimer in comparison with γδ dimer that the former successfully competes for a limiting amount of CD3 components necessary for surface Ti expression. The apparently disproportional decreases in γ and δ protein levels as compared with mRNA levels may thus be due to degradation, because others have already shown that Ti chains not fully assembled into transportable Ti-CD3 complexes are rapidly destroyed (47).

In contrast to the results obtained with PEER, the presence of complete αβ dimers did not prevent simultaneous γδ expression in the case of Ti αβ-transfected mouse γδ hybridomas. In fact, the surface level of αβ dimer is much lower than γδ dimer in this case, supporting the conclusion above that the extinction of γδ expression in Ti α-transfected PEER is the result of a low level of γδ and a much higher level of αβ dimer. The ability of low levels of αβ dimers in DN1-β.α-1.3 to successfully compete for CD3 components with a larger amount of γδ dimers might reflect a higher affinity of αβ dimers for T3 components, a notion consistent with the change in the biochemical properties of CD3 noted in γδ- vs. αβ-expressing human (though not mouse) T cells (48). Whether these effects of heterodimer isotype on CD3 relate to differences in efficiency of assembly or rate of transport remains to be determined, however.

Our biochemical analysis of both α-transfected PEER and Tiαβ-transfected mouse γδ hybridoma cells indicates that Tiαβ-CD3 and Tiγδ-CD3 complexes are expressed independently on the cell surface and that receptors containing mixed dimers such as αγ, αδ, βγ, or βδ are not transported to the cell membrane under these conditions. For example, βγ and βδ dimers were not observed using anti-β chain (βF1) and anti-δ chain (anti-TCRδ1) antibodies and labeled PEER or transfected PEER cells, and αγ, αδ, or βγ dimers were not observed using anti-α chain (A2B4) and anti-γ chain antibodies in studies with αβ-transfected mouse γδ cells. It is unlikely that our failure to observe mixed dimers is the result of the particular genes used for these experiments, as we have analyzed two independent systems and several possible combinations of chains. Limited analysis of biosynthetically labeled material additionally suggests that such mixed dimers are also not present in significant amounts within the cell, implying that either these dimers do not form at all, or, perhaps due to problems with full assembly with CD3 components, are rapidly degraded.

One caveat to this conclusion that receptor chain mixing does not occur comes from studies we have carried out on the assembly and transport of class II MHC-

encoded molecules (Ia molecules). Using an L cell transfection model, interisotypic pairing of Aβ with Eα, followed by transport of the assembled dimer to the membrane, has been observed (49, 50). Despite this, such dimers cannot be demonstrated in or on normal Ia-expressing cells of the appropriate H-2 haplotype (51). Recent results with transfectants containing Eα together with both Aβ and Eβ indicate that there is a definite isotype preference in assembly/transport of class II molecules (A. Sant and R. Germain, manuscript in preparation). Thus, the absence of the mixed Ia molecules in normal cells is probably due to consumption of available chains by preferential pairing with isotypically matched partners, even though the isotype-mixed dimer can be formed and transported when the proper chains are present in a cell by themselves. Similar effects may preclude our detection of mixed receptor dimers that might be seen if the component chains were present without the usual partner in the cell. We are currently transfecting a Ti^- target cell with the appropriate gene combinations to address this question directly.

Based on sequence homology to Igs, all four TCR chain types have been predicted to have a similar structure consisting of two Ig-like domains. Given this presumed close correspondence in structure, it is somewhat surprising that there is such a strict segregation of partner chains in the expression of TCRs by a single cell expressing functional products of all four receptor loci. What part of each receptor chain mediates the preferential formation or transport of αβ and γδ complexes has yet to be determined. Although preservation of pairing among the V region-diversified populations of receptor chains with the same C region suggests a C region rather than a V region-controlled event, we have previously found that the allelically polymorphic (nonconserved) portions of class II MHC molecules play critical roles in determining the efficiency of dimer formation and transport (50, 52). Furthermore, preliminary results indicate that the level of expression of certain pairs of α and β chains seems to depend predominantly on the V region sequences, indicating that these variable segments can have a significant role in either the assembly or transport efficiency of TCR (T. Saito and R. Germain, manuscript in preparation). Because a separate set of V regions is used by each receptor chain (with perhaps rare exceptions for certain α and δ chains), the pattern we have observed could be in large measure controlled by these regions. Analysis of the expression patterns of recombinant chains in transfected cells will provide a means for exploring these issues.

Overall, the results presented here suggest that the chains of the two known types of Ti dimers, αβ and γδ, have evolved to generate two distinct sets of receptor structures. Their expression appears to be mutually exclusive and mixing of chains usually found in the prototypic receptor dimers is inefficient or nonexistent. Because the effector functions (cytolytic activity, lymphokine production) of T cells bearing either of the two receptor types are similar if not identical, these results suggest that these two receptor types most likely function to provide their respective cells with diverse and probably minimally overlapping sets of specificities, enabling a larger antigenic universe to elicit immune effector activity than if only one or the other dimer-mediated recognition for all T cells.

Summary

Surface expression of TCR dimers by cells synthesizing three or four distinct types of receptor chains was analyzed. Cells containing intact γ, α, and β chains had only

γδ dimers on the cell surface. In human PEER cells, addition of a functional α chain led to the loss of γδ dimer expression and expression of only αβ dimers. This result was not due to transcriptional down-regulation of the γ or δ loci. In murine cells expressing all four chains, both γδ and αβ dimers could be demonstrated on a single cell. No other chain combinations (αγ, αδ, βγ, or βδ) were detected. Thus, there is stringent control of assembly and/or transport of TCR heterodimers, such that functional receptors consist only of αβ and γδ pairs, and no additional repertoire diversity is generated by cross pairing.

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