## *Brief Definitive Report*

# EXPRESSION OF TWO DISTINCT T CELL RECEPTOR α/β HETERODIMERS BY AN ANTIGEN-SPECIFIC T CELL CLONE

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MHC-restricted antigen-specific recognition by T lymphocytes is mediated by a clonally distributed disulfide-linked receptor heterodimer (TCR) (1). Analogous to Ig H and L chains, both the α and β proteins of this receptor are encoded by distinct germline gene segments (variable [$V_\alpha$, $V_\beta$], diversity [$D_\beta$], joining [$J_\alpha$, $J_\beta$]) that undergo in-frame rearrangements during differentiation to form complete functional genes (2).

The evidence available suggests that, like Ig expression on B lymphocytes (3), the principle of allelic exclusion also applies to TCR expression. Thus, molecular analysis of TCR-α and -β genes in a limited number of clonal T cell populations has revealed that in most cases there is only a single productive $VJ_\alpha$ or $VDJ_\beta$ rearrangement (2, 4).

Here we present an antigen-specific, MHC-restricted T cell clone with two productive rearrangements of the same $V_\beta$ gene segment as evidenced by the cell surface expression of two distinct TCR-α/β heterodimers. The potential implications of this finding with regard to the mechanism of allelic exclusion and the generation of diversity in the T cell repertoire are discussed.

## Materials and Methods

*T Cell Clones.* The generation and propagation of pigeon cytochrome *c*-specific T cell clones have previously been described (5). Clones 5C.C6 (5) and 1.F8 (6) have been reported. Clone C.D2 was derived by limiting dilution from an antigen-specific T cell line established after immunization of B10.S(9R)→B10.A radiation-induced bone marrow chimeric mice with 100 μg of pigeon cytochrome *c*, and was propagated by serial in vitro stimulation as described (5) with pigeon cytochrome *c* and B10.A APC. The chimeric mice were constructed by injection of anti-Thy-1 + complement-treated B10.S(9R) ($H\text{-}2^{t4}$) bone marrow cells into lethally irradiated (925 rad)B10.A ($H\text{-}2^{a}$) host mice as previously described (7). Subclones were derived from C.D2 by limiting dilution cloning at 0.5 cells/well. Proliferation assays were performed as described previously (5).

*Southern Blot Analysis.* Southern analyses were performed as described previously (8). The $V_\beta1$ and $V_\alpha11.1$ DNA probes have been described (8).

*Antisera and Antibodies.* The rabbit anti-$C_\beta1$ and anti-$C_\beta2$ antisera were raised by immunization with KLH-coupled peptides corresponding to the COOH-terminal sequences of the mouse $C_\beta1$ (CAMVKRKNS) and $C_\beta2$ (CAMVKKKNS) chains, respectively.

*Radiolabeling, Immunoprecipitation, and Gel Electrophoresis.* Cells were surface labeled with

$^{125}$I by the lactoperoxidase method, and immunoprecipitations and gel electrophoreses were performed as described previously (9, 10).

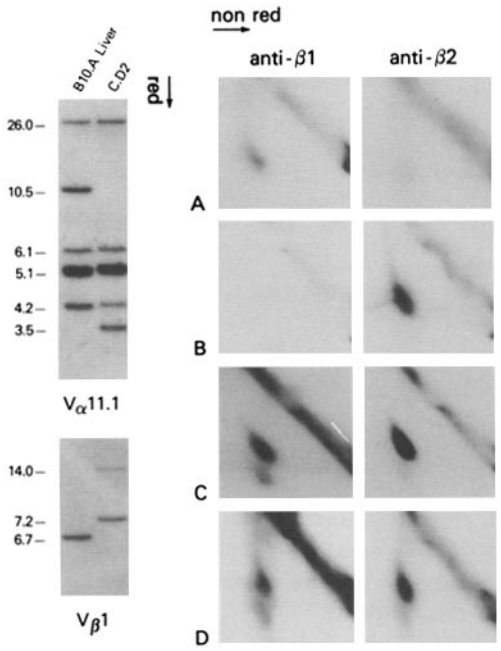
## Results

TCR expression in the murine response to the antigen pigeon cytochrome *c* has been extensively characterized (5, 6, 8, 11, 12). Among many cytochrome *c*-specific T cell clones examined, a limited number of germline $V_\alpha$ and $V_\beta$ genes are expressed. Data from Southern hybridization analyses with $V_\beta$ gene segment probes indicate that 10–20% of cytochrome *c*-specific clones have undergone two complete $VDJ_\beta$ rearrangements (5, 8; Matis, L., unpublished observations). This estimate is accurate because it is based both on the direct demonstration of two $V_\beta$ rearrangements in individual T cell clones as well as on the frequency with which the germline restriction fragment hybridizing to the $V_\beta 3$ gene segment probe (5) has been deleted. Mapping studies have shown that the $V_\beta 3$ gene segment is one of the most downstream (3′) $V_\beta$ elements in the $V_\beta$ gene cluster located 5′ of the TCR $C_\beta$ genes (13), and therefore, most clones that have rearranged two $V_\beta$ gene segments will have deleted $V_\beta 3$.

One clone, C.D2, was of particular interest because it displayed two distinct rearrangements of the $V_\beta 1$ gene segment, which is frequently expressed in I-E-restricted pigeon cytochrome *c*-specific T cell clones (6, 8, 12). The Southern analysis in Fig. 1 shows two rearranged bands of 14.0 and 7.2 kb after hybridization of the $V_\beta 1$ probe to Hind III-digested C.D2 DNA. Subclones of C.D2 all displayed the same two $V_\beta 1$ rearranged bands as the parent clone (data not shown).

C.D2 also rearranges the $V_\alpha 11$ gene segment common to most pigeon cytochrome *c*-specific T cells (Fig. 1) (5, 8, 11, 12). The 3.5-kb rearranged Hind III band hybridizing to the $V_\alpha 11.1$ probe corresponds exactly to a previously reported $V_\alpha 11$-$J_\alpha C7$ rearrangement in cytochrome *c*-specific clones from which TCR-α genes have been sequenced (11), suggesting that C.D2 may also express the $J_\alpha C7$ element.

Based upon previous examination of $V_\beta 1$ gene rearrangements in cytochrome *c*-specific T cell clones (8), the 14.0-kb and 7.2-kb $V_\beta 1$-hybridizing bands (Fig. 1) should represent $V_\beta 1$ rearrangements to the $J_\beta 1$-$C_\beta 1$ and $J_\beta 2$-$C_\beta 2$ clusters, respectively (13). This prediction was confirmed in C.D2 using genomic probes spanning each of the $J_\beta$ clusters (data not shown). Therefore, the possible expression by C.D2 of two TCR-α/β heterodimers with different TCR β chains was examined with two antipeptide antisera directed against the COOH-terminal portions of either the $C_\beta 1$- or $C_\beta 2$-encoded TCR-β proteins, respectively (Fig. 2). Immunoprecipitations were performed on lysates of $^{125}$I-labeled T cell clones and samples were analyzed on diagonal nonreducing-reducing two-dimensional gels. The specificity of each antiserum was first established with T cell clones with single productive TCR-β gene rearrangements. Only the anti-$C_\beta 1$ antiserum precipitated a TCR heterodimer from clone 5C.C6 ($V_\beta 3$-$J_\beta 1.2$-$C_\beta 1$) (Fig. 2 *A*) (5, 11) and conversely, only the anti-$C_\beta 2$ antiserum precipitated TCR-α/β from clone 1.F8 ($V_\beta 1$-$J_\beta 2.1$-$C_\beta 2$) (6) (Fig. 2 *B*). In contrast, distinct TCR heterodimers were precipitated by both anti-$C_\beta 1$ and anti-$C_\beta 2$ antisera in the C.D2 clone and a subclone C.D2.C3 (Fig. 2 *C* and *D*). The two heterodimers were distinguished in appearance by the different relative intensities of the two spots (lower vs. upper) below the diagonal. Thus, the intensity of the lower spot relative to the upper spot was reproducibly greater for the anti-$C_\beta 1$ than for




FIGURES 1 and 2. (*Fig. 1, left*) Southern blot analysis of TCR $V_\alpha 11$ and $V_\beta 1$ gene rearrangements in T cell clone C.D2. DNA was digested with Hind III according to the manufacturer's specifications and then loaded at 10 μg/lane onto 0.8% agarose gels as in Materials and Methods. The $V_\alpha 11.1$ and $V_\beta 1$ probes have been described previously (5, 8). The unrearranged germline bands are shown in the lanes containing control B10.A liver DNA. The sizes of the bands were determined with Hind III-digested bacteriophage λ DNA. After hybridization to the $V_\beta 1$ probe, the filter was stripped and then successively hybridized to genomic $J_\beta$ probes spanning the $J_\beta 1$ and $J_\beta 2$ gene clusters, respectively (5). The $J_\beta 1$ probe hybridized to the 14.0-kb band and the $J_\beta 2$ probe hybridized to the 7.2-kb band (data not shown). (*Fig. 2, right*) Two-dimensional nonreduced-reduced SDS-gel analysis of TCR expression in antigen-specific T cell clones. Immunoprecipitation of surface-labeled cell lysates with the anti-$C_\beta 1$ (*left*) and anti-$C_\beta 2$ (*right*) antisera were performed as described (9, 10), and then run nonreduced in the first (*horizontal*) dimension and reduced in the second (*vertical*) dimension. Precoated protein A-agarose (PAA) beads (Bethesda Research Laboratories, Bethesda, MD) were prepared by incubating 20–40 μl of antiserum with 40–80 μl of beads for 1 h at room temperature. For specific precipitations, 20–40 μl of antiserum-precoated PAA beads were added to 100–200 μl of NP-40 cell lysate for 60 min. (*A*) $C_\beta 1$-expressing clone 5C.C6 (9), (*B*) $C_\beta 2$-expressing clone 1.F8 (10), (*C*) C.D2, (*D*) subclone C.D2.C3.

the anti-$C_\beta 2$ immunoprecipitate (Fig. 2, *C* and *D*, *left vs. right*), providing evidence that the two antisera were precipitating distinct heterodimers. These differences were quantitatively confirmed in several experiments by densitometry of the autoradiographs (data not shown). Finally, immunoprecipitation by each antiserum was inhibited only by the corresponding immunizing $C_\beta$ peptide (data not shown). These data cannot determine whether each β chain pairs with the same $V_\alpha 11.1$-encoded α chain or if different α chains are used. NEPHGE-SDS-PAGE two-dimensional electrophoresis of anti-CD3 immunoprecipitated samples after neuraminidase treatment failed to resolve the individual β chains (data not shown). This was not surprising because both chains use the same $V_\beta$ gene element and there are no charge differences between $C_\beta 1$ and $C_\beta 2$.

The proliferation data shown in Fig. 3 demonstrate a unique pattern of MHC molecule recognition by both C.D2 and the C.D2.C3 subclone. C.D2 responds to both the pigeon cytochrome *c* peptides 81–104 and 81–103 presented by I-$E^k$-expressing B10.A APC, preferentially to the 81–104 peptide when presented by B10.S (9R) (I-$E^s$) APC, and is alloreactive to the I-$E^b$ Ia molecule expressed on B10.A (5R) APC. This particular pattern of recognition of the three homologous I-E alleles has not been reported previously in any cytochrome *c*–specific T cell clones (5, 6, 8, 11, 12).

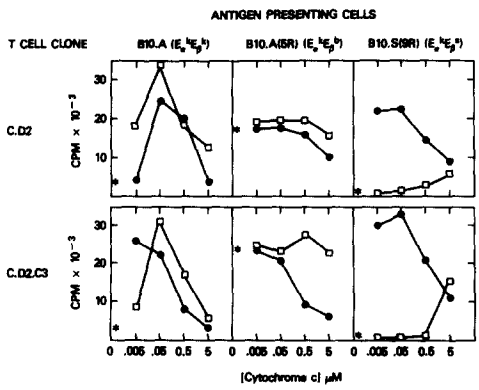



FIGURE 3. Specificity analysis of clone C.D2 and subclone C.D2.C3. Proliferation (cpm) was measured in response to various concentrations (μM) of pigeon cytochrome *c* fragments 81–104 (●) and 81–103 (□). Fragment 81–103 elicits proliferation similar to that of tobacco hornworm moth cytochrome *c* 81–103 (5). Both cytochrome *c* fragments were generously provided by R. H. Schwartz, NIH. Proliferation was measured in the presence of irradiated (3,300 rad) spleen APC from B10.A-($E_\alpha^k E_\beta^k$), B10.A(5R)($E_\alpha^k E_\beta^b$), and B10.S(9R)($E_\alpha^k E_\beta^s$) mice expressing three homologous I-E subregion-encoded I-Eβ alleles. The I-E restriction of the proliferative response was determined by demonstrating no proliferation in the presence of APCs from I-E$^-$ B10.A(4R)($A_\alpha^k A_\beta^k$), B10($A_\alpha^b A_\beta^b$), and B10.S($A_\alpha^s A_\beta^s$) mice, respectively. cpm represent the mean of duplicate cultures. The proliferative responses measured in the presence of APC alone (no antigen) are shown (*).

## Discussion

We have shown directly the expression of two distinct TCR heterodimers on a single antigen-specific T cell clone. Thus, TCR-α/β receptors are not invariably allelically excluded. Recent data from transgenic mice suggest a possible mechanism for allelic exclusion of TCR-β proteins in the majority of T lymphocytes (14). In these animals, the expression of a functional TCR-β transgene inhibited the progression of endogenous TCR-β genes to complete $VDJ_\beta$ rearrangements, analogous to results reported in mice bearing Ig transgenes (15). However, this mechanism cannot explain allelic exclusion in the significant percentage (10–20%) of T cells that have rearranged two $V_\beta$ gene segments. In T cells with two $VDJ_\beta$ rearrangements, allelic exclusion would still occur in most cells by a stochastic process because two-thirds of $VDJ_\beta$ rearrangements should result in an improper translational reading frame for the $J_\beta$ segment (2). However, our data indicate that in some cases two in-frame $VDJ_\beta$ rearrangements, perhaps occurring concurrently and thereby escaping the inhibitory mechanism suggested by the transgene experiment, can result in the expression of two TCR-β proteins and consequently two receptor heterodimers. The frequency with which this occurs is unknown, but may not be exceedingly rare. For example, two in-frame TCR-β cDNAs have been recently described in an antigen-specific human T cell clone (16). Moreover, Southern blot analyses with various $V_\alpha$ gene segment probes have shown germline band deletions in most T cell clones, (5, 6, 8; Matis, L., unpublished data), suggesting that the majority of antigen-specific murine T cells have two $VJ_\alpha$ rearrangements. Thus, it is possible that some T cell clones may also express two TCR-α proteins.

It has not yet been determined whether each of the heterodimers expressed on C.D2 contributes to the observed overall clonal specificity. However, this seems possible because both express a $V_\beta 1$ element frequently associated with I-E-restricted cytochrome *c*-specific T cells (6, 8, 12). Moreover, the C.D2 clone was derived from a T cell line established after immunization of B10.S(9R)→B10.A ($E^s \rightarrow E^k$) allogeneic radiation-induced bone marrow chimeric mice. In these animals, the T cells mature in the presence of host B10.A thymic epithelium bearing I-$E^k$ Ia molecules, while

the peripheral APC are derived from I-E$^s$-expressing B10.S(9R) donor bone marrow cells. Thus, all the cytochrome *c*-specific T cells derived from these mice were intentionally selected for MHC-restricted recognition of both the I-E$^k$ and I-E$^s$ alleles. Previous analyses have shown that multiple independent cytochrome *c*-specific B10.A T cell clones restricted by both the I-E$^k$ and I-E$^s$ Ia molecules express identical $VJ_\alpha$ and $VDJ_\beta$ gene elements different from those expressed by C.D2 (5, 11), implying a very limited germline TCR repertoire for generating this MHC specificity pattern. In addition, the C.D2 clone was alloreactive to the I-E$^b$ Ia molecule (Fig. 3), thus displaying a unique response pattern not previously described in any cytochrome *c*-specific T cell clones (5, 6, 8, 11, 12). Therefore, the isolation of a T cell clone with two functional receptors could have resulted from the selection for a rare specificity. Gene cloning and transfection experiments are in progress to address this possibility.

## Summary

A cytochrome *c*-specific, MHC-restricted T cell clone with two complete rearrangements of the same $V_\beta1$ gene element was shown to express two different TCR-$\alpha/\beta$ heterodimers. Antipeptide antisera specific for TCR $C_\beta1$ and $C_\beta2$ peptides each immunoprecipitated distinct disulfide-linked cell surface heterodimers. The clone was derived from immunized allogeneic chimeric mice, and displayed multiple Ia specificities, including the ability to recognize antigen in association with both I-E$^k$ and I-E$^s$ Ia molecules, as well as alloreactivity to the I-E$^b$ molecule. It will be important to determine whether each receptor contributes independently to the overall specificity of the clone.

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