Brief Definitive Report

HUMAN TCR-γ^+/δ^+, CD8$^+$ T LYMPHOCYTES RECOGNIZE TETANUS TOXOID IN AN MHC-RESTRICTED FASHION

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Although the TCR-γ/δ complex contains relatively few germline V gene segments (1, 2), the TCR-γ diversity is increased by N nucleotide incorporation at the V-J junction (1), and TCR-δ junctional diversity is high, with the use of at least two tandemly arranged Dδ elements and extensive N nucleotide incorporation (2). Because adult TCR-γ/δ shows the most extensive dichotomy with respect to the spectrum of V region diversity compared with junctional diversity, Davis and Bjorkman (3) have suggested that the ligand for γ/δ heterodimer will consist of a peptide fragment of antigen in association with an MHC-like molecule. γ^+/δ^+ T cells with allogenic reactivity have been established (4, 5), indicating that the TCR-γ/δ is capable of self-non-self MHC discrimination. However, the contribution of γ^+/δ^+ T cells to an antigen-specific immune response has never been demonstrated. We have established human γ^+/δ^+, CD8$^+$ T cell clones that specifically responded to tetanus toxoid (TT) and autologous APC with proliferation and IFN-γ production, and the response was restricted by HLA-DR4-related element. These results represent the first experimental demonstration that γ^+/δ^+ T cells contribute to the immune response to a nominal antigen.

Materials and Methods

Cell Culture. Purified TT (lot TAS 239) was obtained from Connaught Laboratories Ltd. (Willowdale, Canada). Purity was confirmed by HPLC analyses. The TT-specific T cell line DF was obtained by stimulation with TT (10 μg/ml) and irradiated (4,000 rad) autologous APC, and maintained in RPMI 1640 medium with 10% heat-inactivated FCS (Flow Laboratories, Inc., McLean, VA) and 20 U/ml of rIL-2 (Amgen Biologicals, Thousand Oaks, CA). Cells were restimulated with antigen and autologous APC every 3 wk.

Antigen-induced Proliferation and IFN-γ Production. T cells were seeded in flat-bottomed microtiter plates (Linbro; Flow Laboratories) at 2×10^4 cells/200 μl in the presence of irradiated APC after plastic adherence of 4×10^5 cells/200 μl. Cultures were stimulated for 36 h with 10 μg/ml of TT or TNP-BSA. Production of IFN-γ was measured by RIA, using standard solutions of IFN-γ as controls. For proliferation, cultures were pulsed for 12 h with 1 μCi/well [^{3}H]TdR (Amersham Corp., Amersham, UK).

Southern and Northern Blotting. DNA samples from DF, from EBV-transformed autologous

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B cell clone, B6, and from γ^+/δ^+ cloned cells were cleaved with restriction enzymes and subjected to electrophoresis in an agarose (Sigma Chemical Co., St. Louis, MO) gel. Cytoplasmic RNA from DF, from γ^+/δ^+ and α^+/β^+ clones, and from γ^+/δ^+ PEER cell line (6) was denatured, electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled TCR probes (6): the cDNA TCR-α (Tα2), TCR-β (Cβ), TCR-δ (0-240), and Tγ-1. The PMI19 genomic TCR-δ probe corresponds to a region 5–11 kb upstream of Cδ (7).

Results and Discussion

TT-specific T Cell Line. The TT-specific T cell line DF was derived from lymphocytes of a hyperimmunized donor by in vitro stimulation with TT (10 μg/ml) and autologous APC. Two-color staining with anti-TCR δ1 (6) and anti-CD8 mAbs revealed a percentage of γ^+/δ^+, CD8$^+$ T cells that fluctuated between 30 and 50% of the total T cell number during the 2-yr culture period. No γ^+/δ^+ T cells were detected in three other TT-specific T cell lines obtained from unrelated donors (data not shown). Therefore the presence of γ^+/δ^+ T cells in the TT-specific cell line may be related to the immunization history of donor D.F., who had received seven TT inoculations. Lymphocytes of donor D.F. showed ~1% of T cells reacting with anti-TCR δ1 antibody, and an autologous T cell line specific for TNP-hapten did not contain γ^+/δ^+ T cells. Immunoprecipitation of ^{125}I-labeled γ^+/δ^+ DF cells with the anti-TCRδ1 antibody revealed a non-disulfide-linked 55-kD TCR-γ and a 40-kD TCR-δ chain after SDS-PAGE (not shown). Cloning of DF cells by limiting dilution yielded several α^+/β^+ T cell clones and three clones that were homogeneously positive for TCR-γ/δ, CD8 and CD2 surface antigens (not shown). One of the γ^+/δ^+ clones was analyzed for rearrangement and expression of the TCR-δ gene (Fig. 1). The Hind III digest of DNA revealed a germline configuration of the Cβ2 complex (7.5 kb) in γ^+/δ^+ clone 4, whereas the same blot hybridized to the Jδ genomic probe PMI19 (7) showed one rearranged band of 12 kb and two germline bands of 6.2 kb and 3.3 kb due to a Hind III site within the PMI 19 probe. An additional Jδ rearrangement of 4.7 kb was detected in DF cells indicating presence of another γ^+/δ^+ T cell population (Fig. 1 *A*). TCR-δ transcripts of 2.2 and 1.5 kb, as well as a 1.6-kb TCR-γ message, were present in DF cells, in clone 4, and PEER cells (Fig. 1 *B*). The 1.5-kb TCR-α transcript was detected in clones 7 and 9 as well as the DF population. Analysis of TCR-β expression revealed 1.3- and 1.0-kb transcripts in DF cells

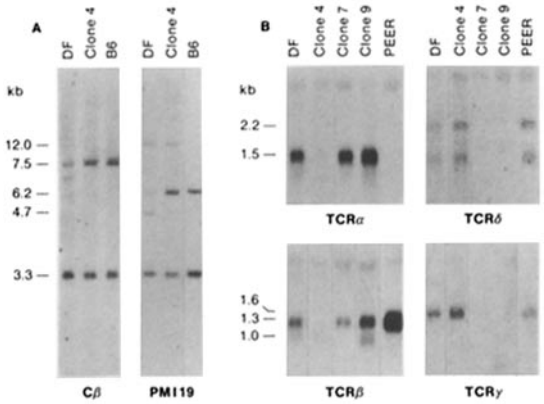

FIGURE 1. Southern (*A*) and Nothern (*B*) blot analysis of the TCR complex in γ^+/δ^+ clone 4. (*A*) Southern blotting of Hind III-digested DNA (10 μg) with a probe for constant regions of TCR-β (Cβ), and Jδ probe (PMI19). (*B*) Northern blotting of 10 μg of RNA/lane with probes for TCR-α (Tα2), TCR-δ (0-240), TCR-β, and TCR-γ (Tγ-1). Clones 7 and 9 are α^+/β^+

and in clone 9, whereas clone 7 and PEER cells showed a 1.3-kb message. A trace of the 1.0-kb message was detected in the γ^+/δ^+ cells.

Antigenic Specificity and MHC Restriction of the γ^+/δ^+ T Cell Clones. Proliferation and IFN-γ production by DF cells and by the α^+/β^+ and γ^+/δ^+, $CD8^+$ T cell clones analyzed was optimal using 10 μg/ml of TT (not shown). In α^+/β^+ T cells, stimulated with TT and autologous APC, [^{3}H]TdR incorporation and IFN-γ production was ~10-fold and ~5-fold higher, respectively, than in the γ^+/δ^+ T cell clones (Fig. 2 *A*). The autologous TNP-specific T cell line used as a specificity control did not respond to stimulation with TT, and none of the TT-specific T cell clones responded to stimulation with TNP-BSA (10 μg/ml). No proliferation or INF-γ production was detected when TT-specific T cell clones were stimulated with either TT or autologous APC only. Addition of IL-2 (10 U/ml) for 36 h to cultures of γ^+/δ^+ cells (2×10^4 cells/200 μl) containing TT (10 μg/ml) and autologous APC increased both proliferation and IFN-γ production (~3-fold) in three γ^+/δ^+ clones (Fig. 2 *A*). To determine whether the antigenic specificity of γ^+/δ^+, $CD8^+$ T cells was MHC restricted, DF T cells, α^+/β^+ clones 7 and 9 and γ^+/δ^+ clone 4 were stimulated with TT and allogeneic APC and assayed for proliferation and IFN-γ production. The D.F. donor

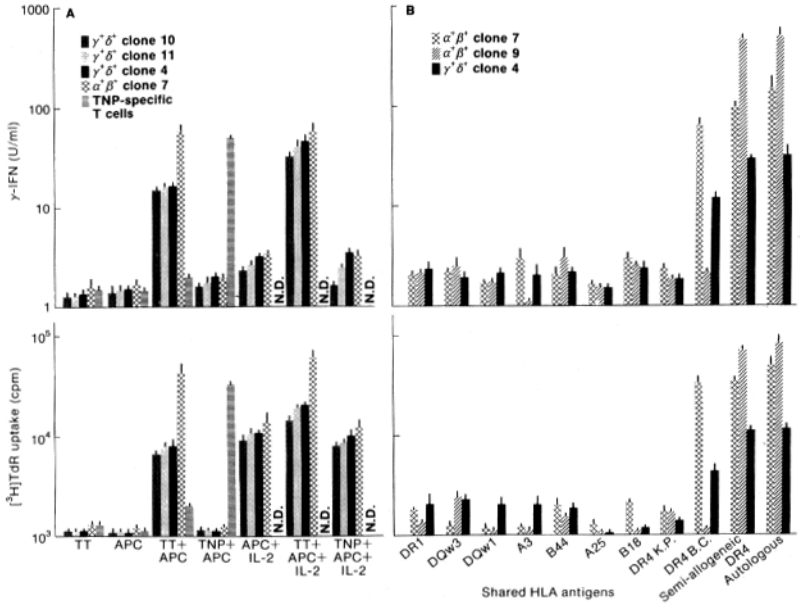

FIGURE 2. Antigen specificity (*A*) and HLA restriction (*B*) of α^+/β^+ and γ^+/δ^+ T cell clones. (*A*) 2×10^4 cells/well of α^+/β^+ T cell clone 7, γ^+/δ^+ T cell clones 4, 10, and 11, and a TNP-specific autologous T cell line were stimulated with TT, TNP-BSA, and autologous APC with or without IL-2 (10 U/ml) for 36 h. Proliferation of cells was monitored by [^{3}H]TdR uptake (*bottom panel*), and IFN-γ was measured by RIA (*top panel*). (*B*) HLA restriction was studied by stimulation of the T cells with TT and APC from 14 $DR4^-$ donors sharing one HLA-determinant with the autologous donor (two donors per case), from 5 $DR4^+$ donors, and from a $DR4^+$ sister of the autologous donor (semi-allogeneic DR4). The response was monitored by [^{3}H]TdR uptake (background ~1,500 cpm; cultures with T cells and APC, or APC and TT, or T cells and TT), and IFN-γ production. $DR4^+$ APC from donor B.C. were able to present TT to T cells, and APC from $DR4^+$ donor K.P. stimulated no response.

HLA phenotype was: A3, A25, B18, B44, DR1, DR4, DQw1, and DQw3. Semi-allogeneic APC derived from a sister of the autologous donor with the following phenotype: A3, A26, B18, B49, DR1, DR4, DQw1, DQw3, presented TT to all α^+/β^+ and γ^+/δ^+ T cells, and the response was identical to that obtained with autologous APC (Fig. 2 *B*). When allogeneic APC from 19 donors, each sharing one class I or class II MHC antigen with D.F. were used, antigen presentation was detected only when T cells were stimulated with TT in the presence of DR4-matched APC. APC from all 14 $DR4^-$ donors were unable to stimulate our T cells. Of the five $DR4^+$ donors tested, donor B.C. gave the best response (60–70% of autologous response), three others stimulated T cells up to 20–40% of the autologous response (not shown), and one donor (K.P.) stimulated no response. Although APC obtained from donor B.C. stimulated DF cells, α^+/β^+ clone 7 and γ^+/δ^+ clone 4, they were unable to present antigen to α^+/β^+ clone 9. The association between the expression of DR4 on unrelated APC and the ability to present TT to the γ^+/δ^+ T cells in highly significant (χ^2 14.19, $n = 19$, $p < 0.0004$). Similar results were obtained with γ^+/δ^+ clones 10 and 11 stimulated with APC from autologous, semi-allogeneic and B.C. $DR4^+$ donors, and with APC from a $DR4^-$ donor (not shown). These results indicate that the TCR-γ/δ receptor can recognize non-MHC-encoded foreign antigens in a self-MHC-restricted fashion. There are at least two possible explanations for the variability among the APC derived from different $DR4^+$ donors in presenting TT to the α^+/β^+ and the γ^+/δ^+ T cell clones. The restriction element for the clones might not be DR4 itself, but a gene closely associated and in linkage disequilibrium with it at the population level. Alternatively, these differences might reflect the well known molecular and structural heterogeneity within DR β chains isolated from a single serologically defined DR4 phenotype that presumably contribute to the dominant polymorphisms recognized by T cells in the MLR (8). The differential response of our T cell clones to TT presented on different $DR4^+$ donors might reflect the discriminatory ability of the TCR-γ/δ. The response of all T cell clones was inhibited by >70% by an anti-HLA DR (DI.B6) antibody directed against a monomorphic determinant, but not by an anti-HLA-DQ (G2.53) mAb (not shown). This inhibition, although consistent with class II restriction, is not conclusive because mAbs against monomorphic determinants of HLA-DR antigens block the human MLR and an IL-2-dependent T cell line by direct interaction with these T cells. Although CD8 antigen expression is especially associated with class I MHC recognition, $CD8^+$ T cell clones specific for class II MHC antigens have been described (9). Since anti-CD8 antibody did not affect the activity of class II MHC-specific CTL clones (9) and did not inhibit antigen-induced proliferation (10) these clones apparently interact with the target cells through the antigen-specific receptor and do not use the CD8 molecule as an associative recognition element. Similarly anti-CD4 and anti-CD8 antibodies did not inhibit proliferation and IFN-γ production, respectively, in our α^+/β^+, $CD4^+$ and γ^+/δ^+, $CD8^+$ clones (not shown).

Although the antigenic response of γ^+/δ^+ T cell remains largely unknown, our results indicate that the TCR-γ/δ heterodimer is a functionally distinct TCR, capable of more than the broad self–non-self discrimination. It is therefore possible that the physiological significance of γ^+/δ^+ T cells in the immune response against a foreign antigen resides in their regulatory ability.

Summary

We have analyzed the ability of human γ^+/δ^+ T cells to recognize a nominal antigen in association with MHC molecules. A TT-specific T cell line with ~40% γ^+/δ^+ T cells was established from a hyperimmunized donor, D.F., by stimulation with antigen and autologous APC. Three DF-derived γ^+/δ^+ clones were $CD8^+$ as determined by immunofluorescence staining, and by Southern and Northern blotting with probes detecting δ chain rearrangement and δ and γ chain transcripts, respectively. The γ^+/δ^+ clones responded to stimulation with TT, but not TNP-BSA, and autologous APC by proliferation and IFN-γ production. No proliferation or IFN-γ production was detected when TT-specific T cell clones were stimulated with either TT or autologous APC only. The response to TT was enhanced by addition of exogenous IL-2. The use of allogeneic APC from 19 donors sharing one HLA-determinant with the autologous donor D.F., showed that the γ^+/δ^+ T cells responded to TT with HLA-DR4-related restriction as measured by proliferation and IFN-γ production. These results demonstrate that γ/δ receptors can recognize non-MHC-encoded foreign antigen in a self-MHC-restricted fashion.

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