Activation of Murine Epidermal Vγ5/Vδ1-TCR$^+$ T Cell Lines by Glu-Tyr Polypeptides

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The physiologic role of γδ-T-cell-receptor-bearing cells and the T cell receptor ligands that they recognize is still poorly understood. Previous studies have suggested that one possible antigen for γδ-TCR$^+$ cells is the random copolymer poly-glutamic acid-tyrosine (poly-Glu-Tyr), because poly-Glu-Tyr-reactive γδ-TCR$^+$ hybridoma cells were produced from poly-Glu-Tyr-immunized mice. We have found, however, that clonal Vγ5/Vδ1-TCR$^+$ epidermal T cell lines from nonimmune mice also respond to poly-Glu-Tyr by producing cytokines. Other amino acid homopolymers, copolymers, and tripolymers were not stimulatory for the Vγ5/Vδ1-TCR$^+$ epidermal T cells, except for poly-glutamic acid-alanine-tyrosine (poly-Glu-Ala-Tyr). Of the poly-Glu-Tyr and poly-Glu-Ala-Tyr polymers, only those that contained Glu and Tyr in an equimolar ratio were stimulatory. The cytokine interleukin-2 was strictly required for the responses to poly-Glu-Ala-Tyr, whereas the responses to poly-Glu-Tyr were merely enhanced with interleukin-2. The response to poly-Glu-Tyr was also enhanced by crosslinking CD28 molecules with plate-bound anti-CD28 crosslinking antibody. This finding suggests that the poly-Glu-Tyr response has a partial dependence on CD28-mediated costimulation, a characteristic of TCR-dependent responses. Consistent with this observation, Vγ5/Vδ1-TCR-loss variants of the epidermal T cell line could no longer respond to poly-Glu-Tyr. The unpredicted responses of epidermal γδ-TCR$^+$ T cells to poly-Glu-Tyr and poly-Glu-Ala-Tyr demonstrate that the functions of these cells potentially can be triggered by peptidic ligands, probably through a TCR-mediated process. ***Key words: amino acids/ IL-2/ mouse/ skin/ T lymphocytes. J Invest Dermatol 116:880–885, 2001***

Relative to the αβ-T-cell-receptor (TCR)-bearing population, T lymphocytes expressing the γδ-TCR represent a minor population of T cells in humans and rodents. Moreover, in the murine epidermis, the major population of tissue-specific T lymphocytes expresses an invariant TCR containing Vγ5[1] and Vδ1 chains (Asarnow *et al*, 1988; Haas and Tonegawa, 1992). In contrast to the peptide and major histocompatibility complex (MHC) molecule recognition by αβ-TCRs, the interaction of γδ-TCRs with their ligands may be more similar to that of antibodies (Rock *et al*, 1994), such that small epitopes of soluble or cell-bound ligands are bound directly by the TCR without apparent MHC compatibility requirements (Abdel-Motal *et al*, 1996). The nature of antigens recognized by γδ-TCR$^+$ cells, including the Vγ5/Vδ1-TCR$^+$ of epidermal T cells (ETC), is not well defined. Based on experiments with partially defined bacterial extracts, small carbohydrates may be antigens for some γδ-TCR$^+$ cells (Pfeffer *et al*, 1990). Natural mycobacterial and synthetic nonpeptide ligands, such as monoethyl phosphate, are also recognized by these cells (Pfeffer *et al*, 1990; Tanaka *et al*, 1994). In addition, some proteins were found to activate γδ-TCR$^+$ cells, including tetanus toxoid (Kozbor *et al*, 1989) and staphylococcal enterotoxin A (Rust *et al*, 1990).

Studies of immune responses to copolymers of L-glutamic acid and L-tyrosine (poly-Glu-Tyr) and tripolymers of L-glutamic acid, L-alanine, and L-tyrosine (poly-Glu-Ala-Tyr) began almost 30 y ago (Bluestein *et al*, 1971a–c). Although early investigations evaluated B cell responses to these polymers, the focus later switched to T cells. Here, class II MHC-restricted suppressor cells were thought to be stimulated by poly-Glu-Tyr, a random copolymer of glutamic acid and tyrosine with an average length of 100 amino acids. Subsequent observations, however, showed that the T cell response to poly-Glu-Tyr was not class II restricted (Vidovic *et al*, 1985) and that the nonresponsiveness of T cells from certain strains of mice to poly-Glu-Tyr was caused by self-tolerance (Vidovic and Matzinger, 1988). In the course of these studies, T cell hybridomas were generated from mice immunized with poly-Glu-Tyr. Two of these were found to express γδ-TCRs (Vidovic *et al*, 1989; Vidovic and Dembic, 1991). The γδ-TCR$^+$ T cell hybridomas responded to poly-Glu-Tyr but not to poly-Glu-Ala-Tyr or poly-Glu-Ala. In addition, the response to poly-Glu-Tyr by one of these hybridomas, DGT3, appeared to be restricted to the

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Abbreviations: CFSE, 5- (and 6) carboxyfluorescein diacetate succinimidyl ester; ETC, epidermal T cell; poly-Glu-Tyr, poly-glutamic acid-tyrosine; poly-Glu-Ala-Tyr, poly-glutamic acid-alanine-tyrosine.

[1]Vγ nomenclature introduced by Maeda *et al* (1987).

nonclassical MHC class Ib molecule, Qa-1^b (Vidovic *et al*, 1989; Vidovic and Dembic, 1991). Poly-Glu-Tyr was assumed to be presented in the context of Qa-1^b molecules, as it was noted to bind to these but not to classical MHC class I molecules (Imani and Soloski, 1991; Soloski *et al*, 1995).

Recently, we have shown that splenic γδ-TCR^+ cells and not αβ-TCR^+ cells respond to poly-Glu-Tyr (Cady *et al*, 2000). Prior immunization of mice with poly-Glu-Tyr did not lead to enhanced responses to poly-Glu-Tyr by the spleen cells. The response to poly-Glu-Tyr did not require antigen processing or presentation, as class I and class II molecules, including Qa-1^b, were not necessary for the poly-Glu-Tyr response. The poly-Glu-Tyr response, however, did require interleukin-2 and the TCR. These studies also showed that some murine γδ-TCR^+ hybridomas with γδ-TCRs different from the DGT3 hybridoma described by Vidovic *et al* (1989) also respond to poly-Glu-Tyr, notably those expressing Vγ1.

We show herein that clonal ETC lines that express an invariant γδ-TCR that is different from the cells used in the studies mentioned above also respond to poly-Glu-Tyr. The reactivity to poly-Glu-Tyr is innate to these cells, both because they were not selected by prior immunization with this copolymer and because their TCR is invariant. Nevertheless, our data are consistent with a TCR-dependent mechanism for responses to these polypeptides.

MATERIALS AND METHODS

Reagents Monopolymers, including poly-L-Tyr, poly-Phe, poly-Leu, and poly-Glu, and copolymers, including poly-Lys-Tyr (1:1 or 1:9), poly-Glu-Asp, poly-Glu-Ala, poly-Glu-Lys, poly-Glu-Phe, and poly-Glu-Leu (4:1) (Sigma, St. Louis, MO) were used as controls for assessing T cell responses to poly-Glu-Tyr (1:1 or 4:1) and poly-Glu-Ala-Tyr (1:1:1 or 6:3:1) (Sigma). Other agents used to assess T cell responsiveness were concanavalin A (5 μg per ml final concentration), ionomycin (500 ng per ml), and phorbol-12-myristate-13-acetate (PMA) (10 ng per ml final concentration) (Sigma).

Monoclonal antibodies Purified antibodies used in the experiments herein include pan-anti-γδ-TCR (403A10) from S. Itohara (Itohara *et al*, 1989), anti-CD28 (35.71) from J. Allison (Allison and Krummel, 1995), and anti-FcRγII (2.4G2) from ATCC (Manassas, VA).

T cell culture The ETC clonal cell line 2CBET-3 was maintained in culture in Iscove's modified Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, β-mercaptoethanol, L-glutamine, nonessential amino acids, glucose, penicillin, streptomycin, gentamycin, and recombinant interleukin-2 (IL-2) (20 U per ml). Other ETC clonal lines, 7/17AT and U2B, gifts from R. Tigelaar (Yale University, New Haven, CT), were grown in culture in a similar manner. The cells were washed free of IL-2 before being used in the assays.

Assessment of ETC line reactivity Cell responses to various agents were measured by cytokine production by a cell proliferation bioassay system. In brief, IL-3/granulocyte-macrophage colony-stimulating factor (GM-CSF) dependent DA-1 cells were placed in 96-well flat-bottomed microtiter plates (3×10^5 cells per well) containing titrated supernatants from cultured T cells, as previously described (Reardon *et al*, 1992; 1995). Cell growth and viability of the DA-1 cells was measured by MTT [3(dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromine] uptake and colorimetric modification as determined with an enzyme-linked immunosorbent assay microplate reader (Biotek 312e), as previously described (Roehm *et al*, 1991). Unit values of these cytokines were defined by recombinant IL-3 NCI standards in all assays. In some experiments, the cells were cultured on flat-bottomed microtiter wells that had been incubated for 1 h with anti-CD28 antibody (100 μg per ml) and washed three times with balanced salt solution (BSS) containing 5% fetal bovine serum.

Cytofluorometry and cell sorting 2CBET-3 cells were stained with the pan-anti-γδ-TCR antibodies listed above followed by incubation with Cy3-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell immunostaining was assessed on an Epics Elite cytofluorometer (Beckman Coulter, Fullerton, CA) as previously described (Reardon *et al*, 1995). Cell sorting was performed on an Epics 431 cytofluorometer (Beckman Coulter).

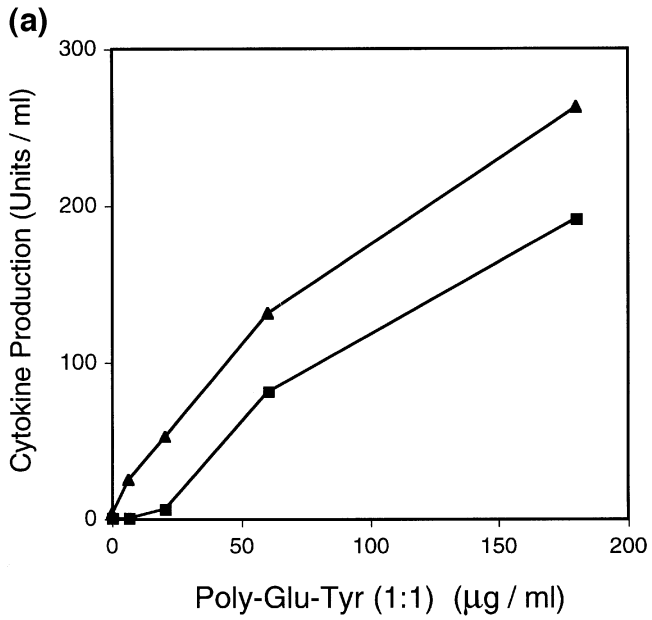
CFSE proliferation assay Fresh suspensions of ETC were prepared as previously described (Reardon *et al*, 1995). Briefly, whole trunk skin from TCR-$\beta^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME), removed aseptically after hair removal with a depilatory, had fat and blood vessels trimmed from the dermis before the skin was cut into approximately 1 cm^2 pieces and trypsinized for 2 h at 37°C. Epidermal cells were gently separated from the dermis and vigorously pipetted to produce single cell suspensions. The cells were enriched for T cells through nylon wool passage to remove non T cells. The cells were then incubated with 200 nM CFSE for 15 min at 37°C (Lyons and Parish, 1994) and washed three times in BSS. The washed cells were resuspended at 2×10^6 cells per ml in complete tissue culture medium with IL-2 as above, plated in 24-well flat-bottomed plates at 1 ml per well, and incubated with poly-Glu-Tyr at 50 or 100 μg per ml, with poly-Glu-Ala-Tyr at 100 μg per ml, or with IL-2 alone for 48 h. At the end of culture, the cells were washed with staining buffer (BSS with 2% fetal bovine serum and 0.1% sodium azide). At the beginning of cell staining, they were preincubated with 2.4G2 Fc receptor-blocking antibody for 15 min on ice, stained for 20 min with biotin-labeled 403A10 monoclonal antibody, and incubated with streptavidin-R-PE (Tago Immunologicals Bio-Source International, Camarillo, CA). The cells were washed with staining buffer and analyzed on a FACSCalibur cytofluorometer (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ) by first gating on live cell populations and then on δ-TCR^+ cells. Percentages of proliferating cells were determined by gating on cells with reduced CFSE content.

Production of TCR-loss cell variants 2CBET-3 cells were given 2000 R of γ irradiation from a ^{60}Co source (Nordian Gamma Beam 150). After allowing the cells to recover for 1–2 wk, they were sorted by flow cytometry to select the most TCR-negative cells and were expanded in culture. The cells were irradiated, stained, sorted, and expanded as before. After repeating this process a third time, TCR-loss cells were subcloned and expanded from single cells. TCR-loss variant candidates were screened for a lack of response to crosslinking anti-γδ-TCR antibody as well as to concanavalin A. Clonal cell lines that no longer were activated through the TCR were tested for their responsiveness to ionomycin and PMA as previously described (O'Brien *et al*, 1989) to ensure that they had not lost signaling components other than the TCR that are necessary for cytokine production. One such TCR-loss clone, 2CBET-3.8, was used in the experiments described herein.

RESULTS

Activation of murine Vγ5/Vδ1-TCR^+ ETC by Glu-Tyr polypeptides We found that a Vγ5/Vδ1-TCR^+ clonal ETC line, 2CBET-3, was responsive to poly-Glu-Tyr. Cytokine production (IL-3/GM-CSF) by this ETC line after 24 h of incubation with poly-Glu-Tyr increased in proportion to the amount of this material added to the cultures (**Fig 1*a***). Others have shown that certain polyclonal γδ-TCR^+ cells are dependent on cytokines derived from αβ-TCR^+ cells (Skeen and Ziegler, 1993; van der Heide *et al*, 1993). To examine this possibility in our system, we added IL-2 back to the assay and reassessed 2CBET-3 responses to poly-Glu-Tyr. IL-2 further enhanced the response to poly-Glu-Tyr particularly at lower doses of the polypeptide (**Fig 1*a***). IL-2 could not be replaced by IL-4 or IL-7, however (data not shown). The response of 2CBET-3 cells to poly-Glu-Tyr was restricted to forms of poly-Glu-Tyr having a 1:1 copolymer ratio, inasmuch as copolymers having a 4:1 poly-Glu-Tyr ratio were not stimulatory even in the presence of IL-2 (**Fig 1*b***). The response to poly-Glu-Tyr was not a unique property of the 2CBET-3 cell line, because other clonal Vγ5/Vδ1-TCR^+ ETC lines, 7-17AT and U2B cells, were also stimulated to produce IL-3/GM-CSF (**Fig 2**). Furthermore, fresh primary epidermal γδ-TCR^+ cells also were stimulated by poly-Glu-Tyr in the presence of IL-2 as measured by the CFSE proliferation assay. After 48 h of incubation with IL-2, 9.6% and 12.8% of the epidermal γδ-TCR^+ cells stimulated with poly-Glu-Tyr at 50 μg per ml and 100 μg per ml, respectively, had undergone at least one cell division relative to 2.9% of the cells incubated with IL-2 alone. In the dose range tested, poly-Glu-Tyr consistently was a weaker stimulus for the ETC lines than the mitogen concanavalin A.

Vγ5/Vδ1-TCR$^+$ ETC line responses to poly-Glu-Ala-Tyr 2CBET-3 cell responses were assessed to a modified form of poly-Glu-Tyr, a tripolymer of poly-Glu-Ala-Tyr. This was done to determine if the presence of other amino acids in the poly-Glu-Tyr polymer could interfere with its ability to stimulate the ETC. One form of poly-Glu-Ala-Tyr (6:3:1) has a 6-fold excess of Glu and previously was found to lack the ability to stimulate some γδ-TCR$^+$ cell populations (Vidovic *et al*, 1989; Vidovic and Dembic, 1991). Another poly-Glu-Ala-Tyr form (1:1:1) has not been tested previously for effects on γδ-TCR$^+$ T cells. This form maintains the equimolar ratio of Glu and Tyr similar to poly-Glu-Tyr in addition to an equimolar ratio of Ala. The 2CBET-3 cells produced cytokines in response to poly-Glu-Ala-Tyr (1:1:1), although only in the presence of IL-2 (**Fig 3**). No stimulation was evident with



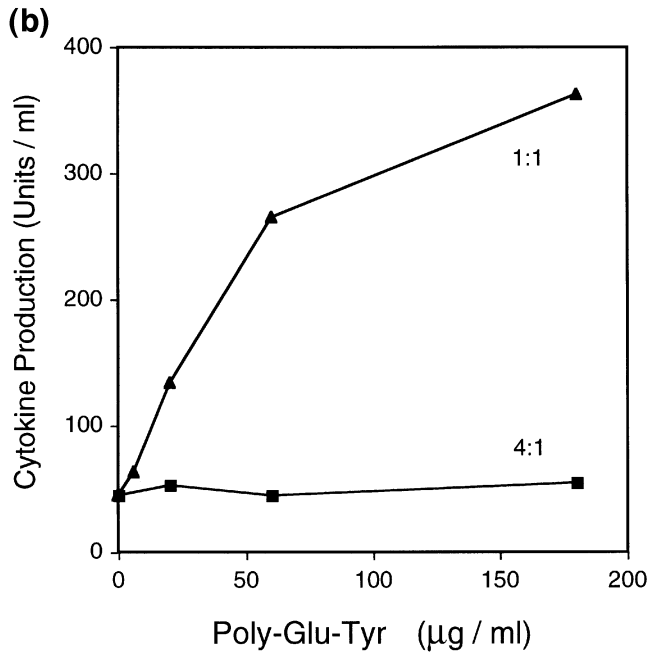


Figure 1. 2CBET-3 Vγ5/Vδ1-TCR$^+$ ETC lines show responses to poly-Glu-Tyr (1:1) but not to poly-Glu-Tyr (4:1) that are enhanced by IL-2. 2CBET-3 cells were incubated with increasing concentrations of the polypeptides with or without IL-2 in the culture medium over a 24 h period prior to the assessment of production of the cytokines, IL-3/GM-CSF. (*a*) 2CBET-3 cells were incubated with poly-Glu-Tyr without IL-2 (■) or with IL-2 (▲). (*b*) 2CBET-3 cells were incubated with IL-2 and poly-Glu-Tyr (4:1) (■) or poly-Glu-Tyr (1:1) (▲).

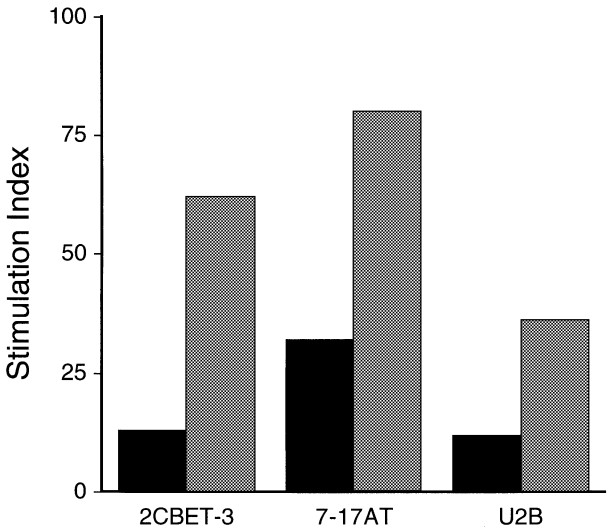


Figure 2. Murine Vγ5/Vδ1-TCR$^+$ ETC clonal cell lines, 7-17AT and U2B cells, also produce cytokines in response to poly-Glu-Tyr. 2CBET-3, 7-17AT, and U2B cells were incubated with poly-Glu-Tyr (1:1) (100 μg per ml) (*black bars*) compared to concanavalin A (*gray bars*) in the presence of IL-2 in the culture medium over a 24 h period prior to the assessment of production of the cytokines, IL-3/GM-CSF. Data are shown as a stimulation index of the amount of cytokine produced by the stimulatory agents divided by the amount of background cytokine production by the ETC lines alone to show the relative degree of cell stimulation by poly-Glu-Tyr compared to a T cell mitogen.

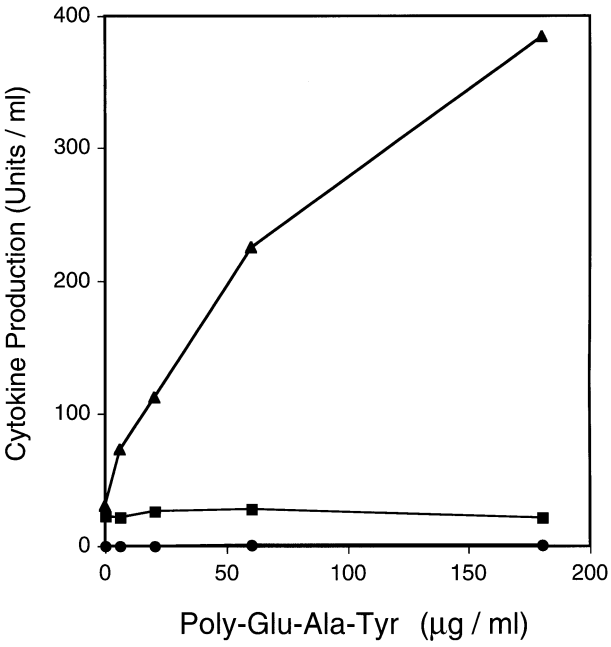


Figure 3. 2CBET-3 ETC lines show responses to poly-Glu-Ala-Tyr (1:1:1) but not to poly-Glu-Ala-Tyr (6:3:1) that strictly require IL-2. 2CBET-3 cells were incubated with increasing concentrations of the polypeptides with or without IL-2 in the culture medium over a 24 h period prior to the assessment of production of the cytokines, IL-3/GM-CSF. 2CBET-3 cells were incubated with poly-Glu-Ala-Tyr (1:1:1) without IL-2 (■) or with IL-2 (▲). The ETC lines were also incubated with IL-2 and poly-Glu-Ala-Tyr (6:3:1) (●), which is representative of the similar values seen following incubation with poly-Glu-Ala-Tyr (6:3:1) in the absence of IL-2.

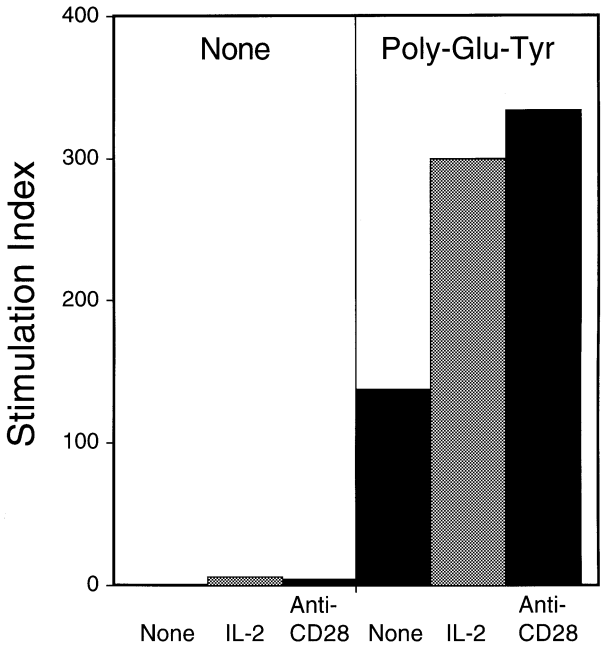


Figure 4. CD28 crosslinking replaces the effect of IL-2 on the 2CBET-3 response to poly-Glu-Tyr. 2CBET-3 cells were incubated without (*left panel*) or with (*right panel*) poly-Glu-Tyr (200 μg per ml) over a 24 h period with medium alone, with IL-2 in the culture medium, or with incubation on anti-CD28 monoclonal-antibody-coated flat-bottomed microtiter plates for CD28 crosslinking prior to the assessment of production of the cytokines, IL-3/GM-CSF. Data are shown as a stimulation index of the amount of cytokine produced under all of these conditions divided by the amount of background cytokine production by the ETC lines alone to show the relative degree of cell stimulation.

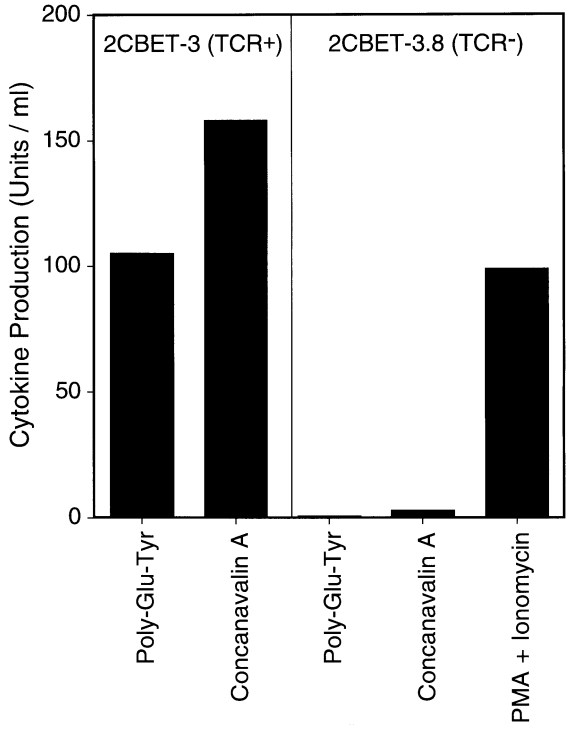


Figure 5. Loss of reactivity to poly-Glu-Tyr by 2CBET-3 TCR-loss variant cells suggests that poly-Glu-Tyr mediates its effects through the TCR. 2CBET-3.8 TCR-loss cells were incubated with poly-Glu-Tyr, concanavalin A, or PMA and ionomycin over a 24 h period in the presence of IL-2 in the culture medium prior to the assessment of production of the cytokines, IL-3/GM-CSF (*right panel*). Responses of 2CBET-3 cells to poly-Glu-Tyr and concanavalin A are shown for comparison as TCR-positive cell controls (*left panel*).

poly-Glu-Ala-Tyr (6:3:1) and IL-2 (**Fig 3**) or with poly-Glu-Ala-Tyr (6:3:1) alone (data not shown). After 48 h of incubation with IL-2, fresh primary epidermal γδ-TCR$^+$ cells that responded to poly-Glu-Tyr did not respond to poly-Glu-Ala-Tyr (1:1:1) at 100 μg per ml, showing percentages of proliferating cells in the CSFE assay that were similar to the IL-2 control at 2.9%. Taken together, these data suggest that poly-Glu-Ala-Tyr (1:1:1) is a weaker stimulus for epidermal γδ-TCR$^+$ cells than is poly-Glu-Tyr (1:1).

Costimulation with anti-CD28 antibody in the response to poly-Glu-Tyr Because the response to poly-Glu-Tyr appeared to be less dependent on auxillary cytokine stimuli, we tested next whether or not the response could be enhanced by accessory molecule interactions, such as between CD28 and B7, which tend to be required for primary TCR-mediated responses of αβ-TCR$^+$ cells. Also, others have shown that at least some γδ-TCR$^+$ cell responses depend on CD28 costimulation (Sperling *et al*, 1993). In our cultures of cloned γδ-TCR$^+$ cells, CD28 is expressed but cells expressing natural B7 ligands are missing (data not shown). As a substitute, we used crosslinking anti-CD28 monoclonal antibodies. We found that crosslinking CD28 on the 2CBET-3 cells with plate-bound anti-CD28 antibody further enhanced the response to poly-Glu-Tyr (**Fig 4**). In contrast, neither IL-2 nor CD28 crosslinking alone were sufficient to stimulate the ETC lines.

TCR-loss variants of ETC lines lose responsiveness to poly-Glu-Tyr Dependence on costimulation via CD28 is a characteristic feature of primary T cell responses to antigens. To further examine the importance of the TCR in the response of ETC lines to poly-Glu-Tyr, we generated TCR-loss variants of the 2CBET-3 cells. The TCR$^-$ 2CBET-3.8 cells did not respond to poly-Glu-Tyr nor to the T cell mitogen, concanavalin A (**Fig 5**). The loss of response to poly-Glu-Tyr in the TCR-loss variant was not due to a defect in downstream signaling pathways as these cells still responded normally to PMA and ionomycin (**Fig 5**).

DISCUSSION

Vγ5/Vδ1-TCR$^+$ ETC lines from unprimed mice show an innate response to the random polypeptides poly-Glu-Tyr and poly-Glu-Ala-Tyr (**Figs 1*a*, 3**). These molecules appear to be unique in their ability to stimulate the 2CBET-3 cells. Primary epidermal γδ-TCR$^+$ ETC also innately respond to poly-Glu-Tyr but do not respond to poly-Glu-Ala-Tyr as discussed further below.

In evaluating what the important characteristics are for these peptidic polymers to be stimulatory, some aspects are apparent. Equimolar combinations of Glu and Tyr in the poly-Glu-Tyr (1:1) and poly-Glu-Ala-Tyr (1:1:1) polymers appears to be important for stimulation, because the copolymers with 4:1 ratios and 6:1 ratios, respectively, of poly-Glu-Tyr were not stimulatory (**Figs 1*b*, 3**). The data also suggest that glutamic acid is not sufficient for γδ-TCR$^+$ cell stimulation. In support, other glutamic-acid-containing copolymers did not stimulate the ETC lines, including poly-Glu-Asp, poly-Glu-Ala, poly-Glu-Lys, poly-Glu-Phe, poly-Glu-Leu, and poly-Glu homopolymers. Additionally, the tyrosines alone are not sufficient as well for γδ-TCR$^+$ cell stimulation, as poly-L-Tyr and other tyrosine-containing polymers, such as Lys-Tyr (1:1) and Lys-Tyr (1:9), did not activate the ETC lines (data not shown). Similar findings are seen in our recent paper using γδ-TCR$^+$ hybridomas (Cady *et al*, 2000), although a few hybridomas showed small responses to poly-Glu-Leu and poly-Glu-Phe but not to other Glu-containing polymers, including poly-Glu-Ala-Tyr (6:3:1). Alanines in the poly-Glu-Tyr polymers appear to make them become a weaker stimulant, as the 2CBET-3 ETC lines were stimulated by poly-Glu-Ala-Tyr (1:1:1) only if IL-2 was present

(**Fig 3**). Moreover, primary epidermal γδ-TCR$^+$ ETC do not respond to poly-Glu-Ala-Tyr, in contrast to their response to poly-Glu-Tyr, again suggesting that the presence of alanines in poly-Glu-Tyr polymers decreases their ability to stimulate γδ-TCR$^+$ cells.

Previous studies have shown that certain polyclonal γδ-TCR$^+$ cells are dependent on cytokines derived from αβ-TCR$^+$ cells (van der Heide *et al*, 1993; Skeen and Ziegler, 1993). We similarly found that a T-cell-derived cytokine, IL-2, was required for the epidermal γδ-TCR$^+$ cell response to poly-Glu-Tyr. Additionally, we found that γδ-TCR$^+$ splenic T cells from TCR-β$^{-/-}$ mice also responded to poly-Glu-Tyr in a manner that was highly dependent on IL-2 (Cady *et al*, 2000).

As shown for αβ-TCR$^+$ cells, costimulatory signals mediated by CD28–B7 interactions normally occur during T cell activation by an antigen (Allison and Krummel, 1995). Although such costimulation is clearly important for αβ-TCR$^+$ cell responses to antigens, and in some cases for responses by γδ-TCR$^+$ cells (Sperling *et al*, 1993), it is less clear whether IL-2 serves to replace such CD28–B7 interactions for γδ-TCR$^+$ cell responses. When a deficiency of CD28–B7 accessory molecule interactions is present during αβ-TCR engagement, the addition of exogenous IL-2 rescues the T cells from entering an anergic state (Clements *et al*, 1993; Sperling *et al*, 1993; Kuiper *et al*, 1994; Seder *et al*, 1994; Loetscher *et al*, 1996; Walunas *et al*, 1996). No CD28–B7 interactions exist in our experimental system, as no B7-bearing antigen-presenting cells are present. IL-2 enhanced the ETC line response to poly-Glu-Tyr (**Fig 1*a***), however, and was required for the response to poly-Glu-Ala-Tyr (**Fig 3**). Thus, IL-2 may play a role in our ETC cultures similar to that in accessory-deficient αβ-TCR$^+$ cell cultures. Furthermore, from the degree of stimulation occurring in the absence of IL-2, poly-Glu-Tyr may have a limited ability to replace the IL-2 signal, perhaps through some weak interactions with receptors, such as the IL-2 receptor or CD28. Fluorescence-tagged poly-Glu-Tyr has been found to bind multiple sites on the γδ-T cell surface (Cady, unpublished).

The mechanism of γδ-TCR$^+$ cell stimulation with poly-Glu-Tyr polypeptides is not fully resolved. Crosslinking CD28 enhanced the response to poly-Glu-Tyr, however (**Fig 4**). As costimulation through CD28 molecules is a characteristic of a TCR-dependent process, this finding suggests that poly-Glu-Tyr stimulates Vγ5/Vδ1-TCR$^+$ ETC also through the TCR. In support of this notion is the observation that the 2CBET-3 TCR-loss variant cells no longer responded to poly-Glu-Tyr even when incubated with IL-2 (**Fig 5**). These cells maintain strong expression of IL-2 receptors (data not shown) and internal signaling pathways, as shown by the response to PMA and ionomycin (**Fig 5**). Moreover, consistent with a TCR-dependent mechanism, we also found that a TCR-negative hybridoma, 58$^{\alpha-\beta-}$, became responsive to poly-Glu-Tyr following transfection with and cell surface expression of Vγ1/Vδ6λ12-TCR genes (Cady *et al*, 2000).

We cannot rule out that the loss of another receptor, in addition to the γδ-TCR, may be responsible for the loss of the response to poly-Glu-Tyr. This could explain why one of our concanavalin-A-responsive Vγ5/Vδ1-TCR$^+$ ETC hybridomas, 709BET-49, does not respond to poly-Glu-Tyr (Cady *et al*, 2000) even in the presence of exogenous IL-2 or with CD28 crosslinking (data not shown). Other γδ-TCR$^+$ hybridomas expressing Vγ4 and Vγ6 also do not respond to poly-Glu-Tyr, however (Cady *et al*, 2000). Poly-Glu-Tyr-responsive hybridomas differ from the nonresponsive γδ-TCR$^+$ hybridomas by existing in a somewhat activated or "spontaneously reactive" state, as we have described previously (O'Brien *et al*, 1989; Reardon *et al*, 1992; Lahn *et al*, 1998). Thus, the possibility exists that the poly-Glu-Tyr response may require cells to be in a preactivated state, either through IL-2 or CD28 accessory effects as seen for the ETC lines or, in the case of the hybridomas, through weak self-reactivity.

As previously discussed above, the responses to poly-Glu-Tyr by the Vγ5/Vδ1-TCR$^+$ ETC line, 2CBET-3, are innate, not requiring prior immunization. This is further supported by our recent paper (Cady *et al*, 2000), which shows that Vγ1-TCR$^+$ splenocytes and to a lesser degree Vγ4-TCR$^+$ splenocytes from mice, not previously immunized to poly-Glu-Tyr, also respond to this polymer. No increases in responses to poly-Glu-Tyr were found in splenocytes from poly-Glu-Tyr-immunized mice. The innate response to poly-Glu-Tyr by the 2CBET-3 ETC line was not based on double-TCR expression with similar Vγ1-TCRs or other γδ-TCRs (data not shown). Additionally, innate responses to poly-Glu-Tyr were seen with two other clonal ETC lines, 7-17AT and U2B (**Fig 2**). As the ETC lines were developed in different laboratories from mice that were also not previously immunized to poly-Glu-Tyr, this finding suggests that all Vγ5/Vδ1-TCR$^+$ ETC lines may be responsive to this copolymer. This is surprising, because the previously reported poly-Glu-Tyr-responsive DGT3 hybridoma from poly-Glu-Tyr-immunized mice (Vidovic *et al*, 1989) expresses a different γδ-TCR (Cady *et al*, 2000). Thus, it is unlikely that poly-Glu-Tyr is recognized as a specific antigen as previously suggested (Vidovic *et al*, 1989; Vidovic and Dembic, 1991). In fact, the DGT3 cells also may have been innately responsive to poly-Glu-Tyr. As αβ-TCR$^+$ cells did not respond polyclonally to poly-Glu-Tyr (Cady *et al*, 2000), the innate responses to poly-Glu-Tyr may be restricted to γδ-TCR$^+$ cells. Moreover, because not all T cells are responsive to this agent (Vidovic and Matzinger, 1988; Cady *et al*, 2000), poly-Glu-Tyr apparently does not function as a nonspecific TCR-crosslinking T cell mitogen, such as concanavalin A.

Previous studies have implied that the γδ-TCR$^+$ cell response to poly-Glu-Tyr may be restricted to the nonclassical MHC class Ib molecule, Qa-1^b, based on studies with the γδ-TCR$^+$ hybridoma DGT3 (Vidovic *et al*, 1989; Vidovic and Dembic, 1991). Poly-Glu-Tyr was assumed to be presented in the context of Qa-1^b molecules, as it was noted to bind to these but not classical MHC class I molecules (Imani and Soloski, 1991; Soloski *et al*, 1995). As we showed in our recent paper, however (Cady *et al*, 2000), the response to poly-Glu-Tyr by γδ-TCR$^+$ cells does not require such presentation.

Poly-Glu-Tyr appears to interact with the γδ-TCR, which we have shown by the development of poly-Glu-Tyr responses in a γδ-TCR$^+$ transfectoma (Cady *et al*, 2000) and by the lack of poly-Glu-Tyr responsiveness by our TCR-loss ETC lines (**Fig 5**). It is uncertain whether or not poly-Glu-Tyr binds the γδ-TCR through the antigen-binding site, however. In fact, binding of these polyanionic ligands by the Vγ5/Vδ1-TCR may be through invariant pattern recognition, and in this regard the Vγ5/Vδ1-TCR and perhaps other γδ-TCRs may function as pattern recognition receptors (Janeway, 1992). Consistently, the earliest B cell receptors (BCR) or γδ-TCRs to appear in the mouse have canonical sequences that lack the diversity generated when random nucleotides are placed between the rearranged BCR or TCR genes. Such BCR or TCR antigen-recognition receptors may be evolutionarily conserved so that the immune system of the newborn is able to innately respond to antigens that have certain amino acid, lipid, or carbohydrate patterns. Poly-Glu-Tyr may mimic a chemical pattern that is inherently recognized by γδ-TCR$^+$ cells, including the first γδ-TCR$^+$ cells, the ETC, that appear in the fetal murine thymus and in the periphery (Havran *et al*, 1991).

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