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Cloning and large-scale expansion of epitope-specific equine cytotoxic T lymphocytes using an anti-equine CD3 monoclonal antibody and human recombinant IL-2

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Abstract

Cytotoxic T lymphocytes are involved in controlling intracellular pathogens in many species, including horses. Particularly, CTL are critical for the control of equine infectious anemia virus (EIAV), a lentivirus that infects horses world-wide. In humans and animal models, CTL clones are valuable for evaluating the fine specificity of epitope recognition, and for adoptive immunotherapy against infectious and neoplastic diseases. Cloned CTL would be equally useful for similar studies in the horse. Here we present the first analysis of a method to generate equine CTL clones. Peripheral blood mononuclear cells were obtained from an EIAV-infected horse and stimulated with the EIAV Rev-QW11 peptide. Sorted CD8⁺ T cells were cloned by limiting dilution, and expanded without further antigen addition using irradiated PBMC, anti-equine CD3, and human recombinant IL-2. Clones could be frozen and thawed without detrimental effects, and could be subsequently expanded to numbers exceeding 2×10^9 cells. Flow cytometry of expanded clones confirmed the CD3⁺/CD8⁺ phenotype, and chromium release assays confirmed CTL activity. Finally, sequencing TCR beta chain genes confirmed clonality. Our results provide a reliable means to generate large numbers of epitope-specific equine CTL clones that are suitable for use in downstream applications, including functional assays and adoptive transfer studies.

Introduction

Cytotoxic T lymphocytes are involved in the control of several important intracellular pathogens of horses, including equine herpes virus-1 (1-7), equine arteritis virus (8), and *Rhodococcus equi* (9,10). In horses infected with equine infectious anemia virus (EIAV), a lentivirus with a world-wide distribution, CTL are critical in the control of viral replication and clinical disease (11-22). Several factors contribute to the protective effects of CTL against EIAV, including epitope specificity and functional avidity (15,20,21,23,24). Importantly, individual EIAV-specific CTL epitopes can be presented by more than one MHC class I molecule, but subtle changes in class I molecules can enhance, diminish, or abolish recognition of these epitopes by CTL (25).

In humans and animal models, studies using cloned CTL have enhanced the understanding of the fine specificity of epitope presentation and recognition. In addition, adoptive transfer studies have evaluated the immunotherapeutic effects of infusions of cloned CTL against

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viruses such as cytomegalovirus and HIV-1, and neoplastic diseases like metastatic melanoma (26-32). Cloned CTL would be equally valuable for similar studies in the horse, but methods to clone equine CTL have not been described. Before the contributions of epitope specificity, MHC class I presentation, functional avidity, and TCR affinity to CTL-mediated protection against intracellular pathogens of horses can be rigorously dissected, a reliable method to clone epitope-specific equine CTL is needed.

In this report, we provide the first description of a culture method to clone and expand equine CTL. We chose to clone CTL specific for the conserved EIAV Rev-QW11 epitope, which is recognized by high avidity CTL (20), and is presented by the equine leukocyte antigen (ELA)-A1 haplotype-associated MHC class I molecules 7-6 and 141 (17,25). Because the ELA-A1 haplotype is well-represented in our research herd of Arabian horses that carry the severe combined immunodeficiency (SCID) trait (33-36), Rev-QW11-specific CTL clones would be excellent candidates for evaluating protective effects by adoptive transfer to SCID foals (19, 37). In addition, this clone would be valuable for investigating the differential recognition efficiencies of Rev-QW11 when presented by the 7-6 and 141 class I molecules (25). The cloning procedure utilized an anti-equine CD3 mAb (38,39) and human recombinant IL-2 (rhuIL-2), which stimulates equine lymphocytes (15,19,22,40), such that autologous APC and specific antigen were not required during expansion in culture. Our results demonstrated that large numbers of CD3+/CD8+ Rev-QW11-specific CTL clones could be generated, and that they retained their phenotype and function following cryopreservation. This cloning procedure should be useful for generating equine CTL clones for fine dissection of antigen specificity and for determining protective effects in adoptive transfer studies.

Materials and Methods

CTL Cloning

The CTL cloning procedure was performed as described (41), with several modifications. PBMC were isolated from horse A2150 blood (20,25) and initially stimulated in vitro with the Rev-QW11 peptide (20) at a concentration of 2 μ M, which was added to PBMC in 10% FBS. Peptide and PBMC were incubated for 2 hours at 37°C with occasional mixing before centrifugation at $250 \times g$ for 10 min. PBMC were resuspended to 2×10^6 /ml in RPMI 1640 medium with 10% FBS, 20 mM HEPES, 10 μ g/ml gentamicin, and 10 μ M 2-ME (culture medium). One ml was added to each well of a 24-well plate and incubated for 1 week at 37°C. Viable cells were then isolated by layering over Ficoll-Paque Plus (GE Healthcare), and fluorescence-activated cell sorting (FACS) was used to obtain CD8+ T lymphocytes. Cells were labeled indirectly with anti-equine CD3 mAb F6G (IgG1) (38,39) (obtained from Dr. Jeffery Stott, University of California, Davis) and anti-equine CD8 mAb ETC91A (IgG3) (42). Fluorecein-conjugated isotype-specific goat anti-mouse IgG1 (Caltag Laboratories, Burlingame, CA) and phycoerytherin-conjugated goat anti-IgG3 (Southern Biotech, Birmingham, AL) were used to detect labeled cells, and a FACS Vantage Cell Sorter (Becton Dickinson, San Jose, CA) was used for cell sorting. Selective gates were placed on lymphocytes (side vs. forward light scatter), to exclude extraneous signal, and CD3+/CD8+ double positive lymphocytes to isolate CD8+ T cells. These cells were sorted into sterile tubes, counted, and plated at limiting dilution (0.3 cells/well) into U-bottom 96-well plates in 50 μ l culture medium containing 50 IU/ml rhuIL-2 (Roche Diagnostics, Indianapolis, IN). The 96-well plates had been previously coated with anti-CD3 by adding 50 μ l/well PBS containing 1.5 μ g/ml F6G and left overnight at room temperature before removing excess PBS just prior to adding cells. Two hours after adding the sorted CD3+/CD8+ cells, 10^3 irradiated allogeneic PBMC in 50 μ l culture medium were added to each well. To prepare the irradiated feeder cells, PBMC were isolated from an ELA-A mismatched horse as determined serologically (43,44), and irradiated with 3,000 rad using the cobalt-60 irradiation unit at the Washington State University Nuclear

Radiation Center. Cloning plates were incubated at 37°C in 5% CO₂ for 7-14 days without manipulation, depending on rate of growth. Wells positive for clonal growth were first split 1:4 into similar 96-well plates, then further expanded and transferred into 24-well flat-bottom plates, then 6-well flat-bottom plates. The 24-well plates were pre-coated with 0.5 ml/well F6G-PBS as above, 10⁴ irradiated allogeneic PBMC per well were added, and each well contained 2 ml of culture medium with 50 IU/ml of rhuIL-2. The 6-well plates were pre-coated with F6G-PBS (2 ml/well) as above. Each well contained 3 ml of culture medium with 50 IU/ml of rhuIL-2. At this stage, 6-well plates contained approximately 3-4 × 10⁶ cloned cells/well and an equal number of irradiated allogeneic PBMC feeder cells. Due to cell adherence, 1 ml of 3 mM EDTA in culture medium was added to the wells of the 6-well plates for 2 min to facilitate lifting of cells during passage. After sufficient numbers of cells were generated, flow cytometry and CTL assays were performed to confirm phenotype and CTL activity (Fig. 1).

CTL Assays

CTL assays were performed as described (21,25). Briefly, direct CTL activity of cloned CTL was measured using a ⁵¹Cr release assay with a 17-h incubation period using autologous equine kidney (EK) target cells. Target cells were pulsed with various amounts of Rev-QW11 peptide as indicated in the figures. The formula, % specific lysis = [(E-S)/(M - S)] × 100, was used, where E is the mean of three test wells, S is the mean spontaneous release from three target cell wells without effector cells, and M is the mean maximal release from three target cell wells with 2% Triton X-100 in distilled water. The E:T cell ratio was 20:1, and each well contained ~30,000 target cells. Only assays with a spontaneous target cell lysis of <30% were used. The SE of percent specific lysis was calculated using a formula that accounts for the variability of E, S, and M (45). Significant lysis was defined as the percent specific lysis of peptide-pulsed target cells that was >10% (after subtracting background lysis of target cells not pulsed with peptide) and also >3 SE above the nonpulsed target cells.

TCR Beta Chain Gene Amplification and Sequencing

The 5' RACE System (46) for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA) was used according to manufacturers instructions to amplify the genes encoding the variable segment of the TCR beta chain (TCRβ) from Rev-QW11-specific CTL clones and from bulk PBMC. Total RNA was extracted from 5 × 10⁷ cells using an RNeasy mini kit (Qiagen, Chatsworth, CA), and first strand synthesis was performed with reverse transcriptase, 30 ul of RNA, and reverse primer 147R (5' ATTCACCCACCAGCTCAGC 3'), chosen based on the conserved equine TCRβ 3' constant region (47). A homopolymeric tail was added at the 5' end with dCTP and terminal deoxytransferase followed by size fractionation to remove smaller cDNAs, and PCR with primer 74R (5' TCGGTGCGGGAGATCTCTGC 3') and the Abridged Anchor Primer from the 5' RACE System. Reverse transcription was carried out at 48°C for 50 minutes, followed by PCR (40 cycles: 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min). The 450 bp PCR product was gel isolated and extracted (Qiagen, Chatsworth, CA), then used for TOPO TA cloning into the pCR4-TOPO vector for sequencing (Invitrogen). Isolated colony minipreps were digested and electrophoresed to screen for inserts and those with inserts of correct size were sequenced by the Laboratory for Biotechnology and Bioanalysis, Washington State University, using dye-labeled dideoxynucleotide cycle sequencing with an ABI 377 automated sequencer. All unique TCRβ sequences have been submitted to GenBank under accession numbers EF514533-EF514545.

Results and Discussion

Horse A2150 is an eight-year-old Arabian mare that has been infected with the WSU5 strain of EIAV for seven years (20,25). This horse has maintained an EIAV-specific CTL response directed against the Rev-QW11 epitope (20). The Rev-QW11 epitope was chosen for CTL

cloning because it occurs in a conserved region of EIAV Rev (48,49), it does not vary during infection (20), and it induces high avidity CTL (20). Moreover, Rev-QW11 is presented by the 7-6 and 141 MHC class I molecules, both of which are associated with the ELA-A1 haplotype (25). The ELA-A1 haplotype is well-represented in our breeding herd of SCID carrier Arabian horses. Therefore, Rev-QW11-specific CTL clones would be desirable for more detailed studies on the fine specificity of TCR recognition and MHC class I presentation, as well as for rigorously dissecting the requirements for CTL-mediated protection against EIAV by adoptive transfer to SCID foals.

To begin with, A2150 PBMC were stimulated for one week with the Rev-QW11 peptide before sorting CD8⁺ T lymphocytes using FACS. The FACS sorting procedure yielded a 90% CD3⁺/CD8⁺ cell population (Fig. 2). Purity less than 100% could have been due to adherence of non CD3⁺/CD8⁺ cells during the sorting procedure. The sorted cells, enriched for Rev-QW11-specific CD8⁺ T lymphocytes, were then cloned by limiting dilution into the wells of 96-well round bottom plates that had been previously coated with an anti-equine CD3 mAb. The use of anti-CD3 to provide TCR stimulation simplified the culture requirements because there was no further need for specific antigen or for autologous APC. This allowed allogeneic irradiated PBMC to be used as feeder cells. Although the specific functions of feeder cells in culture are not fully known, they likely support proliferation by providing additional signals via cytokines or by binding accessory molecules (41). Clones were expanded in culture medium containing 50 IU/ml rhuIL-2. This concentration was used because 50 IU/ml resulted in greater proliferation than 10 or 20 IU/ml, and 100 IU/ml offered no further advantage in earlier experiments (data not shown). The absence of specific peptide during CTL clone expansion did not lead to loss of peptide specificity since CTL clones expanded as above had the same level of Rev-QW11-specific lysis as did the same clones when re-stimulated with peptide, irradiated autologous PBMC, and rhuIL-2 (data not shown).

For each CD3⁺/CD8⁺ Rev-QW11-specific CTL clone, approximately 10^8 cells were obtained within seven weeks (Fig. 1). These cloned CTL could be used immediately in functional assays, further expanded, or cryopreserved for future use. Individual clones displayed different functional characteristics, presumably due to TCR differences. For example, levels of maximum specific lysis differed for each clone, as did Rev-QW11 recognition efficiency (Fig. 3a). Detrimental effects were not observed for CTL clones that were frozen and subsequently thawed. For instance, clones R8-0603 and R5-0603 were cryopreserved and maintained in liquid nitrogen for one month and one year, respectively. Both were thawed and expanded in culture as before, and within four weeks, large numbers of both clones were obtained (1.5×10^9 cells for clone R8-0603 and 2.1×10^9 cells for clone R5-0603). These cryopreserved expanded clones retained their CD3⁺/CD8⁺ phenotype and Rev-QW11-specific CTL activity (Fig. 3b,c), making them suitable for additional functional assays or for infusion into recipients for adoptive transfer studies. Although a small percentage of cells for each clone were not CD3⁺/CD8⁺, it is likely that these few cells were residual viable irradiated feeder cells that were not removed by layering over Ficoll-Paque Plus prior to analysis.

To confirm that the CTL cloning procedure was actually producing clones, the genes encoding the variable segment of the TCR β chain were amplified by 5' RACE from cDNA derived from representative Rev-QW11-specific CTL clone R17-1005, and the RT-PCR products were cloned and sequenced. The 5' RACE procedure was used to obtain the variable region because the 5' portion of TCR β genes is not conserved. The 3' reverse primers were chosen based on the conserved sequence in the equine TCR β chain constant region (47). Fifteen cloned TCR β genes from CTL clone R17-1005 were sequenced, and all were identical (Fig. 4a). The 5' RACE procedure did not yield the entire 5' portion of the leader sequence for all the cloned genes, but the variable, diversity, and joining segments were obtained in each case, and all were identical. Sequencing of cloned TCR β genes from CTL clones R3.3-1006 and R7.2-1006

also confirmed clonality, and indicated that each had a unique TCR (Fig. 4b). Although TCR β sequencing was not performed for CTL clones R5-0603 and R8-0603, the different functional characteristics (Fig. 3a) suggested different TCR β usage. To further confirm that the 5' RACE amplification was not yielding biased results, RNA was extracted from bulk PBMC, and the obtained TCR β genes sequenced. In contrast to CTL clones, all TCR β gene sequences obtained from a heterogeneous population of lymphocytes were unique (Fig. 4b). These results confirmed that the CTL cloning procedure was in fact yielding T cell clones.

Ten round-bottom 96-well plates were set up at 0.3 cells per well each time cloning was initiated, resulting in 288 potential clones. Of these, 20% of the wells usually contained proliferating cells at the first passage. A typical cloning run produced approximately 20 individual CD3⁺/CD8⁺ Rev-QW11-specific CTL clones when completed, giving an overall cloning efficiency of 7%. This efficiency could likely be improved by increasing the initial PBMC stimulation time to further enrich the starting population for Rev-QW11-specific cells. In addition, coating the bottom of the culture plates with anti-CD3 resulted in cell adherence, presumably due to TCR binding. This could have resulted in an unintentional loss of antigen-specific cells upon passage. To aid in lifting cells from the plates during passage, a brief treatment with dilute EDTA was used. Although this treatment did not appear to have detrimental effects, experiments are currently underway to evaluate the addition of anti-CD3 to the culture medium directly, without coating the plates.

In summary, these results provide a reliable method to generate large numbers of antigen-specific CTL clones in the horse. Greater than 2×10^9 cells could be obtained from a single clone. Advantages of the procedure include the use of anti-CD3 which obviates the need for specific peptide and autologous APC during expansion, and the fact that banks of frozen clones can be established for convenient later use. Epitope-specific CTL clones should prove useful for dissecting the fine specificity of TCR recognition of peptide/MHC complexes, and for determining the contribution of TCR affinity and functional avidity to CTL-mediated protection against viral and intracellular bacterial pathogens of horses. Importantly, epitope-specific CTL clones, such as the EIAV Rev-QW11-specific CTL clones described herein, could be used in adoptive transfer studies to rigorously evaluate the protective effects of CTL against infectious challenge.

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Fig 1.

Time-line and general strategy for producing Rev-QW11-specific CTL clones. In the middle of each week starting with week 3, rapidly growing clones were often split 1:2 and fed with fresh media and rhuIL-2.

Fig 2.

Flow cytometric analysis of A2150 PBMC after one week of stimulation with the Rev-QW11 peptide. **a.** Stimulated PBMC immediately prior to sorting. **b.** Phenotype of cells sorted by FACS using anti-equine CD3 and CD8 monoclonal antibodies. Sorted cells were subsequently cloned by limiting dilution.

Fig 3.

Functional and phenotypic characteristics of representative Rev-QW11-specific CTL clones.

a. Rev-QW11-specific CTL activity of five different CTL clones was determined on A2150 EK targets pulsed with increasing concentrations of Rev-QW11 peptide. E:T cell ratio was 20:1. Error bars are standard error. **b.** CD3⁺/CD8⁺ phenotype of CTL clones R8-0603 and R5-0603 following cryopreservation and four weeks of additional expansion to large numbers. **c.** Rev-QW11-specific CTL activity of CTL clones R8-0603 and R5-0603 assayed at the same time as b. Percent specific lysis was determined on A2150 EK targets pulsed with 10⁴ nM Rev-QW11 peptide. E:T cell ratio was 20:1. Error bars are standard error.

Fig 4.

TCR β chain amino acid sequences encoded by cDNA clones derived from **a.** Rev-QW11-specific CTL clone R17-1005, **b.** CTL clones R3.3-1006 and R7.2-1006 (with the R17-1005 sequence shown for comparison), and **c.** bulk PBMC (with the R17-1005 sequence shown for comparison). For each, total RNA was extracted and the TCR β variable segment genes amplified by RT-PCR using the 5' RACE procedure with a 3' primer in the constant region. RT-PCR products were TA cloned, sequenced, and aligned. The sequences shown for R3.3-1006 and R7.2-1006 represent 16 and 15 TA cloned sequences, respectively. Leader sequence, variable, diversity, joining, and constant domains were assigned based on published equine and human TCR β sequences.