EVALUATION OF PEPTIDE BASED VACCINES 
AND INHIBITORS TO PREVENT THE ONSET 
OF HTLV-1 ASSOCIATED DISEASES 

DISSERTATION 

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* * * * * 

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ABSTRACT

The human T-cell lymphotropic virus (HTLV-1) is the etiologic agent of an aggressive T-cell malignancy, adult T-cell leukemia (ATL). HTLV-1 infected individuals can also develop a chronic inflammatory condition affecting the central nervous system termed HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), as well as other inflammatory disorders. Although only \( \leq 10\% \) of infected individuals develop some type of disease state and the majority of infected individuals remain asymptomatic, the underlying causes for disease predilection and predictive markers for disease progression are poorly understood. In addition there is a lack of effective treatment against the serious ailments associated with HTLV-1 infection.

The purpose of this dissertation is to show three potential peptide-based modes of treatment to combat HTLV-1 infection. First prophylactic vaccination was investigated by using the squirrel monkey model of HTLV-1 to evaluate the protective efficacy of two B-cell epitope vaccine candidates MVF175-218 and WCCR2T. The chimeric MVF175-218 peptide containing a promiscuous T-cell epitope derived from the measles virus (MVF) derived from gp46; induced high titered antibody responses which bound to Env on the surface of HTLV-1 infected cells. WCCR2T, derived from the coiled-coil region, residues 347-374, of gp21 induced high titered antibodies in 1 of 4 immunized animals and 2 of 4 were shown to have antibodies that bound Env on the surface of HTLV-1 infected MT-2 cells.
Monkeys were challenged with the EVO/1540 cell line; however all animals were able to lower the proviral load to undetectable levels in all but one animal by 31 weeks after challenge; therefore, the protective effects of the vaccines were unable to be evaluated.

The second methodology investigated for use against HTLV-1 associated diseases was the identification and improvement of possible MHC I epitopes that could be used for CD8+ therapeutic vaccination. Predictive computer algorithms were used to identify the highest ranking HLA-A*0201 restricted epitopes from the HTLV-1 Gag Pol, and Tax proteins. High ranking epitopes with sub-optimal anchor residues were evaluated along with mutant peptides of the epitope containing optimal anchor residues. Relative peptide affinity was measured using the T2 binding assay and immunogenicity was measured in HLA-A*0201 transgenic HHD II mice. In vitro cytotoxicity assays with effector CTL from peptide immunized mice were combined with HTLV-1 infected HLA-A*0201 positive Hut102 A2-GFP cells to determine which epitopes were expressed on the surface of HTLV-1 infected cells enough to sensitize the cells to lysis. Results from this assay and ELISPOT with PBMC from a HAM/TSP patient show that even though enhancement improves the immunogenicity of some of the peptides; the only epitope examined that was expressed enough on the surface to sensitize the cells to lysis was the well defined Tax 11-19 epitope. These findings have important implications for further studies that may want to pursue CD8+ immunotherapy to control HTLV-1 infection.

The final mode investigated was peptide fusion inhibitors. To improve the half-life of potential HTLV-1 fusion inhibitors a retro-inverso peptide mimetic RI P400 was made was synthesized and compared to the previously described native P400 peptide. Circular dichroism measurements showed that the peptides had very similar mirror image secondary
structure and % helicity. P400 and RI P400 both showed strong syncytia inhibition at 100 µM peptide concentration. However the efficacy of RI P400 declined quickly with decreasing concentration. A longer peptide encompassing the P197 region was made covering residues 175-218 to see if extra length would increase syncytia inhibition in the gp46 peptide. However it was shown that P197 was sufficient to inhibit syncytia, and the larger peptide had slightly lower inhibitory capacity than P197. Next P197 and P400 were combined 1:1 in and compared with equal molar concentrations the individual peptides. It was shown that there was no synergism, or additive effect, when the peptides were combined. Finally rational mutants in the 401 position of P400 were evaluated. Shortened peptides 22 aa in length were synthesized on a multiple peptide synthesizer. These peptides were better than the negative control but lost their inhibitory capacity. Therefore, rational mutant inhibitors should be evaluated further as full length 30 aa peptide inhibitors, as smaller peptides were ineffective. This dissertation shows three different peptide-based approaches that could be used to combat HTLV-1 infection and associated diseases including prophylactic as well as therapeutic approaches.
Dedicated to my parents and to any child who is misunderstood by their teachers
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CHAPTER 1

INTRODUCTION

History and epidemiology

The human T-cell lymphotropic virus type 1 (HTLV-1) was the first human retrovirus to be isolated, and is the etiologic agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) as well as other inflammatory disorders. Approximately 10-20 million people are infected with HTLV-1 world-wide, and about 4% develop one of the above diseases. Endemic areas of the world include regions of Japan, Central and South America, the Caribbean, Africa and the Middle East. In the United States, HTLV-1 infection has been detected in immigrants from these endemic areas, south eastern regions, and among intravenous drug users [1]. HTLV-1 transmission is believed to be almost exclusively via cell-to-cell contact \textit{in vivo} and \textit{in vitro} and major modes of transmission include breast feeding, sexual transmission, and transmission via contaminated blood products. [1-7].

The discovery and characterization of HTLV-1 occurred from the joint findings of investigators in the US and Japan. ATL was first described by Uchiyama \textit{et al.}, and later viral particles were isolated and an antigen on the surface of ATL cells was found to be reactive with antisera from ATL patients, eventually establishing a viral etiologic cause for ATL [8, 9]. Adult T-cell leukemia virus (ATLV) as referred to by researchers in Japan was eventually shown to be the same human retrovirus (HTLV-1) described by US researchers who
identified a human retrovirus based on the presence of reverse transcriptase in the Hut102 cell line and fresh tissue isolated form a patient with a cutaneous T-cell lymphoma [10-12]. HAM/TSP was also identified independently by Japanese scientists who described the condition as HAM [13] and French scientists who described the same disease in Jamaican patients as TSP [14]. The combination of these findings reflect the condition currently referred to as HAM/TSP. HTLV-1 infection has also been linked to many other inflammatory disorders such as polymyositis, chronic arthropathy, uveitis infective dermatitis, respiratory disease and co-infection with *Strongyloides stercoralis*; however, clear links to disease have yet to be established [15, 16].

**Genetic organization of HTLV-1**

HTLV-1, like other retroviruses, is an enveloped virus with a two copies of a plus stranded RNA genome. The genome is 9032 bp and encodes for the structural proteins Env and Gag; enzymes such as reverse transcriptase, integrase, and RNase H (Pol); and a unique pX region consisting of regulatory proteins [17, 18]. Proviral transcription, termination and polyadenylation signals are controlled by the U3 region of the long terminal repeats (LTR). LTR are located at the 5' and the 3' ends of the genome and are divided into the U3, R, and the U5 regions. Proviral transcription is specifically regulated by the Tax responsive elements (TRE) in the U3 region which consist of three imperfect 21 bp-enhancer repeat elements [18]. The Full length mRNA encodes the Gag, Pol, and Env proteins, in addition to open reading frames (ORFs) I through IV. ORF III and IV encode the Rex protein, which regulates HTLV-1 genes post-transcriptionally by splicing events, and the transcriptional activator (Tax) protein respectively [18]. A full length mRNA encodes the Gag protein precursor (p55), and a full length mRNA modified by ribosomal frame shifting encodes Pol.
The viral protease (Pro) is also produced by a ribosomal frame shifting event at the end of the Gag protein precursor. Env expression is governed by a single mRNA splicing event, while the Tax and Rex proteins require two splicing events [18]. The double spliced Rex protein stabilizes unspliced and single spliced mRNA increasing their expression [19]. Tax and Rex predominate in the cell during early viral gene expression; as Tax initiates HTLV-1 transcription and Rex is responsible for upregulating the expression of the viral structural proteins Gag, Pol, and Env. Three accessory proteins (p12, p13 and p30) are encoded by pX ORF I and II. The functions of these proteins are continuing to become increasingly elucidated and appear to be generally important for the establishment of successful in vivo infection [20].

**Major target proteins for HTLV-1 vaccines and therapeutics**

- **Tax**

  The HTLV-1 Tax protein is a 353 amino acid, 40kDa multifunctional transcriptional activator protein which indirectly induces cellular genes involved in proliferation and down-modulates genes involved in apoptosis. When activating genes Tax does not bind the LTR region directly, rather it interacts with many cellular transcription factors capable of binding various promoter and enhancer elements. Tax interacts with cAMP responsive elements binding proteins (CREB)/activating transcription factor (ATF) proteins which form homo or heterodimers which bind to either cAMP responsive element (CRE), or change binding specificity for the closely related promoter sequence of the TRE [21]. Tax also interacts with serum response factor (SRF) which binds the CarG box promoter of c-fos, erg-1, and erg-2. Tax expression upregulates many genes including IL-2 and the IL-2Rα chain, TNF-α, TNF-β, GM-CSF, ICAM-1 and c-myc [22]. Tax has been shown to mediate cell proliferation by
upregulating NF-κB, particularly by manipulating the activity of IκB kinase (IKK) in multiple ways [23]. The Tax protein also has been shown to increase cyclin synthesis, cyclin/Cdk interactions, liberate E2F, induce hypophosphorylated Rb degradation, and inhibit DNA repair/apoptotic mechanisms including the tumor suppressor p53 [24]. As the major target of CD8⁺ immune response Tax is a key target for CTL vaccination [25-27]. As the major cause for HTLV-1-mediated cellular transformation, the effects of Tax have also been targeted by chemotherapy as the use of NIK-333 a novel synthetic retinoid was recently shown to be selectively toxic for HTLV-1 infected cells by inhibiting the NF-κB pathway [28]. Dehydroxymethylepoxyquinomicin (DHMEQ) has also been shown to selectively induce apoptosis in HTLV-1 infected cells via NF-κB inhibition [29].

**Gag**

The Gag precursor protein (p55) is cleaved by the viral protease to generate the matrix (MA, p19, aa 1-130), capsid (CA, p24, aa 131-344) and the nucleocapsid (NC, p15, 345-429) proteins. The MA protein is myristylated at the N-terminus anchoring the protein to the membrane facilitating assembly and transmission [30]. MA has also was shown to interact with tetraspanin microdomains on the inner surface of the plasma membrane which may facilitate intracellular membrane trafficking [31]. Although Gag has been generally neglected as a target for therapeutics and vaccines, the recent discovery of HIV-1 assembly inhibitors may eventually be translated into HTLV-1 therapy [32].
Pol

Pol is composed of a heterodimer of two major subunits p62/p49 [33]. In vitro studies have shown HTLV-1 Pol to be sensitive to nucleoside reverse transcriptase inhibitors (NTRIs); however, in a different order than HIV-1 suggesting slightly different catalytic properties between the enzymes [34].

Protease

Since the formation of mature virions depends on the cleavage of Gag p55 by the HTLV-1 protease (Pro), the use of protease inhibitors to combat HTLV-1 is an obvious approach for therapy [35]. The use of protease inhibitors has been shown to be effective in reducing HIV-1 viral load. Modified peptides and organic molecules have been identified as effective inhibitors against HTLV-1 protease [36-38]. Furthermore the recently published crystal structure of HTLV-1 Pro should help the development of HTLV-1 protease inhibitors gain additional momentum [39].

Envelope

The envelope glycoprotein (Env) was identified early on as a prime target for HTLV-1 vaccine and therapeutic development due to the fact that Env is responsible for cell mediated fusion (syncytia), and the transmission of virions from infected cells to non-infected cells [40]. HTLV-1 has little sequence variation throughout the genome including Env; therefore, escape mutants are of far less concern for individuals pursuing the development of an HTLV-1 vaccine when compared to those interested in HIV-1 vaccine development [41]. Env is synthesized as a 488 amino acid 62 kDa precursor protein which is eventually cleaved into the final 312 and 176 amino acid end products gp46 and gp21 respectively [42]. The gp46 surface (SU) subunit interacts noncovalently with the gp21
transmembrane region (TM). The gp46 subunit is believed to interact with the host cell receptor while gp21 is involved in post binding fusion events and anchoring the SU-TM complex to the viral envelope [43, 44]. The Env protein has 5 sites for N-linked glycosylation at residues 140, 222, 244, 273, and 404 which may need to be taken into consideration when developing vaccine constructs, although there seems to be no direct evidence that antibodies from HTLV-1 infected individuals bind directly to the carbohydrate moieties [45, 46]. Interestingly, the HTLV-1 envelope glycoprotein does not tolerate mutations in structure very well. Sequence alterations in many regions of Env lead to a loss in infectivity or improper precursor processing and subsequent expression [44].

Transmission of HTLV-1, more so than HIV-1, appears to require the passage of infected cells between individuals, and is believed to occur between CD4+, and to a lesser extent CD8+, T-cells via a specialized cell-to-cell contact described as the virological synapse [47, 48]. A number of cell surface molecules have been reported to be involved in Env-mediated fusion including VCAM-1, ICAM-1, and HSC-70, membrane glycoprotein C33, type 2 adenosine receptors; although none of these molecules are believed to be the primary receptor [49-54]. It seems likely that in addition to a primary receptor multiple cell surface molecules aid in Env mediated transmission of HTLV-1 [55].

Recently, the first identified protein which may be capable of functioning as a receptor for HTLV was reported [56]. Glucose transporter-1 (GLUT-1), an isoform of the 12 transmembrane glucose transporter family, was shown to bind soluble forms of the HTLV SU proteins and was critical for efficient entry of HTLV pseudotyped virions [56]. Another report demonstrated that the overexpression of GLUT-1 in an HTLV-1 resistant cell line, MDBK, lead to increased susceptibility to HTLV-1 and HTLV-2 pseudotyped virus
particles and HTLV-1 and HTLV-2 Env mediated syncytia formation [57]. While these observations characterized GLUT-1 as a receptor for HTLV-1 as defined by assays of pseudotyped virion entry in adherent non-T cell lines, the precise mechanism of binding and cell-to-cell spread is unclear. Binding domains of both HTLV-1 and HTLV-2 Env inhibit glucose transport by interacting with GLUT-1. It was later shown that the HTLV receptor-binding domain (RBD) lies in the amino terminus of the SU (residues 1-160), immediately upstream of a central immunodominant proline rich region (Env residues 180 to 205)[58].

Heparin sulfate proteoglycans (HSPGs) have also been shown to play an important role in HTLV binding and entry. HSPGs, are a type of glycosaminoglycan (GAG) consisting of a core protein with O-linked heparin sulfate (HS) polysaccharide chains, and are ubiquitously expressed on the surface of mammalian cells. Both the binding of soluble HTLV-1 SU and the titer of HTLV-1 and HTLV-2 pseudotyped viruses were dramatically reduced when HSPGs were removed from the cell surface of non-human T cell lines [59, 60].

Disease association and pathogenesis

- **Adult T-cell Leukemia/Lymphoma**

Adult T-cell leukemia/lymphoma (ATL), is a hematological malignancy associated with HTLV-1 infection in which approximately 6% of infected males and 2% of infected females will develop clinical disease [61]. ATL progresses rapidly and is resistant to conventional chemotherapeutic agents and death may result within 6 months after onset [61]. ATL is classified into four subtypes: acute, chronic, smoldering and lymphoma; depending on the clinical manifestations. Acute ATL comprises about 55% of reported cases and is characterized by leukemic cells with convoluted nuclei (flower cells), lymphadenopathy, hepatosplenomegaly, cough, fever, rash, general malaise, abdominal fullness, jaundice, lytic
bone lesions, increased lactate dehydrogenase (LDH) in the serum and soluble IL-2Rα are observed. Chronic ATL (20% frequency) symptoms are less severe and clinical symptoms develop slower. Lymphoma (20% frequency) displays a characteristic enlargement of lymph nodes in the periphery and body cavity, medium-sized lobulated lymphoid cells, and has a high incidence of cutaneous involvement. Smoldering ATL (5% frequency), patients display less lymphocytosis and aberrations in lymphoid cell morphology in the periphery, but present skin lesions and erythema. Impaired cellular immunity and hypercalcemia (30-60% frequency) is also associated with ATL [15, 18, 62]. Many modes of treatment including chemotherapy, nucleotide analogues, IFN-α and arsenic, and monoclonal antibodies have been tried in ATL patients. However, none of these has been shown to be completely effective, or in the case of bone marrow transplantation practical [61].

HTLV-1 associated myelopathy or tropical spastic paraparesis (HAM/TSP)

HAM/TSP is a chronic, progressive, demyelinating, disease of the central nervous system (CNS). Virus infected cells in the CNS lead to chronic inflammation and subsequent tissue damage. Clinical manifestations are quite similar to those seen in multiple sclerosis: spasticity, hyperreflexia, muscle weakness in the lower extremities, and urinary bladder and bowel dysfunction. Usually HAM/TSP progress slowly over many years, but rarely acute cases have been observed. The age of onset is usually 35 to 45 years, but can be as early as 12 years of age and is three times more prevalent in women than men. The incubation period from time of infection to onset of disease ranges from years to decades, but can be as short as 18 weeks following blood transfusion with HTLV-1 contaminated blood [1, 63-65]. A high proviral load has been identified as a dominant risk factor for developing HAM/TSP and rapid disease progression [66]. HAM/TSP is also characterized by strong immune
responses and some of these responses are thought to contribute to the immunopathology of the disease [67-72]. Elevated levels of HTLV-1 specific IgG are found in the cerebrospinal fluid (CSF) of HAM/TSP patients [73, 74]. HAM/TSP patients have elevated levels of CTL specific for Tax antigen which have been found in the spinal cord lesions of HAM/TSP patients [67]. CD4+ T-cells have also been implicated in HTLV-1 pathogenesis. High frequencies of CD4+ PBMC form HAM/TSP patients secrete IL-2 and TNF-α as well as IFN-γ also implicating them in pathogenesis [75-77]. High levels of the chemokines CXCL9 and CXCL10, but low levels of CCL2 appear to be indicative of HAM/TSP when compared to asymptomatic carriers [78]. Recent studies have also shown that HAM/TSP patients have fewer CD4+CD25+ regulatory T-cells in their PBMC than asymptomatic carriers or healthy donors. Ectopic Tax expression was also shown to reduce the level of Foxp3 mRNA expression in the regulatory CD4+CD25+ T-cells [79]. HAM/TSP treatment includes the use of corticosteroids and azothioprine which induce general immunosuppression and cannot be used consistently without long-term consequences [62]. The use of reverse transcriptase inhibitors such as zidovudine and lamivudine have been shown to have little effect on the symptoms and provirus load of HAM/TSP patients [62, 80]. IFN-β therapy has shown to induce modest results in HAM/TSP patients since it was shown to reduce tax mRNA expression and the frequency of CD8+ T-cells; in fact clinical progression was halted during the study with some patients improved motor function [81]. As there is no acceptable effective therapies additional methodologies must be pursued.
Cytotoxic T-cell responses

Both HAM/TSP patients and asymptomatic carriers mount a strong CD8+ T-cell response against the virus, which is involved in controlling infection, and is readily detected in the PBMC of infected individuals [82-84]. Interestingly protection has been observed in the absence of an antibody response [85, 86], suggesting the importance of cell-mediated immunity. A majority of infected individuals suffering from HAM/TSP exhibit chronically activated virus specific CTL that are directed predominantly against the Tax antigen, specifically to amino acids 11-19 (LLFGYPVYV) [73, 87-91]. The Tax 11-19 peptide binds to HLA-A2 with very high affinity, and in fact the crystal structure has been determined for Tax 11-19 in the peptide binding pocket of HLA-A2 [92]. CD8+ T-cells are seen both in the peripheral blood as well as in the cerebrospinal fluid and the CNS of HAM/TSP patients and are thought to play a role in the pathogenesis. In addition to the well defined CTL target Tax, there is a growing body of evidence that other HTLV-1 proteins may serve as effective targets for controlling or preventing HTLV-1 associated diseases [93-95]. Recently Goon et al., demonstrated the immunodominance hierarchy with regard to the frequency of CD8+ T-cells generated in asymptomatic and HAM/TSP patients as Tax > Pol > Env > Gag [96]. There was no significant difference in the frequency of CD8+ T-cell specificities (by antigen), between HAM/TSP patients, or asymptomatic carriers, although the cytotoxic ability of CTL generated appear to play a major role in controlling infection [97].

The importance of CD8+ T-cells in controlling proviral load in HTLV-1 diseases

There is a large body of information on HTLV-1 pathology, disease association, and cellular immune reactivity which has set the framework for our understanding of HAM/TSP pathogenesis. A high proviral load (PVL) is correlated with increased risk of developing
HAM/TSP; although, many patients remain asymptomatic with a high PVL and some develop disease with a relatively low PVL [66]. The proviral load of HTLV-1 is thought to reach a stable equilibrium “set point”, which generally does not fluctuate more than 2 to 4-fold over a period of years [98]. The median proviral load in a Japanese population was 5% of PBMC in patients with HAM/TSP and 0.3% in asymptomatic HTLV-1 carriers [99]. And certain MHC I alleles, such as HLA-A*02 and Cw*08 associated with strong CD8+ T-cell responses were shown to significantly reduce PVL and the risk of developing HAM/TSP. Individuals who were heterozygous at all three HLA class I loci were also less likely to develop HAM/TSP than those who were homozygous at one or more loci demonstrating the importance of a diverse CD8+ repertoire for antiviral control [82, 100]. Infected individuals who expressed increased mRNA levels of lytic factors such as perforin, granzymes A, B, H, K,M, granulysin, NKG2D, and CD8a also have a lower PVL [82]. Finally and most recently, Asquith et al., developed an ex vivo assay to measure the effect of CD8+ T-cells from infected patients on the lifespan of autologous CD4+ T-cells. HTLV-1 positive individuals varied significantly in the rate at which their CD8+ T-cells could clear infected cells, and their efficiency of clearing infected CD4+ T-cells was inversely correlated with PVL [84].

**Epitope enhancement strategies**

As the importance of CTL in host defense becomes increasingly apparent there has been an effort to optimize CTL responses in vaccine preparations. The use of epitope enhanced peptides has been shown to be a promising methodology to increase the potency of vaccine induced CTL responses. Epitope enhancement operates on the premise that there is a direct correlation between peptide-MHC I affinity and the potency of the CTL response
generated. By optimizing the anchor residues of an antigenic peptide, stable peptide-MHC I complexes are more likely to form, promoting the signaling cascades necessary for activation and differentiation of naïve CTL into effectors [101]. Much work has been performed with tumor antigens as high affinity CTL immunogens are more likely to break the immunological tolerance observed in many CTL responses against tumor antigens [101-103]. A relevant recent example by Okazaki et al., demonstrated that HLA-A*0201 transgenic mice immunized with an epitope enhanced peptide against a highly conserved, but low affinity epitope of HIV-1 RT were capable of significantly reducing the viral titers when challenged with a recombinant vaccinia virus expressing HIV-1 RT. Mice immunized with the WT peptide were not able to significantly reduce titers upon challenge [104].

**CD4⁺ (helper) T-cell response to HTLV-1**

The CD4⁺ T-cell response has been more difficult to study, because HTLV-1 Tax protein activates both IFN-γ transcription and T-cell proliferation, which are the basis of the two most widely used assays for antigen-specific CD4⁺ T-cell responses. Tax-induced CD4⁺ T activation occurs spontaneously *in vitro* i.e. in the absence of extrinsic antigenic stimulation. However, Goon *et al.*, have shown that a short (6 h) ELISPOT assay can detect antigen-induced cytokine production before the spontaneously expressed Tax protein results in host gene transactivation or T-cell activation [76, 77, 96]. Use of this technique has demonstrated that HTLV-1 specific CD4⁺ T-cell responses are dominated by proinflammatory Th1 cells that produce IFN-γ, IL-2, and TNF-α [76]. The frequency of IFN-γ producing CD4⁺ HTLV-1 specific T-cells was found to be between 10 and 25 times greater in HAM/TSP patients than in asymptomatic carriers with a similar proviral loads [75, 77]. Finally, the majority of these CD4⁺ IFN-γ producing T-cells are specific for Env as compared to Tax for CD8⁺ T-
cells. In addition to aiding CTL and B-cell activation and differentiation, CD4+ T-cells can also function as potent effectors capable of directly inhibiting viral/tumor spread [105]. Most recently, it was observed that the expression level of Foxp3, a specific marker of regulatory T-cells, was lower in CD4+ CD25+ T-cells from HAM/TSP patients as compared to normal controls. Interestingly, HTLV-1 Tax was shown to have a direct inhibitory effect on Foxp3 expression in CD4+CD25+ regulatory T-cells that inhibited their suppressive function, suggesting another mechanism for the hyper-inflammatory state of HAM/TSP [79].

**Epitope mapping of Env gp46 with synthetic peptides and antibodies**

Humoral immune responses during natural infection are generally believed to be protective against HTLV-1, resulting in asymptomatic disease status in most individuals [106, 107]. Most HAM/TSP patients have increased antibody titers with increasing PVL [67]. During the natural course of infection, Gag specific antibodies are the first detected in sera, followed by Env specific antibodies (gp21 then gp46) which appear about two months after infection. Tax specific antibodies can also be found in some infected individuals, which appear months later [107, 108].

Since most individuals infected with HTLV-1 remain asymptomatic carriers who contain neutralizing antibodies involved in containing the infection; many groups have made it a priority to identify the neutralizing regions of Env. The gp46 SU protein has served as a major target for epitope mapping due to its involvement in initial host cell receptor binding. Several linear neutralizing determinants have been mapped using synthetic peptides, monoclonal antibodies (mAb), as well as sera from HTLV-1 infected individuals and immunized animals. Matsushita et al., generated an early monoclonal antibody from an ATL
patient with neutralizing capabilities (0.5α) [109]. The corresponding epitope was mapped to residues 186-195 [110]. Soon after, many other reports surfaced of neutralizing regions immediately adjacent to or encompassing this region. Tanaka et al., generated a mAb (LAT-27) capable of neutralizing infectivity mapped to residues 191-196. To show the importance of this region, rabbits were immunized with a LAT-27 reactive peptide (190-199) conjugated to OVA. Antisera from rabbits immunized with the peptide conjugate demonstrated neutralizing activity [111]. In addition to identifying a neutralizing epitope corresponding to residues 90-98, Palker et al., also expanded the residues involved in the central neutralizing epitope of gp46 to residues 176-209 using synthetic peptide conjugates to induce neutralizing antibodies in goats and rabbits [112]. The potential of the central neutralizing region of gp46 was further demonstrated when two mAbs were derived from HAM/TSP patients recognizing overlapping regions in the central domain. One mAb, which bound aa 187-193, was able to inhibit syncytia formation and transformation. The other mAb reacted strongly with 187-196, and to a lesser degree residues 191-196. This mAb was also shown to induce antibody dependant cell-mediated cytotoxicity against an HTLV-1 infected cell line [113]. Inoue et al., mapped 10 regions with neutralizing antibodies derived from HTLV-1 seropositive blood donors. The regions identified from gp46 were residues 20-49, 89-115, 136-160, 175-199, 213-236, 235-254, and 277-292 [114]. Central residues 190-209 have also been shown to induce complement-mediated cytotoxicity [115]. In addition to mapping additional epitopes corresponding to residues 213-236 and 288-317, with mAbs derived from HAM/TSP patients, Baba et al., fine mapped epitopes within aa 175-199 to four distinct epitopes: 187-193, 191-196, 193-199, and a continuous conformational epitope that required the entire region for reactivity. This study also demonstrated a crucial point in favor
of synthetic peptide vaccination in that the neutralizing mAbs generated were capable of binding peptides and native gp46 with comparable affinity as shown by competitive binding experiments. It was also established that synthetic peptides possessed adequate size and other properties needed to mimic conformational epitopes adopted by gp46 [116]. Patient-derived antibodies were used to refine the C-terminal neutralizing epitope to residues 287-311 as well as the identification of a neutralizing epitope in the amino-terminus of gp46 residues 53-75 [117]. As shown above there are numerous well-defined linear epitopes from gp46. Studies performed by Hadlock et al., also demonstrated that there are many neutralizing and immunodominant conformational epitopes found within gp46 [118-120]. This poses a unique challenge to individuals interested in developing various modes of vaccination against HTLV-1 as no crystal structure exists for gp46.

In addition to the mapping of neutralizing epitopes, immunodominant epitopes of gp46 have also been mapped. It is important that one makes the distinction between epitopes that are immunodominant and those that have neutralizing activity. Vaccine preparations designed around immunodominant epitopes without demonstrated neutralizing activity should be avoided as high titer antibody responses with little desired activity may result [121]. In addition immunodominant epitopes present in vaccine preparations containing many epitopes designed to induce a polyclonal antibody response may skew the immune response towards irrelevant epitopes, thus diminishing the efficacy of a vaccine. Palker et al., demonstrated that the central neutralizing region of gp46 (190-209) as well as the C-terminal neutralizing epitope defined by Desgranges et al., as residues 296-312 were also immunodominant epitopes [122]. Horal et al., reported that the majority of gp46 from the central neutralizing domain to the C-terminus contains immunodominant epitopes.
including regions 176-199, 190-212, 224-244, 240-262, and 292-314 [123]. An immunodominant epitope was also described (residues 242-256), which fell within one of the regions described by Horal et al., [123, 124]. Additional immunodominant epitopes have been mapped to an amino-terminal region (86-107) as well as the central region residues 175-199 and residues 239-261. Region 239-261 was further mapped to show that within residues 252-261 there is a linear epitope and two conformational epitopes corresponding to residues 244-249 and 244-257 respectively [125, 126].

**Epitope mapping of Env gp21**

The gp21 TM subunit is an essential component of the fusogenic processes mediated by Env after the initial binding of gp46 to the cellular receptor. Upon binding gp21 is believed to undergo conformational changes into a fusion activated state leading to penetration of the host cell membrane by the TM fusion peptide and subsequent conformational changes which deliver the viral core into the host cell [127, 128]. Preventing generation of the fusion activated state, or inducing premature conformational changes in the absence of uninfected host cells currently serve as attractive targets for new generation retroviral vaccines and therapeutics. Information obtained from the recent crystal structure determination of gp21 should facilitate the design of vaccines and therapeutics that interfere with the autogenic processes between the viral and host cell membranes [128]. A combination of findings from early epitope mapping and applied analysis of the crystal structure may prove useful in the rational design of peptide therapeutics and vaccines. Desgranges et al., reported a neutralizing epitope found within in the coiled coil region of gp21 (346-368) [117]. Five additional neutralizing epitopes were described by Inoue et al., the first of which begins just N-terminal of the coiled coil region leading into the coiled coil
region (332-352), the next is within the coiled coil region (350-386), and the beginning one at the C-terminus of the coiled-coil, extending just beyond the disulfide bonded region (382-403), as well as 2 other C-terminal regions 426-448, and 458-488 [114]. The C-terminal region of gp21 (467-489) was also described as immunodominant [123]. Three more immunodominant regions of gp21 have been described corresponding to residues 361-404, 397-430, and 374-392 [122, 129].

In addition to the epitope mapping studies mentioned for gp46 and gp21, several mutagenesis studies have been performed to elucidate crucial residues that may serve as therapeutic targets or give additional insight into vaccine preparations [40, 44, 130].

**HTLV-1 vaccine studies and their animal models**

The use of appropriate animal models is essential for the preclinical evaluation of HTLV-1 vaccines and therapeutics. Over the years many animal models of HTLV-1 infection have been developed for the purpose of testing various vaccine candidates as well as to study pathogenesis and host-virus interactions. The rabbit model developed by Miyoshi et al., [131] demonstrated that rabbits were susceptible to HTLV-1 infection either by injection with HTLV-1 positive human or rabbit cell lines or from HTLV-1 infected human or rabbit blood. Takehara et al., was unable to protect rabbits immunized with a heat-inactivated HTLV-1 transformed rabbit cell line and a synthetic peptide corresponding to 175-196 of Env coupled to carrier protein, but demonstrated the protective effects of passive immunization in rabbits with hyperimmune sera [106]. Additional studies using recombinant viral vectors were able to demonstrate some level of protection to rabbits immunized and challenged, although all animals became infected after a second challenge [86, 132]. Shida et al., immunized rabbits with recombinant vaccinia virus and was able to
show protection after challenge by the lack of detectable viral antigens after culture [133]. Rabbit models of HTLV-1 infection have proved to be useful for evaluation of vaccine candidates that are targeted for humoral responses although cellular responses have been poorly defined.

Another animal model for HTLV-1 infection is the rat model [134]. However, HTLV-1 infection in rats is generally highly suppressed and the low levels of provirus in the circulating lymphocytes decline rapidly with time, leading to low titers of neutralizing antibodies [135, 136]. A nude rat model of ATL-like symptoms has been used to assess cytotoxic T-cell responses against Tax-encoding DNA vaccination [137, 138]. Rats immunized with recombinant adenovirus vector expressing Env have been demonstrated to elicit CTL and detectable humoral immune responses but without protective effects [139]. To evaluate CTL responses most relevant to human application, we have focused on developing CTL peptide immunogens in HLA-A*0201 transgenic mice, and have used a recombinant vaccinia virus challenge model to evaluate protective efficacy [26, 140, 141]. Recent reports describing real-time PCR methods in the Rabbit and Rat models of HTLV-1 infection have made these models more useful for the future application of accurately evaluating therapeutics and partial protection afforded by vaccines [142-144].

Cynomolgus macaques (Macaca fascicularis) were established early as a non-human primate model for HTLV-1 infection. This model was used to show recombinant gp46 produced in E. coli was able to induce neutralizing antibodies capable of protecting cynomolgus macaques from challenge with MT-2 cells. This was shown by the lack of HTLV-1 antigens detected by culture PBMC from immunized monkeys. Cynomolgus macaques were also used to demonstrate the long-term protection of a recombinant vaccinia
virus vector expressing gp46. Immunized animals challenged with 6C cells generated antibody and CTL responses capable of protecting the immunized monkeys as shown by the lack of viral antigen in the PBMC and PCR [145].

The squirrel monkey *Saimiri sciureus* is also susceptible to experimental infection, by injection with either syngenic or allogeneic HTLV-1 immortalized cells [85]. As in humans, such experimental inoculation leads to chronic infection, and HTLV-1 provirus can be detected in PBMC and lymphoid organs by PCR. Infection in this model also demonstrates the “two-step” nature of HTLV-1 infection, in that upon infection there is an initial burst of viral replication followed by strong immune responses that suppress viral replication, followed by a reemergence and persistence of the virus [146-148]. Further, immunization with attenuated vaccinia virus derived NYVAC vaccine candidates encoding the Env and Gag proteins lead to the protection of squirrel monkeys and cell mediated responses against Env and Gag that could be detected in these animals [149]. These results indicate that the squirrel monkey can be a useful animal model for studying the pathogenesis of HTLV-I and for evaluating new treatments and vaccine candidates against HTLV-1.

The most recent non-human primate model of HTLV-1 infection described, has demonstrated that pig-tailed macaques (*Macaca nemestrina*) are susceptible to HTLV-1 infection upon challenge with homologous cells containing the infectious molecular clone ACH. This model is most notable for the high frequency of morbidity and mortality exhibited by infected animals such as squirrel monkeys and cynomolgus macaques chronically infected with HTLV-1 that have not been shown to develop signs of disease. This model could be extremely useful in evaluating vaccines or other therapeutics by monitoring reductions in mortality or pathologic conditions [150, 151].
Synthetic peptide vaccination against HTLV-1

In 1995 Baba et al., described two synthetic peptide vaccines derived from the central linear neutralizing domain of gp46, one corresponding to aa 181-203, the other to aa 181-210. Both peptides were synthesized with a branched polylysine oligomer. The synthetic peptide spanning residues 181-210 induced higher levels of neutralizing antibodies than the shorter peptide immunogen. The 181-210 peptide was also capable of eliciting antibody titers comparable to peptide conjugated with OVA. Furthermore, it was shown there was a major helper T-cell epitope in residues 194-210, perhaps explaining the marginal results of the 181-203 peptide. This study demonstrated that peptide immunogens containing an adequate helper T-cell epitope and the appropriate size to stabilize its conformation were capable of eliciting high titer neutralizing antibodies against HTLV-1[152].

One of our major projects has revolved around translating structural and immunogenic principles uncovered while developing synthetic peptide immunogens against the lactate dehydrogenase C4 (LDHC4) protein (reviewed in [153]) into efficacious peptide immunogens directed against HTLV-1. Much attention has been focused on neutralizing determinants found within gp46. We began to employ novel strategies with peptide vaccines that made use of promiscuous helper T-cell epitopes capable of overcoming MHC II restriction in mice, rabbits, and humans. Promiscuous T-cell epitopes such as those derived from the measles virus F protein (aa 288-302) (MVF) and tetanus toxoid (aa 580-599) (TT) allow one the flexibility to design highly immunogenic conformation-driven peptide constructs without the use of bulky carrier proteins which could contain potentially immunodominant, irrelevant B-cell epitopes. One of the first of such studies employed the use of promiscuous T-cell epitopes co-linearly synthesized with a GPSL turn and either a B-
cell epitope corresponding to SP2 (aa 86-107) or SP4a (aa 190-209). A third immunogen employed a β-template consisting of 2 strands of alternating Gly-Leu residues. The B-cell epitopes SP2 and SP4a and the two promiscuous T-cell epitopes TT and MVF were grafted onto the four Lys residues of the template via their sidechain ε nitrogens. Based on biochemical and immunologic data it was shown that the SP4a epitope was structurally conserved in the chimeric peptide constructs, while SP2 was poorly stabilized in the peptide chimeras. It was also demonstrated that the use of promiscuous T-cell epitopes can provide necessary T-cell help as well as adequate structural stability to the free SP4a B-cell epitope, leading to the generation of strong antibody responses that were capable of recognizing the native protein [154].

Based on the results in this first study the next gp46 antigen designed was MVF175-218 (MVFMF2), which included additional residues covering more of the central neutralizing epitopes of gp46 in addition to helper and cytotoxic T-cell epitopes within the region selected [155, 156]. Also based on the previous study, only one immunogen containing MVF as the promiscuous T-cell epitope was synthesized. MVF175-218 exhibited CD spectra that was consistent with the predicted secondary structure elements, and was able to elicit high titered antibodies in outbred mice and rabbits with a high affinity for the native protein. Antisera generated in mice and rabbits were able to inhibit syncytia, but were unable to protect rabbits against challenge with a rabbit cell line infected with HTLV-1 [157]. The MVF175-218 construct was used to evaluate a novel encapsulation method generating poly (D,L-lactide-co-glycolide) microspheres to enhance antigen presentation. MVF175-218 containing microspheres were capable of inducing high antibody titers after a single boost that was not significantly different from rabbits who received microspheres plus the adjuvant
nor-MDP. Encapsulation had no negative effect on the specificity of the antibodies generated as shown by competitive ELISA [157, 158]. In order to elicit a potent multi-valent neutralizing antibody response against Env, we have begun to investigate peptides derived from gp21 in addition to peptides from gp46.

Sundaram et al., described the design and evaluation of two peptides against gp21. One of these peptides corresponds to residues 392-415, which lies between the fusogenic region and anchorage domain of gp21. The peptide was co-linearly synthesized with the promiscuous T-cell epitope TT3 corresponding to amino acid residues 947-967 from the tetanus toxoid. This B-cell epitope overlaps with a region that has been shown to have neutralizing activity [159]. CD analysis showed that the peptide possessed the desired helical character and rabbits immunized with the peptide generated very high titers to the immunogen and native protein as shown by whole virus ELISA. The antibodies generated in the rabbits were also able to bind the native protein expressed on the surface of HTLV-1 infected MT-2 and ACH cells. TT3-392-415 also showed very strong neutralizing activity [160].

The second set of gp21 peptides described by Sundaram et al., required a different template strategy to assemble a single epitope into a trimeric structure to mimic the crystal structure of gp21. In that approach, a β-template consisting of alternating Gly-Lys residues was synthesized with the promiscuous helper epitope TT at the N-terminus. After deprotection of the lysine sidechains three identical B-cell epitopes corresponding to residues 347-374 of gp21 were added to the ε NH₂ of the lysine side chains. The template strategy was used as a scaffold to facilitate hydrophobic interactions between individual strands to allow folding into a trimeric coiled-coil structure. Upon cleavage from the resin all
N-termini were acetylated and the C-terminus was amidated to minimize charge repulsions. This construct containing the wild type sequence B-cell epitopes was referred to as WCCR2T. A mutant peptide (CCR2T) was also designed to containing leucine residues substituted at the $a$ and $d$ positions of the heptad repeats contained in the B-cell epitope to maximize hydrophobic interactions in the center of the simulated coiled-coil in an attempt to better mimic the overall three-dimensional structure. Structural analysis revealed that the mutant peptide had a much higher helical character, although the wild type peptide was more immunogenic in outbred mice. Antisera generated against both peptides were able to recognize gp21 expressed on the surface of HTLV-1 infected ACH cells, and were able to inhibit syncytia formation [161].

In order to develop an effective multivalent vaccine against HTLV-1, studies aimed at inducing a broad highly immunogenic CTL response against HTLV-1 was also performed. Tax was the first antigen of interest as it is expressed early in infection and has been shown to elicit a strong natural CTL response. It has also been demonstrated that there is a negative correlation between Tax expression and provirus load [162].

The generation of a multivalent Tax-oriented CTL response involved the synthesis of previously defined Tax epitopes with a high affinity for the human MHC I allele HLA-A*0201. The three best epitopes were synthesized into a single construct which separated each epitope with intervening double arginine residues to facilitate proteasomal cleavage and subsequent antigen presentation. Based on the proteasome and cytolytic data of the triple epitope construct, it was apparent that immunogenicity, proteasome liberation, and epitope orientation of the triple epitope construct were intertwined [26]. The importance of epitope orientation was then investigated. The construction of the triple epitope peptides were
identical, with the exception of the third epitope, corresponding to residues 233-241, was substituted for residues 306-315 of Tax. The epitopes were shifted between 4 different orientations. Similar proteasomal, cytolytic and cytokine secretion assays were performed and a positive correlation between processing rates and immunogenicity were observed. The two most immunogenic individual epitopes still dominated the cytolytic responses irrelevant of orientation. Interestingly the middle and lowest affinity peptides appeared to have a strong role in IFN-γ production supporting the need for multivalent, multiepitope CTL vaccines. Immunization of HLA-A*0201 transgenic mice with the most immunogenic triple epitope peptide construct led to a significant reduction in viral titers when mice were challenged with recombinant vaccinia virus expressing Tax. Mice immunized with a mixture of the individual epitopes did not significantly reduce virus titers [141].

Recently the protective efficacy of peptide vaccination has been demonstrated in the squirrel monkey model of HTLV-1 infection [163]. Kazanji et al., demonstrated complete protection in one monkey and partial protection in another monkey immunized with a combination of MVF175-218 and Tri-Tax peptides described above. Strong humoral responses and CD4+ T-cell responses against the 175-218 epitope were present however weak cellular responses to the individual Tri-Tax epitopes were demonstrated, suggesting the presence on neutralizing antibodies were the likely mode of protection.

**Peptide inhibitors of virus-cell fusion**

As vaccination may one day provide a preventative option against HTLV-1 infection, the use of peptide fusion inhibitors may aid those already infected with HTLV-1 to prevent the onset of HTLV-1 associated diseases. Peptide fusion inhibitors may be especially useful in the case of HAM/TSP patients who generally have elevated proviral loads and
autoimmune-like inflammatory responses. These patients may benefit from therapeutics capable of minimizing provirus load and lessening the need for strong immune responses to control infection and hence the subsequent inflammation. Recently the 36 aa peptide Enfuvirtide (T20, Fuzeon; Trimeris Inc.) has been approved by the FDA for treatment-experienced HIV patients who still have evidence of viral replication. This peptide is the first approved in a new class of antiretroviral drugs targeted against the fusion and entry process [164]. Enfuvirtide specifically targets the heptad repeats in the coiled-coil region of HIV gp41 TM subunit preventing the formation of the hairpin structure necessary for fusion [164]. T20 is derived from the C-terminal HR2 region of gp41. The mechanism of T20 activity is by binding to the N-terminal HR1 region of gp41 interfering with the post fusion formation of a six helix bundle (which results in HR1 and HR2 interaction). T20 occupies the space that HR2 would occupy in the interaction resulting in steric hindrance and inhibition of further Env transitional states needed to fuse virus and target cell membranes [165].

HTLV-1 fusion inhibitors were originally described by Sagara et al., who performed syncytia inhibition assays that incubated cells with overlapping synthetic peptides covering the entire Env region. Out of the pool of overlapping peptides only two peptides were found to inhibit fusion. These peptides corresponded to residues 197-216 (P197) and 400-429 (P400) of gp46 and gp21 respectively. The essential residues responsible for the inhibitory effect were then fine mapped to peptides corresponding to residues 197-208, and 397-406 [159]. Jinno et al. later demonstrated that residues 407, 408, and all leucine residues found in the P400 peptide were essential to the syncytia inhibition mediated by the peptide by alanine scanning [166]. Although P400 is believed to have the same inhibitory mechanism
that T20 has due to their structural homology. In the absence of a resolved crystal structure and of other necessary data, the exact mechanism of P197 inhibition is unknown. However, it is currently assumed that P197 also functions to inhibit fusion in a post-binding or transient state, as P197 cannot competitively inhibit recombinant HTLV-1 SU from binding target cells [167]. This finding has also been confirmed by Kim et al., who showed the HTLV receptor-binding domain (RBD) lies in the amino terminus of the SU, immediately upstream of Env residues 180 to 205 [58].

P400 was recently revisited and shown that its inhibitory capacity could be increased when three residues were mutated to mimic the CR strain of HTLV-1. When residues Arg401Cys, Pro403Leu, and Pro411Ser were mutated the neutralizing capacity of the peptide (PcR400) and its ability to bind a recombinant protein mimicking the coiled-coil region of HTLV-1 were better than that of P400 [59]. This study demonstrated the possibility of improving fusion inhibitors from the P400 region by rational mutations.

Retro-inverso peptides

One major drawback of T20 therapy is that 90 mg must be administered subcutaneously twice daily, resulting in astronomical cost ($20,000/year). Therefore, the development of fusion inhibitors that would possess increased half-life could reduce the frequency amount and ultimately cost of the treatment. On such peptide modification is the use of Retro-inverso (RI) peptides [168, 169]. The premise of retro-inverso peptides is that the retro-inverso peptide analog is synthesized using D-amino acids with the amino acid sequence in reverse order, such that the resulting peptide mimetic has a reversal of the peptide backbone but a topochemical equivalence to the parent peptide in terms of side-chain orientation. RI peptide modifications have been shown to work successfully in vitro and
*in vivo* [170, 171]. Retro-inverso peptides and other peptidomimetics rely on the premise that the key residues of the binding epitope, in particular side-chain functional groups responsible for a significant portion of the binding affinity to a given receptor/ligand, may be transferred to a much smaller molecule with the binding contributions largely intact [168].
CHAPTER 2
EVALUATE THE PROTECTIVE EFFICACY OF ENV B-CELL EPITOPES IN THE SQUIRREL MONKEY MODEL OF HTLV-1

RATIONALE

In our laboratory we have previously designed and characterized the B-cell epitope vaccines: MVF175-218 derived from gp46, WCCR2T derived from gp21 and the triple epitope CTL vaccine Tri-Tax derived from the Tax antigen. MVF175-218 is a linear peptide that incorporates multiple overlapping immunodominant and neutralizing epitopes identified from the gp46 central neutralizing region. WCCR2T is composed of a promiscuous T-cell epitope containing template peptide that supports three copies of a B-cell epitope corresponding to amino acid residues 347-374 to mimic the coiled-coil region of gp21[161]. Tri-Tax is a triple epitope construct consisting of three immunodominant HLA-A*0201 restricted CTL epitopes. Recent reports have demonstrated the value of the squirrel monkey model of HTLV-1 infection as it mimics many characteristics in human infection such as the two step nature of infection, persistent infection and even multinucleated flower cells in one animal. One of the most important aspects is that squirrel monkeys can be challenged with a human derived HTLV-1 clone, not STLV-1, as is the case in the macaque model. In addition squirrel monkeys are relatively inexpensive and easier to obtain when compared to other non-human primate models. A recent report has shown that two squirrel monkey immunized with a combination of MVF175-218 and Tri-Tax were protected from challenge
with the HTLV-1 producing and expressing HTLV-1 cell line EVO/1540. One monkey displayed partial protection the other monkey showed complete protection. Although the protection conferred to the monkeys was most likely due to the presence of neutralizing antibodies and possibly helper T-cells against Env we wanted to assess the relative contribution of each of these peptide to the protection. In addition the number of animals involved in the study were quite limited. Because consistent protection in an outbred population will likely require the inclusion of multiple epitopes with some level of demonstrated efficacy we also evaluated the protective efficacy of the WCCR2T template peptide in the squirrel monkey model.

**SUMMARY**

Sixteen squirrel monkeys were immunized: Four monkeys were immunized with MVF175-218, 4 with WCCR2T, 4 with Tri-Tax (and T\text{H} epitope TT3), four with irrelevant peptides. MVF175-218 and WCCR2T monkeys were immunized a total of 5 times, Tri-tax monkeys were immunized a total of 4 times. Monkeys immunized with MVF175-218 had antibody titers as high as 128,000, but all 175-218 monkeys had a titer of 64,000 at the time of challenge. One WCCR2T immunized monkey had a titer as high as 64,000 at the time of challenge, while the lowest titer was 8000. All MVF175-218 monkeys produced antibodies that recognized Env as shown by flow cytometry. Two of four WCCR2T monkeys showed marginal recognition of Env. The presence of neutralizing antibodies was barely detectable due to high levels of non-specific inhibition shown by control monkey sera (at best \( \sim 10-15\% \) inhibition difference between experimental and control samples). Monkeys were challenged intravenously with \( 10^8 \) Evo/1540 cells two weeks after the 5\text{th} boost time point. These monkeys were kept for 31 weeks before sacrifice and were bled every three weeks.
One day after challenge, the six monkeys were bled. Each monkey had detectable provirus (between 0.62 and 2.97%) in their PBMC. By 10 weeks all but 4 monkeys had detectable provirus in the PBMC. Two were immunized with MVF175-218, one with Tri-tax and one a control. By 19 weeks after challenge 10 monkeys had no detectable provirus. By 25 weeks 14 of 16 monkeys had no detectable provirus (the two infected had only 0.005%). By 31 weeks only one monkey had detectable provirus in the mesenteric LN (0.01%). This unexpected phenomenon was also confirmed by the lack of detectable Gag antibodies by western blot in all animals (Ch+28).

**MATERIALS AND METHODS**

**Peptide synthesis and characterization**

MVF175-218, WCCR2T and Tri-Tax were synthesized by solid phase peptide synthesis using Fmoc/t-But chemistry on a fully automated peptide synthesizer (Model 9600 Peptide Synthesizer MilliGen/Biosearch) as described previously [161]. The WCCR2T template peptide (GKGKGKG) was synthesized with Lys side chain protected (ivDde) (BACHEM California) on Rink-Amide-CLEAR Resin (substitution 0.32 mmol amino groups/g, (Peptides International Louisville, KY). The N-termini were acetylated using Acetyl-Imidazole (4x) in DMF for 4 hr. Negative Kaiser test confirmed the completion of the acetylation reaction. The Lys side chain deprotection (ivDde) was achieved using 2% hydrazine hydrate in DMF (3 min. and 10 min, positive Kaiser Test). The gp21 (residues 347-374) peptides were assembled on the template using peptide synthesis protocol described above and acetylated at N-terminus. The peptides were cleaved from support using reagent B (Trifluoroactic acid:Phenol:Water:Triisopropyl silane 90:4:4:2).
All peptides were characterized by Matrix Assisted Laser Desorption Ionization mass spectroscopy (MALDI). (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, Ohio).

**Animal immunization, PBMC isolation and challenge**

Three groups of four squirrel monkeys (Saimiri sciureus) were immunized with MVF175-218, WCCR2T, or irrelevant peptide. Each monkey was immunized with 1 mg of peptide and 100 µg nor-MDP (N-acetyl-glucosamine-3 yl-acetyl L-alanyl-D-isoglutamine) dissolved in water and emulsified 50:50 in ISA 720 (Squalene/Arcecel A 4:1 respectively) (Seppic, Paris) [172]. All monkeys were boosted 4 times after the primary immunization (1y). Monkeys immunized with Tri-Tax and the Tri-Tax control peptide were immunized a total of four times with 1 mg of Tri-Tax or control peptide and 1.4 mg of promiscuous helper epitope TT3 derived from the tetanus toxoid residues 947-967 and 100 µg nor-MDP dissolved in water emulsified 50:50 in ISA 720.

Monkeys were bled via the femoral vein prior to each immunization and monthly after challenge. Blood was collected in tubes containing heparin (Vacutainer). Plasma was separated from whole blood by centrifugation for 10 min at 1700 RPM. Plasma was then removed and heat inactivated by heating to 56°C for 30 minutes. The cellular component was washed with an equal volume of RPMI containing 2% Penicillin/Streptomycin (Gibco). The cells were then layered on a ficol-hypaque gradient and centrifuged for 30 min at 1700RPM and no brake to separate the PBMC. Isolated PBMC were collected and washed three times with RPMI 2%P/S. PBMC were then stored in liquid nitrogen for further analysis.
Two weeks after the fifth and fourth immunization for the B-cell epitopes and Tri-Tax respectively the monkeys were challenged intravenously with $1 \times 10^8$ EVO/1540 via catheterization in the femoral vein. EVO/1540 cells were grown in RPMI containing 20% fetal bovine serum and 1% P/S 1% glutamine and 10% recombinant human IL-2. Thirty-one weeks after challenge the monkeys were sacrificed and tissues collected.

Genomic DNA was isolated by phenol chloroform extraction using standard methods briefly PBMC and tissue

**ELISA**

Antibody titers were determined using ELISA as previously described [173]. Ninety-six well plates were coated overnight with 100µl of a 2µg/ml solution of peptide in PBS at 4°C. The plates were then washed three times with PBS-Tween 0.05% and 1% horse serum (wash buffer) then blocked for 1 h with 200µl PBS-1% BSA. Plates were then washed and monkey plasma diluted in wash buffer was then added to the antigen coated plates in duplicate wells. Two-fold serial dilutions were then performed in wash buffer and incubated 2 hours at room temperature. Plates were then washed and 100µl of 1:500 goat anti-human IgG conjugated to horseradish peroxidase [173] was added to each well and further incubated for 1 hour. After washing, the bound antibody was detected using 50µl of 0.15% $\text{H}_2\text{O}_2$ in 24mM citric acid, 5mM sodium phosphate buffer, pH 5.2, with 0.5mg/ml 2,2'-aminobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Color development was allowed to proceed for 10 minutes and the reaction stopped with the addition of 25µl of 1% SDS. Absorbance was determined at 415nm using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA). Titers were defined as the highest dilution of sera with an absorbance greater than 0.2 after subtracting the background. All data represent the average of duplicate samples.
Flow cytometry

The ability of peptide induced antibodies to bind the surface of HTLV-1 infected cells was analyzed by flow cytometry. Methods derived from Hudziak et al., were used [174]. MT-2 cells were grown in RPMI 1640 with 10% FCS and 1% pen/strep. 0.5 X 10⁶ cells were incubated with a 1/20 dilution of the peptide antibodies for 1 hr at 4°C. The same dilution of pre-immune serum was used as negative controls. Cells were washed with 500µl of PBS-2% FCS. The cells were then washed twice with FACS buffer and stained by incubation with FITC labeled F(ab)_2 fragment goat anti-human polyvalent secondary antibody (1:50 dilution) for 45 minutes at 4°C in 500µl of PBS-2%FCS. Cells were then washed three times in FACS buffer then fixed in PBS containing 2% paraformadehyde and analyzed by BD FACS Caliber flow cytometer (BD). A total of 10,000 gated events were collected. Debris, cell clusters and dead cells were gated out by light scatter assessment before single parameter histograms were drawn.

Syncytia inhibition

In 24 well plates 5 x 10⁴ HTLV-1 infected MT-2 cells were incubated in 400 µl MT-2 culture medium containing various dilutions of antibody for 1 h at 37°C and 5% CO2. Following incubation, 5 x 10⁴ CosZ28 (containing lacZ under the control of the HIV-1 LTR) and 5 x 10⁴ Hela-Tat (expressing HIV-1 Tat transactivator) cells [175] will be added to each well in a final volume of 1 ml. Plates were then incubated for 20 h at 37°C and 5% CO2. Media was then aspirated and 100 µl of lysis buffer was added to each well and β-galactosidase production was detected using the Galactolight Plus® kit (Tropix). Cell lysate was incubated in 96 well plates for 30 min with substrate then read using an Lmax® luminometer (Molecular Devices). Following the injection of 100 µl/well of accelerator, plates are read for
10 sec after a 2 s delay. The % of syncytia inhibition will be defined as: \[
\frac{[(\text{luminescence of positive control wells without antibodies - background}) - (\text{luminescence of experimental wells - background})]}{\text{luminescence of positive control wells - background}} \times 100\%.
\]
Positive control wells contain MT-2, CosZ28, and Hela-Tat cells in the absence of antibodies. Background was defined as β-galactosidase production in wells containing CosZ28 and Hela-Tat cells, but not MT-2 cells. For some experiments the MT-2 culture medium contained a 1/50 dilution of mouse sera to block non-specific inhibition.

**Measurement of HTLV-1 proviral load by Quantitative PCR**

HTLV-I provirus load was measured by using an ABI PRISM 7700 Sequence Detector (Perkin Elmer/Applied Biosystems). Briefly 100ng of DNA were applied per well and analyzed. Samples were run in triplicate and the mean values of HTLV-1 pX and β-actin standards were used for calculation. The primer set for HTLV-I pX region was 5′-ACAAAGTTAACCATGCTTATTATCAGC-3′ positioned at nt 7276–7302 and 5′-ACACGTAGACTGGGTATCCGAA-3′ positioned at nt 7355–7334. (Nucleotide coordinates are numbered according to the HTLV-I reference sequence on the Genbank database). The primer set for β-actin was 5′-CACACTGTGCCCATCTACGA-3′ positioned at nt 2146–2165 and 5′-CTCAGTGAGGATCTTCATGAGGTAGT-3′ positioned at nt 2250–2225. The TaqMan fluorescent probe was 5′-TTCCCAGGGTTTGGACAGAGTCTTCT-3′ positioned at nt 7307–7332 for HTLV-I pX region and was 5′-ATGCCCTCCCCCATGACATCCTGCGT-3′ positioned at nt 2171–2196 for β-actin. DNA standards were extracted from HTLV-I–negative PBMC for b-actin and TARL-2 for pX to make a standard curve. All samples were performed in triplicate. Amplification was carried out at 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for
15 sec and 60°C for 1 min in a total volume of 50 ml. The human β-actin gene primers and probe set (Applied Biosystems) was used to calculate the HTLV-1 proviral load per 100 cells. The HTLV-1 proviral load was calculated by the following formula: HTLV-1 proviral load = [(mean values of pX) / (mean values of β-actin/2)] X 100.

**Immunoblot Analysis**

Western blot analysis was performed using the western blot diagnostic kit form with monkey plasma. Strips were placed in tray containing 2 ml wash buffer for 5 min then removed and replaced with 2ml of blotting buffer and 20 μl of sera and incubated for 1 h with constant agitation. Wells were then washed with 2 ml of wash buffer 3X for 5 min on rocker. 2 ml of working conjugate solution (1:1000 dilution of conjugate into blotting buffer). Incubated for 1 hr washed 3X with 2 ml wash buffer. 2ml of substrate was added directly to wells and incubated for 15 min on a rocker. Then washed 5x with ddwater, and dried with paper towels.

**RESULTS**

**Immunogenicity of B-cell epitopes**

Monkeys immunized with B-cell epitopes were boosted four times and bled at the time of boosting. Antibody responses to MVF175-218 and WCCR2T were determined by ELISA. As shown in Fig.2.1 a strong antibody titer (128000) was generated against MVF 175-218 by monkey 370 by the 2y+1 (1 week after the 2nd immunization) time point and dropped and remained at 64000 at the time of challenge. Monkeys 400, 401 and 402 started with more modest titers; however they were all boosted to 64000 by the time of challenge.
As shown in Fig.2.2 one WCCR2T immunized monkey (380) reached a titer of 64000 by the time of challenge while the other monkeys reached titers of 32000, 16000, and 8000 for monkeys 371, 381, and 387 respectively.

**Recognition of native Env on surface of HTLV-1 infected cells**

To determine if the peptide immunogens MVF175-218 and WCCR2T were capable of inducing antibodies that can recognize Env in the native form FACS analysis was used. As shown in Fig.2.3 each of the four monkeys immunized with MVF175-218 generated antibodies that were able to recognize native Env expressed on the surface of HTLV-1 infected MT-2 cells. However, only monkeys 371 and 380 were able to recognize the gp21 region of Env on the surface of MT-2 cells, while 381 and 387 did not exhibit any specific reactivity.

**Neutralizing capacity of squirrel monkeys immunized with B-cell epitopes**

To measure neutralizing capacity of antibodies elicited in immunized monkeys a syncytia inhibition assay was used. Two assay conditions were used, one containing horse serum to block non-specific interactions, as described in Sundaram et al., and one without horse serum [161]. As shown in Fig.2.4 sera from MVF175-218 and WCCR2T immunized monkeys inhibited syncytia better than the irrelevant immunized monkey 374. Although the modest response is due to high non-specific inhibition, the largest differences were at the 1/1600 and 1/6400 dilution. In Fig.2.5 MVF175-218 immunized monkeys were evaluated for their ability to inhibit syncytia in the presence of a 1/50 dilution of horse sera to block non-specific interactions. All MVF175-218 immunized monkeys inhibited syncytia better than the control immunized monkey (399) except for monkey 401 at the 1/100 dilution.
**Proviral load as measured by quantitative PCR**

Provirus load was measured in the monkeys to give a quantitative measure of vaccine protection by each of the immunogens. The animals were sacrificed at 31 weeks post challenge and organs were harvested. As seen in Table 2.1, six animals were bled one day after challenge to confirm a successful intravenous injection by monitoring the presence of HTLV-1 in the venous blood supply. A successful inoculation occurred as cells containing HTLV-1 provirus were detected and varied between 2.97% and 0.62% of the circulating PBMC. Ten weeks after challenge provirus was detected in all but two MVF175-218 immunized monkeys. However, by 19 weeks after challenge, ten monkeys had no detectable provirus. By 25 and 28 weeks after challenge proviral load levels dropped and were undetectable in all animals. Upon necropsy only monkey 385 had detectable provirus (0.01%) in the mesenteric LN by 31 weeks after challenge. These results suggest the monkeys resolved the infection, or were not chronically infected.

**Lack of seroconversion shown by Immunoblot**

Monkeys chronically infected with HTLV-1 should possess antibodies directed against Gag and Env proteins. Western blot was performed with sera 28 weeks after challenge. As shown in Fig.2.6, monkeys immunized with MVF175-218 (370, 400, 401, and 402 respectively) were able to recognize recombinant gp46. However, all monkeys in each group MVF175-218, WCCR2T (371, 380, 381, and 387), control (374, 399, 386, and 378), and Tri-Tax (375, 385, 391, and 403) did not have gp21 or Gag (p24 and p19) reactive antibodies. The lack of detectable antibodies by western blot is consistent with the proviral load data shown in Table 2.1, demonstrating that the monkeys were in fact not chronically infected with HTLV-1.


**DISCUSSION**

MVF175-218 was able to induce strong humoral responses in all of the immunized monkeys at the time of challenge that were able to recognize Env on the surface of HTLV-1 infected MT-2 cells. The WCCR2T peptide did not induce as strong of an antibody response and only the monkeys with the two highest antibody titers were able to recognize Env on the surface of MT-2 cells. The lower antibody titers observed in WCCR2T may be due to several reasons including the use of TT as a promiscuous T-cell activating species. The choice was governed by the fact that the promiscuous epitope should not have a lysyl residue which could interfere with the synthesis of the coiled-coil epitopes. The TT epitope is among the weaker promiscuous epitopes that we have used and has been shown to have varying efficacy in various mouse strains; therefore, monkeys 381 and 387 may express alleles that are poorly compatible with TT [176]. This may explain the low antibody titers in the monkeys and also low gp21 recognition shown by FACS. It was difficult to observe the neutralizing abilities of antibodies derived from MVF175-218 immunized monkeys or the highest titered WCCR2T (380) monkey because of high levels of non-specific inhibition in the control monkey plasma. This was resolved previously with mouse sera by adding horse serum to the media to block non-specific interactions; however, that approach was not successful with the monkey plasma [161].
Two monkeys immunized with MVF175-218, 370 and 402, interestingly had no detectable provirus after the 1d post challenge time point. However, based on the unexpected phenomenon of the monkeys resolving the infection; one cannot conclude as to whether or not the lack of detectable provirus was related to the protective effects of MVF175-218, or whether the monkeys spontaneously cleared the infection for reasons related to the transient nature of the proviral load observed in this study.

Based on studies published by Kazanji et al., squirrel monkeys can be experimentally infected with squirrel monkey PBMC lines containing HTLV-1 derived from co-culture with irradiated MT-2 cells. Intravenous injection of HTLV-1 infected homologous (allogeneic) cells are most effective for infection [85]. The presence of HTLV-1 inoculum is detectable 1 d after inoculation, but is undetectable in the blood at 3 days after inoculation. However HTLV-1 provirus can be found in the spleen and mesenteric and submaxillary lymph nodes as early as 12d after infection [148]. Tax/Rex mRNA was undetectable between 1d-12d in the PBMC of two experimentally infected monkeys, (but only detectable in the spleen of one monkey at 12d). In this model, antibody responses against HTLV-1 antigens can be detected 3-6 weeks after infection and should be detectable for at least ~ 30 weeks after infection [85, 148]. Therefore, it was surprising to see that by 28-31 weeks after challenge we were unable to detect provirus in the PBMC and mesenteric LN of the animals or the presence of a detectable antibody response except against rgp46 in the MVF175-218 immunized animals.

There are a few possible reasons why the challenge may have not worked as expected. First, the EVO/1540 cell line is not truly a “cell line,” but rather PBMC that were infected in vitro by co-culture with MT-2 cells. Only some of the cells in EVO/1540 were infected with HTLV-1 and others of course were not. We started our challenge stock of cells
with one vial provided by Dr. Kazanji and began to culture the cells. To challenge the
monkeys, we used twice as many cells as described previously in vaccine evaluation reports
and 10 times as many cells as described in the first report by Kazanji et al., to avoid the
situation that we were eventually indeed faced with. In order to challenge the monkeys with
$10^8$ one must grow ~ ten 75 cm$^2$ culture flasks per animal in the upright position with a thick
mat of cells at the bottom of the flask. Based on recommendations by Dr. Kazanji
EVO/1540 cells should be grown for about 2 weeks before the cells are split, if the cells are
split too frequently they can spontaneously die. Therefore, to prepare for challenging 16
monkeys the equivalent of ~ 200 flasks of cells (some were frozen periodically to serve as a
backup) were grown. Therefore, it is possible that during the extensive in vitro cultivation of
EVO/1540 the composition of cell populations may have changed over time. Different cell
types may have changed the cell composition over time or even less infective HTLV-1+ cell
clones may have increased their proportion in vitro.

It is also possible that the during the long in vitro co-culture period with NK cells in
the culture may have selected for HTLV-1 clones that opt for a more proviral state, as
HTLV-1 has been shown to alter the expression of MHC on the surface of infected T-cells
[20]. Lowered expression of HTLV-1 viral proteins which may include Env would decrease
the infectivity of the cells used for challenge. And although demonstrated by measuring the
efficacy of CD8$^+$ reports by Asquith et al., have shown that the phenomenon of in vitro
killing of infected cells can be a highly dynamic and variable process [84].

However, it is definite that the monkeys were in fact still challenged with a large
measurable amount of HTLV-1 resulting in ~0.5-3% of the circulating PBMC infected with
HLV-1 one day after challenge. Besides changing the composition of cell populations it is
possible that another in vitro viral attenuation mechanism was at work for the loss of infectivity seen by EVO/1540 from the multiple passages. An attenuated HIV-1 strain designed for use in vaccination has been obtained by knocking out the accessory protein Nef [177, 178]. HTLV-1 has similar accessory proteins that are dispensable for \textit{in vitro} growth, but are indispensable for infection [20]. If such a parallel exists here, some type of mutation/attenuation mechanism could have happened during culture that did not affect the growth of the cells in vitro, but may have reduced their \textit{in vivo} virulence.

Another possible explanation for the poor infectivity of EVO/1540 is that challenge with a cell line derived from an animal of the opposite sex, as would occur by sexual contact, results in more successful infection (personal communication M. Kazanji). This could be a strong argument for our results as 12 of the 16 monkeys were male monkeys, as EVO/1540 was derived from a male monkey, however this does not explain the lack of detectable provirus in the four female animals used in the study.

Regardless of the mechanism, the inability to have a strong reliable challenge is a major limitation for the squirrel monkey model of HTLV-1 infection. Because Real-time PCR methods are now available for the rabbit and rat model of HTLV-1 infection, these more established methods are more appealing for future vaccine efficacy studies.
**Fig. 2.1. Antibody responses in squirrel monkeys immunized with MVF175-218.** Plates were coated with the immunogen MVF175-218. Peptide-bound antibodies were detected with a HRP-conjugated goat anti-human secondary antibody. An arrow depicts the time point at which the immunized monkeys were challenged with $10^8$ EVO/1540 cells (an HTLV-1-transformed and producing squirrel monkey PBMC cell line). The number preceding Y represents the number of immunizations, while the number the following the Y is the number of weeks after the immunization. Therefore $5Y+2$ represents two weeks after the fifth immunization.
Fig. 2.2. Antibody responses in squirrel monkeys immunized with WCCR2T. Plates were coated with the immunogen WCCR2T. Peptide-bound antibodies were detected with HRP-conjugated goat anti-human secondary antibody. An arrow depicts the time point at which the immunized monkeys were challenged with $10^8$ EVO/1540 cells (an HTLV-1-transformed and producing squirrel monkey PBMC cell line).
Fig. 2.3. Env reactive antibodies in monkeys immunized with MVF175-218 and WCCR2T. Antisera were used to determine the ability of MVF175-218 (A), WCCR2T (B), and irrelevant peptide (C) to induce Env reactive antibodies. MT-2 cells were incubated with a 1/20 dilution of 5Y+2 monkey antisera. Antibodies bound to Env expressed on MT-2 cells were detected with FITC-conjugated goat anti-human polyvalent Ig. Serum from an HTLV-1 positive asymptomatic individual was used as a positive control (unshaded grey line). Presera and immune sera are represented by shaded and unshaded bold lines respectively.
Fig. 2.4. Induction of neutralizing antibodies in monkeys immunized with MVF175-218, WCCR2T, and irrelevant peptide. Sera from the 5Y+2 time point of immunized monkeys were evaluated for their neutralizing ability in a syncytia inhibition assay, as described in [161] without horse serum. Immunized animals are as follows: MVF175-218 (370), WCCR2T (380), irrelevant peptide (374) Infected patient positive control serum (P7). HTLV-1 infected MT-2 cells ($5 \times 10^4$) were incubated in culture medium containing four fold serial dilutions of squirrel monkey antisera, for 1 h. Following incubation, $5 \times 10^4$ CosZ28 (containing lacZ under the control of the HIV-1 LTR) and $5 \times 10^4$ Hela-Tat cells (expressing HIV-1 Tat) were added to each well. Plates were then incubated for 20 h at 37°C and 5% CO$_2$. β-galactosidase production was detected by chemiluminescence.
Fig. 2.5. Induction of neutralizing antibodies in monkeys immunized with MVF175-218. Sera from the 5Y+2 time point was used to determine the neutralizing ability of antisera to the peptide immunogens as described in Sundaram et al., [161] with the use of horse serum to block non-specific interactions. MVF175-218 immunized monkeys (370, 400, 401, 402) were evaluated and an irrelevant peptide (399) immunized monkey was used for a negative control and an infected asymptomatic individual (P9) was used as a positive control.
<table>
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<th>PVL% Ch+1d</th>
<th>PVL% Ch+10</th>
<th>PVL% Ch+19</th>
<th>PVL% Ch+22</th>
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*Sex of the monkey is shown in parenthesis

**Table 2.1. Proviral load of challenged squirrel monkeys.** Provirus load in the PBMC was determined at various time points and in the mesenteric lymph node after challenge by real-time PCR to evaluate protection.
Fig.2.6  Detection of HTLV-1 reactive antibodies by western blot. Sera from 28 weeks after challenge was used to determine seroconversion to HTLV-1 antigens. Monkeys were immunized with MVF175-218 (lanes 3, 4, 5, and 6 were monkeys 370, 400, 401, and 402 respectively) (WCCR2T (lanes 7, 8, 9, 10 were 371, 380, 381, and 387) irrelevant peptide (lanes 11, 12, 13, and 14 were 374, 399, 386, and 378) and Tri-Tax (16, 17, 18, and 19 were 375, 385, 391, and 403) Lanes 1 and 2 were positive and negative controls respectively.
CHAPTER 3

EVALUATE THE IMMUNOGENICITY AND IMMUNE CROSS-RECOGNITION OF EPITOPE ENHANCED CD8+ T-CELL EPITOPES DERIVED FROM GAG, TAX, AND POL ANTIGENS

RATIONALE

HTLV-1 infection seems to tread a thin line between disease induction in some individuals and effective viral control in other individuals. The contributing factors and the pathogenic mechanisms of HTLV-1-associated diseases are still not well understood, which has complicated the development of vaccines and therapies against HTLV-1. Several lines of evidence have supported the conclusion that possibly the most important determinant in controlling infection and not progressing to an inflammatory diseased condition is the presence of a strong cytolytic response to HTLV-1 [84, 97].

Genetic polymorphisms in MHC I and differences in the expression of CD8+ lytic factors as well as *in vitro* clearance of infected CD4+ T-cells in asymptomatic individuals compared to HAM/TSP patients have demonstrated the importance of a strong CD8+ response to control HTLV-1 infection. However, many reports have shown the production of pro-inflammatory cytokines, such as IFN-γ and TNF-α by both CD4+ and CD8+ T-cells, and some chemokines are associated with inflammatory disease. Therefore, it appears that a trade-off between this beneficial CTL response and the simultaneous production of inflammatory cytokines exists which results in pathology in a minority of patients [25, 82].
The immunodominance hierarchy reported by Goon et al., shows that although CD8\(^+\) T-cell responses in HTLV-1 infected individuals are mainly directed against the Tax antigen CD8\(^+\) T-cells are also directed Env, Pol and Gag antigens [96]. However while there have been many reports of immunity against the Tax protein and to a lesser extent other proteins; only a few reports have tried to identify specific CD8\(^+\) epitopes from other HTLV-1 proteins [94, 95].

One potential way to increase cytolytic responses against HTLV-1 antigens is the use of epitope enhanced peptides. Epitope enhanced peptides are peptides that are rationally modified to possess optimal residues for the desired MHC I molecule. This increases the affinity of the peptide for MHC I and promotes CTL activation and ultimately increased immune responses to peptides that have naturally low immunogenicity. Epitope enhanced peptides have been shown to increase cytolytic responses and cytokine producing cell frequencies against low affinity MHC I antigens better than the wild type sequence they are derived from [101, 104].

Increasing the cytolytic response of individuals infected with HTLV-1 with epitope specific therapeutic vaccination may reduce the risk of disease developing HTLV-1 from the associated inflammatory disease. The enhancement of T-cell immunity to HTLV-1 through the development of therapeutic vaccines could be of significant value, however the identification of HTLV-1 CD8\(^+\) T-cell epitopes is essential to the development of therapeutic vaccines using this strategy. Therefore, relevant subdominant epitopes involved in controlling infection must be identified; and possible improvements in immunogenicity should be investigated through epitope enhancement.
SUMMARY

To find and improve potential candidate epitopes for CD8⁺ T-cell vaccination, computer predicted algorithms were used to identify the top ranking HLA-A*0201 restricted epitopes from the HTLV-1 Gag, Tax and Pol proteins. To improve immunogenicity an epitope enhancement strategy was used. Epitopes with high scores that did not have the optimal anchor residues in positions 2 and 9 (Leu and Val respectively) of the nonameric epitopes were mutated and new predictions were made for the binding score. Additional Gag mutants were designed to include an optimal Tyr in the 1 position to evaluate the effects of immunogenicity and cross-reactivity of 1Y9V double mutants. A T2 binding assay was used to determine the experimental binding affinities of the predicted WT and mutant epitopes. The two highest affinity wild-type Gag epitopes 410-418 and 245-253, which had relatively low predicted scores responded well to 9V and 1Y9V mutations which lead to large increases in binding affinity with each mutation. The Tax epitopes 155-163 and 307-315 also showed strong increases in binding affinity by using 9V mutants while the Tax 233-241 epitope did not. Pol epitopes 264, 673, 128, and 562 showed similar moderate wild type binding. A 128 2L enhanced mutant and a 562 9V enhanced mutant showed increases in experimental binding. HLA-A*0201 transgenic mice (HHD II) were immunized with wild-type and enhanced peptides to measure increases in immunogenicity by the mutants. Gag 410 WT epitope had low specific lysis of WT pulsed target cells which was increased ~2 fold by immunizing with the 9V mutant. Immunization with the 410 1Y9V mutant only led to a small increase in 410 WT specific lysis. Immunization with 410 9V also lead to large increases in frequency of IFN-γ producing CD8⁺ T-cells when compared to 410 WT or 410.
1Y9V immunized mice. Immunization with Gag enhanced peptides 245 9V and 245 1Y9V were both able to increase cytolysis of 245 WT pulsed target cells compared to mice immunized with 245 WT; however, enhancement of the 245 epitope was unable to increase the frequency of IFN-γ producing CD8+ T-cells.

Immunization of HHD II mice with 9V enhanced peptides against Tax 155-163 and 307-315 epitopes did not result in increased cytolysis; however, modest increases in IFN-γ producing CD8+ T-cells recognizing the WT peptide were detected. Strong cytolytic responses were detected against the 264-272 Pol epitope with >60% of cytolysis, while other wild-type Pol epitopes induced cytolytic responses >30% specific lysis. Surprisingly CTL from mice immunized with various Gag, Pol, and Tax wild-type and enhanced epitopes were unable to induce specific lysis of Hut 102 A2-GFP cells. The only peptide capable of inducing specific lysis of the HTLV-1 infected HLA-A*0201 cell line was the Tax 11-19 epitope. ELISPOT performed with PBMC from a HAM/TSP patient also showed that the only epitope specifically recognized by this patient was Tax 11-19. These results suggest that Tax 11-19 may be the only major HLA-A*0201 restricted epitope from the Gag, Tax, and Pol proteins involved in controlling HTLV-1 infection.

MATERIALS AND METHODS

Prediction of CTL epitopes

The HTLV-1 Gag, Pol, and Tax protein sequences [17] were analyzed for potential HLA-A*0201 restricted epitopes using the NIH BIMASS website created by Parker et al. http://bimas.dcert.nih.gov/molbio/hla_bind/. Epitopes with the highest predicted binding score for each protein were selected for analysis. A T₁/₂ cut-off score of 10 was used for the prediction. Based on the prediction, epitopes were prioritized by score and epitopes with
suboptimal anchor residues were mutated and reanalyzed for improved scoring. To evaluate mutant peptides, sequences a single amino acid “filler” repeat was used to increase the peptide to a size that will permit analysis.

**Peptide Synthesis and Purification**

Nonameric peptides were synthesized on either Milligen/Biosearch 9600 solid-phase peptide synthesizer (Bedford, MA) or MPS 396 (Advanced Chemtech Louisville, KY) using Fmoc/t-But chemistry. CLEAR ACID resin (0.32 mmol/gm) was used for synthesis (Peptides International, Louisville, KY). All peptides were cleaved from the resin using the cleavage reagent B (Trifluoroacteic acid:Phenol:Water:Triisopropyl silane 90:4:4:2) and crude peptides were purified as reported earlier [161]. All peptides were characterized by Matrix Assisted Laser Desorption Ionization mass spectroscopy (MALDI) (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, Ohio).

**T2 Binding Assay**

In 96 well plates, \(3 \times 10^5\) 174xCEM.T2 cells (T2 cells) were incubated in various concentrations of peptide diluted in T2 assay medium (IMDM containing 2% FBS and 100 IU/mL penicillin, 100µg/mL streptomycin) for 20 h. Cells were harvested, washed twice in FACS buffer (PBS 2% FBS and 0.1% sodium azide) and stained with 1:1000 dilution of FITC conjugated HLA-A2 (BD) antibody for 30min. Cells were then washed 3X in FACS buffer and Fixed in 1% paraformaldehyde in PBS then measured by FACS analysis. Fluorescence index was defined as: (the geometric mean of the sample with peptide) / (the geometric mean of the background consisting of stained T2 cells without peptide).
Animals and Immunizations

Wild type and enhanced predicted epitopes were evaluated in HLA-A*0201 transgenic mice (HHD II) provided by Dr. Francois Lemonnier (Pasteur Institute, France) [140]. Each mouse received 100 µg of peptide + 140 µg of promiscuous T-helper epitope TT3 (derived from tetanus toxoid aa 947-967), and 100 µg of the adjuvant N-acetyl-glucosamine-3-acetyl-L-alanyl-D-isoglutamine (nor-MDP) [179] (Peninsula Laboratories, Belmont, CA) dissolved in water. The water fraction was emulsified 1:1 in ISA 720 (Seppic, France). Emulsions were injected subcutaneously at the base of the tail. One booster immunization was given three weeks after the primary immunization. Mice were immunized in groups of three and spleens were pooled when sacrificed for experiments 10 days after the booster immunization. Single cell suspensions from spleens were RBC depleted for 5 min in a 0.83% NH₄Cl solution then washed in RPMI containing 10% FBS, 2mM glutamine, 100 IU/mL penicillin, 100µg/mL streptomycin 25mM HEPES (CTL Wash buffer). Isolated spleenocytes were then used for functional assays. Mice were bred and housed at the Ohio State University in an AAALAC accredited facility. All animal procedures were approved by the institutional laboratory animal care and use committee.

Cell Lines

The murine lymphocyte cell line EL4/HHD was obtained from Dr. Francois Lemonnier and has been described elsewhere [140]. EL-4 cells have β2 macroglobulin and H-2D<sup>b</sup> genes knocked out and are stably transfected with the HLA-A*0201/D<sup>b</sup> (HHD) chimeric gene. The EL4/HHD cell line was maintained in RPMI 1640 medium supplemented with 10% FBS, 2mM glutamine, 100 IU/mL penicillin, 100µg/mL streptomycin, and 0.5mg/ml
Geneticin. T2.CEM (T2) cells were obtained from ATCC and cultured in IMDM containing 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin. Hut102-A2GFP cells were obtained from Dr. Steve Jacobson and were derived by transfecting the HTLV-1 infected cell line Hut102 with a previously described HLA-A*0201-GFP fusion construct [180]. Hut102-A2GFP cells were grown in RPMI 1640 containing 10% FBS, 2 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 1 mg/ml Geneticin. Frozen human PBMC from an HLA-A*0201 positive HAM/TSP patient were provided by Dr. Steve Jacobson and were cryopreserved until they were used for the ELISPOT assay. All cell culture components were purchased from Gibco.

**Purification of murine CD8^+ T-cells**

Isolated spleenocytes were washed once in PBS containing 0.5% BSA and 2 mM EDTA (MACS buffer). The CD8^+ T-cells from spleenocytes were positively selected using CD8α (Ly-2) MACS micro beads and LS+ positive selection columns according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Ninety µl of micro beads were incubated with 10^8 cells in MACS buffer for 15 minutes at 6°C. Cells were then washed in MACS buffer and applied onto LS+ columns placed in a magnetic field. Negative cells passed through the column by rinsing with buffer. The positive cells were then expelled out of the column after removing the column from the magnetic field.

**Human CD4^+ T cell enrichment**

PBMC were depleted of CD4^+ T cells by use of magnetic micro beads (Miltenyi Biotec), according to the manufacturer's instructions, and run through LD columns. PBMC were washed once in MACS buffer. The CD4^+ T-cells from spleenocytes were negatively selected using human CD4 MACS micro beads (Miltenyi Biotech, Auburn, CA). Incubated for 15
min at 6°C. Cells were then passed through LD+ columns placed in a magnetic field and washed with MACS buffer. CD4+ cells passed through the column by rinsing with buffer and were used for ELISPOT experiments.

**Enzyme Linked Immunospot (ELISPOT) Assay for IFN-γ Detection**

Ninety-six well flat-bottom multiscreen plates (Millipore, Bedford, MA) were be coated with 15 µg/ml of anti-IFN-γ mAb AN18 (Mabtech, Marimont, OH) overnight at 4°C. The plates were washed 5 times with PBS and blocked with ELISPOT culture medium (RPMI 1640 containing 5% FBS, 2mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 25mM Hepes buffer) for 3 h. ELISPOT media was discarded and 10^5 purified CD8+ T-cells were added along with 2.5 X 10^5 naïve syngeneic splenocytes irradiated at 5000 rads in the presence or absence of 10 µM peptide. Cells were incubated for 48 hours at 37°C and 5% CO₂ of peptide. Spots were detected using the Mabtech mouse IFN-γ detection kit as per the manufacturer’s instructions. Following the incubation plates were washed 5 times with PBS before adding a 1:1000 dilution of R4-6A2-biotin in PBS containing 0.5% FBS to each well at room temperature for 2 h at room temperature. Plates were then washed with PBS and a 1:1000 dilution of strepavidin-ALP in PBS containing 0.5% FBS to each well at room temperature for 1 h at room temperature. Plates were then washed with PBS and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) alkaline phosphatase substrate [173] was be used to develop spots for 10 min. The reaction was stopped after 10 min by washing with cold tap water and air dried before counting the spots on an Immunospot Image Analyzer (Zeiss, Oberkochen, Germany).
Patient IFN-γ ELISPOT

Cellular responses to CTL peptides were measured using methods similar to those described in [76, 96]. Flat-bottomed 96-well nitrocellulose multiscreen plates (MAIPS45, Millipore). Each well was then be precoated with the primary capture antibody to IFN-γ 1-D1K (Mabtech, Marimont, OH) at a concentration of 15 µg/mL in sterile PBS overnight at 4°C. Plates were then washed 6 times with sterile PBS. Complete medium (CM), which is RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine (Gibco), 100 IU/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) was then added to block nonspecific binding of cytokines and antibodies, and incubated at 37°C for 3 hours. The blocking solution was discarded and peptide solution (10 µM/peptide) and cells (3 x 10⁵ CD4 depleted PBMC) were to each well. All assays were performed in duplicate wells. The plates were incubated for 6 hours at 37°C in 5% CO₂. The cells were then discarded and plates were washed 6 times with PBS and then Plates incubated with 7-B6-1-biotin detection antibody for 2 hours to detect IFN-γ, streptavidin-alkaline phosphatase conjugate diluted 1:1000 in sterile PBS containing 0.5% FBS. After 1 hr of incubation at room temperature the plates were washed again with PBS and spots were developed with NBT/BCIP substrate as described above.

Measurement of cytolytic activity

Once spleenocyte suspensions were obtained from immunized HHD II mice 4x10⁶ spleenocytes were stimulated with 1x10⁶ spleenocytes from the same set of animals pulsed with 10 µM peptide for 1 h at 37°C and 5% CO₂. Target cells were washed once after the pulse with CTL wash media. Cells were cultured in RPMI 1640 with 10% FCS, 2mM glutamine, 100 IU/mL penicillin, 100µg/mL streptomycin, and 25mM Hepes buffer supplemented with 5% Rat-T-Stim without ConA (BD Labware, Bedford, MA).
refed with 1 ml of culture medium after 3 and 6 days was replaced every 48 hours. Expanded spleenocytes were harvested on day 7 the cells for use as effectors in a standard 4-hour $^{51}$Cr release assay. EL4/HHD target cells were radiolabeled with 100 $\mu$Ci Na$_2$$^{51}$CrO$_4$ (Perkin Elmer, Boston, MA)/$10^6$ cells for 1 hour. The target cells were also pulsed with 100$\mu$g relevant peptide at the time of radiolabeling. The cells were then washed 3 times with CTL wash buffer. Target cells were plated 5000 cells / well in 96-well round bottom tissue culture plates and used at different effector to target ratios (E:T) in triplicate. After 4 hours the supernatants were collected and counted on a $\gamma$ counter (Beckman model 5500). Spontaneous and maximum (100%) release was determined from wells containing either medium alone or 5% SDS. Specific Lysis was calculated in triplicate as (experimental release - spontaneous release) / (maximum release - spontaneous) X 100.

**Epitope expression and cytotoxicity assay.**

*In vitro* expanded spleenocytes described above from immunized HHD II mice were used as effectors cells in a standard $^{51}$Cr release assay. HTLV-1 infected and HLA-A*0201-GFP expressing Hut102A2-GFP target cells (kind gift for Dr. Steve Jacobson, NIH) were radiolabeled, but were not pulsed with peptide as described above. Lysis of Hut102A2-GFP cells were measure using the same methods described above.

**RESULTS**

**Selection and screening of HLA-A*0201 restricted Epitopes from the Gag, Tax, and Pol Proteins of HTLV-1**

Potential HLA-A*0201 binding peptides from the Gag, Tax, and Pol proteins of HTLV-1 were predicted using the computer program at the Bioinformatics and Molecular Analysis Section website (http://bimas.cit.nih.gov/molbio/index.html) developed by Parker
et al., [181]. Once the best binding epitopes were predicted they were then manually analyzed for the presence of optimal anchor residues at positions 2 and 9 (Leu and Val respectively. The 1 position is involved in MHC recognition as well as TCR recognition, aromatic residues (tyrosine, phenylalanine, and tryptophan) are shown to be optimal at this position. Gag peptides were also evaluated for enhancement at position 1 where Tyr is the optimal residue. Top ranking, but still suboptimal, peptides that could be mutated to optimize anchor residues were mutated and re-evaluated by the program for the potential increase in their binding score. As shown in Table 3.1 the two highest scoring 9-mer epitopes for the Gag protein are the 410 WT and 245 WT epitopes, and although these were the highest scoring epitopes they do not have relatively high scores as compared to the Tax 11-19 epitope (T2) which has a binding score of 2406 (Table 3.2). As neither epitope has Val in position 9 both epitopes increased their score greatly designing 410 9V and 245 9V mutants and both epitopes were able to increase their binding score even further by mutating the Tyr in position 1 to make double mutants as shown by 410 1Y9V and 245 1Y9V (Table 3.1). Previously reported by Okazaki et al., mutants containing tyrosine in the 1 position display the expected increased binding affinity, however they can forfeit CTL cross-reactivity and ultimately protection. This is due to the dual interaction of position 1 with the MHC and TCR. The side chains in positions 2 and 9 side chains are buried deep in the MHC I binding groove and are not believed to interact with the TCR (Fig.3.1). Therefore, we decided to only synthesize 9V and 1Y9V mutants to be evaluated with the low affinity Gag protein and chose not to synthesize 1Y single mutants for the Gag epitopes.
The Tax 11-19 epitope was the highest predicted Tax epitope and is naturally optimized at positions 2 and 9 (Table 3.2). The second highest affinity epitope Tax 178-186 (T3) has a score of 608 which also has optimized residues at positions 2 and 9 (Table 3.2). Mutants in the 9V position were then predicted for the next three highest scoring wild type epitopes 307 WT, 233 WT, and 155 WT; which were increased in score by ~3-6 fold.

As shown in Table 3.3 Pol has two strong epitopes 264 and 673 which already contain optimal residues in positions 2 and 9, however the next two highest scoring epitopes predicted were able to be enhanced. The 128 WT peptide was mutated to make a 128 2L mutant and the 562 WT was mutated to make a 562 9V mutant.

**Experimental binding of wild-type and enhanced peptides**

To determine the relative experimental binding affinities of each of the predicted epitopes the T2 binding assay was used. T2 is an HLA-A*0201 positive human lymphocyte line that is TAP deficient leading to a reduced pool of available peptides and subsequently reduced MHC I expression on the cell surface. Exogenously added nonameric peptides can bind and stabilize MHC I molecules leading to their increased surface expression. The higher the affinity of the peptide for HLA-A*0201, the more MHC I molecules are stabilized on the surface of T2 cells. As shown in Fig.3.2 Gag peptides 410 WT, 410 9V, and 410 1Y9V were able to incrementally increase HLA-A*0201 expression on the surface of T2 cells as expected based on the predicted scores. The Gag 245 WT, 9V, and 1Y9V are also in accord with the predicted binding affinities as these peptides also incrementally increased with respect to the predicted score.
The immunodominant Tax 11-19 peptide (T2) was the strongest binding wild type epitope out of any of the proteins tested as expected (Fig.3.3). The two Tax epitopes 155 WT and 307 WT were shown to benefit strongly from enhancement in position 9 as predicted. Surprisingly the wild-type epitopes 178-186 (T3) and 306-315 (T6) as well as 233 WT and its enhanced mutant 233 9V did increase HLA-A*0201 expression on the surface of T2 cells even in the presence of 100 µM peptide. Relative binding affinities of Pol peptides also did not work completely as expected either. As shown in Fig3.4. the 264 peptide which had the highest predicted affinity for a wild-type epitope only induced a modest increase in HLA-A*0201 expression. The 673 peptide had a slightly higher induction of affinity while 128, 562, and 533 all had somewhat similar comparable WT fluorescence index values. Pol 128 WT and 562 WT were enhanced strongly by the 2L and 9V mutations respectively, while the 533 9V mutant was not able to increase the affinity of the 533 epitope.

Immunogenicity and cross-reactivity of epitope enhanced peptides against HTLV-1 Gag

To evaluate whether epitope enhancement of the Gag peptides could overcome their naturally weak immunogenicity, standard 51Cr release and ELISpot assays were used. As shown in Fig.3.5A mice immunized with Gag 410 WT were able to mount a moderate cytolytic response against the immunogen and Gag 410 9V pulsed target cells, but showed little cross-reactivity with 410 1Y9V, or an HLA-A*0201 restricted irrelevant peptide (SVRD). As expected, mice immunized with the 410 9V epitope enhanced peptide were capable of generating strong cytolytic responses to the immunogen as well as the wild-type peptide (Fig.3.5B). Cytolytic responses by 410 9V CTL were actually slightly higher for WT peptide pulsed cells than 9V pulsed cells. The 410 1Y9V mutant was also able to increase
peptide affinity; however, it negatively affected cross-reactivity of the CTL with WT as well as 9V pulsed target cells (Fig.3.5C). The 410 derived peptides showed a similar trend with the frequency of peptide specific CD8\(^+\) IFN-\(\gamma\) producing cells. As shown in Fig.3.6 mice immunized with 410 WT or 410 1Y9V were able to elicit CTL capable of producing IFN-\(\gamma\), although only at moderate levels. Immunization with the 410 9V enhanced peptide markedly increased the frequency of IFN-\(\gamma\) producing cells which could be triggered by the wild-type peptide or the immunogen, the other Gag epitope analyzed, 245 WT, also responded well to epitope enhancement. Low cytolytic responses were seen in the natural epitope, however, greater than 2 fold increase in recognition of WT or 9V peptides were seen in mice immunized with 245 9V. Interestingly immunization with 245 1Y9V also showed strong increases in cytolytic responses that were able to recognize the immunogen as well as cross-react equally with 245 WT and 9V peptide pulsed targets (Fig.3.7). Although enhancement of the 245 peptide demonstrated strong increases of cytolytic responses neither of the 245 enhanced peptides was unable to increase the frequency of IFN-\(\gamma\) producing CD8\(^+\) T-cells capable of recognizing the WT peptide (Fig.3.8).
Sub-optimal Tax peptides were analyzed for their ability to increase immunogenicity using the epitope enhancement strategy. As shown in Fig.3.9 and Fig.3.10 mutation of the 155 WT peptide to 155 9V did not increase cytolytic responses, however immunization with 155 9V lead to a small increase in the frequency of IFN-γ producing CD8+ T-cells that recognize 155 WT. The second Tax epitope evaluated, 307, also showed similar results to those of the 155 epitope. Immunization of mice with the 307 9V epitope did not lead to any detectable increases in cytolytic responses (Fig.3.11); however, detectable increases in the frequency of IFN-γ producing CD8+ T-cells that recognize 307 WT were observed in mice immunized with 307 9V (Fig.3.12).

The cytolytic responses of four wild-type Pol epitopes were also evaluated using °°Cr release. When balancing the computer based predictions and the T2 binding assay it appears that the peptides epitopes had cytolytic properties correlated similar closer related to their T2 binding scores than their computer predicted scores except for the 264 epitope which elicited stronger cytolytic responses similar to the Tax 11-19 (T2) control peptide as (Fig.3.13) as suggested by the predicted binding score (Table 3.3). As shown in Fig.3.14 the 673 Pol immunogen induced a cytolytic response which was only less than half as strong as the T2 immunogen The Pol epitopes 128 and 562 also had similar cytolytic levels of cytotoxicity compared to the Tax 11-19 epitope (Fig3.15 and Fig 3.16).

To measure the level of epitope expression on the surface the HTLV-1 infected HLA-A*0201 cell line was used as a target cell line for a cytotolytic assay. In Fig.3.17 the susceptibility of the Hut 102 A2-GFP cells to lysis mediated by spleenocytes from HHD II mice immunized with Tax 11-19 (T2) is shown. Hut 102 A2-GFP cells were about half as susceptible to lysis mediated by T2 splenocytes as EL4/HHD cells pulsed with peptide.
However as shown in Fig.3.17 mice immunized with 410 WT or the more immunogenic 9V mutant or 1Y9V mutant were unable to induce cell mediated lysis of Hut 102 A2-GFP cells. Furthermore neither of the Tax 155 or 307 WT or 9V epitopes were able to induce any detectable levels of CTL mediated lysis of the Hut 102 A2-GFP cells (Fig.3.18 and Fig.3.19). CTL from mice immunized with the high scoring wild-type Pol epitopes 264 and 673 were also unable to specifically lyse Hut 102 A2-GFP cells (Fig.3.20 and Fig.3.21). And finally the wild-type and enhanced mutants of Pol epitopes 128 and 562 were also unable to induce Hut 102 A2-GFP specific lysis (Fig.3.22 and Fig.3.23).

ELISPOT was performed to detect whether an HLA-A*0201 positive HAM/TSP patient developed immune responses to any of the WT epitopes used in this study. Detecting the immune responses by ELISPOT was previously been a problem due to the high background of IFN-γ producing cells in HTLV-1 infected individuals. Background IFN-γ production was minimized using a 6 hr incubation [96]. The only responses noticeably detectable over background in the patient were made were against The Tax 11-19 epitope (Fig.3.24). All other epitopes seem to be comparable with media alone. These results have been confirmed by our collaborator, Dr. Eduardo Gotuzzo, in Lima, Peru who has observed similar trends in a larger number of A2 patients (data not shown).

DISCUSSION

The development of epitope based CTL vaccination is sometimes criticized because of the high binding specificity of MHC I and the diversity among the alleles in human populations. However, as we continue our efforts to develop a therapeutic vaccine to augment immune responses capable of controlling HTLV-1 infection, the data derived from the use of HHD II mice can in fact be translated easily to patients in an endemic country.
such as Peru, as ~ 50% of the patients are HLA-A2 seropositive (Dr. Eduardo Gotuzzo, UMPCH, personal communication). Although HLA-A02 and HLA-A*0201 differ slightly in preferred epitopes there is much redundancy in peptide binding, and algorithms have shown that some of the predicted epitopes investigated here could bind the A2 motif with even higher affinity than HLA-A*0201 (Data not shown).

The binding score was determined using a predictive computer algorithm that calculates the relative contribution of finding each amino acid sidechain to binding stability irrespective of the overall sequence [181]. Therefore, it is possible that certain amino acid residues located in direct succession could have an additional positive or negative effect on the affinity of a peptide for HLA-A*0201, but are not considered in this algorithm. This may explain discrepancies between the predicted score and binding affinity of certain peptides. Both Gag 410 and 245 WT and enhanced peptides showed progressively increased affinity consistent with strong increases in predicted binding score and subsequent immunogenicity. It is currently unclear why the Gag peptides showed stronger correlations between the predicted binding score and the T2 experimental binding affinity than some of the Tax and Pol peptides. These results are also somewhat conflicting with reports of experimental binding affinities by Pique et al., which have reported that there are many wild type epitopes that should bind HLA-A*0201 with similar or better affinity than Tax 11-19. However much of this data is not supported by our binding data, computer predicted scores, or basic observations of anchor residue binding motifs [182]. However reports such as these and others were responsible for forming our original hypothesis that there should be many relevant epitopes that could be important for CTL mediated control of HTLV-1 and could be improved by epitope enhancement [95, 96, 182, 183].
The use of predictive computer algorithms to identify HLA binding motifs within a protein of interest is an extremely useful tool to begin a search for relevant epitopes for peptide based vaccination. This is essential because all CTL epitopes must in fact bind the specific MHC I allele to some degree to be expressed on the surface of virus infected cells and therefore be selected as a possible vaccine candidate [184]. However, the processing of MHC I restricted peptides is an extremely complicated and dynamic process and many other factors are involved in the generation MHC I epitopes in addition to the presence of appropriate MHC I binding motifs. When generating peptides for MHC I presentation, many potential epitopes with optimal binding motifs are destroyed, and are not available for presentation. The presentation of antigenic peptides is highly inefficient. Only 0.1% of cellular peptides that remain intact after cellular degradation are presented on MHC I molecules on the surface of cells [185]. Ubiquitinated peptides degraded by the proteasome result in a defined C-terminus, but a highly undefined amino terminus and peptides of varying length 4-20 aa [186]. Peptide cleavage sites and peptide frequencies can also be influenced by the expression of the IFN-γ inducible immunoproteasome in contrast to the 26S constitutive proteasome. One study demonstrated that the generation of the immunodominant epitope from lymphocytic choriomeningitis virus was increased upon induction of the immunoproteasome. At the same time, the generation of a subdominant epitope from the same protein was significantly reduced [187]. It is highly likely that the immunoproteasome plays a particularly important role in the generation of HTLV-1 epitopes, as HTLV-1 infection leads to spontaneous IFN-γ production. The amino-terminus of larger peptides are then trimmed by aminopeptidases such as TPPII and TOP which cleave peptides that are greater than 16 aa and 8-17 aa respectively [186]. Once these
peptides are cleaved to 8-12 aa in length, they can be transported into the ER lumen where peptides are loaded onto MHC I. However, newly generated peptides only have about 8 seconds to bind TAP for transport into the ER before being degraded, limiting potential epitopes. Peptides transported into the ER can be loaded on to MHC I, or are further trimmed by aminopeptidases that reside in the ER such as ERAP1 before being loaded onto MHC I; also contributing to the generation or destruction of potential MHC I epitopes [186]. Because of the continued presence of peptidases in the ER, high affinity peptides could have an advantage in the ER as lower affinity peptides may be more likely to dissociate and be degraded than higher affinity epitopes. However many low affinity epitopes are found on the surface in higher abundance than high affinity epitopes as there as numerous factors contribute to peptide antigenicity [188]. All of these processes contribute to epitope presentation, and are driven by the specific sequence of the epitope; as well as the sequence around the epitope. These are major factors involved that can determine what peptides are available in the pool before MHC I binding motifs are involved at all in determining the presented repertoire of epitopes for any antigen [188].

Other factors have also been shown to affect the rate of epitopes on the surface including rate of protein synthesis. It is estimated that one MHC I complex is formed per 2,000 to 10,000 protein molecules degraded. Therefore, the relative abundance of antigens within a cell can be a contributing factor that determines the likelihood of epitopes presented on the surface [188]. This can be readily observed with self-antigens as those most commonly presented on MHC I are ribosomal and heat-shock antigens [185].
Based on reports by Goon et al., and others is was quite surprising that some of the highest predicted binding epitopes from Gag, Tax and Pol and their increased affinity mutants were unable to elicit CTL capable of killing the Hut 102 A2-GFP cell line. It is possible that although the Pol peptide had some strong binding epitopes, one molecule of Pol is expressed for every \( \sim 100 \) molecules of Gag [33]. This and other sequence-based processing factors described earlier in this section may help to explain the immunodominance of Tax 11-19. Although only Hut 102 A2-GFP experiments with 264 and 673 were repeated and further studies must be performed to draw definitive conclusions; it appears that Tax 11-19 is the only epitope from Gag, Tax, and Pol, bound to HLA-A*0201 molecules in a high enough concentration to sensitize the cells for CTL mediated lysis. This is at first glance contradictory with reports by Goon et al., that Tax, in addition to Gag and Pol should be involved in CD8\(^+\) responses. However, these experiments were carried out for each patient using pools of overlapping peptides combined per antigen. Peptide pools and patient data were compiled by antigen and were irrespective of MHC I type therefore the responses detected to a protein such as Gag were likely to have been against another MHC I allele other than HLA-A*0201.

Another possible explanation for the lack of epitopes other than Tax 11-19 sensitizing the Hut102 A2-GFP cells to lysis is that endogenous generation of MHC I epitopes has been shown to be more efficient in inducing presentation of MHC I epitopes than exogenously supplied epitopes, particularly in dendritic cells, effecting immunogenicity [189]. However, this also does not completely explain the lack of activity by other epitopes, since Tax 11-19 was capable of inducing an adequate CTL response against Hut102 A2-GFP cells. Therefore, the delivery mode of immunogen does not seem to be problematic.
This is the first report to our knowledge of combining CTL derived from HHD II mice with HLA-A*0201 target cells instead of HHD target cells, which were engineered for compatibility with mouse CD8. However, it has been shown that high affinity peptide interactions can overcome the need for CD8 engagement to exert effects such as cell mediated lysis and production of cytokines such as TNF-α and IFN-γ [190]. Although Tax 11-19 CTL were able to induce detectable lysis of the Hut 102 A2-GFP cells, these were only about half the level detected with EL4/HHD peptide pulsed target cells. HTLV-1 Tax based disruptions in the cell include anti-apoptotic pathways/cell cycle arrest pathways (some described in introduction). It is therefore possible that Hut 102 A2-GFP cells could be inherently resistant to CTL mediated lysis making them less sensitive to lysis the peptide pulsed EL4/HHD cells. However, it is just as plausible that the peptide pulsed EL4/HHD cells could have been have a higher frequency of Tax 11-19 pMHC expressed on the surface compared to Hut 102 A2-GFP cells due to the high pulsing concentration. It is also difficult to make direct comparisons between the two cells lines because the relative expressions of HLA-A*0201 on the surface of the cells is unknown; as well as the presence of human, not mouse, α1 domain on the HLA-A*0201 molecule of the Hut102 A2-GFP cells, which could have some level of incompatibility with mouse derived CD8+ T-cells.

The results for the patient ELISPOT seem to support the hypothesis that Tax 11-19 may be the only major epitope derived from the Gag, Tax, or Pol proteins for HLA-A*0201 patients. Taken alone the ELISPOT results suggest that although Tax 11-19 is the only epitope inducing an immune response because it is immunodominant, however it would not necessarily mean that other epitopes are not making it to the surface in some concentration, just that they are not expressed on the surface enough to elicit an immune response.
However, when combined with the Hut102 A2-GFP results, it is more likely to conclude that they are not feasible targets to pursue for effective multivalent vaccination. However, therapeutic vaccination and epitope enhancement should not be completely abandoned as these strategies may be important for other MHC I alleles.

In conclusion, epitope enhancement can be a highly effective strategy to increase cytolytic responses against specific CTL epitopes of desired MHC I alleles. However, the “enhanceability” is specific to each epitope and a priori considerations must be confirmed experimentally. The effectiveness of epitope enhancement ultimately is in the adequate expression of a low affinity epitope on the surface of target cells to render them sensitive to lysis. Therefore it is possible for highly enhanceable epitopes could still have either no or inadequate efficacy. Tax 11-19 has optimized anchor residues so epitope enhancement is not a viable strategy to increase this peptide’s cytolytic character; however, as controversy revolves around the epitope’s role in protection and disease onset, much work can be done to develop this epitope as a therapeutic vaccine candidate. One such approach is to pursue immunization strategies with various adjuvants shown to specifically increase cytolytic responses. Vaccine preparations that included either IL-2 or IL-27 have been shown to directly increase cytolytic responses, and may have future application in a therapeutic vaccine designed to increase cytolysis [191, 192].
<table>
<thead>
<tr>
<th>Start Position</th>
<th>Sequence</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>245 1Y9V</td>
<td>YLWLAAFAV</td>
<td>15402</td>
</tr>
<tr>
<td>245 9V</td>
<td>QLWLAAFAV</td>
<td>3348</td>
</tr>
<tr>
<td><strong>245 WT</strong></td>
<td>QLWLAAFAA</td>
<td>239</td>
</tr>
<tr>
<td>410 1Y9V</td>
<td>YLLDLPADV</td>
<td>4853</td>
</tr>
<tr>
<td>410 9V</td>
<td>LLLDLPADV</td>
<td>1794</td>
</tr>
<tr>
<td><strong>410 WT</strong></td>
<td>LLLDLPADI</td>
<td>269</td>
</tr>
</tbody>
</table>

**Table 3.1.** Predicted binding scores of enhanced Gag CTL epitopes (HLA-A*0201)
<table>
<thead>
<tr>
<th>Start Position</th>
<th>Sequence</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>307 9V</td>
<td>LLFEEYTNV</td>
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<tr>
<td>307 WT</td>
<td>LLFEEYTNI</td>
<td>403.57</td>
</tr>
<tr>
<td>233 9V</td>
<td>GLLPFHSTV</td>
<td>977.90</td>
</tr>
<tr>
<td>233 WT</td>
<td>GLLPFHSTL</td>
<td>300.35</td>
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<tr>
<td>155 9V</td>
<td>YLYQLSPPV</td>
<td>1023.80</td>
</tr>
<tr>
<td>155 WT</td>
<td>YLYQLSPPI</td>
<td>153.57</td>
</tr>
<tr>
<td>Tax 11-19</td>
<td>LLFGYPVYV</td>
<td>2406.15</td>
</tr>
<tr>
<td>Tax 178-186</td>
<td>QLGAFLTNV</td>
<td>607.88</td>
</tr>
</tbody>
</table>

**Table 3.2.** Predicted binding scores of enhanced Tax CTL epitopes HLA-A*0201
### Table 3.3. Predicted binding scores of enhanced Pol CTL epitopes (HLA-A*0201)

<table>
<thead>
<tr>
<th>Start Position</th>
<th>Sequence</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>264</td>
<td>ALLGEIQWV</td>
<td>3255</td>
</tr>
<tr>
<td>673</td>
<td>TLYRLHVWV</td>
<td>1224</td>
</tr>
<tr>
<td>128 2L</td>
<td>FLPYFAFTV</td>
<td>7051</td>
</tr>
<tr>
<td>128 WT</td>
<td>FQPYFAFTV</td>
<td>715</td>
</tr>
<tr>
<td>562 9V</td>
<td>LLSRKVYV</td>
<td>1496</td>
</tr>
<tr>
<td>562 WT</td>
<td>LLSRKVYL</td>
<td>459</td>
</tr>
<tr>
<td>533 9V</td>
<td>YLYHYLRTV</td>
<td>471</td>
</tr>
<tr>
<td>533 WT</td>
<td>YLYHYLRTL</td>
<td>145</td>
</tr>
</tbody>
</table>
Fig. 3.1 Hypothetical structures of HLA-A*0201 restricted epitopes derived from residues 410-418 from HTLV-1 Gag. 410 WT, 410 9V, and 410 1Y9V peptide structures were generated using Swiss PDB viewer beginning with coordinates described in [193]. MHC I binding anchor residues at positions 2 and 9 are shown in blue. Residues in position 1 which interacts with the HLA-A*0201 MHC molecule and the T-cell receptor (TCR) are shown in yellow. Residues in the “bulge” of the epitope comprising the main residues involved in T-cell recognition are shown in Red.
Fig. 3.2. T2 binding assay of Gag 410 and 245 wild-type and enhanced peptides. 3 x 10⁵ 174CEM.T2 cells were incubated in 100 μM or 25 μM peptide diluted in T2 assay medium for 20 h. Cells were harvested washed twice in FACS buffer and stained with 1:1000 dilution of FITC conjugated HLA-A2 antibody for 30 min. Cells were then washed and fixed in 1% paraformaldehyde in PBS. Cells were then measured by FACS analysis. Fluorescence index (FI) is defined as: (The geometric mean of the sample with peptide) / (the geometric mean of the background consisting of stained T2 cells without peptide).
Fig. 3.3. T2 binding assay of Tax wild-type and enhanced peptides. $3 \times 10^5$

174CEM.T2 cells were incubated in 100 µM or 25 µM peptide diluted in T2 assay medium for 20 h. Cells were harvested washed twice in FACS buffer and stained with 1:1000 dilution of FITC conjugated HLA-A2 antibody for 30 min. Cells were then washed and fixed in 1% paraformaldehyde in PBS. Cells were then measured by FACS analysis. Fluorescence index (FI) is defined as: (The geometric mean of the sample with peptide) / (the geometric mean of the background consisting of stained T2 cells without peptide). The epitopes T2, T3, and T6 correspond to Tax epitopes 11-19, 178-186, and 306-315 respectively.
Fig. 3.4. T2 binding assay of Pol wild-type and enhanced peptides. $3 \times 10^5$ 174CEM.T2 cells were incubated in 100 µM or 25 µM peptide diluted in T2 assay medium for 20 h. Cells were harvested, washed twice in FACS buffer, and stained with 1:1000 dilution of FITC conjugated HLA-A2 antibody for 30 min. Cells were then washed and fixed in 1% paraformaldehyde in PBS. Cells were then measured by FACS analysis. Fluorescence index (FI) is defined as: (The geometric mean of the sample with peptide) / (the geometric mean of the background consisting of stained T2 cells without peptide).
Fig. 3.5. Cytolytic responses and cross-reactivity of epitope enhanced peptides derived from Gag 410-418. Groups of three mice were immunized twice, with peptides 410 WT (A) 410 9V (B) or 410 1Y9V (C) against aa 410-418 of Gag. After 6 days of *in vitro* expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCro4 (100 µCi/10⁶ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.6. Immunogenicity and cross-reactivity of epitope enhanced peptides derived from Gag 410-418 as shown by ELISPOT. Frequency of IFN-γ producing cells were measured by the co-culture of $10^5$ CD8$^+$ purified spleenocytes from HHD mice immunized with: 410 WT (A), 410 9V (B), or 410 1Y9V (C) with $2.5 \times 10^5$ naïve irradiated feeder cells/well for 48 h in the presence of $10 \mu M$ peptide. Anti-mouse CD3ε (2 µg/well) was used as a positive control and SVRD peptide was used as a negative control. Detection of spots were performed using the mouse IFN-γ ELISPOT kit by Mabtech as per manufacturer's instructions.
Fig. 3.7. Cytolytic responses and cross-reactivity of epitope enhanced peptides derived from Gag 245-253. Groups of three mice were immunized twice, with peptides 245 WT (A) 245 9V (B) or 245 1Y9V (C) against aa 245-253 of Gag. After 6 days of *in vitro* expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCrO₄ (100μCi/10^⁶ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.8. Immunogenicity and cross-reactivity of epitope enhanced peptides derived from Gag 245-253 as shown by ELISPOT. Frequency of IFN-γ producing cells were measured by the co-culture of $10^5$ CD8⁺ purified splenocytes from HHD mice immunized with: 245 WT (A), 245 9V (B), or 245 1Y9V (C) with $2.5 \times 10^5$ naïve irradiated feeder cells/well for 48 h in the presence of 10 μM peptide. Anti-mouse CD3é (2 μg/ well) was used as a positive control and SVRD peptide was used as a negative control. Detection of spots were performed using the mouse IFN-γ ELISPOT kit by Mabtech as per manufacturer’s instructions.
Fig. 3.9. Cytolytic responses and cross-reactivity of epitope enhanced peptides derived from Gag 155-163. Groups of three mice were immunized twice, with peptides 155 WT (A) or 155 9V (B) against aa 155-163 of Tax. After 6 days of in vitro expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCrO₄ (100µCi/10⁶ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig.3.10. Immunogenicity and cross-reactivity of epitope enhanced peptides derived from Tax 155-163 as shown by ELISPOT. Frequency of IFN-γ producing cells were measured by the co-culture of $10^5$ CD8$^+$ purified spleenocytes from HHD mice immunized with: 155 WT (A), 155 9V or (B) with $2.5 \times 10^5$ naïve irradiated feeder cells/well for 48 h in the presence of 10 µM peptide. Anti-mouse CD3ε (2 µg/well) was used as a positive control and SVRD peptide was used as a negative control. Detection of spots were performed using the mouse IFN-γ ELISPOT kit by Mabtech as per manufacturer’s instructions.
Fig. 3.11. Cytolytic responses and cross-reactivity of epitope enhanced peptides derived from Tax 307-315. Groups of three mice were immunized twice, with peptides 307 WT (A) or 307 9V (B) against aa 307-315 of Tax. After 6 days of in vitro expansion spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCrO₄ (100µCi/10⁶ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.12. Immunogenicity and cross-reactivity of epitope enhanced peptides derived from Gag 307-315 as shown by ELISPOT. Frequency of IFN-γ producing cells were measured by the co-culture of $10^5$ CD8$^+$ purified spleenocytes from HHD mice immunized with: 307 WT (A) or 307 9V (B) with $2.5 \times 10^5$ naïve irradiated feeder cells/well for 48 h in the presence of 10 μM peptide. Anti-mouse CD3ε (2 μg/well) was used as a positive control and SVRD peptide was used as a negative control. Detection of spots were performed using the mouse IFN-γ ELISPOT kit by Mabtech as per manufacturer’s instructions.
Fig. 3.13. Cytolytic responses induced by the 264 Pol Immunogen. Groups of three mice were immunized twice, with peptides against aa 264-272 of Pol. After 6 days of in vitro expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCro₄ (100μCi/10⁶ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig.3.14. Cytolytic responses induced by the 673 Pol Immunogen. Groups of three mice were immunized twice, with peptides against aa 673-681 of Pol. After 6 days of \textit{in vitro} expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCro$_4$ (100\,$\mu$Ci/10$^6$ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of $\gamma$-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.15. Cytolytic responses induced by the 128 Pol Immunogen. Groups of three mice were immunized twice, with peptides against aa 128-136 of Pol. After 6 days of \textit{in vitro} expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCro\textsubscript{4} (100\textmu Ci/10\textsuperscript{6} cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of \gamma\textsuperscript{-}irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.16. Cytolytic responses induced by the 562 Pol Immunogen. Groups of three mice were immunized twice, with peptides against aa 562-570 of Pol. After 6 days of *in vitro* expansion, spleenocytes were then incubated with EL4/HHD target cells pulsed with NaCro₄ (100µCi/10⁶ cells) and relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.17. Cytolytic responses of mice immunized with wild-type and enhanced peptides derived from Gag 410-418 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides 410 WT, 410 9V, or 410 1Y9V against aa 410-418 of Gag. After 6 days of in vitro expansion, spleenocytes were then incubated with Hut 102 A2-GFP target cells pulsed with NaCrO₄ (100µCi/10⁶ cells). Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector:target (E:T) ratios.
Fig.3.18. Cytolytic responses of mice immunized with wild-type and enhanced peptides derived from Tax 155-163 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides 155 WT or 155 9V against aa 155-163 or 11-19 (T2) of Tax as a positive control. After 6 days of in vitro expansion, splenocytes were then incubated with Hut 102 A2-GFP (A2) target cells pulsed with NaCrO₄ (100μCi/10⁶ cells) or EL4/HHD cells pulsed with relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.19. Cytolytic responses of mice immunized with wild-type and enhanced peptides derived from Tax 307-315 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides 307 WT or 307 9V against aa 307-315 or 11-19 (T2) of Tax as a positive control. After 6 days of in vitro expansion, splenocytes were then incubated with Hut 102 A2-GFP (A2) target cells pulsed with NaCro₄ (100µCi/10⁶ cells) or EL4/HHD cells pulsed with relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.20. Cytolytic responses of mice immunized with Pol 264-272 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides against aa 264-272 of Pol or Tax 11-19 (T2) as a positive control. After 6 days of in vitro expansion, spleenocytes were then incubated with Hut 102 A2-GFP (A2) target cells pulsed with NaCro4 (100µCi/10^6 cells) or EL4/HHD cells pulsed with relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.21. Cytolytic responses of mice immunized with Pol 673-681 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides against aa 673-681 of Pol or Tax 11-19 (T2) as a positive control. After 6 days of *in vitro* expansion, spleenocytes were then incubated with Hut 102 A2-GFP (A2) target cells pulsed with NaCro₄ (100µCi/10⁶ cells). Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig.3.22. Cytolytic responses of mice immunized with wild-type and enhanced peptides derived from Pol 128-136 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides 128 WT or 128 2L against aa 128-136 of Pol or Tax 11-19 (T2) as a positive control. After 6 days of in vitro expansion, spleenocytes were then incubated with Hut 102 A2-GFP (A2) target cells pulsed with NaC3O4 (100µCi/10⁶ cells) or EL4/HHD cells pulsed with relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector : target (E:T) ratios.
Fig. 3.23. Cytolytic responses of mice immunized with wild-type and enhanced peptides derived from Pol 562-560 against the Hut 102 A2-GFP cell line. Groups of three mice were immunized twice, with peptides 562 WT or 562 9V against aa 562-560 of Pol or Tax 11-19 (T2) as a positive control. After 6 days of in vitro expansion, spleenocytes were then incubated with Hut 102 A2-GFP (A2) target cells pulsed with NaCrO$_4$ (100µCi/10$^6$ cells) or EL4/HHD cells pulsed with relevant or irrelevant (SVRD) peptide. Cytolysis was measured by the amount of γ-irradiation present in the supernatant after 4 hr incubation of various effector:target (E:T) ratios.
Fig. 3.24. Frequency of IFN-γ producing cells against HLA-A*0201 restricted peptides derived from HTLV-1 Gag, Tax and Pol protein in an HTLV-1 infected HAM/TSP patient. PBMC from and HTLV-1 infected patient were depleted of CD4+ T-cells and a 4hr ELISPOT was performed with 3 X 10^5 CD4 depleted PBMC.
CHAPTER 4

EVALUATE THE EFFICACY OF FUSION INHIBITORS DERIVED FROM HTLV-1 ENV

RATIONALE

It is well established that HAM/TSP patients generally have an elevated provirus load; however, it is not fully understood whether or not the elevated provirus load drives the disease state or if it is a hallmark of a well established infection [82]. Although it has been suggested that HTLV-1 may rely more heavily on cell division than HIV; it is unknown how much of a contribution cell-cell transmission within an individual has on the maintenance of provirus load versus the division of infected cells. If one were to assume that cellular transmission plays a significant role in the maintenance of provirus load and that elevated proviral load is driving the inflammation associated with HAM/TSP, then strategies to reduce cell-cell transmission of HTLV-1 could have great value in treating HAM/TSP patients or patients with other HTLV-1 associated inflammatory diseases. The use of HTLV-1 fusion inhibitors is one possible strategy to reduce continuous cell-to-cell transmission of HTLV-1. The effective clinical application and FDA approval of Fuzeon for HIV-1 treatment has recently created a new class of drugs for retroviral treatment. Many methodologies to inhibit viral fusion have appeared in the literature which most frequently consist of peptide based strategies, but small organic molecules have also been used [194]. T20 is a linear 36 aa peptide derived from the HR2 region of HIV-1. Coincidently a 30 aa
peptide with fusion inhibiting properties was described 10 years ago from an analogous region of the HTLV-1 Env (P400), in addition to a 25 aa peptide derived from the SU subunit of Env (P197); however, these peptides have never been tested in vivo [159]. One drawback of Fuzeon therapy is that treatment consists of two 90 mg subcutaneous injections daily and therefore is extremely expensive [164]. Although P400 and P197 have never been evaluated in vivo, one could assume that low half-life and high cost would also be associated with these peptides if used as a treatment for HTLV-1 associated inflammatory diseases. The use of retro-inverso peptides is one strategy to address the problem of peptide half-life. Retro-inverso peptides are peptides made from D- amino acids in a reversed sequential order. The use of D-amino acids makes the peptide resistant to cellular proteases but reverses the peptide backbone. The reversal of backbone orientation is negated by reversing the peptide sequence which restores the sidechain spatial orientation, but leaves a non-natural backbone. Therefore, this strategy is employed assuming the sidechains contribute to the majority of a peptide’s binding character [195].

It has also been reported that readily measurable improvements in efficacy can be produced by mutating 3 key residues in the P400 peptide to a natural Env sequence variant (Pcr400). These results suggest that rationale mutant inhibitors may yield P400 derived peptides with improved neutralizing capacity and thus should be further investigated [59].

SUMMARY

To identify and improve the properties of HTLV-1 peptide fusion inhibitors, several aspects were investigated. To improve the half-life of potential HTLV-1 fusion inhibitors retro-inverso peptide mimetics were used to evaluate the in vitro efficacy. The previously described P400 and Pcr400 were first synthesized and evaluated as endblocked L-amino acid...
inhibitors. Based on syncytia inhibition experiments P400 inhibited syncytia better than Pcr400; therefore, this sequence was used to make a retro-inverso analogue (RI P400). Circular dichroism (CD) measurements showed that the peptides had very similar mirror image secondary structure and % helicity. P400 and RI P400 both showed strong syncytia inhibition at 100 µM; however, the efficacy of RI P400 declined quickly with decreasing concentration. To improve the fusogenic capacity of the gp46 derived fusion inhibitor, additional amino acid residues were added to P197 to see if a longer version of the peptide corresponding to the region 175-218 (P175) would improve the inhibitory capacity of P197 as use of this peptide as a B-cell vaccine includes additional neutralizing epitopes [196]. Syncytia inhibition experiments showed that the increased length slightly lowered the ability of the peptide to inhibit syncytia, most likely due to steric constraints blocking the association of the P197 region with gp46. Once P197 was shown to be better than a longer gp46 peptide, we hypothesized that the use of P197 and P400 may work synergistically as they inhibit syncytia at two important and independent steps in fusion. However, syncytia inhibition experiments showed that the presence of P197 seemed to antagonize the activity of P400. Finally, to begin to improve P400 based inhibitors by rational mutation, a shortened P400 (Pm400) peptide as well as Pm401C and Pm401S mutants were synthesized by mutating the Arg residue in the 401 position of the Env sequence to Cys and Ser respectively. Twenty-two amino acid peptides were made on a multiple peptide synthesizer so that the mutants could be rapidly screened; however the shortened peptides were found to have much lower neutralizing capacity due to the decreased length. Differences could not be readily observed between the Pm401C and Pm401S peptides, however because all of the peptides were equally better than the scrambled control peptide. The Ser401 mutation may
yet have a useful application and should be further investigated as a full length inhibitor. The studies shown and conclusions drawn here demonstrate that there are many approaches that can be used to modify and improve fusion inhibitors and that therefore may be used to eventually treat HTLV-1 associated diseases; therefore, continued efforts are needed to develop the full potential of such therapies.

MATERIALS AND METHODS

Peptide synthesis

Peptide synthesis was performed on a Milligen/Biosearch 9600 solid-phase peptide synthesizer (Bedford, MA) or a 40 well multiple peptide synthesizer (MPS 396, Advanced Chem Tech, Louisville, KY) using Fmoc/t-But chemistry. CLEAR amide resin (0.32 mmol/gm) (Peptides International, Louisville, KY) was used, resulting in the amidated C-terminus of all the peptides. After the synthesis and removal of the Fmoc-group from the N-terminus, confirmed by Kaiser test (positive), the N-terminus was acetylated on resin using N-acetyl-imidazole in DMF (4x for 4h) negative Kaiser Test confirmed completion of acetylation. All peptides were cleaved from the resin using the cleavage reagent B (Trifluoroacteic acid:Phenol:Water:Triisopropylsilane 90:4:4:2) and crude peptides were purified on RP-HPLC (Water’s Associates) as previously described [161]. All peptides were characterized by Matrix Assisted Laser Desorption Ionization mass spectroscopy (MALDI) (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, Ohio).

Circular dichroism measurements

Circular dichroism measurements were taken using an AVIV model 62A DS CD (Lakewood, NJ). A range of peptide concentrations (25-250 µM) were prepared in water and 50 % trifluoroethanol [197]. Spectral measurements were obtained for each peptide
concentration using a 0.1 cm path length quartz cuvette at 25 °C and under continuous nitrogen purging. Readings were taken every 1 nm between the wavelengths 260 nm to 190 nm with a 5 s averaging time. The measurements expressed in millidegrees were subtracted from water and 50 % TFE blank readings. Mean residue ellipticity (θ) was calculated using the following formula: θ = millidegrees x M_w / 10 x c x l x n, where M_w is the molecular weight of peptide, c is the concentration of peptide used in mg / ml, n is the number of amino acids, and l is the path length [198]. Percentage helicity was calculated using Chen's equation [199] with reference to the mean residue ellipticity at 222 nm for 100 % α-helix is = -35,700. 100% helicity for a particular peptide at 222 nm (Xh°) was calculated using -35,700 / (1-K / n), where k is a constant that = 2.57, and n is the number of amino acids in the chain. The calculated value of θ at 222 nm in both solvents were divided by the Xh° value of the corresponding peptide.

**Viral Fusion assay (Syncytia inhibition assay)**

Syncytia inhibition assay was performed as described in chapter 2. For these experiments horse sera was not added to the media to reduce nonspecific inhibition.

**Env transmission assay**

Construction of the HA-GLUT1 plasmid has been described previously [200, 201]. For transfection, 2 x 10^6 cells/100 ml COS-7 cells (grown in DMEM containing 10% FBS, 100U/ml penicillin, 100ng/ml streptomycin, and 2mM L-glutamine) were washed once in PBS and resuspended in electrophoration buffer (Amaxa Nucleofector™ solution V). Then 5 µg of HA-GLUT1 were added to the cells and transfected using Nucleofector™ I (Amaxa) according to the manufacturer’s instructions. After electrophoration, the cells were immediately suspended in 10 ml of culture medium in 75ml tissue culture culture flask and cultured
in 37°C / 5% CO₂ incubator. After twenty-four hours, the cells were immunostained with anti-hemagglutinin (HA) antibodies (HA.11; Berkeley Antibody Co.) to confirm GLUT expression on the cell surface. Then cell-cell Env transmission was detected by co-culturing 6.75 x 10⁵ HTLV-1 infected Hut102 cells and 6.75 x 10⁵ COS-7-GLUT-1 target cells in media containing 50 µM peptide for 48 h. After incubation, COS-7 cells were analyzed by FACS for the expression of GLUT-1-HA and Env on the surface. Cells were immunostained with anti-HA antibody (Immunogen), anti-HTLV-1 Env antibody (NIH AIDS Research and Reference Reagent Program; #309). Secondary antibodies used to detect bound primary antibodies were PE conjugated goat anti-mouse antibody [173] and goat anti-human APC. Flow cytometric analysis was performed on a FACS Caliber™ cytometer (BD Biosciences) after gating on cells by size and the absence of GFP. Data analysis was performed using CELLQuest™ software (Becton Dickinson). Background staining controls values were subtracted from the % infected values.

RESULTS

Selection of P400 region for use as a retro-inverso fusion inhibitor

Based on the seminal study performed by Sagara et al., [159] and later studies by Jinno et al., [166] and Pinon et al., [59] peptides corresponding to two linear regions of Env have been shown to inhibit syncytia formation and they were examined for further improvement; one from gp46 (P197 residues 197-216 of Env) and one from gp21 (P400 residues 400-429 of Env). The first region to be evaluated was the P400 region as it has been shown to be a more potent viral fusion inhibitor than P197. It is derived from the C-terminus of the TM region, and is similar to the HIV-1 fusion inhibitor T20. In addition, rational analysis can be performed with this region because the resolved crystal structure of
gp21 is available. As shown in Fig.4.1 the C-terminal P400 region non-covalently interacts with the coiled-coil region in the post-fusion state. Therefore, the presence of a peptide fusion inhibitor interacting with the coiled-coil region will inhibit the viral P400 region from completing the post binding conformational changes necessary to complete fusion by competitive inhibition [128]. Since the low peptide half-life of T20 is a major drawback for HIV-1 inhibitors, we wanted to address this potential problem with HTLV-1 fusion inhibitors using RI peptides mimetics. Retro-inverso peptides are modified peptide composed of D-amino acids in a reversed sequential order from the parent peptide. The use of D amino acids results in inverted chirality and the reversed order of amide bonds (-NHCO- instead of –CONH-) creates an analogue that regenerates both the planarity of peptide bonds and spatial orientation of side chains closely resembling the parent peptide. Therefore retro-inverso peptides are essentially a mirror image of a mirror image with respect to amino acid sidechain orientation (Fig.4.2) [202].

Before the RI P400 peptide was synthesized, it was necessary to decide which of the two reported sequences would be used. The earlier described P400 corresponding to the ATK strain of the virus, or the sequence corresponding to the Pcr400 peptide. The latter was described as having stronger neutralizing capacity than P400, derived from the CR strain of HTLV-1. Both peptides were synthesized in the native L-amino acid conformation. Peptides were acetylated at the N-terminus and amidated at the C-terminus for stability (Table 4.1). They were then tested for their ability to inhibit fusion using our three cell syncytia inhibition assays. Contrary to the results reported by Pinion et al., P400 was shown to inhibit fusion better than Pcr400 (Fig.4.3). Based on these findings it was decided to synthesize the RI form of P400 (RI P400) to evaluate as a fusion inhibitor (Table 4.1).
Characterization of P400 and RI P400 by circular dichroism measurement

Circular dichroism (CD) measurements were taken to observe the similarities in the secondary structure of P400 and RI P400. As shown in Fig.4.4 the two peptides show almost identical mirror image conformations when dissolved in water or 50% trifluoroethanol [197] at a concentration of 100 µM over a range of wavelengths. The change in chirality (negative $\theta$ for L-amino acids and positive $\theta$ for D-amino acids) should be expected by the use of D-amino acids, but otherwise the CD profiles show a high degree of similarity, demonstrating similar secondary structural elements between the RI peptide and the parent peptide such as helicity. Percent helicity calculations from the CD measurements [199] revealed that the % helicity of P400 in water and in 50% TFE were 8.07% and 14.32% respectively, while % helicity measurements for RI P400 were 7.40% and 14.7% for water and 50% TFE respectively, confirming the similarity in structure elements (Data not shown).

RI P400 mediated fusion inhibition

The RI P400 peptide was then evaluated for its ability to inhibit syncytia and compared with positive control peptides P400, P197 and the negative control peptide SCD28. RI P400 effectively inhibited syncytia (~80 %) at 100 µM peptide (Fig.4.5). The efficacy of RI P400 decreased sharply to 24% inhibition at 10 µM, whereas P400 maintained 94 % inhibition at the same concentration. By 1 µM RI P400 inhibited only 11% while P400 remained at 43 %. However P197 had a neutralizing character very similar to RI P400, and at the 100 µM concentration P197 only inhibited syncytia 68% and quickly dropped off thereafter.
To further evaluate the inhibitory function of the peptide inhibitors, we tested a novel assay developed by our collaborator Steve Jacobson at the NIH. This assay is based on the recent finding that increased expression of GLUT-1 increases the susceptibility of target cells to infection with HTLV-1. COS-7 cells were transfected with a GLUT-1 expression vector and incubated with HUT 102 A2-GFP cells in the presence of peptide for 48 hr. As shown in Fig.4.6 P400, RI P400, and P197 were able to reduce the amount of newly infected cells compared to the control peptide (SCRCD40L). RI P400 showed the highest inhibition of Env transmission out of all of the Env peptides.

**Increased length of P197 does not improve activity**

The P197 region, located within the central region of gp46, not only has been shown to be one of the two linear peptide fusion inhibitors, but is also a major target for the humoral response suggesting the importance of this region for Env mediated fusion. Adjacent regions of P197 are also targeted by the immune system. Therefore, we wanted to evaluate if extending the length of the peptide to aa 175-218 could increase the inhibitory efficacy of P197 [117, 157]. We also felt that the additional residues may add conformational stability to the peptide increasing the neutralizing capacity of the peptide. As shown in Fig.4.7 P197 showed ~10% greater inhibition from 100 μM to 10 μM then the 175-218 peptide. The additional N-terminal residues of 175-218 do not seem to provide any additional inhibitory capacity to P197. In the absence of a gp46 crystal structure and because the exact mechanism of P197 based inhibition is unknown, it is difficult to conclude exactly why the additional residues did not increase fusion inhibition. However, a peptide corresponding to the N-terminal residues 175-199 has been shown to have poor neutralizing capacity, as an inhibitor although antibodies to this region can inhibit syncytia [117, 159].
Therefore it seems that the N-terminal residues make the peptide slightly more bulky, and reduce its ability to fit into whatever inhibitory site it binds. Based on previous studies, the inhibitory action of this peptide occurs in the post binding stages [167]. Based on the findings in Fig.4.7, and the fact a smaller peptide is less expensive; P197 was considered as a better candidate than P175 as a fusion inhibitor.

The combination of P400 and P197 does not improve inhibition

Similar to our strategy to produce a multivalent vaccine, we wanted to see if the combination of fusion inhibitors from P197 and P400 would have a synergistic effect on fusion, which would warrant the use of a combined regimen of P197 and P400 or RI P197 and RI P400 as a treatment option. As shown in Fig.4.8 the synergistic interactions between the two peptides did not occur. Fusion by P400 at 2.5 µM resulted in ~100 % inhibition while fusion of the Mix (1:1 P197:P400) sample at 5µM which contains 2.5 µM of both P197 and P400 inhibited syncytia at 82 %. This is also shown again as P400 inhibits syncytia 60 % at 0.5 µm, while the mixture of P197 and P400 at the same concentration only inhibit syncytia at 13 %. While the mix at 1 µM only inhibited syncytia at 38 % although it contained 5 µM of both peptides. Therefore it appears that in fact the combination of the two peptides leads to reduced inhibitory effects. Due to the reduced ability of combined peptides we did not pursue a retro-inverso version of P197; as the parent peptide does not inhibit syncytia as well as parent P400, combined with the lack of synergism suggests there would be no benefit in using a mixture of these two RI peptides for treatment.
Evaluation of mutant inhibitors of P400

The comparison of P400 and Pcr400 fusion inhibitors derived from natural sequences have shown that three different amino acids mutations at residues 401, 403, and 411, in a P400 derived 30 aa peptide can lead to measurable differences in syncytia inhibition. This suggests that the use of rational mutations could result in increased inhibitory activity. To evaluate this hypothesis mutant inhibitor peptides were synthesized on a multiple synthesizer so that ~ 10 mg of mutant P400 derived peptides could be quickly screened for inhibitory properties and a larger batch of peptide could be synthesized for more analysis. One drawback of the use of the multiple peptide synthesizer is the small size of the well volume. Based on resin swelling only a peptide of \( \leq 22 \) amino acids in length (residues 400-421) could be evaluated. Four shortened peptides [203] were examined: Pm400, Pm401C, Pm401S, and sPm400; which correspond to a shortened version of P400, a Cys mutant in position 401, a Ser mutant in position 401, and a scrambled P400 peptide respectively (Table 4.2). The Pm400 was synthesized as the positive control derived from the ATK strain. The Pm401C peptide was evaluated as a single mutant from the Pcr400 peptide. Analysis of the crystal structure of gp21 shows that in the post fusion state the Arg401 residue of the ATK strain protrudes out into the solvent. This is most likely due to the position of the complementary Arg379 at the C-terminus of the coiled-coil which leads to repulsions between the two positively charged residues. Computer modeling showed that the Pm401C mutation can relieve this repulsion and initiate H-bonding interactions between Cys401 and Arg379 as well as a more complimentary fit. Based on this analysis a Pm401S mutant was designed with consideration based on the fact that the Ser and Cys residues would occupy very similar space; however, the hydroxyl sidechain of Ser may perform better
H-bonding interactions with Arg379 than the sulfhydryl sidechain of a Cys residue. The syncytia inhibition assay showed that all of the Pm peptides had greatly reduced inhibitory activity compared to the full length P400 (Fig.4.9). This was obviously due to the shortened sequence of the Pm peptides. As shown in Fig.4.9 due to the reduced activity of the shortened peptides, improvements in syncytia inhibition could not be detected between the Pm401C and Pm401S mutants and Pm400. Therefore full length mutants will need to be synthesized in further studies.

DISCUSSION

Based on results by Pinion et al., P400 was not expected to inhibit syncytia better than the Pcr400 peptide; however, there are several explanations for our different results. First the assay used by Pinion et al., was similar to the assay we used, with important differences. The syncytia inhibition assay they described used a similar β-Gal system, however X-Gal staining was used to observe syncytia and individual blue spots were counted as syncytia. For the appearance of blue spots, at least two cells, COS-Z28 (Transfected with and Env expression vector) and Hela-Tat, must fuse but there are no other considerations for normalizing the extent of “total fusion”. In other words two cells fusing could count as one blue spot, and ten cells fusing could count as one blue spot, resulting more β-Gal produced than in a fusion between two cells. This could explain the reported results. One would have to see the fields in the wells counted to know if P400 may have had more numerical fusions than Pcr400, but of smaller size (less cells involved) to validate this explanation. By quantifying β-Gal production our assay measures the total amount of overall fusion inhibited.
In addition, the P400 and Pcr400 peptides analyzed by us were acetylated and amidated at the N and C-termini respectively. Two possibilities could have arisen from these changes that could have led to inconsistencies with the data inconsistent with the data previously reported. First, there could be slightly different structure and hence interactions between the peptides with the charged free end groups described by Pinion et al., and the relatively neutral ends of end group blocked peptides used by us. Although we did not synthesize peptides with free ends, this would be useful to see if free peptide trends similar to Pinion et al., could be observed. A second possibility is that the endblocked peptides could have been influenced by the three mutated amino acid residues in the Pcr400 peptide, making it noticeably less soluble than P400. This peptide was so insoluble that we could not take CD measurements of Pcr400 because small quantities of DMSO were the only way to solubilize the peptide. It has been shown in HIV-1 inhibitors that increases in solubility increase inhibitory activity [194]. Although, the endblocking rationale is unnecessary and might negatively affect the activity of the peptide, it should be noted that Fuzeon is similar to our peptides in that it is endblocked to improve stability. The in vivo half-life of Fuzeon is 3.8 hours [204]. The half-life of the free hydroxy metabolite (M-20) only has an in vivo half-life of \( \leq 15\% \) of T20 half-life and \( \sim 20\% \) efficacy of T20 [205]. Therefore even if a free peptide has efficacy equal to or better than an end blocked peptide in vitro, one could not assume that it would have the same efficacy in vivo.
Here we describe the use of a novel fusion assay developed by our collaborator Dr. Jacobson. This assay is another interesting way to detect the activity of fusion inhibitors. Nevertheless one must keep in mind that this assay can have similar, but somewhat different application than that of the β-Gal based syncytia inhibition assay. The β-Gal assay measures the ability of the contents of one cell (Hela-Tat) to be combined with the intracellular contents of another cell (Cos-Z28) via the presence of Env (MT-2 cells). This assay therefore is a very good indicator of syncytia. The minimum requirements needed for infection are still poorly understood. The Env transfer assay does not measure fusion or the mixture of cellular components. It essentially measures the transfer of gp46 from the surface of the infected cell to an uninfected target cell. Therefore this assay could be considered more of a binding assay than a fusion assay, as the process of fusion includes gp21, which is also transferred from cell-to-cell, although the transfer of gp21 is not being examined in this assay. Interestingly, this assay still showed that infected cells treated with gp21 fusion inhibitors somehow interfered with the transfer of gp46, which may reflect changes in Env or changes in the non-covalent interaction between gp46 and gp21 when bound with a fusion inhibitor. Less surprising is the effect of P197 on reducing gp46 transmission to the COS cells. However P197 is also not believed to inhibit the initial binding of gp46, but rather function at some post binding step [167]. One important consideration of the Env transfer assay is that it uses COS-7 cells which are adherent. This is not as much of a problem when working with L-peptide inhibitors, but does pose some problem with RI peptides, as assays with RI P400 have shown that harvesting the cells with trypsin is impossible and cell scrapers must be used which compromises the integrity of the cell for FACS analysis. One possible way to improve this assay is replace COS-7 with Jurkat cells.
The use of a T-cell line should simplify harvesting the cells and give additional relevance to the data as CD4+ T-cells are the major target for HTLV-1. In conclusion although the Env transfer assay shows sensitivity to gp21 derived peptide fusion inhibitors, the syncytia inhibition assay is a better indicator of fusion inhibition. However the Env transfer assay may have very useful application in identifying and evaluating effective binding inhibitors from Env and various host cell receptor mimetics.

When making RI peptides one assumes that similar activity between the parent peptide and the RI peptide will be exerted \textit{in vitro}. If this occurs then the RI peptide would be expected to be superior \textit{in vivo} because of the reduced turnover of the peptide. P400 was chosen to make an RI homologue (RI P400) because the peptide inhibited syncytia better than Pcr400. Results of the syncytia inhibition assay have shown that P400 and RI P400 did not have the same neutralizing capacity. RI P400 inhibited syncytia at 100 µM, but quickly lost efficacy at 10 µM. However one could still argue that RI P400 still reduced a significant level of fusion as it was able to induce slightly higher levels of syncytia inhibition than the second documented fusion inhibitor P197. It must also be considered that RI P400 was highly effective at the highest concentration (100 µM) and the reduction of RI peptide degradation should lead to a higher available plasma concentration of peptide, increasing with the duration and frequency of administration. Although there is limited data on the actual half-life of RI peptides, it has been shown that they can be very stable \textit{in vivo}, and it is possible that the largest limiting factor of RI P00 availability in the blood after administration could be the actual binding of the peptide to gp21 [171, 206-208]. Therefore \textit{in vivo} experiments are necessary to validate and elucidate the complex relationship of the cost benefit analysis of this peptide, which would include several variables such as the
increased cost of RI peptides, the lowered inhibition activity (affinity), compared with the possible accumulation of RI peptide due to reduced turnover, which could result in lower dosage and frequency of dosage during long-term administration and higher average plasma concentrations.

Preclinical studies could use the rabbit or more recently the rat model of infection as real-time PCR methods have now been established [142-144]. Based on Fuzeon studies in Juveniles a 14 day study could be designed. Rats or rabbits could be infected with HTLV-1 and once stable proviral loads are established the animals could be treated intravenously with relevant natural and RI peptide as well as irrelevant natural and RI peptide controls. During various time intervals, blood samples could be taken to detect reductions in proviral load. These studies would yield valuable efficacy information as well as additional information about tolerable levels of RI peptides.

Before one attempts such important but expensive in vivo studies mentioned above, the best possible fusion inhibitor needs to be identified. We selected peptides based on the hypothesis that better in vitro activity should have better in vivo inhibitory activity. This assumption should continue be our major criteria for validating whether or not an altered peptide has been improved. Therefore, molecular modeling was used to further analyze the mechanism behind the differences in syncytia inhibition between P400 and RI P400. As shown in Fig.4.10 and Fig 4.11 an RI Pcr400 peptide may in fact make a better RI peptide fusion inhibitor than the P400 sequence since the predicted structures of P400 and RI P400 do not directly mirror each other as well as the Pcr400 and RI Pcr400 predicted structures. This observation may reflect the presence of a Pro 411 in the sequence of P400 which is replaced by Ser411 in the Per sequence. Although many RI peptides made containing proline
have been found to be effective, other peptides have lost their efficacy [171, 195]. It is important to keep in mind that P400 does have two additional Pro residues that do not appear to be incompatible with RI design based on the molecular modeling results, however the modeling does suggest that Pro 411 may be the reason for the reduced inhibitory capacity of RI P400 and mutant residues at this position should be investigated further.

Although we were not able to detect any noticeable improvement or reduction in the neutralizing capacity of the Pm401S mutant peptide, the use of this mutant may still have useful application as a fusion inhibitor. Based on concerns of Pcr400 solubility and considering the delivery of a final drug product, the serine mutant could be used to increase the solubility of a mutant inhibitor peptide. It is also worth noting that the shortened inhibitors were much more soluble than their longer counterparts. Therefore, we were unable to see any solubility differences between Pm401S and Pm401C. Future studies must be conducted with full length (30 aa) peptides to fully evaluate the contributions of binding each mutated residue makes on the peptide. Listed here are only a few of the possible mutants that can be made and careful analysis of the crystal structure shows that there are more interesting candidates in the P400 region. Once an exhaustive analysis has been preformed, one should move on to in vivo efficacy studies for L and RI peptides form of P400.
Based on our goal to identify a multivalent vaccine against HTLV-1 it was disappointing to see that the fusion inhibitors did not work synergistically, but instead worked antagonistically. These results could have been due to the nature of the non-covalent interactions between gp46 and gp21. Once P197 binds to gp46 it may cause a conformational change which may alter the interaction of gp46 with gp21 and may limit access of P400 based peptides to gp21. It is unlikely that the reverse occurs, since one would expect the results of the mixture to exactly replicate those of P400.

One final limitation of approach concerning HTLV-1 fusion inhibitors is the fact that HTLV-1 is believed to depend much more on proviral replication than HIV-1 [209]. However, to what extent these two retroviruses differ in modes of replication is not known. If HTLV-1 only depends on cell-cell transmission for the maintenance of 20% of the infected cell population \textit{in vivo} and 80% of infected cells are derived from Tax driven proviral replication via cell division, then one could assume that fusion inhibitors could reduce proviral load by only a maximum of 20%. Although such results from \textit{in vivo} studies would be somewhat discouraging compared to HIV viral load reductions; such a finding at least answer this currently elusive central question in HTLV-1 biology, and would help to shape the future directions of other treatment methods such as an increased focus on CTL based immunotherapy.
Fig.4.1. Structure of residues 338-421 of HTLV-1 gp21 in the fusion activated state.

The N-terminal coiled-coil domain and hinge region are shown in blue. Residues incorporating the P400 (400-429) peptide are shown in red. Image was generated using Swiss PDB viewer based on coordinates described by Kobe et al.,[128]
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<tr>
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<tr>
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<td>STEP CD28</td>
<td>Ac-KIPTGLNP RMSMEYEDFP1Y-NH2</td>
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**Table 4.1.** Sequences of full length gp21 (P400 peptides), gp46 (P197 and P175) fusion inhibitors. SCRCD40 and STEPCD28 peptides were used as negative controls.
Fig.4.2. Schematic representation of the effects of retro-inverso peptides [202].

Natural orientation of parent L-amino acid peptide (1). Reversed sidechain orientation in D-amino acid peptide (2). Retro-inverso peptide with restored sidechain orientation (3).
**Fig.4.3. P400 inhibits syncytia better than Pcr400.** To measure the neutralizing capacity of the P400 and Pcr400 peptide fusion inhibitors a three cell line based fusion assay was used. $5 \times 10^4$ HTLV-1 infected and producing MT-2 cells were incubated for 2 hr with various peptides prior to the addition of $5 \times 10^4$ CosZ28 (containing HIV-LTR-lacZ) and Hela-Tat (expressing HIV Tat). After a 20 hr incubation of all three cells, the reduction in β-galactosidase production from peptide-mediated fusion inhibition was determined by chemiluminescence.
Figure 4.4. CD spectra of peptide fusion inhibitors P400 and RI P400. CD spectroscopy measurements were made of 100 µM peptide solutions in either (A) water or 50% TFE (B). Changes in the spectra indicate the presence of secondary structure in the peptides. Spectra characteristic of α-helices comprised of L-amino acids contain minima at 208nm and 222nm and a maximum at 193nm. Spectra of D-amino acid peptides are expected to be an exact mirror image.
Fig. 4.5. Neutralizing capacity of RIP400. To measure the neutralizing capacity of the RI P400 it was compared to positive controls P400 and P197. A peptide from CD28 was used as a negative control. A three cell line based fusion assay was used. 5 x 10^4 HTLV-1 infected and producing MT-2 cells were incubated for 2 hr with various peptides prior to the addition of 5 x 10^4 CosZ28 (containing HIV-LTR-lacZ) and Hela-Tat (expressing HIV Tat). After a 20 hr incubation of all three cells, the reduction in β-galactosidase production from peptide-mediated fusion inhibition was determined by chemiluminescence.
Fig.4.6. Measurement of inhibition of GLUT-1 aided improved Env transfer by peptide fusion inhibitors. Based on the finding that up-regulation of the ubiquitous glucose transporter GLUT-1 causes cells to be more permissive to HTLV-1 infection, Cos-7 cells were transfected with 5 μg /2 x 10^6 cells of GLUT-1-HA expression vector using Amaxa® nucleofection. Twenty-four hours after nucleofection 6.75 x 10^5 Cos-7-GLUT-1 cells were incubated with 6.75 x 10^5 HTLV-1 infected Hut102-A2-GFP cells in 50 μM peptide. 48 h after incubation, Cos-7 cells were analyzed by FACS for the expression of Env.
Fig.4.7. Neutralizing capacity of gp46 derived fusion inhibitors. To measure the neutralizing capacity of P197 and P175 syncytia inhibition assay was used to compare the peptides to the positive control P400. A peptide from CD28 was used a negative control.
**Fig.4.8. Measurement of synergism between P400 and P197.** To measure possible synergism when P400 and P197, a 1:1 mixtures of the two peptides was compared to an equal molar equivalent of the individual peptides. A three cell line based fusion assay was used to measure fusion inhibition. $5 \times 10^4$ HTLV-1 infected and producing MT-2 cells were incubated for 2 hr with various peptides prior to the addition of $5 \times 10^4$ CosZ28 (containing HIV-LTR-\textit{lacZ}) and Hela-Tat (expressing HIV Tat). After a 20 hr incubation of all three cells, the reduction in $\beta$-galactosidase production from peptide-mediated fusion inhibition was determined by chemiluminescence.
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<td>Scrambled ATK sequence</td>
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</tr>
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</table>

**Table 4.2** Sequences of shortened 22 amino acid residue peptide synthesized using a multiple peptide synthesizer.
**Fig. 4.9. Neutralizing capacity of mutant peptide Pm401S.** To measure the neutralizing capacity of a P400 derived peptide containing 22 aa mutant peptides (Pm401S Pm401C) were used to measure the effects if individual mutation at the 401 position. Pm400 was a shortened P400 positive control and SPm400 is a scrambled sequence used for negative control.
Fig. 4.10 Molecular modeling comparisons of hypothetical structures of P400 and RI P400. Molecular modeling of P400 (bottom) and RI P400 (top) was used to predict the conserved structural homology of the parent and RI peptides. Raw sequences were loaded and in Hyperchem 5.01 and Polik-Rebiere energy minimization computations were performed in vacuo, and the structures were loaded into Pymol for viewing.
Fig. 4.11 Molecular modeling comparisons of hypothetical structures of Pcr400 and RI Pcr400. Molecular modeling of Pcr400 (bottom) and RI Pcr400 (top) was used to predict the conserved structural homology of the parent and RI peptides. Raw sequences were loaded and in Hyperchem 5.01 and Polik-Rebiere energy minimization computations were performed in vacuo, and the structures were loaded into Pymol for viewing.
CONCLUDING REMARKS

The underlying goal of this project is to prevent HTLV-1 infection by prophylactic vaccination and/or to alleviate disease in HTLV-1 infected individuals by using various peptide modalities; which would result in reversion back to asymptomatic status or keep asymptomatic individuals from progressing to a disease status. Based on the results shown here it the importance of humoral prophylactic vaccination and the application of MVF 175-218 is further confirmed. Also the essential role of immunity against the Tax 11-19 epitope in HLA-A*0201 positive infected individuals is also shown. Further clinical studies should be carefully preformed to see if manipulation of immune responses against this epitope would increase cytolytic propensity and leading to suppression of HTLV-1 infection, ultimately resulting in a therapeutic effect. It has also been shown here that retro-inverso peptide inhibitors can inhibit HTLV-1 mediated fusion. This is a barely researched field and as suggested here much can still be done to identify and improve peptides derived from P197 and P400. In addition much can be done to identify and improve fusion inhibitors from the list of potential host cell receptors that are still actively debated. Only by continued improvements in preclinical models and cooperative efforts in coordinating clinical evaluations, combined with continued active design of vaccines, immunotherapeutics and therapeutics can such treatments come to fruition.
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