Chimeric peptide vaccine composed of B- and T-cell epitopes of human T-cell leukemia virus type 1 induces humoral and cellular immune responses and reduces the proviral load in immunized squirrel monkeys (Saimiri sciureus)

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A squirrel monkey model of human T-cell leukemia virus type 1 (HTLV-1) infection was used to evaluate the immunogenicity and protective efficacy of a chimeric peptide vaccine composed of a B-cell epitope from the envelope region (aa 175–218) and three HLA-A*0201-restricted cytotoxic T-lymphocyte epitopes derived from Tax protein (Tri-Tax). These selected Tax peptides induced secretion of gamma interferon (IFN-γ) in peripheral blood mononuclear cells obtained from monkeys chronically infected with HTLV-1. After immunization, a high titre of antibodies and a high frequency of IFN-γ-producing cells were detected against the Env and the Tri-Tax immunogens, but not against the individual Tax peptides. This might indicate that epitope(s) distinct from those recognized by humans are recognized by responder monkeys. After challenge, it was shown by competitive PCR that partial protection against HTLV-1 infection could be raised in immunized animals. Further studies should be developed to determine the duration of this protection.

Human T-cell leukemia/lymphoma virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATLL) (Yoshida et al., 1984) and of tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (Gessain et al., 1985). It has also been associated with a number of inflammatory diseases (Ijichi et al., 1990; Lagrenade et al., 1990; Mochizuki et al., 1992; Morgan et al., 1989). The overall prevalence of severe HTLV-1-associated disease is 2–8% among HTLV-1-infected persons, estimated to comprise 15–25 million people worldwide, mostly in Central and South America, Equatorial Africa and Asia (de Thé & Kazanji, 1996). With the exception of a few cases surviving after bone-marrow transplantation, no treatment has been shown to prevent eventual relapse of aggressive ATLL effectively. Thus, the development of an HTLV-1 vaccine is crucial.

The genomic stability of HTLV-1 and the presence of neutralizing antibodies in HTLV-1-infected individuals are favourable factors for the design of an efficient HTLV-1 vaccine. A major obstacle to the development of an HTLV-1 vaccine, however, has been the lack of an appropriate animal model. Many models of HTLV-1 infection, including rabbits (Cockerell et al., 1990; Lairmore et al., 1992; Miyoshi et al., 1985), rats (Ibrahim et al., 1994; Ishiguro et al., 1992; Kazanji et al., 1997b; Yoshiki et al., 1987) and monkeys ( Ibuki et al., 1997; Kazanji, 2000; Nakamura et al., 1986), have been used to test vaccine candidates and to study pathogenesis and virus–host interactions. Over the past few years, we have shown that the squirrel monkey, Saimiri sciureus, is susceptible to experimental infection with HTLV-1-immortalized cells. As in humans, experimental inoculation leads to chronic infection (Kazanji et al., 1997c). The adequacy of the model is supported further by specificity for the target cell, the CD4 T helper lymphocyte, and by a replication strategy
that is similar to that of HTLV-1 in humans (Mortreux et al., 2001). As in humans, HTLV-1 infection of squirrel monkeys, after an extended latency, leads to continuous expansion of a restricted number of abundant HTLV-1 cellular clones (Mortreux et al., 2001). We showed recently that some chronically infected monkeys had high CD4+ T-cell counts concomitant with an increased total lymphocyte population; a significant proportion of these lymphocytes were infected and flower cells were found in peripheral blood (Debacq et al., 2005). The squirrel monkey thus appears to be a suitable model for studying the pathogenesis of HTLV-1 and for evaluating candidate vaccines (Kazanji, 2000; Kazanji et al., 2001).

Peptide-based vaccines have been used as candidates for HTLV-1 vaccines. Frangione-Beebe et al. (2000) showed that a peptide construct comprising aa 175–218 (FLNTEPSLP-TAPPLLPHSNLDHILEPSIWKSCLLTQLVQTL) of gp46 linked by a four-residue turn (GPSL) to a promiscuous T-cell epitope from the measles virus fusion protein (MVF) aa 288–306 (KLLSIKGIVHRLEGV) with the adjuvant N-acetylglucosamine-3yl-acetyl-l-alanyl-d-isoglutamine was immunogenic in outbred populations of rabbits and mice. Sundaram et al. (2003) designed a novel multivalent peptide construct comprising three HLA-A*0201-restricted CTL epitopes located on aa 11–19 (LQLGAFLTNV), 178–186 (QLGAFSLTV) and 306–315 (HLLFEEYTNI) of the HTLV-1 Tax protein, with double arginine residues intervening in tandem (Tri-Tax). These peptides were selected because of their ability to induce secretion of gamma interferon (IFN-γ) in peripheral blood mononuclear cells (PBMCs) obtained from HLA-A2-infected persons. Immunization of HLA-A*0201 transgenic mice with this construct elicited cellular responses to each intended epitope and protected the mice against challenge with recombinant vaccinia virus containing the tax gene (Sundaram et al., 2004a).

In the study reported here, we evaluated the immunogenicity and protective efficacy in squirrel monkeys of a B-cell epitope peptide located on aa 175–218 of the env gene and of a new, selected T-cell multiepitope derived from Tri-Tax, both conjugated to promiscuous T-cell epitopes as described above.

To evaluate the frequency of circulating effector T cells against the three selected Tax peptides (located at aa 11–19, 178–186 and 306–315 of the Tax protein) in HTLV-1-infected monkeys, we used enzyme-linked immunospot (ELISPOT) assays, performed as described by Sundaram et al. (2003), with anti-IFN-γ monoclonal antibody 1-D1K (Mabtech) as the coating antibody and biotinylated anti-IFN-γ monoclonal antibody 7-B6-1 (Mabtech) as the secondary antibody, as described in the manufacturer's instructions. These anti-human IFN-γ antibodies recognized squirrel monkey IFN-γ (data not shown). PBMCs (2 × 10⁶) were added to anti-IFN-γ-coated ELISPOT 96-well plates and stimulated in triplicate wells with 10 μmol each peptide or irrelevant peptide ml⁻¹ for 40 h. IFN-γ-secreting cells were counted with an ELISPOT image analyser. PBMCs from two monkeys chronically infected with HTLV-1 (nos 1715 and 1491) were used in the assay. In monkey 1715, only two of the three tested peptides, aa 11–19 and 178–186, induced a high level of IFN-γ, with 222 ± 15 and 158 ± 8.8 spots (10⁶ cells)⁻¹, respectively (irrelevant peptide induced 15 ± 3.5 spots). In the second infected animal, monkey 1491, all three peptides induced IFN-γ, with 259 ± 62 spots for the 11–19 peptide, 113 ± 5.5 for the 178–186 peptide and 297 ± 42 for the 306–315 peptide. The irrelevant peptide induced 33 ± 1.7 spots (10⁶ cells)⁻¹. These results indicated the presence of virus-specific circulating effector cells in HTLV-1-infected monkeys that can produce IFN-γ in the presence of the selected Tax epitopes.

To evaluate the immunogenicity and protective efficacy of these Tax peptides and of a selected B-cell epitope, we used four 6-year-old male squirrel monkeys. Two monkeys (A047C and A064C) were injected intramuscularly twice, at 0 and 4 weeks, with the Env B-cell epitope aa 175–218 (500 μg per monkey) linked to the promiscuous T-helper cell epitope MVF (700 μg per monkey), as described previously (Frangione-Beebe et al., 2000). Six weeks after the first immunization, the monkeys were injected with another construct, consisting of the three Tax CTL epitopes (aa 11–19, 178–186, 306–315), as described previously (Sundaram et al., 2003, 2004a). Monkeys were boosted twice at weeks 9 and 16 with both B- and T-cell epitopes (Fig. 1). Two control animals (A078C and A103C) were injected with irrelevant B- and T-cell peptides. We determined the antibody response every 2–3 weeks throughout the study by direct ELISA, as described previously (Frangione-Beebe et al., 2000).

As seen in Fig. 1(a), antibodies against both the B-cell epitope peptide and the immunogen MVF–175–218 were detected in the two immunized monkeys after the first boost and the response increased further with successive boosts. The antibody titre to the immunogen was higher than that to the B-cell epitope. Furthermore, the Env chimeric peptide was highly immunogenic in monkey A047C, eliciting a higher antibody response than in monkey A064C. No antibody response was detected in the two control monkeys (data not shown).

For peptide antibodies to be effective in neutralizing viral infection, they must cross-react with the native protein from which the epitope is derived. Binding of the peptide antibodies to HTLV-1-infected (MT-2) cells was tested by flow cytometry, as described previously (Sundaram et al., 2004b). As seen in Fig. 1(b), the antisera from both immunized monkeys bound to the surface of the MT-2 cells, indicating that the antibodies recognized the native protein. Pre-immune serum from the same monkeys and sera from the control monkeys (A078C and A103C), used as negative controls, showed no binding, as expected (Fig. 1b).

To evaluate the cellular immune response against Env (aa 175–218) and Tax peptides, the ELISPOT assay was performed 1 week after the last boost in the immunized
monkeys and controls. In the control monkey A103C, the number of IFN-\(\gamma\)-producing cells in the presence of 175–218 peptide was equivalent to that observed in the presence of irrelevant peptide or media alone (Fig. 2). Similar results were also obtained in a second control animal, A078C (data not shown). In contrast, PBMCs from the two immunized monkeys showed a three- to sevenfold increase in IFN-\(\gamma\)-producing cells, indicating a robust immune response to the selected antigen (aa 175–218). Depletion of CD4\(^+\) lymphocytes reduced the number of IFN-\(\gamma\)-producing cells from immunized monkeys to a level similar to that of the control animals (Fig. 2).

The cellular immune response was also evaluated against the Tax peptides. As shown in Fig. 2, a high frequency of IFN-\(\gamma\)-producing cells was detected in the presence of the immunogen construct (Tri-Tax peptide) in the two immunized animals but not in the control. When PBMCs were stimulated with the three selected peptides (aa 11–19, 178–186 and 306–315) individually, however, no difference was found between the immunized and control animals (Fig. 2). Interestingly, in the case of the anti-Tax response, depletion of CD4\(^+\) lymphocytes only slightly reduced IFN-\(\gamma\) production (Fig. 2).

The immunized monkeys were challenged 15 days after the last boost with an intravenous injection of \(5 \times 10^7\) cells of a squirrel monkey HTLV-1-transformed cell line (EVO/1540), as described previously (Kazanji et al., 1997c). Another control monkey (A119C), which was free of HTLV-1 and was not immunized, was also included in the study. The serum levels of specific HTLV-1 antibodies were determined by ELISA (Cobas Core Anti-HTLV-I/II EIA; Roche) and confirmed by Western blot analysis (HTLV-1 blot 2.3; Diagnostic Biotechnology). Five months after challenge, the two immunized monkeys (A047C and A064C) had not undergone seroconversion against HTLV-1, while the two controls animals (A078C and A103C) immunized with irrelevant peptide and the new control monkey (A119C) developed an HTLV-1 antibody response (Fig. 3a). Western blot analysis performed at 5 months after challenge showed that one of the two immunized animals (A047C) had not undergone seroconversion against HTLV-1 antigens (Fig. 3b, lane 3), while the second animal (A064C) showed only a low response against the recombinant gp-21 protein (Fig. 3b, lane 4). Seroconversion against HTLV-1 Env and Gag proteins was found in the three control animals, similar to that observed in one animal chronically infected with HTLV-1 (Fig. 3b, lanes 5–8).

Six months after challenge, the five challenged monkeys were killed and the HTLV-1 proviral load was evaluated in PBMCs and spleen by quantitative PCR. The proviral load was measured with an ABI PRISM 7700 Sequence Detector (Perkin Elmer/Applied Biosystems), as described elsewhere (Kubota et al., 2000). No HTLV-1 provirus copy could be detected in the PBMCs or the spleen of immunized monkey A047C. In the second animal (A064C), 20 HTLV-1 copies were found in 1 \(\mu\)g DNA from PBMCs and 40 copies in spleen. These values were much lower than the copy numbers detected in the two control animals, A078C (370 copies in PBMCs and 150 in spleen) and A103C (1110 copies in

**Fig. 1.** Humoral immune responses to the B-cell the B-cell epitope 175–218 peptide and against epitope in two immunized monkeys. (a) Antibody titres against the B-cell epitope 175–218 peptide and against the corresponding immunogen (MVF–175–218) in sera were determined by direct ELISA. Arrows represent the immunization protocol with boosters and challenge. (b) Relative binding of 175–218 antisera to Env protein on the surface of HTLV-1-immortalized cell lines as determined by indirect immunofluorescence staining and then analysed by flow cytometry. Filled histograms represent staining with pre-immune serum; open histograms represent sera from immunized monkeys or controls. Monkeys A047C and A064C were immunized with HTLV-1 B-cell epitope combined with T-cell epitopes; monkeys A078C and A103C were immunized with irrelevant peptides.
PBMCs and 220 in spleen), immunized with irrelevant peptide or in the unimmunized monkey A119C (2420 copies in PBMCs and 400 in spleen).

In this study, we have shown that immunization of squirrel monkeys with a peptide construct containing B- and T-cell epitopes can elicit a high titre of antibodies, a high frequency of specific IFN-γ-producing cells and partial protection.

We showed first that PBMCs from chronically HTLV-1-infected monkeys could produce IFN-γ in the presence of the Tri-Tax peptide. We showed previously that HTLV-1-infected squirrel monkeys develop a cell-mediated immune response against target cells infected with recombinant vaccinia expressing the whole p40 Tax protein (Kazanji et al., 2000). In the present study, we confirmed our previous data by demonstrating a high frequency of IFN-γ-producing cells in two HTLV-1-infected monkeys.

The CTL response in our squirrel monkey model appears to be comparable to that observed in asymptomatic human HTLV-1 carriers. In carriers and patients with TSP/HAM, the CTL response is also directed mainly against the p40 Tax protein (Jacobson, 2002). It has been shown recently that the HTLV-1 proviral load is a strong predictor of disease progression and that patients with TSP/HAM or ATLL have a higher proviral load than asymptomatic HTLV-1 carriers (Jacobson, 2002; Yamano et al., 2002). It has been suggested that this cellular response plays a major role in controlling HTLV-1 replication and then maintaining a low viral load.

**Fig. 2.** Frequency of epitope-specific IFN-γ-secreting T cells in monkeys after immunization with B-cell epitope combined with multivalent Tax peptide. PBMCs were cultured in the presence of 10 μmol of the relevant peptide ml⁻¹. IFN-γ-releasing cells were evaluated in an IFN-γ ELISPOT assay. Filled bars indicate ELISPOT assays conducted with PBMCs or CD4⁺-depleted cells from immunized monkeys and cultured with different Env or control peptides. Hatched bars indicate ELISPOT assays conducted with PBMCs or CD4⁺-depleted cells cultured with different Tax or control peptides. Monkeys A047C and A064C were immunized with HTLV-1 B-cell epitope combined with T-cell epitopes; monkey A103C was immunized with irrelevant peptide. Background levels were measured in wells containing irrelevant peptide or medium only. Each bar represents the mean ± SD of the number of IFN-γ spots in triplicate wells. T2 is the Tax epitope located at aa 11–19 (LLFGYPVYV), T3 is aa 178–186 (QLGAFLTNV) and T6 is aa 306–315 (HLLFEEYTN). 236, Tri-Tax peptide; TT, tetanus toxoid (aa 947–967) peptide (promiscuous T-helper epitope); IR, irrelevant peptide.
In our study, the immunized monkeys developed a strong cellular immune response, as detected by IFN-\(\gamma\)-producing cells, in the presence of selected peptides. Furthermore, a significant reduction in the proviral load was seen in these immunized monkeys after challenge. These results provide a rational background for clinical use of such a vaccine, not only for controlling infection, but also for preventing associated diseases. However, further efforts should be directed towards elucidating the duration of this protection.

Administration of the peptides with adjuvant induced both a high antibody response and a cellular immune response, and the antibody response against Env peptide was increased in immunized animals after boosting. Furthermore, these antibodies recognized the native protein in MT-2 cells. The Env protein has been reported to confer partial protection against HTLV-1 infection in various animal models, but the mechanisms of immunity associated with the protection remain unclear (Franchini et al., 1995; Kazanji et al., 1997a).

In our study, a cell-mediated immune response was also detected, which was strong in the presence of the 175–218 B-cell epitope; however, depletion of CD4\(^+\) lymphocytes reduced the number of IFN-\(\gamma\)-producing cells from immunized monkeys to a level similar to that in controls. Therefore, the CD4\(^+\) subset of lymphocytes, which give the T-cell help required for high antibody titres, contributes to the vaccine-induced cellular immune response (Goon et al., 2002). In contrast, the reduction in the number of cells producing IFN-\(\gamma\) in response to the Tri-Tax T-cell epitope peptide was much lower than with the B-cell epitope peptide, indicating the involvement of other virus-specific T-cell subsets in this cellular immune response.

Although we found a potent response to the peptide containing the three Tax epitopes, individual Tax peptides did not induce IFN-\(\gamma\) production. This absence of response might indicate that epitope(s) distinct from those recognized by humans are recognized by responder monkeys because of differences in major histocompatibility complex class I molecules. In order to characterize further the immune response involved in HTLV-1 infection and in response to administration of this vaccine in squirrel monkeys, we intend to investigate the genetic structure and polymorphism of this gene in our colony of squirrel monkeys.

The monkey that showed the highest antibody titre and the highest level of specific IFN-\(\gamma\)-producing cells was totally protected after challenge. As we could not demonstrate a specific cellular immune response against an individual Tax peptide, we cannot draw any conclusions on the effective role of this combined vaccination in the induction of this complete protection. However, we reported previously that monkeys primed with naked DNA containing the HTLV-1 env gene and then boosted with the vaccinia virus vector NYVAC containing the env and gag genes developed both humoral and cell-mediated immune responses to Env and Gag after boosting. Furthermore, protection against challenge was observed in monkeys receiving the vaccine for both Env and Gag, but not in monkeys immunized with Env alone (Kazanji et al., 2001). Thus, Gag components are also

**Fig. 3.** Antibody response to HTLV-1 antigens after challenge. (a) Antibody response as detected by ELISA in monkeys immunized with HTLV-1 B-cell epitope combined with T-cell epitopes (A047C, A064C) or with irrelevant peptide (A078C, A103C). An additional control monkey (A119C) not immunized with any peptide was included in the challenge study. (b) Western blot analysis of sera from immunized monkeys before and after challenge with HTLV-1-producing cells. Lanes: 1 and 2, sera from monkeys A047C and A064C 1 week after the last boost (before challenge); 3 and 4, sera from monkeys A047C and A064C 5 months after challenge; 5 and 6, sera from the two control monkeys (A078C, A103C) 5 months after challenge; 7, serum from the unimmunized control monkey A119C after challenge; 8, serum from a chronically HTLV-1-infected animal. MTA-1, recombinant gp46-I protein incorporated in the Western blot.

(Bangham, 2003; Bangham et al., 1996; Jacobson, 2002).
important in HTLV-1 vaccine design. Further studies including the use of different immunogens and a larger number of animals should be directed towards elucidating complete and long-term protection against HTLV-1 infection.

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References


