Identification of a cytotoxic T-lymphocyte (CTL) epitope recognized by Gag-specific CTLs in cynomolgus monkeys infected with simian/human immunodeficiency virus

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INTRODUCTION

Non-human primate models play a major role in the preclinical development of human immunodeficiency virus 1 (HIV-1) vaccine strategies (Johnson, 1996). In particular, Macaca mulatta (rhesus monkeys) and Macaca fascicularis (cynomolgus monkeys) infected with simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV) are the most commonly used animal models for assessing the immunogenicity and protective efficacy of different vaccine protocols.

Current data indicate that antigen-specific CD8+ T-cell responses play a critical role in controlling SHIV/SIV replication in infected macaques (Schmitz et al., 1999; Barouch et al., 2000), as well as in controlling HIV in humans (Ogg et al., 1998). In addition, several authors have suggested that control of HIV infection is characterized by polyfunctional [secreting interleukin-2 (IL-2) plus gamma interferon (IFN-γ)] CD4+ and CD8+ T-cell responses, reflecting the presence of memory T cells at different stages of differentiation (Appay et al., 2002; Pantaleo & Koup, 2004; Harari et al., 2005). These data indicate that the efficacy of virus-specific immune responses is not based solely on the quantity of the T-cell response, but is also affected by the quality of the response. For these reasons, it is important to analyse immune responses in detail in the animal models used in the experimental infection of AIDS.

The most widely used techniques for monitoring specific CD8+ T-cell responses [ELISPOT assay, intracellular staining and analysis of tetrameric major histocompatibility complex (MHC)–peptide complexes] in human trials, as well as in the simian animal models of AIDS (Kuroda et al., 1998; Bercovici et al., 2000; Donahoe et al., 2000; Sun et al., 2003), employ peptides with an optimal length of 8–10 aa (based on knowledge of MHC class I alleles) or pools of 9–15mers spanning a specific region of interest of a viral antigen when the peptide matching the MHC class I allele or the MHC class I allele itself is unknown. In the latter case, several peptide pools are required, with a consequent decrease in sensitivity and the requirement for a large number of cells from the sample under analysis. Moreover, the high cost of multiple peptide synthesis is an obstacle to...
routine use, especially when multiple antigen-specific CD8\(^+\) T cells need to be determined.

The detection of MHC-restricted CD8-specific responses in rhesus monkeys has been facilitated firstly by the definition of a dominant SIV Gag cytotoxic T-lymphocyte (CTL) epitope referred to as p11C presented to CD8\(^+\) T cells by the common MHC class I Manu-A\(^*\)01 allele (Miller et al., 1991) and later by the discovery of other virus epitopes presented in the context of Manu-A\(^*\)01, Manu-A\(^*\)02 and Manu-B\(^*\)17 alleles (Robinson et al., 2001; Mothé et al., 2002; Loffredo et al., 2004). Recently, Uda et al. (2004, 2005) identified MHC class I A and B loci in cynomolgus monkeys, and other authors are studying MHC class II alleles (Leuchte et al., 2004). Thus, the identification of optimal epitopes in combination with the knowledge of MHC class I alleles of cynomolgus monkeys will improve our understanding of CD8\(^+\) T-cell-specific responses during SIV/SHIV infection.

We previously demonstrated the feasibility of using autologous B lymphoblastoid cell lines (BLCLs) transduced with retroviral vectors expressing SHIV89.6P viral antigens as source of antigen-specific CD8\(^+\) T cells with cytolytic activity (Negri et al., 2004). In this report, we have shown that these CTLs, when properly stimulated with Gag-expressing BLCLs, specifically recognize an epitope of 9 aa included in the SIV Gag protein. In addition, further screening using an IFN-\(\gamma\) ELISPOT assay of six SHIV-infected monkeys showed positive reactivity against this newly identified Gag CTL 9mer epitope in one of the animals.

**METHODS**

**Animals.** Cynomolgus macaques (M. fascicularis) from the Mauritius breeding colony were housed in single cages within Level 3 biosafety facilities in accordance with European guidelines for non-human primate care (EEC Directive 86/609, 24 November 1986). For challenge experiments, six monkeys (Mk) were infected intravenously with 15 monkey infectious doses (MID\(_{50}\)) of SIVmac239 whilst Mk 783 was infected intravenously with 20 MID\(_{50}\) of the SHIV89.6P virus. Clinical observation, weight measurements and bleedings for haematological analysis and immunological assays were taken whilst monkeys were under ketamine hydrochloride anaesthesia (10 mg kg\(^{-1}\), intramuscular). Bleedings were performed by collection of 10 ml whole blood in acid citrate-containing tubes.

**Cell lines.** In order to produce stimulator/target cells expressing the SIV Gag protein, herpesvirus papio-transformed BLCLs derived from cynomolgus monkeys were transduced with the FB-Gag and FB-Neo retroviral vectors, as described previously (Negri et al., 2004). Briefly, BLCLs were incubated for 2 h at 37 °C in 5% CO\(_2\), whilst Mk 783 was infected intravenously with 20 MID\(_{50}\) of the SHIV89.6P virus. Clinical observation, weight measurements and bleedings for haematological analysis and immunological assays were taken whilst monkeys were under ketamine hydrochloride anaesthesia (10 mg kg\(^{-1}\), intramuscular). Bleedings were performed by collection of 10 ml whole blood in acid citrate-containing tubes.

**Generation of CTL cultures.** Peripheral blood mononuclear cells (PBMCs) isolated from the animals were cultured in 24-well plates at a concentration of 2 × 10\(^6\) cells ml\(^{-1}\) in a volume of 2 ml per well. Stimulation with autologous BLCLs transduced with retroviral vector expressing SIV Gag was performed as described previously (Negri et al., 2004). Briefly, on day 0, irradiated (3000 rad) Gag-expressing BLCLs were cultured with PBMCs at an effector:stimulator ratio of 20:1. Human recombinant IL-2 (Proleukin; Chiron) was added at a concentration of 20 U ml\(^{-1}\) on day 8 and every following 2 days. In some cases, cells received a further stimulation with irradiated FB-Gag BLCLs on day 14. On day 14 (after one stimulation) or day 28 (after two stimulations), an IFN-\(\gamma\) ELISPOT assay, a \(^{51}\)Cr-release assay and intracellular staining for tumour necrosis factor alpha (TNF-\(\alpha\)) were performed.

**Gag peptides.** Overlapping 15 aa peptides spanning the entire region of the SIVmac239 Gag protein (p53969) were provided by the NIH AIDS Research and Reference Reagent Program (cat. #6204). Pools of 10–62 peptides at a final concentration of 2 μg ml\(^{-1}\) for each peptide were used in the IFN-\(\gamma\) ELISPOT assay. Peptides of 8, 9 and 10 aa were synthesized by UFPepitides (Ferrara, Italy) and used for the *in vitro* assays at various concentrations.

**IFN-\(\gamma\) ELISPOT assay.** An IFN-\(\gamma\) ELISPOT assay was performed using reagents from Mabtech AB. Briefly, PBMCs isolated from infected monkeys, stimulated as described above, were resuspended at a concentration of 5 × 10\(^6\) cells in 100 μl and seeded in a MultiScreen-IP microtitre plate (Millipore) coated with a monoclonal antibody against monkey IFN-\(\gamma\). Pools of 15mer SIV Gag peptides overlapping by 11 aa or 8-, 9- or 10mer peptides were added at different concentrations. Medium alone and an unrelated peptide (FLU peptide [GILGFVFTL], an epitope of influenza virus, HLA-I A*0201-restricted; Prolimmune) were added to the assay as negative controls. Phytohaemagglutinin at a final concentration of 2 μg ml\(^{-1}\) was added as a positive control. In some experiments, stimulator cells (FB-Neo or FB-Gag BLCLs) were resuspended in 100 μl medium and added in duplicate to the plate at an effector:target (E:T) ratio of 25:1. After overnight incubation at 37 °C in 5% CO\(_2\), cells were removed and a biotinylated antibody against monkey IFN-\(\gamma\) was added to the wells, followed by the addition of streptavidin–alkaline phosphatase and then the chromogenic substrate NBT/BCIP (Sigma). After development, spot-forming cells (SFCs) were counted using an ELISPOT reader (AELVIS). A positive response was defined as being more than two times the mean of the negative controls and > 50 SFCs per 10\(^5\) cells.

**\(^{51}\)Cr-release assay.** A standard chromium-release assay was performed using PBMCs as effector cells, following stimulation for 14 days in the presence of irradiated FB-Gag BLCLs, as described above. Target cells (FB-Neo and FB-Gag BLCLs) were labelled with 50 μCi (1-85 MBq) \(^{51}\)Cr (Perkin Elmer Life Sciences) per 10\(^5\) cells for 1 h and washed with cold medium. FB-Neo BLCLs were pulsed with or without peptides at a concentration of 5 μg ml\(^{-1}\) in serum-free medium for 1 h at 37 °C. Target cells (5 × 10\(^5\)) were then seeded in duplicate wells for each E:T ratio (from 40:1 to 5:1). \(^{51}\)Cr release was assayed after 4 h of incubation at 37 °C in 5% CO\(_2\) by harvesting 40 μl supernatant from each well on to wells of a LumaPlate-96 (Perkin Elmer Life Sciences). Emitted radioactivity was measured using a MicroBeta counter (Wallac). Spontaneous release and total release were determined from wells containing target cells and medium alone or with the addition of 0.5% Triton X-100 (Sigma), respectively. The percentage specific cytotoxicity was
calculated as \[ \frac{([\text{test release} - \text{spontaneous release}]) \times 100}{\text{total release} - \text{spontaneous release}} \]. Spontaneous release of target cells was < 15% in all assays.

**Intracellular staining for TNF-α.** Intracellular staining for TNF-α was performed as initially described in humans with modifications as described previously for optimal performance using macaque PBMCs (Kaur et al., 2002; Gauduin et al., 2004). PBMCs stimulated with FB-Gag BLCLs for 28 days were stimulated for 6 h with phorbol myristate acetate (PMA; 10 ng ml\(^{-1}\)) and ionomycin (1 µg ml\(^{-1}\)), with or without the specific viral antigen (GAG3 9mer peptide) at a concentration of 5 µg ml\(^{-1}\). For optimal co-stimulation, cells were stimulated in the presence of monoclonal antibody specific for the cytokine under analysis.

Identification of SIV Gag protein (FB-Gag BLCLs) (Negri et al., 2004). Anti-Gag-specific CTL cultures were generated from a SHIV89.6P-infected cynomolgus monkey (Mk 783) infected with SHIV89.6P using PBMCs stimulated with autologous BLCLs transduced with a retroviral vector expressing the full-length SIV Gag protein (FB-Gag BLCLs) (Negri et al., 2004).

**RESULTS**

**Identification of SIVmac239 Gag CTL epitope in a SHIV89.6P-infected cynomolgus monkey**

Anti-Gag-specific CTL cultures were generated from a cynomolgus monkey (Mk 783) infected with SHIV89.6P using PBMCs stimulated with autologous BLCLs transduced with a retroviral vector expressing the full-length SIV Gag protein (FB-Gag BLCLs) (Negri et al., 2004).

Importantly, 14 days after the first stimulation, cytofluorimetric analysis showed that 80% of cells were CD8\(^+\) T cells, which approached 90% when the cells received a second stimulation and were maintained in culture for 28 days (data not shown). Following two stimulations, CTL cultures were used for a detailed analysis of the reactivity against SIV Gag antigen. As shown in Fig. 1(a), CTLs specifically recognized the SIV Gag antigen presented by the FB-Gag BLCLs, as described previously (Negri et al., 2004). In order to identify the region of the Gag protein containing the epitope recognized by the Gag-specific CTLs, three pools of peptides covering the p17 (pool A), p27 (pool B) and p9 + p6 (pool C) regions of the Gag protein (Fig. 1) were prepared and used to perform a preliminary screening by IFN-γ ELISPOT assay on effector cells. Using this assay, Gag-specific CTLs demonstrated a strong functional reactivity to pool B covering the p27 Gag protein (Fig. 1b). Consequently, pool B was divided into six smaller pools (pools 4–9), each containing 10–11 peptides, and used for further characterization of the specificity of Gag-specific CTL cultures by IFN-γ ELISPOT assay. Analysis indicated strong reactivity with pool 5, comprising 10 peptides of 15 aa, covering aa 161–212 in the protein sequence of SIV Gag (Fig. 1c). When the single 15mer peptides included in pool 5 were tested by IFN-γ ELISPOT assay, two peptides (peptides 47 and 48) showed strong reactivity (Fig. 2a). To characterize the Gag CTL epitope specifically, three 9mer peptides were synthesized spanning the region shared by these two reactive 15mer peptides (Fig. 2a) and used to perform an IFN-γ ELISPOT assay on the Gag-specific T cells. Of the three 9mers tested, two (GAG2 and GAG3) showed strong reactivity (Fig. 2b).

Finally, in order to identify the minimal CTL epitope, two further peptides were synthesized, an 8mer and a 10mer (Fig. 3a), based on the sequence of the GAG2 and GAG3 9mers, and tested using tenfold dilutions ranging from 10 to 0.01 µg ml\(^{-1}\) in an IFN-γ ELISPOT assay, along with the GAG2 and GAG3 9mers. As shown in Fig. 3(a), both the...
10mer and the two 9mers were strongly recognized, showing a dose-dependent curve, whilst no reactivity was observed when using the 8mer peptide or the FLU control peptide. Interestingly, GAG3 was more reactive than GAG2 at all concentrations tested (Figs 2b and 3a), whilst the reactivity of the 10mer peptide was similar to that of GAG3.

To confirm that these peptides corresponded to a Gag CTL epitope, a ⁵¹Cr-release assay was performed using autologous BLCLs pulsed with or without the 8-, 9- or 10mer peptides. As shown in Fig. 3(b), a higher level of specific lysis of target cells (% lysis of the sample minus % lysis of the relative control) was detected in the presence of GAG3 9mer.

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**Fig. 2.** PBMCs from SHIV89.6P-infected Mk 783 were stimulated for 28 days in the presence of autologous FB-Gag BLCLs and tested for IFN-γ production by ELISpot assay. The number of IFN-γ-producing T cells is shown, expressed as SFCs per 10⁶ cells, in the presence of single 15mer peptides (Pep 41–Pep 50) corresponding to pool 5 (a) and the 9mer peptides (GAG1–GAG3) corresponding to the overlapping region of Pep 47 and Pep 48 (bold) (b). Data are representative of three independent experiments. Error bars represent SD between duplicate samples.

**Fig. 3.** PBMCs from SHIV89.6P-infected Mk 783 stimulated for 14 days in the presence of autologous FB-Gag BLCLs were tested for IFN-γ production by ELISpot assay (a) and for CTL activity by ⁵¹Cr release assay (b). (a) The number of IFN-γ-producing T cells in the presence of the 8-, 9- or 10mer peptides at different concentrations is expressed as SFCs per 10⁶ cells. Medium alone (M) and the unrelated FLU peptide were used as negative controls. Error bars represent SD between two experiments. The sequences of the 8mer and 10mer peptides are shown. (b) The percentage of specific lysis was determined by a standard ⁵¹Cr release assay using FB-Neo BLCLs (○), FB-Gag BLCLs (●), FB-Neo BLCLs pulsed with GAG2 9mer (△), GAG3 9mer (●), 8mer (◇) or 10mer (▲) peptides or unrelated FLU peptide (□) as target cells. Data are representative of three independent experiments.
peptide (54% specific lysis at an E:T ratio of 20:1) and in the presence of the 10mer peptide (55% specific lysis at an E:T ratio of 20:1), indicating that the GAG3 9mer peptide represents the minimal CTL epitope corresponding to the epitope naturally presented on BLCLs transduced with retroviral vector expressing Gag protein (FB-Gag, 53% specific lysis at an E:T ratio of 20:1). In addition, the GAG2 9mer peptide was also able to induce cytotoxic activity in the 51Cr-release assay, although to a lesser extent (40% specific lysis at an E:T ratio of 20:1) than the GAG3 9mer and the 10mer peptides, as previously seen in the IFN-γ ELISPOT assay. The 8mer and the unrelated FLU peptide did not show any specific activity.

To assess the functional reactivity of the Gag3 9mer further and to validate an additional immunological assay in cynomolgus monkeys, TNF-α production was evaluated by intracellular staining of Gag-specific CD8+ T cells. As expected with fully competent effector cells, a high percentage of CD8+ T cells was capable of the production of TNF-α in response to the GAG3 9mer peptide (15% vs 1.9% of non-stimulated cells; Fig. 4).

Analysis of the response to GAG3 9mer in other SHIV89.6P-infected monkeys

In order to evaluate the reactivity of this newly identified Gag CTL epitope in infected cynomolgus monkeys, additional SHIV-infected monkeys with a positive response to the Gag pool B, as measured by IFN-γ ELISPOT, were screened for responsiveness to GAG3 9mer. After in vitro stimulation with autologous BLCLs transduced with retroviral vector expressing Gag protein, effector cells were used in an IFN-γ ELISPOT assay in the presence of Gag pool B and the GAG3 9mer. As shown in Fig. 5(a), all of the monkeys were reactive against the Gag pool B, but only Mk 273 revealed IFN-γ production in the presence of GAG3 9mer peptide, demonstrating specific recognition of the CTL Gag epitope. This result was further confirmed for Mk 273 at another time point after infection (Fig. 4b).

For further confirmation of the MHC restriction of the epitope, CTLs from Mk 783 as effector cells and BLCLs from Mk 273 as stimulator cells were used in an IFN-γ ELISPOT assay. As shown in Fig. 6(a), Gag-specific T cells from Mk 783 were able to produce IFN-γ when the Gag epitope was
presented by GAG3-pulsed BLCLs or by FB-Gag BLCLs constitutively expressing SIV Gag from Mk 273, in amounts comparable to those obtained in the autologous system, showing only a slightly higher background (Fig. 6a). Similar results were obtained using CTLs from Mk 273 as effector cells and BLCLs from Mk 783 as stimulator cells (Fig. 6b), indicating that both monkeys had identical epitope specificity restricted by the same MHC class I allele.

DISCUSSION

In the study presented here, we have reported on the identification of a novel CD8$^+$ T-cell epitope derived from SIVmac239 p55$^{gag}$ recognized by CTLs in SHIV89.6P-infected cynomolgus monkeys. As described previously (Negri et al., 2004), we efficiently generated anti-Gag CTLs using autologous BLCLs transduced with a retroviral vector expressing the SIV Gag protein as stimulator cells. We further characterized the Gag-specific response in an SHIV-infected monkey by specifically defining the minimal CTL epitope recognized by the CTLs. In particular, a Gag CTL 9mer epitope (GAG3) was demonstrated at aa 192–200 in the p27 region of the SIVmac239 p55$^{gag}$ protein. In addition to GAG3, a 10mer (aa 191–200) was also able to induce a positive response in functional assays, suggesting a flexibility of the MHC I molecule in adapting to the nature of the bound peptide, as described previously in biochemical studies on MHC class I–peptide complexes (Reid et al., 1996; Menssen et al., 1999). Unfortunately, a lack of information about the MHC class I molecule does not allow us to hypothesize on the conformation of the peptide–MHC complex and on the binding affinity of the key residues of the two peptides. Further studies using peptides mutated at single amino acid residues should help in the design of possible MHC class I-binding motifs and clarification of the best reactivity relative to both the 9mer and 10mer peptides.

The usefulness of this newly defined epitope to AIDS investigators will be determined, in part, by the degree to which this particular Gag sequence is conserved among different SIV and SHIV isolates. In particular, the sequence of the defined GAG3 epitope is highly conserved among gag genes encoded by the SIV and SHIV viruses that are commonly employed in non-human primate research. Specifically, SIVmac251, SIVmac239 and SIVmac32H gag genes encode an identical 9 aa sequence used to construct the peptide identified in this study (NCVGDHQAA). Moreover, all of the SHIV chimeras in use by various investigators incorporate the SIVmac239 gag gene and therefore are predicted to express this epitope.

A further aspect that still awaits investigation concerns the accurate analysis of the MHC class I allele presenting the defined Gag CTL epitope in cynomolgus monkeys. We were able to examine a limited number of infected animals showing a positive response in the presence of the Gag peptide pools used in this study. Although this prevented us from performing a more thorough statistical analysis, we found that two out of the seven screened monkeys (Mk 273 and Mk 783) exhibited positive reactivity against the 9mer peptide, as demonstrated by IFN-$\gamma$ production in an ELISPOT assay. Further genetic analysis needs to be performed to confirm the characteristics and frequency of the particular MHC class I allele presenting this defined GAG3 epitope.

In this context, several studies have been carried out on rhesus monkeys in order to characterize MHC class I molecules and their corresponding restricted SIV/SHIV epitopes. Indeed, a methodology to screen rhesus monkeys

![Fig. 6.](image)
for the expression of Mamu-A*01, Mamu-A*02 and Mamu-B*17 alleles has been described extensively (Robinson et al., 2001; Mothé et al., 2002; Loffredo et al., 2004). So far, to our knowledge, only one paper has described a Gag CTL epitope in cynomolgus monkeys (Geretti et al., 1997), but, to date, no information is available describing the MHC class I allele responsible for the epitope restriction. Importantly, however, two studies have recently reported on the initial molecular characterization of MHC class I A and B loci in M. fascicularis, allowing a more detailed analysis of the MHC class I/epitope interplay during natural infection or immunization (Uda et al., 2004, 2005).

The two monkeys that were responsive to the GAG3 9mer epitope were both long-term non-progressors (LTNPs) with low or undetectable viremia, and with stable numbers of CD4 T-cell counts at all of the time points analysed (data not shown). Viral mutations that escape CTL recognition have been described previously in humans infected with HIV (Goulder et al., 1997) and monkeys infected with SIV and SHIV (Goulder & Watkins, 2004). Indeed, escape mutants have been commonly detected in the Mamu-A*01-restricted Gag epitope p11C in rhesus monkeys and the response to this epitope has been associated with the effective control of viraemia, whilst escape from this epitope seemed to be coincident with loss of immune-mediated control (Barouch et al., 2002). In this setting, it becomes important to evaluate over time the GAG3-specific response in the two LTNP monkeys described herein in order to verify whether this particular Gag epitope is associated with control of viraemia and, in the case of mutations, with loss of control.

This information will be essential in expanding and improving the evaluation of precise immune responses during vaccination and infection in the cynomolgus monkey model of AIDS.

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