Specificity of helper T-cells generated from macaques infected with attenuated simian immunodeficiency virus

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Deletion of the simian immunodeficiency virus (SIV) nef gene leads to an attenuated virus phenotype in vivo. We have previously shown that these viruses induce a potent cellular immune response in macaques. To extend these studies, we established virus-specific short-term T-cell lines from four rhesus macaques infected with a nef deletion mutant of SIV. These T-cell lines proliferated upon restimulation with whole SIV or SIV gp140 antigen in vitro. The proliferating cells were characterized as CD4 helper T-cells (TH) and their antigen recognition was MHC class II DR-restricted. After antigenic stimulation, they transcribed mRNA for various TH1- and TH2-like cytokines. Using these SIV-specific cell lines, a variety of helper T-cell epitopes in the SIV Env protein were determined with overlapping peptides. TH epitopes were identified throughout the whole SIV Env including both constant and variable regions. Although the recognition of TH epitopes was heterogeneous among different animals, five more broadly reactive T-cell epitopes were identified. As expected, recognition was associated with the MHC class II DRB background of the animals. This is the first report on helper T-cell epitopes in SIV-infected monkeys. Such studies should be of considerable significance for AIDS/vaccine research.

Introduction

Since the discovery of human immunodeficiency virus (HIV) as the causal agent of AIDS, the infection of macaques with simian immunodeficiency viruses (SIV) has been the main approach to study vaccination. Although some success has been achieved in this model using viral antigens as vaccines (Hu et al., 1992; Lüke et al., 1995), the most promising results have come from experiments with attenuated SIV (Marthas et al., 1990; Daniel et al., 1992; Stahl-Hennig et al., 1996). Such viruses have been experimentally attenuated by the deletion of regulatory genes. Deletion of the SIV nef gene (SIVΔnef) leads to an attenuated phenotype of the virus characterized by reduced replicative ability in macaques (Kestler et al., 1991). Although a few cases of SIVΔnef-induced immunodeficiency have been found (Cohen, 1997), attenuated SIV does not usually cause disease but induces a potent antiviral immune response. To understand the mechanism of protection, the immune response of macaques infected with attenuated SIV has to be investigated in detail. We and others have previously reported on the SIV-specific helper T-cell (TH) and cytotoxic T-cell (CTL) responses of animals infected with SIVΔnef (Dittmer et al., 1995b; Gallimore et al., 1995; Gundlach et al., 1997). The identification of T-cell epitopes on SIV proteins is a second important step towards the understanding of SIV immune recognition. Several groups have previously characterized SIV-specific TH epitopes from animals immunized with different prototype vaccines based on the core (Mills et al., 1991; Lehner et al., 1993; Brookes et al., 1995) or envelope protein (Jones et al., 1992; Voss et al., 1993b) of SIV. However, since pathogenic SIV leads to early TH-dysfunction (Voss et al., 1993a; Dittmer et al., 1994) TH epitopes have not been described in infected animals until now. The infection of macaques with attenuated SIV provides a unique model to determine TH-cell specificities in infected animals. We have used short-term T-cell lines from four SIVΔnef-infected rhesus macaques to determine TH epitopes in the Env protein of SIV. Five broadly reactive epitopes were identified which seem to be associated with the major histocompatibility complex (MHC) class II DRB background of the animals.
Table 1. SIV-specific proliferation of STCL from SIV nef-infected macaques

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Virus</th>
<th>Antigen:</th>
<th>[H]Thymidine incorporation (c.p.m. ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SIV†</td>
<td>SIV gp140†</td>
</tr>
<tr>
<td>7755</td>
<td>SIVANU</td>
<td>3013 ± 108</td>
<td>1470 ± 430</td>
</tr>
<tr>
<td>7756</td>
<td>SIVANU</td>
<td>4531 ± 103</td>
<td>1922 ± 78</td>
</tr>
<tr>
<td>7761</td>
<td>SIVANU</td>
<td>1895 ± 127</td>
<td>1016 ± 175</td>
</tr>
<tr>
<td>7763</td>
<td>SIVANU</td>
<td>1287 ± 282</td>
<td>1190 ± 640</td>
</tr>
<tr>
<td>7743</td>
<td></td>
<td>497 ± 179</td>
<td>667 ± 49</td>
</tr>
<tr>
<td>8307</td>
<td></td>
<td>701 ± 57</td>
<td>741 ± 262</td>
</tr>
<tr>
<td>8313</td>
<td></td>
<td>663 ± 26</td>
<td>401 ± 78</td>
</tr>
<tr>
<td>8322</td>
<td></td>
<td>704 ± 177</td>
<td>371 ± 104</td>
</tr>
<tr>
<td>7759</td>
<td>SIVg</td>
<td>427 ± 110</td>
<td>630 ± 359</td>
</tr>
</tbody>
</table>

* Mean of triplicate assays; SD calculated by the formula σ x (n − 1).
† STCL were stimulated with inactivated SIV (0.5 or 2.5 µg/ml) or recombinant SIV gp140 (2.5 or 5.0 µg/ml) in the presence of antigen-presenting cells (APC). The c.p.m. are shown for the antigen concentrations that gave the higher result.
‡ Irradiated PBMC (APC) alone.
§ STCL and APC without antigen (medium).
¶ Animal 7759 was infected for 90 weeks with pathogenic SIVmac239.

Methods

Animals and viruses. The rhesus macaques (Macaca mulatta) used for this study were housed at the German Primate Center in Göttingen, Germany. Handling of the monkeys and collection of specimens were performed according to institutional guidelines as described previously (Stahl-Hennig et al., 1990). The four rhesus monkeys, Mm 7755, 7756, 7761, and 7763, were infected intravenously with 300 TCID₅₀ of the apathogenic SIVANU containing deletions of 513 bp in the nef gene (described in Gundlach et al., 1997). Virus replication was detected in all animals but they did not develop any signs of an immunodeficiency over 70 weeks post-infection (Gundlach et al., 1997; C. Stahl-Hennig, unpublished data). During the course of infection with SIVANU, all four animals developed virus-specific TH responses (Gundlach et al., 1997). This enabled us to use peripheral blood mononuclear cells (PBMC) from these animals to establish SIV-specific T-cell lines and to study T-cell specificity.

T-cell proliferation assay and epitope mapping. At weeks 10 and 34 after infection with SIVANU, we generated SIV-specific short-term cell lines (STCL) from PBMC of the four animals essentially as described (Lehner et al., 1993). Briefly, 3 x 10⁶ PBMC/ml were cultured in RPMI 1640 cell growth medium (1:1; supplemented with 10% FCS) for 14 days in the presence of 2.5 µg/ml purified inactivated SIV antigen (Dittmer et al., 1994). In addition, STCL from four uninfected controls and one animal infected with pathogenic SIV were established. After 14 days, 0.5 or 2.5 µg/ml inactivated SIV or 2.5 or 5 µg/ml recombinant SIV gp140 (Program EVA) were used as antigen to restimulate 2 x 10⁸ T-cells in the presence of 1 x 10⁵ autologous irradiated PBMC (40 Gy) as antigen-presenting cells (APC). The 5 day proliferation assays were performed in triplicate using Cell Growth Medium (Vitromex) supplemented with 1% human AB serum. [H]Thymidine (0.5 µCi per well) was present during the last 6 h of cultivation, and the incorporated radioactivity was determined in a β-counter. For the epitope mapping, 2 x 10⁵ STCL were cultured for 5 days in duplicate with 10 µg/ml SIVmac251/32H Env peptides covering the whole SIV Env protein (Program EVA; McBride et al., 1993) and 1 x 10⁵ irradiated autologous PBMC. The 46 peptides used were 20-mer synthetic peptides overlapping by 10 amino acids. Two unrelated HIV-1 Env peptides (no. 10 and no. 30; Program EVA) served as control antigens. Proliferative responses were considered as positive if the mean counts per minute (c.p.m.) of cultures containing antigen was greater than twice the background (mean c.p.m. without antigen; Dittmer et al., 1995a).

Phenotypic characterization of T-cells. To determine the phenotype of the proliferating cells, CD4 subsets were isolated with immunomagnetic beads from STCL generated 34 weeks after SIVANU infection as described (Voss et al., 1993b). The remaining cells, together with the cells bound to the beads, were then restimulated with 2-5 µg/ml inactivated SIV antigen in the presence of autologous irradiated PBMC (40 Gy) as APC.

The MHC restriction of the antigen presentation for the proliferating cells was assessed by adding monoclonal antibodies (1:1000) specific for MHC class I (W6/32) or class II DR (Q513) molecules as described (Voss et al., 1993b). The proliferation assay was then performed for both as described above.

To determine which T-cell subsets were present in the STCL after 14 days of culture, we characterized the phenotype of STCL cells by flow...
cytometry using labelled CD2- (55.2; Becton Dickinson), CD4- (OKT4; Orthomune) and CD8-specific (3B5; Medac) antibodies (Stahl-Hennig et al., 1990).

- Cytokine transcription. Cytokine transcription was measured after SIV-specific restimulation of STCL in vitro using a semiquantitative RT–PCR (Spring et al., 1997). The PCR was optimized for every cytokine transcript to generate signals that could be evaluated within a linear range of the PCR process. The resulting cycle numbers were: 34 for IL-2, IL-4 and INF-γ; 32 for IL-6 and IL-10; 30 for β-actin. The intensity of the resulting band was quantified with the Gelprint documentation system (MWG-Biotech) for each cell line and compared with the intensity of the respecting β-actin band (housekeeping gene).

- MHC background of the rhesus macaques. The MHC class II DRB background of the rhesus macaques (Mamu-DRB) was investigated by gel electrophoretic analysis. The second exon of the Mamu-DRB genes was amplified by PCR as described (Slierendregt et al., 1994). The PCR products were separated on a precast polyacrylamide gel according to their GC nucleic acid content (GenGel Excel; 12% polyacrylamide) using the GenPhor-System (20 mA, 5 h; Pharmacia). Bands were then visualized by standard silver staining.

**Results and Discussion**

At 10 and 34 weeks after SIVΔNU-infection of four rhesus macaques, STLC were established to determine SIV-specific T-cell epitopes. After 2 weeks of virus-specific stimulation, the STCL showed a proliferative response after restimulation with purified SIV, as well as against the recombinant SIV gp140, that was greater than twice that of cultures without antigen (Table 1). The SIV gp140-specific response was lower than the response to whole virus, possibly due to the antigen preparation or to specificities against other SIV antigens. Three out of four STCL from SIVANU-infected macaques developed SIV-specific proliferative responses 4-fold greater than background. The STCL from SIVANU-infected animals showed no reactivity against unrelated control antigens (Fig. 1). In addition, none of the STCL from four uninfected controls or an animal infected with pathogenic SIV showed a SIV-specific proliferation (Table 1). However, the STCL of all investigated animals responded (c.p.m. were 3.9–11.2-fold greater than background) after phytohaemagglutinin stimulation (1%, v/v).

To determine which T-cell populations were proliferating in response to SIV antigen, we characterized the phenotype of STCL. Approximately 60% of the cells from STCL were CD2+ lymphocytes. Up to 25% CD4+ and 45% CD8+ cells were detected in the STLC. However, the SIV-specific proliferation was exclusively linked to the CD4+ cells. CD4-expressing cells isolated from the STCL proliferated after restimulation with soluble SIV and APC, whereas CD4-depleted STCL did not respond. The CD4+ cell reactivity could be blocked by an MHC class II DR-specific antibody (Fig. 1). Therefore, the specifically proliferating T-cells were characterized as helper T-cells.

![Fig. 1. Phenotypic characterization of the proliferating T-cells.](image-url)
Fig. 2. Proliferative response of STCL from SIVΔnef-infected macaques after restimulation with SIV Env peptides. STCL were generated at week 34 after SIVΔNU infection from the four macaques (7755, 7756, 7761 and 7763). After 14 days of SIV-specific stimulation, cells from the STCL were cultured with 10 µg/ml synthetic SIVmac251/32H Env peptides covering the whole SIV Env protein and irradiated autologous PBMC. [3H]Thymidine uptake was measured in c.p.m. The c.p.m. of cultures with SIV peptides (numbers), unrelated HIV-1 control peptides (Ca, Cb) and without peptides (M) are shown. SIV Env peptides 5, 38 and 46 were not available. A proliferation assay with STCL generated 10 weeks after infection revealed the same results. The horizontal line represents the experimental cut-off (2-fold greater than background).
Table 2. Cytokine transcription of SIV-specific stimulated STCL from SIVΔnef-infected macaques

Cytokine transcription was measured in a semiquantitative RT–PCR (Spring et al., 1997). The intensity of the resulting band compared with the intensity of the respective β-actin band of each cell type is indicated as follows: −, no PCR product visible; + / −, only half of the samples showed a low intensity band; +, the intensity of the band was lower than the intensity of the β-actin band but was visible for all samples; + +, similar intensity of the cytokine and the β-actin band.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>STCL*</th>
<th>APCI†</th>
<th>PBMC‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>IL-4</td>
<td>+ / −</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IL-6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-10</td>
<td>+ +</td>
<td>+</td>
<td>+ / −</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td>−</td>
<td>+ / −</td>
</tr>
<tr>
<td>β-Actin</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* STLC (2 × 10⁶ cells) of SIVΔnef-infected macaques after restimulation with irradiated autologous PBMC (APC) presenting SIV antigen (n = 4).
† Irradiated PBMC (APC) of SIVΔnef-infected macaques alone (n = 4).
‡ PBMC (2 × 10⁶) isolated from naive macaques without any in vitro culture served as a control (n = 10).

To investigate the functional properties of the virus-specific STCL, we determined their cytokine transcription after in vitro restimulation with autologous APC and inactivated SIV antigen. We detected increased transcription of the cytokines IFN-γ and IL-2, -4 and -10 for all four virus-specific STCL in comparison to APC alone (Table 2). In addition, transcription of the same cytokines, except IL-2, was enhanced compared to unstimulated monkey PBMC. This implied that the SIV-specific proliferating TH consisted of both TH1-, TH2- and/or perhaps TH0-like T-cell populations.

Table 3. Sequence of SIV Env epitopes recognized by STCL from several SIVΔnef-infected animals

All peptides that were recognized by the STCL of at least two out of four animals are listed. The epitope in the C2 region seems to be in the overlapping region of peptides 25 and 26. The STCL from four uninfected control animals and one animal infected with pathogenic SIV did not recognize any of these peptides.

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Amino acid sequence</th>
<th>Region of SIV Env</th>
<th>No. of animals responding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>YCTQYVTVFYGVPAWRNAT1</td>
<td>C2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>TWGTDQCLPDNGDYSELALN</td>
<td>C1</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>TVTEQAEVDVWQIFETSIKP</td>
<td>C1</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>WGLTKSLTPTAPAPTAASK</td>
<td>V1</td>
<td>2</td>
</tr>
<tr>
<td>25</td>
<td>SCRRMMETQSTSTWFGNGTR</td>
<td>C2</td>
<td>3</td>
</tr>
<tr>
<td>26</td>
<td>STWFGFNGTRAENRTTIYWH</td>
<td>C2</td>
<td></td>
</tr>
</tbody>
</table>
Visualized by standard silver staining. The individual animals are indicated.

The MHC class II DRB background of the rhesus macaques (Mamu-DRB) was investigated by gel electrophoretic analysis. The second exon of Mamu-DRB genes was amplified by PCR. The PCR products were then separated according to their GC nucleic acid content on a polyacrylamide gel. Bands were visualized by standard silver staining. The individual animals are indicated.

Interestingly, over 75% of the TH epitopes were identified in regions that were genetically identical between the two closely related SIV isolates.

Since the proliferative activity in our assay was MHC class II DR-restricted, the heterogeneity of the TH recognition might be due to MHC class II DRB polymorphism of the animals. The MHC class II DRB locus (Mamu-DRB) is known to be highly polymorphic in rhesus macaques (Slierendregt et al., 1994). Therefore, denatured Mamu-DRB PCR products were analysed by gel electrophoresis. This analysis revealed differences between the animals, except for no. 7761 and no. 7763 which showed a very similar pattern of Mamu-DRB genes (Fig. 3). These findings were confirmed by sequence analysis of cloned PCR products (data not shown). Interestingly, the STCL of these two monkeys recognized three identical peptides. Furthermore, TH epitopes shared by other animals might also be associated with identical MHC class II genes. It has also been suggested that individual peptides may be recognized by T-cells from animals of more than one haplotype (Jones et al., 1992; Brookes et al., 1995). Although an association between MHC genes and TH epitopes was found, the MHC restriction of the TH epitopes described was not defined in this study. Furthermore, other MHC class II genes beside those of the DR locus might have contributed to peptide recognition in our animals.

The demonstrated ability to elicit TH responses to diverse peptides in MHC-disparate animals by attenuated SIV suggests that a similar vaccine strategy might be successful against HIV in humans. Furthermore, identification of TH epitopes recognized by T-cells from animals of more than one MHC class II DR haplotype can be useful for different vaccine approaches. In this context, the TH response raised during infection of macaques with apathogenic SIV could be of great importance to understanding retroviral immunity.

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