Induction of anti-simian immunodeficiency virus cellular and humoral immune responses in rhesus macaques by peptide immunogens: correlation of CTL activity and reduction of cell-associated but not plasma virus load following challenge

Thorsten U. Vogel,1,3 Brigitte E. Beer,¹ Jan zur Megede,¹ Hans-Georg Ihlenfeldt,² Günther Jung,² Silke Holzammer,¹ David I. Watkins,³ John D. Altman,⁴ Reinhard Kurth¹ and Stephen Norley¹

¹Paul Ehrlich Institute, 63225 Langen, Germany
²Institut für Organische Chemie, University of Tübingen, 72076 Tübingen, Germany
³Wisconsin Regional Primate Research Center, University of Wisconsin, Madison, WI 53715-1299, USA
⁴Emory University Vaccine Center at Yerkes, Atlanta, GA 30329, USA

Lipopeptides which carry the N-terminal moiety tripalmitoyl-S-glceryl-cysteinyl-seryl-seryl (P₃CSS) have been shown to have effective adjuvant and transmembrane carrier properties. To test the ability of these constructs to immunize against simian immunodeficiency virus [(SIV)mac] infection, rhesus macaques, prescreened for expression of the Mamu-A*01 MHC class I molecule, were immunized at regular intervals with lipopeptides corresponding to known SIVmac CTL epitopes alone or in combination with multiple antigenic peptides corresponding to neutralizing epitopes. Both humoral and CTL responses were elicited and the monkeys, along with non-immunized control animals, were challenged intravenously with 20 MID₅₀ of the homologous, uncloned SIVmac251-32H grown in rhesus monkey PBMC. Although none of the monkeys were protected from infection, most demonstrated an anamnestic CTL response with epitope-specific CTL precursor frequencies reaching as high as 1 in 20 total PBMC as measured by limiting dilution CTL assay or 25% of all CD₈⁺ T-cells using tetrameric MHC-I/peptide complexes. A significant inverse correlation between the levels of CTLp and the number of infected cells in circulation was observed. However, no such correlation with the plasma viral load (RNA copies/ml) was evident.

Introduction

Attempts to induce immunity in macaques against vigorous simian immunodeficiency virus [(SIV)mac] challenge using purified or recombinant viral proteins have, with very few exceptions, failed to protect animals from infection (Norley & Kurth, 1996), often despite the generation of high levels of neutralizing antibodies. The potential for vaccines that induce only the humoral arm of the immune response using such immunogens would therefore appear to be limited. Vaccine research presently tends to concentrate on the stimulation of both cellular and humoral immune responses using combina-

tions of subunit proteins, live recombinant vectors and/or plasmid DNA; the latter two can readily stimulate antiviral CTL as a result of endogenous processing of protein and expression of peptides in the context of MHC class I molecules. However, CTLs can also be induced by the administration of peptide immunogens (Aichele et al., 1990; Deres et al., 1989; Hart et al., 1991; Kast et al., 1991; Mortara et al., 1999; Sastry et al., 1992; Schild et al., 1991; Watanari et al., 1987), demonstrating that the requirement for intracellular protein synthesis can be circumvented. In particular, lipopeptides, which carry the N-terminal moiety tripalmitoyl-S-glceryl-cysteinyl-seryl-seryl (P₃CSS) induce CTL without the need for additional adjuvants (BenMohamed et al., 1997; Deres et al., 1989; Schild et al., 1991) and have recently been shown to act via the toll-like receptor-2 (Aliprantis et al., 1999). Indeed, the induction of CTLs in humans using lipopeptide immunogens
has been demonstrated for a number of systems (Agrawal et al., 1998; Livingston et al., 1997; Vitiello et al., 1995). Similarly, multiple antigenic peptides (MAP; Tam, 1988) are able to induce antibodies without the need of coupling to a carrier protein (Posnett et al., 1988; Troalen et al., 1990; Vogel et al., 1994; Wang et al., 1991).

Vaccines based on peptides have a number of potential advantages over other forms of vaccine. First, there are none of the risks associated with using live attenuated virus vaccines. Second, epitopes stimulating a protective immune response can be selected. Third, potentially detrimental epitopes (e.g. those enhancing HIV infection; Robinson et al., 1987, 1990) or those with sequence homology to cellular proteins (molecular mimicry; Beretta et al., 1987; Brenneman et al., 1988; Reiter et al., 1986; Vega et al., 1990; Young, 1988) can be excluded. We therefore designed this study to assess the ability of peptide immunogens to induce both humoral and CTL responses against HIV and to protect against challenge. As the restricting MHC class I molecule for only one SIVmac CTL epitope (p11C) was known at initiation of this study, rhesus monkeys were prescreened for expression of the corresponding Mamu-A*01 molecule (Vogel et al., 1995).

Methods

**Peptides.** Peptides were synthesized in an Abimed AMS 422 multiple peptide synthesizer or in an LKB Biochrom Biosyn 4170 peptide synthesizer by Fmoc chemistry according to the manufacturer’s protocols. Multiple antigenic peptides (MAPs) were synthesized on an Fmoc-β-Lysomer-Lys-βAla–Wang-Resin, and then cleaved and purified in the same manner as the single peptides. Peptide-PPS peptides were prepared using protocols described previously (Prass et al., 1987; Wiesmüller et al., 1988) and are now available from EM Microcollections GmbH, Tübingen, Germany.

Peptide sequences were as follows. SIVmac neutralization epitopes: gp130[287-296] (V2), KMTNGMLRDKTKEYNET (Benichou et al., 1992); Kent et al., 1992); gp130[290-297] (V2), KEDQRTNYPRKRHER RNYPV (Torres et al., 1993); gp130[290-297] (V2), KEDQRTNYPRKRHER RNYPV (Torres et al., 1993); SIVmac CTL epitopes Gag[95-102] (p17), WVAANLDRFGLAELLENKEGCQK (Yamamoto et al., 1990); Gag[160-167] (p27 = peptide 11), VPGFAQICFGTVPGTINQMLMVCVDG (Miller et al., 1991); Yamamoto et al., 1990); Gag[160-167] (p27 = p11C,C → M), CTPYDINQ (Allen et al., 1998); Nef[98-105] LRMTYSKYLAD MSHF (Bourgaux et al., 1992); Nef[153-159] DQWQDYTSGPGRYKPTFG WLVKLV (Bourgaux et al., 1992). The p11C,C → M peptide is the minimal epitope contained within the P11 sequence and is required for preparation of the Mamu-A*01/peptide tetramers.

**Cell lines.** MT-4 cells were obtained from the ATCC and C8166 cells from the MRC AIDS Reagent Project (UK). Molt-4/8 cells were kindly donated by Dr M. Hayami (Kyoto, Japan). Rhesus monkey B-lymphoblastoid cell lines (B-LCL) were generated as described previously (Vogel et al., 1995) by incubating PBMC with herpesvirus papio produced by S94 cells (kindly provided by Dr Letvin, NERPC, USA). All permanent cell lines were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 100 μg neomycin, 100 U penicillin and 100 μg streptomycin per ml.

**PBMC.** Peripheral blood mononuclear cells (PBMC) were separated from whole heparinized blood by Ficoll–dextranize (Histopaque, Sigma) density-gradient centrifugation. The PBMC were either used immediately or stored in liquid nitrogen. PBMC were cultured in PBMC medium [RPMI 1640 supplemented with 20% FCS, 4 mM l-glutamine, 15 mM HEPEs buffer, 2.5 x 10^{-3} M 2-mercaptoethanol, 100 μg neomycin, 100 U penicillin, 100 μg streptomycin and 500 IU recombinant IL-2 (Proleukin, EuroCetus) per ml]. Phytohaemagglutinin (PHA) blasts were prepared by addition of PHA (54 μg/ml; Murex Diagnostics) to the culture medium for the first 3 days of culture. The cytotoxicity assay was carried out in PBMC medium.

**Virus reisolation and cell-associated virus load.** On the day of challenge and subsequent bleedings 1.5 x 10^8 PBMC from each monkey were cocultivated with 5 x 10^6 C8166 cells. After 28 days the cultures were analysed for the presence of SIVmac infection by immunoperoxidase staining (Norley et al., 1993).

For determining the virus load 10-fold dilutions of PBMC (10^6–10^1 cells) were cocultivated with 1 x 10^6 C8166 cells in triplicate and analysed as above. The numbers of infected cells per 10^6 PBMC were then calculated using a Poisson distribution fitting model.

**Rhesus macaques, immunization and challenge schedule.** Mamu-A*01-positive rhesus macaques (Macaca mulatta) were selected by 1-dimensional isoelectrofocusing (1-D IEF) as described previously (Watkins et al., 1988) and immunized according to the schedule described in Table 1. Two weeks after the last immunization all animals, including the two control animals, were challenged with 200 MID_50 of the in vitro titrated SIVmac stock 5, prepared by passaging the European SIVmac239 stock 11/68 challenge virus once through rhesus PBMC.

**Neutralization test.** This was performed as described previously (Vogel et al., 1994).

**In vitro stimulation of PBMC.** The presence of peptide-specific CTL was determined after in vitro stimulation of PBMC with peptide-pulsed stimulator cells. Briefly, stimulator cells were generated by incubation of 5 x 10^5 autologous B-LCL or PHA-activated PBMC with 50 μM peptide at 37 °C in a 5% CO_2 humidified atmosphere. After 1 h, the cells were γ-irradiated (3000 rad), washed twice with PBS and added to 5 x 10^6 fresh autologous PBMC. After 12–14 days, during which time the cultures were refed five times with PBMC medium, the in vitro stimulated PBMC were used as effectors in the cytotoxicity assay.

**Limiting dilution analysis (LDA).** To estimate the peptide-specific CTL precursor frequency, PBMC were titrated (initial cell number 10^1 per well; 12 dilution steps at 1:2; 12 replicates per dilution) in microtitre plates. 10^6 peptide-pulsed stimulator cells were added to give a final volume of 200 μl per well. After 12–14 days in vitro stimulation, well contents were analysed by cytotoxicity assay (see below) for the presence of peptide-specific CTL. Frequency calculations were made using the zero order term Poisson equation as described previously (Donnenberg et al., 1989).

**Cytotoxicity assay.** Target cells were prepared by incubating autologous B-LCL or PHA-activated PBMC with synthetic peptides at a final concentration of 50 μM and Na_2HCO_3 at 0.5 mM/ml for 3 h at 37 °C in a 5% CO_2 humidified atmosphere followed by six washes in RPMI 1640. The in vitro stimulated PBMC were used as effector cells. The chromium release assay was performed using standard procedures in U-bottom 96-well microtitre plates. Briefly, autologous B-LCL or PHA-activated PBMC target cells were plated at 1 x 10^4 or 2 x 10^4 cells per well, respectively, effector cells were added and, after centrifugation at
### Table 1. Immunization and challenge protocol

<table>
<thead>
<tr>
<th>Week</th>
<th>Adjuvant</th>
<th>Controls</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Antigen dose (per peptide)</th>
<th>Injection route*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>–</td>
<td>CTL‡</td>
<td>CTL‡</td>
<td>100 µg</td>
<td>s.c.</td>
</tr>
<tr>
<td>2</td>
<td>RIBI+</td>
<td>–</td>
<td>V2-, V417-MAP</td>
<td></td>
<td>100 µg</td>
<td>i.m.</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>CTL</td>
<td>CTL</td>
<td>100 µg</td>
<td>s.c.</td>
</tr>
<tr>
<td>6</td>
<td>RIBI</td>
<td>–</td>
<td>V2-, V417-MAP</td>
<td></td>
<td>100 µg</td>
<td>i.m.</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
<td>V2-, V417-MAP</td>
<td>V2-, V417-MAP</td>
<td>100 µg</td>
<td>s.c.</td>
</tr>
<tr>
<td>10</td>
<td>RIBI</td>
<td>–</td>
<td>V2-, V417-MAP</td>
<td>V2-, V417-MAP</td>
<td>100 µg</td>
<td>i.m.</td>
</tr>
</tbody>
</table>

**Immunization section A**

| 15   | –        | –        | CTL     | CTL + V2-P_CSS| 1 mg + lipid§ | s.c.            |
| 17   | RIBI     | –        | V2-, V417-MAP| V2-, V417-MAP| 1 mg + lipid | s.c.            |
| 19   | –        | –        | CTL     | CTL + V2-P_CSS| 1 mg + lipid | s.c.            |
| 21   | RIBI     | –        | V2-, V417-MAP| V2-, V417-MAP| 1 mg + lipid | s.c.            |
| 23   | –        | –        | V2-, V417-MAP| V2-, V417-MAP| 1 mg + lipid | s.c.            |
| 25   | RIBI     | –        | V2-, V417-MAP| V2-, V417-MAP| 1 mg + lipid | s.c.            |

**Immunization section B**

| 41   | Montanide‡ | – | CTL     | CTL + V2-P_CSS + V2-, V417-MAP | 1 mg | s.c. |
| 43   | Montanide  | – | CTL     | CTL + V2-P_CSS + V2-, V417-MAP | 1 mg | s.c. |
| 46   | Montanide  | – | CTL     | CTL + V2-P_CSS + V2-, V417-MAP | 1 mg | s.c. |
| 50   | Montanide  | – | CTL     | CTL + V2-P_CSS + V2-, V417-MAP | 1 mg | s.c. |
| 52   | Montanide  | – | –       | –                                 | –    | i.v. |

**Immunization section C**

| 52   | Montanide  | – | –       | –                                 | –    | i.v. |

**Blood sampling only**

| 2    | –        | –        | –       | –                                 | –    | i.v. |

* s.c., subcutaneous; i.m., intramuscular; i.v., intravenous.

† The controls and Group 1 received only RIBI without peptides.
‡ CTL, CTL epitopes as P3CSS peptides.
§ V2 epitope as P1CSS peptide.
∥ V2 = gp130, 168–190; V4 = gp130, 410–430 (sequences listed in Methods).
§ P1CSS peptides were resuspended in a lipid solution (Intralipid; Pfrimmer Kabib, Erlangen, Germany).
● The controls only received the mineral oil adjuvant Montanide ISA 51 without peptides.

2000 r.p.m. for 5 min, the plates were incubated for 4 h at 37 °C. Spontaneous and total release were determined by incubating targets with media or detergent, respectively. Radioactivity in harvested supernatants was measured using a γ-counter and the percent specific chromium release calculated using the following formula: %specific lysis = c.p.m.(effectors) – c.p.m.(spontaneous)/c.p.m.(total) – c.p.m. (spontaneous) × 100.

#### Tetramer staining

1 × 10⁶ thawed PBMC were washed three times in FACS-buffer (PBS, 2% FCS) in a 96-well U-bottom plate. Cells (100 µl) were incubated for 40 min at room temperature in the dark with the Mamu-A*01/p11C,C→M-tetramer labelled either with PE or APC (1:200 dilution of stock), an anti-rhesus CD3–FITC monoclonal antibody (10 µl; BioSource) and an anti-CD8–PerCP monoclonal antibody (3 µl; BD). The Mamu-A*01 molecule was refolded with the p11C,C→M peptide and the tetramers were made as described previously (Altman et al., 1996). Plates were then washed four times with FACS-buffer. Finally, 450 µl 2% paraformaldehyde (PFA) in PBS was added to fix the stained cells. A CTL clone specific for these tetramers was stained in parallel with isotype controls (mouse IgG1–FITC (Biosource); mouse IgG2a–PE (ImmunoTech); mouse IgG1–PerCP (BD); mouse IgG1–APC (ImmunoTech), anti-CD3–FITC, anti-CD8–PE (ImmunoTech), anti-CD8–PerCP, or CD8–APC (ImmunoTech) to establish compensation parameters]. Sample data were acquired on a Becton Dickinson FACSCalibur instrument and analysed using CellQuest software. Background staining of fresh PBMC from naive Mamu-A*01+ animals with tetrameric complexes was routinely less than 0.07%.

#### PCR

- Nested PCR analysis of rhesus PBMC was performed using standard protocols (Saiki et al., 1988) and the following SIVmac239 nef primers: SN9044N (5’ GACCTACCTACATATGGG 3’) and SN9866C (5’ TCAGGAGTTTCTCCTTGT 3’) for the first 40 cycles and SN9272N (5’ GAATCTACCATGGGAAACC 3’) and SN9763C (5’ GGGTATCTACATATGCTC 3’) for the second 40 cycles. To determine the frequency of proviral DNA carrying cells in peripheral blood, different amounts of PBMC (10³–10⁷ cells) were analysed by PCR.

#### p27 assay

This was was performed using the commercial SIV Core Antigen Assay (Coulter) according to manufacturer’s protocol.

#### Plasma virus load

The number of SIV RNA copies per ml of...
plasma was measured using real-time quantitative RT–PCR. Briefly, viral RNA was isolated from 200 µl plasma using the High Pure Viral RNA kit (Roche) according to the manufacturer’s instructions. Eluted RNA was then subjected to a one-step RT–PCR using the OneStep RT–PCR kit (Qiagen) with the following reagent mix (volume: 40 µl mix plus 10 µl RNA sample): 1× Qiagen OneStep RT–PCR buffer; 1.5 µM forward and reverse primers; 1 µM probe; 400 µM of each dNTP; 3 mM MgCl₂; 20 U rRNasin; 2 µl Qiagen Enzyme Mix and 0.5 µM Rox dye. Primers and probe, synthesized by Eurogentec, Belgium, were as follows: Forward primer 5′-CTAGTGGTGAAACAGGAAAC-3′; reverse primer 5′-TGTCTCGGGCTTAATGGCA-3′; probe 5′-F CCAACAGCACCATCTAGCGAGG-3′. The probe was 3′-phosphorylated to prevent extension. The RT reaction was carried out at 95 °C for 30 min followed by an initial denaturation step of 15 min at 95 °C and 45 PCR cycles (95 °C 15 s; 60 °C 2 min). Reactions were performed in an ABI Prism 7700 Sequence Detector (Applied Biosystems). The number of SIV RNA copies was calculated by comparing the Threshold Cycle (Cₚ) value with those of a dilution series of standard SIVmac RNA included in each reaction. All samples and standards were run in triplicate and means were used for calculations.

Results

Peptide immunization induced humoral and cellular immune responses

After three immunizations with an antigen dose of 0.1 mg/peptide, rhesus monkeys developed antibodies against both the neutralization epitopes delivered as MAP (group 2) and against some of the CTL epitopes (Table 1). In the majority of the macaques it was also possible to detect antibodies binding native SIVmac antigen. After a further three immunizations with 1 mg/peptide the antibody titres against all peptides increased and two macaques of group 2 that had been immunized with the neutralizing epitopes developed antibodies against rgp140 in ELISA and against gp130 in Western blot (data not shown). However, in none of the animals was it possible to detect neutralizing antibodies against SIVmac. After a break in the immunization schedule of 16 weeks, during which time antibody titres against all immunogens decreased, the animals were again immunized three times, this time using the mineral oil adjuvant Montanide ISA 51 in conjunction with both kinds of peptides (MAP and Peptide-CS peptides). In most cases antibody titres again increased and the sera from one macaque of group 2 was shown to neutralize SIVmac in vitro (Fig. 1).

After three immunizations with the low antigen dose (0.1 mg/peptide) no peptide-specific CTL could be detected (data not shown), although it was possible to demonstrate p11-specific CTL in one monkey (Rh 67) following immunization with the high antigen dose (1 mg/peptide; Table 2). However, after one immunization utilizing the mineral oil adjuvant Montanide ISA 51, p11-specific CTL could be demonstrated in five out of six Mamu-A*01-positive rhesus macaques (Rh 67, 80, 84, 85, 86; Table 2). Despite being immunized a further three times it was not possible to demonstrate CTL at every bleeding. Indeed, on the day of challenge p11-specific CTL could only be demonstrated in macaques 67, 80 and 85. None of the animals developed CTL against the other CTL epitopes used even after infection (data not shown). It is likely that they did not possess the MHC class I molecule that binds these epitopes. The Mamu-A*01-negative rhesus 66 never developed p11-specific CTL.

Secondary antibody response after virus infection

With the exception of Rh 67, the monkeys showed little or no anamnestic antibody response to the peptides following challenge. In contrast, all animals showed a strong increase in antibody titres against rgp140 and whole SIVmac antigen after challenge. Rhesus monkey 67 showed an increase in anti-rgp140 antibodies as soon as 2 weeks post-challenge whereas in most others the titres increased from week 3 after challenge. Animals of group 2 (Rh 80 and 88), which possessed anti-rgp140 responses at the day of challenge, showed the strongest and fastest response after challenge. The pattern of antibody development against whole SIVmac antigen was very similar to that seen for rgp140. Most animals showed an increase in titre beginning 3–4 weeks after challenge and levelling off at 20 weeks.

As Fig. 1 shows, all animals had detectable neutralizing antibodies at 3 weeks post-challenge, although in contrast to the others rhesus monkey 66 showed a weak neutralizing response that had disappeared by week 8. Rhesus monkey 80, in which neutralizing antibodies had been detectable on the day of challenge, showed an anamnestic response with the titre already rising in the second week after challenge.

Post-challenge virus load measurements

After challenge with SIVmac32H all immunized animals and the two control animals became infected as demonstrated by
**Table 2.** Demonstration of p11-specific CTL after immunization with P3CSS peptides by *in vitro* stimulation of PBMC with p11-pulsed stimulator cells*

<table>
<thead>
<tr>
<th>Immunization section B</th>
<th>Week 17†</th>
<th>Week 21</th>
<th>Week 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus macaque no.</td>
<td>in vitro stimulations</td>
<td>in vitro stimulations</td>
<td>in vitro stimulations</td>
</tr>
<tr>
<td></td>
<td>1 × †</td>
<td>2 × §</td>
<td>1 ×</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>66</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>67</td>
<td>65</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>84</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>85</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>86</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>88</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>130</td>
<td></td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Immunization section C</th>
<th>Week 43†</th>
<th>Week 46</th>
<th>Week 50</th>
<th>Week 52 (challenge day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus macaque no.</td>
<td>in vitro stimulations</td>
<td>in vitro stimulations</td>
<td>in vitro stimulations</td>
<td>in vitro stimulations</td>
</tr>
<tr>
<td></td>
<td>1 × †</td>
<td>2 × §</td>
<td>1 ×</td>
<td>2 ×</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>66‡</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>67</td>
<td>30</td>
<td>56</td>
<td>13</td>
<td>NT</td>
</tr>
<tr>
<td>80</td>
<td>11</td>
<td>66</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>84</td>
<td>0</td>
<td>21</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>85</td>
<td>3</td>
<td>44</td>
<td>4</td>
<td>46</td>
</tr>
<tr>
<td>86</td>
<td>5</td>
<td>23</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>88</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Freshly isolated PBMC (2–5 × 10⁶) were stimulated with p11-pulsed stimulator cells.
† Weeks after the first immunization (Table 1), when the PBMC were isolated for the *in vitro* stimulation.
‡ The presence of p11-specific CTL was determined after 12–14 days *in vitro* stimulation by CTL assay (= 1st stimulation).
§ After the first stimulation the PBMC cultures were stimulated once again with p11-pulsed stimulator cells and analysed after 12–14 days (= 2nd stimulation).
‖ Specific lysis above background. Values over 10% specific lysis are in bold type.
¶ Rhesus monkey 130 died in the 23rd week as a consequence of complications following narcotization.
** Frozen PBMC were used in the assay.
NT, Not tested.

virus reisolation, quantitative RT–PCR (Fig. 2, Fig. 3) and provirus PCR (data not shown). The immunized, Mamu-A*01-positive macaques 67, 80 and 84 had a reduced cell-associated virus load in the peripheral blood as determined by limiting dilution virus reisolation. One immunized animal (Rh 86) showed a delayed primary peak in the number of infected cells and the Mamu-A*01-negative rhesus macaque 66 yielded the highest virus load. Although the p27 assay (Fig. 2) and the quantitative PCR showed a different picture from the cell-associated virus load, the reduced virus load of rhesus macaque 67 was confirmed. The discrepancy between the virus load as measured for infected cells in culture and that indicated by p27 assay and quantitative PCR may be caused by the fact that the latter methods also detect disrupted virus or defective proviruses, respectively.

Nevertheless the quantitative PCR also confirmed the delayed primary viraemia of rhesus macaque 86 (data not shown). The Mamu-A*01-negative rhesus macaque 66, which showed prolonged p27 viraemia and had the highest virus load (which increased again 15 weeks after challenge) finally
developed AIDS-like symptoms during week 18 and was euthanized at week 20 after challenge.

In contrast, no beneficial effect of immunization was evident when assessed by the levels of viral RNA in the plasma (Fig. 3). Indeed, the two control macaques (Rh 14 and Rh 20) had the lowest ‘set points’ (average virus load from week 8 onwards) of all animals tested ($10^{6}$ and $10^{7}$ RNA copies/ml plasma respectively) compared to a mean of $10^{8}$ for most (omitting Rh 86) of the immunized macaques ($p = 0.12$). Rh 86 showed a higher set point of $10^{6}$ copies/ml and in agreement with the results of the cell-associated virus load measurements, the Mamu-A*01-negative macaque (Rh 66) which rapidly developed simian AIDS showed the highest degree of plasma virus load (set point $10^{7}$ RNA copies/ml plasma).

**Correlation between CTL response and virus load**

Although most animals had shown a positive p11-specific CTL response at one or more time points before challenge as measured by bulk culture, it was only possible to detect CTL using the limiting dilution assay in monkeys 67 and 85 (Fig. 4A). However, in contrast to the control animals, all immunized, Mamu-A*01-positive monkeys with the exception of Rh 86 had measurable frequencies of p11-specific CTL 1 week after challenge (Fig. 4A). By 2 weeks after challenge all immunized and control animals, except the Mamu-A*01-negative Rh 66, demonstrated p11-specific CTL. In Rh 67 the frequency of CTLp in the second week after challenge was extremely high, with 1 in 20 of all circulating PBMC being a p11-specific CTLp and in the ensuing weeks the frequency of CTLp in this animal remained in the range 1:20–1:50. Similarly, at 2 weeks post-challenge the frequency of circulating p11-specific CTLp in rhesus 80 was already 1:250 of the total PBMC.

With the advent of tetramer technology (Altman et al., 1996) it became possible to confirm these extraordinarily high CTLp frequencies. PBMC frozen at the time of sampling were thawed and tested in FACS for binding of the Mamu-A*01/p11C,C → M tetramer. The levels of tetramer-positive
Fig. 4. Frequency of p11-specific CTLp upon and after challenge with 20 MID50 SIVmac32H. (A) Frequencies as determined by limiting dilution CTL assay as described in Methods. The frequency of 0-2 (Rh 66) is the lower limit of sensitivity and indicates that no CTL could be demonstrated. (B) Frequencies of Mamu-A*01/p11C,C→M tetramer-positive T-cells. Control animals: Rh 14 (●), Rh 20 (△). Animals of immunization group 1: Rh 66 (●), Rh 67 (▲), Rh 84 (■), Rh 86 (■). Animals of immunization group 2: Rh 80 (○), Rh 85 (△), Rh 88 (□). Rh 85 died in week 52 for reasons unrelated to the study.

cells were, as indicated by previous reports (Tan et al., 1999), generally higher (1- to 10-fold) than the functional CTLp frequencies measured previously. In particular, for the two animals with the highest CTLp frequency as measured by LDA (Rh 67 and Rh 80) the proportion of CD8+ T-cells specific for the Mamu-A*01/p11C,C→M tetramer peaked at 25-4% and 19-5% respectively (Fig. 4B).

It was striking that Rh 67, which demonstrated the highest CTLp frequency (LDA and tetramer staining), also had the lowest peripheral cell-associated virus load, suggesting a possible correlation between the two. Indeed, comparison of the cell-associated virus load and the frequency of Mamu-A*01/p11C,C→M tetramer-positive cells at 4 weeks post-challenge revealed an inverse relationship with a correlation coefficient of 0-788 (Fig. 5A). Similarly, plotting the available data for 4 weeks post-challenge gave an R# value of 0-782 for CTLp frequency (LDA) vs cell-associated virus load and 0-707 for tetramer-positive cells vs provirus containing cells (data not shown). These correlations broke down at later time-points as cell-associated virus loads became negligible.

However, the correlation between CTLp frequency and virus load was not seen at the level of plasma viraemia (Fig. 5B, R# = 0-0). Even animals with extraordinarily high levels of CTLp fared no better than those with lower levels. For example, macaques 67 and 80, with post-challenge tetramer-positive cell frequencies of 25-4% and 9-5%, respectively, had plasma RNA set points as high, if not higher, than most other macaques (including controls) with tetramer-positive cells in the range 0-6 to 3-9%.

**Discussion**

The aim of this study was to stimulate SIVmac-specific antibodies and CTL in rhesus macaques using peptides and to assess the vaccine effect of such immunogens by challenge. Using MAPs and PCSS-peptides as immunogens it was indeed possible to induce SIVmac-specific antibodies and in most cases CTL in rhesus monkeys. The PCSS lipid tail appeared to function as an adjuvant, as reported previously (Lex et al., 1986), with the monkeys developing antibody responses to the CTL epitope peptides, responses not seen in non-immunized, infected monkeys. The peptide-specific antibodies induced by immunization could not only bind the relevant peptides but also virus proteins as demonstrated in ELISA and Western blot (data not shown). In addition, neutralizing epitopes as MAP stimulated a neutralizing response against SIVmac (Fig. 1) in at least one monkey. Although the V2 and V4 domains are known neutralizing epitopes (Schlienger et al., 1994; Torres et al., 1993), in this study they did not seem to play a major role in the neutralization of SIVmac. Although all animals developed strong neutralizing activity against SIVmac by the third week after virus challenge (Fig. 1) many developed no or low titre antibodies specific for the V2 and/or V4 peptides.

The SIV-specific antibody response after virus challenge was very complex, differing from monkey to monkey. Sera
from rhesus macaque 67, which had a reduced virus load, were very reactive against some viral peptides and proteins. In addition, this monkey developed soon after challenge a reaction against gp130 in Western blot (data not shown). However, two of the best responders in terms of anti-gp140 antibodies, Rh 80 and Rh 88, had low and high virus loads respectively after challenge (Fig. 2). This indicates that antibodies against the envelope protein were not by themselves responsible for a reduction in virus load although possibly the neutralizing antibodies which were induced by immunization in rhesus macaque 80 (Fig. 1) played a part in reducing the subsequent load.

In comparison to the induction of CTL in mice (Defoort et al., 1992; Deres et al., 1989; Nardelli et al., 1992; Schild et al., 1991) the P3CSS-peptides alone did not perform as well in the rhesus monkeys, with p11-specific CTL detectable in only one monkey at one time-point (Table 2). However, after using the mineral oil adjuvant Montanide ISA 51 in conjunction with the P3CSS-peptides, p11-specific CTL could be demonstrated at one or more time-points prior to challenge in five out of six Mamu-A*01-positive rhesus monkeys (Table 2). It would therefore appear that whereas P3CSS modification is sufficient to induce CTL in mice, a stronger ‘adjuvant’ is needed for CTL induction in rhesus monkeys.

Of the four CTL epitopes used as immunogen it was only possible to induce a response to the p11 peptide of SIVmac Gag. This is almost certainly because the remaining three epitopes are not restricted by Mamu-A*01, as shown by the lack of CTL against these epitopes even after infection.

In four of the five immunized Mamu-A*01-positive macaques the p11-specific CTL activity was quickly boosted after infection and active CTLp were readily detectable in the LDA at 1 week post-challenge (Fig. 4A). This apparent anamnestic CTL response was not seen in the control animals which first became positive at 2 weeks post-challenge. The levels of p11-specific CTLp in circulation reached levels in some animals which had never previously been observed in SIV (or HIV) infection (up to 5% of the total PBMC). Such levels were at the time considered unlikely and raised questions about the validity of the limiting dilution CTL assay used. Since then, others have shown levels of CTL approaching these values (Borrow et al., 1997) and in particular the development of tetramer technology has made it clear that the frequency of virus-specific CTLp in circulation is much higher than was previously thought (Jordan et al., 1999; Kuroda et al., 1998, 1999). Upon re-examining frozen PBMC for peptide-specific cells using tetramer staining, the high frequencies could indeed be confirmed, with up to 25% of all CD8+ T-cells binding the Mamu-A*01/p11C,C→M tetrameric complex. These are amongst the highest reported levels of tetramer-positive cells, with Jordan et al. (1999), for example, finding up to 22% Mamu-A*01/p11C,C→M tetramer-positive CD8+ T-cells in the semen of SIV-infected macaques. Comparing the percentage of CD8+ T-cells binding the tetrameric complexes with the percentage of CD8+ T-cells with functional CTLp activity (calculated from CTLp frequency and percentage CD8+ cells) yielded only a 1-to 10-fold difference for tetramer-positive cells, which is in closer agreement than the average 24-fold difference observed by others (Tan et al., 1999).

Using tetramer technology it was also possible to detect Mamu-A*01/p11C,C→M-specific cells in some animals (Rh 67, Rh 80 and Rh 84) on the day of challenge, albeit at low levels (0.3–100%; data not shown). Others have been unable to detect Mamu-A*01/p11C,C→M tetramer staining in fresh PBMC after two DNA immunizations (Hanke et al., 1999) or after one immunization with recombinant MVA in most of their animals (Seth et al., 1998). This suggests that the vaccine regimen used in this study was indeed able to efficiently prime CTLs, allowing the animals to react with a strong anamnestic immune response upon infection with SIV. More recently, pre-challenge levels of over 1% tetramer-positive cells have been achieved by SIV gag DNA vaccination in combination with IL-2/1g plasmid or protein (Barouch et al., 2000). Indeed, post-challenge levels in these animals reached as high as 40% and, more significantly, animals were protected from high virus loads and disease.

Although all immunized animals in this study were shown to be productively infected with SIVmac after challenge, three of the five Mamu-A*01-positive immunized animals (Rh 67, 84 and 80) yielded peak cell-associated virus loads 10-fold lower than the non-immunized macaques (Fig. 2). Interestingly, it was in these three monkeys that tetramer-positive cells were detected prior to challenge. Moreover, it was possible to show an inverse correlation between the cell-associated virus load and the frequency of p11-specific CTL soon after the infection (Fig. 5A), indicating a role for the CTL in controlling the number of infected cells during this phase. A similar inverse correlation between the levels of circulating CTL induced by an attenuated virus vaccine and the cell-associated virus load following challenge with wild-type SIVmac had been reported as early as 1995 (Gallimore et al., 1995). However, as with all vaccine studies which do not achieve sterilizing immunity, it is not possible in the study presented here to conclude with certainty that the correlation between CTLs and the reduction in the number of infected cells is directly the result of the immunizations, although the high frequencies observed in animal 67 and 80 (LDA and tetramer) are normally not observed in the PBMC of naive animals after challenge (Kuroda et al., 1998).

Given the correlation with infected cell numbers, the lack of correlation between the levels of CTLp and the plasma virus load as measured by RT–PCR is interesting. It is possible that the circulating CTLp were able to recognize and eliminate those infected cells which were also in circulation but not the infected cells present in the gastrointestinal tract, the main reservoir for SIV replication (Veazey et al., 1998). Indeed, it appears that CTL induced systemically are less likely to migrate to mucosal tissues (Eo et al., 2001; Gallichan &
Rosenthal, 1996). Irregardless of whether the very high levels of post-challenge CTLp in some animals were the consequence of peptide immunization or not, these cells were apparently unable to influence the plasma virus load. Indeed, the two non-immunized control animals appeared to control the virus more efficiently than those immunized with the lipopeptides. It is therefore possible that peptide immunization had the effect of focusing the post-challenge CTL response on a single dominant epitope (p11C,C → M) to the detriment of other epitopes in Gag (or elsewhere). If such a focused response is less efficient at controlling virus production than a weaker but broader response, then monoeptopic immunogens would have to be used and analysed with caution.

In conclusion, the results presented here show that by peptide immunization it was possible to induce humoral and CTL immune responses against SIVmac which were quickly boosted upon challenge. Two animals had remarkably high CTLp frequencies of 1:20 and 1:250 total PBMC. These two animals also had the highest Mamu-A*01/p11C,C → M tetramer staining values (25-4% and 19-5% of their CD8+ T-cells). The inverse correlation between the CTLp frequency (as measured by both functional CTLp limiting dilution assay and Mamu-A*01/p11C,C → M tetramer staining) and the peripheral cell-associated virus load indicates that the p11-specific CTL might have played a role in limiting the initial spread of virus. However, the apparent failure of high frequencies of epitope-specific CTLp to reduce plasma RNA levels may indicate that a broader response is required. Since the study was started the number of CTL epitopes with known MHC class I restriction element has dramatically increased (Allen et al., 2001; Evans et al., 1999, 2000) and it is possible that the use of peptides representing multiple CTL epitopes in macaques expressing appropriate MHC class I molecules would be more effective, particularly if used in conjunction with strong adjuvants and the concurrent stimulation of T-help. Although the relatively weak antiviral responses achieved before challenge suggests that peptide immunogens alone would have limited use as a vaccine, the use of lipopeptides to specifically boost responses induced by priming with live recombinant vectors or DNA vaccines certainly merits further investigation.

This work was supported by contract no. 1506/TG04 to R. K. from the Federal Ministry of Health. D.I.W is an Elizabeth Glaser Scientist.

We would like to thank Nicole Norley, Manuela Schütze and Anne Reckziegel for their excellent technical assistance. We would also like to thank Program EVA for donating many of the reagents used in this study.

References


