Immunological changes in simian immunodeficiency virus (SIV$_{agm}$)-infected African green monkeys (AGM): expanded cytotoxic T lymphocyte, natural killer and B cell subsets in the natural host of SIV$_{agm}$

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The African green monkey (AGM) model system for simian immunodeficiency virus (SIV$_{agm}$) has been used to examine why prolonged infection with the relevant virus does not result in the development of immunodeficiency in its natural host. Blood lymphocyte subset values were determined in uninfected (n = 88) and naturally SIV$_{agm}$-infected AGMs (n = 74). A number of blood cell subsets, such as CD8$^+$CD3$^+$CD28$^{neg}$, CD8$^+$CD3$^+$ and CD20$^+$ cells, were expanded significantly in clinically asymptomatic animals carrying a relatively high plasma load of viral RNA ($10^5$–$10^7$ RNA copies/ml plasma). The expanded CD8$^+$CD3$^+$CD28$^{neg}$ subpopulation (1094±868 cells/µl blood in infected animals versus 402±364 cells/µl blood, P = 0.03) comprised cells that resembled terminally differentiated effector CD8 T cells (CD27$^{neg}$ and CD11a$^+$). In SIV$_{agm}$-infected animals, the expanded CD8$^+$CD3$^+$ cell subset shared identity with the CD16$^+$ population (natural killer cells). These results demonstrate for the first time that apathogenic SIV$_{agm}$ infection causes significant changes in the immune system of its natural host. Although previous studies had indicated that noncytotoxic mechanisms might play an important role in the suppression of virus replication in the natural host of SIV$_{agm}$, this study sheds new light on the possible role of cytotoxic T lymphocytes, the innate immune system and double-positive T helper cells (CD4$^+$CD8$^+$CD3$^+$) in suppressing virus replication in this animal model of AIDS.

Introduction

One of the many natural host species of simian immunodeficiency virus (SIV) is the African green monkey (AGM) (Kraus et al., 1989; Ohta et al., 1988). AGMs, which belong to the Old World monkeys (Cercopithecidae species) (Allan et al., 1991; van der Kuyl et al., 1995), have been classified into four different subspecies: grivet monkeys (Chlorocebus aethiops), vervet monkeys (C. a. pygerythrus), tantalus monkeys (C. a. tantalus) and sabaeus monkeys (C. a. sabaeus) based on their phenotypic differences and geographical distribution (Allan et al., 1991; van der Kuyl et al., 1995). The immune system of AGMs is characterized by extremely high numbers of blood CD8$^+$ T cells in apparently healthy and uninfected AGMs and by an extraordinarily high number of mature T helper cells coexpressing the CD8 $\alpha$-chain (Beer et al., 1998; Murayama et al., 1997, 1999). AGMs are frequently infected with SIV in the wild (Daniel et al., 1988; Hendry et al., 1986; Jolly et al., 1996; Kanki et al., 1985; Krugner-Higby et al., 1990; Lowenstine et al., 1986; Ohta et al., 1988; Otusyla et al., 1996; Phillips-Conroy et al., 1994), with each subspecies of monkey infected by specific strains of SIV, referred to as SIV$_{agm-gri}$ (grivet monkey), SIV$_{agm-ver}$ (vervet monkey), SIV$_{agm-tan}$ (tantalus monkey) and SIV$_{agm-sab}$ (sabaeus monkey) (Allan et al., 1991; Bibollet-Ruche et al., 1997; Jubier-Maurin et al., 1995; Muller et al., 1993; Johnson et al., 1990). Despite long-term infection with these viruses, the natural hosts of SIV do not develop simian AIDS. Indeed, significant virus-associated changes within blood lymphocyte subsets have not been observed in SIV$_{agm}$-infected AGMs (Norley et al., 1999; Whetter et al., 1999). However, SIV$_{agm}$ isolates have been shown to have the capacity to cause immunodeficiency syndrome in experimentally infected pig-tailed macaques (Macaca nemestrina) (Hirsch et al., 1995; Johnson et al., 1990; Norley et al., 1999), suggesting mechanisms of natural
resistance in the natural host species (Norley et al., 1999). Double-positive (DP) T cells from AGMs downregulate expression of the CD4 gene upon in vitro activation and are resistant to SIV<sub>agm</sub> infection (Murayama et al., 1999), a phenomenon contributing possibly to the natural resistance of AGMs (Murayama et al., 1999). It has been shown recently that heterologous host systems can control, at least temporarily, SIV replication via the action of CD<sup>8+</sup> lymphocytes (Jin et al., 1999; Schmitz et al., 1999). However, selective pressure exerted possibly by cytotoxic T lymphocytes (CTLs) may result in the emergence of virus ‘escape mutants’ and this ongoing process may lead ultimately to an exhaustion of the immune system (Evans et al., 1999). Human immunodeficiency virus type 1 (HIV-1) (Dalod et al., 1999; Fiorentino et al., 1996; Giorgi et al., 1999; Lewis et al., 1999; Mugnaini et al., 1999; Posnett et al., 1999; Weekes et al., 1999) and SIV-specific (Kuroda et al., 1998, 1999) cytotoxic T lymphocyte (CTL) responses have been more difficult to demonstrate than in other species (Kurth & Norley, 1994). Interestingly, there is evidence that SIV<sub>smm</sub>-infected sooty mangabeys, another natural host of SIV, have increased numbers of activated CD<sup>8+</sup> T cells (Villinger et al., 1999). Until now, this has not been shown in SIV<sub>agm</sub>-infected AGMs.

We have performed three-colour flow cytometric analyses to study the role of CD<sup>8+</sup> T cells in SIV<sub>agm</sub>-infected AGMs and applied marker combinations (CD28, CD27, CD45RA and CD11a) shown to enumerate the whole effector CD<sup>8+</sup> T cell subset. We also evaluated DP T cells in infected animals and determined their numbers in different subspecies. We included a population of animals living on one of the Caribbean islands (Barbados), which are known to be free from SIV (Daniel et al., 1988; Hendry et al., 1986); the monkey population of Barbados phenotypically resemble African sable monkeys. As these animals are the descendants of only a few, probably SIV-free (Daniel et al., 1988; Lekutis & Letvin, 1995), AGMs brought from the African continent to the Caribbean islands (Denham, 1981) in the 17th century, it is likely that their immune systems have developed for several hundred years in the absence of SIV (Daniel et al., 1988).

### Methods

#### Animals and specimens.

Uninfected (n = 65) and SIV<sub>agm</sub>-infected grivet, vervet, tantalus and sable monkeys (n = 74) were used for this study: all these animals were descendants of animals that had been imported originally from Ethiopia and Kenya. In addition, we examined blood samples from 23 uninfected Caribbean sable monkeys, which were imported from Barbados. All animals were kept together in mixed groups, i.e. infected animals together with uninfected animals, allowing natural modes of virus transmission, at the Paul-Ehrlich-Institute, Langen, Germany, or at the Primate Center of Chiron-Behring, Marburg, Germany, under specified-pathogen-free conditions: negative for simian T cell lymphotropic virus type I, type D retrovirus, cercopithicine herpesvirus-2 (SA8, herpes papionis)/herpes simplex virus and tuberculosis/paratuberculosis. Of the SIV<sub>smm</sub>-infected and uninfected vervet monkeys, 34 were, in addition, free from spumavirus infection. All animal experiments were performed in accordance with section 8 of the German Animal Protection Law (Tierschutzgesetz) in compliance with EC Directive 86/609, which states that it is an offence to carry out any scientific procedure on an animal except under license. Blood samples (EDTA-anticoagulated and citrated blood) were collected under anaesthesia (2.5 mg/kg xylazine–HCl and 6 mg/kg ketamine–HCl) administered intramuscularly between 8 and 9 am in order to exclude diurnal variations. Furthermore, we performed each of the studies on at least two

<table>
<thead>
<tr>
<th>Panel</th>
<th>mAb-FITC (clone, source)</th>
<th>mAb-PE (clone, source)</th>
<th>mAb-PerCP (clone, source)</th>
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<tbody>
<tr>
<td>A</td>
<td>CD3 (FN18, Labg)</td>
<td>CD4 (Leu3, BD)</td>
<td>CD8α (Leu2, BD)</td>
</tr>
<tr>
<td>B</td>
<td>CD3 (FN18, Labg)</td>
<td>CD8β (2ST8.5H7, IT)</td>
<td>CD8α (Leu2, BD)</td>
</tr>
<tr>
<td>C</td>
<td>CD3 (FN18, Labg)</td>
<td>CD28 (Leu28, BD)</td>
<td>CD8α (Leu2, BD)</td>
</tr>
<tr>
<td>D</td>
<td>CD16 (Leu11, BD)</td>
<td>CD56 (Leu19, BD)</td>
<td>CD8α (Leu2, BD)</td>
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<tr>
<td>E</td>
<td>CD20 (Leu16, BD)</td>
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<td>F</td>
<td>CD27 (M-T271, Pha)</td>
<td>CD28 (Leu28, BD)</td>
<td>CD8α (Leu2, BD)</td>
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<tr>
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<td>CD11a (25.3.1, IT)</td>
<td>CD28 (Leu28, BD)</td>
<td>CD8α (Leu2, BD)</td>
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<tr>
<td>H</td>
<td>CD45RA (Leu18, BD)</td>
<td>CD28 (Leu28, BD)</td>
<td>CD8α (Leu2, BD)</td>
</tr>
<tr>
<td>I</td>
<td>TCRVδ1 (TS8.2, DPC)</td>
<td>CD4 (Leu3, BD)</td>
<td>CD8α (Leu2, BD)</td>
</tr>
<tr>
<td>J</td>
<td>TCRVδ2 (15D, DPC)</td>
<td>CD4 (Leu3, BD)</td>
<td>CD8α (Leu2, BD)</td>
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Table 2. Relative and absolute blood lymphocyte subset values determined on two different occasions for SIV<sub>agm</sub>-seronegative AGMs (grivet and vervet subspecies) and Caribbean sabeaus monkeys

(a) Cell counts in each set are indicated as follows: mean/median (indicated in bold and expressed together when the two values were identical), 25–75th percentile and 10–90th percentile.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Mean age (range)</th>
<th>No. AGMs</th>
<th>T cells</th>
<th>B cells</th>
<th>NK cells</th>
<th>CD4 cells</th>
<th>CD8 cells</th>
<th>CD8 cell subset</th>
<th>DP T cells</th>
<th>DN T cells</th>
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<tr>
<td>African AGMs (1:4–3:5)</td>
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<td>14</td>
<td>72/76</td>
<td>18/14</td>
<td>7/13</td>
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<td>68–79</td>
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<td>41–55</td>
<td>26–32</td>
<td>7–19</td>
<td>7–20</td>
</tr>
<tr>
<td>Caribbean sabeaus Adult</td>
<td>2.2</td>
<td>22</td>
<td>74/22</td>
<td>12/8</td>
<td>12/15</td>
<td>14/15</td>
<td>53/50</td>
<td>24/27</td>
<td>45/41</td>
<td>0/4–0.7</td>
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<td></td>
<td></td>
<td></td>
<td>71–81</td>
<td>9–15</td>
<td>9–14</td>
<td>12–17</td>
<td>37–64</td>
<td>19–33</td>
<td>29–51</td>
<td>0–3–0.8</td>
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<td></td>
<td></td>
<td></td>
<td>63–83</td>
<td>8–18</td>
<td>6–21</td>
<td>9–30</td>
<td>10–21</td>
<td>28–68</td>
<td>19–60</td>
<td>0–1–1.6</td>
</tr>
</tbody>
</table>

(b) Cell counts (× 10<sup>9</sup> cells/µl blood) in each set are indicated as follows: mean/median (indicated in bold and expressed together when the two values were identical), 25–75th percentile and 10–90th percentile.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Mean age (range)</th>
<th>No. AGMs</th>
<th>T cells</th>
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<th>DN T cells</th>
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<tr>
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<td>2.5</td>
<td>14</td>
<td>3/5</td>
<td>0/9</td>
<td>0/4–0.65</td>
<td>1/4</td>
<td>2/1</td>
<td>0/35</td>
<td>0/8</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2/9–4/2</td>
<td>0/6–1/1</td>
<td>0/2–0.55</td>
<td>1/25–1/7</td>
<td>1/45–2/3</td>
<td>0/25–0.5</td>
<td>0/6–1/0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2/3–5/6</td>
<td>0/44–1/5</td>
<td>0/18–2/4</td>
<td>0/8–2/9</td>
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<tr>
<td>African AGMs (3:6–9:8)</td>
<td>5.6</td>
<td>49</td>
<td>2/1</td>
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<td>0/25</td>
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<td>0/5–0.6</td>
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<td></td>
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<td>1/4–2/7</td>
<td>0/2–0.5</td>
<td>0/2–0.4</td>
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<td>0/68–2/8</td>
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<tr>
<td>Caribbean sabeaus Adult</td>
<td>6.6</td>
<td>22</td>
<td>3/4</td>
<td>0/5</td>
<td>0/45</td>
<td>0/6</td>
<td>2/55</td>
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<td>2/1–4/4</td>
<td>0/3–0.8</td>
<td>0/3–0.65</td>
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<td>1/0–3/7</td>
<td>0/55–2/35</td>
<td>0/2–0.5</td>
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<td></td>
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<td>1/4–6/2</td>
<td>0/2–1/1</td>
<td>0/22–1/0</td>
<td>0/36–1/0</td>
<td>0/73–5/3</td>
<td>0/3–3.5</td>
<td>0/15–0.8</td>
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</tr>
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</table>

* CD4<sup>+</sup>CD<sub>3</sub><sup>+</sup>/CD8<sup>+</sup>CD3<sup>+</sup>CD4<sup>neg</sup>.
determined restriction to the use of a mAb against CD20. For the purpose of our study, lymphocyte subsets were defined as follows: CD8αβ T cells (CD8αβCD3+), DP T cells (CD4+CD3−CD8αβ), single-positive (SP) CD4 T cells (CD4+CD3+CD8αβ−) and natural killer (NK) cells (CD3−CD8αβ+, either CD16+ or CD16−). Antibodies were diluted according to the manufacturer’s guidelines. Aliquots of 50 µl EDTA-anticoagulated blood from each animal were incubated with each of these reagent combinations for 20 min at room temperature before lysis and fixation using the Coulter Immunoprep Reagent system (Coulter), as described elsewhere (Macey et al., 1997). Lysed and fixed samples were washed with 1 ml PBS solution (PBSS) and centrifuged for 5 min at 300 g. The supernatants were removed, cells were resuspended in 300 µl PBSS and analysed within 2 h.

Flow cytometry. Samples were analysed on a FACScan analyser using CellQuest software (Becton & Dickinson). The lymphocyte population was determined using forward scatter versus side scatter. For analyses, 10,000 (Table 1, antibody panels A–E) and 50,000 (Table 1, antibody panels F–J) lymphocytes were used. Positive cut-off values for fluorescence were set to the first log step to include less than 1% of nonstaining cells. Absolute numbers of lymphocyte subsets were calculated using routine diagnostic lymphocyte counts obtained from the same blood specimens analysed on a CellDyn CD3500SL (Abbott Diagnostics). Tube-to-tube variability was assessed routinely. The CD3 lineage marker in mAb panels A–C was used to validate the lymphocyte gate by assessing the coefficient of variation (CV) in the percentage of CD3+ cells. Samples showing a CV greater than 2% were not used. In addition, we calculated routinely the lymphosum for each animal, i.e. the sum of the percentages of CD3+CD8αβ+ cells (panel A), CD3+ cells (panel B) and CD20+ cells (panel E). Samples were not used when the lymphosum was below 95% or above 105%. For negative (isotype) controls, we followed the recommendations outlined by the Centers for Disease Control and Prevention (CDC, 1997).

Determination of the virus load. Peripheral blood lymphocytes (PBLs) were separated by Ficoll–Hypaque density centrifugation. After washing, PBLs were stored at −80 °C as 5 × 10⁶ cell pellets for subsequent PCR studies. Cells were removed from the plasma by centrifugation and 500 µl aliquots frozen at −80 °C until used for quantitative RT–PCR assays. RNA was isolated from 200 µl plasma using the High Pure Viral RNA kit (Roche), according to the manufacturer’s instructions, eluting in 50 µl RNase-free water. DNA was isolated from 5 × 10⁶ cells using the QIAamp DNA Blood Mini kit (Qiagen). The proviral and viral RNA loads were quantified as described previously (Holzammer et al., 2001).

Serology. Titres of virus-specific antibodies were determined using standard ELISA protocols. Sera were diluted 1: 100 in PBSS, 2% (w/v) milk powder and 0.05% (v/v) Tween 20 and were tested on both uncoated ELISA plates and plates coated with detergent-disrupted sucrose gradient-purified whole SIVagm. Sera were scored as positive when the OD value against antigen minus the OD value against empty plates was greater than 0.2.

Statistical analyses and data presentation. The flow cytometric parameters of the groups were analysed for significant differences (P < 0.05) by the Mann–Whitney test (H₁W). Correlations were calculated and expressed as the Spearman coefficient of correlation (r). Data were displayed using the box-and-whisker plot method. In all figures displaying box plots, the box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median, and the whiskers extend down to the smallest value and up to the largest value.

Results

No clinical signs of immunodeficiency (chronic or unusual diseases, wasting or persistently enlarged lymph nodes) were seen during the whole observation period of 2 years in any of the SIVagm-infected monkeys examined.

The RNA virus load in the animals tested (n = 18, mean age 6–2 years, range 2.5–9–1) ranged from 4.7 × 10⁴ to 1.1 × 10⁵ RNA copies/ml plasma (mean value 2.24 × 10⁴). All animals were SIVagm-seropositive for at least 2 years.

When the haematological parameters (expressed as median, 25–75th percentile and 10–90th percentile) of infected (n = 15 female animals, 5.5 ± 1.6 years old) and uninfected (n = 16) age- and sex-matched vervet monkeys were analysed by the Mann–Whitney test, increased absolute lymphocyte counts, expressed as × 10³ cells/µl blood, of 4.2, 3.2–5.5 and 3.0–7.5

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at most, 4% of T cells (Table 2) in either infected or uninfected double-negative (DN) T cells. This population accounted for, African and Caribbean AGMs concerned the population of

We determined the normal composition of PBL subsets by three-colour flow cytometric analysis of EDTA-anticoagulated whole blood samples from uninfected AGMs (Table 2). High numbers of DP T cells were found in AGM subspecies from the African continent but not in Caribbean sabaeus monkeys (Fig. 1 and Table 2). DP T cells did not express the CD8 $\alpha$-chain, only the $\beta$-chain (Fig. 1). Another striking difference between African and Caribbean AGMs concerned the population of double-negative (DN) T cells. This population accounted for, at most, 4% of T cells (Table 2) in either infected or uninfected animals of African origin, but up to 22% of T cells in Caribbean sabaeus monkeys (Fig. 1 and Table 2). Unfortunately, cross-reactive antibodies against common epitopes of simian $\alpha$ or $\alpha\beta$ T cell receptor (TCR)-positive cells, which would allow the identification of DN T cells as TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$, were not available. However, two mAbs recognizing subsets of human TCR$\gamma\delta^+$ cells were found to cross-react with simian homologues. TCR$\delta^+$ and TCRV$\delta^+$ PBLs were negative for CD4 and normally expressed the CD8 $\alpha$-chain in all AGM subspecies examined (data not shown).

The SP CD8 T cell subset, i.e. CD8 T cells that do not coexpress CD4, was formed by cells expressing either the CD8 $\alpha\alpha$-heterodimer (55 ± 11.8% of all SP CD8 T cells, $n = 29$ animals tested) or the CD8 $\alpha\beta$-heterodimer (45 ± 11.7% ; Fig. 2). As a rule, CD16+ PBLs had identity with the subset of CD3$^{\text{neg}}$CD8$^{\text{neg}}$ cells. However, we obtained better lymphosum values (sum of all lymphocytes) when we used CD3$^{\text{tot}}$CD8$^{\text{+}}$ percentages (data not shown). This was due to 10% of all samples tested having extraordinarily high values of CD3$^{\text{neg}}$CD8$^{\text{+}}$ cells compared to lower CD16+ cells.

We also determined the effect of ageing on blood lymphocyte subsets (percentages) in uninfected grivet and vervet monkeys ($n = 64$). Significant inverse correlations were found between age and CD4$^+$CD3$^+$ cell counts (relative numbers $r_a = -0.31$, $P = 0.001$; absolute numbers $r_a = -0.45$, $P = 0.01$), between age and CD4/CD8 ratios ($r_a = -0.55$, $P = 0.0001$) and between age and CD20+ cell counts (absolute numbers $r_a = -0.42$, $P = 0.01$). A moderate but statistically not significant (ns) decrease was also found for DP T cell counts [relative numbers $r_a = -0.28$ (ns); absolute numbers $r_a = -0.35$ (ns)]. However, the relative numbers of CD8$^+$CD3$^+$CD4$^{\text{neg}}$ cells [relative numbers $r_a = 0.58$, $P < 0.0001$; absolute numbers $r_a = -0.11$ (ns)] and CD8$^+$CD3$^+$
CD28neg cells (relative numbers \( r_s = 0.56, P < 0.0001 \); absolute numbers \( r_s = 0.2, P = 0.02 \) were correlated positively with ageing. The numbers of NK cells did not show any differences between uninfected AGMs of different ages (data not shown).

Finally, we examined the effect of long-term SIVagm infection on the composition of blood lymphocyte subsets (Figs 3–6). Female SIVagm-infected vervet monkeys (\( n = 17 \), 5.7 ± 1.6 years old) and age- and sex-matched uninfected (\( n = 17 \) ) vervet monkeys were selected randomly to determine the effect of SIVagm infection on the composition of blood lymphocyte subsets. A number of relative (Figs 3 and 5) and absolute lymphocyte subset counts (Figs 4 and 6) was significantly different between the two groups. Significantly higher absolute CD8α+ T cell numbers were found in the infected monkeys (2775 ± 1510 cells/μl blood versus 1771 ± 1160 cells/μl blood, \( P_{MW} = 0.03 \)) (Fig. 4). This was due mainly to a significantly higher level of CD8α+CD3+CD28neg PBLs [1094 ± 986 cells/μl blood (Fig. 4) versus 402 ± 364 cells/μl blood (Fig. 5), \( P_{MW} = 0.03 \)]. When gated on CD8αbright+ PBLs, which were identical with CD8α+ T cells (Fig. 2d), the absolute numbers of CD28negCD27neg cells within this CD8 subset were higher in SIVagm-infected AGMs (133 ± 168 cells/μl blood) than in uninfected AGMs (39 ± 90 cells/μl blood, \( P_{MW} = 0.0006 \)) (Fig. 6). Similar differences were found for the CD8αbright+CD28negCD11abright+ cell subset (197 ± 255 cells/μl blood versus 83 ± 170 cells/μl blood, \( P = 0.02 \)) (Fig. 6), but not for the CD8αbright+CD28neg CD45RAbright+ cell counts (Fig. 6). The absolute CD20+ cell counts were significantly higher in the infected animals (644 ± 330 cells/μl blood versus 437 ± 345 cells/μl blood, \( P_{MW} = 0.03 \)) (Fig. 4), as were the absolute counts of CD8α+CD3+ PBLs (692 ± 561 cells/μl blood versus 323 ± 197 cells/μl blood, \( P_{MW} = 0.05 \)) (Fig. 4). To confirm our results obtained from the female vervet monkeys, we investigated a larger number of samples collected from SIVagm-infected (\( n = 55 \), 6.2 ± 1.9 years old) and uninfected grivet, vervet and tantalus monkeys (\( n = 48 \), 5.5 ± 2.4 years old). However, it was not possible to match exactly the control group with regard to age, sex and subspecies. We could confirm significantly higher absolute CD8α+CD3+CD28neg counts in the infected group compared with the uninfected group (818 ± 751 cells/μl blood versus 371 ± 330 cells/μl blood, \( P_{MW} = 0.0005 \)). Changes among other subsets were not significantly different (data not shown).
Finally, there were no correlations between RNA virus load \((n = 25\) animals tested\) and numbers of cell subsets associated with major histocompatibility complex class I (MHC-I)-restricted or non-MHC-restricted cytotoxicity.

Discussion

In the present study, we examined immunological parameters in the natural host of SIV\(_{agm}\) using improved laboratory methods. Density-gradient purified PBLs have been used routinely for immunophenotypic analysis of blood lymphocyte subsets in AGMs (Beer et al., 1998; Murayama et al., 1997, 1998). However, we obtained significantly different results when we used EDTA-anticoagulated whole blood for analysis: lower B cells counts and increased CD8\(^{low+}\) cell counts in purified PBLs compared to EDTA-anticoagulated whole blood (data not shown), as described previously for human blood (Renzi & Ginns, 1987). To avoid density-based losses and enrichments, we used only the EDTA-anticoagulated whole blood lysis technique for immunophenotyping blood lymphocyte subsets.

The most prominent differences between the AGM and the human immune systems are the physiologically high frequency of CD8\(^+\) T cells and the finding that the majority of CD4\(^+\)CD3\(^+\) PBLs coexpress the CD8\(_x\) molecule (Kuroda et al., 1998; Beer et al., 1998; Murayama et al., 1997, 1998). Surprisingly, Caribbean sabaeus monkeys had only a few DP T cells but large numbers of DN T cells. Our data suggested that the DN T cells may not belong to the TCR\(\gamma\delta\) cells, since subsets of \(\gamma\delta^+\) cells expressed the CD8 \(\alpha\)-chain in AGMs, similar to rhesus monkey TCR\(\gamma\delta\) cells (Gan et al., 1995).

A further novel finding is the higher frequency of blood CD8\(^+\) T cells expressing the \(\alpha\)\(\alpha\) homodimer, in contrast to human CD8\(^+\) T cells, the majority of which express the \(\alpha\)\(\beta\) heterodimer (Schmitz et al., 1999).

We found significant changes with age in the proportion of different lymphocyte subsets, changes that were similar to those reported for ageing humans (Fagnoni et al., 1996; Hanjet et al., 1992; Sopper et al., 1997).

The mean plasma RNA virus load in long-term SIV\(_{agm}\)-infected animals was not significantly different from values published for asymptomatic HIV-1-infected individuals (Ogg et al., 1999) and naturally SIV\(_{agm}\)-infected sooty mangabeys (Rey-Cuille et al., 1998). A more detailed description of the provirus and virus loads was published recently (Holzammer et al., 2001).

The observation of significantly low platelet counts in long-term SIV\(_{agm}\)-infected AGMs, which, until now, has not been reported, is corroborated by the fact that (i) SIV\(_{agm}\) can also cause severe thrombocytopenia within 1–2 years after experimental infection in a heterologous host system (pig-tailed macaques) (Hirsch et al., 1995) and (ii) low platelet counts are found frequently in HIV-infected patients (Moses et al., 1998).

HIV-1 infection of humans (Rosenberg et al., 1998) and SIV\(_{mac}\) infection of rhesus macaques (Letvin et al., 1999) induce a CD8\(^+\) T cell lymphcytosis caused mainly by CD8\(^{neg}\) cells (Borthwick et al., 1994; Monteiro et al., 1996; Mugnaini et al., 1999). Surprisingly, we found the same in long-term SIV\(_{agm}\)-infected AGMs. However, there was no difference between uninfected and infected monkeys when the proportion of CD8\(^+\) T cells expressing the \(\alpha\)\(\alpha\)-heterodimer and those expressing the \(\alpha\)\(\beta\)-heterodimer was compared (data not shown). In contrast, HIV-1-infection is associated with a significant drop in CD3\(^+\)CD8\(^{neg}\) T cell counts (Schmitz et al., 1999).

The CD8\(^{neg}\)CD28\(^{neg}\) T cell subset was characterized further by the use of mAbs directed against CD27, CD11a and CD45RA. CD11a is the \(\alpha\)-subunit of the leukocyte function-associated molecule-1 (LFA-1) (Hamann et al., 1999). LFA-1 is increased on memory T cells and mediates adhesion of CTLs to target cells and lymphocyte adhesion to endothelial cells, i.e. involved in lymphocyte trafficking (Hamann et al., 1999). The function of CD27 is not clear, but prolonged stimulation of T cells switches off CD27 expression irreversibly (Hamann et al., 1999). CTLs were shown to be CD27\(^{neg}\) but to re-express CD45RA and to be stained brightly with CD11a (Hamann et al., 1999). HIV-1 (Dalod et al., 1999; Giorgi et al., 1999; Hamann et al., 1999; Ogg et al., 1999; Lewis et al., 1999; Mugnaini et al., 1999; Posnett et al., 1999; Weeks et al., 1999) and SIV\(_{mac}\)-specific CTLs (Kuroda et al., 1998) are found among these CD3\(^+\)CD8\(^{neg}\)CD28\(^{neg}\)CD27\(^{neg}\)CD11a\(^{bright+}\) cells. It is very likely that this subset also comprised the CTL subset in AGMs. However, it is not yet possible to functionally characterize and enumerate virus-specific CTLs in AGMs.

Significantly increased NK and B cell counts have not been described previously in SIV\(_{agm}\)-infected AGMs. Interestingly, progression towards AIDS is associated with decreasing NK cell numbers, both in HIV-1 (De Souza et al., 2000; Margolick et al., 1991; Peruzzi et al., 2000; Vuillier et al., 1988) and in SIV\(_{mac}\) infection (Powell et al., 1989; Vowels et al., 1990).

Taking the virological, haematological and immunological data together, there are at least three common characteristics of the natural SIV\(_{agm}\) system and the heterologous lentivirus systems: comparable plasma virus loads, thrombocytopenia and an increase in CD8\(^+\)CD28\(^{neg}\) T cell counts. In contrast to pathogenic lentivirus infections, however, we found significantly elevated NK cell numbers and normal absolute CD4 T helper cell counts in infected AGMs. Despite the lack of overt disease progression, the lowered platelet counts suggest that AGMs are not completely protected from the deleterious effects of SIV\(_{agm}\) infection.

Lentiviruses can prevent CTL-mediated killing of infected cells by a nef-mediated downregulation of MHC-I molecules (Piguet et al., 1999) and concurrently lowered NK cell numbers allow the escape from nonMHC-restricted cytotoxicity (Powell et al., 1989; Vowels et al., 1990). Although it has yet to be demonstrated formally that the SIV\(_{agm}\) Nef protein is able, like that of HIV, to downregulate expression of MHC-I molecules,
elevated NK cell numbers may contribute to the natural resistance in AGMs.

In pathogenic lentivirus infections, CTLs appear to be effective in the short- and medium-term containment of the virus but are ultimately unable to control the infection (Goulder & Walker, 1999). As innate immunity has the function of nonMHC-restricted killing of infected cells (Fearon & Locksley, 1996; Medzhitov & Janeway, 1997; Peruzzi et al., 2000) and is involved in the initiation of adaptive immune responses (Fearon & Locksley, 1996; Levy, 2001; Medzhitov & Janeway, 1997) and secretion of chemokines (Levy, 2001), it is tempting to hypothesize that virus escape mutants (Evans et al., 1999) may be recognized more effectively by the adaptive arm of the immune system in long-term infected AGMs, whereas the decrease in NK cell numbers during progression towards human and simian AIDS may support the generation of newly produced SIV variants, at least due to a lower interferon-γ concentration produced by NK cells (Peruzzi et al., 2000) and lower chemokines (Levy, 2001). Similarly, the loss of CD4 T cells in AIDS patients also impairs the generation and function of newly produced CTLs (Goulder & Walker, 1999). Since the absolute CD4 T cell counts are stable in long-term-infected AGMs, one may argue that this may allow the maintenance of an effective CTL response. A third possible explanation for the persistent CTL response in AGMs is the maintenance of lymph node architecture throughout the course of infection (Beer et al., 1999), which would enable the immune system to continue producing CTLs by peripheral expansion. Despite their elevated levels, there was no evidence that the CD8 T and NK cells contributed to a lower RNA virus load and we cannot exclude the possibility that the high CTL counts observed resulted from unspecific bystander activation (Tough & Sprent, 1996; Welsh et al., 2000).

To conclude, long-term SIV<sub>agm</sub> infection of its natural AGM host, although inducing no signs of overt AIDS, does have profound effects on the immune system of the animal. Significant increases in cells of the NK, CTL and B cell phenotypes indicate continuous and vigorous immune activation. Whether or not these cells, or indeed the naturally high level of CD4/CD8 DP cells in circulation, play a decisive role in preventing disease remains to be elucidated.

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References


Monteiro, J., Batliwalla, F., Ostrer, H. & Gregersen, P. K. (1996). Shortened telomeres in clonally expanded CD28+CD8+ T cells imply a replicative history that is distinct from their CD28−CD8+ counterparts. *Journal of Immunology* 156, 3587–3590.


+ p 107–118.


teria against simian immunodeficiency virus (SIV) and simian T-lymphotropic virus (STLV) in a colony of non-human primates in Kenya, East Africa. *Annual of Tropical Medicine and Parasitology* **90**, 65–70.


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