Mucosal Exposure to Subinfectious Doses of SIV Primes Gut-Associated Antibody-Secreting Cells and T Cells: Lack of Enhancement by Nonneutralizing Antibody

Natasha Polyanskaya,* 1 Lesley A. Bergmeier,† Sally A. Sharpe,* Nicola Cook,* Sharon Leech,* Graham Hall,* Mike Dennis,* Peter ten Haaf,‡ Jonathan Heeney,‡ Fabrizio Manca,§ Thomas Lehner,† and Martin P. Cranage*

*Centre for Applied Microbiology and Research (CAMR), Salisbury, United Kingdom; †Department of Immunobiology, GKT Guy’s Hospital, University of London, London, United Kingdom; ‡Biomedical Primate Research Centre, Rijswijk, The Netherlands; and §Unit of Retroviral Immunology, Advanced Biotechnology Center, Genoa, Italy

Received June 2, 2000; returned to author for revision July 27, 2000; accepted October 10, 2000

INTRODUCTION

Human immunodeficiency virus (HIV) is transmitted primarily via mucosal surfaces and this can be modeled in macaques using simian immunodeficiency virus (SIV) (Cranage et al., 1992). To develop rational vaccine and therapeutic strategies, it is important to understand how the virus is transmitted across the intact mucosal surface and what local immune responses are generated, as these may modulate subsequent spread of the virus and confer resistance to subsequent exposure. Mucosal transmission may involve direct infection of lymphoid cells, perhaps through small breaks in the mucosa. Another possibility is a non-CD4-dependent entry mechanism into epithelial cells. Fcγ2 and Fcγ3 receptors have been identified on human and simian rectal epithelial cells (Hussain et al., 1991; Hussain and Lehner, 1995), raising the possibility that SIV or HIV may cross the epithelial barrier by means of virus–IgG antibody complexes binding to these receptors. In natural infection, the individual may be exposed to virus that is complexed to nonneutralizing antibody, either derived from the transmitted partner (Witkin et al., 1980) or arising in the recipient as a result of previous low-dose exposure to virus. Furthermore, in response to mucosal vaccines, immunized subjects may develop antibodies at the virus portal of entry and, if the antibodies are nonneutralizing, they may facilitate virus entry. In addition, it was previously demonstrated in vitro, that significant enhancement of virus replication occurs in cells infected with HIV-1 in the presence of subneutralizing concentrations of serum antibodies (Takeda et al., 1988; Takeda and Ennis, 1990). However, enhanced antibody-mediated HIV or SIV replication has not yet been demonstrated in vivo.

Mucosal exposure to virus may result in a self-limiting or occult infection, giving rise to selective immune responses, as indicated by studies in humans and monkeys, and has in some cases been associated with protection from subsequent exposure. A significant number of commercial sex workers exposed to HIV-1 (Rowland-Jones et al., 1995, 1998) or who have HIV-infected partners (Langlade-Demey et al., 1994; Clerici, 1992) appear to resist infection. HIV-specific T-helper and cytotoxic T lymphocytes (CTL) (Rowland-Jones et al., 1993, 1995; Langlade-Demey et al., 1994; Shearer and Clerici, 1996), mucosal HIV-1-specific IgA antibody responses (Mazzoli et al., 1997; Kaul et al., 1999), and CD8+ antiviral activity (Stranford et al., 1999) were detected in subjects...
who were not infected, despite being exposed to HIV. Similar observations were reported in the SIV model following inoculation of low doses of pathogenic virus by the intrarectal (Pauza et al., 1993; Clerici et al., 1994; Trivedi et al., 1996; Murphey-Corb et al., 1999), oral (Van Rompay et al., 1998), intravaginal (McChesney et al., 1998), or intravenous (Dittmer et al., 1995) routes. There are, however, only limited data on the stimulation of local T cells and antibody-secreting cells (ASC) following subinfectious exposure.

In the study described here, we sought to establish whether rectal infectivity in macaques can be facilitated (infection with subinfectious dose) or enhanced (higher virus loads upon infection) by nonneutralizing anti-SIV gp120 IgG antibodies. Furthermore, in this study we examined gut-associated and systemic ASC and T-cell function in virus-exposed animals that remained uninfected by the criteria of virus isolation, proviral DNA detection in blood and tissue mononuclear cells (MNC), and detection of viral RNA in plasma.

RESULTS

Outcome of immunization of macaques with antibody-complexed gp120 or gp120 alone

We compared the immunogenicity of affinity-purified SIV gp120 given alone or as immune complexes with a nonneutralizing IgG fraction of a serum pool from macaques immunized with gp120. Two macaques (50 and 51) were immunized intrarectally, first with immune complexes in antibody excess, then in equilibrium, followed by complexes in antigen excess and boosted by the intramuscular (im) route at about monthly intervals. Another macaque (24) received gp120 alone, following the same schedule, and two animals (86 and 87) were immunized im once with gp120 alone. Intrarectal inoculations were given in the absence of adjuvant, although alum was used in the im administration. Secretory and serum IgA and IgG antibody responses to the complexed gp120 were enhanced as compared with gp120 alone after the three rectal inoculations (Fig. 1). A T-cell-proliferative response was detected in only one animal (50) that received the gp120–IgG complex [stimulation index (SI) = 3.3]. A single intramuscular boost stimulated a further increase in the antibody levels in the gp120–IgG complex immunized animals; T-cell-proliferative responses were detected in both animals (SI = 3.1 for animal 50, and 27.7 for animal 51). In contrast, the macaque immunized with gp120 alone showed no increase in rectal or serum antibodies, although the T-cell-proliferative response reached a significant level (SI = 4.6).

In vivo infectivity titer of SIVmac220

To test whether anti-gp120 IgG antibodies could facilitate or enhance SIV infection in vivo, the titer of infectivity of the virus stock was determined by intrarectal titration in eight macaques. Two of the two animals...
receiving the highest dose of SIV (6000 TCID₅₀), two of
two receiving 1200 TCID₅₀, and one of the two animals
receiving 240 TCID₅₀ became infected (Table 1). Virus
was not isolated from the remaining two animals; they
also failed to seroconvert and PCR analysis of PBMC
failed to reveal proviral DNA (Table 1). Thus, within the
limitations of the titration, one median infectious dose
(MID₅₀) of SIVmac220 was found to be equivalent to 240
TCID₅₀ following intrarectal inoculation.

Outcome of in vivo exposure to virus and immune-
complexed virus

Virus infectivity. Having determined that the dose of
virus required to infect 50% of animals by rectal inocu-
lation was 240 TCID₅₀, another four macaques were
inoculated rectally with 120 TCID₅₀ alone and another
four animals received the same dose of SIV but com-
plexed to the nonneutralizing IgG antibodies. This "sub-
infectious" dose of SIV, either in the presence or in the
absence of antibody, failed to infect macaques. All ani-
mals remained negative for virus isolation and PCR, nor
did they develop serum or rectal antibodies or a T-cell-
proliferative response to SIV. The same animals were
rechallenged rectally at Week 17 with SIV–immune com-
plexes or SIV alone as previously, but using a 10-fold
higher concentration of SIV (1200 TCID₅₀). By 2 weeks
after the second SIV challenge, two of four macaques in
each group had become overtly infected as indicated by
virus isolation (Table 2). As the number of infected ani-
mals in each group was the same, there was no evi-
dence that the nonneutralizing macaque anti-SIV gp120
IgG facilitated infection via the rectal mucosa. However,
the cell-associated virus loads were higher at Week 2 in
animals given the SIV–immune complexes, compared to
those receiving virus alone, which showed a higher load
at Week 4 (Table 2). This difference was not reflected in
the plasma RNA loads; indeed, the highest load was
detected in an animal infected with virus alone (70T) and
this was the only animal in which RNA was detected at
1 week after challenge.

To determine whether exposed, "uninfected" (EU) ani-
mals had local sequestration of challenge virus, various
lymphoid tissues (iliac, mesenteric, and axillary lymph
nodes; spleen; PBMC) were analyzed post mortem.

### Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Inoculum</th>
<th>TCID₅₀</th>
<th>Virus isolation from PBMC [PCR]</th>
<th>Autopsy (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10T</td>
<td>6000</td>
<td>+</td>
<td>+</td>
<td>+ (13)</td>
</tr>
<tr>
<td>4T</td>
<td>6000</td>
<td>+</td>
<td>+ [+ ]</td>
<td>+ (13)</td>
</tr>
<tr>
<td>14T</td>
<td>1200</td>
<td>+</td>
<td>+</td>
<td>+ (10)</td>
</tr>
<tr>
<td>19T</td>
<td>1200</td>
<td>+</td>
<td>+</td>
<td>+ (10)</td>
</tr>
<tr>
<td>28S</td>
<td>240</td>
<td>+</td>
<td>+ [+ ]</td>
<td>+ (9)</td>
</tr>
<tr>
<td>25T</td>
<td>240</td>
<td>-</td>
<td>- [+ ]</td>
<td>- (9)</td>
</tr>
<tr>
<td>5T</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>- (10)</td>
</tr>
<tr>
<td>18T</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>- (10)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Animal</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PBMCᵃ</td>
<td>Plasmaᵇ</td>
<td>PBMCᵃ</td>
<td>Plasmaᵇ</td>
</tr>
<tr>
<td>SIV-immune complex</td>
<td>54T</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
<td>312.5</td>
<td>120,000</td>
</tr>
<tr>
<td></td>
<td>147T</td>
<td>&lt;1.6</td>
<td>nt</td>
<td>3,509</td>
<td>680,000</td>
</tr>
<tr>
<td></td>
<td>V14</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
</tr>
<tr>
<td></td>
<td>V2</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
<td>&lt;1.6</td>
<td>nt</td>
</tr>
<tr>
<td>SIV alone</td>
<td>73T</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
</tr>
<tr>
<td></td>
<td>70T</td>
<td>&lt;1.6</td>
<td>1,800</td>
<td>2.5</td>
<td>1,500,000</td>
</tr>
<tr>
<td></td>
<td>51T</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
</tr>
<tr>
<td></td>
<td>60T</td>
<td>&lt;1.6</td>
<td>&lt;140</td>
<td>140</td>
<td>67,000</td>
</tr>
</tbody>
</table>

ᵃ No. of SIV infected cells/10⁶ PBMC.
ᵇ SIV RNA Eq/ml plasma; nt = not tested.
weeks after the second exposure to the virus. All tissues were negative for virus isolation and for the presence of provirus by PCR.

**SIV-specific immune responses.** All four infected animals seroconverted and serum antibody titers to whole virus, gagp27, and gp140 were not enhanced by infection with immune-complexed virus (Fig. 2). Thus, immune-complexed SIV did not enhance systemic SIV-specific antibody responses.

To determine whether rectal mucosal exposure to virus, in the presence and absence of specific IgG antibodies, had stimulated local or systemic cellular immune responses, cells from blood and tissues were analyzed post mortem. In the exposed, uninfected animals, SIV antigen-specific T-cell-proliferative responses were evident in the cells recovered from blood and lymphoid tissues (Table 3). Proliferation with stimulation indices (SI) to p27 and gp120 between 2.6 and 10.2 was detected in jejunal IEL (V14), jejunal LPL (51T), iliac lymph nodes (73T and V2), mesenteric lymph nodes (51T, 73T, and V2), axillary lymph nodes (51T, 73T, and V2), and blood (V2). Infected animals failed to exhibit SIV-specific T-cell-proliferative responses in the gut, iliac, and mesenteric lymph nodes and spleen, although p27-driven proliferation was detected in axillary lymph nodes in one macaque (147T) and in blood in the other (54T). Maximum proliferative responses were stimulated with different concentrations of antigen that varied between the tissues (Table 3). Lamina propria lymphocytes from animal 73T and PBMC from animal 51T showed nonsignificant proliferative values at single concentrations of antigen. In contrast, MNC from axillary lymph nodes of animal 51T were reactive at every concentration of gp120 tested (1, 5, 10 \( \mu \text{g/ml} \)) but with high replicate variability. Cells from blood (n = 12) and different tissues (gut, iliac, mesenteric, and axillary lymph nodes, and spleen; n = 3) of naive macaques were also assayed under the same conditions. In no instance did the level of stimulation reach a value of 2.5.

In addition to lymphoproliferative responses, we examined CTL activity against autologous target cells expressing SIV antigens from recombinant vaccinia viruses. Previous observations in our laboratory showed that cells from GALT frequently die in culture and, therefore, cells were analyzed directly for CTL effector activity. Levels of direct net SIV-specific CTL of 10% or greater were detectable at an effector-to-target ratio of 200:1 in two of four EU animals and two of three infected animals (Fig. 3). Systemic CTL were found in two EU animals (V2; axillary lymph node and spleen, 73T; PBMC) and in one infected macaque (147T; spleen and PBMC). Local CTL to SIV env were detected in one EU animal (73T; jejunal IEL) and to SIV nef in one infected animal (60T; iliac lymph node). MNC from the spleen of the SIV-infected animal 147T maintained nef-specific killing at 25:1 and env-specific killing at 50:1.

Antibody-secreting cells within MNC from two EU macaques (51T and 73T) and two infected macaques (60T and 70T) were analyzed by the ELISPOT assay (Fig. 4). Both EU and infected animals demonstrated significant frequencies of SIV-specific IgG ASC in the rectum, iliac, and axillary lymph nodes, spleen, and PBMC. Surprisingly, the IgA ASC were negligible.

Thus, rectal mucosal exposure to SIV, in the absence of overt infection, primed both virus-specific cell-mediated immune responses and antibody-secreting cell activity detectable at systemic and mucosa-associated sites.

**DISCUSSION**

Little is known about early events in rectal mucosal infection with HIV, despite the fact that this is a frequent mode of virus transmission. The possibility of antibody-
facilitated uptake of virus via Fcy receptors was previously suggested by several in vitro studies (Homsy et al., 1989; Takeda and Ennis, 1990; Robinson et al., 1990). It was shown that IgG antibodies engaging FcγR3, unlike FcγR1, can enhance HIV infection of macrophages, independently of CD4 (Trischmann et al., 1995). Immune-complexed virus may also be taken up in vitro via complement receptors if complement is available (Montefiori et al., 1991). Interestingly, FcγR3 may associate with complement receptor type 3 (CR3) and co-cap on the plasma membrane, suggesting that the two receptors cooperate in mediating biological function (Zhou et al., 1993).

The significance of antibody and/or complement mediated facilitation in enhancement of infection is difficult to study in man, although this is a matter of obvious concern in vaccine design. In FIV infection of cats, enhancement was previously seen following vaccination (Siebelink et al., 1995; Osterhaus et al., 1996). In the SIV model we observed increased infection in one study of macaques immunized with SIVgp120 and gag p27 following rectal inoculation of the animals with a low dose of virus (Cranage et al., unpublished data), suggesting the possibility of antibody-facilitated enhancement of infection.

Although only a small number of animals were used in the pilot immunogenicity experiment, there was a consistent trend for rectally administered IgG immune complexes of SIV gp120 to elicit higher titers of secretary and serum antibodies compared to titers in animals given gp120 alone, either intrarectally or intramuscularly. This suggests that the presence of Fcy and complement receptors expressed by rectal epithelial cells (Hussain et al., 1991; Hussain and Lehner, 1995) may have facilitated antigen uptake. Alternatively, immune complexes may have bound directly to macrophages, thus leading to enhanced immune presentation.

A marked difference was found between the enhanced immunogenicity of immune complexes, consisting of SIV antigens and corresponding IgG antibodies, and the lack of any effect of these antibodies on the infectivity or immunogenicity of live SIV. The failure of the nonneutralizing anti-SIV gp120 IgG either to facilitate or to enhance infection with SIV has several possible explanations. First, complexed virus may not be taken up any more efficiently than virus alone. Second, the antibody used in the production of the challenge virus (Schutten and Moore, 1995). However, although the antigen-antibody ratio was carefully worked out, we were unable to do a similar titration for the virus:antibody ratio and we used the ratios calculated for the antigen which may not have been optimal for the virus. Third, antibodies can neutralize or enhance HIV-1 infection, depending on the phenotype of the virus used and/or the cell substrates used in the production of the challenge virus (Schutten et al., 1994; Osterhaus et al., 1996). In our study with SIV, the virus was derived ex vivo without being cultured on cell lines and, therefore, should have been close to a biologically relevant phenotype. Splenocyte-derived virus, however, may not represent a naturally transmitted phenotype. Finally, as only one type of antibody was used, we cannot be confident that other antibodies directed to

---

### Table 3

SIV-Stimulated Proliferation of MNC from SIV-Exposed Macaques

<table>
<thead>
<tr>
<th>Animal</th>
<th>Exposed to</th>
<th>Status</th>
<th>Antigen</th>
<th>IEL</th>
<th>LPL</th>
<th>Iliac lymph node</th>
<th>Mesenteric lymph node</th>
<th>Axillary lymph node</th>
<th>Spleen</th>
<th>Peripheral blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>V2</td>
<td>SIV</td>
<td>EU</td>
<td>p27</td>
<td>nt</td>
<td>1.1</td>
<td>3.6 (8)</td>
<td>3.4 (4)</td>
<td>5.9 (8)</td>
<td>2.2</td>
<td>10.2 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gp120</td>
<td>nt</td>
<td>1.2</td>
<td>2.5 (0.5)</td>
<td>1.8</td>
<td>3.5 (0.5)</td>
<td>2.1</td>
<td>8.0 (1)</td>
</tr>
<tr>
<td>73T</td>
<td>SIV</td>
<td>EU</td>
<td>p27</td>
<td>1.8</td>
<td>5.3</td>
<td>6.8 (8)</td>
<td>5.6 (1)</td>
<td>4.4 (1)</td>
<td>2.0</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gp120</td>
<td>1.0</td>
<td>1.0</td>
<td>5.8 (2)</td>
<td>5.6 (4)</td>
<td>2.3</td>
<td>1.3</td>
<td>nt</td>
</tr>
<tr>
<td>51T</td>
<td>SIV-IC</td>
<td>EU</td>
<td>p27</td>
<td>nt</td>
<td>3.2 (5)</td>
<td>1.4</td>
<td>4.3 (10)</td>
<td>2.7 (10)</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gp120</td>
<td>nt</td>
<td>5.0 (5)</td>
<td>1.6</td>
<td>3.9 (5)</td>
<td>3.9</td>
<td>1.4</td>
<td>3.4</td>
</tr>
<tr>
<td>V14</td>
<td>SIV-IC</td>
<td>EU</td>
<td>p27</td>
<td>2.6</td>
<td>2.5</td>
<td>1.2</td>
<td>1.9</td>
<td>1.5</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gp120</td>
<td>1.5</td>
<td>1.5</td>
<td>1.1</td>
<td>1.7</td>
<td>1.5</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>147T</td>
<td>SIV-IC</td>
<td>Infected</td>
<td>p27</td>
<td>1.0</td>
<td>1.1</td>
<td>1.5</td>
<td>1.0</td>
<td>3.3 (2)</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gp120</td>
<td>1.0</td>
<td>1.3</td>
<td>1.4</td>
<td>1.6</td>
<td>1.4</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>54T</td>
<td>SIV-IC</td>
<td>Infected</td>
<td>p27</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>1.5</td>
<td>1.6</td>
<td>1.4</td>
<td>5.7 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gp120</td>
<td>2.3</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
<td>1.4</td>
<td>1.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**Note.** Significant ($P < 0.05$) stimulation indices ≥ 2.5 are shown in bold; the other SI > 2.5 showed considerable variations between triplicate cultures. nt, not tested.

a IC, immune complex.
b EU, exposed, uninfected.
enhancing regions, such as were described for gp41 (Robinson et al., 1990), might not have yielded enhancement. We believe this to be the first prospective attempt to study antibody-mediated facilitation of rectal mucosal infection in vivo using the SIV model and, with the above-noted caveats, the failure to observe an effect is encouraging from the standpoint of vaccine development.

Our study also showed priming of both T- and B-cell responses following mucosal exposure to low doses of virus, in the absence of overt infection. In the macaques remaining seronegative the T-cell-proliferative responses were detected predominantly in the gut-associated iliac and axillary lymph nodes but not in the spleen or PBMC. This is consistent with the mucosal immune responses found after local immunization, as compared with systemic immunization (Lehner et al., 1994). Others reported systemic T-cell responses in macaques that were transiently infected but which remained seronegative (Salvato et al., 1994; Trivedi et al., 1996; McChesney et al., 1998). In one study (Clerici et al., 1994), SIV-specific responses were detected in the circulation of macaques that were exposed to low doses of pathogenic virus but were apparently uninfected. More recently, Murphy-Corb et al. (1999) reported the generation of CTL responses detectable in the circulation and the jejunal lamina propria following colonic inoculation of macaques with SIV, resulting in either transient or silent infection. In our study, SIV-specific proliferative responses may have arisen from direct exposure to the inoculum (although this contained only approximately 0.5 ng p27) or may have resulted from limited transient infection, despite the fact that no evidence was obtained of local sequestration of virus. Surprisingly, we demonstrated low levels of direct CTL activity in exposed uninfected macaques, including envelope-specific activity in jejunal intraepithelial lymphocytes in one animal. This finding further supports the notion that limited viral replication may have occurred, since in one animal a CTL response to nef, a nonstructural protein, was detected. It is unclear how a direct effector activity is maintained in the absence of a persisting viral infection and experiments are now in progress to analyze the generation of this activity using a specific CTL epitope marker.

As well as T-cell immune priming, low-dose exposure to SIV was found to generate SIV-specific IgG antibody-secreting cells detectable in the rectum, iliac and axillary lymph nodes, spleen, and PBMC. This extends and, in the case of overtly infected animals, confirms the previous observation in SIV-infected macaques (Bergmeier et al., 1998). In both studies exposure to or infection with SIV appeared to stimulate IgG-specific ASC. This finding is of particular interest, given the recent observations that SIV-specific IgG may be able to protect from mucosal infection (Baba et al., 2000; Mascola et al., 2000). The apparent absence of IgA-specific ASC in tissues from infected animals was surprising, given that such cells were previously detected in our other studies (Bergmeier et al., 1998; Polyanskaya et al., unpublished data). However, in the present study, tissues were frozen prior to analysis and this may have reduced the sensitivity of the assay.

The significance of immune responses induced by low-dose exposure in protection against subsequent challenge is yet to be fully resolved. In one study, repeated low-dose intravenous exposure of macaques to SIV induced boostable SIV-specific proliferative T-cell responses, although the animals were not protected against subsequent intravenous challenge with a higher dose of virus (Dittmer et al., 1995). Likewise, we were

### FIG. 3. SIV-specific CTL in mononuclear cells from tissues. All values show net killing corrected for nonspecific antivaccinia virus activity at an effector:target ratio of 200:1. Solid bars show killing of cells from exposed, uninfected animals. Empty bars represent overtly infected animals. Results are shown for gag (g), nef (n), and env (e) net-specific release for each animal in the same order: g, n, e. The symbols for each antigen appear above bars where net-specific killing is ≥10%.
able to infect animals that had failed to be infected in an intrarectal titration when they were reexposed to a higher dose of virus (Cranage et al., 1997 and this study). In contrast, in other studies, protection against rectal mucosal challenge was associated with T-cell responses generated following both intravenous low-dose exposure (Clerici et al., 1994; Putkonen et al., 1997) and intrarectal exposure (Trivedi et al., 1996; Murphey-Corb et al., 1999). Indeed, in the latter study, protection correlated with the presence of envelope-specific CTL in the lamina propria. Protection was previously shown to be associated with the generation of CD8-suppressor factors, $\beta$-chemokines, and SIV-specific ASC following immunization with p27 and gp120 by subcutaneous injec-

FIG. 4. Tissue distribution of SIV-specific antibody-secreting cells (ASC). ASC were enumerated by ELISPOT assay in mononuclear cells from the tissues indicated. In each panel the left-hand side graphs show results from exposed, uninfected macaques (73T, 51T) and right-hand side graphs show results from overtly infected macaques (60T, 70T). ASC frequencies are shown for SIV p27 (open bars), SIV gp120 (solid bars), and KLH (control, shaded bars). Data are presented as means ± SEM; nd = not done.
tion in the region of the internal and external iliac lymph nodes (Lehner et al., 1996; Bergmeier et al., 1998). Gut-associated lymphoid tissue is a primary site for virus replication following infection with SIV, even when administered intravenously (Veazey et al., 1998). Although infection with virulent virus is associated with a rapid depletion of CD4+ cells in GALT, infection with attenuated virus induces protection against subsequent rectal challenge and induces SIV-specific immune responses detectable in lymphoid tissue associated with the virus portal of entry (Cranage et al., 1997). These observations suggest that local mucosal and regional lymph node immune responses contribute to protection against rectal challenge, as previously discussed (Lehner et al., 1999).

Further investigation of the low-dose exposure effect will define the conditions necessary to stimulate protective responses and the precise nature of such responses, including durability and specificity of protection against antigenically and phenotypically diverse virus stocks. Despite the absence of enhancement in the present study, further investigations are required to exclude this potentially important mechanism of mucosal infection.

MATERIALS AND METHODS

Virus
SIVmac220 is a cell-free pool of virus derived from the culture of spleen cells, taken from rhesus macaque 220, 167 days after infection with SIVmacJ5 (Polyanskaya et al., 1997). Rhesus macaque 220 had been infected intravenously with 100 median monkey infectious doses (MID50) of cell-free simian peripheral blood mononuclear cell (PBMC)-grown SIVmacJ5, a pathogenic molecular clone derived from SIVmac32H (Rud et al., 1994). The virus pool SIVmac220 6/94 had a p27 antigen content of 4.4 ng/ml and an in vitro titer of 10^4 TCID50/ml on C8166 cells.

SIV gp120 and antibody

Baculovirus expressed-SIV gp120 was produced and purified to near homogeneity by monoclonal antibody affinity chromatography as described by Doyle et al. (1995). Rhesus anti-gp120 serum was obtained from three macaques previously immunized with purified SIV gp120. The serum pool had no virus infectivity-neutralizing activity. An immunoglobulin fraction was precipitated with 50% ammonium sulfate and dialyzed against 0.01 M Tris–phosphate buffer, pH 7.0. The IgG fraction was separated by DEAE cellulose ion-exchange chromatography and purity demonstrated by immunoelectrophoresis against anti-monkey IgG and anti-monkey whole serum (Nordic Immunologicals, The Netherlands). Specificity for gp120 was confirmed by ELISA.

Immune complexes

Immune complexes of SIV gp120 and IgG were prepared in antibody excess (gp120:lgG, 4:412 µg), at equivalence (gp120:lgG, 8:200 µg), and in antigen excess (gp120:lgG, 16:50 µg) and these were used in sequence for the immunization of macaques. Antigen-antibody equivalence was determined by preparing immune complexes, followed by overnight incubation at room temperature (total volume 0.2 ml). The complexes at different ratios were centrifuged for 5 min at 6000 rpm. The supernatants were tested for free antibody on ELISA plates coated with antigen and developed with rabbit anti-monkey IgG–HRP conjugate (Sigma, Poole, UK). The first IgG dilution that was negative for free antibodies was taken as equivalence. The reason for using different antigen-antibody ratios for optimal uptake and presentation of complexed antigen was previously discussed (Manca et al., 1991). For infectivity experiments an equal volume of virus stock at a subinfectious dose (see below) was mixed with an equal volume of IgG fraction at 1.85 mg/ml (approximately equivalent to 10% serum).

Animals

Juvenile Indian rhesus macaques were bred within the United Kingdom and housed according to the Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedure (1989). Primates were anesthetized with ketamine hydrochloride for all procedures requiring their removal from cages. Intrarectal inoculations with SIV were performed without trauma using a soft polypropylene 6FG catheter; each animal received 3 ml of cell-free virus stock or SIV–IgG complexes.

Animals were killed humanely by ketamine sedation followed by intravenous injection of pentobarbitone (Euthatal; Rhône Mérieux, Harlow, UK). Heparinized blood was collected during terminal anesthesia, and spleen, iliac lymph node, mesenteric lymph node, axillary lymph node, sections of small intestine (jejunum/ileum), and colon (including rectum) were collected immediately post mortem.

Virus isolation, virus loads, PCR

Virus isolation was performed on PBMC from heparinized blood. To determine cell-associated virus loads, PBMC from each challenged animal were diluted from 10^9 to 4 × 10^5 and subsequently in fivefold steps to 130 cells and cocultivated in duplicate with C8166 human T-cell line. Medium and cells were replenished every 3 to 4 days and the total culture volume was maintained at approximately 15 ml. All cultures were maintained for 28 days or until cytopathic effect was apparent. Culture results were confirmed by indirect immunofluorescence staining for SIV antigen in acetone/methanol-fixed cells.
using simian polyclonal anti-SIV serum. Fifty percent end points were calculated using the Kärber formula and the results expressed as the number of virus-infected cells per $10^6$ PBMC. Viral isolation on C8166 cells was performed for PBMC and mononuclear cells from tissues taken post mortem.

PCR for SIVmac proviral DNA was performed using env- and nef-specific primers on DNA extracted from PBMC and tissues (Kitchin et al., 1998). Quantitative competitive (QC) PCR for plasma RNA was performed as described previously (ten Haaft et al., 1997). The assay had a dynamic range between 40 and $5.6 \times 10^7$ RNA Eq/ml plasma.

Preparation of mononuclear cell suspensions from blood and tissues

PBMC were isolated by Ficoll–Paque (Pharmacia, Milton Keynes, UK) density centrifugation from heparinized blood and washed in culture medium RPMI 1640 (Gibco, Paisley, UK) supplemented with L-glutamine (2 mM; Gibco), penicillin (50 U/ml)/streptomycin (50 μg/ml; Gibco), and 10% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, Oxon, UK). Splenic and lymph node MNC were obtained by mechanical and enzymatic dissociation. Tissue remaining after removal of IEL was cut into smaller pieces and incubated at 37°C for 2 h in 0.75 mM EDTA (Sigma). The supernatant was filtered into 44/67% Percoll gradients for 20 min at 500

ELISA

ELISA was performed using standard methods (Kitchin et al., 1998b). Antigens were either recombinant SIV p27 or recombinant SIV gp120 or SIV gp 140 (MRC AIDS Reagent Project, EVA643, EVA655, and EVA641, respectively), or a detergent lysate of virus-infected C8166 cells (whole SIV). Bound IgG antibody was detected using rabbit anti-monkey IgG-AP or IgG-HRP conjugate (Sigma). IgA antibody was detected by incubation with rabbit anti-monkey IgA (Fc) (Nordic Immunologicals) followed by goat anti-rabbit IgG-AP (Sigma).

Lymphoproliferative assay

Mononuclear cells from blood and tissues were assayed in triplicate at $2 \times 10^5$ cells per well in 96-well plates in the presence of SIV antigens (p27-GST and baculovirus-produced gp120; MRC AIDS Reagent Project, EVA655 and EVA643, respectively) at a concentration range of 0.05–10 μg/well for 6–7 days or concanavalin A (1 μg/well; Sigma) for 3 days. $[^{3}H]$Thymidine incorporation of cells stimulated with antigen/mean in-

CTL assay

Fresh mononuclear cells from mucosa-associated lymph nodes, spleen, and peripheral blood, isolated on density gradients, were assessed for direct CTL activity. Target cells were prepared from autologous Herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCL) by overnight infection with 5 PFU/cell of recombinant vaccinia viruses expressing either SIV nef (v9011), SIV- env gp160 (v9002), SVV gag (ARP274, ARP270, EVA261; Centralised Facility for AIDS Reagents, NIBSC, Potters Bar, UK), or influenza A protein PB2 (a kind gift from Professor F. Gotch, Chelsea and Westminster Hospital, London). The following day, the cells were labeled with 150 μCi of Na$_2^{35}$Cr (Amersham) for 1 h at 37°C. Effector cells were combined with labeled target cells at effector-to-target ratios ranging from 200:1 to 12.5:1 in 96-well U-bottom plates. Following incubation at 37°C for 4 h,
supernatants were collected with a harvester (Skatron, Oslo, Norway) and release of chromium was measured in a gamma counter. Total $^{51}$Cr incorporation was determined by incubation of targets with 2.5% (v/v) Triton X-100 and spontaneous release determined after incubation of targets with medium alone. The tests were performed in triplicate and the geometric mean was determined. CTL activity was expressed as: % specific release = 100 × (experimental release, cpm − spontaneous release, cpm)/(total release, cpm − spontaneous release, cpm). Net SIV-specific activity was calculated by subtraction of the % specific release from autologous targets infected with vaccinia virus expressing influenza PB2 from the % specific release from targets expressing SIV proteins.

**Enumeration of antibody-secreting cells (ELISPOT assay)**

ELISPOT assay was performed on frozen cells. Antibody-secreting cells (ASC) were assayed in Microtitre Multiscreen HA plates (Millipore UH, Watford, Herts, UK) with nitrocellulose bases and coated with SIV recombinant p27 or gp140 (MRC AIDS Reagent Project, EVA655 with nitrocellulose bases and coated with SIV recombinant p27 or gp140 (MRC AIDS Reagent Project, EVA655 and EVA643, respectively), or keyhole limpet hemocyanin (KLH; Sigma) as a control, all at 10 μg/ml (Möller and Borrebbeck, 1985; Bergmeier et al., 1998). An amplified ELISPOT assay for the detection of macaque ASC was used (Ericksson et al., 1992; Bergmeier et al., 1998). The numbers of cells used in the antigen-specific ASC assay, in triplicates, were: tissue-derived cells, 1 × 10⁶, 5 × 10⁶, and 10⁷ cells per well; PBMC, 5, 2, and 1 × 10⁶ cells per well. The cells were incubated for 16 h at 37°C in 5% CO₂. Glutaraldehyde-inactivated and -washed plates were incubated with biotinylated Fc-specific goat anti-human IgG (Tago Immunologicals, Camarillo, CA) or biotinylated Fc-specific goat anti-monkey IgA (Nordic Immunologicals) (Bergmeier et al., 1998), then treated with avidin-conjugated peroxidase (Sigma), followed by biotinylated rabbit anti-peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA). The reaction was amplified by repeating the incubation with avidin-conjugated peroxidase. Plates were developed with AEC/hydrogen peroxide (Sigma). The spots were enumerated under low magnification (X20) with a binocular microscope. The results were expressed as the means ± SEM of the number of spots per 1 × 10⁶ cells. Total immunoglobulin-secreting cells were also assayed as a positive control (data not shown).

**ACKNOWLEDGMENTS**

This work was supported by the U.K. Department of Health and European Vaccine against AIDS (Programme EVA; Contract No. BM/H 97/2515). We are grateful to Dr. Harvey Holmes (AIDS Reagent Repository, NIBSC, UK) and Dr. Ian Jones (IOV, Oxford) for the provision of many of the reagents and to Kate Pratt for technical help.

**REFERENCES**


Kaul, R., Trabattoni, D., Bwayo, J. J., Arienti, D., Zagliani, A., Mwangi,


