Co-expression of interleukin-5 influences replication of simian/human immunodeficiency viruses in vivo

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The positive effect of the co-expression of T helper (Th) cell type 2 cytokine interleukin-5 (IL-5) on nef-deleted simian/human immunodeficiency virus (SHIV) replication in vitro has been observed previously. To analyse whether the growth advantage of IL-5-containing SHIV (NI-IL5) in vitro would be relevant in vivo, the virus was inoculated into monkeys. Three rhesus macaques were inoculated intravenously with 10^4 TCID50 of NI-IL5. Results were compared with those obtained previously from SHIV NM-3rN (intact) and SHIV-dn (nef-deleted)-infected monkeys. Cytokine production, analysed by IL-5 ELISA, showed a twofold increase in IL-5 concentration in the plasma soon after the peak of virus replication. Virus replication and antibody production were greater in monkeys inoculated with IL-5-expressing SHIV than in monkeys inoculated with nef-deleted SHIV without IL-5. These findings show a stimulation of SHIV replication by co-expression of IL-5 and suggest the important role of Th2-type cytokines in human immunodeficiency virus type 1 infection.

Introduction

Cytokine production by immune system cells plays an important role in the regulation of the immune response. On their first encounter with an antigen, naive CD4⁺ T helper (Th) cells differentiate into cytokine-producing effector cells. Two types of effector cells, Th1 and Th2, which are characterized by distinct cytokine profiles, have been described. In many pathological cases, the balance between Th1 and Th2 immune responses determines the outcome of diverse, immunologically mediated clinical syndromes, including infectious, autoimmune and allergic diseases. A progressive decrease in the production of Th1-type cytokines in favour of the production of Th2-type cytokines (the Th1- to Th2-type cytokine shift) during AIDS progression has been postulated recently (Klein et al., 1997; Meroni et al., 1996; Clerici & Shearer, 1993). However, it is well known that T-cell activation is required for efficient T-lymphotropic virus replication; such kinds of activation could be caused by cytokines. Thus, Th2-type cytokines might have an influence on human immunodeficiency virus (HIV) replication. The aim of the present study was to see if a Th2-type cytokine produced along with virus replication could influence virus replication and pathogenicity in vivo.

Interleukin-5 (IL-5) is a Th2-type cytokine with pleiotropic functions. It exhibits biological activity on cells of diverse haemopoietic lineages. Its many functions include: (1) enhancement of mediator release from human basophils; (2) induction of activated B-cell proliferation; (3) involvement in the chemoattraction, proliferation, differentiation, survival and activation of eosinophils (including stimulation of CCR3 production in eosinophils); (4) induction of immunoglobulin (Ig) secretion in primed B-cells and BCL1 (T-cell replacing factor); and (5) induction of IL-2 receptors on T- and B-cells. Th2 T-cells, mast cells, basophils and eosinophils are the known cellular sources of this cytokine (Paul, 1989; Takatsu et al., 1987; Sanderson, 1992).

A model for determining the roles of Th2-type cytokines during the course of T-lymphotropic virus replication in vivo could be provided by a recently constructed simian/human immunodeficiency chimeric virus (SHIV). SHIV was generated in order to obtain a human immunodeficiency virus type 1 (HIV-1)-related virus that would be infectious to macaque monkeys. Briefly, the virus has the HIV-1 vpr, tat, rev, vpu and env genes on a simian immunodeficiency virus (SIV) (strain mac) background. Monkeys infected with the constructed
virus have been shown to be a very useful animal model for HIV infection. Gene-deleted SHIVs, which are candidates for live-attenuated vaccines (Haga et al., 1998; Ui et al., 1999), can serve as a representative model of virus replication as well as a vector that expresses cytokine genes along with virus replication. However, the replication ability of these SHIVs was significantly decreased due to the gene deletions (Kuwata et al., 1995; Haga et al., 1998; Ui et al., 1999).

Recently, we have succeeded in the construction and in vitro investigation of replication-competent SHIV NI-IL5 (Fig. 1), which has the nef gene replaced with the human IL-5-coding sequence (Kozyrev et al., 2001). Co-expression of IL-5 seemed to confer a growth advantage to SHIV during replication in vitro. To analyse whether the growth advantage of NI-IL5 in vitro would be relevant in vivo, the virus was inoculated into rhesus monkeys.

**Methods**

**Cells and viruses.** The construction of infectious SHIV NM-3rN was reported previously (Kuwata et al., 1995). Briefly, this chimeric virus contains the cpr, tat, rev, vpu and env genes of HIV-1 (NL432 isolate) in an SIV (strain mac, clone 239) (nef-open) background. SHIV-dn is an NM-3rN-derived virus which lacks the nef gene open reading frame. The construction of this virus was described by Ui et al. (1999). Infectious SHIV-NI (∆nef vector) was constructed by the insertion of short polylinkers containing unique endonuclease cleavage sites in place of the deleted nef gene of SHIV-dn. NI-IL5 has the nef gene replaced by the human IL-5-coding sequence (Fig. 1). Viruses were constructed using SHIV-NI as the base virus. An infectious stock for each virus was prepared using M8166 cells and stored in liquid nitrogen until use.

**Inoculation of rhesus monkeys with SHIV.** Monkeys were treated in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University, Japan. Three rhesus macaques (MM175, MM204 and MM249) were inoculated with $10^3$ TCID$_{50}$ of NI-IL5. All animals were housed in a F3 level, monkey storage facility; health conditions were examined weekly. Blood samples, treated with sodium citrate to prevent coagulation, were collected weekly for the first 4 weeks and subsequently at 2 week intervals.

**Collecting and processing of blood samples.** PBMCs and plasma samples were isolated by centrifuging the collected blood with sodium citrate anticoagulant agent. Isolated plasma samples were frozen at $-80^\circ$C and subsequently used for viral RNA, IL-5 and antibody titre quantification. Isolated PBMCs (10$^6$cells) were used to evaluate the CD4$^+$ and CD20$^+$ cell ratio by FACS analysis and another 10$^6$ PBMCs were used for virus isolation.

**Plasma viral RNA quantification.** SHIV RNA quantitative assays were performed by RT–PCR for the SIV gag region using the primers SIVII-696F (5’ GAAATTTGGAAATAAACTAG 3’) and SIVII-784R (5’ CTTACATTTATCCGCTTA 3’) and a TaqMan RT–PCR kit (Perkin-Elmer). Virion RNA was extracted from plasma samples using a Viral RNA kit (QIAGEN). A labelled probe, SIVIL-7311T (5’-GATGTCCACCTGCCATTAAGCCGG-3’), was used for the detection of PCR products. For each run, a standard curve was generated from dilutions of a standard sample, measured by the Branched DNA method (Bayer Diagnostics).

**Lymphocyte immunophenotyping and haematological evaluation.** CD4$^+$/CD3$^+$ and CD20$^+$/CD3$^+$ cell populations in PBMCs isolated from the macaques were calculated before and after infection with NI-IL5. PBMCs were treated with anti-CD3 (EN-18/FITC) (Becton-Dickinson), anti-CD8 (Leu-2a/PerCP) (Becton-Dickinson) and either anti-CD4 (Nu-TH/1-PE) (NICHIREI) or anti-CD20 (Leu-16/PE) (Becton Dickinson) antibody and examined on a FACScan analyser (Becton-Dickinson), according to the manufacturer’s recommendations. Absolute lymphocyte counts in the blood were determined with an automated blood cell counter (F-820) (Sysmex).

**Human IL-5 immunoassay (ELISA).** IL-5 levels in the plasma were determined using a commercially available Quantikine Human IL-5 ELISA kit (R&D Systems).

**Anti-SHIV antibody titres.** Anti-SHIV antibody titres were determined using a commercial Serodia-HIV kit (Fujirebio). Plasma samples were isolated from sodium citrate anticoagulated blood, serially diluted and assayed routinely. The end-point titre was determined as the highest dilution to give a positive result.

**Results**

To analyse whether the growth advantage of SHIV NI-IL5 in vitro would be relevant in vivo, three rhesus macaques were inoculated intravenously with $10^4$ TCID$_{50}$ of NI-IL5. The data
IL-5 enhances SHIV replication in vivo

Fig. 2. Changes in CD4⁺ and CD20⁺ lymphocyte counts, IL-5 concentrations and viral RNA with time in rhesus monkeys inoculated with NI-IL5 (MM175, MM204 and MM249), SHIV-dn (MM155 and MM156) and NM-3rN (MM125, MM126, MM203 and MM205). Data are expressed as the mean ± SD of results from each virus-infected monkey. (A) CD4⁺ T-lymphocyte counts; (B) CD20⁺ B-lymphocyte counts; (C) IL-5 concentration in plasma (shaded area indicates normal concentration ranges of IL-5 in plasma); (D) plasma viral RNA concentration.

obtained previously from SHIV-dn (nef-deleted) and NM-3rN inoculations (Kuwata et al., 1995; Ui et al., 1999) served for comparison.

Since a drop in the population of CD4⁺ T-cells is an early prognostic marker for the onset of immunodeficiency in both humans and macaques, the number of these cells was determined (Fig. 2A). However, the levels of CD4⁺ cells showed no significant change during the early stages of infection and recovered to the pre-infection state soon after 4 weeks post-infection.

Since the functions of IL-5 include the induction of B-cell proliferation, the number of CD20⁻/CD3⁻ cells (B-lymphocytes) was determined as well (Fig. 2B). In our experiment, no significant change in the number of CD20⁻ cells was detected.

The concentration of human IL-5 in the plasma of NI-IL5-infected macaques was examined by human IL-5 ELISA (Fig. 2C). All monkeys showed detectable levels of IL-5, with peaks of more than 14 pg/ml at 2–4 weeks post-infection; this was not detected in SHIV-dn-infected monkeys. The IL-5 concentration at the time of peak production was about twofold higher than the normal IL-5 concentration in plasma (< 7.4 pg/ml) and may indicate IL-5 expression by NI-IL5 in vivo.

Plasma viral RNA loads were almost the same in all NI-IL5-infected monkeys, reaching peak values of more than 20000 RNA copies/ml at 1 week post-infection (Fig. 2D). As was observed in the in vitro experiments (Kozyrev et al., 2001), NI-IL5 showed a detectable growth advantage during the early phase of infection in comparison with SHIV-dn (nef-deleted) without IL-5. NI-IL5 replication peaked 1 week earlier than SHIV-dn and was significantly higher. Furthermore, the viral RNA titre of NI-IL5 at 1 week post-infection was higher than that of NM-3rN (which has an intact nef gene). Although the viral RNA titre of NI-IL5 at 1 week post-infection was higher than that of NM-3rN, the viral titre of NI-IL5, like that of SHIV-dn, declined to an undetectable level after 4 weeks, while the viral titre of NM-3rN persisted at high levels for up to 10 weeks.

The virus could also be isolated from CD8-depleted PBMCs of each NI-IL5-infected monkey for up to 4 weeks after infection. Subsequently, no virus could been isolated. The
Table 1. Antibody responses of NI-IL5-infected monkeys

The effect of IL-5 over-expression on the humoral immune response was analysed by an HIV-1 particle agglutination assay. Antibody titres are expressed as the highest dilution to give a positive result.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Monkey</th>
<th>Time post-infection (weeks)</th>
<th>Pre</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>13</th>
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<td>–</td>
<td>–</td>
<td>1:32</td>
<td>1:64</td>
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<td>1:1024</td>
<td>1:2048</td>
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<tr>
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<td>MM205</td>
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<td>–</td>
<td>–</td>
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<td>1:128</td>
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<td>1:1024</td>
<td>1:2048</td>
</tr>
<tr>
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<tr>
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nt, Not tested.

Discussion

The idea for this study was based on the recent finding of a progressive decrease in the production of Th1-type cytokines in favour of the production of Th2-type cytokines during the progression of AIDS (Klein et al., 1997; Meroni et al., 1996; Clerici & Shearer, 1993). Since Th2-type cytokine production increases during the progression of AIDS, this type of cytokine might influence the replication HIV. Thus, the co-expression of IL-5 seemed to confer a growth advantage to SHIV during replication in vitro (Kozyrev et al., 2001).

Similar to the in vitro results, NI-IL5 showed a detectable growth advantage during the early phase of infection in comparison with SHIV-dn (nef-deleted) without the IL-5 gene. NI-IL5 replication peaked 1 week earlier than SHIV-dn replication and was significantly higher. However, the highest peaks of replication were shown by NM-3rN, which has an intact nef gene, suggesting that IL-5 can not functionally replace the nef gene. The twofold increase in IL-5 in the plasma of NI-IL5-infected monkeys at the time of peak virus replication, as detected by ELISA, suggests the successful expression of IL-5 by NI-IL5. Despite the higher replication ability and co-expression of the Th2-type cytokine IL-5, the percentage of CD4+ cells in infected monkeys remained stable, thus providing further evidence that NI-IL5 is less capable of causing disease than the parental viruses NM-3rN and SHIV-dn.

Two of the three NI-IL5-infected monkeys developed a very strong antiviral antibody response, with titres much higher than those in SHIV-dn-infected monkeys. This result, taken together with the finding that Ig secretion is induced in primed B-cells (Paul, 1989; Takatsu et al., 1987), suggests that IL-5 stimulated an antiviral antibody response in the NI-IL5-infected monkeys. If this is correct, recombinant viruses such as NI-IL5 may be useful in the development of live-attenuated vaccines.

Although antibody production was increased, no significant increase in CD20+ positive cells was observed. A non-significant increase in B-cell number, which could not be detected by flow cytometry due to its low sensitivity, can lead to a significant increase in antibody titers.

Although there have been several attempts to clarify the role of Th1-type cytokines during the course of SIV and SHIV virus replication in vivo (Giavedoni & Yilma, 1996; Gundlach et al., 1997; Kuwata et al., 2000), no similar study has been done with Th2-type cytokines. Therefore, this report is the first to describe a positive effect of a Th2-type cytokine (IL-5) on the replication of a nef-deleted SHIV chimeric virus in vivo. Furthermore, this finding, taken together with the results of replication of viruses containing Th1-type cytokines, provides direct evidence of an essential role of Th2-type cytokines during the early stages of HIV-1 replication, which was...
suggested by the Th1- to Th2-type cytokine shift. However, the mechanisms of this interaction are still unclear.

IL-5 has been shown recently to be a potent inducer of CCR3 co-receptor expression on the surface of eosinophilic cells (Tiffany et al., 1998; Zimmermann et al., 2000). Because CCR3 can interact with most primary and T-cell line-adapted HIV-1 Env proteins (Alkhatib et al., 1997; Bazan et al., 1998), it could also be used by a virus to enter eosinophils. This may occur in HIV-1 infections, since HIV-1 has been detected in bone marrow eosinophils from certain HIV-1-infected individuals (Tiffany et al., 1998; Diagbouga et al., 1999). Other well-known effects of IL-5 on eosinophils are that it stimulates their proliferation and differentiation, activates them and increases their survival (Paul, 1989; Takatsu et al., 1987). These two separate functions of IL-5 (stimulating CCR3 expression and stimulating eosinophils) could provide the virus with an expanded population of target cells.

IL-5 can induce the proliferation of activated B-cells and cytotoxic T-lymphocytes and the secretion of Ig in primed B-cells (Paul, 1989; Takatsu et al., 1987; Wasik et al., 1997). Taken together with results of serology assays, these facts suggest that IL-5 stimulated an antiviral antibody response in NI-IL5-infected monkeys. If this is correct, recombinant viruses such as NI-IL5 may be useful as models for live-attenuated or half-live DNA vaccines (Akahata et al., 2000) because of their ability to improve the antiviral immune response.

In conclusion, we have shown that incorporation of a Th2-type cytokine (IL-5) in a gene-deleted SHIV chimera results in the over-expression of the cytokine and increases the replication and immunogenicity of immunodeficiency viruses in vivo. Our results indicate that the postulated Th1- to Th2-type cytokine shift might stimulate HIV-1 replication. Also, if the immunogenicity of a vaccine candidate such as nef-deleted SHIV can be enhanced by the co-expression of a Th2-type cytokine, it may be useful as a model for live-attenuated or half-live DNA vaccines against AIDS.

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References


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