Broadening of coreceptor usage by human immunodeficiency virus type 2 does not correlate with increased pathogenicity in an in vivo model

M. E. van der Ende,1, 2 C. Guillon,1 P. H. M. Boers,1 R. A. Gruters,3 P. Racz,4 K. Tenner-Racz,4 A. D. M. E. Osterhaus1 and M. Schutten1

1 Department of Virology, Erasmus Medical Centre Rotterdam, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
2 Department of Internal Medicine, Erasmus Medical Centre Rotterdam, Dr Molewaterplein 40, 3015 GD Rotterdam, The Netherlands
3 UMR103 CNRS BioMerieux, ENS Lyon, Lyon, France
4 Bernhard Nocht Institut für Tropen Medicin, Körber Labor für AIDS Forschung, Bernhard Nocht Straße 74, 2000 Hamburg 36, Germany

The pathogenic properties of four primary human immunodeficiency virus type 2 (HIV-2) isolates and two primary HIV-2 biological clones were studied in an in vivo human-to-mouse chimeric model. The cell-associated viral load and the ability to reduce the severity of the induced graft-versus-host disease symptoms, the CD4/CD8 ratio and the level of repopulation of the mouse tissues by the graft, were determined. All HIV-2 strains, irrespective of their in vitro biological phenotype, replicated to high titres and significantly reduced graft-versus-host disease symptoms as well as the CD4/CD8 ratios. Reduction of graft repopulation caused by infection with the respective HIV-2 strains showed that the in vitro replication rate, syncytium-inducing capacity and ability to infect human macrophages did influence the in vivo pathogenic potential whereas broadening of coreceptor usage did not.

Introduction

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are the causative agents of AIDS (Barre-Sinoussi et al., 1983; Clavel et al., 1986; Levy et al., 1986). The most prominent feature of the pathogenesis of AIDS is the quantitative and qualitative deterioration of the CD4+ T cell subset. Although CD4+ T cells are already affected soon after infection, it may take from 1 to more than 15 years before immune failure leads to overt clinical symptoms (Gruters et al., 1990, 1991). Both virological and host-delimited factors have been shown to determine the length of the asymptomatic period (Cheng-Mayer et al., 1988; Dean et al., 1996; Fenyo et al., 1988; van Baalen et al., 1997). Epidemiological studies have shown that, although progression to AIDS may in specific cases be relatively fast, disease progression in HIV-2-infected individuals is in general much slower than disease progression in HIV-1-infected individuals (Marlink et al., 1994; Whittle et al., 1994; van der Ende et al., 1996). Virological factors which underlie this difference between HIV-1- and HIV-2-induced disease progression are presently not known. Studies in in vitro and in vivo systems, specifying viral characteristics which determine the pathogenicity of HIV strains, may aid in the understanding of HIV-induced pathogenesis.

The characteristics used to classify HIV strains in vitro include replication rate, ability to induce the formation of multinucleated cells (syncytium-inducing (SI) capacity), coreceptor requirement and ability to infect different target cells (T cell lines versus macrophages) (Asjö et al., 1986; Berger et al., 1998; Cheng-Mayer et al., 1988; Schuitemaker et al., 1992b; Tersmette et al., 1989). For HIV-1 it has been shown that these in vitro characteristics exhibit a high degree of correlation. For example, SI strains in general have a high replication rate, infect target cells via x and β chemokine receptors, may infect immortalized T cell lines and only infect macrophages inefficiently. NSI strains, on the other hand, have a low replication rate, require expression of β chemokine receptors, do not infect T cell lines and replicate efficiently in macrophages (Berger et al., 1998; Schuitemaker et al., 1991). It
should, however, be noted that individual virus strains may also display intermediate biological phenotypes (Groenink et al., 1991; Sabri et al., 1996; Schuitmaker et al., 1992a). Several differences in in vitro characteristics of HIV-1 and HIV-2 have become apparent. The replication rate, as defined by the time required to detect virus after initiation of standard virus culture from donor peripheral blood mononuclear cells (PBMC), is generally lower for HIV-2 than for HIV-1 (Albert et al., 1990; van der Ende et al., 1996). Furthermore, we and others have recently shown that clear differences exist in coreceptor requirements (Guillon et al., 1998; McKnight et al., 1998). The linkage between HIV-1 SI phenotype and CXCR-4 coreceptor usage was not observed for HIV-2. Furthermore, HIV-2 strains in general have a broader coreceptor usage than HIV-1. Taken together, these observations suggest that in vitro usage of the CXCR-4 coreceptor and broadening of the coreceptor usage by HIV-2 do not result in enhanced in vivo pathogenicity.

To further address this issue we studied the in vivo pathogenic potential of HIV-2 isolates and biological clones in a chimeric human-to-mouse model for in vivo HIV infection (the xenogenic GVHD [graft-versus-host disease] mouse model) (Huppes et al., 1992, 1993; Schutten et al., 1996). In this model, high numbers of human PBMC are grafted into the peritoneal cavity of immune-deficient mice. In these mice, an acute graft-versus-host reaction develops within 7 to 14 days. The human lymphocyte population that repopulates the mouse tissues is characterized by high CD4/CD8 ratios (Schutten et al., 1996). Depletion of human CD4+ T cells from the graft results in a complete abrogation of the acute xenograft reaction. Depletion of antigen-presenting cells (APC; macrophages) from the human graft results in lower CD4/CD8 ratios and a concomitant delay in appearance of the xenograft symptoms (Huppes et al., 1993). We therefore set out to study in this model the direct (killing of infected CD4+ T cells) and indirect (inhibition/modulation of antigen presentation) pathogenic effect of different HIV-2 strains and isolates on the development of acute xenograft symptoms, CD4/CD8 ratio and the ability of the graft to repopulate mouse tissues.

Methods

**Virus strains and xenograft mice.** Xenograft mice were prepared as described previously (Huppes et al., 1992, 1993; Schutten et al., 1996). Briefly, 3- to 4-week-old XID mice (CBA/HNOlaHsd, Harlan Netherlands) were given total body irradiation (9 Gy) with syngeneic bone marrow support (5 x 10^5 cells per mouse, intravenously). Subsequently, Ficoll-gradient-isolated human PBMC from HIV-seronegative individuals (blood type A, Rhesus +) were given at 2 x 10^5 per gram mouse body weight intraperitoneally (i.p.). These xenograft mice were challenged i.p. with HIV-2 within 1 h of being grafted with human PBMC. Animals grafted with human PBMC from one and the same HIV-seronegative blood donor were equally distributed over groups of five mice receiving different HIV-2 strains. The primary HIV-2 strains used in this study had been isolated in mitogen-stimulated PBMC only and the in vitro characteristics have been described previously (van der Ende et al., 1996; Guillon et al., 1998). Relevant virus characteristics are given in Table 1. The doses of challenge virus were equilibrated on the basis of c.p.m. measured in an RT assay (van Baalen et al., 1998) and equaled approximately 30 mouse infectious doses 50% per mouse.

**Parameters of acute xenograft reaction and HIV-2 infection.** Directly after the onset of acute xenograft symptoms (furry coat, breathing problems and wasting), mice were sacrificed and lymphocytes were isolated from the peritoneal cavity. Human-to-mouse chimerism was confirmed and the CD4/CD8 ratio of the human graft was calculated, using FACS analysis with human CD3 and CD8 conjugates (Becton Dickinson) according to the manufacturer’s instructions. The viral loads of the lymphocytes isolated from the peritoneal cavities were determined in an infectious centre assay (ICT) (Schutten et al., 1996). Briefly, human lymphocytes isolated from the peritoneal cavity were counted and titrated in duplicate from 2 x 10^5 to 2 cells per well onward using fivefold dilution steps in 96-well (round-bottomed) plates. PBMC, pre-stimulated with mitogen for 3 days, were added as feeder cells (5 x 10^4 per well). The cells were cultured for 7 days in RPMI 1640 (BioWhittaker) supplemented with 10% foetal calf serum (BioWhittaker), penicillin (100 U/ml), streptomycin (10 µg/ml) and 50 IU recombinant human IL-2/ml (Euracetus), after which virus was detected by RT assay. The minimal number of cells isolated from the peritoneal cavity required for detection of virus was taken as a measure of the cell-associated viral load.

**HIV-2 in situ hybridization and immunohistochemistry.** Xenograft mouse tissues, including spleen, lung, bowel, heart, liver and bone, were prepared from groups of mice which had been grafted with PBMC isolated from the same individual. Tissues from two mice infected with the same HIV-2 isolate/strain were analysed pairwise. Tissues were fixed overnight with 4% formalin and subsequently embedded in paraffin. For routine immunohistochemistry, dewaxed 5 µm paraffin sections were heat-denatured with 0.1 M buffered sodium citrate (Norton et al., 1994). The sections were incubated with CD45 (LCA) (Dakopatts) according to the manufacturer’s instructions (Tenner-Racz et al., 1998). Binding of antibodies was visualized by the alkaline phosphatase anti-alkaline phosphatase technique using New Fuchsin as red chromogen. We have previously shown that this conjugate does not stain mouse cells (Schutten et al., 1996). After immunostaining the sections were either counter-stained with haematoxylin and mounted, or dehydrated and subjected to HIV RNA in situ hybridization. 32P-labelled, single-stranded, anti-sense HIV-2 RNA probes (Lofstrand Laboratories), which contained 1-4 to 7 kb fragments, collectively representing approximately 90% of the HIV-2 genome, were used as described previously (Embretson et al., 1993; Fox et al., 1991). Briefly, paraffin sections were either treated with Proteinase K (0.01 mg/ml) for 8 min at room temperature or heat denatured. The sections were incubated with prehybridization mixture (50% formamide, 0.5 M NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.02% Ficoll–polyvinylpyrrolidone–BSA and 7 mg of tRNA/ml) for 2 h at 37°C and then covered with hybridization mixture (prehybridization mixture + 10% dextran sulfate and 2 x 10^4 d.p.m. of probe/ml) overnight at 45°C. The sections were washed, RNase treated (Boehringer Mannheim) for 40 min at 37°C and further developed, counter-stained with haemalaun and mounted as previously described. As a negative control, sections were hybridized with a radiolabelled sense probe. The sections were examined with a microscope equipped with epiluminescent illumination (Axioskop; Carl Zeiss). Cells were considered positive for viral gene expression if the number of grains overlying a cell was more than six times the background.
Table 1. Characteristics of the HIV-2 strains

<table>
<thead>
<tr>
<th>HIV-2 strain</th>
<th>Replication rate*</th>
<th>Biological phenotype†</th>
<th>Coreceptor usage‡</th>
<th>Macrophage-tropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH2-1</td>
<td>42</td>
<td>Early NSI</td>
<td>R5</td>
<td>+</td>
</tr>
<tr>
<td>RH2-2</td>
<td>&lt; 7</td>
<td>Late SI</td>
<td>R1,R3,R5,X4</td>
<td>-</td>
</tr>
<tr>
<td>RH2-6</td>
<td>&lt; 7</td>
<td>Late SI</td>
<td>X4</td>
<td>+</td>
</tr>
<tr>
<td>PH2-1 E6</td>
<td>14</td>
<td>Intermediate NSI</td>
<td>R1,R3,R5,X4</td>
<td>+</td>
</tr>
<tr>
<td>PH2-1 D5</td>
<td>14</td>
<td>Intermediate SI</td>
<td>R5</td>
<td>+</td>
</tr>
</tbody>
</table>

* First day of a positive signal in p24 antigen ELISA after start of virus isolation (van der Ende et al., 1996).
† Biological phenotype according to van der Ende et al. (1996).
‡ Coreceptor usage according to Guillon et al. (1998) and Berger et al. (1998).

Results

Acute xeno-GvHD symptoms and CD4/CD8 ratio

Grafting human PBMC from HIV-seronegative individuals at $2 \times 10^7$ per gram mouse body-weight i.p. has been shown to induce an acute xeno-GvHD reaction within 14 days in approximately 100% of the animals (Huppes et al., 1992; Schutten et al., 1996) (Table 2). In general, the development of acute xeno-GvHD symptoms is fast and little variation is observed between animals grafted with PBMC from donors with the same ABO/Rhesus factor. The acute xeno-GvHD symptoms observed in the HIV-2-infected xeno-GvHD mice were in general less severe than in the control mice (Table 2). No significant differences in absolute numbers of human lymphocytes isolated from the peritoneal cavity (data not shown) or in the number of infected centres in the ICT (Table 2) were observed between the different groups of HIV-2-infected mice. Quite unexpectedly, no significant differences in symptoms or CD4/CD8 ratio were observed between xeno-GvHD mice infected with the early HIV-2 RH2-1 (R5) isolate or late isolate HIV-2 RH2-2 (R1,R3,R5,X4). Acute xeno-GvHD symptoms were not observed in the mice infected with the late HIV-2 subtype B isolate RH2-6 (X4) and CD4/CD8 ratios from these mice were lower compared to those in mice in the other groups (Table 2). Although the differences between mice infected with the SI and NSI biological clone from PH2-1 were minor, the xeno-GvHD mice infected with the SI biological clone PH2-1 D5 (R5) did show less severe xeno-GvHD symptoms as compared to the mice infected with the NSI biological clone PH2-1 E6 (R1,R3,R5,X4) (Table 2).

Table 2. Acute xeno-GvHD symptoms in HIV-2-infected mice

<table>
<thead>
<tr>
<th>HIV-2 strain</th>
<th>Xeno-GvHD*</th>
<th>ICT†</th>
<th>CD4/CD8 ratio‡</th>
<th>CD45⁺ cells§</th>
<th>HIV-2-infected cells</th>
<th></th>
<th>CD45⁺ cells§</th>
<th>HIV-2-infected cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>+</td>
<td>–</td>
<td>4–9</td>
<td>+</td>
<td>–</td>
<td>NA</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RH2-1</td>
<td>+</td>
<td>1:6 $\times 10^3$</td>
<td>0:6–1:2</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>RH2-2</td>
<td>+</td>
<td>4 $\times 10^3$</td>
<td>0:5–1:0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>RH2-6</td>
<td>–</td>
<td>3 $\times 10^3$</td>
<td>0:3–0:5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PH2-1 E6</td>
<td>+</td>
<td>2 $\times 10^3$</td>
<td>0:7–1:2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PH2-1 D5</td>
<td>+</td>
<td>1:6 $\times 10^3$</td>
<td>0:6–0:8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Severity of the xeno-GvHD symptoms: +++, severe; +, minor; –, no symptoms observed within the observation period (11 days).
† Number of infectious centres per $4 \times 10^3$ cells isolated from the peritoneal cavity.
‡ Range of the CD4/CD8 ratio within each group of mice.
§ Numbers of CD45⁺ cells in the organ: +++, very high; ++, high; +, low; –, occasional positive cell; NA, not applicable due to xeno-GvHD induced necrosis of the organ.
|| Numbers of HIV-2-RNA-positive cells: +++, very high; ++, high; +, low; –, occasional positive cell.
Immunohistochemistry of tissues from xeno-GvHD mice

In order to study the ability of the human PBMC graft to repopulate the mouse tissues after infection with HIV-2, tissues from the xeno-GvHD mice (spleen, lung, heart, bone, bowel) were prepared and examined to determine the numbers human cells present as indicated by immunohistochemical staining for human CD45. Variation in the distribution of the human lymphocytes was low between mice grafted with the same donor PBMC (data not shown). High numbers of human cells, especially in highly vascularized tissues like the lung, were observed in xeno-GvHD mice which were uninfected (Table 2). Only small remnants of necrotized spleen could be obtained from these mice, in which the mouse tissue was almost completely replaced by human cells (Fig. 1A). Within these tissues only CD45+CD68− cells (T cells) were found and CD45+CD68+ cells (macrophages) were exclusively found adhered to and migrating into tissues isolated from the peritoneal cavity (Schutten et al., 1996). In all HIV-2-infected xeno-GvHD mice, relatively high numbers of human T cells and macrophages could be observed in the tissues isolated from the peritoneal cavity. Similar to what was found for uninfected mice, macrophages were exclusively found migrating into and adhered to the tissues isolated from the peritoneal cavity (data not shown). The numbers of human

Fig. 1. Comparison of the numbers of human lymphoid cells and productively HIV-2-infected cells in xeno-GvHD mice as determined by CD45 staining (red alkaline phosphatase staining) and HIV-2 RNA in situ hybridization (black grains). (A) Uninfected control; (B) HIV-2 RH2-1 (R5); (C) HIV-2 RH2-2 (R1,R3,R5,X4); (D) HIV-2 RH2-6 (X4); (E) HIV-2 PH2-1 E6 (R1,R3,R5,X4); (F) HIV-2 PH2-1 D5 (R5).
cells in the lungs of the xeno-GvHD mice infected with HIV-2 RH2-1 (R5), RH2-2 (R1,R3,R5,X4) and PH2-1 E6 (R1,R3, R5,X4) was significantly lower than the number of human cells in the lungs of the control mice (Table 2). The structure of the spleen obtained from these mice was relatively intact and contained many CD45<sup>+</sup> cells, mainly within the periarтерiolar lymphocyte sheets (Fig. 1B, C, E; Table 2). Hardly any CD45<sup>+</sup> cells were found in the lung and spleen of mice infected with HIV-2 RH2-6 (X4) (Fig. 1D; Table 2). Similarly, the lungs of HIV-2 PH2-1 D5 (R5)-infected mice was almost completely devoid of human cells and low numbers of human cells were observed in the spleen (Fig. 1F, Table 2).

**HIV-2 infection of xeno-GvHD mice**

The ability of the HIV-2 isolates and biological clones to productively infect cells expressing the macrophage marker CD68 was determined by double labelling (HIV-2 RNA in situ hybridization with CD68 immunohistochemistry) (Table 1). Productively infected macrophages were observed in all HIV-2-infected xeno-GvHD mice except for those infected with the late isolate RH2-2 (R1,R3,R5,X4) (Table 1). Also, the relative number of productively infected cells was determined in the tissues with high numbers of human lymphocytes (spleen and lung) (Table 2). HIV-2 RNA in situ hybridization signal above background was absent in the negative control mice. High numbers of HIV-2 RNA<sup>+</sup> cells were observed in the peritoneal cavity of all mice infected with the different HIV-2 isolates and biological clones (data not shown). High numbers of productively infected cells could be observed in the lungs and spleens of xeno-GvHD mice infected with HIV-2 RH2-1 (R5) (Fig. 1C). Despite the fact that no significant differences in the numbers of CD45<sup>+</sup> cells were observed between xeno-GvHD mice infected with HIV-2 RH2-1 (R5) (Fig. 1C) or HIV-2 RH2-2 (R1,R3,R5,X4) (Fig. 1D), a significantly lower number of productively infected cells was found in the tissues of xeno-GvHD mice infected with HIV-2 RH2-2 (R1,R3,R5,X4) (Table 2). Similar to HIV-2 RH2-1 (R5)-infected xeno-GvHD mice, many productively infected cells were found in the tissues of xeno-GvHD mice infected with HIV-2 PH2-1 E6 (R1,R3, R5,X4) (Fig. 1E, Table 2). Although the numbers of cells and therefore also the numbers of productively infected cells in the peripheral tissues of xeno-GvHD mice infected with HIV-2 PH2-1 D5 (R5) (Fig. 1F) were significantly lower as compared to HIV-2 RH2-1 (R5), RH2-2 (R1,R3,R5,X4) and PH2-1 E6 (R1,R3,R5,X4), a relatively high percentage of the cells observed in the peripheral tissues of these mice were still productively infected (Table 2).

**Discussion**

In the present paper we have shown that the SI capacity, replication rate and ability to infect macrophages influenced the pathogenic properties of HIV-2 strains in the xeno-GvHD mouse model for HIV infection. Moreover, an SI macrophage-tropic HIV-2 biological clone that only uses CCR-5 as coreceptor proved to be more pathogenic than a highly homologous NSI macrophage-tropic biological clone with a potentially broader coreceptor usage (CCR-1, CCR-3, CCR-5 and CXC5-4). These data suggest that broadening of the potential cellular host-range does not necessarily increase the pathogenic potential of HIV-2 and probably HIV-1.

Both direct and indirect mechanisms have been suggested to contribute to the pronounced CD4<sup>+</sup> T cell depletion observed in HIV-infected individuals (for review see Zinkernagel et al., 1994). The direct mechanisms include acute single cell lysis of infected cells (Samson et al., 1996) and depletion of uninfected CD4<sup>+</sup> cells by fusion with infected cells (syncytium formation) (Tersmette et al., 1993). It has also been shown that cells of the monocyte/macrophage lineage do not function properly, which may result in dysregulation of activation and/or maturation of CD4<sup>+</sup> T cells due to functionally disturbed antigen presentation (Meyaard et al., 1993). This may also indirectly contribute to the decline of CD4<sup>+</sup> T cells.

Two observations suggest that this last phenomenon contributed to the HIV-2-induced CD4<sup>+</sup> T cell depletion in our xeno-GvHD mouse model. First, no significant differences were observed in the CD4/CD8 ratio and the number of CD4<sup>+</sup> cells in spleens and lungs of mice infected with the early, macrophage-tropic NSI isolate RH2-1 (R5) and the late non-macrophage-tropic SI isolate RH2-2 (R1,R3,R5,X4). This is despite the fact that the late SI isolate is highly cytopathic for CD4<sup>+</sup> T cells in vitro and the early NSI isolate does not induce single cell lysis to a significant extent (unpublished observations). The fact that the percentage of infected cells was high in the RH2-1 (R5)-infected mice and low in the RH2-2 (R1,R3,R5,X4)-infected mice also suggests that these virus strains cause CD4<sup>+</sup> T cell depletion by two different mechanisms. It seems appropriate to assume that HIV-2 RH2-2 (R1,R3,R5,X4)-infected cells are directly killed and therefore do not reach the peripheral tissues studied, whereas RH2-1 (R5)-infected cells are not killed directly, but seem rather to be functionally affected. Second, RH2-2 (R1,R3,R5,X4) and RH2-6 (X4) are both highly cytopathic for CD4<sup>+</sup> T cells in vitro. The macrophage-tropic isolate RH2-6 (X4) proved to be far more pathogenic in vivo with respect to abrogating GvHD symptoms and inhibiting migration of T cells to peripheral tissues than the non-macrophage-tropic isolate RH2-2 (R1,R3,R5,X4).

SI capacity, replication rate and coreceptor requirements have all been shown to positively correlate with rapid disease progression in HIV-1-infected individuals. Because, for HIV-1, these factors are generally linked, it has not been possible to distinguish which factors have a causal relation with disease progression. Since SI capacity and coreceptor usage are not linked for HIV-2, we could study these factors independently. The observation that in vitro coreceptor usage of HIV-2 strains is broader than that of HIV-1 has already suggested that broadening of coreceptor usage does not necessarily lead to increased in vivo pathogenicity. Indeed, in the xeno-GvHD
HIV-1 strains (Schutten et al., 1996). It therefore seems that in the absence of a substantial humoral and cellular antiviral immune response both in vitro and in our human-to-mouse chimeric model, HIV-1 and HIV-2 may have comparable replicative and pathogenic potential. We therefore hypothesize that the lower viral load and pathogenicity observed in HIV-2-infected individuals as compared to HIV-1-infected individuals is primarily related to a difference in interaction of the virus with the specific immune response.

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