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Comparison of early events during infection of human and chimpanzee peripheral blood mononuclear cells with HIV-1

K. Pischinger[1,*], **K. Zimmermann**[1,*], **M. M. Eibl**[2], and **J. W. Mannhalter**[1]

[1]IMMUNO AG, Vienna, Austria
[2]Institute of Immunology, University of Vienna, Vienna, Austria

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Summary. Differences in early events during entry and integration of HIV-1 into peripheral blood mononuclear cells (PBMC) might contribute to the absence of AIDS-like disease in chimpanzees as compared to humans. To address this question, we first tested the in vitro susceptibility of human and chimpanzee PBMC for infection with the two HIV-1 isolates III B and RF. The results of these studies revealed that chimpanzee PBMC had a slightly lower capability to support the growth of HIV-1 as compared to human PBMC. This was accompanied by a delayed accumulation of proviral HIV-1 DNA in cultures of HIV-1-infected chimpanzee PBMC. However, no differences between cells of the two species were observed when very early events of HIV-1 infection were studied. Shortly (20 h) after infection chimpanzee and human cells harbored similar amounts of proviral HIV-1 DNA and PBMC of both species behaved comparably with respect to pre-integration latency (i.e. the ability to activate extrachromosomal HIV-1 intermediates in HIV-1 infected quiescent cells at various times after infection). These results strongly suggest that the absence of AIDS-like disease in chimpanzees cannot be correlated with defects in early events of the HIV-1 replicative cycle.

Introduction

Among the non-human primate disease models for AIDS, the chimpanzee (*Pan troglodytes*) model plays a prominent role [11, 15]. The human immunodeficiency virus type 1 (HIV-1) readily infects chimpanzees, but HIV-1-inoculated animals do not normally develop AIDS-like disease (with two possible exceptions reported by Novembre et al. [21] and Fultz et al. [12]). The reasons for the lack of progression to AIDS in chimpanzees are still incompletely understood.

*Both authors contributed equally to this work.

CD8-positive cells with the capacity to suppress HIV-1 replication, or HIV-1 suppressive factors released by such cells have been suggested to be involved [3, 7, 17, 29]. It can, however, at present not be ruled out that chimpanzee cells per se are less susceptible to HIV-1 infection; especially since Shibata et al. [26] could recently demonstrate that only a small number of HIV-1 isolates derived from AIDS patients had the capacity to infect chimpanzee lymphocytes in vitro.

In the present study the question of susceptibility of chimpanzee cells for HIV-1 infection has been addressed, and we focused our attention on the early events following HIV-1 infection of peripheral blood mononuclear cells (PBMC) in vitro. In human PBMC, HIV-1 entry into the cell is followed by uncoating of the viral particle, formation of the pre-integration complex and initiation of reverse transcription [4]. In quiescent cells, incompletely reverse-transcribed HIV-1 genomes can persist for several days and the replicative cycle can be completed following activation [32]. Proviral HIV-1 DNA is then integrated into the genome [4] and cells harboring this DNA accumulate in the course of an in vitro infection of activated PBMC [5]. In order to assess if chimpanzee cells behave differently compared to their human counterparts with respect to HIV-1 infection, different steps in the viral life cycle have been evaluated in both human and chimpanzee PBMC.

Materials and methods

Animals

The chimpanzees were housed individually in the primate unit of IMMUNO AG (ISIS code 110 308 900) under conditions meeting the requirements of the appropriate animal biosafety level.

Isolation of cells

Chimpanzee and human PBMC were isolated by buoyant density gradient centrifugation according to the procedure described by Bøyum [2] with slight modifications. Briefly, heparinized (40–50 IU/ml) venous blood (Liquemin; Hoffmann-La Roche, Basel, Switzerland) was layered over Lymphoprep (Nycomed A/S, Oslo, Norway) and centrifuged at $400 \times \mathbf{g}$ for 30 min at room temperature. The mononuclear cells from the interphase were aspirated, washed three times in Hanks' balanced salt solution (HBSS) and resuspended at a concentration of 2×10^6 cells/ml in culture medium [RPMI-1640; Flow Laboratories, Irvine, UK containing 2mM L-glutamine (GIBCO BRL, Bethesda, MD, USA), antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin; GIBCO)], and supplemented either with 10% pooled human serum derived from blood group AB-positive donors (human AB serum) or fetal calf serum (FCS, Flow Laboratories) (both sera were previously heat-inactivated at 56 °C for 30 min).

Phytohemagglutinin-induced lymphocyte activation

PBMC were stimulated with 0.01 mitogenic units/ml of phytohemagglutinin (PHA HA16, Wellcome, Dartford, UK) in culture medium plus 10% FCS in a CO_2 incubator (5% CO_2, 95% humidity) for 3 days at 37 °C. Following stimulation, the cells were washed three times with HBSS and adjusted to a final concentration of 2×10^6 cells/ml in culture medium plus 10% FCS containing 20 U of interleukin 2 (IL-2)/ml (Biotest, Frankfurt, Germany).

The activation of PBMC was checked by assessing cell proliferation. Briefly, cells were incubated for 16 h with 1 μCi/ml [^{3}H]methylthymidine (DuPont NEN, Bad Homburg, Germany). Cells were then lysed in H_2O and harvested onto glass fiber filters by means of a harvesting machine (Tomtec, Wallac Oy, Turku, Finland). The radioactivity incorporated into DNA was measured in a scintillation counter (1450 MicroBeta, Wallac Oy).

Preparation of viral stocks

Stocks of HIV-1 strains IIIB and RF were prepared by culture of infected AA2 cells (a CD 4-positive lymphoblastoid B cell line, previously shown to be especially permissive for HIV replication [8]) in RPMI 1640 culture medium containing 10% FCS and 1% L-glutamine without antibiotics. When immunofluorescence analysis showed most of the cells to be positive for viral antigen, the cells were pelleted by centrifugation and the supernatant was concentrated by ultrafiltration through Minitan filters (cut-off range 300 kDA; Schleicher & Schuell, Kassel, Germany). The virus was then pelleted through a 20% sucrose cushion at 28 000 rpm and 4 °C for 3 h in a SW28 rotor (Beckman, Palo Alto, CA, USA). The pellet was resuspended in 0.01 M Tris-HCl, pH 7.2.

To remove HIV-1 DNA contamination from the viral stocks, they were mixed with an equal volume of a buffer containing 2000 U/ml of RNase-free DNaseI (Boehringer Mannheim, Mannheim, Germany) in 100 mM Tris-HCl, pH 8 and 10 mM NaCl and incubated for 1 h at 37 °C.

Virus titrations

1×10^5 chimpanzee or human PBMC or 5×10^4 AA2 cells per microtiter well were infected with semi-logarithmic dilutions of HIV-1 IIIB or RF isolates. After incubation for 28 days, viral titers of AA2 cells were assessed directly by induction of syncytia, whereas viral titers of PBMC were determined by transfer of cell culture supernatants to AA2 indicator cells and subsequent assessment of syncytia. Titers were determined by the method of Reed and Muench [24] and expressed as $TCID_{50}$.

Infection of cells with HIV-1

2×10^6 lymphocytes/ml were infected by incubation for 2 h in the presence of 1×10^5 $TCID_{50}$ HIV-1/ml in RPMI medium containing 10 μg/ml polybrene (Sigma, Deisenhofen, Germany). Then, free virus was removed by washing three times with HBSS and 2×10^6 infected cells/ml were resuspended in culture medium supplemented either with 10% human AB serum (resting cells) or with 10% FCS and 20 U IL-2 (activated cells). For PCR quantitation of HIV-1, lymphocytes were first activated with PHA as described, adjusted to 2×10^6 cells/ml and then infected with 2×10^6 $TCID_{50}$ HIV-1/ml. Viabilities of infected cells, which were measured by trypan blue exclusion, were usually higher than 80%.

Analysis of cell culture supernatants by antigen capture ELISA

Culture supernatants were analyzed for the presence of HIV-1 antigen by HIV-ELISA (Integrated Diagnostics, Baltimore, MD, USA) according to the supplier's instructions, using horseradish peroxidase-coupled secondary antibodies and *o*-phenylenediamine as substrate. Color development was determined by OD_{490} measurement after 10 minutes. Positive (culture supernatant of HIV-1-infected AA2 cells) and negative (culture medium containing 10% FCS and 20 U IL-2, as well as culture supernatant of uninfected AA2 cells) controls were included in each plate.

Quantitation of HIV-1 DNA by PCR

Crude cell lysates were used for PCR analysis. For this purpose, 10^6 cells/ml were digested with lysis buffer containing 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 0.01% Nonidet P40 and 200 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for 1 h at 65 °C. The enzyme was inactivated for 15 min at 94 °C. A newly developed quantitative multiple competitive PCR (QMC-PCR) was then employed for determination of HIV-1 DNA copy number [33]. By introducing varying amounts of four different-sized competitive DNA templates to the sample containing the wild-type fragment, the HIV-1 copy number could be determined in a single reaction tube. Each PCR was carried out in 50 μl of a solution containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP, 1 U DynaZyme (Finnzymes Oy, Espoo, Finland), 250 ng each of SK68 (5′-AGCAGCAGGAAGCACTATGG-3′) and SK69 (5′-CCAGACTGTGAGTTGCAACAG-3′), the sample to be determined and the respective competitive templates. The samples were overlaid with mineral oil and amplified 40 cycles: step 1, 1 min at 94 °C; step 2, 1 min at 60 °C; step 3, 1 min at 75 °C (extended 1 second per cycle); and final incubation at 75 °C for 1 min.

Samples were analyzed on a 3.5% NuSieve GTG Agarose gel (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide and scanned with a video densitometer (Hirschmann GmbH, Taufkirchen, Germany). Peak areas of the competitor fragments (each multiplied by a specific correction factor [33] and wild-type fragment were analyzed in each lane, and the copy number of the sample was determined using a single linear regression plot [23].

Results

In vitro titration of HIV-1 IIIB and RF in chimpanzee and human lymphocytes

The capacity of the two HIV-1 strains IIIB and RF to complete a replicative cycle and be released was assessed in activated PBMC of four individual chimpanzees and four different human subjects by determination of virus titers. In parallel, the two viral strains were also titrated twice in AA2 cells. The viral titers, expressed as $TCID_{50}$, are depicted in Table 1. As could be expected from laboratory-adapted HIV-1, the highest titers were found in AA2 cells ($TCID_{50}$ of $10^{7.6}$ to $10^{7.8}$ for IIIB and $10^{7.8}$ to $10^{8.0}$ for RF). In human PBMC the HIV-1 titers were slightly lower (mean titers $10^{7.0}$ for IIIB and $10^{7.2}$ for RF) and chimpanzee PBMC supported HIV-1 growth to an even lesser extent (mean titers for IIIB $10^{6.6}$ and for RF $10^{5.9}$). Thus, although chimpanzee PBMC could clearly be infected with HIV-1 IIIB and RF, they were slightly less susceptible to infection with these viral strains than human cells.

Progression of HIV-1 replication in PBMC at early stages of infection determined by increase of DNA copy numbers

To determine if this slightly decreased release of viral particles by chimpanzee PBMC correlated with a reduced accumulation of proviral HIV-1 DNA in infected cells, we determined proviral DNA copy numbers at 20, 48 and 72 h after infection by QMC-PCR [33]. Accumulation of HIV-1 IIIB DNA is shown in Fig. 1A (humans) and Fig. 1C (chimpanzees). Figures 1B and D show the same parameters for HIV-1 RF-infected cells. As can be seen, chimpanzee as well as

Table 1. In vitro titration of HIV-1-IIIB and -RF

Cells	HIV-1 strain	
	IIIB	RF
AA2	$10^{7.8}$	$10^{7.8}$
	$10^{7.6}$	$10^{8.0}$
Mean	$10^{7.7}$	$10^{7.9}$
Human subjects		
hu #1	$10^{7.0}$	$10^{7.2}$
hu #2	$10^{6.6}$	$10^{6.8}$
hu #3	$10^{6.9}$	$10^{7.2}$
hu #4	$10^{7.4}$	$10^{7.5}$
Mean	$10^{7.0}$	$10^{7.2}$
Chimpanzees		
ch #1	$10^{6.8}$	$10^{5.8}$
ch #2	$10^{6.5}$	$10^{6.3}$
ch #3	$10^{6.1}$	$10^{5.8}$
ch #4	$10^{6.9}$	$10^{5.7}$
Mean	$10^{6.6}$	$10^{5.9}$

Viral titers are expressed as $TCID_{50}$ according to Reed and Muench [24]

human PBMC appear to have nearly the same in vitro susceptibility for both viral strains. Before the first completion of a viral replication cycle (20 hours post infection) approximately 100 000 copies/10^5 cells were determined for both strains in human and chimpanzee PBMC (as was to be expected with an MOI of 1). After a period of 3 days, the HIV-1 DNA copy number in human PBMC usually rose by a factor of 5 to 10 for both virus strains, whereas in chimpanzee PBMC 2- to 5-fold increases were observed. Statistical analysis (t test) revealed a significant difference in HIV-1 copy numbers after infection and subsequent cultivation of human and chimpanzee cells with HIV-1 IIIB ($p < 0.032$), whereas the differences observed after HIV-1 RF infection and cultivation did not reach the level of statistical significance.

Activation of extrachromosomal replicative intermediates in quiescent PBMC at various times after HIV-1 infection

It has been previously shown that HIV-1 can enter quiescent primary human lymphocytes and persist there as an incompletely reverse-transcribed intermediate for several days. Upon subsequent activation of these cells, the viral replication cycle is completed and HIV-1 is released [32]. In order to test for possible differences in this type of pre-integration latency between PBMC of both species,

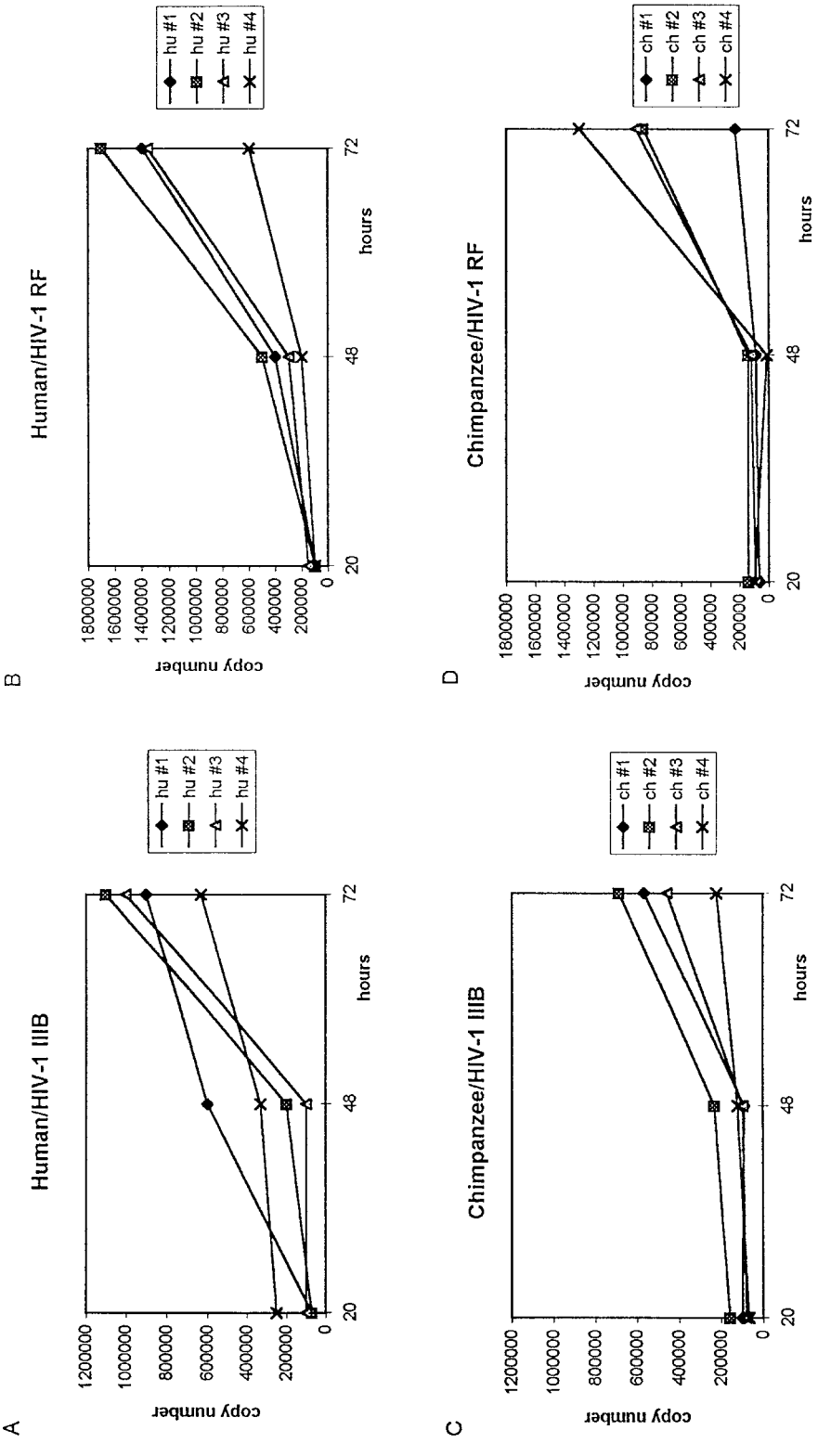

Fig. 1. HIV-1 copy number in HIV-1 IIIB and RF-infected PBMC. PBMC of four humans (hu #1 – hu #4) and chimpanzees (ch #1 – ch #4) were infected with HIV-1 IIIB and RF isolates and HIV-1 copy number per 100 000 cells was determined by QMC-PCR after 20, 48 and 72 hours (results depict average of two determinations). **A** Human PBMC infected with HIV-1 IIIB, **B** human PBMC infected with HIV-1 RF, **C** chimpanzee PBMC infected with HIV-1 IIIB, **D** chimpanzee PBMC infected with HIV-1 RF

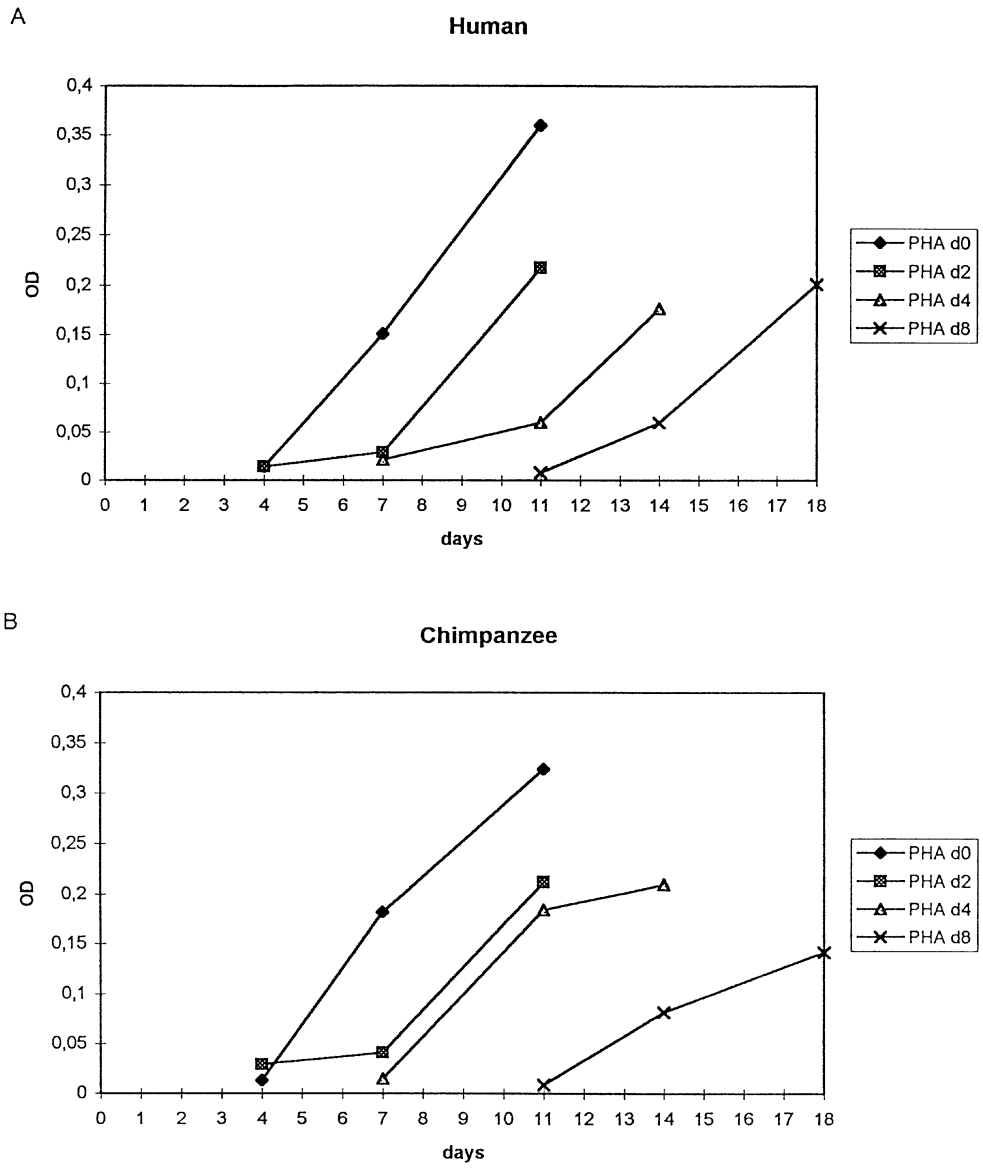

Fig. 2. Determination of viral antigen in supernatants of quiescent PBMC activated at various time points after infection. Human (**A**) and chimpanzee PBMC (**B**) were infected with HIV-1 IIIB and activated with PHA after 0, 2, 4 or 8 days (PHA d0 – PHA d8). Culture supernatants were analyzed for the presence of HIV-1 antigen at the indicated time points and results are expressed as OD

human and chimpanzee PBMC were infected with 0.05 MOI of HIV-1 IIIB isolate. After removal of free virus, aliquots of the cells were activated with PHA either immediately or 2, 4, or 8 days after infection. The ability of the cells to become activated at these time points was demonstrated by assessing lymphocyte proliferation and was found to be comparable between chimpanzee and human

cells (data not shown). Furthermore, no major difference in the magnitude of the proliferative response was observed, when human or chimpanzee PBMC were stimulated with PHA either immediately after HIV-1 infection or after various days of incubation (data not shown). To assess if chimpanzee or human cells display similar HIV-1 pre-integration latency, the amount of antigen released by PBMC activated after various days of quiescence was determined. As could be expected when analyzing cells of outbread populations, the results obtained in the different experiments varied substantially with respect to the amount of virus released at different times. Therefore, a representative experiment of four, each performed with human and chimpanzee PBMC, is depicted in Fig. 2. As can be seen in Figs. 2A and 2B, the results obtained with human and chimpanzee PBMC were basically the same. These experiments do, however, clearly demonstrate that replicative HIV-1 intermediates may remain as long as 8 days in a state of pre-integration latency in resting PBMC from both humans and chimpanzees. If activated at the end of this period, they produced comparable amounts of HIV-1 particles.

Discussion

The reasons for the absence of disease in HIV-1-infected chimpanzees are still not fully understood. Possible explanations for this phenomenon are (a) the ability of $CD8^+$ lymphocytes to suppress virus replication [7, 9, 12, 17, 19], (b) specific anti-HIV-1 antibody-dependent cellular cytotoxic (ADCC) reactivities [10], (c) relative resistance to the systemic effects of HIV-1 on T-cell dysfunction [14, 25], (d) the preservation of MHC class II-APC-Th cell microenvironment [13], and (e) increase in NK cell activity [19]. Furthermore, in contrast to humans, no differences in T-cell receptor (TCR)-variable region β (Vβ) chain repertoire were found between naive and HIV-1-infected chimpanzees [1].

Alternatively to these immunological explanations, the biological properties of the virus strains selected for inoculation might be responsible for not producing a rapid and pathogenic infection in chimpanzees. For example, Shibata et al. [26] screened HIV-1 isolates from patients with AIDS for infectivity in chimpanzee PBMC. Only three of 23 isolates were infectious, and of these three, only one was able to productively infect all PBMC of the 25 chimpanzees tested. Considering these problems, we used two HIV-1 isolates, HIV-1 IIIB and HIV-1 RF to directly compare early stages of viral integration in PBMC of both chimpanzees and humans. HIV-1 IIIB is the viral isolate used most frequently for HIV-1 challenge studies in chimpanzees immunized with AIDS candidate vaccines and both HIV-1 IIIB and RF are known to readily infect human lymphocytes. By employing PBMC instead of CD4-positive cells for HIV-1 infection studies we hoped to more closely mimic the in vivo situation and to include into our results possible effects of other immune competent cells.

We found that the susceptibility of PBMC to both viral strains was somewhat lower in chimpanzees as compared to humans. When accumulation of proviral HIV-1 DNA in infected cell cultures was studied over time, it was observed that both chimpanzee and human cells integrated similar amounts of HIV-1 DNA

shortly (20 h) after first contact with the virus. This indicates a similar initial susceptibility of cells of both species for HIV-1 infection. In human cells DNA copy numbers increased slightly at 48 h and rose to levels of up to more than 10 copies/cell at 72 h. This high DNA copy number is most likely due to accumulation of unintegrated HIV-1 DNA [32], similar high HIV-1 DNA copy numbers were also observed by Karageorgeous et al. [18]. In chimpanzees the increase in HIV-1 DNA following infection was somewhat lower. These data correlate well with results reported by Watanabe et al. [30], who also showed that HIV-1 strains IIIB, RF and MN did not infect chimpanzee PBMC as efficiently as human cells, and are in contrast to findings of Nara et al. [20], who found similar titers of HIV-1 IIIB and RF in human and chimpanzee PBMC. The contradictory results in these studies may be explained by different experimental setups or changes in pathogenicity of the respective viral isolates during cultivation.

Zack et al. [32] demonstrated that replicative viral intermediates may remain in PBMC in an unintegrated quiescent state for up to several days. As HIV-1-infected quiescent T cells have been shown to be an important reservoir of inducible HIV-1 [16], differences between the two species at this pre-integration stage of infection may contribute to the discordant course of HIV-1-infection in humans and chimpanzees. However, our experiments with HIV-1-infected quiescent cells of a number of chimpanzees and humans, activated at various times (up to 8 days) post infection showed no significant differences of HIV-1 production. This result makes it very unlikely that differences in pre-integration latency might play a role in this context.

At present it can not be completely ruled out that even a slightly lower support of chimpanzee PBMC for HIV-1 growth might contribute to the absence of AIDS-like disease in these animals (in this context it is interesting to note that in chimpanzees the percentage of circulating CD 8-positive cells is higher and the percentage of circulating CD 4-positive cells lower than in humans [17]). Recent studies in HIV-1-infected humans have clearly demonstrated a rapid in vivo turnover of plasma virions and infected cells [16, 22]. The progression of these individuals towards AIDS depends on a highly dynamic and delicately balanced process of viral replication and elimination of HIV-1 and infected cells by a potent immune system. It might be speculated that a somewhat lower susceptibility of chimpanzee PBMC for HIV-1 might, especially after several rounds of viral replication, lead to a lower viral load in these animals. This speculation might be supported by the significantly lower HIV-1 copy number found in this study after in vitro HIV-1 IIIB infection of chimpanzee PBMC. Interestingly, HIV-1-infected chimpanzees present with a viral load that is decreased by ten-fold or more as compared to HIV-1 seropositive humans [27] and low viral load has been shown to be associated with absent disease progression, as demonstrated in several studies investigating long-term nonprogressors [6, 28]. However, both human and chimpanzee PBMC did not behave differently with respect to viral uptake. Thus, it appears that restrictions at very early stages of the HIV-1 replicative cycle are not responsible for the absence of AIDS-like disease in chimpanzees.

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Authors' address: Dr. J. W. Mannhalter, IMMUNO AG, Industriestrasse 67, A-1221 Vienna, Austria.

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