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In Vitro Susceptibility to Infection With SIVcpz and HIV-1 Is Lower in Chimpanzee Than in Human Peripheral Blood Mononuclear Cells

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This study was undertaken to evaluate and compare the susceptibility of chimpanzee versus human peripheral blood mononuclear cells (PBMCs) to infection with SIVcpz and HIV-1 non-syncitium inducing primary isolates. The results demonstrate clearly that chimpanzee PBMCs have a lower capacity to support viral replication as compared to human PBMCs. There was no experimental evidence that this difference was due to a lower availability of target cells for viral infection (PBMCs positive for CD4 and CCR5 molecules) or to a differential susceptibility to apoptosis (PBMCs positive for CD4 and CD95 molecules). A lower capacity of chimpanzee PBMCs to support SIVcpz and HIV-1 replication in vitro is related to a post-entry barrier to virus replication. ***J. Med. Virol. 67:301–311, 2002.***

KEY WORDS: primate; susceptibility to infection; viral replication

INTRODUCTION

Immunologic and virologic evidence on HIV-1- and SIVcpz-infected chimpanzees suggests that viral factors are unlikely to be the cause for their long-term AIDS-free infection. There is no consistent evidence that vigorous specific humoral or cellular immune responses are responsible for delayed disease progression in infected chimpanzees [Peeters et al., 1995; Nyambi et al., 1997; Kestens et al., 1998; Ondoa et al., 2001]. In HIV-1-infected humans and the SIVmac-infected macaque model, it has been established that the post-acute viral replication set point has an important prognostic significance [Mellors et al., 1995, 1996; ten Haaft et al., 1998]. There are many observations suggesting that host factors different from specific immunity exert their effect during the early stages of infection and play a critical role in establishing both the initial and post-acute level of viral replication [Phillips et al., 1991; Daar et al., 1991; Clark et al., 1991]. A previous study has suggested that there might be a correlation between the intrinsic susceptibility of host target cells and the level of early in vivo viral replication [Lifson et al., 1997]. This may possibly explain why the natural hosts for SIV infections or some human long-term non-progressors are relatively refractory to disease development. Given this position, the decline of infectious virions during the post-acute phase of infection would be related to viral population dynamics rather than specific immune responses [Phillips, 1996]. It has been proposed that restriction in host cell susceptibility is related to: (1) determinants of viral entry, e.g., the availability of cells bearing the CD4 and CCR5 molecules (target cells) [Ashorn et al., 1990; Deng et al., 1996; Maddon et al., 1998], (2) determinants for anti-viral activity, e.g., the levels of β-chemokine secretion [Cocchi et al., 1995], or (3) susceptibility of the target cells to the cytopathic effect of the virus (e.g., syncitium formation or programmed cell death) [Heeney et al., 1993; Gougeon et al., 1997]. Others have reported that restriction of viral replication in chimpanzee cells occurs later in the replication cycle, compared to human cells, and is independent of cell entry [Pischinger et al., 1998]. In addition to a possible poor susceptibility of the chimpanzee target

All protocols are approved by the Ethical Review Boards of the Institute of Tropical Medicine and the BPRC and are implemented according to international guidelines.

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cells, viral factors such as the number of infectious virus particles in the inoculum per number of cells (multiplicity of infection) may also influence the outcome of infection. Previous findings suggest indeed that a large proportion of SIVcpz cell-free virions in the chimpanzees is non-infectious [Ondoa et al., 2001], which in part may help explain the low pathogenicity of SIVcpz in vivo.

The present study was undertaken to compare and evaluate the susceptibility of chimpanzee and human peripheral blood mononuclear cells (PBMCs) in vitro. Limiting dilution susceptibility culture assays on PBMCs from multiple chimpanzee and human donors were done using SIVcpz and HIV-1 primary isolates. Comparisons were made between the in vitro intrinsic susceptibility of the cells, the proportion of target cells positive for the virus receptors CD4 and CCR5, the marker for susceptibility to apoptosis CD95 [Jiang et al., 1997; Moretti et al., 2000] and the level of β-chemokine secretion. In order to assess the presence of entry or post-entry barriers to virus replication, the output of virus as measured by the $TCID_{50}$ was quantitated as a function of the number of provirus present in the PBMCs.

MATERIALS AND METHODS

Isolates

SIVcpz isolates. Stocks of SIVcpz primary isolates were prepared from freshly purified CD4+ lymphocytes isolated from a naturally infected chimpanzee as previously described [Peeters et al., 1989a]: SIVcpz-ant_{T4} and SIVcpz-ant and SIVcpz-gab strains isolated from PBMCs of infected chimpanzees [Peeters et al., 1989b; vanden Haesevelde et al., 1996] and passaged only twice through phytohemagglutinin (PHA)-stimulated human PBMCs: SIVcpz-ant_{PBMC}, SIVcpz-gab_{PBMC}.

HIV-1 isolates. Stocks of non-syncytium inducing (NSI) HIV-1 primary isolates were obtained directly from purified CD4+ lymphocytes isolated from a patient attending the Institute of Tropical Medicine of Antwerp (ITM) clinic ($VI2085_{T4}$, group M) and from CA20 and CA5 [Nkengasong et al., 1994], group M and ANT-70 group O [vanden Haesevelde et al., 1994] strains isolated from the PBMCs of infected patients and passaged only twice in PHA-stimulated human PBMCs.

The CD4+ T cells were purified by positive selection using immunomagnetic beads (Dynal, Oslo) as previously described [Ondoa et al., 2001] from 3 days-PHA-stimulated PBMCs. Virus was subsequently isolated from cultured CD4+ lymphocytes without any further addition of human or chimpanzee donor PBMCs.

PBMCs

Chimpanzee and human non-infected PBMCs were separated from heparin-treated venous blood over a Ficoll-Hypaque gradient. Five HIV and SIVcpz seronegative chimpanzees were included in the study: Ch-Ja, Ch-Ro, Ch-Re, Ch-Fr, Ch-Ch. Eight HIV seronegative human donors were included in the study: Hum-A, Hum-B, Hum-C, Hum-E, Hum-F, and Hum-G, Hum-01, and Hum-08. Freshly obtained PBMCs were used for the in vitro PBMC susceptibility assay. PBMCs were stored at −70°C and thawed for the in vitro virus infectivity assay.

Limiting Dilution Culture Assays for PBMC In Vitro Susceptibility Testing

One hundred and twenty microliters of virus supernatants were titrated on the same batches of fresh PBMCs from 4 uninfected chimpanzees (Ch-Ro, Ja, Re, and Fr) and 6 uninfected human donors (Hum-A, B, C, E, F, G). Briefly, freshly obtained PBMCs were stimulated with PHA for 3 days. Cells were then resuspended in RPMI 1640 medium supplemented with 15% heat inactivated fetal calf serum, 0.03% glutamine (all purchased from Gibco, UK), recombinant human interleukin-2 (IL-2, 10U/ml, R&D Systems, Minneapolis, MN) and antibiotics, and transferred to 96 well plates (7.5×10^4 cells per well). PBMCs of chimpanzees and humans were infected with virus supernatant diluted serially in 3 lots, each with 4 duplicates. PBMCs were incubated for 3 days in the presence of virus and washed 3 times to remove virus supernatant. At that time moment (day 3 post-infection), two of the lots were stopped, one was used for lymphocyte immunophenotyping whereas the 4 cultures at each viral dose of the second were pooled and stored at −20°C for further analysis. The third lot was maintained for 1 to 3 weeks. Approximately 60% of culture medium (125 μl) were replaced twice a week. Part of the culture supernatant was used for monitoring viral antigen release by an in-house antigen assay [Beirnaert et al., 1998] and calculating $TCID_{50}$ values by the Reed and Muench method [Reed and Muench, 1938]. The remaining supernatant was stored at −80°C for further analysis.

To examine whether CD8+ T cells affect the number of virions required to infect a culture, the virus were titrated on CD8+T-depleted PBMCs. CD8+T cells were removed from the PBMCs using magnetic beads (Dynal, Oslo), according to the manufacturer's instructions and as described previously [Kestens et al., 1995].

Limiting Dilution Culture Assay for Virus In Vitro Infectivity Testing

To compare SIVcpz and HIV-1 virus infectivity, the same batches of PBMCs from 2 chimpanzee (Ch-Re and Ch-Ch) and 2 human donors (Hum-01 and Hum-08) infected with 2 SIVcpz (SIVcpz-ant_{PBMC} and SIVcpz-gab_{PBMC}) and 3 HIV-1 isolates (CA5, CA20, and ANT-70) at equal multiplicities of infection (4×10^{-4} MOI). For this experiment, PBMCs had to be frozen and thawed in order to determine the volume of infectious dose to use for infecting the PBMCs and to

carry out the actual limiting dilution culture assay on the same batches of cells.

PBMCs of each donor were infected with logarithmic dilutions of equal MOI of each isolate in 2 series of quadruplicates. One series of quadruplicates was stopped after 3 days of exposure to the virus, the cells were washed, counted, pooled as a function of virus dilution, and stored as dry pellets at −20°C for further analysis. The second series of quadruplicates was maintained for 2 to 3 weeks to measure the virus capacity to infect the cells and then replicate. Viral antigen released in culture supernatant was monitored once a week with an antigen assay [Beirnaert et al., 1998]. $TCID_{50}$/ml were calculated with the Reed and Muench method [Reed and Muench, 1938] and represented virus infectivity.

Immunophenotyping of PBMC Cultures

In order to assess chimpanzee and human PBMC determinants for virus entry and for cell susceptibility to apoptosis, cell phenotyping was performed on fresh PBMCs and 72 hr after exogenous infection of PHA-stimulated PBMCs. The percentages of CCR5 and CD95 expressing CD4 positive memory cells were determined using a combination of CD4-peridinin chlorophyll protein (PerCP, Becton-Dickinson, Erembodegem, Belgium), CCR5-phycoerythin (PE, Pharmingen, San Diego, CA), or CD95-phycoerythin (PE Becton-Dickinson, Erembodegem, Belgium) and CD45RO-fluorescein isothiocyanate (FITC, DAKO, Glostrup, Denmark). Briefly, cell surface staining was performed by incubating 50 μL of PBMCs suspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA from Acros, London, UK), with 5 μl of each monoclonal antibody for 15 min at room temperature. The whole blood specimen was treated with 2 ml of lysing solution from Becton-Dickinson (Erembodegem, Belgium) for 10 min in the dark to lyse the red blood cells, centrifuged and washed with PBS 1% BSA. Finally, the cells were fixed with 1% paraformaldehyde in PBS and analyzed by FACScan (Becton Dickinson).

Quantification of β-Chemokines in Culture Supernatants

PBMC culture supernatants collected after 1 week and at 1-week intervals thereafter were stored at −80°C and thawed for the determination of "regulated on activation normal T expressed and secreted" (RANTES), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β concentrations by an ELISA technique (Quantikine EIA-kit, R&D System, Minneapolis, MN) according to the instructions of the manufacturer. A standard curve generated by serial dilution of a known amount of recombinant human RANTES, MIP1-α or MIP1-β (R&D System, Minneapolis, MN) was incorporated into each ELISA assay. Optical densities (OD) were used to calculate the concentration of released β-chemokines against the standard curve. Results are given in pg/ml.

HIV-1 Group M Quantitative DNA Load

PBMCs infected with $VI2085_{T4}$ and CA20 were washed, counted and pelleted. The number of viral DNA copies in infected PBMCs was determined using the Amplicor Quantitative HIV-1 DNA Monitor test (Roche, Branchburg, NJ). The test is an in vitro assay for the quantification of HIV-1 DNA in PBMCs and detects both integrated and circular intracellular viral DNA. The primers are specific for HIV-1 group M strains. The test has an analytical sensitivity of 10 input copies per amplification reaction with a 3-log_{10} detection range and uses HIV-1 viral DNA as quantification standard. It is a non-infectious DNA fragment that contains the identical primer binding site as the HIV-1 target and a unique probe binding region that allows standard amplicons to be distinguished from HIV-1 amplicons. The HIV-1 DNA standard is incorporated into a fixed amount of specimen cells at a known copy number and is co-amplified with the HIV-1 target. The HIV-1 DNA level in the specimen tested can be calculated by comparing the HIV-1 signal to the amplification standard signal for each specimen.

SIVcpz and HIV-1 Semi-Quantitative DNA Load

DNA from human and chimpanzee PBMCs infected with serial dilutions of virus ($SIVcpz\text{-}ant_{PBMC}$, $SIVcpz\text{-}gab_{PBMC}$, CA5, CA20, and ANT-70) at equal MOI was subjected to a semi-quantitative *pol* DNA PCR [Fransen et al., 1994]. This method was used to compare SIVcpz and HIV-1 isolates in the same test since the *pol* gene is relatively conserved between SIVcpz, HIV-1 group M and group O. Figure 1 depicts the position of the PCR internal and external *pol* primer sets on HIV-1 and SIVcpz genome. In a previous investigation, comparable quantitative amplification was reported of DNA derived from HIV-1 group M, group O, and group N isolates by the pol PCR (Nasako et al., personal communication). SIVcpz-ant and SIVcpz-gab genomes present less mismatches at the 5′ end as compared to group O ANT-70 and group N YBF30 strains. Therefore, it was assumed that DNA derived from SIVcpz isolates could be amplified by PCR as efficiently as HIV-1 isolates with our *pol* primers. Briefly, cultured PBMCs were pelleted, treated by proteinase K and screened for a positive PCR following the protocol described by Fransen and colleagues [Fransen et al., 1994]. Logarithmic dilution of the DNA from cells infected with the lowest dilution of virus giving a positive PCR signal were then subjected to a second PCR in 4 duplicates. The number of DNA molecules present in the diluted sample was calculated on the basis of the Reed and Muench cumulative method [Reed and Muench, 1938] and used to deduce the DNA copy number in the undiluted samples.

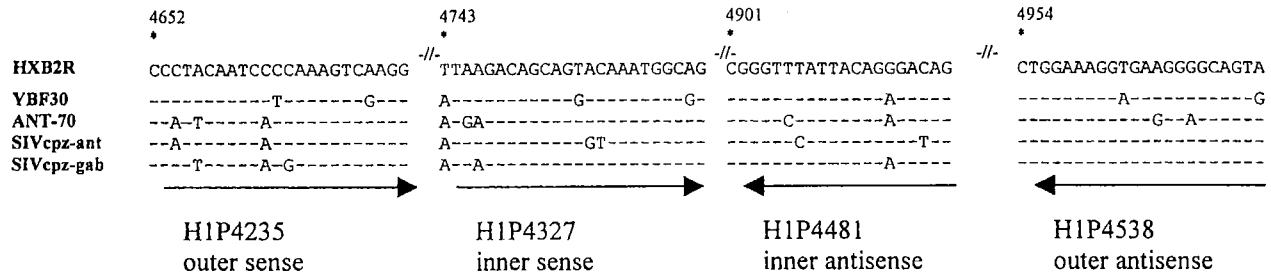

Fig. 1. Position of the outer and inner *pol* primer sets on HIV-1 and SIVcpz isolates genome. Sequence alignment of 3 HIV-1 [group M (HXB2R), N (YBF30), O (ANT-70)] and 2 SIVcpz (cpz ant and cpz gab) isolate *pol* gene and derived from the Los Alamos data base. Interruptions of the nucleotide sequences are indicated by (-//-). The asterisks above the alignment indicate nucleic acid positions on the viral genome. Only nucleotide differences between genomic sequences are shown. Similarities are indicated by (-). The arrows symbolize positions of the inner and outer *pol* primer sets on the viral genome.

Quantitation of Viral Antigen Release in Culture Supernatants

A standard curve generated with serial dilutions of HIV-1_{IIIB} (for the quantitation of HIV-1 antigen) or SIVcpz-ant (for the quantitation of SIVcpz antigen) cultured in human PBMCs was incorporated into each antigen assay. Optical densities were used to calculate concentrations of released SIVcpz or HIV-1-specific antigen against the standard curve. Results are given in $\log_{10}$ arbitrary units of viral antigen. The in-house antigen assay is prepared from polyclonal sera of HIV-infected patients [Beirnaert et al., 1998]. It is 4 to 8 times less sensitive than the commercial assay Vironostika HIV-1 Antigen Microelisa System (Organon Teknika, Boxtel, The Netherlands) in detecting p24 antigen in culture supernatant with the virus strains used in this study [Beirnaert et al., 1998].

Statistical Analysis

Differences between groups of means were tested for statistical significance with the Mann-Whitney U test. Correlation between two sets of data (e.g., correlation between $TCID_{50}$ and DNA copy number) was performed with the Spearman rank order correlation technique. The level of statistical significance was set at $P < 0.05$.

RESULTS

Susceptibility of Human and Chimpanzee PBMCs to Infection With SIVcpz and HIV-1

Differences in susceptibility of human and chimpanzee PBMCs to infection with HIV-1 and SIVcpz isolates are presented in Table I. Virus titers were highest at week 1 for SIVcpz isolates and comparable at weeks 1, 2, and 3 for HIV-1 isolates (data not shown). Therefore, we used data from week 1 for statistical analysis. $TCID_{50}$/ml were significantly lower in chimpanzee PBMCs [median of SIVcpz and HIV-1 $TCID_{50}$/ml at week 1 = $\log_{10}$ 0.52 ($\log_{10}$ −4.0* and $\log_{10}$ 2.0**)] as compared to human PBMCs [median = $\log_{10}$ 3.35 ($\log_{10}$ 2.0* and $\log_{10}$ 4.18**)] irrespective of the isolate ($P < 0.0001$). This indicated that for comparable virus inputs, chimpanzee PBMCs were less susceptible to infection than human PBMCs. SIVcpz-ant_{PBMC} and SIVcpz-gab had $TCID_{50}$/ml comparable to those of the 3 HIV-1 isolates in PBMCs irrespective of the species ($P = 0.275$). SIVcpz-ant_{T4} had significantly lower titers than HIV-1 isolates in chimpanzee and human PBMCs ($P = 0.0001$, data not shown). CD8+T depleted PBMCs of Ch-Re and Ch-Fr (less than 10% of remaining cells were CD8+, data not shown) had similar susceptibility to viral infection in vitro with the 3 SIVcpz and the 3 HIV-1 isolates as compared to unfractionated PBMCs of Ch-Ja, Ch-Ro, Ch-Re, and Ch-Fr ($P = 0.248$, lowest rows of Table I).

*25th percentile.

**75th percentile.

Lymphocyte Immunophenotyping

Chimpanzee PBMC counts varied from 0.3 to 1.1 × 10^6 and human PBMC count from 0.3 to 1.5 × 10^6 per 4 wells, 72 hr after incubation with the virus. The proportions of fresh PBMCs positive for the CD4 as well as PBMCs positive simultaneously for CD4/CD45RO/CCR5 or CD4/CD45RO/CD95 were comparable between humans and chimpanzees (Table II). In chimpanzees but not in humans, PHA stimulation followed by exogenous infection resulted in a significant reduction in the proportion of PBMCs positive simultaneously for CD4/C45RO/CCR5 molecules ($P = 0.029$, data not shown) and in a significant increase of PBMCs expressing simultaneously CD4/CD45RO/CD95 molecules ($P = 0.29$, data not shown). The percentage of PBMCs positive for the CD4 molecule remained comparable between chimpanzees and humans after exogenous infection ($P = 0.114$, data not shown). The percentage of PBMCs positive for CD4/CD45RO/CCR5 was significantly lower in chimpanzee than in human infected PBMCs ($P = 0.038$), whereas percentages of PHA-stimulated PBMCs positive for CD4/CD45RO/CCR5 and CD4/CD45RO/CD95 were comparable between the two species ($P = 0.114$ and 0.171, respectively).

TABLE I. In Vitro Susceptibility of Human and Chimpanzee PBMCs to 3 SIVcpz and 3 NSI HIV-1 Primary Isolates

		Infectious virus titers (in log10 of TCID50/ml)									
		SIVcpz			HIV-1			Median values			*P* values
PBMCs	Subjects	$SIVcpz_{-ant}$ (T4)	$SIVcpz_{-ant}$ (PBMC)	$SIVcpz_{-gab}$ (PBMC)	VI2085 (T4)	CA20(PBMC)	ANT-70 (PBMC)	Total SIVcpz[a] (n = 3)	Total HIV-1[b] (n = 6)	Total SIVcpz/ HIV-1[c] (n = 6)	SIVcpz vs HIV-1[h] (n = 3) vs (n = 3)
Chimpanzee PBMCs	Ch-Ja	*	0.52	2.22	*	2.52	2.02	0.52	2.02	1.73	0.658
	Ch-Ro	*	*	1.87	*	2.02	2.02	*	2.02	1.56	0.239
	Ch-Re	*	*	2.72	*	2.02	2.02	*	2.02	1.72	0.814
	Ch-Fr	*	*	2.72	*	2.02	2.02	*	2.02	1.72	0.812
Human PBMCs	Hum-A	*	2.22	4.32	4.75	4.19	4.19	2.22	4.19	4.19	0.268
	Hum-B	*	2.8	4.78	4.36	4.19	4.52	2.80	4.19	4.28	0.513
	Hum-C	*	0.52	3.27	2.86	3.19	3.19	0.52	3.19	3.05	0.507
	Hum-E	*	3.36	4.70	3.52	4.19	3.96	3.36	3.96	3.79	0.513
	Hum-F	*	1.99	3.98	3.02	5.00	4.19	1.99	4.19	3.72	0.127
	Hum-G	*	1.99	3.85	2.52	4.02	4.02	1.99	4.02	3.56	0.121
Chimpanzee CD8-depleted PBMCs	Ch-Re	*	*	1.87	*	*	2.02	*	*	*	0.796
	Ch-Fr	*	*	1.87	0.52	2.02	2.02	*	2.02	0.98	0.116
Median values	Total chimp PBMCs[d] (n = 4)	*	*	2.53	*	2.02	2.02	*	2.02	0.52	0.43
	Total human PBMCs[e] (n = 6)	*	2.12	4.18	3.34	4.19	4.02	2.12	4.02	3.35	**0.006**
	Total human + chimp PBMCs[f] (n = 10)	*	0.52	3.07	2.22	2.97	2.91	*	2.50	2.02	**0.004**
	Total chimp CD8-depleted[g] (n = 2)	*	*	1.87	−0.3	1.13	2.02	*	1.13	−0.38	0.17
P values	Human PBMCs vs chimp PBMCs[I] (n = 6) vs (n = 4)	1	**0.013**	**0.011**	**0.008**	**0.009**	**0.034**	**0.049**	**<0.0001**	**<0.0001**	
	Chimp PBMCs vs CD8-depleted[j] (n = 4) vs (n = 2)	1	0.800	**0.06**	0.157	**0.049**	0.200	0.616	0.216	0.248	

Each isolate was titrated in PBMCs of 4 chimpanzee (e.g., Ch-Ja) and 6 human (e.g., Hum-A) blood donors. Viruses were isolated either from unfractionated PBMCs (PBMC) or from purified CD4 + T cells (T4). Virus titers are expressed in $\log_{10}$ $TCID_{50}$/ml and have been calculated at weeks 1, 2, and 3 of culture. Only data of week 1 were used for statistical analyses. The minus signs (−) correspond to a total absence of viral antigen release (0 $TCID_{50}$/ml). *P* values are highlighted in grey.

Calculation of the median titers (at week 1):

[a]Median titers of the 3 SIVcpz isolates on PBMCs from each donor or group of donors e.g: median of 3 SIVcpz isolates for 1 Ch-Ja is calculated from 3 data sets (SIVcpz-ant(T4), SIVcpz-ant(PBMC), SIVcpz-gab). Median of the 3 SIVcpz isolates for the group of 4 chimpanzee (Ch-Ja, Ch-Ro, Ch-Re, Ch-Fr) donors is calculated from 12 data sets). (See 9th column).

[b]Median titers of the 3 HIV-1 isolates on PBMCs from each donor or group of donors.

[c]Median titers of the 3 SIVcpz and the 3 HIV-1 isolates (n = 6) on PBMCs from each donor or group of donors.

[d]Median titers of each isolate on PBMCs from the group of 4 chimpanzee donors.

[e]Median titers of each isolate on PBMCs from the group of 6 human donors.

[f]Median titers of each isolate on PBMCs from 4 chimpanzee and 6 human (n = 10) donors. e.g: median titers of SIVcpz-ant(PBMC) are calculated on 10 data sets. (See 18th row).

[g]Median titers of each isolate on CD8-depleted PBMCs from the group of 2 chimpanzee donors.

Calculation of the *P* values (calculated on data from week 1):

[h]Comparison of mean rank values between SIVcpz and HIV-1 titers for each donor or group of donors. e.g: *P* value for the difference between titers of the SIVcpz (n = 3) and HIV-1 (n = 3) isolates in Ch-Ja has been calculated on 6 data sets. (See the last grey column).

[i]Comparison of mean rank values of each isolate's titers between human and chimpanzee donors. e.g: *P* value for the difference of SIVcpz-ant (PBMCs) titers between human (n = 6) and chimpanzee (n = 4) donors has been calculated on 10 data sets. (See the last but one grey row).

[j]Comparison of mean rank values of each isolate titers between unfractionated (n = 4) and CD8-depleted (n = 2) PBMCs chimpanzee donors.

Significant *P* values (< 0.05) are indicated in bold.

TABLE II. Percentages of PBMCs Positive for CD4, and Simultaneously Positive for CD4/CD45RO/CCR5 and CD4/CD45RO/CD95 From Chimpanzee and Human Donors*

	CD4 (%)		CD4/CD45RO/CCR5 (%)		CD4/CD45RO/CD95 (%)	
Subjects	Fresh	Infected	Fresh	Infected	Fresh	Infected
Chimpanzee PBMCs						
Ch-Ja	32.5	11.84	31.6	9.97	24.48	92.07
Ch-Ro	32.9	25.96	18.1	2.59	13.17	97.18
Ch-Fr	35.8	21.01	40.1	4.075	65.2	68.098
Ch-Re	33.7	34.11	20.97	3.45	52.03	73.325
Medians chimpanzee PBMCs						
Median	33.3	23.48	26.28	3.7	38.25	82.69
25th percentile	32.6	14.13	18.81	2.8	15.99	69.4
75th percentile	35.27	32.07	37.97	8.49	61.9	95.9
Human PBMCs						
Hum-A	47.9	40.87	24.1	8.2	54.89	83.91
Hum-B	33	34.92	21.8	14.4	54.18	56.6
Hum-C	57	74.93	0.3	6.81	17.09	44.82
Hum-E	33.8	45.9	27	18.5	73.02	70.43
Hum-F	57.39	20.16	10.24	10.9	47.78	91.47
Hum-G	55.18	28.14	8.18	46.49	55.49	43.09
Median human PBMCs						
Median	51.54	37.89	16.02	12.64	54.53	63.51
25th percentile	33.6	26.14	6.21	7.84	40.1	44.38
75th percentile	7.09	5.15	4.82	2549	59.7	85.79
P values						
P (chimpanzees vs. humans)	0.67	0.114	0.257	0.038	0.476	0.171

*Lymphocytes immunophenotyping has been performed on fresh and on PHA-stimulated PBMCs, 72 hr after exogenous infection with SIVcpz and HIV-1 strains (infected PBMCs). For infected PBMCs, percentages of cells for each subject represent the median values of 6 data sets corresponding to different exogenous infection with 6 strains of virus per donor PBMCs. *P* values were obtained from the analysis of mean rank values of lymphocyte subset percentages between chimpanzees (n = 4) and humans (n = 6).

The level of fluorescence for each cell surface marker used in this study was comparable between chimpanzee and human PBMCs (Fig. 2).

Levels of RANTES, MIP1-α, and MIP1-β in Culture Supernatants

PBMC culture supernatants harvested at week 1 were used for the determination of MIP1-α, MIP1-β, and RANTES concentrations (in pg/ml). The level of β-chemokines released into the culture supernatants of infected PBMCs did not differ significantly from that of non-infected human and chimpanzee control PBMCs as illustrated in Figure 3. MIP1-α and RANTES but not MIP1-β concentration tended to increase as a function of virus input. Secretion of β-chemokines tended to be higher in human as compared to chimpanzee PBMCs. The differences were significant for MIP1-α and for RANTES.

Determination of Proviral HIV-1 DNA Loads in PBMC

Determination of viral DNA load in PBMCs was limited to $VI2085_{T4}$ and CA20 infected cells since the commercial Roche DNA quantitation assay primers can only amplify viral genomic fragments conserved within HIV-1 group M. All the DNA VL results were within the detection range. For the same volume of CA20 input virus, the number of DNA copies/million PBMCs was comparable in chimpanzee and human PBMCs, 72 hr of incubation with the virus (see Fig. 4). However, when $VI2085_{T4}$ was used, the number of intracellular DNA copies/million PBMCs was significantly lower in chimpanzee than in human PBMCs ($P = 0.011$ for virus dilution = 1). There was a significant correlation between the number of intracellular viral DNA copies/million cells and the $TCID_{50}$/ml in human PBMCs (Spearman rank order correlation: $R = 0.609$, $P = 0.035$, data not shown). The ratio of $TCID_{50}$/DNA copy per culture well was significantly lower in chimpanzee as compared to human cells for VI2085 [median = $\log_{10}$ −7.09 ($\log_{10}$ −7.14* and $\log_{10}$ −6.80**); for chimpanzees; median = $\log_{10}$ −1.63 ($\log_{10}$ −2.03* and $\log_{10}$ −1.27**) for humans with $P = 0.011$] and for CA20 [median = $\log_{10}$ −3.79 ($\log_{10}$ −3.86* and $\log_{10}$ −3.72**) for chimpanzees; median = $\log_{10}$ −1.98 ($\log_{10}$ −2.32* and $\log_{10}$ −1.41**) for humans with $P = 0.011$]. The ratio of viral antigen units released into culture SN per DNA copy number per well was also significantly lower in chimpanzee as compared to human PBMCs, irrespective of the isolate [median = $\log_{10}$ 0.12 ($\log_{10}$ −0.35* and $\log_{10}$ 0.29**, respectively) for chimpanzees; median = $\log_{10}$ −2.07 ($\log_{10}$ 1.4* and $\log_{10}$ 2.98**) for humans with $P < 0.0001$]. These results suggest that a blockage of virus replication in chimpanzee cells occurs after virus entry (see Fig. 4).

Quantitation of Proviral DNA by Semi-Quantitative Pol PCR

Analysis of data from PBMCs infected with virus at 4×10^{-4} MOI indicated that for comparable infectious

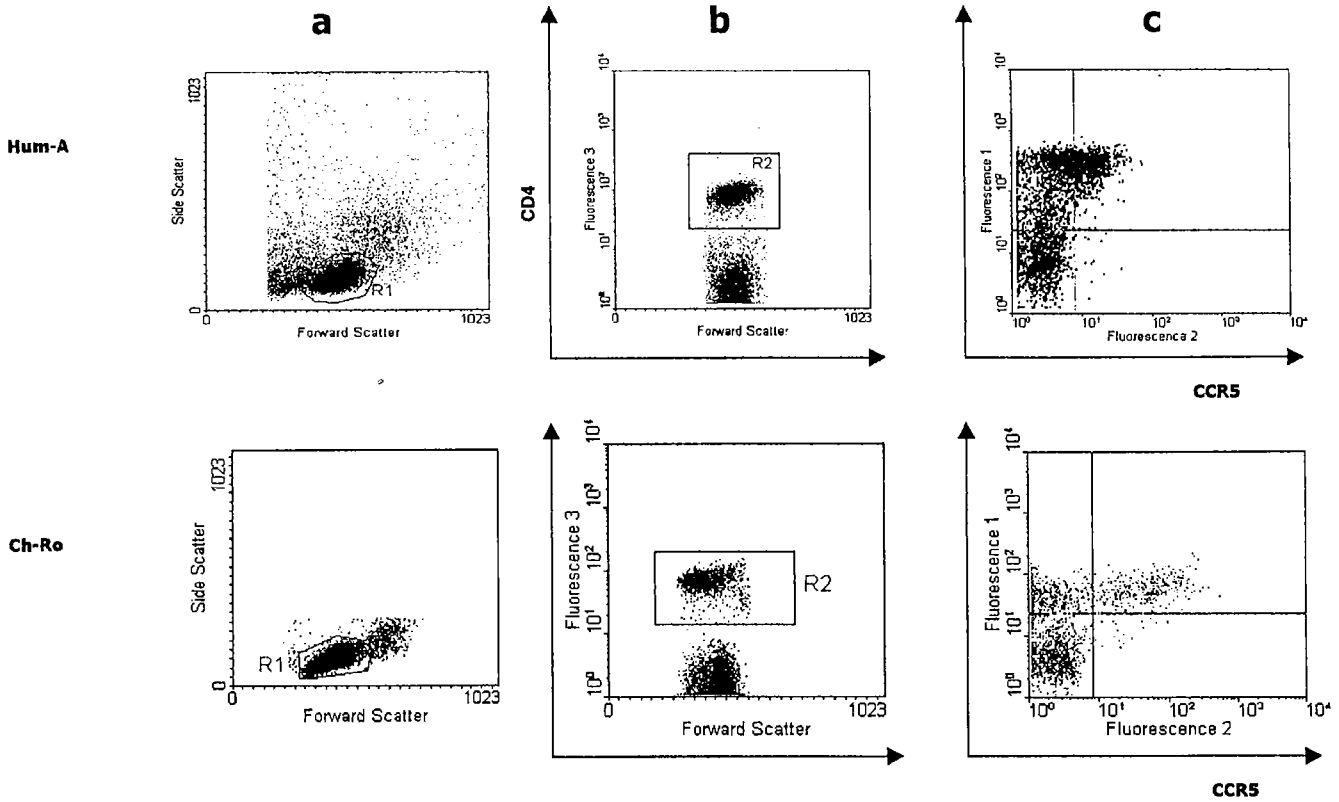

Fig. 2. Representative experiment of immunophenotyping by flow cytometry analysis of PBMCs (Hum-A). PHA-stimulated PBMCs were infected with SIVcpz-ant and collected after 72 hr of exposition to the virus. PBMCs were stained with a triple combination of monoclonal antibodies (CD4-PerCP, CD45R0-FITC, and CCR5-PE). Flow cytometry analysis was performed immediately. Approximately 5,000 cells were gated and analyzed for each sample. **a:** Dot plots of PBMCs as defined by forward (FS) and side (SS) scattering. Viable lymphocytes are gated according to their size(R1). **b:** Dot plots of lymphocytes as defined by fluorescence 3 (CD4-PerCP) and FS. Lymphocytes positive for CD4 are gated in R2 (45.77% of lymphocytes); **c:** CD4 positive lymphocytes as gated in b were analyzed for fluorescence 1 and 2 signal. Lymphocytes that triple express CD45RO, CCR5, and CD4 are plotted in the upper right quadrant (22.17%).

virus inputs, SIVcpz tended to accumulate less DNA than HIV-1 in PBMCs from chimpanzees [for SIVcpz: medians (per million of PBMCs) $=\log_{10}$ 1.97 ($\log_{10}$ 1.81* and $\log_{10}$ 3.07**); for HIV-1: median $=\log_{10}$ 3.00 ($\log_{10}$ 2.30* and $\log_{10}$ 6.00**) with $P=0.134$]. In PBMCs from humans [for SIVcpz: median $=\log_{10}$ 1.45 ($\log_{10}$ 0.30* and $\log_{10}$ 1.92**); for HIV1: median $=\log_{10}$ 2.35 ($\log_{10}$ 1.89* and $\log_{10}$ 2.70**) with $P=0.054$] although the differences were not significant (data not shown).

DISCUSSION

The results obtained in the present study demonstrate clearly that chimpanzee PBMCs differ significantly from human PBMCs in their capacity to support virus replication of both HIV-1 and SIVcpz strains. This important observation may itself explain why chimpanzees are relatively refractory to disease progression. Extrapolation of the data to the in vivo situation suggests that the lower susceptibility of chimpanzee species to infection is likely to affect the level of early viral replication, which is predictive of the post-seroconversion set point plasma viremia level and disease progression [Lifson et al., 1997].

Notably, the data suggest that the relative susceptibility of chimpanzee cells to support in vitro virus replication is determined more by the CD4 positive lymphocytes (target cells) rather than by possible antiviral activity of the CD8 positive lymphocytes. Neither the presence of CD8+T in PBMCs nor the level of β-chemokine secretion correlated with the restriction of in vitro virus replication. These findings are supported by several lines of evidence [Mackewicz et al., 1997; Kakkanaiah et al., 1998; Ondoa et al., 2001]. Indeed, the results of the present study do not show any consistent correlation between lower in vitro susceptibility of PBMCs to infection and a lower constitutive availability of T cells bearing the two main surface determinants for cell entry of NSI viruses, the CD4 and CCR5 receptors [Deng et al., 1996; Maddon et al., 1998]. The CD4 gene of humans and chimpanzees shares 98% homology [Fomsgaard, 1992] and SIVcpz and HIV-1 NSI primary isolates can both use chimpanzee or human CCR5 for cell entry with equal efficiency [Prétet et al., 1997]. None of the PBMC donors included in the study were homozygous for the Δ32 CCR5 deletion phenotype associated with resistance to infection [Samson et al., 1996; Liu et al., 1996] as confirmed by cell surface expression of CCR5 in every subject

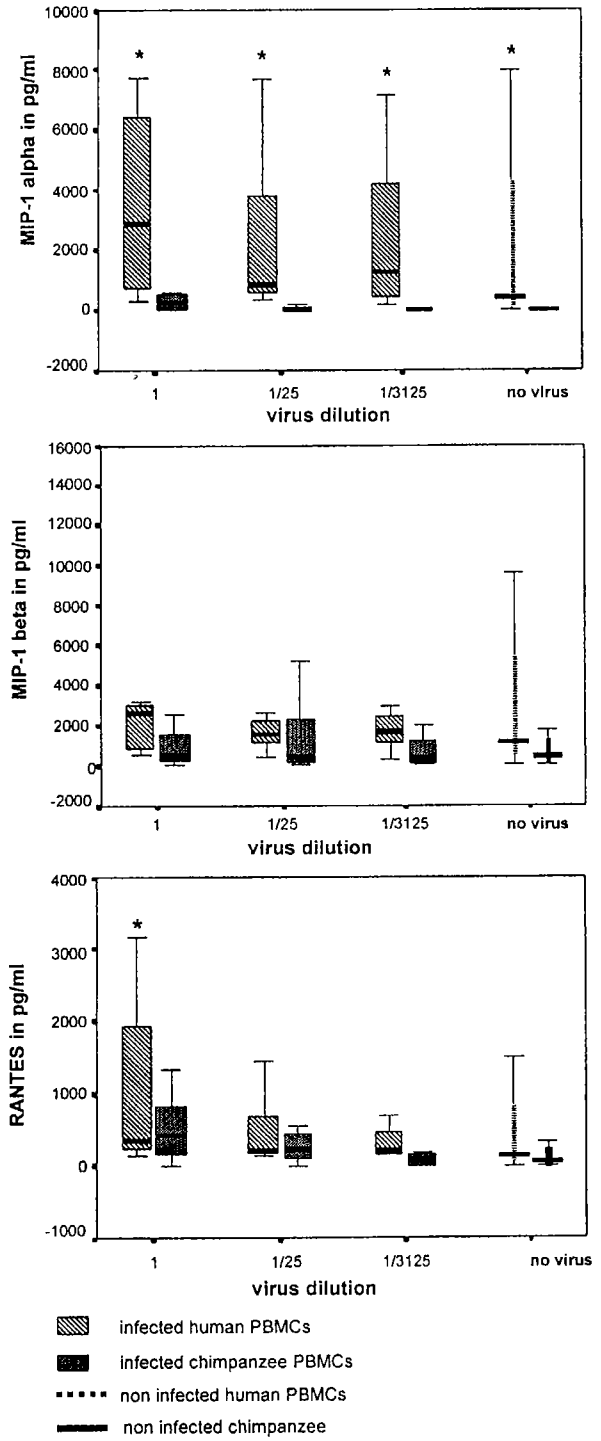

Fig. 3. β-chemokine concentrations in culture supernatant of infected PBMCs from human and chimpanzee blood donors. Levels of β-chemokines were measured in culture supernatants of PBMCs from humans (n = 6) and chimpanzees (n = 4) infected with serial dilutions of HIV-1 (n = 3) and SIVcpz (n = 3) isolates as well as in non-infected control PBMCs of each subject. Dosage of MIP-1α, MIP-1β, and RANTES was determined upon culture medium exchange at week 1. Levels of β-chemokine secretion were comparable between HIV-1- and SIVcpz-infected PBMCs irrespective of the species (data not shown). The boxes include the median, 25th and 75th percentiles, and the whisker caps represent the maximum and minimum values. A star (*) symbolizes significant differences between chimpanzee and human PBMCs.

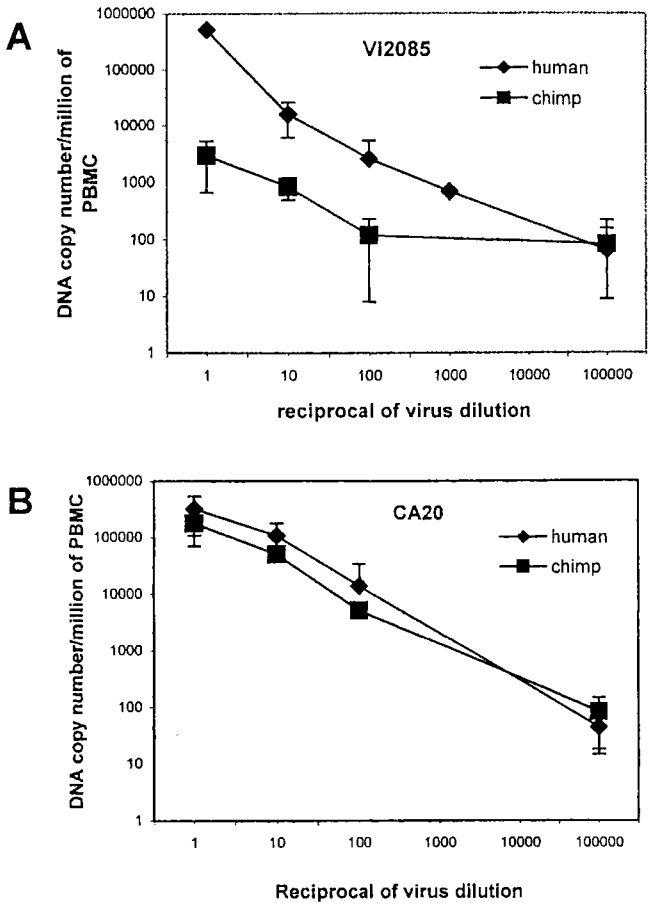

Fig. 4. Number of accumulated viral DNA copy per million PBMCs from human and chimpanzee blood donors 72 hr after exogenous infection. Proviral DNA loads were measured in PBMCs infected with serial dilutions of VI2085 (**A**) and CA20 (**B**). The values are plotted on a logarithmic scale and represent mean values for the 4 chimpanzees and the 6 human donors. Proviral load for SIVcpz and the HIV-1 goup O (ANT-70) strains could not be measured with the commercial Roche test. The amount of proviral DNA accumulated in PBMCs was equal (CA20) or lower (VI2085, $P = 0.011$ for virus dilution = 1) in chimpanzee PBMCs as compared to human PBMCs.

[Siciliano et al., 1999]. Furthermore, PBMCs from chimpanzees and humans have comparable intensities of CD4 and CCR5 expression at the cell surface. Therefore, availability of target cells for viral infection was quantitatively and qualitatively comparable for both chimpanzees and humans. Our data are consistent with the idea that relative resistance to infection of chimpanzee PBMCs does not depend on low availability of target cells expressing the appropriate virus co-receptor. There was no correlation between the degree of target cell permissiveness to infection and their susceptibility to apoptosis.

Quantitation of proviral DNA load in the PBMC after 72 hr of exposure to the virus indicated that chimpanzee and human PBMCs had a comparable permissiveness to infection with CA20. Whether proviral DNA is integrated in the genome or remains in a circular form has yet to be determined. The results

indicate that although chimpanzee and human PBMCs differ significantly in their susceptibility to infection with CA20 (2 $\log_{10}$ difference in $TCID_{50}$/ml), this is not related to a lower capacity of the chimpanzee PBMCs to accumulate viral DNA as compared to human PBMCs. Lower proviral DNA of $VI2085_{T4}$ in chimpanzee as compared to human PBMCs, might be related to a less efficient viral uptake or impairment of the replication cycle in chimpanzees. Comparison of equal amounts of infectious virus particles of SIVcpz and HIV-1 indicated that SIVcpz tended to have a lower capacity to infect PBMCs from humans and chimpanzees than HIV-1 isolates. The difference, however, was not significant. In addition to species-related cellular factors, viral determinants may, therefore, also participate in the poor susceptibility of chimpanzee PBMCs to infection.

There is a significantly lower production of infectious virus ($TCID_{50}$/ml) associated with a lower production of viral antigen per viral DNA copy in PBMCs of chimpanzees. This suggests a less permissive expression from proviral DNA following virus entry. Zack et al. [1988, 1990] demonstrated previously that replicative viral intermediates may persist in human PBMCs in an unintegrated quiescent state for several days They proposed that differences between the two species at the pre-integration level might contribute to the discordant course of infection in HIV-1 infected chimpanzees and humans. Conversely, Pischinger et al. [1998] reported that differences at the pre-integration level were very unlikely to play a role in this context. Whichever step(s) of the viral replication are restricted in chimpanzees, this blockage of virus replication likely results in a decline of virus production, compatible with the control of infection and the absence of disease progression in chimpanzees.

It has been shown that the activation state of the host cell represents an important determinant that can influence the efficiency of infection by HIV-1 [Zack et al., 1988; Stevenson et al., 1990]. An optimal degree of T-cell activation is required for completion of HIV-1 reverse transcription and migration of the preintegration complex into the nucleus [Korin and Zack, 1998]. In another study, it was shown that in contrast to HIV-1 infected humans, productive SIVcpz infection of 2 chimpanzees was not associated with an increased level of cellular immune activation [Kestens et al., 1998]. These observations raise the hypothesis that low cellular immune activation of the chimpanzee target cells upon in vivo infection leads to the production of non-fully infectious virus as a result of blockage of the reverse transcription process. Production of defective virus particles could explain by itself the discrepancy between the relatively high number of cell-free virions and the low infectious virus titers observed in SIVcpz-infected chimpanzees [Ondoa et al., 2001].

There is no evidence that in culture conditions, PHA followed by IL-2 treatment is less efficient for PBMCs from chimpanzees than from humans. The results of various previous experiments using PHA stimulation, indicate that chimpanzees peripheral blood lymphocytes proliferate, express markers for maturation, and perform specific effector activities in a way comparable to human peripheral lymphocytes [Kestens et al., 1995; Kestens et al., personal observation; Ondoa et al., 2001]. Whether some discrete steps of the chimpanzee cell cycle that are necessary for efficient virus replication are inherently lacking or are not optimally completed under PHA stimulation, remains to be determined.

Although HIV and SIV replication occur mainly in activated cells in the lymph nodes and not in quiescent PBMCs, the observations presented here are of importance. In chronically infected chimpanzees, failure of SIVcpz to induce overt immune activation of peripheral blood lymphocytes [Kestens et al., 1998] is associated with low levels of virus-producing cells in lymph nodes as well as absence of virus trapping in the germinal centers [Koopman et al., 1999].

In conclusion, the data show a significantly lower permissiveness of chimpanzee PBMCs to in vitro infection with both HIV-1 and SIVcpz as compared to human PBMCs. This lower susceptibility is dependent on CD4+ T cells and is not related to reduced levels of viral receptors on the CD4+ T cells. The site of post entry barrier(s) to viral replication in chimpanzees remains to be determined. It may involve absence of promoting [Bogerd et al., 1995; Wei et al., 1998; Stutz et al., 1995] or presence of inhibitory [Simon and Malin, 1996; Madani and Kabat, 1998] species-specific cellular factors. Identification of cellular determinant(s) that may restrict virus replication is relevant for elucidating the cause of the relative resistance to disease in chimpanzees.

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Studies on chimpanzees were approved by the Ethics Committee of the Center.

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