

Inhibition of replication of primary HIV-1 isolates in huPBL-NOD/Scid mice by antibodies from HIV-1 infected patients

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Abstract

Although a limited number of HIV-infected patients have broadly neutralizing antibodies, it has not been examined whether these antibodies can protect against infection with primary virus *in vivo*. Here we screened the plasma of 23 HIV-1-infected patients for broadly neutralizing antibodies. Purified antibodies from subjects with broad and more narrow responses were administered to huPBL-NOD/Scid mice that were subsequently challenged with primary viruses of clade A, B and CRF01_AE. Although we observed a lack of correlation between the data from the *in vitro* neutralization assay and the results from the passive immunization experiments, we report for the first time that antibodies from HIV-infected persons can inhibit replication of primary virus isolates in an animal model.

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1. Introduction

The development of an HIV vaccine is probably one of the most difficult challenges confronting biomedical research today. To accelerate the global effort to develop a safe and effective HIV vaccine, an international group of scientists proposed the creation of a Global HIV Vaccine Enterprise in June 2003 (Ho, 2005; Klausner et al., 2003). This strategic plan identifies a number of unanswered scientific questions along the critical path for vaccine discovery. A successful HIV vaccine needs to induce potent, broadly reactive neutralizing antibodies as well as T cell immune responses against HIV strains circulating in different parts of the world. There has, however, been limited progress towards the development of immunogens able

to induce such antibodies. Success will likely require a deeper understanding of the interactions between the HIV envelope and neutralizing antibodies. To achieve this goal, numerous well-characterized, broadly neutralizing monoclonal antibodies are needed.

So far, a few rare human monoclonal antibodies have been isolated that neutralize primary HIV-1 isolates from different genetic subtypes *in vitro*. The first broadly neutralizing monoclonal antibody identified was IgG1b12 (Burton et al., 1994). This antibody is directed against the CD4 binding site on gp120 (Barbas et al., 1993; Roben et al., 1994; Saphire et al., 2001). A second monoclonal antibody 2G12 (Trkola et al., 1995, 1996) recognizes a cluster of oligomannose residues on gp120 through a unique antibody structure (Calarese et al., 2003; Sanders et al., 2002; Scanlan et al., 2002). The antibodies 2F5 (Conley et al., 1994; D'Souza et al., 1997; Trkola et al., 1995; Zwick et al., 2001) and 4E10 (Zwick et al., 2001) recognize epitopes on the membrane-proximal region of the gp41 ectodomain (Muster et al., 1993; Stiegler et al., 2001; Zwick et al., 2001). Recently, it has been suggested that 2F5 and 4E10 are polyspecific

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auto-antibodies, which may explain why these types of antibodies are induced rarely during infection or by vaccination (Haynes et al., 2005). The monoclonal antibody IgG1b12 was isolated from the bone marrow of an asymptomatic patient that had been HIV positive for 6 years (Burton et al., 1991). The monoclonal antibodies 2G12, 2F5 and 4E10 were isolated from HIV-positive volunteers in CDC stage II or III with CD4 cell counts higher than $500 \mu\text{l}^{-1}$ and with high anti-HIV antibody titers (Buchacher et al., 1994).

It has been estimated that approximately 10% of HIV-1 infected patients can neutralize primary HIV-1 isolates from different clades *in vitro* (Beirnaert et al., 2000; Donners et al., 2002). Frequently, these patients are long-term non-progressors and/or long-term survivors (Cao et al., 1995; Cecilia et al., 1999; Pilgrim et al., 1997). To increase the number of neutralizing monoclonal antibodies, several research groups have tried to isolate such antibodies from bone marrow or peripheral B lymphocytes derived from patients whose plasma display broadly neutralizing activity against primary viruses. However, whether the polyclonal antibodies from such patients might inhibit replication of primary HIV viruses in animal models has never been investigated.

Here, we selected asymptomatic patients from the HIV outpatient clinic currently not on antiretroviral therapy. The neutralizing activity of their plasma was characterized in a highly sensitive GHOST cell-based assay against primary isolates of clades A, B, C, D and CRF01_AE (<http://hiv-web.lanl.gov/content/hiv-db/CRFs/CRFs.html>).

To assess the *in vivo* protective ability of antibodies, purified IgG from subjects with broad and more narrow responses were administered to huPBL-NOD/Scid mice which were subsequently challenged with primary viruses of clade B, A and CRF01_AE.

2. Material and methods

2.1. Human samples

Pooled plasma samples from healthy seronegative (SN) volunteers were kept frozen at -80°C and served as a negative control for *in vitro* and *in vivo* assays. Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats of healthy blood donors (Belgian Red Cross) by Ficoll–Hypaque centrifugation (density 1.077 g/ml) (Nycomed Pharma) and stored in liquid nitrogen. Aliquots of these PBMC were used to produce the chimeric mice. HIV-1-infected subjects (CV) were recruited at the HIV outpatient clinic of the Ghent University Hospital. Plasma and PBMC were prepared from EDTA-anti-coagulated blood. The human immunoglobulin G (IgG) plasma concentration was determined using an in house ELISA as described previously (Tournoy et al., 2000). The subtype of the viral strain was determined by phylogenetic analysis of the protease and reverse transcriptase gene and/or the viral envelope (V1–V3) gene with group M reference sequences. Informed consent was obtained from all participants and the study protocol was approved by the ethical committee of the Ghent University Hospital, Belgium (2002/109).

2.2. HuPBL-NOD/Scid mice

NOD/LtSz-Prkdcscid/Prkdcscid (NOD/Scid) mice were bred and maintained under specific pathogen-free conditions. The mice were housed in individually ventilated cages and fed with autoclaved food and water. Mice were used between 8 and 12 weeks of age. The study was approved by the animal ethical committee of the Faculty of Medicine, University Ghent, Belgium (ECP 01/16). To generate chimeric animals, NOD/Scid mice were conditioned by the injection of $0.5 \text{ mg TM}\beta 1$, a rat monoclonal antibody directed against the murine IL-2 receptor β -chain (Tanaka et al., 1993), and total body gamma irradiation (1 Gy) generated by a linear accelerator. Twenty-four hours later, 1×10^7 PBMC from healthy donors were transferred into the peritoneum (*i.p.*). To confirm successful engraftment, mouse plasma was analyzed for increasing human IgG as described (Tournoy et al., 2000).

2.3. Virus stocks

Virus working stocks were prepared by thawing a frozen aliquot of virus culture supernatant. This was used to infect 5×10^6 phytohemagglutinin (PHA)-stimulated human PBMC from a seronegative donor. Fresh PBMC and fresh medium with recombinant interleukin-2 (IL-2) (20 units/ml) (Roche) were added twice a week. Virus growth was monitored with an in house HIV-antigen capture ELISA as described previously (Beirnaert et al., 1998). All *in vitro* and *in vivo* experiments were performed with primary virus isolates. VI 1031, VI 829, VI 882, VI 693, VI 656, VI 1888 and CA10 were isolated at the Institute of Tropical Medicine (ITM), Antwerp from subjects either attending clinics at ITM (VI) (Beirnaert et al., 1998; Louwagie et al., 1993) or Cameroon (Nkengasong et al., 1994). The viruses 93US143 (original donor M. Robb) and 92US077 (original donor J. Sullivan) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. SF162 (Original donor J. Levy) and 92UG037 (originally donated by The UNAIDS Network for HIV isolation and characterization, and the DAIDS, NIAID) were obtained from the Centralised Facility for AIDS Reagents, the European Programme EVA, NIBSC. MN (original donor R. Gallo) was obtained from S. Zolla-Pazner and W61D (original donor H. Schuitemaker) was obtained from GlaxoSmithKline (Rixensart, Belgium).

2.4. Purification of polyclonal immunoglobulins

Immunoglobulins were purified from heat-inactivated plasma with a HiTrapTM Protein G column (Amersham Biosciences). After washing the column with phosphate-buffered saline (PBS), 1/10 diluted samples were added to the column and the run-through fractions were reloaded twice. After washing, the columns were pre-eluted with $0.1 \text{ M glycine HCl pH } 3.6$ to remove possibly antibody-bound antigens. Finally, IgG fractions were eluted with $0.1 \text{ M glycine HCl pH } 2.6$ and neutralized with $1 \text{ M Tris-HCl pH } 8.0$. The eluting buffer was exchanged to PBS and the purified immunoglobulins were concentrated

using Ultrafree-15 centrifugal filter devices (Biomax-50 membrane) (Millipore). The IgG content was measured as described (Tournoy et al., 2000).

2.5. *In vitro* neutralization assay

Human plasma and purified immunoglobulins were screened for neutralizing activity with a highly sensitive GHOST cell-based assay (Donners et al., 2003). These cells are derived from human osteosarcoma cells and are transfected with the gene coding for human CD4, one of the HIV co-receptors (CCR5 or CXCR4) and green fluorescent protein under the control of the HIV-2 LTR promoter. The number of infected cells was measured by FACS. Plasma samples were diluted 1/20 and purified IgGs to a concentration of 500 µg/ml. The format of the neutralization assay was 24/24/48 where 24/*x*/*x* is the time in hours during which antibody and virus were pre-incubated, *x*/24/*x* is the time in hours during which cells were exposed to these mixtures and *x*/*x*/48 is the time in hours between the start of viral inoculation and the FACS analysis. The percentage neutralization was calculated as $100 - [(\# \text{ infected cells of tested sample} / \# \text{ infected cells of seronegative control}) \times 100]$.

2.6. *Passive immunization in mice*

To assess virus-inhibiting activity *in vivo*, human polyclonal immunoglobulins were administered to huPBL-NOD/Scid mice 6 days after reconstitution and 1 day before viral challenge. All injections were given intraperitoneally (i.p.). Each experimen-

tal group consisted of four mice. The minimal viral inoculum needed to infect all mice was determined in preliminary titrations. The chimeric mice that survived the graft-versus-host reaction (82%) were sacrificed 14 days after challenge and viral load was measured in their plasma using COBAS Amplicor HIV-1 Monitor™ version 1.5 (Roche) according manufacturer's instructions. Due to the limited availability of mouse plasma, these were diluted 1/100 and therefore the lower limit of detection of this assay was 3.70 log gequiv./ml.

3. Results

3.1. *Description of the study population*

Twenty-three HIV-1 infected subjects (CV1 through CV23) were selected over a period of 10 months (2002–2003) from the 550 patients visiting the HIV outpatient clinic at the Ghent University Hospital (Fig. 1). The criteria used for selection were the presence of a good clinical condition and the absence of antiretroviral therapy at the moment of blood sampling. Seven of the 23 subjects received such treatment in the past, but always more than 10 months before current blood sampling. For three of these subjects (CV6, CV10 and CV16), therapy was limited to a very short period (3 weeks to 6 months). For two subjects (CV3 and CV17) who were diagnosed with HIV more recently, the seroconversion date was unclear and therefore the time since infection unknown. As expected from the selection criteria, none of the subjects had a CD4 count below 200 cells/µl and all subjects presented with low to intermediate viral levels (range <1.70–4.81 log gequiv./ml).

	age	sex	ethnicity	transmission	time since HIV diagnosis	ART in the past	CD4 count	viral load	viral subtype
subject	years				years	>10 months ago	# / µl	log geq / ml	
CV1	44	M	caucasian	MSM	10	+	346	4.81	B
CV2	46	M	caucasian	MSM	18	-	311	4.52	B
CV3	37	M	caucasian	MSM	0.42	-	424	4.34	B
CV4	42	M	caucasian	MSM	5	+	319	4.31	B
CV5	31	F	african	HE	5	+	490	4.39	CRF02_AG
CV6	44	M	caucasian	HE	10	+	518	4.24	B
CV7	24	F	caucasian	HE	2	-	1,090	3.92	B
CV8	44	M	caucasian	MSM	15	-	345	3.92	B
CV9	58	M	caucasian	MSM	6	+	697	3.93	B
CV10	30	F	african	?	4	+	387	4.19	G
CV11	39	F	caucasian	HE	15	-	370	3.15	C
CV12	38	M	hispanic	MSM	14	-	557	3.79	B
CV13	54	F	caucasian	HE	5	-	542	3.27	NA
CV14	34	M	caucasian	MSM	5	-	580	4.10	NA
CV15	54	F	caucasian	HE	7	-	525	3.81	NA
CV16	74	F	caucasian	HE	4	+	663	<1.70	NA
CV17	44	M	caucasian	HE	0.75	-	1,070	1.80	NA
CV18	26	M	caucasian	?	3	-	336	4.63	B
CV19	40	F	caucasian	HE	3	-	1,040	2.57	CRF02_AG
CV20	37	F	caucasian	HE	7	-	496	4.11	A
CV21	34	F	caucasian	HE	7	-	469	3.62	NA
CV22	47	M	caucasian	?	3	-	278	4.54	CRF01_AE
CV23	27	F	african	HE	2	-	683	2.66	CRF02_AG

M male; F female; MSM men who have sex with men; HE heterosexual transmission; ? unknown; ART antiretroviral therapy; + yes; - no; NA not available

Fig. 1. Description of the study population.

subject	HIV-1 viral isolates												NEUT mean	CLADES #	PRODUCT mean × #
	92UG037 R5 clade A	VI 1031 R5	93US143 X4/R5	92US077 X4/R5 clade B	MN X4	SF162 R5	VI 829 R5 clade C	VI 882 R5	VI 693 R5 clade D	VI 656 X4/R5	CA 10 X4 CRF01_AE	VI 1888 R5			
CV11	54.3	82.6	87.1	93.7	98.0	99.5	93.4	98.6	83.4	92.2	95.4	85.2	87.7	4	351
CV12	41.1	92.4	97.0	98.6	98.7	98.5	92.1	85.5	68.4	95.4	89.8	73.7	83.6	4	334
CV20	96.8	95.1	NA	98.8	98.1	NA	85.9	84.9	65.3	71.2	62.0	90.7	84.9	3	255
CV22	92.1	94.5	NA	93.4	97.8	NA	47.5	92.5	56.3	38.3	61.1	43.8	71.7	3	215
CV8	91.3	68.4	94.1	96.8	98.9	99.1	73.1	0.0	0.0	74.3	68.4	94.0	66.5	3	200
CV15	76.0	62.8	87.4	84.2	98.7	NA	89.3	24.4	90.7	74.3	55.3	76.0	73.2	2	146
CV13	58.3	77.6	65.3	73.1	97.0	98.4	94.8	43.8	58.3	70.5	66.1	81.4	72.1	2	144
CV14	76.0	82.2	78.1	83.4	97.2	NA	66.1	45.0	85.2	75.5	78.1	81.8	77.1	1	77
CV18	78.1	80.9	74.9	85.2	98.3	NA	53.2	0.0	88.5	66.9	65.3	69.1	68.6	1	69
CV17	69.8	73.1	49.9	74.3	92.8	NA	62.8	24.4	71.2	73.1	68.4	67.6	67.8	1	68
CV2	80.0	0.0	97.2	96.1	98.7	98.5	78.6	2.3	85.2	77.1	88.0	45.0	65.1	1	65
CV4	24.1	62.8	85.9	76.0	98.9	99.2	61.1	46.3	58.3	62.0	69.1	55.3	61.4	1	61
CV1	0.0	30.8	87.4	91.3	98.4	99.1	64.5	53.2	79.6	85.9	56.3	47.5	60.8	1	61
CV16	73.7	53.2	86.2	61.1	94.2	NA	54.3	0.0	76.6	66.1	70.5	56.3	60.6	1	61
CV10	53.2	75.5	54.3	65.3	97.5	98.4	86.8	24.4	0.0	86.8	27.6	70.5	58.8	1	59
CV23	0.0	36.9	NA	58.3	94.6	NA	69.1	63.7	70.5	4.5	76.0	62.0	53.6	1	54
CV9	0.0	74.9	76.0	72.5	93.4	98.7	46.3	10.9	64.5	70.5	62.8	33.9	53.0	1	53
CV21	46.3	35.4	NA	97.4	98.2	NA	10.9	0.0	68.4	63.7	72.5	33.9	52.7	1	53
CV7	22.4	53.2	91.7	85.5	98.1	98.8	47.5	0.0	60.2	54.3	60.2	33.9	51.5	1	52
CV5	16.8	52.1	84.2	80.5	90.2	99.4	49.9	0.0	47.5	82.2	57.3	35.4	51.2	1	51
CV6	0.0	66.1	90.7	74.3	97.7	98.8	0.0	0.0	49.9	69.8	60.2	0.0	41.8	1	42
CV19	47.5	36.9	NA	95.2	93.8	NA	20.6	0.0	10.9	0.0	36.9	53.2	39.5	1	40
CV3	27.6	0.0	82.2	73.7	98.6	98.6	12.9	0.0	65.3	53.2	32.4	0.0	36.4	1	36

Fig. 2. Neutralizing activity of subject plasma in a 24/24/48 GHOST assay against primary HIV-1 isolates from five clades. The co-receptor (X4 and/or R5) used by these isolates is printed below the virus code. The neutralizing activity is presented as the percentage reduction in infectivity at a 1/20 sample dilution. To facilitate reading, the neutralization results are colour coded: a yellow box indicates neutralization between 0 and 49%, an orange box between 50 and 79%, a green box between 80 and 89% and a blue box between 90 and 100%. To capture the information about potency and breadth of neutralization, the mean of the individual neutralization percentages (except for 93US143 and SF162 for which the results are incomplete) and the number of clades neutralized at a 90% level were calculated for each subject and multiplied. This product was used for ranking of the individuals. NA: not available. NEUT: neutralization.

The predominance of subtype B viruses is in accordance with the European epidemiology (Osmanov et al., 2002).

3.2. Screening of the plasma for neutralizing activity in vitro

The human plasma samples were screened with a highly sensitive 24/24/48 GHOST neutralization assay at a 1/20 dilution against 12 primary HIV-1 isolates from 5 clades (Fig. 2). The high sensitivity of this assay stems from the extended incubation times (24 h) for the antibodies with virus and for this mixture with GHOST cells, subsequently. All plasma samples neutralized the primary isolates SF162 and/or MN, both known to be very neutralization-sensitive (Binley et al., 2004), at a 90% level. The strongest and broadest responses were observed for plasma CV11 and CV12 which both neutralized 7 of 12 isolates from 4 clades at levels exceeding 90%. Plasma CV8 neutralized (>90%) 6 of 12 isolates and, plasma CV20 and CV22 5 of 10 isolates from 3 clades. Plasma CV13 and CV15 neutralized (>90%) 3 of 12 and 2 of 11 isolates from 2 clades, respectively. Neutralization (>90%) by the other plasma samples was limited to viruses from clade B.

3.3. Screening of the purified antibodies for neutralizing activity in vitro

To examine whether neutralization was antibody-mediated, IgG was purified from the plasma of four subjects and a seroneg-

ative control. CV11 and CV12 were chosen for their broadly cross-neutralizing profile *in vitro* and, CV3 and CV4 for their limited neutralizing profile. From the panel of viruses used for plasma screening, five strains were chosen that each belonged to another clade (A, B, C, D and CRF01_AE). The purified antibodies and the plasma samples were tested in a 24/24/48 GHOST assay at an IgG concentration of 500 µg/ml and a 1/20 dilution, respectively. The latter corresponded to an IgG concentration of ~450 µg/ml for CV3, ~660 µg/ml for CV4, ~346 µg/ml for CV11, ~260 µg/ml for CV12 and ~500 µg/ml for SN. The neutralizing activity present in the plasma of these subjects was also present in the enriched IgG fractions thus demonstrating that the neutralizing activity was at least antibody-mediated (Fig. 3).

3.4. Passive immunization and challenge with clade B isolates in huPBL-NOD/Scid mice

The ability of polyclonal antibodies to interfere with viral replication *in vivo* was studied in huPBL-NOD/Scid mice. In these chimeras, the primary isolates SF162 and W61D replicated rapidly to high levels. Inoculation of five (W61D) to six (SF162) log genome equivalents (gequiv.) sufficed to reach plasma levels exceeding 7 log gequiv./ml 14 days later. A limitation of this model is that the reconstituted animals die within 2–3 weeks due to fatal graft-versus-host disease (GVHD).

In a preliminary experiment (data not shown), chimeric mice were immunized with 1 mg of polyclonal antibodies (SN,

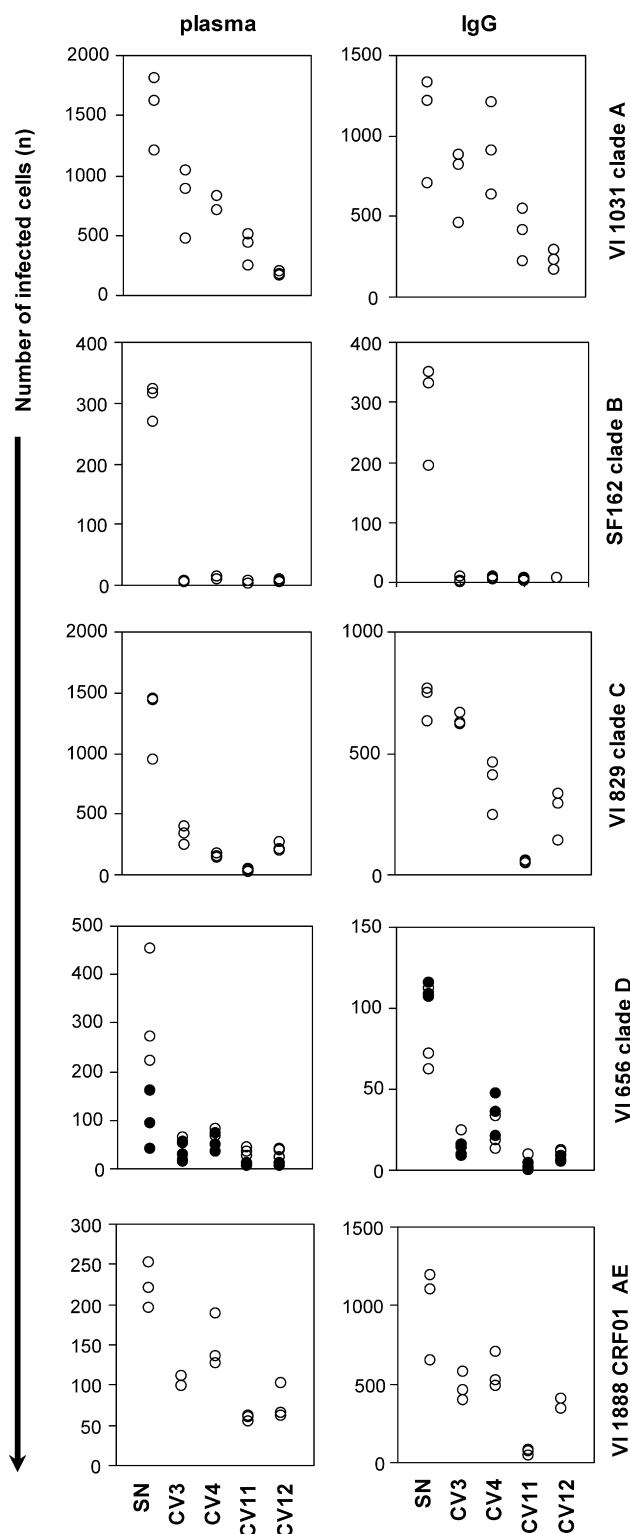


Fig. 3. Neutralizing activity of subject plasma and purified immunoglobulin G (IgG) in a 24/24/48 GHOST assay. IgG was purified from the plasma of HIV-1 infected subjects (CV) and a seronegative control (SN). Triplicate data are presented as the absolute number of infected cells (n). Open circles represent analyses with CCR5 expressing and closed circles with CXCR4-expressing GHOST cells.

CV3, CV11 and CV12) 24 h before injection of SF162. At that time, human IgG was circulating at an average concentration of 305 $\mu\text{g/ml}$ (95% confidence interval 246–364 $\mu\text{g/ml}$). The contribution of immunoglobulins produced by the PBMC expanding in the mice was at average 68 $\mu\text{g/ml}$ (95% confidence interval 58–78 $\mu\text{g/ml}$). Two weeks after challenge, high levels of virus (>6 log gequiv./ml) were detected in all mice treated with control and CV11 antibodies. CV3 antibodies inhibited viral replication in one of three mice and CV12 antibodies in two of two mice (<3.70 log gequiv./ml). One CV12 mouse that died earlier due to GVHD showed low level viral replication (4.02 log gequiv./ml).

To investigate whether a higher dose of antibodies may protect a higher proportion of mice, the amount of antibodies was increased from 1 to 3 mg. This resulted in higher circulating IgG levels (875 $\mu\text{g/ml}$ with a 95% confidence interval of 833–918 $\mu\text{g/ml}$). In addition, IgGs from five other patients (CV2, CV4, CV6, CV20 and CV22) were evaluated for their protective capacity in mice. CV2 was chosen for its long term HIV history without antiretroviral therapy and its strong *in vitro* neutralizing activity ($>96\%$) against all clade B isolates tested. CV20 and CV22 were chosen for their strong *in vitro* neutralizing activity ($>92\%$) against both clade A isolates tested. CV4 and CV6 were included as two subjects with limited *in vitro* neutralizing activity.

CV12 antibodies inhibited replication of SF162 (<3.70 log gequiv./ml) in seven of eight mice (Fig. 4A). Antibodies that were purified from a sample taken 2 years later (CV12-2004) also prevented replication in three of four mice (Fig. 4E). CV11 antibodies of which only 2.3 or 3 mg were administered because of limited availability, protected four of eight mice. Antibodies that were purified from a sample taken 3 years later (CV11-2005) also prevented replication in two of three mice (Fig. 4E). CV6 and CV22 antibodies protected two of four mice. With control and CV3 antibodies, viral replication was present in all but one mouse. With CV2, CV4 and CV20 antibodies, viral replication was detected in all mice.

In a subset of subjects (CV3, CV4, CV11 and CV12), the virus-inhibiting activity against another primary clade B isolate (W61D) was examined. None of the IgG preparations inhibited replication of this isolate (Fig. 4B).

3.5. Passive immunization and challenge with a clade A and a CRF01_AE isolate in huPBL-NOD/Scid mice

To examine whether polyclonal antibodies can protect mice against non-clade B isolates, VI 1031 (clade A) and VI 1888 (CRF01_AE) were included. These viruses replicated rapidly to high levels, and inoculation with 5 log gequiv. (VI 1888) to 6 log gequiv. (VI 1031) sufficed to reach levels exceeding 7 log gequiv./ml. Three mg of SN, CV2, CV3, CV4, CV6, CV11, CV12, CV20 and CV22 antibodies were administered 1 day before viral inoculation.

None of the IgG preparations protected mice from viral challenge with VI 1031 (Fig. 4C). In contrast, several mice were protected against VI 1888 (Fig. 4D). CV11 antibodies inhibited viral replication in two of three mice and CV22 antibodies in

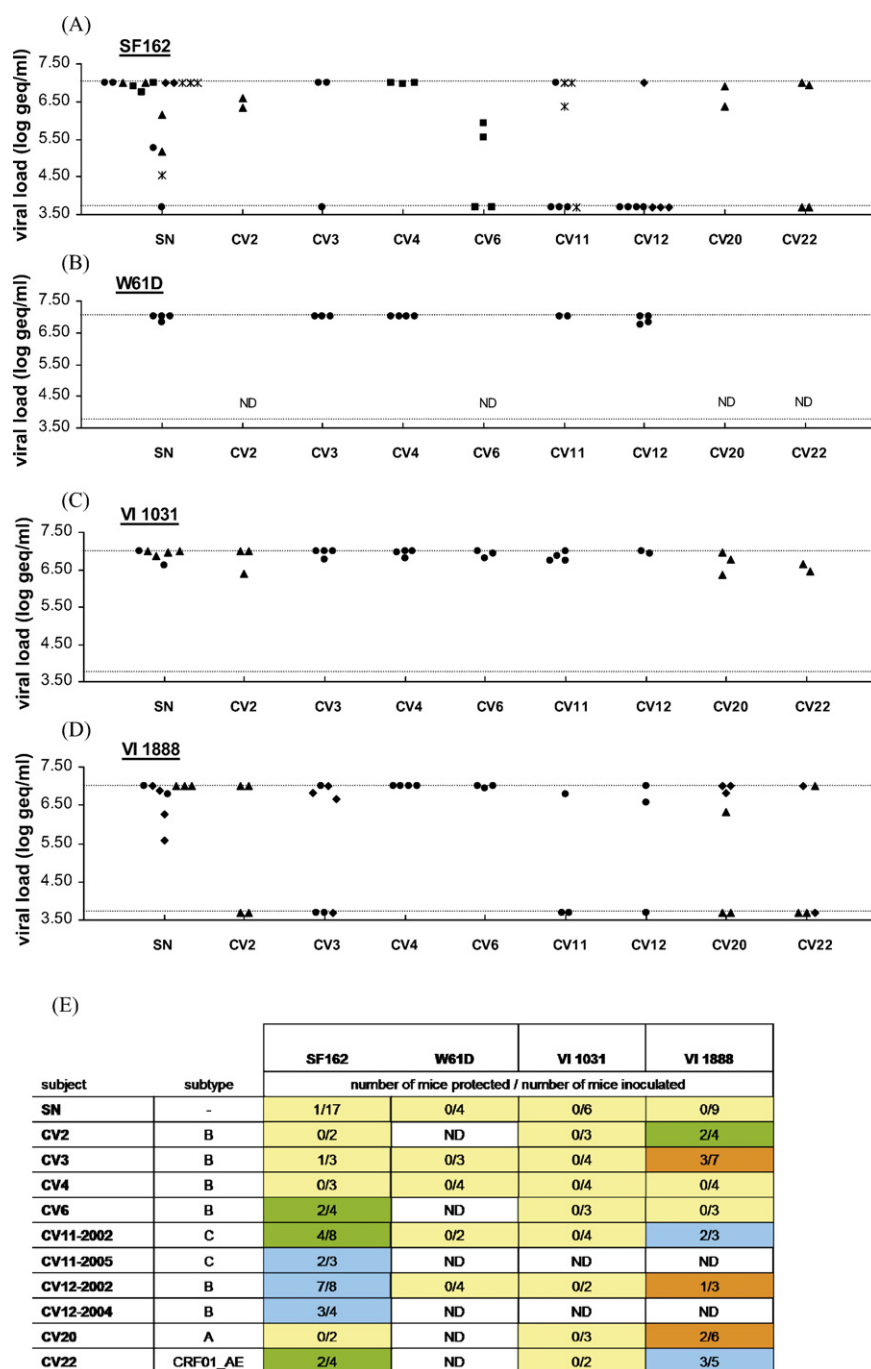


Fig. 4. Passive immunization in huPBL-NOD/Scid mice with human polyclonal antibodies. Immunoglobulins were purified from HIV-1 infected subjects (CV) and a seronegative control (SN). Mice were challenged with the clade B primary isolates SF162 (A) and W61D (B), the clade A isolate VI 1031 (C) and the CRF01_AE isolate VI 1888 (D). Viral load (log gequiv./ml) was measured in mouse plasma 2 weeks after challenge. The lower and upper limit of detection was 3.70 and 7.00 log gequiv./ml, respectively (—). Each experimental group is represented by another symbol and each point represents a mouse. In Fig. 4E, a summary of these data is shown. To facilitate reading, the results are colour coded: a yellow box indicates viral inhibition in none or one (SF162) of the mice, an orange box in less than half of the mice, a green box in half of the mice and a blue box in more than half of the mice. ND: not determined.

three of five mice. For the other IgGs, protection was observed in two of four mice (CV2), in three of seven mice (CV3) and in one of three mice (CV12). Viral replication was detected in all mice treated with SN, CV4 or CV6. An overview of the number of mice protected by human polyclonal antibodies against these four primary isolates is given in Fig. 4E.

3.6. Antibodies from CV12 interfere with viral replication in mice reconstituted with PBMC from CV12

Since the antibodies isolated from subject CV12 had remarkable protective activities *in vitro* and *in vivo*, we examined his clinical files more in depth. CV12 had been infected with HIV

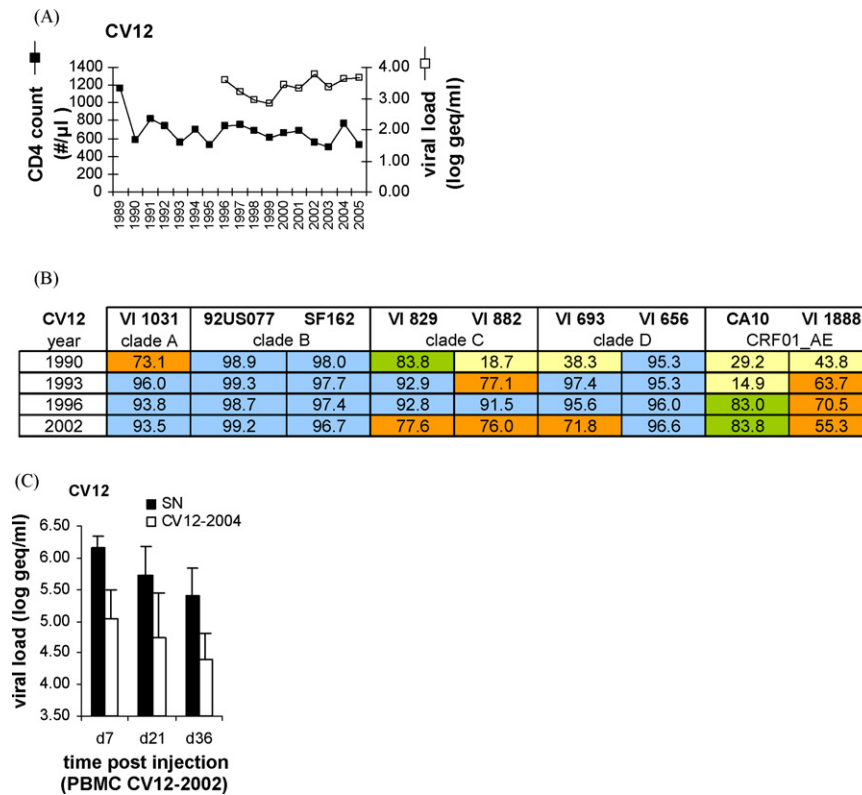


Fig. 5. Evolution of CD4 count, viral load and plasma neutralizing activity in subject CV12. (A) Yearly CD4 count (#/μl) and viral load (log gequiv./ml) where available, (B) neutralizing activity of CV12 plasma in a 24/24/48 GHOST assay against primary HIV-1 isolates from five clades at different time points. The neutralizing activity is presented as the percentage reduction in infectivity at a 1/20 sample dilution. To facilitate reading, the neutralization results are colour coded: a yellow box indicates neutralization between 0 and 49%, an orange box between 50 and 79%, a green box between 80 and 89% and a blue box between 90 and 100% and (C) antibodies from CV12 interfere with replication of autologous archived virus. Three NOD/Scid mice were reconstituted with PBMC from CV12-2002, 24 h after passive immunization with 3 mg purified CV12-2004 antibodies. Viral load was measured in mouse plasma at days 7, 21 and 36. Data are presented as mean + standard deviation.

for more than a decade and has remained clinically stable until the present day without antiretroviral therapy. CV12 has been controlling his viral load below 4 log gequiv./ml and his CD4 cell counts have remained stable over years (Fig. 5A).

Serum aliquots of CV12 have been stored at regular time intervals (1990, 1993, 1996), which allowed us to characterize the qualities of these samples in addition to the sample (2002) that has been used in all experiments described before (Fig. 5B). High level neutralization (>90%) of the clade B isolates 92US077 and SF162, and the clade D isolate VI 656 was present in all samples even the earliest one (1990). The strongest (>90%) and broadest responses were observed in the sample taken 7 years after diagnosis (1996). These results suggested that broadly neutralizing antibodies were generated early after infection and remained present until now and that these antibodies might contribute to the control of viral replication. This was investigated by injection of three mg IgG purified from SN and CV12-2004 to optimally conditioned NOD/Scid mice. Twenty-four hours later, all these mice were reconstituted i.p. with 9×10^6 PBMC from CV12-2002. Direct transfer of infected PBMC into mice provides the advantage that the endogenous virus is replicating without any *in vitro* manipulation (Boyle et al., 1995). CV12-2004 antibodies reduced the viral load with one log compared to control antibodies (Fig. 5C), thereby sup-

porting the idea that antibodies in this patient contribute to the control of viral replication.

4. Discussion

Protection against primary HIV by passive immunization with patient-derived polyclonal antibodies in humanized mice or macaques has never been observed (Gauduin et al., 1997; Mascola et al., 1999; Schutten et al., 1996). To our knowledge, this is the first report showing that polyclonal antibodies from HIV-1-infected subjects are able to inhibit replication of primary HIV-1 isolates in huPBL-NOD/Scid mice. Remarkably, in several cases the antibody-mediated inhibition was not restricted to the virus belonging to the same clade a subject was infected with. Antibodies of 5 different patients, together inhibited replication in 16 of 34 SF162-inoculated animals. In particular, antibodies of CV12 were very potent, inhibiting replication of SF162 in seven of eight mice. However, even antibodies of CV11 and CV12 did not inhibit replication of another primary clade B isolate W61D. Perhaps these different results are not surprising since W61D is known to be relatively resistant to antibody-mediated neutralization and resists for example *in vitro* neutralization by the monoclonal antibodies IgG1b12 and 2F5 (Beddows et al., 2005). In contrast, SF162 is among the most neutralization-sensitive

primary isolates and its use in (passive) immunization and challenge experiments (Cherpelis et al., 2001; Parren et al., 2001; Veazey et al., 2003) has been questioned (Moore and Burton, 2004). Antibodies of 6 different patients inhibited replication in 13 of 35 mice inoculated with VI 1888, a CRF01_AE virus. The most potent responses were observed with antibodies of CV11 and CV22 that inhibited replication of VI 1888 in two of three and three of five mice, respectively. None of the tested patient-derived antibody preparations conveyed protection against the clade A isolate VI 1031 (clade A). Finally, for CV11 and CV12, it was demonstrated that the antiviral activity against SF162 was still present in a sample taken 2 or 3 years after the first. In addition, patient CV12 has now been infected for more than 16 years, controlled his viral load below 4 log₁₀ equiv./ml and his CD4 cell counts have remained stable over the years. Our observation that CV12 antibodies reduced viral replication of this patient's own archived virus, suggests that the antibodies in this patient contribute to the control of viral replication.

It is assumed that the inhibition of replication in these PBMC reconstituted mice can be attributed to direct neutralization of viral infectivity and/or antibody-dependent cytotoxicities. However, replication of SF162 was still inhibited by CV12 antibodies when mice were reconstituted with NK cell-depleted PBMC (data not shown), which suggest that antibody-dependent cellular cytotoxicity is not required to inhibit replication. Complement-mediated effector functions are also unlikely since NOD/LtSz-Scid mice lack complement activity (Shultz et al., 1995). The inhibition of viral replication by the polyclonal antibodies was achieved at an IgG concentration of 115–150 mg/kg. Because only a fraction of the administered antibodies can be envelope-specific (Binley et al., 1997), the protective capacity of this neutralizing fraction must be rather strong. For comparison, the broadly neutralizing monoclonal antibody IgG1b12 completely protected mice against two primary clade B isolates only at 50 mg/kg, whereas a dose of 10 mg/kg offered only partial protection (Gauduin et al., 1997). In the same study, a HIVIG preparation at 150 mg/kg did not significantly protect huPBL-Scid mice.

Passive immunizations with HIV-1-neutralizing monoclonal antibodies rapidly selected escape mutants in huPBL/Scid mice (Andrus et al., 1998; Poignard et al., 1999). Escape mutants were also rapidly generated after passive transfer of human broadly neutralizing antibodies in HIV-infected humans (Trkola et al., 2005). This demonstrates the neutralizing potential of broadly neutralizing antibodies but also reveals their limitations for therapeutic use. In addition these observations demonstrate that a universal HIV vaccine will need to elicit high levels of exceptionally broadly and potently neutralizing antibodies. We sequenced most of the *env* gene of “breakthrough” viruses of SF162 and VI 1888 inoculated mice, to see whether these viruses carried mutations that would suggest possible antibody escape. Sequence analysis of SF162 virus in CV11, CV12 and CV22-immunized mice in which replication was not inhibited, revealed amino acid differences at four positions (Fig. 1S, supplementary data). However, these amino acid differences were also found in SN-immunized mice and were probably differentially present in the two different SF162 preparations used to

infect these mice. This limited analysis suggest that no antibody escape mutants of SF162 were generated. Sequence analysis of VI 1888 replicating in CV3, CV12, CV20 or CV22-immunized mice revealed the following unique amino acids: 270N, 272I and 364R in the CV3 mouse, 270N, 272I in the CV20-1 mouse, 51N in the CV22 mouse and 168V and 270Y in the CV22-1 mouse. However we can neither confirm nor exclude that these mutations are linked to escape since these altered amino acids might be due to polymorphisms present in the primary isolate. Population sequencing of VI 1888 primary isolate-derived amplicons resulted in an unreadable sequence, demonstrating indeed the polymorphisms present in this primary isolate.

Finally, it should be noted that the results from the sensitive *in vitro* neutralization assay did not correlate well with the outcome of passive immunizations for several antibody-virus combinations. For example, antibodies from subjects CV2 and CV4 neutralized SF162 at levels >98% but no *in vivo* inhibition was observed. CV12, CV20 and CV22 neutralized VI 1031 and CV20 neutralized VI 1888 at levels >90% *in vitro*, but no inhibition of replication was observed in mice. CV3 antibodies did not neutralize VI 1888 *in vitro*, but inhibited replication of this isolate in three of seven mice. The possible reasons for these divergences are currently under investigation, but it seems that the 24/24/48 neutralization assay format is not representative for the *in vivo* interaction between antibodies and virus. Consequently, this format is no longer used in our laboratory. Nevertheless, this assay allowed us to identify HIV-1-infected subjects CV11, CV22 and especially CV12, who seem to have antibodies with interesting *in vitro* and *in vivo* characteristics, either quantitative or qualitative. These HIV-1-infected subjects might be appropriate cell donors for the generation of neutralizing monoclonal antibodies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2006.10.013.

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