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Detection, quantification and sequencing of HIV-1 from the plasma of seropositive individuals and from factor VIII concentrates

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A highly sensitive and reliable RNA polymerase chain reaction method has been developed which has been used to detect, quantify and sequence cell-free HIV RNA directly from the plasma of seropositive individuals. Plasma from 10 out of 12 haemophiliacs tested was found to contain detectable levels of HIV-1 RNA [log mean value: 1.2×10^3 copies for Centers for Disease Control (CDC) group II patients, 5.5×10^3 copies for CDC group IV patients]. The presence of cell-free circulating virus in both symptomatic and asymptomatic individuals suggests that viral replication continues throughout the course of infection. The same procedure has been used to detect and sequence HIV-1 RNA in two batches of unheated commercial factor VIII concentrate distributed in 1981 and 1983. The sequences obtained revealed a closer relationship to North American than to African strains of HIV-1.

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Introduction

Using the polymerase chain reaction (PCR), HIV provirus can be not only detected [1–4], but also accurately quantified directly in patient's peripheral blood mononuclear cells (PBMC) [5,6]. However, detection of HIV DNA in PBMC does not indicate whether such cells are expressing viral RNA sequences or whether free virus is present in plasma or other body fluids. Several investigators have coupled a reverse transcriptase (RT) reaction step to the PCR (RNA PCR) [7–9] and have successfully detected HIV RNA both in cultured HIV-infected cell lines and in PBMC from seropositive subjects. Unfortunately these reports have not included an assessment of the sensitivity of the methods used and therefore have not determined absolute quantities of RNA.

Using the nested PCR method, we have developed a highly sensitive and quantitive RNA PCR assay. After reverse transcription, complementary (c) DNA was amplified in two sequential PCRs. As the nested PCR can detect single molecules of target DNA sequence, quantitation of HIV-specific cDNA, and by implication of HIV RNA sequences present in the original sample, can be achieved by limiting dilution as described previously for provirus quantification in PBMC [5]. The efficiency of the RT reaction was estimated by measurement of the yield of cDNA from known amounts of specific RNA sequences after reverse transcription. We have used the methods to estimate the plasma virus load in a group of HIV-infected individuals including both symptomatic and asymptomatic patients.

We have also investigated the presence of HIV-1 RNA sequences in eight batches of unheated factor VIII concentrate distributed between 1981 and 1984. HIV-1 RNA was detected in two batches of commercial factor VIII concentrate distributed in 1981 and 1983. The identity of the RNA was confirmed by nucleotide se-

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quencing the PCR product. Sequences obtained in the *pol* and *env* regions from these concentrates provide some information on the geographical origins of the infected blood donors.

Materials and methods

Plasma

Plasma samples were obtained from 12 HIV-1-infected haemophiliacs who had been exposed to HIV-contaminated factor VIII early in 1984 [10]. All 12 individuals seroconverted for antibody 3–10 months after receiving the factor VIII [10,11]. Patients 56, 70, 82, 83 and 84 have been classified as stage II according to Centers for Disease Control (CDC) clasification, while patients 72, 74, 77, 79, 95 and 87 are in CDC group IV and have been suffering from a range of opportunistic infections and constitutional symptoms of HIV infection. Patient 28 died in 1988. Apart from patient 72, all CDC group IV patients but none of the CDC group II patients have been receiving antiviral treatment (zidovudine). Three samples from a seronegative individual, who has no known risk factors for HIV infection, were used as negative controls. Plasma samples and cell culture supernatant were assayed for HIV-1 p24 core antigen with a commercial enzyme immunoassay (Du Pont, Stevenage, Herts, UK) according to the manufacturer's instructions. This assay was capable of detecting 7 pg/ml p24 antigen.

Factor VIII concentrates

Eight batches of factor VIII concentrate, including both commercial and National Health Service-produced material, were obtained from the National Institute of Biological Standards (Potters Bar, UK). All batches were unheated and prepared before the introduction of donor screening for anti-HIV antibodies. They were distributed in the UK between 1981 and 1984.

HIV and *tk* primers

HIV primers were synthesized by the Oswel DNA Service, Department of Chemistry, University of Edinburgh, and purified by high-pessure liquid chromatography (HPLC). The primers were based on the consensus of several published HIV sequences (HIV_{BRU}; HIV_{ELI}; HIV_{IIIB}; HIV_{MAL}; HIV_{MN} and HIV_{RF}). The primer sites were chosen for greatest conservation between these published sequences. No more than one mismatch with any of the above published HIV sequences was permitted, nor was any mismatch near the 3′ terminus. The primers for herpes simplex virus (HSV) *tk* gene were provided by R. Al-Shawi (Centre for Genome Research, University of Edinburgh, Edinburgh, UK). The sequences of the primers for both HIV template and transcribed HSV *tk* template are given here and the coordinates listed in the brackets of HIV primers are from HIV_{HXB2} (+, sense; −, antisense). HIV *pol* primers: (a) 5′-CCCAAAAGTTAAACAATGGCC (+, 2602), (b) 5′-AGAAATTTGTACAGAGATGG (+, 2653), (c) 5′-CCATTTATCAGGATGGAGTTC (−, 3245), (d) 5′-GCTGTCTTTTTCTGGCAGCAC (−, 3281); V4-V5 primers: (e) 5′-TCAGGAGGGGACCCCAGAAATT (+, 7316), (f) 5′-GGGGAATTTTTCTACTGTAAT (+, 7360), (g) 5′ CTTCTCCAATTGTCCCTCATA (−, 7665), (h) 5′-CCATAGTGCTTCCTGCTGCT (−, 7814). HSV *tk* primers: 5′-GCCAGTAAGTCATCGGCTCGGG (+), 5′-CCATCAACACGCGTCTGCGTTCG (−).

Viral RNA extraction

Five hundred microlitres of patient plasma or 500 µl HIV-infected C8166 culture supernatant was mixed with 8.5 ml phosphate-buffered saline (PBS); alternatively, 3 ml factor VIII reconstituted with the recommended volume of water was mixed with 6 ml PBS. In both cases, virus was pelleted at 45 000 **g** in a swing out rotor (Sorvall SH80, Du Pont) at 4°C for 2 h. The pellet was resuspended in 1.2 ml of a denaturing solution [2 mol/lguanidinium thiocynate, 12.5 mmol/l sodium citrate (pH 7.0), 0.25% sarcosyl, 0.05 mol/l2-mercaptoethanol, 50% v/v water-saturated distilled phenol] and RNA purification was continued as described [12].

cDNA synthesis

RNA was incubated with ribonuclease (RNase)-free deoxyribonuclease(DNase; Boehringer-Mannheim, Lewes, UK) at 37°C for 20 min in a 10 µl volume of DNase reaction buffer [50 mmol/l Tris-Cl pH 7.5, 10 mmol/l $MgCl_2$, 4 mmol/l DTT, 10 units RNAsin, 1 µg carrier RNA (sheep fibroblast cell total RNA) and 15 units RNase-free DNase]. The sample was then incubated at 80°C for 10 min to terminate the reaction. cDNA synthesis was carried out by adding an equal volume of RT reaction buffer [50 mmol/l Tris-Cl pH 8.0; 5 mmol/l $MgCl_2$; 5 mmol/l DTT; 50 mmol/l KCl; 0.05 µg/µl bovine serum albumin (BSA); 600 µmol/l each of dATP, dGTP, dTTP, dCTP; 20% dimethyl sulphoxide (DMSO); 1.5 µmol/l outer antisense primer (d or h); 10 units RNAsin (Promega, Southampton, UK) and 10 units AMV reverse-transcriptase (Promega) to the digested HIV RNA sample and incubating at 42°C for 30 min.

Measurement of RT reaction efficiency using double PCR method

The HIV RNA for cDNA synthesis was serially diluted in twofold steps, slot-blotted and quantified by hybridization with HIVBH10R.3 plasmid in comparison with a dilution series of known amounts of HIVBH10R.3 DNA; 50 ng HIVBH10R.3 plasmid DNA was used to make an HIV-specific probe using the Pharmacia Oligolabelling Kit (Milton Keynes, UK). Blot hybridization was carried out following the method of

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Church and Gilbert [13]. cDNA samples were diluted, amplified in the PCR with products sequenced as described previously [5].

Measurement of RT reaction efficiency by plasmid RNA transcription

Construct pSV2gpt, containing a mouse promoter region and coding region of the HSV type 1 thymidine kinase gene (HSV *tk*) was obtained from R. Al-Shawi [15]. RNA was transcribed *in vitro* from 100 ng of the construct plasma DNA at 37°C for 1 h (20 μl volumes of 4 mmol/l Tris-Cl pH 8.0; 8 mmol/l $MgCl_2$; 2 mmol/l spermidine; 50 mmol/l NaCl; 0.01 mol/l DTT; 0.4 mmol/l each of rATP, rCTP and rGTP; 30 units of RNAsin; 100 ng/μl BSA; 10 units of T7 RNA polymerase). The concentration of pSV2gpt RNA transcripts used for subsequent cDNA synthesis and HSV *tk* plasmid DNA used for quantitative comparison was estimated by spectrophotometry at 260 nm. Twofold serial titrations of *tk* cDNA after reverse transcription with antisense primer and of HSV *tk* plasmid DNA were made prior to PCR amplification with HSV *tk* specific primers (spacing = 260 bp). Twenty-five cycles were employed and the product of PCR was analysed by agarose gel electrophoresis and ethidium bromide staining. The amount of cDNA was estimated by reference to a dilution series of HSV *tk* DNA after amplification with the same primers. This was then compared with the number of RNA sequences from which the cDNA was made.

Controls for PCR

In order to remove any possible HIV DNA contamination, either from plasmid itself [18] or from other sources, all HIV RNA samples were treated with RNase-free DNase (Boehringer-Mannheim) prior to reverse transcription. All experiments included appropriate negative controls. In order to confirm that carrier RNA itself did not generate results in the experiment, carrier RNA was subjected to double PCR with V4-V5 primers and *pol* primers. None of the samples was positive in any assay. HIV RNA samples amplified without RT also gave negative double PCR results (data not shown) demonstrating that the positives obtained were from HIV cDNA, not from HIV DNA contamination.

Results

RT reaction efficiency from plasmid RNA transcription

A known amount of RNA transcript from a cloned *tk* gene was reverse transcribed, and the cDNA titrated prior to amplification in the PCR with *tk*-specific primers. This was compared with the results of amplifying a dilution series of cloned *tk* DNA. The cut-off point, below which amplified DNA was not detectable by agarose gel electrophoresis and ethidium bromide staining, was 5.12 fg for the *tk* plasmid. Using the known molecular mass of the *tk* plasmid DNA (7270 bp × 660 g/mol per bp), this figure corresponded to 630 molecules of *tk* plasmid, or 1260 copies of target sequence (630 × 2 for double-stranded DNA). cDNA synthesized from 8 ng of HSV *tk* transcript was serially diluted prior to amplification. A dilution containing cDNA synthesized from 16 fg RNA gave a positive result and the next dilution containing cDNA from 8 fg was negative. Using the estimated molecular mass of the RNA transcript (2400 bases × 330 g/mol per base), the minimum detectable amount of cDNA corresponded to an input of 1.2×10^4 copies of RNA. The efficiency of the RT reaction, in terms of the number of molecules of amplifiable cDNA synthesized from the RNA template, is the ratio of the two figures obtained above (1260/12 000), or approximately 10%.

RT reaction efficiency using HIV RNA

Six HIV-1 RNA samples were extracted from culture supernatant of C8166 cells infected with HIV_{RF}, HIV_{IIIB} and HIV_{HXB2}. The viral RNA was slot-blotted and quantified by hybridization with HIVBH10R.3 plasmid probe, in comparison with a dilution series of known amounts of HIVBH10R.3 DNA; 19.8, 9.9, 7 and 0.6 pg HIV_{RF} viral RNA, 3.0 and 1.5 pg HIV_{IIIB} and HIV_{HXB2}, respectively, were then reverse transcribed with an HIV V4-V5 outer antisense primer (h). The number of copies of cDNA in each sample after reverse transcription was estimated by limiting dilution and amplification with nested primers. The frequency of positive reactions at limiting dilution was used to calculate the molecular concentration of cDNA using a Poisson correction for positive reactions which contain more than one template molecule. [The mean number of template molecules per reaction (m) is equal to $-\ln(f_o)$, where f_o is the fraction of negative reactions.] The molecular mass of one copy of HIV-1 RNA was 6.5 ag [5] from which the RT reaction efficiency was calculated. In each case, 20 (or 24) replicates were tested at limiting dilution, and the estimates were reasonably accurate. For the first sample for instance, at a 1 in 78 000 dilution, there were two PCR positives out of 24 replicates. Using the Poisson formula to correct for multiple positives, the total number of molecules of HIV RNA detected at this dilution was estimated to be 2.1. As the molecular mass of single HIV RNA was 6.5 ag, the total amount of cDNA synthesized by the reverse transcription reaction was therefore equivalent to 1.06 pg ($2.1 \times 6.5 \times 10^{-6} \times 78000$). The ratio of the amount of HIV RNA reverse transcribed and the initial amount of HIV RNA available for reverse transcription gave the RT efficiency (1.06/19.8 = 5.3%). We show the results

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of dilution and distribution of six independent cDNA reactions after double PCR amplification in Table 1, along with the calculated RT efficiencies.

Quantification of HIV virus particle in the plasma or serum of seropositive individuals

Plasma from 10 out of 12 haemophiliacs contained detectable levels of HIV-1 RNA. The threshold of detection can be calculated to be 200 copies of RNA per ml plasma, based on the volume of plasma from which RNA was extracted, the input volume of the reverse transcription reaction, the efficiency of reverse transcription and the proportion of cDNA used in the first PCR reaction. The estimated concentration of virus particles in plasma ranged from 1×10^3 to 3×10^4 copies per ml in the positive samples (Table 2), with a logarithmic mean value of 1.2×10^3 copies for CDC group II patients, and 5.5×10^3 copies for CDC group IV patients. There was a significant positive association between the concentration of viral RNA in plasma and the proviral abundance in PBMC DNA. The correlation coefficient between the log-transformed RNA and DNA estimates is 0.74 ($0.01 < P < 0.02$), indicating that over 50% of the variance in DNA proviral abundance can be explained by the RNA concentrations. Thus the patients with the lowest proviral abundance (83 and 84 with an average of one provirus in 14000 and 10000 PBMC, respectively) had less than 200 copies of RNA per ml in their plasma. On the other hand in patients 82 and 87, relatively higher concentrations of virus (8.5×10^3 and 3.0×10^4 per ml plasma) were associated with high frequencies of provirus-bearing PBMC (one in 700 and one in 589 PBMC infected, respectively). However, no correlation was found, regardless of the stage of infection, between the amount of virus in plasma and the level of p24 antigen. Five patients who were negative for p24 antigen (70, 72, 74, 77 and 95) contained over 1.0×10^3 virus particles per ml plasma. Further, no correlation between CD4+ lymphocyte depletion and amount of circulating virus was found in these individuals. For example, relatively normal CD4 counts were found in p82 despite containing 8.5×10^3 copies of viral RNA per ml. Conversely, p84 had low CD4 counts (0.05×10^9/l) yet no detectable circulating virus. Zidovudine treatment appeared to have little effect on the levels of circulating virus. Those on long-term treatment (74, 77 and 79) contained comparable levels

Table 1. Titration of HIV cDNA after reverse transcriptase (RT) reaction by dilution, distribution and double polymerase chain reaction (PCR) amplification.

Sample (pg)	Amount of RNA in RT reaction (pg)	Dilution factor of cDNA (no. positive cells/no. tested)						Calculated amount of cDNA (pg)	% RT efficiency
		125	620	3100	15600	78000	390000		
RF1	19.8	ND	4/4	4/4	3/4	2/24	0/4	1.06	5.0
RF2	9.9	4/4	4/4	4/4	4/24	0/4	0/4	0.44	4.0
RF3	7	4/4	4/4	4/4	4/24	0/4	0/4	0.44	6.0
RF4	0.6	3/4	5/24	0/4	0/4	0/4	0/4	0.02	3.3
IIIB	3.0	4/4	3/4	1/4	2/20	0/4	ND	0.22	7.0
HXB2	1.5	2/4	2/4	4/20	0/4	0/4	ND	0.09	6.0

ND, not done.

Table 2. Comparison of the amount of circulating virus from patients' plasma with the amount of provirus in peripheral blood mononuclear cells (PBMC), plasma p24 antigen concentration, CD4+ counts, and clinical status.

Patient	Virions per ml in plasma	Number of cells per single provirus	p24 antigen (pg/ml)	CD4+ lymphocytes ($\times 10^9$/l)	Zidovudine treatment (months)	Disease stage (CDC)
p83	$< 10^2$	14000	–	0.27	–	II
p84	$< 10^2$	10000	–	0.05	–	II
p77	1.26×10^3	2500	–	0.07	14	IVA
p56	1.29×10^3	ND	15	0.51	–	II
p74	3.49×10^3	2000	–	0.38	10	IVC2
p95	3.50×10^3	455	–	0.06	–	IV
p28	3.97×10^3	2718	300	0.09	–	IV
p79	4.39×10^3	3300	63	0.21	15	IVC2
p70	6.12×10^3	ND	–	0.39	–	II
p82	8.53×10^3	700	53	0.65	–	II
p72	2.76×10^4	2720	–	0.33	–	IVC
p87	2.96×10^4	589	20	0.05	17	IVC/E

–, less than 7 pg/ml; ND, not done; CDC, Centers for Disease Control.

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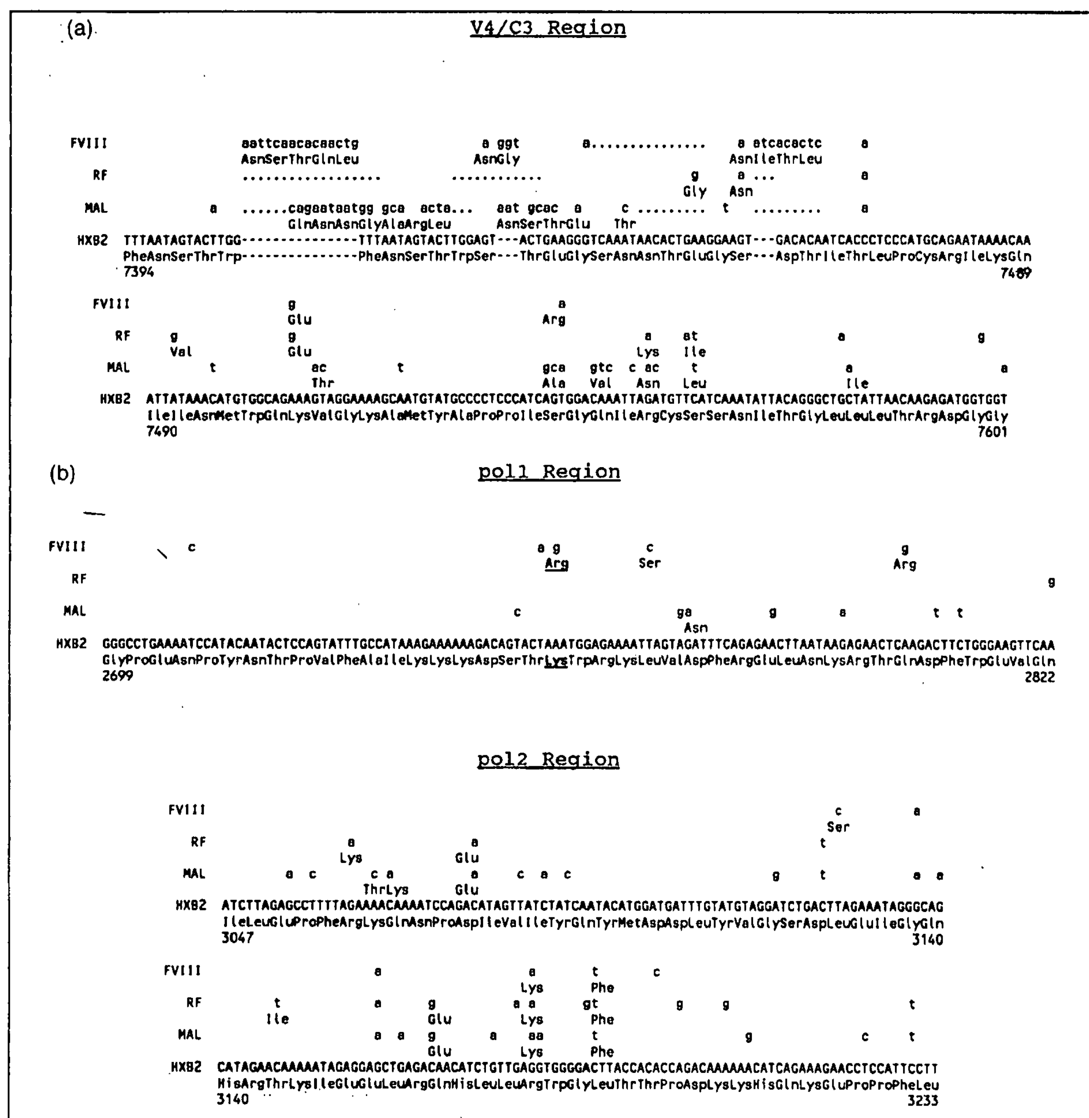

Fig. 1. Comparison of sequences detected in factor VIII with those of known geographical variants of HIV-1. (a) Nucleotide and amino acid sequences of the V4 and C3 region (*env*) of RNA detected in factor VIII batch no. 1. (b) Sequences in two regions of *pol* of RNA from batch no. 8. The location of these sequences in the genome of the HIV_{IIIB} isolate (clone HXB2, Genbank accession number K03455) is indicated. Differences between the factor VIII sequences and those of the HIV_{RF} and HIV_{MAL} isolates from that of HIV_{IIIB} are shown in the body of the figure.

to the two untreated symptomatic individuals (95 and 72).

Detection of HIV-1 in factor VIII concentrate

RNA was prepared from eight different batches of factor VIII (distributed between 1981 and 1984), by high-speed centrifugation and solubilization of the pellets as described previously. One-third of each RNA sample was reverse transcribed with primers d and h (*pol* and *env* genes), and one-quarter of the cDNA was amplified by PCR with primers a–d or e–h (see Methods). Two factor VIII batches (both commercially derived) out of eight tested gave positive results: in one case with the *env* primers, the other with the *pol* primers. Single molecules of target DNA were isolated by limit-

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ing dilution of the cDNA and were directly sequenced as previously described (Fig. 1) [5,15]. In the *env* region, two HIV RNA sequences, obtained from batch no. 1, were identical in the V4 and C3 regions. The sequence was distinct from those of all published HIV isolates and from any HIV sequence obtained previously in our laboratory [15,16]. This is particularly apparent in the V4 hypervariable region, which is clearly distinct from all published sequences, including those illustrated in Fig. 1. The C3 region showed 92% homology with HIV_{HXB2}, 90% with HIV_{RF}, and 77% with $HIV_{SF\text{-}2}$ (Fig. 1). In the *pol* region, two HIV RNA sequences obtained from batch no. 8 were also identical and distinct from any published sequence (Fig. 1). In this region, the sequence was 96% identical to HIV_{HBX2}, 94% with HIV_{RF} and $HIV_{SF\text{-}2}$ and 92% with HIV_{Z6}. The amount of RNA present in this material was close to the threshold of sensitivity for the RNA PCR method used. Allowing 5% efficiency of reverse transcription with these primers, the calculated amount of HIV RNA in both batches of reconstituted factor VIII was only 2.5 copies per ml.

Discussion

Detection and quantification of circulating HIV RNA in plasma

An efficiency of 5% was obtained in the RT reaction for HIV-1 template and primers *e–b* (spacing 480 bp). The overall efficiency of the procedure declined with wider primer spacing; amplification of cDNA using primer pairs separated by 858 bp gave an efficiency of 1.8% (data not shown). We are therefore able to obtain a direct estimate of the amount of cell-free HIV in plasma in absolute terms.

All plasma samples used in these experiments were obtained during 1988 and 1989 from HIV-seropositive haemophiliacs who were infected in 1984 [10]. Five were asymptomatic and seven had AIDS or AIDS-related complex.

Of the five asymptomatic (CDC group II) patients, three were RNA PCR-positive, while all seven CDC group IV patients were positive for RNA PCR (Table 2). All samples that were p24 antigen-positive were positive in the RNA PCR. High levels of viral RNA sequences were found even in some p24 antigen-negative plasma samples (patients 70, 72, 74 and 77) while similar or lower amounts of circulating RNA have been found in other plasma samples that were antigen-positive. The RNA PCR method provides a direct way to detect and quantify virus production regardless of immune complex formation, hence it may provide a better marker of the progression of disease.

On average, HIV RNA was more abundant in the plasma of patients with more advanced disease compared with asymptomatics (Table 2). However, a wide range in the amount of cell-free HIV RNA was found among patients in similar stages of disease. In other studies, a similar lack of correlation between p24 antigen and titers of infectious virus in plasma has been reported [19,20]. These results can be explained by a variation in the level of p24 antibody as concluded following a recent study of antigen levels after dissolution of immune complexes [21]. Total levels of p24 antigen, both free and immune complexed, have been found to vary little during the course of primary infection and subsequently [21].

The levels of HIV in patients' plasma, whether they were from CDC group II or from patients undergoing antiviral treatment, were much higher than previously estimated (Table 2). Zidovudine treatment has previously been shown to decrease the amount of cell-free circulating HIV virus initially both in plasma of infected humans [19] and of severe combined immunodeficiency infected mice [22]. The high concentration of cell-free circulating HIV in our patients, who have been undergoing zidovudine therapy for over 10 months, may imply that some resistant viral strains have emerged.

The most striking feature of the results is the high level of cell-free HIV found in some CDC group II patients (patient 56, 70 and 82; Table 2). Serial samples from one CDC group II patient (patient 82) without antiviral treatment showed persistently high levels of plasma virus for several years and rapid turnover of sequence variants (Simmonds *et al.* in preparation). The detection of high levels of cell-free HIV from the plasma of both CDC group II and IV patients suggests that viral replication occurs continuously throughout the course of an HIV infection. We find no evidence for a virological 'latent' period.

Detection of HIV RNA from factor VIII

Two out of eight batches of factor VIII concentrate were positive for HIV-1 RNA by PCR; in one case with *env* primers and the other with *pol* primers. Both were confirmed by sequencing the PCR product. The amount of HIV RNA present in factor VIII is very low (2.5 copies per ml), and close to the threshold of detection. These two factor VIII sequences, which were both found in commercial products, are distinct from those of any published HIV isolates, but are more closely related to North American strains than to African ones.

We believe that this is the first direct demonstration of contamination of factor VIII by HIV-1. Previous studies by ourselves [23] and others [24] have detected hepatitis C virus (HCV) RNA sequences in factor VIII using similar methods. In fact, out of the eight batches of factor VIII concentrate tested for HIV-1 RNA in this study, all six of the commercially-derived batches also contained detectable amounts of HCV RNA, including the two positive for HIV RNA (the two National Health

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Service-derived batches were negative for HCV RNA). The amounts of HCV RNA were higher than HIV; in two batches that were quantified by limiting dilution, between 30 000 and 100 000 copies of HCV RNA per ml were found [23]. There are many possible explanations for the difference in the concentration of HIV and HCV RNA. Comparison of the levels of HIV viraemia with that of HCV shows that HCV is present in 10–100-fold greater amounts in infected individuals. Second, the prevalence of HCV infection may be higher in paid donors. Third, HIV may be less stable during the factor VIII fractionation process than HCV, or may be excluded with greater efficiency. The last possibility could be the most likely if most plasma HIV is held in immune complexes.

We note that the *pol* sequence obtained from batch no. 8 contains an arginine residue at position 70 in the RT domain. This substitution was one of four found to be associated with resistance to zidovudine [25, 26]. The discovery of this substitution in factor VIII concentrate prepared before the use of zidovudine as an antiviral agent suggests that it was a pre-existing polymorphism.

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