VIROLOGY 204, 223–233 (1994)

The Natural History of HIV-1 Infection: Virus Load and Virus Phenotype Independent Determinants of Clinical Course?

SUZANNE JURRIAANS,*,[1] BOB VAN GEMEN,† GERRIT JAN WEVERLING,‡ DIANNE VAN STRIJP,† PETER NARA,§ ROEL COUTINHO,¶ MAARTEN KOOT,‖ HANNEKE SCHUITEMAKER,‖ AND JAAP GOUDSMIT*

Human Retrovirus Laboratory, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; †Organon Teknika, Boseind 15, 5281 RM Boxtel, The Netherlands; ‡Department of Clinical Epidemiology and Biostatistics, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; §Virus Biology Section, Laboratory of Tumor Cell Biology, National Cancer Institute, Frederick, Maryland 21701; ¶Department of Public Health and Environment, Municipal Health Service, Nieuwe Achtergracht 100, 1018 WT Amsterdam, The Netherlands; and ‖Department of Clinical Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands

Received April 6, 1994; accepted June 14, 1994

Virus load and virus phenotype have both been indicated as major determinants of disease progression in HIV-1 infection. In this study HIV-1 RNA copy numbers in serum, virus phenotype, and CD4+ cell counts were analyzed longitudinally in a group of 20 seroconverters progressing to AIDS within 5.5 years. In this group 12 individuals developed AIDS without syncytium-inducing (SI) viruses ever being isolated, while 8 individuals showed a non-SI (NSI) to SI phenotypic switch prior to AIDS development. HIV-1 RNA copy numbers in sera of all progressors were stable and high from seroconversion until development of AIDS. Twenty-one seroconverters remaining asymptomatic for more than 5.5 years were selected as nonprogressing controls, and both progressors and nonprogressors were evaluated at seroconversion and early in infection (3 years post seroconversion). Comparative analysis revealed that at the point of seroconversion HIV-1 RNA copy numbers in sera from NSI progressors, SI progressors, and nonprogressors were not significantly different, nor were their CD4+ cell counts. At seroconversion all individuals harbored viruses with an NSI phenotype. In contrast to the progressors, HIV-1 RNA copy numbers in sera of nonprogressors had declined significantly during the early period of infection. At the second time point RNA copy numbers in the sera of NSI progressors and nonprogressors differed significantly ($P = 0.0005$), while RNA copy numbers in the sera of SI progressors and nonprogressors did not. However, at this time point the CD4+ cell counts of SI progressors were significantly lower than those from nonprogressors ($P = 0.002$), while the CD4+ cell counts of NSI progressors and nonprogressors did not differ significantly. These results show that early in HIV-1 infection progressors and nonprogressors are distinguishable. NSI progressors can be distinguished from nonprogressors on the basis of serum HIV-1 RNA load and SI progressors on the basis of CD4+ cell decline. In addition, a significant decrease in the number of HIV-1 RNA copies in the early phase of infection seems to postpone the development of AIDS.

INTRODUCTION

In early HIV-1 infection a large number of infectious particles as well as viral genomic RNA copy numbers have been reported (Clark *et al.*, 1991; Daar *et al.*, 1991; van Gemen *et al.*, 1993). The acute phase of infection is followed by the emergence of anti-HIV antibodies and a clinical asymptomatic carrier state, which can last several years. The existence of HIV-1 latency on a molecular level has been debated for a long time (Baltimore and Feinberg, 1989). In several studies it has been shown that the blood of individuals with AIDS contains more HIV-1 RNA or infectious viral particles (Ho *et al.*, 1989; Zhang *et al.*, 1991; Holodniy *et al.*, 1991; Bagnarelli *et al.*, 1991; Scadden *et al.*, 1992; Aoki-Sei *et al.*, 1992; Piatak *et al.*, 1993) and more HIV-1 infected cells (Psallidopoulos *et al.*, 1989; Schnittman *et al.*, 1990; Simmonds *et al.*, 1990; Bagasra *et al.*, 1992; Jurriaans *et al.*, 1992; Connor *et al.*, 1993) than the blood of individuals without symptoms. The number of genomic HIV-1 RNA copies as well as the number of proviral HIV-1 DNA copies in peripheral blood appears to be inversely related to the number of CD4+ cells (Michael *et al.*, 1992; Gupta *et al.*, 1993; Piatak *et al.*, 1993).

Prognostic markers have helped to distinguish the progressors from nonprogressors during the asymptomatic period. Decline of CD4+ cell counts is a universal characteristic of people progressing to AIDS and may be the hallmark of disease (Phillips *et al.*, 1991). In addition, the conversion from p24 antigen negativity to positivity during the asymptomatic period of infection has been interpreted as a virological marker for disease progression (de Wolf *et al.*, 1988; Keet *et al.*, 1993). Indeed, p24 antigen positivity is strongly related to immunodeficiency and AIDS among HIV-1 seropositive individuals (Goudsmit *et al.*, 1986; Allain *et al.*, 1986, 1987), although many individuals progress to AIDS without p24 antigen positivity. HIV-1 virus variants that induce syncytia in primary

[1] To whom reprint requests should be addressed.

0042-6822/94 $6.00

cell culture and are transmissible to permanent cell lines have been shown to correlate strongly with low CD4+ cell counts and HIV-1 related disease (Åsjö *et al.*, 1986; Tersmette *et al.*, 1989a,b). The risk of a rapid CD4+ cell decline and onset of AIDS rises significantly with the appearance of these syncytium-inducing (SI) variants in the peripheral blood of asymptomatic and seropositive individuals (Koot *et al.*, 1993; Connor *et al.*, 1993). As a corollary, individuals who are seropositive but symptom-free for an extended period of time are persistently p24 antigen negative and carry stable non-SI (NSI) viruses (Sheppard *et al.*, 1993). However, the majority of individuals progressing to immunodeficiency and AIDS are p24 antigen negative and SI negative during the first years of seropositivity, and only about half of the progressors convert to p24 antigen positivity and/or SI positivity before symptoms of AIDS are diagnosed (Tersmette *et al.*, 1989a,b; Sheppard *et al.*, 1993).

Among 109 seroconverters from the Amsterdam Cohort Studies (de Wolf *et al.*, 1988) 20 have developed AIDS without antiretroviral therapy and with adequate follow-up time. This group was studied longitudinally for HIV-1 RNA copy numbers and levels of p24 antigen in serum, CD4+ cell counts, and virus phenotype. Based on the length of the individual symptom-free follow-up time, 21 seroconverters were selected as nonprogressing controls. In this group the different parameters were determined at seroconversion and 3 years later.

In this study a competitive quantification method for RNA, based on specific RNA amplification (Q-NASBA) (Kievits *et al.*, 1991; Bruisten *et al.*, 1993; van Gemen *et al.*, 1993), was used. The accuracy of the method was determined using a well-characterized *in vitro* cultured HIV-1 viral stock (Layne *et al.*, 1992). Subsequently, we used the Q-NASBA for analysis of serum samples of seroconverters who either progressed rapidly to immunodeficiency and/or AIDS or remained symptom-free.

MATERIALS AND METHODS

Patients

The study population consisted of seroconverters in a cohort of homosexual men participating in a study on the natural course of HIV-1 infection in Amsterdam (de Wolf *et al.*, 1988). One hundred nine individuals seroconverted for HIV-1 antibodies during 7 years of follow-up in the period from October 1984 through October 1991. HIV-1 antibodies were tested every 3 months, and the seroconversion moment was considered as the first moment HIV-1 antibodies were detected and confirmed by immunoblotting. Within six weeks following the first seropositive sample another sample was tested to confirm the initial findings. Twenty-nine seroconverters developed AIDS within the follow-up period. Twenty of these were included in this study (the other were excluded because of antiretroviral treatment or inadequate follow-up) and sera taken at regular intervals during the complete symptom-free period were tested for changes in quantity of HIV-1 RNA and levels of p24 antigen. Furthermore, numbers of CD4+ cells and the phenotype of viruses present in patient peripheral blood mononuclear cells (PBMC) were determined. Patient characteristics and the clinical diagnoses are shown in Table 1.

Comparative analysis

Twenty-one seroconverters from the same cohort were selected as nonprogressing controls. These individuals were symptom-free for a period of 5 years or more and had persistently normal T-cell function (anti-CD3 response $>$ 1000 cpm). The mean follow-up time of the nonprogressors, defined as the minimum symptom-free interval, was 76.1 (SD $\pm$ 11.4) months. This group was analyzed for HIV-1 RNA copy numbers in serum at seroconversion, i.e., 3.1 (SD $\pm$ 0.9) months, and 39.2 (SD $\pm$ 2.4) months after the last seronegative test. Levels of p24 antigen in serum, CD4+ cell counts, and virus phenotype were also determined at these time points and at the end of study follow-up. Characteristics of all individuals are presented in Table 2. The group of nonprogressors did not differ in mean age (36.0 years) from the group of progressors (35.4 years). None of the patients received antiretroviral therapy during the period of study.

Serological, immunological, and virological studies

HIV-1 p24 antigen in serum was measured by a solid-phase, sandwich-type enzyme immunoassay (EIA, Abbott Laboratories, North Chicago, IL). CD4+ cells were counted by an indirect immunofluorescence technique using monoclonal antibodies (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) and a flow cytometry system (Coulter EPICS, Luton, Bedfordshire, UK). Virus phenotype was determined by cocultivation of patient PBMC with MT-2 cells (Koot *et al.*, 1992).

Virus stock

Physical, chemical, and biological properties of an HIV-1 viral stock were examined by Layne *et al.* (1992). Briefly, this included the following methods. Viral stocks were prepared by inoculation of H9 cell cultures with the HIV-1 molecular clone HXB3. Aliquots of supernatant were removed and clarified by centrifugation. Quantitative infectivity assays were performed using human PBMC and CEM-SS as target cells and CEM-SS monolayers as indicator cells. The infection of individual target cells by cell free virus was counted and represented as syncytial forming units (SFU). Quantitative determinations of gp120 envelope and p24 core proteins were carried out by ELISA, and protein concentrations were determined against recombinant HIV-1 IIIB gp120 and p24 antigen. Viral RNA polymerase activity was measured by incorporation of [^{3}H]TTP, counted for beta activity, and quantified against recombinant HIV-1 IIIB polymerase.

TABLE 1

CHARACTERISTICS OF SEROCONVERTERS PROGRESSING TO AIDS

Patient	Months[a]	CD4 (10^6/l)	p24-Ag (pg/ml)	Virus phenotype	RNA (copies/ml)	AIDS defining diagnosis[b]
317	2.9	1300	0	NSI	1.6×10^6	KS
	7.0	600	107	↓	1.9×10^5	(20 months)[a]
	12	500	163		1.3×10^6	
	18	700	1071		1.1×10^6	
239	3.0	700	48	NSI	2.4×10^6	*Candida oesophagitis*
	9.5	500	37	↓	1.1×10^6	(22 months)
	15	300	247		5.0×10^6	
	21	100	209		8.8×10^6	
172	3.0	1100	0	NSI	3.2×10^6	KS
	12	1100	0	↓	2.5×10^6	(29 months)
	18	1400	0		1.1×10^7	
	27*	1300	0		6.3×10^6	
1145	2.9	710	0	NSI	3.9×10^6	HIV encephalopathy
	9.5	1300	0	↓	1.8×10^6	(30 months)
	21	700	0		6.3×10^5	
	27*	400	0		6.3×10^5	
571	3.2	800	0	NSI	1.1×10^5	HIV encephalopathy
	12	700	0	↓	1.1×10^5	(35 months)
	21	500	4		3.8×10^5	
	33*	200	756	SI	2.3×10^4	
411	3.0	1060	0	NSI	5.6×10^4	*C. oesophagitis*
	12	800	0	↓	2.5×10^5	(36 months)
	27*	400	0		1.6×10^6	
	34	430	0		1.5×10^6	
159	3.0	500	0	NSI	8.2×10^5	PCP and *C. oesophagitis*
	9.1	700	303	↓	7.5×10^5	(37 months)
	18	500	343		3.8×10^5	
	24	300	585	SI	6.3×10^5	
	33*	200	108	↓	1.6×10^6	
39	3.4	700	32	NSI	1.1×10^5	*C. oesophagitis*
	6.3	1000	80	↓	5.0×10^4	(42 months)
	13	600	427		1.1×10^5	
	25	640	253	SI	5.0×10^5	
	40*	70	377	↓	2.0×10^6	
537	3.0	400	0	NSI	5.0×10^5	KS
	14	400	0	↓	4.0×10^6	(46 months)
	23	300	0		3.8×10^6	
	39*	330	0		3.0×10^6	
	49	450	0		2.0×10^6	
356	5.8	500	0	NSI	1.0×10^6	PCP
	12	600	0	↓	1.8×10^5	(47 months)
	21	400	0		1.3×10^5	
	33*	300	0		7.5×10^5	
	45	200	56		2.3×10^6	
186	3.0	600	0	NSI	8.8×10^4	PCP
	12	900	0	↓	5.0×10^5	(47 months)
	27	500	0		7.5×10^5	
	36*	300	0		1.8×10^6	
	45	210	0		2.5×10^5	
569	3.2	500	0	NSI	8.8×10^5	KS
	12	1000	0	↓	1.8×10^5	(53 months)
	23	400	0		3.8×10^5	
	35*	300	0		1.9×10^5	
	48	350	9		1.0×10^6	

TABLE 1—*Continued*

Patient	Months[a]	CD4 (10^6/l)	p24-Ag (pg/ml)	Virus phenotype	RNA (copies/ml)	AIDS defining diagnosis[b]
424	10.5	660	0	NSI	4.2×10^4	*C. oesophagitis*
	13	900	0	↓	1.8×10^4	(54 months)
	23	500	0		2.0×10^5	
	33	340	100		3.8×10^5	
	48*	230	69		3.8×10^4	
746	3.0	1200	0	NSI	5.0×10^5	Generalized HSV infection
	12	700	0	↓	3.8×10^4	(56 months)
	24	700	0		3.8×10^4	
	38*	400	0	SI	1.0×10^4	
	53	180	42	↓	3.8×10^4	
495	3.0	400	0	NSI	2.0×10^5	*C. oesophagitis*
	12	300	21	↓	3.8×10^5	(57 months)
	24	400	31		3.0×10^5	
	39*	300	0		7.5×10^4	
	54	160	2020	SI	2.5×10^5	
	56	160	92	↓	5.0×10^6	
208	3.0	600	0	NSI	1.5×10^5	PCP
	9.0	800	0	↓	1.8×10^4	(59 months)
	22	400	0	SI	5.0×10^4	
	42*	130	49	↓	1.1×10^6	
	57	40	104		3.7×10^5	
412	3.3	1100	0	NSI	8.4×10^5	KS
	12	1000	0	↓	5.0×10^5	(61 months)
	28*	900	0		3.8×10^5	
	46	550	0		5.0×10^5	
	61	500	0		3.8×10^6	
224	3.0	500	0	NSI	2.0×10^5	PCP
	21	700	0	↓	1.8×10^4	(65 months)
	42*	580	99		6.3×10^4	
	51	480	61	SI	1.6×10^4	
	63	110	779	↓	2.0×10^4	
450	3.0	300	0	NSI	$<1.0 \times 10^4$	KS
	18	1100	0	↓	$<1.0 \times 10^4$	(69 months)
	43*	500	0		$<1.0 \times 10^4$	
	64	140	0	SI	1.3×10^5	
	67	70	37	↓	1.1×10^5	
140	3.2	900	0	NSI	1.1×10^6	KS
	15	1400	0	↓	3.8×10^5	(72 months)
	30*	500	0		8.8×10^5	
	51	310	0		7.5×10^4	
	69	150	0		2.3×10^4	

Note. * Time point used for comparative analysis with nonprogressing controls.

[a] Months after last seronegative sample.

[b] KS, Kaposi's sarcoma; PCP, *Pneumocystis carinii* pneumonia; HSV, Herpes simplex virus.

Finally, the absolute number of HIV particles was determined by electron microscopy.

Nucleic acid isolation

Nucleic acid was isolated from 100 μl serum stored at −70°C (Boom *et al.*, 1990). Serum samples were lysed in 5.25 *M* guanidinium thiocyanate, 50 m*M* Tris/HCl, pH 6.4, 20 m*M* EDTA, and 1.3% w/v Triton X-100. Nucleic acid was bound by 40 μl activated silica (1 mg/ml size selected suspension in 0.1 *N* HCl). Silica particles were washed twice with 5.25 *M* guanidinium thiocyanate, 50 m*M* Tris/HCl, pH 6.4, twice with 70% ethanol, and once with acetone. Nucleic acid was eluted in 50 μl distilled water, aliquoted in 2-μl portions, and stored at −70°C.

Plasmids and RNA synthesis

Plasmid pGEM3p24, containing a 1491-bp fragment of the HIV-1 pv22 sequence (comprising *gag* and part of

TABLE 2

CHARACTERISTICS OF NONPROGRESSING CONTROLS

Patient	Months[a]	CD4 (10^6/l)	p24-Ag (pg/ml)	Virus phenotype	RNA (copies/ml)	AIDS defining diagnosis[b]
1024	0.2	750	0	NSI	6.5×10^5	
	37	440	0	↓	6.8×10^4	
	52	430	0			
545	4.1	260	0	NSI	4.0×10^5	
	40	370	0	↓	1.0×10^4	
	58	390	1			
138	3.4	460	0	NSI	2.1×10^6	KS
	39	210	0	SI	5.0×10^5	(66 months)[a]
	63	150	1	↓		
170	3.0	570	0	NSI	5.0×10^4	
	40	570	0	↓	3.8×10^4	
	64	580	0			
1160	4.2	840	0	NSI	1.1×10^7	
	43	690	0	↓	1.0×10^4	
	68	630	0			
82	3.1	600	0	NSI	3.1×10^5	
	40	520	12	↓	1.0×10^4	
	73	430	11			
715	1.8	410	0	NSI	5.5×10^5	
	38	460	0	↓	1.0×10^4	
	73	500	0			
1171	4.1	590	0	NSI	6.3×10^5	
	41	460	0	↓	$<1.0 \times 10^4$	
	73	600	0			
171	2.9	750	0	NSI	5.0×10^5	
	39	560	0	↓	3.8×10^4	
	74	370	38			
658	3.3	950	0	NSI	8.1×10^5	
	39	640	40	↓	1.0×10^4	
	74	630	3			
26	3.0	440	0	NSI	8.0×10^4	
	38	390	0	↓	8.0×10^4	
	75	310	0			
1	3.2	770	18	NSI	3.8×10^6	
	40	700	15	↓	1.1×10^6	
	76	490	85			
594	3.2	900	0	NSI	1.0×10^4	CMV colitis
	39	990	0	↓	1.0×10^4	(78 months)
	77	170	0	SI		
1140	3.7	720	0	NSI	1.0×10^5	
	40	400	0	↓	8.5×10^5	
	78	600	0			
207	2.9	730	0	NSI	3.3×10^6	
	40	580	0	↓	2.3×10^5	
	79	340	0			
434	3.0	720	0	NSI	7.5×10^4	
	39	420	0	↓	1.3×10^5	
	87	370	0			
57	4.3	790	0	NSI	4.8×10^4	
	42	540	0	↓	1.0×10^4	
	89	490	0			

TABLE 2—*Continued*

Patient	Months[a]	CD4 (10^6/l)	p24-Ag (pg/ml)	Virus phenotype	RNA (copies/ml)	AIDS defining diagnosis[b]
709	3.3	1110	0	NSI	1.0×10^4	
	31	690	0	↓	$<1.0 \times 10^4$	
	89	770	0			
169	3.0	1140	0	NSI	2.5×10^5	
	38	950	0	↓	6.3×10^4	
	90	390	12			
16	3.0	950	0	NSI	2.5×10^5	
	39	610	0	↓	7.0×10^5	
	92	550	0			
90	2.7	460	0	NSI	$<1.0 \times 10^4$	
	42	880	0	↓	$<1.0 \times 10^4$	
	95	940	0			

[a] *Months after last seronegative sample.*
[b] CMV, cytomegalovirus.

the *pol* region of the HIV-1 genome (nucleotides 1195 to 2686) (Muesing *et al.,* 1985), was used to construct a mutant plasmid, pGEM3RAN. A unique *SphI–PstI* fragment (positions 1428 to 1456) was exchanged for a randomized version of the original HIV-1 *pv22* sequence: 5′ CTG. CAG. ACA. GTG. TAG. ATA. GAT. GAC. AGT. CGC.-ATG.C.

In vitro RNA was generated from pGEM3RAN (Q-RNA) using SP6 RNA polymerase and treated with DNase to remove the plasmid. The Q-RNA was purified by phenol extraction and ethanol precipitation and quantified on slot blot by comparison with a dilution series of denatured plasmid DNA, which had been quantified spectrophotometrically.

Quantitative NASBA

Ten-fold dilutions ranging from 10^2 to 10^6 molecules of Q-RNA were made and mixed with the 2 μl aliquoted nucleic acid isolated from the sera. Reaction mixture (19 μl) containing 40 m*M* Tris/HCl, pH 8.5, 42 m*M* KCl, 12 m*M* $MgCl_2$, 5 m*M* DTT, 15% v/v DMSO, 1 m*M* each dNTP, 2 m*M* each NTP, 4 U RNA guard, 0.2 μ*M* primer 1 (5′ AAT. TCT. AAT. ACG. ACT. CAC. TAT. AGG. GTG.-CTA.TGT.CAC.TTC.CCC.TTG.GTT.CTC.TCA), and 0.2 μ*M* primer 2 (5′ AGT.GGG.GGG.ACA.TCA.AGC.AGC.CAT.G-CA.AA) was added. Samples were incubated at 65°C for 5 min to allow primer annealing and subsequently cooled down to 41°C. Amplification was started by adding 2 μl enzyme mixture containing 2.6 μg BSA, 0.1 U RNase H, 40 U T7 RNA polymerase, and 8 U AMV-reverse transcriptase. Reactions were incubated for 75 min at 41°C in a total volume of 25 μl. For the quantification of every patient sample two negative controls were added: one negative control was included from nucleic acid isolation and a second negative control was added during amplification.

Nonradioactive bead-based detection assay

To detect and determine the NASBA Q-RNA and wild-type RNA amplificate ratio a bead-based, colorimetric assay was performed. Paramagnetic, 2.8-μm polystyrene beads coated with streptavidin (Dynal Inc, Great Neck, NY) were treated as follows. One-hundred microliters ($6-7 \times 10^7$) beads were washed twice with 200 μl 1× PBS/0.1% BSA and resuspended in 100 μl 1× PBS/0.1% BSA. Washed beads were incubated for 1 hr at room temperature with 300 pmol of an HIV-1 specific, biotinylated capture probe (5′ TGT.TAA.AAG.AGA.CCA.TCA.ATG.AGG.A) and subsequently washed once with 200 μl 5× *SSPE* (750 m*M* NaCl, 50 m*M* NaH_2PO_4, 5 m*M* EDTA, pH 7.4)/0.1% SDS and once with 200 μl 1× PBS/0.1% BSA. The beads were resuspended in 100 μl 1× PBS/0.1% BSA.

Five microliter beads, 5 μl of NASBA amplification product, and 50 μl hybridization buffer (5× SSPE, 0.1% SDS, 0.1% blocking reagent, 10 μg/ml salmon sperm DNA) were incubated for 30 min at 45°C. Next, beads were washed twice with 100 μl 2× SSC/0.1% BSA. Hybridization with 1 μl $5-6 \times 10^{-7}$ *M* detection probe of which 10% was HRP (horseradish peroxidase)-labeled was performed in 50 μl hybridization buffer for 30 min at 45°C (wild-type probe, 5′ GAA.TGG.GAT.AGA.GTG.CAT.-CCA.GTG.CAT.G, or Q-probe, 5′ GAC.AGT.GTA.GAT.-AGA.TGA.CAG.TCG).

The bead/capture probe/target/detection probe complex was washed once with 100 μl 2× SSC/0.1% BSA, once with 100 μl TBST (100 m*M* Tris/HCl, pH 7.5, 150 m*M* NaCl, 0.2% Tween 20), and twice with 100 μl TBS (100 m*M* Tris/HCl, pH 7.5, 150 m*M* NaCl). A color substrate (100 μl TMB (3,3′,5,5′-tetramethyl-benzidin-dihydrochlorid-dihydrate)/peroxide solution) was added to the samples and incubated for 3 min. The reaction was stopped with 50 μl of 250 m*M* oxalate. The absorbance of 100 μl of the color reaction was read at 450 nm on a

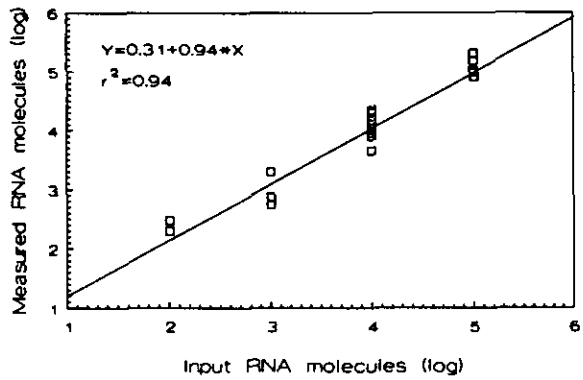

FIG. 1. Quantification of *in vitro* generated HIV-1 wild-type RNA. Known amounts of wild-type HIV-1 RNA (10^2, 10^3, 10^4, and 10^5 molecules) were quantified with multiple replicates for each point using Q-NASBA and the colorimetric detection assay. Line fitting of the input RNA molecules and the measured RNA molecules was done with the program SlideWrite Plus.

plate reader (Micro SLT 510; Organon Teknika, Turnhout, Belgium).

For each sample the wild-type absorbance values were corrected for wild-type background signal (negative controls) and calculated as a percentage of the signal obtained by independently amplified wild-type RNA. The same was done for the absorbance values obtained with the Q-probe. The input amount of wild-type RNA was calculated using the formula

$$\log WT_{input} = (\log Q_{WT=50\%}) + (\log Q_{Q=50\%})/2.$$

Standardization

As standard of assay-to-assay consistency a virus stock was quantified during a number of quantifications of serum samples. RNA was isolated from a 10^{-4} dilution of the virus stock seven times and each isolate was quantified *in duplo*. The mean log RNA of the first set of quantifications was 4.4 (SD ± 0.16). The mean log RNA of the second set of quantifications was 4.5 (SD ± 0.14). The mean difference between these two sets of quantifications was 0.03 log with a standard deviation of 0.09. This difference was insignificant ($P = 0.49$).

The interassay variation of the HIV-1 RNA copy number in the same serum sample was within 0.5 log (mean difference: 0.37 log, SD ± 0.22). This difference was considered not significant according to Graziosi *et al.* (1993).

Statistics

Statistical differences between results were analyzed using the Student's *t* test (two-tailed). A *P* value of 0.05 was considered statistically significant.

RESULTS

Quantification of HIV-1 RNA was first assessed in reconstruction experiments in which known amounts of *in vitro* generated HIV-1 wild-type RNA were mixed with 10-fold serial dilutions of mutant (Q-) RNA and amplified (Fig. 1). Initial wild-type RNA input (10^2, 10^3, 10^4, or 10^5 molecules) gave a 50% reduction in signal for both the wild-type and Q-probe at the addition of an equal number of Q-molecules. The decrease in signals obtained with wild-type and Q-probes was measured with respect to the signal obtained from independently amplified wild-type or Q-RNA. The dynamic range of the procedure was 10^2–10^6 initial RNA molecules with an accuracy of 0.5 log (van Gemen *et al.*, 1993).

The Q-NASBA procedure was further validated by the quantification of a virus stock, which had been extensively characterized by Layne *et al.* (1992). The supernatant of HIV HXB3-infected H9 cell cultures was examined by quantitative electron microscopy, gp120 and p24 antigen ELISA, reverse transcriptase assays, and quantitative infectivity assays (Table 3). RNA was isolated from 100 μl of culture supernatant in duplicate. Thousand-fold, 10,000-fold, and 100,000-fold dilutions of extracted nucleic acid were made and HIV-1 RNA was quantified as described (Fig. 2). The amount of RNA was calculated to be 5.5 (±1.8) × 10^{10} molecules/ml, while the viral stock contained 2.9 (±1.6) × 10^{10} particles/ml as determined by quantitative electron microscopy (Table 3).

Longitudinal follow-up of seroconverters progressing to AIDS

A group of 20 patients progressing to AIDS at 20–72 months after the last seronegative sample was studied longitudinally. Table 1 shows CD4+ cell counts, p24 antigen levels, virus phenotype, the amount of viral RNA, and the clinical diagnosis of all individuals. Virus phenotype analysis revealed that these progressors could be divided into two groups: 12 individuals persistently harboring NSI variants (NSI progressors) and 8 individuals with a conversion from NSI to SI variants (SI progressors). In agreement with a previous publication (Tersmette *et al.*, 1989b) individuals progressing with NSI viruses were more frequently diagnosed with HIV-1 associated malignities, while individuals progressing with SI variants were diagnosed mainly with opportunistic infections.

Within the group of NSI progressors 1 individual was p24 antigen positive (patient 239), 3 switched to p24 antigen positivity (patients 317, 356, and 424), and 8 individuals were persistently p24 antigen negative. CD4+ cell counts declined during follow-up in 10 of the 12 individu-

TABLE 3

PHYSICOCHEMICAL CHARACTERISTICS OF CELL-FREE HIV-1 (HXB3)

Particle density (ml^{-1})	2.9 (±1.6) × 10^{10} [a]
p24 ($g \cdot ml^{-1}$)	1.6 (±0.2) × 10^{-6}
gp120 ($g \cdot ml^{-1}$)	6.9 (±0.4) × 10^{-8}
RT ($g \cdot ml^{-1}$)	6.1 (±0.3) × 10^{-7}
SFU (ml^{-1}) [b]	1.2 (±0.04) × 10^{4}
RNA molecules (ml^{-1})	5.5 (±1.8) × 10^{10}

[a] Mean (±SD).

[b] SFU, syncytial forming units.

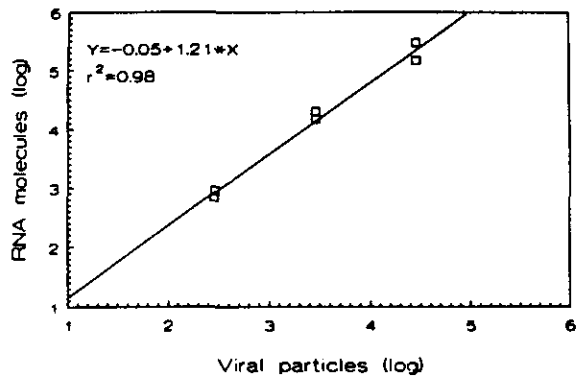

FIG. 2. Quantification of a virus stock produced by HIV HXB3 infected H9 cells. Nucleic acid was extracted from 100 μl culture supernatant in duplicate. Thousand-fold, 10,000-fold, and 100,000-fold dilutions of extracted nucleic acid were subjected to Q-NASBA. Line fitting of input viral particles and measured RNA molecules was done with the program SlideWrite Plus.

als (mean decline 192 ± 141 cells per mm^3/year), but 50% of the individuals still had CD4+ cell counts ≥400 per mm^3 prior to AIDS diagnosis. In all samples obtained from NSI progressors HIV-1 RNA was detected, ranging from 1.8×10^4 (patient 424) to 1.1×10^7 (patient 172) copies/ml serum. In all but 1 of the NSI progressors a rather stable level of viral RNA was observed throughout the infection, with fluctuations within 1 order of magnitude. In patient 140 a decline in HIV-1 RNA copy number of 2 logs was observed during the course of infection. The mean HIV-1 RNA load at seroconversion ($10^{5.78\pm0.70}$/ml) did not differ significantly from the mean RNA load 3 months before AIDS diagnosis ($10^{5.95\pm0.81}$/ml) in this group (Fig. 3).

In the group of SI progressors one individual was persistently p24 antigen positive (patient 39) and all others switched to p24 antigen positivity. CD4+ cell counts declined with a mean 139 ± 82 cells per mm^3/year, but were below 400 per mm^3 in all individuals prior to AIDS diagnosis. HIV-1 RNA could be detected in all but three samples (obtained from one patient (450); Table 1). In all but one individual rather stable levels of viral RNA were observed and no significant changes were found upon conversion of virus phenotype. Sera from individual 450 obtained early in the symptom-free period contained low levels of HIV-1 RNA, but showed a significant rise in HIV-1 RNA coincident with an NSI to SI switch. Comparison of mean RNA load at seroconversion ($10^{4.98\pm1.09}$/ml) and prior to AIDS diagnosis (mean $10^{5.38\pm0.95}$/ml) showed a slight increase, which was not statistically significant (Fig. 3).

Statistical analysis of the serum RNA load at seroconversion and 3 months before AIDS diagnosis revealed no significant differences ($P > 0.05$) between the group of NSI progressors and that of SI progressors (Fig. 3).

Analysis of nonprogressing seroconverters

Twenty-one seroconverters with a mean follow-up time, defined as the minimum symptom-free interval, of 76.1 (SD ± 11.4) months were selected as nonprogressing controls. The first time point tested was at seroconversion, i.e., 3.1 (SD ± 0.9) months following the last seronegative test, and the second test point was 39.2 (SD ± 2.4) months following the last seronegative test. Data obtained at these time points and at the end of study follow-up are given in Table 2. Three individuals (patients 1, 82, and 658) were p24 antigen positive or switched to p24 antigen positivity in the early period of infection and another two individuals (patient 169 and 171) had switched to p24 antigen positivity at the end of follow-up. Sixteen individuals remained p24 antigen negative from seroconversion until the end of follow-up. CD4+ cell counts declined between the first and second test points with a mean 47 (SD ± 67) cells per mm^3/year. All but two individuals persistently harbored NSI virus variants. The PBMC of patient 138 yielded an SI isolate at the second test point, while patient 594 was found to harbor SI virus variants at the end of follow-up. Moreover, these two designated nonprogressors developed AIDS after the end of the study follow-up (see Table 2). The mean RNA level in serum of nonprogressors at seroconversion was $10^{5.33\pm1.04}$/ml. At the later time point HIV-1 RNA copy numbers had declined significantly to a mean $10^{4.42\pm1.10}$/ml ($P = 0.001$) (Fig. 4a).

Comparative analysis of progressors and nonprogressors in the early period of infection

To assess whether progressors and nonprogressors could be distinguished in the early asymptomatic period

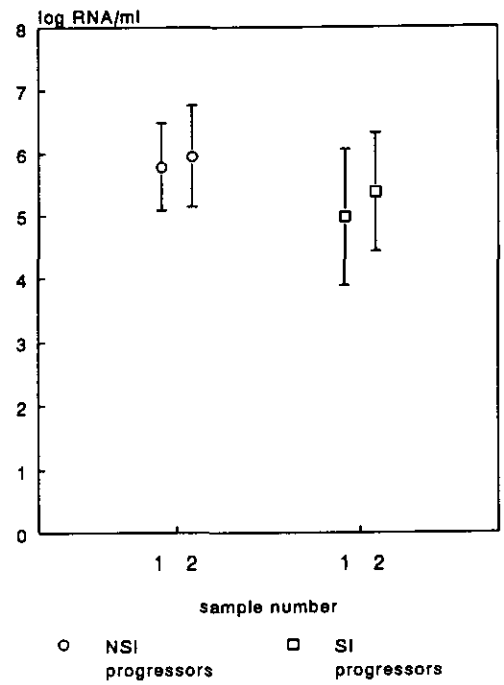

FIG. 3. HIV-1 RNA copy numbers in sera collected at seroconversion (1) and 3 months before AIDS diagnosis (2) from NSI progressors and SI progressors. Error bars indicate the SD.

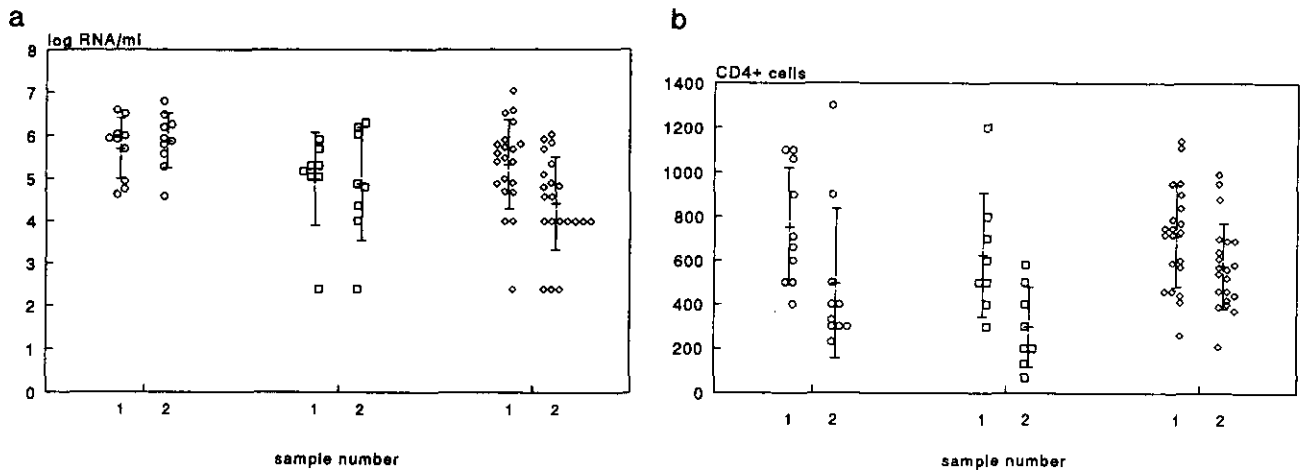

FIG. 4. (a) HIV-1 RNA copy numbers in serum and (b) CD4+ cell counts in blood collected at seroconversion (1) and 3 years thereafter (2) from NSI progressors, SI progressors, and nonprogressors. ○, NSI progressors; □, SI progressors; ◇, non progressors; +, mean ± SD.

of infection on the basis of serum HIV-1 RNA load, virus phenotype, and/or CD4+ cell counts, comparative analysis was performed. The characteristics were compared at seroconversion (i.e., 4.1 (SD ± 2.4) months for NSI progressors, 3.1 (SD ± 0.1) months for SI progressors, and 3.1 (SD ± 0.9) months for nonprogressors after the last seronegative sample) and early in the asymptomatic period of infection (33.0 (SD ± 6.8) months for NSI progressors, 38.8 (SD ± 3.9) months for SI progressors, and 39.2 (SD ± 2.4) months for the nonprogressors following the last seronegative test). Patients 317 and 239 were excluded from analysis because of progression to AIDS within 24 months. In the first three years of follow-up 5 of the progressors (28%) and 1 of the nonprogressors (5%) showed an NSI to SI conversion. Comparison of HIV-1 RNA copy numbers at seroconversion found a mean RNA level of $10^{5.70\pm0.70}$ in NSI progressors, $10^{4.98\pm1.09}$ in SI progressors, and $10^{5.33\pm1.04}$ in nonprogressors, which did not differ significantly ($P > 0.2$) (Fig. 4a). The amount of CD4+ cells was also comparable in the three groups (mean ± SD: 753 ± 267 cells/mm^3 in NSI progressors, 625 ± 282 cells/mm^3 in SI progressors, and 710 ± 231 cells/mm^3 in nonprogressors) (Fig. 4b).

At the later time point HIV-1 RNA copy numbers in the sera of nonprogressors had declined significantly ($P = 0.001$). This change resulted in a substantial difference in the level of RNA between NSI progressors ($10^{5.88\pm0.63}$) and nonprogressors ($10^{4.42\pm1.10}$) ($P = 0.0005$). RNA copy numbers in sera from SI progressors (mean ± SD, $10^{4.87\pm1.33}$) did not differ significantly ($P > 0.2$) from the nonprogressors (Fig. 4a). However, the mean number of CD4+ cells in the peripheral blood of SI progressors (298 ± 181/mm^3) was significantly lower ($P = 0.002$) than the mean number of CD4+ cells of nonprogressors (575 ± 196 cells/mm^3) at this time point, whereas CD4+ cell counts of NSI progressors (496 ± 340 cells/mm^3) and nonprogressors did not differ significantly ($P > 0.2$) (Fig. 4b).

DISCUSSION

Progression to AIDS in seroconverters without established prognostic markers like the isolation of SI variants or p24 antigenemia (NSI progressors) is associated with persistently high levels of genomic HIV-1 RNA copy numbers in serum. Stable HIV-1 RNA copy numbers were also detected in progressors with an NSI to SI phenotypic switch (SI progressors). The conversion of virus phenotype was not accompanied by significant changes in HIV-1 RNA levels, except in one individual. These results indicate that at seroconversion rapid progressors generally retain high HIV-1 RNA levels persisting throughout the symptom-free period. A recent study by Piatak *et al.* (1993) showed increasing numbers of HIV-1 RNA copies with disease progression. This study was performed on transectional samples obtained from different individuals. The samples obtained from symptomatic patients most probably represent a homogeneous group; however, the samples obtained from asymptomatic individuals represent a heterogeneous population because these individuals may progress to AIDS within a wide range of time. The data obtained from six individuals followed longitudinally (Piatak *et al.*, 1993) showed stable HIV-1 RNA copy numbers and are in agreement with the results described in the present study. To establish whether the HIV-1 RNA load was predictive of disease progression, the amount of HIV-1 RNA in serum from nonprogressing controls was determined at seroconversion and early in the asymptomatic period. Previously it was demonstrated that in primary HIV-1 infection high copy numbers of viral RNA and infectious viral particles are present (Clark *et al.*, 1991; Daar *et al.*, 1991; van Gemen *et al.*, 1993; Piatak *et al.*, 1993). HIV-1 RNA levels were shown to peak before seroconversion and decline with the raise of antibodies against HIV-1. The RNA copy numbers found in the present study correspond to the previously described observation that following the acute retroviral syndrome, the

HIV-1 RNA copy number is reduced to a level of about 10^5/ml (van Gemen *et al.*, 1993; Piatak *et al.*, 1993). However, our data strongly suggest that seroconversion signifies only a partial reduction in virus load and that a substantial amount of virus continues to be produced in the initial phase of infection, independent of the immunological or clinical prognosis. At seroconversion, neither the HIV-1 RNA copy number nor the amount of CD4+ cells was predictive of disease progression. Furthermore, all individuals carried NSI virus variants as determined by PBMC culture and sequence analysis of the V3 loop (Koot *et al.*, 1993; Kuiken *et al.*, 1993). Three years later significant changes in HIV-1 RNA load and CD4+ cell counts were observed among progressors and nonprogressors. NSI progressors and nonprogressors had similar CD4+ cell counts in peripheral blood, but these groups could be distinguished on the basis of RNA copy numbers. On the contrary, SI progressors and nonprogressors had comparable levels of HIV-1 RNA in serum, but could be separated on the basis of CD4+ cell count and/or virus phenotype. A recent study by Saksela *et al.* (1994) showed that abundant expression of HIV-1 mRNA in PBMC of infected individuals was predictive of active disease progression. HIV-1 infected individuals with normal levels of CD4+ cells and similar clinical indices were analyzed for expression of HIV-1 mRNA in PBMC and it was found that individuals with low or undetectable amounts of HIV-1 mRNA continued to have normal numbers of CD4+ cells and no signs of clinical disease during the subsequent 5 years, whereas individuals with abundant expression of HIV-1 mRNA in their PBMC showed accelerated disease progression within the next 2 years. These results are in accordance with the differences in serum RNA load found between NSI progressors and nonprogressors in our study.

A high virus load during the entire symptom-free period apparently results in the development of AIDS, judging from the stable and high circulating virus levels in symptom-free individuals who progressed to AIDS rapidly. The well-established rise in HIV-1 infected and HIV-1 producing peripheral blood cells, which heralds disease progression (Schnittman *et al.*, 1990; Bagasra *et al.*, 1992; Michael *et al.*, 1992; Connor *et al.*, 1993; Gupta *et al.*, 1993), has apparently only a minor impact on the overall virus load. In contrast to the high and stable HIV-1 RNA levels of progressors, the number of HIV-1 RNA copies in sera of nonprogressors declined significantly early in infection. Our data indicate that such a decline in HIV-1 RNA copy numbers during the symptom-free period of infection is strongly associated with delayed onset of AIDS. Since CD4+ cell decline was not completely halted by a declining virus load in our study population, we do not expect that the natural host defense against virus multiplication is generally powerful enough to prevent AIDS all together. Our results show that relatively early in HIV-1 infection progressors and nonprogressors can be distinguished: in the case of NSI progressors on the basis of serum HIV-1 RNA load and in the case of SI progressors on the basis of CD4+ cell decline.

ACKNOWLEDGMENTS

The authors thank Margreet Bakker, Geert Haverkamp, Rianne Schukkink, Gemma Goossens, Peter Lens, and Wessel Lageweg for their help with several aspects of this work. The research was supported in part by the Ministerie van Welzijn, Volksgezondheid en Cultuur, Grants 91009, 28-2370 and 94-22.

REFERENCES

ALLAIN, J. P., PAUL, D. A., LAURIAN, Y., and SENN, D. (1986). Serological markers in early stages of human immunodeficiency virus infection in haemophiliacs. *Lancet* **1**, 1233–1236.

ALLAIN, J. P., LAURIAN, Y., PAUL, D., VERROUST, F., LEUTHER, M., GAZENGEL, C., SENN, D., LARRIEU, M. J., and BOSSER, C. (1987). Long-term evaluation of HIV antigen and antibodies to p24 and gp41 in patients with haemophilia. *N. Engl. J. Med.* **317**, 1114–1121.

AOKI-SEI, S., YARCHOAN, R., KAGEYAMA, S., HOEKZEMA, D. T., PLUDA, J. M., WYVILL, K. M., BRODER, S., and MITSUYA, H. (1992). Plasma HIV-1 viremia in HIV-1 infected individuals assessed by polymerase chain reaction. *AIDS Res. Hum. Retroviruses* **8**, 1263–1270.

ÅSJÖ, B., MORFELDT-MÅNSON, L., ALBERT, J., BIBERFELD, G., KARLSSON, A., LIDMAN, K., and FENYÖ, E. M. (1986). Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *Lancet* **2**, 660–662.

BAGASRA, O., HAUPTMAN, S. P., LISCHNER, H. W., SACHS, M., and POMERANTZ, R. J. (1992). Detection of human immunodeficiency virus type 1 provirus in mononuclear cells by in situ polymerase chain reaction. *N. Engl. J. Med.* **326**, 1385–1391.

BAGNARELLI, P., MENZO, S., MANZIN, A., GIACCA, M., VARALDO, P. E., and CLEMENTI, M. (1991). Detection of human immunodeficiency virus type 1 genomic RNA in plasma samples by reverse-transcription polymerase chain reaction. *J. Med. Virol.* **34**, 89–95.

BALTIMORE, D., and FEINBERG, M. B. (1989). HIV revealed: Toward a natural history of the infection. *N. Engl. J. Med.* **321**, 1673–1675.

BOOM, R., SOL, C. J. A., SALIMANS, M. M. M., JANSEN, C. L., WERTHEIM-VAN DILLEN, P. M. E., and VAN DER NOORDAA, J. (1990). A rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**, 495–503.

BRUISTEN, S., VAN GEMEN, B., KOPPELMAN, M., RASCH, M., VAN STRIJP, D., SCHUKKINK, R., BEYER, R., WEIGEL, H., LENS, P., and HUISMAN, H. (1993). Detection of HIV-1 distribution in different blood fractions by two nucleic acid amplification assays. *AIDS Res. Hum. Retroviruses* **9**, 259–265.

CLARK, S. J., SAAG, M. S., DECKER, W. D., CAMPBELL-HILL, S., ROBERSON, J. L., VELDKAMP, P. J., KAPPES, J. C., HAHN, B. H., and SHAW, G. M. (1991). High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N. Engl. J. Med.* **324**, 954–960.

CONNOR, R. I., MOHRI, H., CAO, Y., and HO, D. D. (1993). Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. *J. Virol.* **67**, 1772–1777.

DAAR, E. S., MOUDGIL, T., MEYER, R. D., and HO, D. D. (1991). Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **324**, 961–964.

DE WOLF, F., LANGE, J. M. A., HOUWELING, J. T. M., COUTINHO, R. A., SCHELLEKENS, P. T., VAN DER NOORDAA, J., and GOUDSMIT, J. (1988). Numbers of CD4+ cells and the levels of core antigens of and antibodies to the human immunodeficiency virus as predictors of AIDS among seropositive homosexual men. *J. Infect. Dis.* **158**, 615–622.

GOUDSMIT, J., DE WOLF, F., PAUL, D. A., EPSTEIN, L. G., LANGE, J. M. A., KRONE, W. J. A., SPEELMAN, H., WOLTERS, E. C., VAN DER NOORDAA, J., OLESKE, J. M., VAN DER HELM, H. J., and COUTINHO, R. A. (1986). Expression of human immunodeficiency virus antigen (HIV-Ag) in serum

and cerebrospinal fluid during acute and chronic infection. *Lancet* **2,** 177–180.

GRAZIOSI, C., PANTALEO, G., BUTINI, L., DEMAREST, J. F., SAAG, M. S., SHAW, G. M., and FAUCI, A. S. (1993). Kinetics of human immunodeficiency virus type 1 (HIV-1) DNA RNA synthesis during primary HIV-1 infection. *Proc. Natl. Acad. Sci. USA* **90,** 6405–6409.

GUPTA, P., KINGSLEY, L., ARMSTRONG, J., DING, M., COTTRILL, M., and RINALDO, C. (1993). Enhanced expression of human immunodeficiency virus type 1 correlates with the development of AIDS. *Virology* **196,** 586–595.

HO, D. D., MOUDGIL, T., and ALAM, M. (1989). Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N. Engl. J. Med.* **321,** 1621–1625.

HOLODNIY, M., KATZENSTEIN, D. A., SENGUPTA, S., WANG, A. M., CASIPIT, C., SCHWARTZ, D. H., KONRAD, M., GROVES, E., and MERIGAN, T. C. (1991). Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. *J. Infect. Dis.* **163,** 862–866.

JURRIAANS, S., DEKKER, J. T., and DE RONDE, A. (1992). HIV-1 viral DNA load in peripheral blood mononuclear cells from seroconverters and long-term infected individuals. *AIDS* **6,** 635–541.

KEET, I. P. M., KRIJNEN, P., KOOT, M., LANGE, J. M. A., MIEDEMA, F., GOUDSMIT, J., and COUTINHO, R. A. (1993). Predictors of rapid progression to AIDS in HIV-1 seroconverters. *AIDS* **7,** 51–57.

KIEVITS, T., VAN GEMEN, B., VAN STRIJP, D., SCHUKKINK, R., DIRCKS, M., ADRIAANSE, H., MALEK, L., SOOKNANAN, R., and LENS, P. (1991). NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. *J. Virol. Methods* **35,** 273–286.

KOOT, M., VOS, A. H. V., KEET, R. P. M., DE GOEDE, R. E. Y., DERCKSEN, M. W., TERPSTRA, F. G., COUTINHO, R. A., MIEDEMA, F., and TERSMETTE, M. (1992). HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. *AIDS* **6,** 49–54.

KOOT, M., KEET, I. P. M., VOS, A. H. V., DE GOEDE, R. E. Y., ROOS, M. T. L., COUTINHO, R. A., MIEDEMA, F., SCHELLEKENS, P. T. A., and TERSMETTE, M. (1993). Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Ann. Intern. Med.* **118,** 681–688.

KUIKEN, C. L., ZWART, G., BAAN, E., COUTINHO, R. A., VAN DEN HOEK, J. A. R., and GOUDSMIT, J. (1993). Increasing antigenic and genetic diversity of the V3 variable domain of the human immunodeficiency virus envelope protein in the course of the AIDS epidemic. *Proc. Natl. Acad. Sci. USA* **90,** 9061–9065.

LAYNE, S. P., MERGES, M. J., DEMBO, M., SPOUGE, J. L., CONLEY, S. R., MOORE, J. P., RAINA, J. L., RENZ, H., GELDERBLOM, H. R., and NARA, P. L. (1992). Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virology* **189,** 695–714.

MICHAEL, N. L., VAHEY, M., BURKE, D. S., and REDFIELD, R. R. (1992). Viral DNA and mRNA expression correlate with the stage of human immunodeficiency virus (HIV) type 1 infection in humans: Evidence for viral replication in all stages of HIV disease. *J. Virol.* **66,** 310–316.

MUESING, M. A., SMITH, D. H., CABRADILLA, C. D., BENTON, C. V., LASKY, L. A., and CAPON, D. J. (1985). Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature* **313,** 450–458.

PHILLIPS, A. N., LEE, C. A., ELFORD, J., JANOSSY, G., TIMMS, A., BOFILL, M., and KERNOFF, P. B. A. (1991). Serial CD4 lymphocyte counts and development of AIDS. *Lancet* **337,** 389–392.

PIATAK, M. J., SAAG, M. S., YANG, L. C., CLARK, S. J., KAPPES, J. C., LUK, K. C., HAHN, B. H., SHAW, G. M., and LIFSON, J. D. (1993). High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* **259,** 1749–1754.

PSALLIDOPOULOS, M. C., SCHNITTMAN, S. M., THOMPSON, L. M., III, BASELER, M., FAUCI, A. S., LANE, H. C., and SALZMAN, N. P. (1989). Integrated proviral human immunodeficiency virus type 1 is present in CD4+ peripheral blood lymphocytes in healthy seropositive individuals. *J. Virol.* **63,** 4626–4631.

SAKSELA, K., STEVENS, C., RUBINSTEIN, P., and BALTIMORE, D. (1994). Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of CD4+ lymphocytes. *Proc. Natl. Acad. Sci. USA* **91,** 1104–1108.

SCADDEN, D. T., WANG, Z. Y., and GROOPMAN, J. E. (1992). Quantitation of plasma human immunodeficiency virus type-1 RNA by competitive polymerase chain reaction. *J. Infect. Dis.* **165,** 1119–1123.

SCHNITTMAN, S. M., GREENHOUSE, J. J., PSALLIDOPOULOS, M. C., BASELER, M., SALZMAN, N. P., FAUCI, A. S., and LANE, H. C. (1990). Increasing viral burden in CD4+ T cells from patients with human immunodeficiency virus (HIV) infection reflects rapidly progressive immunosuppression and clinical disease. *Ann. Intern. Med.* **113,** 438–443.

SHEPPARD, H. W., LANG, W., ASCHER, M. S., VITTINGHOFF, E., and WINKELSTEIN, W. (1993). The characterization of non-progressors: Long-term HIV-1 infection with stable CD4+ T-cell levels. *AIDS* **7,** 1159–1166.

SIMMONDS, P., BALFE, P., PEUTHERER, J. F., LUDLAM, C. A., BISHOP, J. O., and LEIGH BROWN, A. J. (1990). Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J. Virol.* **64,** 864–872.

TERSMETTE, M., GRUTERS, R. A., DE WOLF, F., DE GOEDE, R. E. Y., LANGE, J. M. A., SCHELLEKENS, P. Th. A., GOUDSMIT, J., HUISMAN, J. G., and MIEDEMA, F. (1989a). Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. *J. Virol.* **63,** 2118–2125.

TERSMETTE, M., LANGE, J. M. A., DE GOEDE, R. E. Y., DE WOLF, F., EEFTINCK SCHATTENKERK, J. K. M., SCHELLEKENS, P. Th. A., COUTINHO, R. A., HUISMAN, J. G., GOUDSMIT, J., and MIEDEMA, F. (1989b). Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. *Lancet* **1,** 983–985.

VAN GEMEN, B., KIEVITS, T., SCHUKKINK, R., VAN STRIJP, D., MALEK, L. T., SOOKNANAN, R., HUISMAN, H. G., and LENS, P. (1993). Quantification of HIV-1 RNA in plasma using NASBA during HIV-1 primary infection. *J. Virol. Methods* **43,** 177–188.

ZHANG, L. Q., SIMMONDS, P., LUDLAM, C. A., and LEIGH BROWN, A. J. (1991). Detection, quantification and sequencing of HIV-1 from the plasma of seropositive individuals and from factor VIII concentrates. *AIDS* **5,** 675–681.