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Stability in Controlling Viral Replication Identifies Long-Term Nonprogressors as a Distinct Subgroup among Human Immunodeficiency Virus Type 1-Infected Persons

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Long-term nonprogressors (LTNPs) of human immunodeficiency virus type 1 (HIV-1) infection are characterized by low levels of HIV-1 replication and viral load. However, it has not been established whether they differ in this regard from progressors from the very early stage of infection. By studying peripheral blood mononuclear cell (PBMC) specimens from a longitudinally monitored cohort of HIV-1-infected men, we found that HIV-1 proviral copy numbers and HIV-1 mRNA expression levels as low or lower than those seen in seven carefully selected LTNPs were commonly observed in specimens collected soon after seroconversion from 28 subjects who became infected while under observation. However, only the LTNPs were able to stably maintain such an efficient viral control over time. Because of the instability of the early control of HIV-1 replication, the predictive value of HIV-1 mRNA expression in PBMCs at postseroconversion was found to be limited but significantly increased during the first year of infection. Besides their diagnostic implications, these data support the idea that LTNPs may be a pathophysiologically distinct subgroup among persons infected with HIV-1.

The median time to clinical AIDS after becoming infected with human immunodeficiency virus type 1 (HIV-1) is 9 to 10 years (27), but the duration of this period is highly variable. Despite the relatively lengthy incubation period of AIDS, signs of progressing immunodeficiency can be observed in many HIV-1-infected persons much earlier, typically evidenced by a gradual loss of circulating CD4 T lymphocytes (median loss, 60 cells per microliter per year [14]). However, a small proportion of infected persons, referred to as long-term nonprogressors of HIV-1 infection (LTNPs), are able to maintain an apparently healthy immune system and live longer than a decade without showing any signs of HIV-1 disease progression.

It has been hypothesized that LTNPs may differ biologically from other HIV-1-infected persons, instead of representing individuals who are experiencing typical but unusually slow HIV-1 disease progression. Epidemiological studies, however, have thus far not indicated that the survival curves in HIV-1-infected populations would be biphasic (27) and thus rather seem to support a more stochastic model. Nevertheless, examples of an apparently distinct virus-host interaction have been reported among LTNPs by showing that the lack of disease progression may in some cases be due to an attenuated strain of HIV-1 lacking a functional *nef* gene (4, 13). An inactive viral *nef* gene, however, appears to explain the lack of disease progression only in a small proportion of LTNPs (12).

LTNPs have been shown to have lower viral loads than most progressors (1, 19), presumably due to their robust antiviral responses. HIV-1 replication and viral load are generally at their minimum soon after the onset of the acute viremia (3, 7). It has been suggested that the success in the early containment of HIV-1 infection plays an important role in establishing the magnitude of a subsequently relatively stable viral load (9) determined by a dynamic balance between virus production and clearance (10, 26). HIV-1 load in infected persons has considerable pathophysiological and clinical significance, since it is known that HIV-1 mRNA expression and proviral copy numbers in peripheral blood mononuclear cells (PBMCs) as well as the cell-free viral load in plasma correlate with the rate of subsequent disease progression and time to AIDS (6, 11, 15–18, 22, 23).

Although an equilibrium between HIV-1 replication and clearance may be established in some persons early in the course of infection, levels of HIV-1 mRNA in PBMCs of certain individuals may initially be relatively low but dramatically increase thereafter, followed by an accelerated loss of CD4 cells and development of AIDS (22). Such low levels of HIV-1 mRNA in early specimens from progressors might indicate that an efficient suppression of HIV replication is not unique to LTNPs, but rather may be a relatively common occurrence during the early asymptomatic stage of HIV-1 infection.

In this study we show that an apparently efficient control of HIV-1 replication, similar to that observed in LTNPs, was common among recent seroconverters but that only the LTNPs retained such low levels of HIV-1 replication throughout the years of observation, suggesting that they may differ fundamentally from progressors in their virus-host relationship.

Sample selection and quantitative analyses. The specimens tested in this study were collected as a part of a long-term prospective study of a cohort of 408 HIV-1-infected men (Prospective AIDS Study [PAS]) carried out by the New York Blood Center (24, 25). The group of 28 seroconverters for this study was formed by selecting from among the 46 PAS participants who became HIV-1 antibody positive while under observation those who were subsequently monitored for at least 3 years. From each of these 28 men three sets of PBMC specimens were tested, including their first HIV-1 antibody-positive specimen, as well as the specimens collected approximately 1 and 3 years thereafter. Since HIV-1 replication in some of the earliest HIV-1 antibody-positive specimens might

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still be elevated due to the acute infection, in cases when this specimen contained more (>twofold) HIV-1 mRNA than the 1-year specimen, the next available specimen was also tested. In three cases (S17, S22, and S25), this second specimen was found to contain less HIV-1 mRNA than the first one and thus was considered the representative postseroconversion specimen. The LTNP group (n, 7) was formed by selecting from among the 408 PAS cohort members all men whose $CD4^+$ cells count, despite >10 years of serologically verified HIV-1 infection in the absence of any antiretroviral therapies, was still higher than 500/μl and had not showed an apparent decreasing trend during their follow up (see Table 2). From each of these LTNPs four specimens were tested, including the first and last ones available (typically separated by 7 years) as well as two additional specimens between these (see Table 2).

Total cellular RNA and DNA were extracted from PBMC specimens using TRI reagent (Molecular Research Center, Inc., Cincinnati, Ohio). RNA preparations were treated with DNase, purified, and subjected to reverse transcription PCR as described before (21). Conditions used in all PCR assays were identical to those described previously (21, 22), except for the following modifications. To optimize linear quantitation of multiply spliced (MS) mRNA in specimens with low levels of viral replication, a nested PCR strategy was developed in which both rounds of amplification utilized a common antisense primer: 5′-TTC CTT CGG GCC TGT CGG GTC GG-3′, while the outer-sense MS primer was 5′-CTT AGG CAT CTC CTA TGG CAG GAA-3′ and the inner one was: 5′-TCC TAT GGC AGG AAG AAG CGG AG-3′. After the first 25 cycles of amplification 5 μl of this reaction mixture was added into triplicate nested reactions which were cycled for 11, 17, or 22 rounds. The secondary reaction in which the signal was in the optimal linear range of amplification (compared with a standard dilution curve ranging from 100,000 to 64 copies of in vitro-transcribed RNA templates [22] in 1 μg of total PBMC RNA) was subsequently used to estimate its MS HIV mRNA copy number. Unspliced (US) mRNA PCR was performed as described previously (21) but was cycled for 31 rounds of 94°C for 40 s and 69°C for 1 min.

To quantitate cellular proviral DNA load, 1 μg of PBMC DNA ($\sim 1.4 \times 10^5$ cells) in 2.5 μl of water was added to a 50-μl PCR mixture and cycled for 33 cycles of 94°C for 40 s and 69°C for 1 min, using HIV-1 *gag*-specific primers 5′-TCT CTA GCA GTG GCC CCC GAA CA-3′ (sense) and 5′-TTC TTT CCC CCT GGC CTT AAC CG-3′ (antisense). When necessary (specimens with <200 proviral copies per 10^6 PBMCs), PCR amplification was repeated using 38 cycles. Standards for quantitation were generated by mixing DNA from uninfected PBMCs with DNA from ACH-2 cells (5) in ratios ranging from $1{:}10^2$ to $1{:}10^5$. The quantity and quality of DNA was monitored by parallel amplification of a 150-bp GAPDH gene-specific fragment, using primers 5′-TGG TAT CGT GGA AGG ACT CAT GGT-3′ (sense) and 5′-GAA AGG AAA TTA TGG GAA AGC CAG-3′ (antisense). To measure plasma viral load, genomic HIV-1 RNA was extracted from 100 μl of plasma using TRI reagent, followed by reverse transcription PCR and quantitation of amplification products as described for US HIV-1 mRNA. The sensitivity of the assay was determined to be 500 virions per ml of plasma.

Overlap in levels of HIV-1 mRNA and DNA in PBMCs of LTNPs and recent seroconverters. In agreement with our previous results of HIV-1 mRNA expression in PBMCs of asymptomatic subjects (21, 22), notable differences in the relative amounts of MS and US HIV-1 mRNA were rare (>threefold differences in their molarity were seen only in 4 of the 75 total [5.3%] tested specimens which had detectable levels of both)

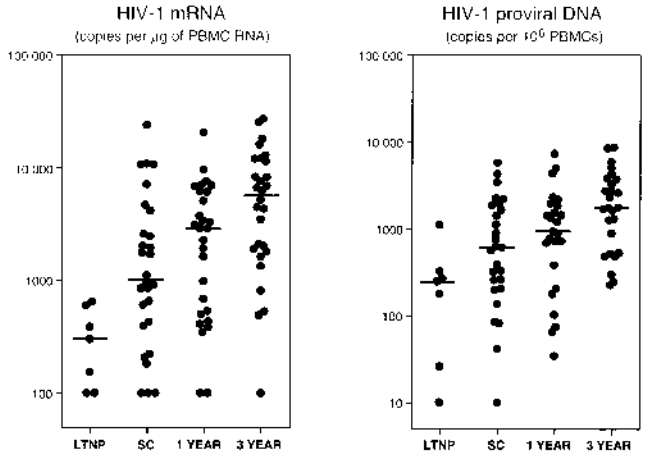

FIG. 1. Comparison of HIV-1 mRNA expression and proviral DNA loads in PBMCs from recent specimens from 7 LTNPs obtained after >10 years of HIV infection and from 28 men collected soon after their seroconversion (SC) and at 1 and 3 years thereafter. The horizontal bars in the figures denote median values in each set of specimens. HIV-1 mRNA expression is shown as the average copy number of US and MS mRNA species per microgram of total RNA in PBMCs, and the HIV-1 proviral load is shown as DNA copy number per million PBMCs. The dots at the bottom of the graphs represent values which are at or below the threshold of linear quantitation of the assays (100/μg for HIV-1 mRNA and $10/10^6$ for HIV-1 DNA).

and did not show an apparent association with any particular subsequent disease pattern. For clarity, HIV-1 mRNA expression is therefore referred to in the following as their average copy number, doubly negative specimens thus having a value <100 per μg.

Figure 1 shows HIV-1 mRNA and proviral copy numbers in the most recent PBMC specimens from the LTNPs collected in each case after more than 10 years of HIV-1 infection (average >145 months) compared with those of the 28 seroconverters collected 0, 1, and 3 years after they became HIV-1 seropositive. The complete results on quantitation of HIV-1 mRNA and DNA in PBMCs are shown in Tables 1 and 2. As evidenced by the horizontal bars in Fig. 1 representing the median mRNA or DNA copy numbers in each set of specimens, the levels of HIV-1 mRNA expression (median 300/μg) and proviral load (median $244/10^6$ cells) in the LTNPs were lower than in PBMCs collected from the 28 seroconverters at any of the examined time points. The median HIV-1 mRNA copy number in PBMCs of all four sets of LTNP specimens combined was 152/μg (ranging from <100 to 992), and the median proviral DNA load was $88/10^6$ cells (ranging from <10 to 1,402; Table 2).

However, despite the generally lower HIV-1 mRNA and DNA copy numbers in PBMCs of the LTNP specimens, considerable overlap was observed in the distribution of these values and those of the early specimens from the seroconverters. Fourteen of the 28 postseroconversion specimens (50%) had HIV-1 mRNA copy numbers which were within the range observed for the seven LTNPs (less than 1,000/μg), and six of them (21%), including PBMCs from a man who developed AIDS only 3.5 years later, had HIV-1 mRNA levels lower than the median value of the LTNPs. Even greater overlap was seen in the PBMC proviral copy numbers of the postseroconversion specimens and those of the LTNPs (Fig. 1, right). Moreover, since the period of minimal viral replication after the acute phase of HIV-1 infection might be relatively short in some cases, it is likely that even lower levels of mRNA and DNA in PBMCs could have been found if sampling during the follow

TABLE 1. Summary of data on seroconverters[a]

Subject	HIV-1 mRNA (copies/μg)			HIV-1 plasma virus load (virions/ml) at 3 yr	HIV-1 DNA (copies/10^6 cells)			Mo from SC without AIDS[b]	End status (AIDS or $CD4^+$ count)
	SC	1 yr	3 yr		SC	1 yr	3 yr		
S1	23,882	9,562	11,292	92,967	1,141	772	296	17	AIDS
S2	2,462	7,162	15,804	13,484	904	1,214	8,688	30	AIDS
S3	4,635	6,889	25,315	191,297	628	937	1,788	34	AIDS
S4	2,059	6,100	12,017		2,258	4,427	5,044	35	AIDS
S5	655	3,258	12,165	88,335	253	1,408	3,873	42	AIDS
S6	927	3,695	27,071	63,302	1,419	2,207	5,930	42	AIDS
S7	<100	6,142	12,807	41,561	<10	672	1,640	42	AIDS
S8	4,126	3,140	7,637	8,156	1,851	5,053	8,423	51	AIDS
S9	1,688	1,640	3,455	6,164	755	1,443	1,296	52	AIDS
S10	1,739	3,394	18,133	50,941	326	721	4,386	56	AIDS
S11	2,618	1,925	6,248	12,507	554	ND	2,663	59	AIDS
S12	10,884	20,509	6,968	7,291	1,662	1,879	1,271	81	AIDS
S13	609	2,267	1,625	<500	321	205	246	84	AIDS
S14	182	346	1,348	<500	85	34	225	87	AIDS
S15	1,976	5,092	4,395	7,401	3,461	7,294	1,680	88	AIDS
S16	10,846	7,650	8,292	7,836	4,246	2,304	2,597	102	AIDS
S17	212	388	1,798	<500	383	176	490	45	282
S18	838	689	4,456	5,806	196	724	2,669	49	927
S19	7,103	6,983	5,174		2,235	1,954	1,773	50	728
S20	393	434	490	<500	257	102	493	50/72	351/ND[c]
S21	10,946	2,939	6,702	3,969	5,754	1,354	3,762	77	387
S22	<100	<100	814		149	ND	ND	77	387
S23	217	502	1,992	<500	203	379	3,225	79	496
S24	1,126	975	2,093	<500	41	65	516	80	255
S25	873	413	8,295	81,663	81	73	881	45/84	219/ND[c]
S26	425	529	533	<500	605	891	535	89	436
S27	959	2,916	1,927	<500	1,907	1,555	2,324	97	522
S28	<100	<100	<100	<500	ND	ND	ND	87/120	734/ND[c]

[a] The 28 seroconverters tested are listed in the order of their relative rate of disease progression. Their level of HIV-1 mRNA expression in PBMCs and proviral DNA loads are expressed as copies per microgram of total cellular RNA or proviral copies per million cells or as <100 or <10 when below the limits of linear detection of the respective assays. In some cases, insufficient amounts of intact DNA in PBMCs were recovered for analysis (ND). Cell-free HIV-1 RNA loads in plasma samples obtained at the time of collection of the 3-year follow-up PBMC specimens were also measured and are expressed as number of virions per ml of plasma.

[b] In cases when an AIDS diagnosis (2) was made (S1 to S16), the time from seroconversion (SC) to AIDS (in months) is indicated. In the remaining cases (S17 to S28), the number of months indicates the time from seroconversion to the last available CD4 cell count, which is shown in the rightmost column as the average $CD4^+$ cell number of the two last follow-up visits.

[c] In three cases (S20, S25, and S28), an AIDS-free status could be confirmed at a later time when these individuals were no longer monitored, although the exact CD4 cell count of at this time was not known (ND).

up of the cohort had been more frequent. Thus, although these data are in good agreement with previous studies reporting low levels of HIV-1 replication and proviral loads in LTNPs (1, 19), they also show that similarly low levels are common early in infection, even in persons who subsequently experience rapid disease progression.

HIV-1 replication increases over time in seroconverters but not in LTNPs. The content of HIV-1 mRNA in PBMCs of the seroconverters increased on average during the follow up, with median values of 1,043, 2,928, and 5,711/μg at 0, 1, and 3 years after seroconversion, respectively. By contrast, the longitudinal patterns of HIV-1 mRNA expression in the PBMCs of the LTNPs revealed no evidence of such an increasing trend (Table 2). Although these data do not prove that the levels of HIV-1 mRNA (and DNA) in PBMCs of the LTNPs have been constant from the time of their seroconversion, they clearly demonstrate that the low levels of HIV-1 replication observed in their PBMCs have remained unchanged for at least the past several years.

Unlike the LTNPs, only one (S28) of the six seroconverters who initially had very low levels of HIV-1 mRNA in PBMCs (less than the median value of LTNPs) continued to do so during 3 years of follow up. Notably, this man subsequently showed no evidence of disease progression and had a CD4 cell count of >600/μl when last seen after more than 7 years of follow up with HIV-1 infection (87 months) and was known to be free of AIDS at least 3 more years after that, suggesting that he might well meet the criteria used to define LTNPs if more follow-up data were available. In support of this possibility, examination of the PBMC specimen collected at his last follow-up visit still showed fewer than 100 copies of HIV-1 mRNA per μg (data not shown).

In addition to this potential LTNP, three other men (S26, S22, and S20) among the 14 seroconverters whose PBMC HIV-1 mRNA values were initially within the range of the LTNPs (less than 1,000 copies/μg) were still in this category after 3 years. Although none of them developed AIDS during the study (monitored for 89, 77, and 72 months from seroconversion), a long-term trend of decreasing CD4 cell counts identified them as slow progressors rather than potential LTNPs. Moreover, the available follow-up data on the remaining 24 seroconverters do not suggest that other persons in this population would be potential LTNPs (Table 1).

Prognostic value of HIV-1 mRNA in PBMCs increases during the first year of infection. Low levels of HIV-1 mRNA in PBMCs of asymptomatic HIV-1-infected individuals have been shown to be a strong predictor of a benign prognosis (23). However, some persons in the current study had very low levels of HIV-1 mRNA in PBMCs at postseroconversion but yet progressed to AIDS relatively soon, suggesting that the prognostic value of HIV-1 mRNA expression in PBMCs is not yet maximal at that stage of the disease. To examine this possibil-

TABLE 2. Summary of data on the LTNPs[d]

LTNP no.	Mo and yr of seroconversion[a]	Duration of follow up (mo)	Specimen[b]	HIV mRNA	HIV DNA	CD4 count (per mm^3 of blood)[c]
1	<2/1979	157	66	<100	<10	797
			98	150	18	1,270
			125	<100	26	1,003
			157	154	<10	1,007
2	4/1980	138	53	638	746	820
			76	789	1,402	1,020
			115	671	925	793
			138	648	1,111	650
3	8/1980	139	55	<100	<10	943
			79	313	45	1,163
			110	148	102	973
			139	599	175	703
4	<3/1979	157	69	<100	48	1,250
			97	239	<10	1,467
			127	477	170	1,000
			157	300	324	1,043
5	<4/1979	156	67	992	664	817
			96	396	1,155	777
			122	268	912	830
			155	385	296	870
6	4/1980	145	52	<100	<10	1,063
			78	<100	37	990
			109	<100	12	963
			144	<100	26	873
7	3/1982	121	27	<100	18	1,160
			60	<100	73	1,210
			86	<100	138	1,143
			121	<100	244	857

[a] <, the first HIV-1 antibody-positive blood specimen. After 1984, the follow up included regular CD4$^+$ T-lymphocyte enumeration and cryopreservation of PBMCs.
[b] The time of sampling of the tested PBMC specimens after the month indicated in the Seroconversion column.
[c] Average value of three sequential counts obtained at the time of collection and at follow-up visits 4 to 5 months before and after that.
[d] See text for plasma viral loads.

ity, we compared the predictive value of HIV-1 mRNA and DNA in the PBMCs collected postseroconversion and 1 year later.

For this purpose we split the seroconverter cohort into two groups (high and low) on the basis of their HIV-1 mRNA or DNA copy numbers in PBMCs either at postseroconversion or 1 year later and compared the subsequent rate of progression to AIDS in these four categories (mRNA at zero year, mRNA at 1 year, DNA at zero year, and DNA at 1 year after seroconversion) by Kaplan-Meier survival analyses (Fig. 2). The values dividing the high and low copy-number groups were chosen in reference to the values obtained from the earliest set of specimens from the LTNPs (Table 2). Thus, we considered all HIV-1 mRNA values of less than 1,000/μg and all HIV-1 DNA values of less than 750/10^6 to be low and values above that to be high.

As can be seen in Fig. 2, the relative magnitude and the statistical significance of the difference in the incidence of AIDS between the low and high copy-number groups were greatest when the analysis was based on the 1-year values of HIV-1 mRNA in PBMCs (Fig. 2B). As noted above, the less apparent difference in the incidence of AIDS between the two groups when based on the postseroconversion HIV-1 mRNA values (Fig. 2A) was not due to the relatively larger size of the low copy-number group in this case (n, 14) but rather because some men with low levels of HIV-1 replication at this point progressed to AIDS relatively soon (S5, S6, and S7 were all diagnosed with AIDS 42 months after seroconversion; Table 1). On the other hand, although the highest levels of HIV-1 mRNA at postseroconversion were seen in the individual who experienced the most rapid progression to AIDS (S1), high expression of HIV-1 mRNA in PBMCs at this time, although an ominous sign, was not diagnostic for rapid progressors.

Similar analyses based on levels of HIV-1 DNA in PBMCs indicated that the proviral load at postseroconversion (Fig. 2C) and at 1 year later (Fig. 2D) also appears to be associated with the subsequent rate of HIV-1 disease progression, albeit less strongly than HIV-1 mRNA expression, such that the differences in the incidence of AIDS between the high and low HIV-1 DNA copy-number groups were not statistically significant.

Plasma samples matching with the tested PBMC specimens were available from the more recent years of the PAS study. Therefore, to facilitate the comparison of our HIV-1 mRNA data in PBMCs with studies on plasma virus load, genomic HIV-1 RNA copy numbers in plasma samples corresponding to the 3-year follow-up PBMC specimens of the 28 seroconverters and the most recent specimens of the LTNPs were also determined. In agreement with the results of other studies (1, 19), very low levels of cell-free virus were detected in the LTNPs. In fact, detectable levels of HIV RNA (>500 virions per ml) were found only in two of the seven plasma specimens from the LTNPs (LTNP2 [1,080 virions per ml] and LTNP3 [505 virions per ml]). Among the seroconverters, plasma viral load generally correlated well with the HIV-1 cellular mRNA data (Table 1).

The present results confirm previous reports of low levels of HIV-1 replication in LTNPs (1, 19) but also show similarly low levels in many individuals who have recently become HIV-1 seropositive. Thus, a very low level of HIV-1 replication cannot be considered a distinctive feature of LTNPs but rather is a relatively common occurrence during early infection. Instead, persistence of such low levels seems to distinguish LTNPs from progressors. Although the possibility cannot be excluded that the favorable virus-host balance in these individuals could change at some point in the future, on the basis of the low copy numbers of HIV-1 mRNA in their PBMCs it can be predicted (23) that the future course of their disease will also be substantially more benign than that in a randomly selected group of HIV-1-infected individuals with similar CD4 cell counts. Therefore, the reasons for the lack of disease progression in the LTNPs during the past >10 years are unlikely to have been only stochastic and probably involve a significant pathophysiological component.

The present data indicate that the utility of HIV-1 mRNA expression in PBMCs as a prognostic marker is not yet maximal when measured soon after seroconversion but is significantly increased by the end of the first year of seropositivity. This improved predictive value appears mainly to derive from a strengthening association over time between low levels of HIV-1 mRNA in PBMCs and a benign prognosis. Thus, this point should be taken into consideration when using HIV-1 mRNA expression in PBMCs for prognostication of the future clinical course of very early HIV-1 infection and may also deserve additional experimental attention regarding the use of cell-free viremia in plasma as an early predictor of HIV-1 disease progression.

The observation that levels of HIV-1 mRNA in PBMCs as low as those in the LTNPs were also seen in early specimens from persons who subsequently experienced rapid disease progression indicates that the extent of the initial elimination of HIV-1 replication does not correlate with the ability to main-

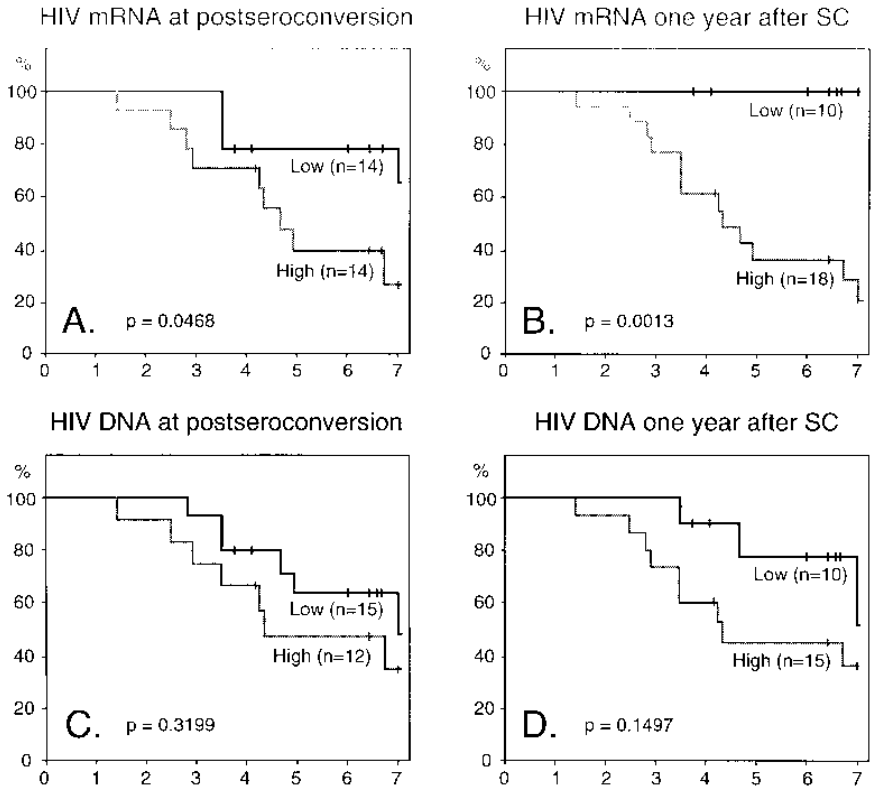

FIG. 2. Incidence of AIDS among subgroups of the seroconverter cohort on the basis of their HIV-1 mRNA expression or proviral copy number at seroconversion or 1 year later. Values in each category were divided into low and high groups as explained in the text, and their subsequent rate of progression to AIDS was compared by Kaplan-Meier survival analyses. The *P* values for the differences observed in the rate of progression to AIDS between the high and low copy-number groups are shown. Years of follow-up are shown on the *x* axis; percentage of the study population living free of AIDS is shown on the *y* axis. Vertical bars indicate losses of persons from the study for reasons other than AIDS.

tain this status over time. Thus, it is possible that early containment of HIV-1 infection and maintenance of such low levels of viral replication involve distinct mechanisms.

Although robust T- and B-cell receptor-mediated anti-HIV-1 immune responses have been demonstrated in LTNPs (1, 8, 19, 20), other forms of antiviral immunity as well as unrelated host- or virus-specific mechanisms could also be critically important for long-term resistance for HIV-1 disease progression. Recent progress in monitoring HIV-1 replication and viral load in vivo is facilitating the uncovering of these mechanisms and will thereby hopefully lead to new opportunities for prevention and treatment of HIV-1 infection.

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