Neutralizing Antibody Responses to Human Immunodeficiency Virus Type 1 in Primary Infection and Long-Term–Nonprogressive Infection

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The role of neutralizing antibodies in human immunodeficiency virus type 1 (HIV-1) infection is poorly understood and was assessed by evaluating responses at different stages of infection. Undiluted sera from long-term nonprogressors (LTNP) had broad neutralizing antibodies against heterologous primary isolates and were more likely to neutralize the contemporaneous autologous isolate than were sera from short-term nonprogressors and progressors. In primary infection, envelope-specific IgG was detected before the initial decline in plasma viremia, but neutralizing antibodies developed more slowly. Here, neutralizing antibodies against strains SF-2 and MN were sometimes the first to be detected, but titers were low for at least 17 weeks from onset of symptoms. Neutralizing antibodies against the early autologous isolate were detected for 4 patients by 5–40 weeks but were undetectable in 2 additional patients for 27–45 weeks. The results indicate that neutralizing antibody responses are slow to develop during primary infection and are uniquely broad in LTNP.

Infection with human immunodeficiency virus type 1 (HIV-1) is accompanied by cellular and humoral immune responses of various magnitudes and specificities that are thought to down-regulate plasma viremia during primary infection [1, 2] and to establish a period of clinical latency that follows [3]. Despite the benefit provided by these responses early in infection, nearly all HIV-1–infected persons progress to AIDS. Specific responses that are at least partially beneficial during the course of infection are poorly understood. Detection of HIV-1–specific cytotoxic T lymphocytes (CTL) correlates well with the initial down-regulation of plasma viremia during primary infection [4–7] and could be a dominant antiviral immune response. Another correlate during primary infection is the production of HIV-1–specific IgM [8] and possibly IgG [2, 9], but it is not clear whether these early antibodies are effective against the virus. For example, neutralizing antibody responses against the early autologous virus have been shown to be delayed relative to when plasma viremia is first down-regulated [5–7, 10]. Similar delays in neutralizing antibody production have been reported for simian immunodeficiency virus (SIV) infection in macaques [11, 12]. This slow development of neutralizing antibodies during primary infection might be one of the reasons the virus is able to establish persistent infection.

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Although most infected persons progress to AIDS within a median time of 10 years from initial seroconversion, ~5%–10% tolerate infection without immune suppression or other clinical manifestations for longer periods of time [13–15]. This latter group of infected persons is referred to as long-term nonprogressors (LTNP). LTNP generally have low virus loads in their plasma, peripheral blood mononuclear cells (PBMC), and lymph nodes and mount robust cellular and humoral immune responses [16–21]. Some cases of long-term nonprogression are associated with infection by attenuated virus [22] or with a heterozygous defect in the CCR5 co-receptor [23]. Many other LTNP are thought to benefit from the overall quality of their immune responses.

Previous studies showed that neutralizing antibody responses in LTNP are greater in magnitude and breadth than they are in other HIV-1–infected persons. For example, the average titer of neutralizing antibodies against HIV-1_{IIIB} and HIV-1_{MN} was significantly higher in sera from LTNP than from persons who had been infected for <7 years and had no signs of immune suppression [17]. In addition, we [17] and others [18] determined that sera from LTNP neutralize heterologous primary isolates more effectively than do sera from progressors. Importantly, it was not clear from these studies whether the greater capacity of sera from LTNP to neutralize primary isolates was merely a reflection of diminished response in immunologically suppressed persons.

The present study aimed to determine whether the broad neutralizing antibody responses seen in LTNP are unique compared with responses in other HIV-1–infected persons, including short-term nonprogressors (STNP) who had been infected for 2–7 years. We took advantage of the fact that virus neutralization was more readily detected with undiluted serum samples. By use of this approach, neutralizing antibodies in sera

from LTNP, progressors, recent seroconverters, and STNP were evaluated with autologous and heterologous primary isolates. We also determined whether low-level neutralizing antibodies might be present during primary infection that had previously gone undetected. Sequential serum samples obtained during and immediately following primary infection were assessed for neutralizing antibodies against the early autologous virus and T cell line–adapted (TCLA) variants of HIV-1, including IIIB, MN, and SF-2.

Methods

Study participants. HIV-1–infected LTNP and progressors were described previously [16, 17]. LTNP had well-documented infection for ⩾10 years, were asymptomatic, and had >600 CD4 T lymphocytes/mm^3 in their peripheral blood; these levels were stable over time. With the exception of 1 LTNP (no. 17), no LTNP received antiretroviral therapy. All progressors had signs of immunosuppression as evidenced by low CD4 T lymphocyte counts (range, 10–371/mm^3). A group of STNP, defined as having documented infection for 2–7 years but remaining healthy, with >600 CD4 T lymphocytes/mm^3, were recruited from the Durham, North Carolina, area. Information about antiretroviral therapy for progressors and STNP was unavailable.

Six persons with primary HIV-1 infection were referred for study by their private physicians. Each of them had developed transient mild clinical symptoms, which brought them to the attention of their physicians. Four of them began antiretroviral therapy within a few days of entry into study. Exceptions were patient 45, who received no antiretroviral therapy during the course of study, and patient 46, who began antiretroviral therapy ~25 weeks from the onset of symptoms. Healthy, HIV-1–negative persons were recruited from local research laboratories.

Primary isolates. Viruses were isolated by coculture of PBMC from infected persons with phytohemagglutinin-stimulated PBMC (PHA-PBMC) from healthy, noninfected persons in the presence of interleukin-2 (IL-2) as described [17]. Primary isolates from acute seroconverters were obtained at the time of their first serum collection. Virus stocks were made cell-free by low-speed centrifugation and passage through 0.5-μm cellulose acetate filters before being stored in aliquots at −70°C. Some stocks were later expanded a single time in PHA-PBMC. $TCID_{50}$ was determined by the method of Reed and Muench [24] after 14 days of incubation with PHA-PBMC. Viral antigen concentration was measured by p24 immunoassay (DuPont, Wilmington, DE). Syncytium-inducing (SI) and non-SI (NSI) phenotypes were determined in MT-2 cells and PHA-PBMC as described [17]. The $TCID_{50}$, p24 concentration, and phenotype of each virus stock are given in table 1. Additional information on isolates obtained from LTNP and progressors has been published [17]. Primary isolates were used as either first- or second-passage stocks produced in PHA-PBMC.

Serologic assays. Neutralizing antibodies against TCLA strains of HIV-1 were measured in MT-2 cells (strains IIIB and MN) or CEMx174 cells (strain SF-2) in 96-well plates by a reduction in virus-induced cell killing as described previously [25]. In brief, cell-free virus (1000 $TCID_{50}$) and serial dilutions of test sera were incubated in triplicate at 37°C for 30 min before the addition of cells (5×10^4/well). Cultures were then incubated for the minimum length of time that allowed for extensive syncytium formation to occur, as observed microscopically (3–4 days). Cell viability was quantified by neutral red staining as described [25]. Percentage of protection was calculated by the difference in absorbance at 540 nm between test wells (cells, serum sample, and virus) and virus control wells (cells and virus) divided by the difference in absorbance between cell control wells (cells only) and virus control wells. Neutralization titers were defined as the reciprocal of the serum dilution required to protect at least 50% of cells from virus-induced killing. Assays were standardized by including positive and negative control sera that had been assayed multiple times and for which the average titer was well-characterized. Virus stocks for these assays were produced in H9 cells.

Antibody-mediated neutralization of primary isolates was measured in PHA-PBMC as described [17] by use of a p24 immunoassay to quantify virus production. For assays with undiluted serum, 20 μL of cell-free virus was incubated with 80 μL of serum for 1 h at 37°C, and 25 μL of this mixture was added to triplicate wells containing PHA-PBMC (4×10^5 cells in 175 μL added per well). For measurement of titer, 50 μL of virus was incubated in triplicate as above with an equal volume of serum samples that were undiluted and diluted 1:2, 1:4, 1:16, and 1:32 (dilutions were made in IL-2 growth medium). Next, 25 μL was transfered to corresponding wells of U-bottom plates containing PHA-PBMC. Cells were incubated with the virus-serum mixtures for 3 h at 37°C and then washed three times with 200 μL of growth medium to

Table 1. Characteristics of primary isolates from HIV-1–infected subjects.

Group, isolate	Phenotype	$TCID_{50}$	p24 (ng/mL)*	$TCID_{50}$/ng of p24
Progressors				
P59423	NSI	5.6×10^4	35.0	1600
W25798	NSI	1.7×10^4	21.0	809
W79290	NSI	7.5×10^3	31.4	239
V89872	SI	1.3×10^5	29.8	4362
V67970	SI	9.4×10^5	32.9	28,571
W179273	SI	3.3×10^5	12.0	27,500
STNP				
P46471	NSI	9.4×10^5	61.0	15,410
T00953	NSI	1.3×10^5	57.9	2245
V91008	NSI	1.6×10^6	109.0	14,679
W97464	NSI	9.4×10^5	39.6	23,738
LTNP				
18	NSI	1.9×10^5	53.5	3551
22	NSI	1.9×10^5	600.0	317
24	NSI	1.3×10^5	67.7	1920
Primary infection				
15	NSI	4.7×10^6	124.8	37,660
23	NSI	1.9×10^5	44.8	4241
27	NSI	2.8×10^5	25.3	11,067
30	NSI	2.1×10^6	60.0	35,000
45	NSI	3.8×10^4	4.7	8085
46	NSI	2.1×10^6	41.0	51,220

NOTE. STNP, short-term nonprogressors; LTNP, long-term nonprogressors; SI, syncytium-inducing; NSI, non–syncytium-inducing.

* Concentration of p24 at time of harvest from initial cocultivation.

remove the virus inoculum and antibodies. Washed cells were suspended in 250 μL of IL-2 growth medium and incubated in 96-well flat-bottom plates for the duration. Culture supernatants (25 μL) were collected on a daily basis and mixed with 225 μL of 0.5% Triton X-100 for the quantification of p24. This volume was replaced with 25 μL of fresh IL-2 growth medium at each collection.

Viral p24 produced in the absence of test sera (virus control) was determined for each daily sampling and was used to construct a viral replication curve. Neutralization was measured at a time when the concentration of p24 in virus control wells exceeded 5 ng/mL (limit of detection was 0.1 ng/mL) but had not reached peak production (4–6 days). Serum samples were considered positive for primary isolate neutralization if they caused >80% reduction in p24 relative to a negative control serum (D01). This cutoff corresponds to a minimum 1 log reduction in infectious virus yield. Standard deviations for a given serum sample were divided by the average p24 value of the virus control and then multiplied by 100 for percentage. An equal volume of undiluted virus stock was used for all assays. While the infectious dose contained within this volume was not the same for all isolates, each serum sample was tested against an equal dose on a per-virus basis. Sera were heat-inactivated at 56°C for 1 h before use.

Antibodies that bound to HIV-1 antigens were assessed by HIV-1_{IIIB} Western immunoblot (Cambridge Biotech, Worcester, MA) and by HIV-1_{MN} gp160 ELISA. Baculovirus-derived HIV-1_{MN} gp160 was obtained from Quality Biologicals (Gaithersburg, MD). ELISAs were done as described previously [26] with alkaline phosphatase–conjugated goat anti-human IgG (whole molecule; Sigma, St. Louis) and *p*-nitrophenylphosphate disodium hexahydrate (Sigma; catalog no. N-9389) used for detection and color development, respectively. Titers are reported as the reciprocal of the highest serum dilution to produce an average absorbance reading of at least twice that of a negative control serum (D01).

Removal of IgG. IgG was removed from serum samples by absorption with protein G–sepharose (GammaBind Plus Sepharose; Pharmacia, Piscataway, NJ). Protein G–sepharose was obtained by the supplier as a suspension in PBS containing thimerosal as a preservative. The resin was washed five times with PBS and collected by low-speed centrifugation as premeasured aliquots after the last wash. Supernatants were removed and replaced with a volume of undiluted serum equal to the volume of packed resin. Suspensions were incubated at room temperature for 1 h with constant gentle mixing. Protein G–sepharose was then separated from the serum by low-speed centrifugation, and the serum was collected by pipette. Serum samples were subjected to two additional absorption steps to ensure the removal of all IgG. Complete removal of HIV-1–specific IgG was confirmed by Western immunoblot analysis.

Measurements of plasma viremia. HIV-1 RNA in plasma samples was quantified by the branched DNA method as described previously [27].

Chemokine assays. Quantification of the chemokines RANTES and MIP-1β in serum samples was done with commercially available ELISA kits as described by the supplier (R&D Systems, Minneapolis). Levels of RANTES and MIP-1β were quantified when sufficient sample volumes were available. Levels of MIP-1α were not measured because of a limited volume of serum samples.

Statistical analyses. The significance of the frequency by which sera from paired groups of infected persons neutralized primary isolates in a panel of 6 isolates was evaluated by χ^2 analysis with 1 *df*. This analysis assumed that the ability of a particular serum sample to neutralize one primary isolate does not predict its ability to neutralize other primary isolates. Yates's correction factor was used to give a more conservative estimate of *P*. Neutralization sensitivity of NSI and SI viruses was also compared by χ^2 analysis, but here sera from all infected persons were grouped as one. The small number of NSI and SI isolates (3 each) did not permit a comparison of neutralization sensitivity between sera from different subgroups of persons.

Results

Autologous and heterologous neutralizing antibody responses at different stages of HIV-1 infection. Serum samples obtained at different stages of HIV-1 infection were assessed for neutralization breadth with a panel of 6 primary isolates. Sera from 6 recent seroconverters (sera obtained 13–53 weeks from onset of symptoms), 5 progressors, 6 STNP, 6 LTNP, and 5 healthy HIV-1–negative persons were used in this evaluation (table 2). Primary isolates were derived from the progressors listed in table 2. These same primary isolates and sera from progressors and LTNP were used in a previous study in which the primary isolates were rarely neutralized by 1:16-diluted serum samples [17]. Undiluted serum was used in the present study to improve the detection of neutralizing antibodies.

No neutralization of primary isolates was detected with sera from HIV-1–negative persons. In some cases, mild enhancement of infection was observed with these sera, but the enhancing effect was never more than a doubling of p24 production, which would not be considered true enhancement [12, 17]. Neutralization of each virus was detected sporadically with serum samples from HIV-1–infected persons. Positive neutralization was detected most often with sera from LTNP. Of the LTNP sera evaluated, 20 (56%) of 36 virus-serum combinations tested positive. Neutralization was detected less frequently with sera from STNP (25% of combinations testing positive), recent seroconverters (17% of combinations testing positive), and progressors (17% of combinations testing positive). The breadth of neutralization was significantly greater for sera from LTNP compared with sera from all other groups ($P = .0014$ compared with recent seroconverters, $P = .0028$ compared with progressors, and $P = .0163$ compared with STNP; 95% confidence level). The breadth of neutralization with sera from STNP was no greater than with sera from recent seroconverters or progressors ($P > .05$). There was also no significant difference in neutralization sensitivity between NSI and SI viruses when sera from all infected persons were grouped as one for the analysis ($P = .124$).

Undiluted sera from LTNP neutralized the autologous virus obtained at the time of serum collection in all 3 cases examined (table 3). These same sera had failed to neutralize the autologous virus at a 1:16 dilution previously [17]. Isolates from

Table 2. Neutralization of primary isolates by undiluted sera obtained at different stages of HIV-1 infection.

Group, serum no. (weeks from symptom onset)	Neutralization of primary isolate					
	P59423	W25798	W79290	V89872	V67970	W179273
Recent seroconverters						
15 (53)	**96 ± 0**	**97 ± 1**	**96 ± 2**	19 ± 30	66 ± 5	71 ± 10
23 (45)	14 ± 34	28 ± 19	**98 ± 1**	4 ± 25	4 ± 17	−3 ± 39
27 (27)	39 ± 16	65 ± 5	**91 ± 2**	44 ± 19	27 ± 19	52 ± 12
30 (13)	17 ± 63	37 ± 31	30 ± 14	9 ± 18	−9 ± 39	−6 ± 32
45 (25)	56 ± 21	69 ± 3	**91 ± 9**	51 ± 19	30 ± 24	51 ± 22
46 (13)	22 ± 23	61 ± 7	80 ± 7	23 ± 20	−11 ± 34	35 ± 18
Progressors						
P59423	67 ± 18	**97 ± 1**	−58 ± 41	9 ± 4	**96 ± 1**	−29 ± 12
W25798	**90 ± 15**	**88 ± 11**	−15 ± 19	−17 ± 38	28 ± 33	−6 ± 21
V89872	12 ± 22	**85 ± 12**	56 ± 16	−51 ± 46	61 ± 21	34 ± 19
V67970	0 ± 44	61 ± 78	70 ± 39	5 ± 30	33 ± 10	−1 ± 43
W179273	22 ± 27	43 ± 36	−85 ± 72	−81 ± 59	8 ± 9	−16 ± 32
STNP						
T00953	**92 ± 11**	**99 ± 1**	**87 ± 10**	36 ± 11	**99 ± 0**	**98 ± 1**
V91008	19 ± 39	17 ± 29	38 ± 18	**91 ± 6**	**99 ± 0**	**86 ± 15**
W97464	−19 ± 29	8 ± 33	26 ± 15	3 ± 8	67 ± 3	−55 ± 61
P46471	44 ± 16	−6 ± 52	23 ± 9	26 ± 44	54 ± 29	79 ± 26
T84136	48 ± 28	20 ± 55	35 ± 16	**93 ± 4**	2 ± 54	1 ± 5
W55819	43 ± 3	20 ± 13	7 ± 19	42 ± 28	17 ± 46	26 ± 44
LTNP						
1	**100**	**100**	**100**	**85 ± 10**	**90 ± 8**	**100**
2	**82 ± 18**	**87 ± 11**	−3 ± 77	38 ± 24	**98 ± 3**	**95 ± 6**
3	**96 ± 5**	**96 ± 4**	47 ± 33	42 ± 21	**100**	**97 ± 2**
9	39 ± 11	51 ± 58	−28 ± 127	31 ± 21	62 ± 19	74 ± 21
10	**100**	**100**	−15 ± 126	53 ± 4	**100**	**83 ± 24**
17	70 ± 12	60 ± 17	**81 ± 23**	11 ± 19	**88 ± 9**	23 ± 14
HIV-1–negative subjects						
D01	−21 ± 42	9 ± 35	23 ± 20	−47 ± 70	24 ± 4	24 ± 20
D02	−44 ± 30	32 ± 14	35 ± 1	−80 ± 28	2 ± 22	−38 ± 31
D03	−48 ± 41	18 ± 14	15 ± 25	−64 ± 35	−11 ± 22	0 ± 9
D04	−40 ± 7	23 ± 9	9 ± 8	−62 ± 13	29 ± 19	−42 ± 37
D05	7 ± 9	14 ± 8	35 ± 10	−32 ± 19	23 ± 22	−3 ± 11

NOTE. Virus neutralization was measured in phytohemagglutinin-stimulated peripheral blood mononuclear cells by using undiluted serum. Values are % reduction in p24 production relative to virus control (no test serum) in HIV-1–negative subjects and relative to p24 production in presence of serum D01 for all others. Results are given as average of triplicate wells ± SD. Boldface type indicates >80% reduction (positive for neutralization). STNP, short-term nonprogressors; LTNP, long-term nonprogressors.

progressors were neutralized by undiluted autologous serum in only 1 of 5 cases examined (table 2), whereas isolates from STNP were neutralized by undiluted autologous serum in 0 of 4 cases examined (table 3).

Virus neutralization by undiluted serum is antibody-mediated. We examined whether high concentrations of antiviral cytokines or chemokines in undiluted serum were at least partially responsible for neutralizing activity. Protein G–sepharose was used to remove the total IgG from 5 serum samples that had previously neutralized 1 or more primary isolates. Samples were negative for anti–HIV-1 antibodies by Western blot analysis after absorption (data not shown). Serum samples were reevaluated for virus neutralization before and after absorption. As can be seen in table 4, samples absorbed with protein G–sepharose no longer had neutralizing activity. Loss of neutralizing activity could not be attributed to sample dilution during absorption, since the volume of processed samples never exceeded the original preabsorption volumes. These results strongly indicate that virus neutralization was mediated by IgG.

Antibody-independent neutralization was further assessed by quantifying the concentration of the anti–HIV-1 chemokines, RANTES and MIP-1β in serum samples (table 5). Many infected persons had moderately elevated levels of RANTES in their serum compared with levels in serum from healthy, noninfected persons (>14.8 ng/mL). Elevated levels were mostly seen in sera from progressors, and these particular serum samples had poor neutralizing activity against primary isolates. Only 1 infected subject had a level of MIP-1β that

Table 3. Neutralization of primary isolates from long-term and short-term nonprogressors (LTNP and STNP) by autologous serum.

Subject	Status	Neutralization of autologous virus
18	LTNP	99 ± 0
22	LTNP	94 ± 3
24	LTNP	99 ± 0
P46471	STNP	30 ± 18
T00953	STNP	45 ± 18
V91008	STNP	52 ± 13
W97464	STNP	32 ± 9

NOTE. Undiluted serum from each subject was evaluated for neutralizing antibodies against autologous isolate obtained at time of serum collection. Values are % reduction in p24 production in phytohemagglutinin-stimulated peripheral blood monocuclear cells and are given as average of triplicate wells ± SD.

was highly elevated relative to all others. This subject (V89872) was a progressor whose serum rarely neutralized primary isolates. Sera with the most potent neutralizing activity (i.e., serum samples 3, 10, and T00953) had lower concentrations of these chemokines than did the remaining sera that had poor or no neutralizing activity. We conclude that virus neutralization using undiluted serum was unrelated to the concentration of anti–HIV-1 chemokines.

CD4 T cells, plasma virus loads, and serum antibody responses during primary infection. CD4 T cells, plasma virus, and serum antibodies were quantified for HIV-1–infected persons starting at 3–10 weeks from onset of symptoms and continuing for an additional 20–57 weeks thereafter (table 6). Four of 6 patients had levels of plasma viremia of $>10^5$ RNA copies/mL at the earliest time point examined. These levels subsequently declined and remained persistently low in 3 of them (patients 15, 23, and 30), all of whom began antiretroviral therapy shortly after the first time point. The fourth patient with high levels of plasma virus on entry continued to have high virus loads for 20 weeks of follow-up (patient 45); this patient received no antiretroviral ther-

Table 5. Analysis of serum chemokine concentrations at different stages of HIV-1 infection.

Group, serum	RANTES (ng/mL)	MIP-1β (pg/mL)
Progressors		
W25798	77.8	NT
V89872	55.5	153.8
V67970	68.0	20.3
STNP		
T00953	19.8	8.5
P46471	47.6	52.5
T84136	10.6	25.0
LTNP		
3	20.5	NT
10	18.3	NT
17	21.7	41.4
18	17.6	58.6
Controls		
D01	9.3	22.2
D02	13.5	54.2
D03	6.8	13.7
D04	14.8	10.2

NOTE. LTNP, long-term nonprogressors; STNP, short-term nonprogressors; NT, not tested.

Table 4. Neutralization of primary isolates after removing IgG from serum samples.

	Neutralization of primary isolate						
Serum, IgG	P59423	W25798	W79290	V89872	V67970	W179273	18
15 (53 weeks)							
+	82 ± 4	95 ± 2	94 ± 4	NT	NT	NT	NT
−	26 ± 16	11 ± 16	59 ± 48	NT	NT	NT	NT
T00953							
+	NT	NT	NT	NT	99 ± 1	97 ± 3	NT
−	NT	NT	NT	NT	0 ± 22	15 ± 52	NT
V91008							
+	NT	NT	NT	89 ± 4	100	NT	NT
−	NT	NT	NT	0 ± 39	0 ± 15	NT	NT
10							
+	NT	100	NT	NT	100	NT	NT
−	NT	0 ± 37	NT	NT	0 ± 19	NT	NT
18							
+	NT	NT	NT	NT	NT	NT	87 ± 7
−	NT	NT	NT	NT	NT	NT	14 ± 45

NOTE. Undiluted sera were evaluated for neutralizing antibiotics against indicated primary isolate. Values are % reduction in p24 production in phytohemagglutinin-stimulated peripheral blood mononuclear cells and are given as average of triplicate wells ± SD. NT, not tested. Samples were evaluated before (+) and after (−) IgG was removed by absorption with protein G–sepharose.

Table 6. CD4 T cells, plasma virus concentration, and antibody titers in primary HIV-1 infection.

Patient, weeks from symptom onset*	CD4 T cells/mm^3	Plasma virus†	ELISA titer‡	Neutralizing antibody titer§			
				Autologous isolate	IIIB	MN	SF-2
Patient 15							
4	704	744,000	150	<2	<10	<10	<10
20	638	13,000	4050	4	<10	24	393
25	542	19,000	4050	8	26	55	490
29	502	11,000	12,150	32	82	125	830
35	655	12,000	36,450	32	67	156	713
53	644	<10,000	36,450	>64	72	149	1932
Patient 23							
10	756	171,000	450	<2	<10	23	14
18	523	137,000	1350	<2	<10	78	136
25	484	19,000	4050	<2	<10	157	520
29	385	31,000	4050	<2	12	200	665
42	363	41,000	4050	<2	<10	342	661
45	391	58,000	12,150	<2	<10	199	1039
Patient 27							
4	441	40,000	150	<2	<10	<10	<10
5	NT	NT	450	<2	<10	<10	<10
6	313	<10,000	450	<2	22	29	<10
11	395	<10,000	1350	<2	<10	72	20
17	359	<10,000	1350	<2	14	24	840
27	357	15,000	4050	<2	24	72	552
Patient 30							
3	558	1,224,000	1350	<2	<10	<10	<10
7	679	37,000	1350	<2	24	16	<10
13	665	<10,000	1350	<2	32	23	226
18	655	12,000	1350	<2	29	71	778
40	525	<10,000	4050	2	48	144	2014
Patient 45							
5	502	594,000	450	2	<10	<10	<10
12	613	377,000	1350	2	<10	<10	<10
20	651	297,000	1350	4	23	200	14
25	722	230,000	4050	16	43	137	20
Patient 46							
5	848	43,000	4050	<2	49	235	46
13	498	70,000	4050	2	120	236	143
30	436	<10,000	1350	8	308	685	2335
51	400	<500	450	32	227	544	762
62	488	<500	1350	32	279	1317	2066

NOTE. NT, not tested.

* Virus was isolated from each patient at first time point indicated.

† Quantified by branched DNA assay. Values are copies of viral RNA/mL of plasma.

‡ Envelope-specific IgG was quantified by ELISA using HIV-1_{MN} gp160 as antigen for detection.

§ Neutralizing antibodies were measured in human peripheral blood mononuclear cells against early autologous isolate obtained ~1 month from onset of symptoms. Neutralizing antibodies to HIV-1_{IIIB} and HIV-1_{MN} were measured in MT-2 cell–killing assay, whereas neutralizing antibodies to HIV-1_{SF-2} were measured in CEMx174 cell–killing assay. Values are reciprocal of highest serum dilution to reduce production of p24 by >80% relative to negative control serum D01 (primary isolates) or to result in 50% reduction in virus-induced cell killing (strains IIIB, MN, and SF-2).

apy. Two remaining patients (27 and 46) had moderate levels of plasma virus at the earliest time point examined; these levels were undetectable by weeks 5 and 30 of follow-up, respectively, and remained low or undetectable thereafter. The last 2 patients began antiretroviral therapy shortly before plasma viremia declined to undetectable levels.

Envelope-specific IgG to HIV-1_{MN} gp160 was detected by ELISA at the earliest time point for all 6 patients (table 6). Seroconversion to the viral envelope glycoproteins at these early time points was confirmed by HIV-1_{IIIB} Western blot analysis (data not shown). Despite early seroconversion, development of neutralizing antibodies to the early autologous iso-

late was delayed in most patients. For example, autologous neutralizing antibodies in sera from patients 23, 27, and 30 were undetectable for 45, 27, and 18 weeks from onset of symptoms, respectively. Neutralizing antibodies in sera from patient 15 were undetectable at 4 weeks but were present at a titer of 1:4 by 20 weeks and rose to a titer >1:64 by 53 weeks. Patient 45 developed low-titer (1:2) autologous neutralizing antibodies by week 5, and the titer increased to 1:16 by week 25. This patient received no antiretroviral therapy and maintained high levels of plasma HIV-1 RNA of 594,000–230,000 copies/mL during this period of time. Patient 46 developed detectable neutralizing antibodies to his early autologous isolate by week 13 (titer of 1:2), and the titer steadily rose to 1:32 by week 51 and was maintained at week 62. This latter patient began antiretroviral therapy at about week 25, which was followed by a drop in plasma HIV-1 RNA to undetectable levels while titers of autologous neutralizing antibodies continued to increase. Neutralizing antibodies to the early autologous isolate were undetectable in sera from patients 23 and 27 throughout the entire period of follow-up (i.e., 45 and 27 weeks from onset of symptoms, respectively).

Neutralizing antibodies were detected within 5–20 weeks from onset of symptoms when TCLA strains of HIV-1 were used for detection (table 6). These neutralizing antibodies were most effective against strain SF-2, followed by MN and, to a much lesser degree, IIIB. Titers of neutralizing antibodies were relatively low before 17 weeks for SF-2 and for a considerably longer period of time for MN and IIIB. For example, average titers of neutralizing antibodies in sera from asymptomatic HIV-1–infected persons infected for 2–7 years are 1:345 and 1:680 for IIIB and MN, respectively [17], and 1:2259 for SF-2 (data not shown) in the same assay as that used above. On the basis of these average titers and the data presented in table 6, the development of high-titer neutralizing antibodies to IIIB and MN usually requires >25–53 weeks of infection.

Finally, 3 of 6 seroconverters showed signs of immune suppression within 1 year from the onset of symptoms, as evidenced by a drop in CD4 T cells below 500/mm^3 (patients 23, 27, and 46). This immune suppression showed no obvious correlation with plasma virus loads or neutralizing antibody responses.

Discussion

Our results show that long-term nonprogressive HIV-1 infection is associated with broad neutralizing antibody responses against primary isolates. Similar results have been reported [17, 18], but those studies did not determine whether broad responses were unique to LTNP. Our results showed that neutralizing antibodies in sera from LTNP are reactive against a broader spectrum of primary isolates than are sera from progressors and non–immune-suppressed asymptomatic persons who had been infected for <7 years (STNP). In addition, sera from LTNP were more likely to neutralize the contemporaneous autologous isolate than were sera from STNP or progressors.

Broadly cross-reactive neutralizing antibodies in sera from LTNP might be explained by responses against highly conserved epitopes, such as those recognized by the human monoclonal antibodies 2G12, IgG1b12, and 2F5 (reviewed in [28]) or by multiple responses to isolate-specific epitopes that accumulated over time [29]. The sporadic neutralization of our primary isolates by many sera suggests that variable, isolate-specific epitopes dominate the neutralizing antibody response. It is reasonable to assume that the virus regularly mutates to escape contemporaneous neutralizing antibodies against these variable epitopes. Consistent with this, Delwart et al. [30] observed a high and constantly changing complexity of viral quasispecies in LTNP over time. This could create a wide range of neutralization epitopes for antigen presentation and eventually lead to broadly cross-reactive neutralizing antibodies after long-term nonprogressive infection.

Early neutralizing antibody responses would have to be long-lived to become cumulative over time as an explanation for the broad responses in LTNP. Our results showed that neutralizing antibodies against early isolates continue to rise in titer for at least 1 year (patients 15 and 46, table 6). Others have shown that isolate-specific neutralizing antibody responses may be maintained for at least 2–3 years [29, 31–33]. Whether these responses can last for even longer periods of time has not been addressed. Isolate-specific neutralizing antibody responses would be maintained if the autologous virus persisted either as a minor population of replicating virus or as immune complexes on follicular dendritic cells. Additional studies are needed to determine more precisely the longevity of isolate-specific neutralizing antibody responses in HIV-1–infected persons and whether neutralization-escape variants arise periodically in LTNP.

It should be noted that the titers of neutralizing antibodies against primary isolates were low. Specifically, neutralizing antibodies detected in undiluted sera from progressors and LTNP were much broader than those detected previously [17] when the same serum samples were evaluated at a 1:16 dilution against this panel of primary isolates. The earlier study also determined that autologous neutralizing antibodies against viruses from 3 of 3 LTNP tested were undetectable at a 1:16 serum dilution, whereas here they were detected for all 3 LTNP when undiluted serum was used. Although the titers of these neutralizing antibodies were low, they may still be significant in the host, where they are undiluted.

Our detection of virus neutralization with undiluted serum raised the possibility that antiviral cytokines, such as the interferons [25], or antiviral chemokines, such as RANTES, MIP-1α, MIP-1β [34], and SDF-1 [35, 36], were partially responsible. Clearly this was not the case for sera from HIV-1–negative persons, since no neutralization was detected. It remained possible that the levels of antiviral cytokines and chemokines in serum are elevated during HIV-1 infection. We examined this

possibility and found that RANTES and MIP-1β were not elevated in serum samples with broad neutralization activity. Others have shown that serum chemokine levels are similar for LTNP and progressors and that these levels are below those reported to block HIV-1 infection in vitro [37]. Additional evidence that neutralization was antibody-mediated came from lack of neutralization when IgG was removed from serum samples. The virus neutralization detected with undiluted serum samples was therefore most likely antibody-mediated.

Our improved detection of primary isolate neutralization with undiluted serum led us to investigate how soon after primary infection autologous neutralizing antibodies could be detected. Most studies agree that neutralizing antibodies are slow to develop and do not correlate with the initial down-regulation of plasma viremia during primary infection [5–7, 10]. The initial down-regulation in plasma viremia in the patients followed here was probably due to a combination of their immune response and antiretroviral therapy. Our results agree that autologous neutralizing antibodies against the early isolate are slow to develop in many persons, even when measured with a 1:2 serum dilution. Possible exceptions were found with 3 patients who developed detectable autologous neutralizing antibodies within 5–20 weeks from onset of symptoms (patients 15, 45, and 46, table 6). These antibodies might have been present sooner, but appropriate samples were not available for analysis. Of particular interest was patient 45; this patient received no antiretroviral therapy and had high levels of plasma viremia (>230,000 viral RNA copies/mL) at all time points even though neutralizing antibodies against the early isolate were detected within 5 weeks from onset of symptoms. Neutralization-escape variants might have rapidly emerged in this person.

In addition to autologous primary isolates, several neutralization-sensitive TCLA strains of HIV-1 were used to assess neutralizing antibodies following seroconversion. Interestingly, neutralizing antibodies against strains MN and SF-2 were detected well before the detection of neutralizing antibodies against the autologous virus in 3 of 6 infected persons. A similar phenomenon has been described for neutralization-resistant variants of SIV in macaques [12, 38]. One possible explanation is that primary isolates contain neutralization epitopes that are shared with TCLA virus and are immunogenic but either are poorly exposed on primary isolate envelope glycoproteins or are not needed for primary isolate infectivity. In the case of poorly exposed epitopes, neutralizing antibodies that are specific for TCLA viruses could be induced when monomeric gp120 and gp160 are released into circulation by infected cells or when infected cells are killed by cytotoxic T lymphocytes. One region that appears to contain neutralization epitopes that are hidden in native, oligomeric gp120 is the V3 loop [39], which also is an important region for co-receptor interactions [40, 41].

We conclude that neutralizing antibodies are slow to develop during primary infection and are uniquely broad in LTNP. The slow development of neutralizing antibodies during primary infection could allow the virus to establish persistent infection. In this regard, any means of accelerating the neutralizing antibody response, such as by vaccination, might provide a clinical benefit that is not realized by the natural response to infection. Neutralization epitopes recognized by sera from LTNP would be interesting to pursue for HIV-1 vaccine development. We also wish to emphasize that envelope-specific IgG was detected very early during primary infection. Similar results have been reported previously [2], and although these antibodies are nonneutralizing, they might still provide a benefit by participating in antibody-dependent cellular cytotoxicity [7, 42] or in complement-mediated clearance [43, 44]. Finally, it may be possible that continuous and high production of circulating virus [45] will sometimes remove a significant portion of neutralizing antibodies and that these antibodies are not replenished at a pace to keep up with their removal. Our studies do not eliminate this as a possible explanation for cases in which neutralizing antibody titers were low or undetectable.

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