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Neutralizing Antibody Responses to Autologous and Heterologous Isolates of Human Immunodeficiency Virus

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Summary: Although laboratory-adapted strains of human immunodeficiency virus (HIV) are generally highly sensitive to neutralization by HIV-positive patient sera, we have found a more complex pattern of cross-neutralization and neutralization resistance among low-passage clinical isolates. These HIV isolates, like many other lentiviruses, resisted neutralization by the patient's own (autologous) antibodies. We assessed the degree of antigenic relatedness between different patient isolates of HIV through cross-neutralization with heterologous sera and virus isolates. Complicated patterns emerged, with variation in breadth of neutralization among individual plasmas and variation in frequency of neutralization among isolates. In longitudinal studies of individuals, we found that some but not all such patients develop a neutralizing response that "catches up" with their earlier isolates after a lag period. Taken together, these data suggest that an individual's immune response broadens with time because of cumulative exposure to multiple antigenic variants that arise throughout HIV disease. **Key Words:** HIV neutralizing antibodies—HIV antigenic variants—Heterologous neutralization—Autologous neutralization—Temporal development of HIV immune response.

Human immunodeficiency virus (HIV) infection causes a progressive, debilitating disease in which destruction of the immune system leads to characteristic opportunistic infections. The progression is usually not prevented even when the initial infection occurs in the presence of a competent, often vigorous, immune response to the virus (1). It has been observed in several laboratories that most HIV isolates resist neutralization by autologous, contemporaneous sera (i.e., sera taken from the same venipuncture as the viral isolate) (2–8). Evasion of the immune response is a phenomenon common to the lentivirus family, of which HIV is a member. These viruses produce slow, progressive diseases in a variety of immunocompetent hosts. The lentiviruses visna (9–11), equine infectious anemia virus (EIAV) (12,13) and caprine arthritis encephalitis virus (CAEV) (14,15), along with feline leukemia virus (FeLV) (16), have been shown to persist, at least in part, by mutating into neutralization-resistant variants. The generation of viral variants throughout the course of HIV infections has also been observed using both immunologic and molecular techniques (5,17–24). Neutralization-resistant HIV variants can furthermore be demonstrated in vitro by selection using monoclonal antibodies to the principal neutralizing domain (25,26).

The purpose of this study was to assess the degree of antigenic relatedness between different patient isolates through cross-neutralization of heter-

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ologous sera and virus isolates and to determine neutralizing antibody titers in HIV-infected individuals against their autologous (homotypic) isolates. We also determined the neutralization titers of sequential (longitudinal) bleeds from several individuals against their own isolates to characterize temporal development of the humoral immune response to HIV. Taken together, these data suggest the development of sequential variants of HIV in the infected individual.

MATERIALS AND METHODS

Subjects and Clinical Specimens

The virus isolates were derived from blood specimens collected from randomly selected long-term infected symptomatic and asymptomatic HIV-infected homosexual men living in California. These subjects were participating in (a) the San Francisco Men's Health Study (27), (b) the University of Southern California's clinical trial of the Salk Immunogen sponsored by the Immune Response Corp., and (c) studies on neurological manifestations of AIDS at the Oak Knoll Naval Hospital. It should be noted that some subjects in the second group received the Salk Immunogen (28) during our studies. There were no detectable changes in neutralizing antibodies, however, in response to inoculation with this envelope-depleted material. Anticoagulated blood specimens were collected from all subjects; the plasma was removed by centrifugation and stored at 4°C; and the patient peripheral blood mononuclear cells (PBMCs) were cultured as described subsequently.

Viruses

Primary clinical isolates of HIV were all recovered from cultured PBMCs as previously described (29). Briefly, HIV isolation was accomplished by incubating fresh or frozen HIV-infected patient PBMCs with seronegative donor PBMCs in RPMI-1640 medium containing 20% heat-inactivated fetal bovine serum, 2 μg/ml polybrene, 5% interleukin-2, and 0.1% anti-human leukocyte interferon. The cultures were fed with fresh donor PBMCs once a week, and the supernatants were assayed for the presence of reverse transcriptase (RT) activity beginning at day 11. The cultures were considered positive if, for 2 consecutive weeks, the RT counts were >10-fold higher than those in cultures of the seronegative donor PBMCs alone.

Forty-seven of the resultant RT-positive virus isolates were tested for cytolysis in the α-4 clone (30) of MT cells (31), and 28% of these were found to be cytolytic, a requirement for viruses usable in the MT2 microplaque assay system described subsequently. Supernatant fluids from the primary PBMC isolation cultures were used to infect expanded cultures of phytohemagglutinin (PHA)-stimulated PBMCs from healthy seronegative blood donors. These infected PBMC cultures were grown in RPMI-1640 medium supplemented with 15% fetal bovine serum, 5% interleukin-2, 0.1% anti-α interferon, 2 μg/ml polybrene, 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin. The crude culture supernatants were harvested after 7 days and frozen as viral stocks at −70°C. HIV_{IIIB}, originally from the supernatant of chronically infected H9 cells (generously supplied by Robert Gallo, NCI), was also grown in PBMCs and harvested as previously described.

Microplaque Neutralization Assay

MT2 (clone α-4) cells were used as indicator cells in a 96-well microtiter plaque reduction assay, which was previously described (30). Briefly, serial twofold dilutions of heat-inactivated patient plasma were combined in quadruplicate with an equal volume containing 10–25 plaque-forming units (PFU) of HIV per well and incubated for 18 h at 37°C. The diluent used for both virus and patient plasma dilutions contained 50% normal human serum pool (prepared by recalicification of human plasma), which had or had not been heat inactivated (56°C, 60 min) to remove complement, depending on the experiment. The presence of the high, constant level of human serum effectively overwhelms variable inhibitory or stimulatory effects of individual patient plasmas (30). Negative control wells also contained 50% normal human serum pool with no patient immune serum. After the 18-h incubation of serum and virus, 90,000 MT2 cells were added per well and incubated at 37°C for 1 h. SeaPlaque Agarose in assay medium at 39.5°C was then added to a final concentration of 1.6%. While the warm agarose was still molten, the microtiter plates were centrifuged at 20°C for 20 min at 500 *g* to form cell monolayers. The plates were incubated for 5 days at 37°C and then stained 18–24 h with 50 μg/ml propidium iodide. The fluorescent plaques were counted with transillumination by a 304-nm ultraviolet light source using a low-power stereo zoom microscope. Neutralization titer is defined as the reciprocal of the plasma dilution giving 50% inhibition of plaque count as compared with controls. This dilution is interpolated between data points by a computer program using a third-order least-squares curve fit. Within an experimental run, the intrinsic statistical error of the interpolated titers averages ±30%.

RESULTS

Table 1 shows neutralization of 11 HIV primary clinical isolates (and HIV_{IIIB}) by 23 plasmas, including autologous, contemporaneous neutralization by the 11 plasmas taken from the same venipuncture as the clinical viral isolates. (Plasmas 11–22 are from patients whose viruses did not induce syncytia in MT2 cells and from whom only the plasmas were therefore used in this study.) A complex pattern of (heterologous) cross-neutralization is seen; however, all plasmas failed to neutralize autologous, contemporaneous isolates. The heterologous reactions reveal some viruses that were neutralized by most plasmas (particularly virus 1) and other viruses that were relatively resistant to neutralization (viruses 6, 9A, and 9B). Similarly, different plasmas had a wide range of neutralization capabilities. Plasmas 10, 13, and 18 neutralized almost all of the isolates, whereas plasmas 1, 2, 15, and 19 had no neutralizing activity against the primary isolates tested.

TABLE 1. *Cross-Neutralization of Heterologous and Autologous HIV Isolates*

	Reciprocal 50% neutralization titer with indicated viral isolate[a]											
Antiserum	1	2	3	4	5	6	7	8	9A[b]	9B[b]	10	IIIB
1	—[c]	—	—	—	—	—	—	—	—	—	—	17
2	—	—	—	—	—	—	—	—	—	—	—	11
3	13	—	—	—	—	—	—	—	—	—	—	75
4	11	—	—	—	—	—	13	—	—	—	—	18
5	98	19	—	12	—	—	—	—	—	12	—	17
6	686	—	—	16	—	—	—	—	—	—	49	77
7	46	—	17	—	30	—	—	—	—	—	21	30
8	11	26	—	11	—	—	18	—	—	—	12	130
9A[b]	22	11	18	—	40	—	83	15	—	—	—	95
9B[b]	25	27	30	—	19	—	53	23	—	—	—	63
10	63	106	15	—	279	15	75	28	11	15	—	288
11	178	—	—	—	—	—	15	—	—	—	11	171
12	25	—	—	—	—	—	12	—	—	—	—	37
13	144	19	95	13	130	28	20	29	15	41	36	86
14	106	—	16	43	—	13	10	—	—	—	20	39
15	—	—	—	—	—	—	—	—	—	—	—	23
16	77	—	—	—	—	—	—	—	—	—	—	166
17	—	—	10	—	14	—	—	—	—	—	—	28
18	77	83	597	279	149	35	184	21	131	49	26	1154
19	—	—	—	—	—	—	—	—	—	—	—	22
20	61	39	—	10	—	—	28	17	17	83	13	36
21	21	—	—	—	—	—	—	—	—	—	—	28
22	—	—	13	—	—	—	19	—	—	—	—	26
Pos control	381	166	20	44	86	23	109	95	77	197	27	408

Boxed areas are autologous contemporaneous virus–antiserum pairs.
[a] Viral isolates grown in normal peripheral blood mononuclear cells.
[b] 9A and 9B are from bleeds 5 months apart from the same individual.
[c] —, negative (<1:10).

Interestingly, virus 1 was neutralized by nearly all of the plasmas, but the contemporaneous plasma from this individual was incapable of neutralizing any of the isolates. Thirty-two of the 40 positive heterologous reactions revealed such unidirectional cross-neutralization.

Mixing experiments were performed with several neutralizing heterologous plasmas in the presence of a high concentration of (nonneutralizing) autologous plasma. No changes in titer of the neutralizing antibody were found in the presence of the nonneutralizing antibody (data not shown), thus ruling out any significant role for "blocking" antibodies or autologous antibodies that could mask neutralization by means of offsetting antibody-mediated enhancement of viral expression.

Figure 1 shows the neutralizing antibody titers obtained with sequential viral isolates from patient 7. Autologous neutralization by contemporaneous antibody is absent. However, ~2–3 months after each viral isolation, autologous neutralizing antibodies appear and then increase in titer. All plasmas obtained at the time of or before a particular isolate not only failed to neutralize that isolate, but 70% of

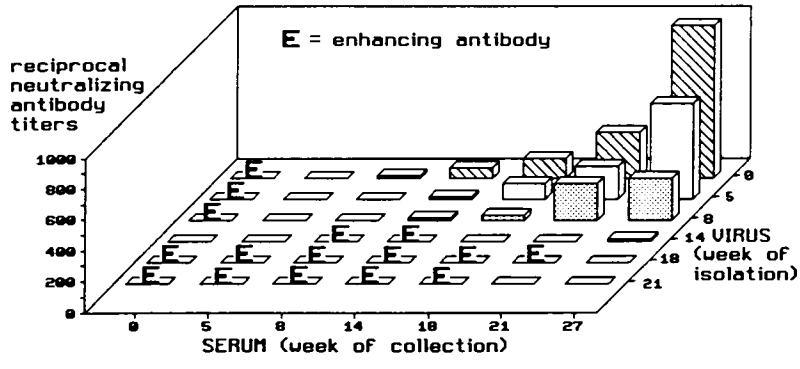


FIG. 1. Autologous HIV cross-neutralization titers in an asymptomatic individual. The vertical scale shows the reciprocal of the interpolated plasma titer giving 50% inhibition of HIV plaque number. E, the plasma enhanced expression of the isolate by at least 150%.

these plasmas also moderately enhanced the expression of the isolate. Figure 2 shows an example of such autologous enhancement of an earlier isolate by a plasma that neutralized a later isolate. The observed enhancement is complement dependent and disappears if the human plasma in the assay diluent is heat inactivated (data not shown).

As shown in Tables 2 and 3, the clear-cut pattern of catch-up neutralization of patient 7 is not typical of all patients: One patient (no. 10, Table 2), starting 4 months after the virus isolation, maintained for several months a constant low positive neutralizing titer to the only autologous isolate tested. Another patient in this cohort (no. 9) failed to develop neutralizing antibody to three autologous isolates, collected over a 14-month period. Two isolates from this latter patient (viruses 9A and 9B) were included in the study of heterologous neutralization (Table 1), in which they showed very similar heterologous neutralization resistance.

Table 3 shows three other patients with sequential viral isolates and serum samples obtained with longer intervals (approximately 6 months) between specimens. From each of two of these patients, two isolates were tested, but from the third individual (no. 25), only a single viral isolate was available for testing. The latter patient had available bleeds only preceding his isolate. This patient's plasmas from the preceding bleeds failed to neutralize this isolate. The neutralization titers in Table 3 were derived from neutralizations performed in the absence of active complement because control experiments indicated that complement-mediated enhancement was masking neutralization in some reactions. Similar control experiments with selected specimens showed that the relative titers in Tables 1 and 2 were unaffected by the presence of complement.

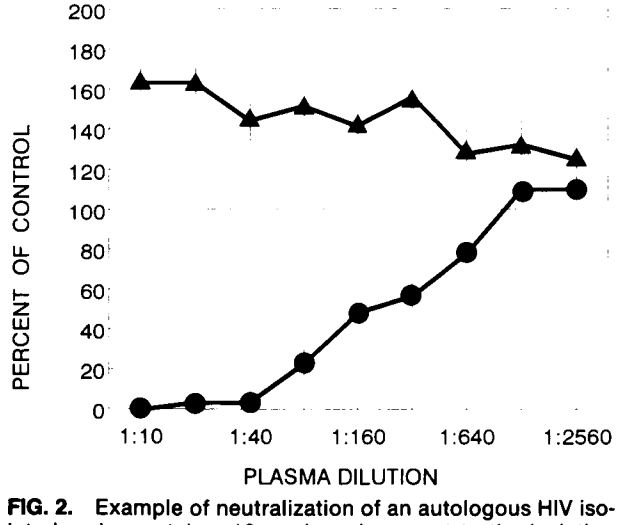


FIG. 2. Example of neutralization of an autologous HIV isolate by plasma taken 16 weeks subsequent to the isolation and enhancement by the same plasma of an isolate derived 3 weeks before the plasma. The subject is the same patient from whom the data of Fig. 1 was obtained. ●, week no. 8 virus; ▲, week no. 18 virus.

TABLE 2. *Neutralization of an isolate of patient 10 by sequential autologous bleeds at short intervals*

Bleed times	Reciprocal titer
Contemporaneous	—
+16 weeks	19
+18 weeks	25
+25 weeks	34
+28 weeks	24
Positive control	10

Positive control is a pool of HIV—positive patient sera.
—, negative (<1:10).

Patient 23 (Table 3) had neutralizing antibody in plasmas from all the bleeds tested, including those predating, post-dating, and contemporaneous with the viral isolate from bleed 23A. The 23B isolate, which was isolated from the same patient 6 months after virus 23A, was neutralized by only one plasma, which postdated the isolate by 1 year. This was a very low level neutralization titer, which reverted to negative at subsequent bleeds.

Patient 24 (Table 3) developed a neutralizing antibody titer to the bleed 24A virus by the next time blood was drawn (~6 months later), but plasmas preceding or contemporaneous with the viral isolate failed to neutralize it. A neutralizing titer was detected against the bleed 24B virus from this patient 18 months after isolation, but the bleeds preceding and contemporaneous with the virus were negative for neutralizing ability.

DISCUSSION

Our data show that (a) there are varying amounts of (heterologous) cross-neutralization among patients' plasmas and virus isolates; (b) such cross-neutralization is overwhelmingly unidirectional; (c) individuals always show little or no autologous neutralization with their contemporaneous plasma; and (d) some individuals exhibit a pattern of catch-up neutralization of emerging autologous viral variants, whereas others do not. We believe that these observations, especially the lack of autologous neutralization, support a theory of HIV pathogenesis in which the generation of neutralization-escape mutants allows the virus to persist in the host.

TABLE 3. *Neutralization of patient isolates by sequential autologous sera at 6-month intervals*[a]

Approximate time of bleed (relative to isolation)	Patient 23 isolates		Patient 24 isolates		Patient 25
	A	B	A	B	A
−24 months	nd	nd	nd	nd	—
−18 months	nd	—	nd	—	—
−12 months	10	nd	—	—	—
−6 months	nd	—	—	nd	—
At virus isolation	21	—	—	—	—
+6 months	28	—	16	—	nd
+12 months	67	11	12	nd	nd
+18 months	24	—	41	211	nd
+24 months	26	—	171	57	nd
+30 months	27	nd	65	nd	nd

For patients with two isolates, isolates A and B were made 6 months apart.
nd, not done; —, negative (<1:10).
[a] Reciprocal 50% neutralization titer with indicated isolate.

The heterologous neutralization data in Table 1 show a complex pattern of cross-reactivity and a predominance of unidirectional cross-neutralizations (i.e., plasma from patient A will neutralize the virus from patient B, but the plasma of patient B will not neutralize the virus from patient A). The data reveal that some viruses are neutralized by most sera from infected individuals whereas other isolates are neutralized by few. A schema based on the pattern of cross-reactivity is presented in Fig. 3. This pattern of cross-reactivity has features in common with that found in the work of Nowak et al. (32), who presented a computer model for the accumulation of viral variants throughout disease. Their model predicts that before suppression by neutralizing immune responses, each new viral variant inflicts cumulative damage to the immune system, thus leading to its eventual collapse after a long period. We suggest a succession or accumulation or both of viral variants, in agreement with the Nowak model. We assume that each virus isolated is the most recent, dominant type to have arisen in the patient to which the patient has not yet mounted a neutralizing immune response. The plasma of each such patient, on the other hand, contains neutralizing antibodies to antigenic types of the virus that have previously arisen in the patient. (It is possible that some nonimmunogenic types have also arisen but failed to evoke a response.) The specificity of such a plasma is thus the total of these prior antigenic types, each being symbolized in Fig. 3 by an alphabet letter. This scheme predicts that there are a number of variants (as defined by neutralization phenotype) and that these emerge at different times in the course of disease. The pattern assumed in Fig. 3 accounts for all of the unidirectionality of neutralization that was observed. The model in Fig. 3 requires hypothesizing multiple viral antigenic types, which, in the case of these data, are as many types as there are patients.

It is notable that among the 11 viruses in Table 1, there are isolates relatively sensitive and isolates relatively resistant to all 23 plasmas tested. This pattern of susceptibility and resistance could reflect either inherent biochemical susceptibility to neutralization or the statistical predominance of certain neutralization epitopes throughout this infected human population.

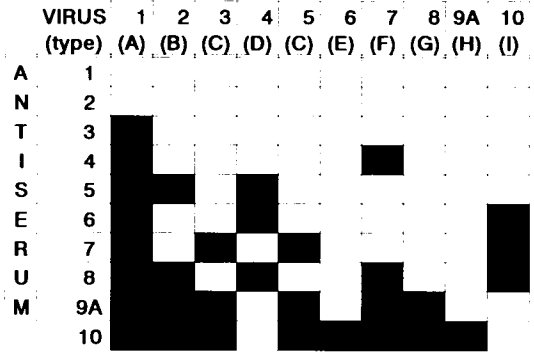
The data in Table 1 suggest that seropositive plasmas may vary greatly in their breadth of neutralization of heterologous isolates, and the data from Tables 2 and 3 show that in sequential bleeds from the same individual, there is an evolution of the immune response over time, which may result in this increased breadth of reactivity. The data and the model that results from analysis of these data (Fig. 3) suggest that an individual who has had many variants arise during the course of an infection will have greater cross-neutralization of heterologous isolates than an individual who has experienced few variants.

Sequential virus isolates and plasmas from several individuals were studied for autologous neutralization. We found several patterns, including patients who developed a neutralizing response to newly emerging variants, patients who did not develop a neutralizing response during the period of observation, and one patient who neutralized an isolate both with contemporaneous plasma and plasma drawn before virus isolation. The different patterns may reflect genetic differences in the infecting viruses, in the individual's immune re-

sponse, or in the stage of infection at the time of our studies. A consistent finding, both in our autologous, longitudinal study and in our heterologous study, was the negative (or, rarely, very low) contemporaneous autologous neutralizing titer.

HIV infection is known to generate neutralization-resistant mutants when under immune pressure. The variants are detected in infected individuals who fail to neutralize their contemporaneous isolates even though they effectively neutralize heterologous isolates [and autologous viruses isolated earlier (2,3,6–8)]. Neutralization resistance can also be generated by in vitro culturing of HIV in the presence of neutralizing antibodies. The use of monoclonal antibodies for this purpose has helped determine some of the regions of the viral envelope gp120, including the principal neutralizing determinant in the V3 loop, as well as the V2 and CD4-binding regions, which are important in neutralization (33–39). New viruses may emerge continually in vivo as a result of immune pressure on the predominant viral quasispecies. In our studies, patients 7, 23, and 24 all generated neutralization escape mutants over time. Patients 7 and 24 were able to respond rapidly to their new variants, but patient 23 failed to develop neutralizing antibody to the second isolate (bleed 23B) within the 2-year study. In addition to that found in our work, an absence of neutralizing response to autologous isolates has also been found in the studies of several other investigators (3,6–8,22). This is not, however, a universal observation; other laboratories have reported positive autologous, contemporaneous neutralization (4,40–43). There is also a lack of agreement among studies as to whether there is a catching-up of neutralizing antibodies in bleeds subsequent to the virus isolation. These discrepancies may be related to the use of different neutralizing antibody assay methodologies, different host cells for virus stock production, patient populations at different stages of HIV disease, or more than one of these.

In addition to evasion of neutralization through mutation, other factors may alter viral properties. The presence of an autologous neutralizing titer that increases over time (patient 7) is evidence that the successive immune responses may not completely eliminate the variants—as with visna, where the prior variant coexists at a new lower level along with the new dominant quasispecies (10,44). Another possibility is that antibodies to the earlier variant are produced in response to newly emerged variants that are very similar to the original (original antigenic sin) (45). This latter phenomenon has been observed both with HIV infections and with experimental HIV immunizations (5,46). Either of these situations may be deleterious to the host: Persistant low-level variants may inflict steady low-level damage or reemerge as a dominant species after the loss of critical immune function. Similarly, an anamnestic response to an earlier variant may damage the host by precluding the development of an effective antibody response to a new mutant. Another mechanism could be the continuing presentation of antigen on the surfaces of follicular dendritic cells in the lymph nodes. These cells are known to concentrate antigen in immune complexes on their cytoplasmic processes for a year or more (47).



1: A
2: B
3: A -> C
4: [A -> F] -> D
5: [A -> B -> D] -> C
6: [A -> D -> I] -> E
7: [A -> C -> I] -> F
8: [A -> B -> D -> F -> I] -> G
9A: [A -> B -> C -> F -> G] -> H
10: [A -> B -> C -> E -> F -> G -> H] -> I

The order of types within square brackets is arbitrary.

FIG. 3. Cross-neutralization of heterologous HIV clinical isolates. A filled square in the figure indicates positive neutralization (>1:10). Each isolate is arbitrarily symbolized by an alphabet letter representing the predominant variant at the time of isolation, and the data are arranged to emphasize the varying degrees of cross-neutralization by the corresponding plasmas. The "specificity" of each plasma, according to this model, is the total of variants to which the plasma donor has been able to mount a neutralizing antibody response. At the bottom of the figure, a hypothetical sequence of such variants for each subject (one of many possible sequences), consistent with the data, is presented. It is assumed in this model that the predominant variant at the time of venipuncture is a neutralization-resistant mutant, which is therefore not included in the cumulative specificity of the corresponding plasma.

Some patients (patients 9 and 23) did not generate neutralizing responses to their autologous viruses. Interestingly, patient 9, from whom three isolates were obtained over 14 months, did not have neutralizing antibody against any of his isolates. The first two of these viruses also have highly similar patterns in their heterologous neutralization sensitivity (the third isolate was not tested with the heterologous sera). As shown in Table 1, these isolates (9A and 9B) were among the most resistant viruses to heterologous neutralization by all 23 plasmas, raising the possibility that these isolates are inherently difficult to neutralize. Alternatively, original antigenic sin could prevent an adequate immune response. These viruses might be expected to inflict severe damage on the host because they can grow unimpeded by neutralizing antibody. It has been observed that individuals whose viruses do not diverge significantly from the infecting inoculum (24) or who fail to develop a neutralizing antibody response to the initial variant (41) progress more rapidly to disease than those who respond immunologically and generate viral divergence. Finally, patients in late disease stage may have a generalized inability to generate responses to antigens, because of loss of immune cell function. Unfortunately, clinical data for the individuals in our study are unavailable.

In early studies, many HIV-infected patients progressed to AIDS in spite of significant neutralizing antibody titers found in their sera (48–51). Most such studies employed laboratory-adapted strains of HIV—notably HIV_{IIIB}—grown in established cell lines. Such viral stocks are readily neutralized by nearly all patients, perhaps because growth in established cell lines renders the virus neutralization sensitive. Our studies were performed using low passage PBMC-grown virus stocks. This may contribute significantly to neutralization differences compared with T-cell–grown laboratory-adapted strains. We have observed very dramatic increases in neutralization titers of antibodies against some isolates grown in H9 cells as compared with the same isolate grown in PBMCs (52). The relative lack of neutralization observed with low-passage isolates, and in particular the lack of neutralization of autologous isolates, may help to resolve the paradox that clinical status does not correlate with neutralizing antibody titers determined using laboratory-adapted virus strains.

This study was conducted using only those viruses that would grow and induce syncytia in MT2 cells. The results may therefore pertain to the subset of viruses that are considered more pathogenic, usually associated with more advanced stages of HIV disease, and have in vitro phenotypes of rapid production of large amounts of virus, growth in established T-cell lines, and syncytium production in PBMC cultures (53–57). Studies of viruses that will not grow in MT2, including those from individuals 11 through 22 in Table 1, are currently under way and are important to determine the relevancy of our observations to a wider range of viral isolates and stages of disease.

It remains to be determined in prospective studies with clinically defined patients whether the neutralization of heterotypic and homotypic viral isolates may have prognostic significance. To avoid artifacts resulting from the possible isolation in culture of minor in vivo forms of the virus, future studies might evaluate neutralization sensitivity before culturing by isolating virus from serial dilutions of a patient clinical specimen in the presence and in the absence of autologous serum.

The data presented here may have implications for the design of HIV vaccines. The effort to define serotypes of HIV on which to base such vaccines may be complex. Serotypes are classically defined by cross-neutralization of infectivity rather than by mere binding to antibodies. It is significant that we saw patterns of heterologous neutralization that suggest multiple phenotypes, indeed, as many phenotypes as there were individuals in the study. Finally, the inability of patients to mount neutralizing antibody responses to contemporaneous autologous virus variants may be one important explanation for viral persistence and disease progression.

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