Antibody-Mediated Neutralization of Primary Isolates of Human Immunodeficiency Virus Type 1 in Peripheral Blood Mononuclear Cells Is Not Affected by the Initial Activation State of the Cells

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Antibody-mediated neutralization of human immunodeficiency virus type 1 (HIV-1) was evaluated with primary isolates and sera from infected individuals, using human peripheral blood mononuclear cells (PBMC) activated with phytohemagglutinin 1 day after virus inoculation (resting-cell assay) or 2 days prior to virus inoculation (blast assay). Assays were performed exclusively with syncytium-inducing (SI) isolates since non-SI isolates replicated poorly or not at all in the resting-cell assay. Ninety percent neutralization was difficult to achieve in both assays for most virus-serum combinations tested. Of particular note, virus replication in the absence of antibody was delayed 2 to 3 days in the resting-cell assay. At least part of this delay was due to a decrease in virus infectivity; the 50% tissue culture infectious dose of primary isolates was 25 to 30 times lower in the resting-cell assay than in the PBMC blast assay. When a broadly neutralizing serum and the same dilution of virus were used in both assays, neutralization was greater in the resting-cell assay than in the blast assay on day 7, but neutralization was equal in both assays when measurements were made 3 days sooner in the PBMC blast assay. Both assays had the same level of detection on day 7 when the amount of virus mixed with antibody and added to cells was standardized according to infectivity for the respective target cells. Thus, when the infectious dose was adjusted, the two assays were equally sensitive for detecting antibody-mediated neutralization of primary isolates of HIV-1. These results indicate that primary isolates of HIV-1 are difficult to neutralize in both assays and that the detection of neutralization is not affected by the initial activation state of PBMC.

The significance of virus-specific neutralizing antibodies detected in biological assays depends upon the extent to which the *in vitro* assay predicts antibody function *in vivo*. Antibody-mediated neutralization of human immunodeficiency virus type 1 (HIV-1) assessed in transformed human CD4 T lymphoblastoid cell lines and mitogen-stimulated peripheral blood mononuclear cells (PBMC) (15) may be considered biologically relevant since CD4 T cells are a major target for infection *in vivo* (28). A current dilemma with these assays is that laboratory strains of HIV-1 are considerably more sensitive to neutralization than primary isolates (17, 19, 21). One possible explanation for the difficulty in neutralizing primary isolates is that cellular proteins on the virus or target cells mask important epitopes or alter the way in which the virus physically interacts with the cell surface so that antibody binding no longer neutralizes virus infectivity. The surface of HIV-1 contains a number of cellular proteins, including major histocompatibility complex molecules, leukocyte adhesion receptors, complement control proteins, and other factors (2, 18, 23) that are likely candidates for producing such effects. Other elements to consider are the density of the major receptor for HIV-1, CD4 (8), and the differential utilization of recently described coreceptors for the virus, such as the chemokine receptors CXCR-4/LESTR (13), CCR-2, CCR-3 (7, 10), and CCR-5 (1, 9, 11), on the cell surface. Lymphocyte stimulation

can dramatically affect the expression of chemokine receptors (16, 22, 32) and surface adhesion molecules (33) and can also influence the association of CD4 with the antigen receptor complex (26). These observations imply that antibody-mediated neutralization might be affected differentially depending on the activation state of the cells used in neutralization assays.

PBMC are the preferred cell type for primary HIV-1 isolate neutralization assays, and most studies agree that lymphocyte activation is required for the productive infection of PBMC *in vitro* (6, 14, 29, 31, 35). A common method used to prepare activated lymphocytes for HIV-1 neutralization assays is to incubate freshly isolated PBMC with mitogens, such as phytohemagglutinin (PHA), prior to virus inoculation (15). Lymphocyte activation might also be important for productive HIV-1 infection *in vivo* (6). The vast majority of CD4 T cells in the body reside in lymphoid organs (31), which have been identified as a major site of HIV-1-infected cells (24), and 25 to 50% of CD4 T cells in the lymph nodes of HIV-1-infected individuals appear to be activated (25). Quiescent cells exposed to HIV-1 *in vitro* harbor viral genomic intermediates with a half-life of approximately 1 day that degrade without producing progeny virus (34), but productive infection can be rescued by stimulating the cells with a mitogen shortly after virus exposure (29, 35). CD4 T cells might be exposed to similar activation signals, intrinsically, shortly after exposure to HIV-1 *in vivo*. It seems prudent, therefore, to include both quiescent and activated lymphocytes in studies of antibody-mediated neutralization of HIV-1 *in vitro*.

It was recently reported that primary HIV-1 isolates are more sensitive to antibody-mediated neutralization in resting PBMC than in PBMC blasts (38). Nonneutralized virus in

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resting PBMC was amplified by stimulating the cells 1 day after the addition of virus-serum mixtures, thereby affording neutralizing antibodies an opportunity to block infection prior to lymphocyte activation. Neutralization that is detected only under these conditions may be taken as evidence that mitogen-induced changes in cell phenotype interfere with virus neutralization. Hence, observations made in this resting-cell assay could have important implications for our understanding of HIV-1 neutralization and the biological relevance of *in vitro* assays.

To investigate this phenomenon further, antibody-mediated neutralization of six primary HIV-1 isolates was evaluated here in the resting-cell assay and in PBMC blasts, with sera from eight infected individuals. All sera contained high-titer neutralizing antibodies to one or more of the laboratory strains IIB, MN, and SF-2 as measured in a cell-killing assay (20). Primary isolates included a stock of PBMC-grown BZ167 obtained from Susan Zolla-Pazner (New York University Medical Center). This stock of BZ167 was used in resting-cell assays described previously (38) and was expanded once in human PBMC for the assays we performed here. The remaining primary isolates have been described elsewhere (19). Each of these isolates had a syncytium-inducing (SI) phenotype in MT-2 cells and human PBMC. Non-SI (NSI) isolates were excluded from the study, since 10 of 10 NSI isolates examined replicated poorly or not at all in the resting-cell assay.

Neutralization assays were performed with PBMC from random HIV-1-negative donors, but cells from multiple donors were never mixed. Cryopreserved and noncryopreserved PBMC came from separate donors. Buffy coats were obtained from the local Red Cross and were processed by centrifugation over lymphocyte separation medium (Organon Teknika/Akzo, Durham, N.C.). PBMC at the interface were washed twice with Hanks' balanced salt solution, suspended at a concentration of 5×10^7 cells/ml in cryopreservation medium (90% fetal bovine serum plus 10% dimethyl sulfoxide), and frozen in liquid nitrogen with the aid of a Gordonier controlled-rate cryostat. PBMC were thawed and used for neutralization assays as resting cells and blasts in parallel, as described elsewhere (38), with the exception that the cells were stimulated with PHA (PHA-P; Sigma Chemical Co., St. Louis, Mo.) at 5 instead of 0.8 μ g/ml; we found that the lower concentration of PHA did not support the replication of most primary isolates in our laboratory. PHA used previously (38) was purchased from Difco, and it is possible that this preparation was more active than ours.

Unless otherwise indicated, viruses were used as 1:5-diluted stocks obtained at the peak of virus production in human PBMC. Virus (50 μ l) was mixed with diluted serum samples (50 μ l each), incubated at 37°C for 1 h, and transferred to 96-well culture plates containing resting PBMC or 2-day PBMC blasts. Virus-serum mixtures were incubated with cells for 1.5 h at 37°C and then removed from the cells by careful aspiration as described elsewhere (38). Fresh growth medium was added, and the cells were incubated at 37°C for 24 h before PHA and interleukin-2 were added to the medium. Cell suspensions (25 μ l each) were collected daily thereafter and were mixed with 225 μ l of 0.5% Triton X-100 for p24 immunoassay (DuPont). An equal volume of growth medium was added in place of the cell suspensions removed each day. Virus production in the absence of test serum (virus control) was measured by p24 immunoassays on each daily sample. Virus production in the presence of test serum (neutralization) was measured on various days as indicated in the text and figure legends.

Neutralization assays were first performed with BZ167 and three additional primary isolates in resting cells and PBMC

TABLE 1. Antibody-mediated neutralization of primary HIV-1 isolates in resting cells and PBMC blasts

% p24 reduction and virus	Assay	Neutralization titer with serum ^a :			
		W86165	V89872	6101	W25798
≥90					
BZ167	Resting cell	160	40	<10	80
	Blast	40	20	<10	40
W179273	Resting cell	—	—	—	—
	Blast	—	—	—	+
V67970	Resting cell	—	—	—	+
	Blast	+	—	—	+
W86165	Resting cell	+	—	—	—
	Blast	—	+	—	—
≥50					
BZ167	Resting cell	>320	160	<10	160
	Blast	160	80	<10	40
W179273	Resting cell	—	—	—	+
	Blast	—	—	—	+
V67970	Resting cell	+	+	—	+
	Blast	+	+	—	+
W86165	Resting cell	+	+	—	—
	Blast	—	+	—	—

^a Neutralization of BZ167 was evaluated with twofold serum dilutions ranging from 1:10 to 1:320 in triplicate. Titers are the reciprocal of the last serum dilution to reduce p24 production by ≥ 90 or $\geq 50\%$. Other viruses were evaluated at a 1:10 serum dilution only, and results are presented as positive (+) or negative (—) for neutralization.

blasts in parallel, as described above, with sera from four HIV-1-infected individuals. BZ167 was evaluated with twofold serum dilutions ranging from 1:10 to 1:320 to determine the threshold dilution at which neutralization was detected in each assay. The remaining primary isolates are difficult to neutralize in PBMC blasts (19) and therefore were tested only at a 1:10 serum dilution. Data were analyzed in two separate ways, with ≥ 90 and $\geq 50\%$ reductions in p24 as the cutoffs for positive neutralization.

The results of this first set of parallel neutralization assays are shown in Table 1. Neutralization was measured in the PBMC blast assay and the resting-cell assay, respectively, on days 4 and 6 for BZ167, on days 5 and 7 for W179273, on days 4 and 6 for V67970, and on days 6 and 8 for W86165. BZ167 was neutralized by three of four sera in both assays; $\geq 90\%$ neutralization was often detected at serum dilutions twofold to fourfold higher than those which neutralized the virus in PBMC blasts. As discussed below, the detection of higher titers of antibody against BZ167 in the resting-cell assay was probably a reflection of the lower infectivity of the virus inoculum in that assay than in the PBMC blast assay. The remaining primary isolates were difficult to neutralize in both assays, where 90% neutralization was detected in only 6 of the remaining 24 tests performed. Of these six positives, two occurred with the same virus-serum combination in both assays, three occurred in blasts only, and one occurred in resting cells only. These few discordant outcomes signify no clear qualitative differences between the two assays and can probably be explained by normal experimental variation for sera having borderline neutralization activity at the dilution tested. This

explanation is supported by the greater concordance obtained with the less stringent 50% cutoff. Here, most virus-serum combinations that tested positive were indeed positive in both assays, with the exception of one combination that was positive only in the resting-cell assay. Neutralization of BZ167 was positive for the same three sera that were positive at the $\geq 90\%$ cutoff, and titers were proportionately higher at the 50% cutoff.

We also performed a set of parallel assays with fresh PBMC which had not been frozen and in which the quiescent-cell phenotype would be less disturbed. This is different from the assays described previously (38) and from all other assays performed here, where cryopreserved PBMC were used. Resting-cell assays with noncryopreserved PBMC were initiated on the day PBMC were isolated. Blast assays were begun 2 days later with PBMC which were from the same batch but which had been incubated with PHA for the 2-day period. Two primary isolates, designated V89872 and H69172, were evaluated with sera from four infected individuals. The sera were tested at threefold dilutions ranging from 1:10 to 1:270. Neutralization ($\geq 90\%$) was detected with only one serum sample. This serum sample neutralized isolate V89872 in PBMC blasts (titer of 1:10) but not in resting cells and neutralized isolate H69172 in both assays, where the titer was 1:10 in the resting-cell assay and 1:30 in the PBMC blast assay. No neutralization was detected in either assay for the remaining virus-serum combinations tested (data not shown).

The above results indicate that it is difficult to achieve 90% neutralization of many primary isolates in resting-cell or PBMC blast assays. BZ167 was an exception because it was neutralized at relatively high dilutions of sera from four of five infected individuals in both assays. Sera that contained high-titer neutralizing antibodies to BZ167 rarely neutralized any of eight primary isolates when tested previously at a 1:16 dilution in PBMC blasts (19). Thus, the detection of neutralizing antibodies with BZ167 does not predict the ability of those antibodies to neutralize most other primary isolates. This underscores the importance of including multiple isolates when the overall quality of neutralizing antibodies is being assessed.

When performing the neutralization assays described above, we noticed that the replication of all primary isolates was delayed in the resting-cell assay. This delay in replication is consistent with incomplete reverse transcription of the viral genome in quiescent cells (34) and also agrees with the difficulty others have had in rescuing virus by mitogenic stimulation after virus exposure (31). A similar delay in the replication of BZ167 in resting-cell assays was reported previously (38); in that study, about 10 times more p24 was detected in PBMC blasts than in resting cells on day 4. We selectively measured neutralization when virus production in the absence of antibodies was in a linear phase of increase, which usually meant that neutralization was measured in the blast assay 2 days before it was measured in the resting-cell assays. This was done in the event that prolonged incubations would permit nonneutralized virus to eventually grow through. The above-described assays used a fixed virus inoculum, and it was possible that the quantity, specificity, and affinity of antibodies in a given serum sample would not be sufficient to neutralize all infectious particles. Many stocks of primary HIV-1 isolates contain a mixture of genetic quaspecies that could have different sensitivities to neutralization by a given serum sample.

We examined the growth of nonneutralized virus during prolonged incubation by measuring neutralization at multiple time points in resting cells and PBMC blasts, using the same dilution of virus (1:5) in both assays. The neutralizing serum used in these assays (serum D75899) came from a 42-year-old

female whose HIV-1 infection for 10 years was well-documented and who was asymptomatic at the time of collection. Assays were performed with four primary isolates in parallel, as before. Replication of each primary isolate was delayed in the resting-cell assay, where virus production in the absence of antibody peaked on day 3 or 4 in PBMC blasts and on day 7 in resting cells. Neutralization was measured in the blast assay on days 3, 4, 5, and 7 and in the resting-cell assay on days 7, 8, and 10. These days were chosen because they corresponded to the initial peak in virus production in the absence of antibody and extended several days beyond the initial peak in both assays. The results are given as neutralization curves in Fig. 1. Neutralization in the PBMC blast assay was most apparent on days 3 and 4 and decreased thereafter. By comparison, neutralization in the resting-cell assay remained relatively constant from days 7 through 10. Of particular note, neutralization measured on day 7 in both assays was consistently greater in the resting-cell assay, but neutralization measured on day 3 or 4 in PBMC blasts was equal to or greater than that which was detected at any time in the resting-cell assay. These results are most likely explained by the eventual growth of nonneutralized virus in the PBMC blast assay and, further, illustrate that optimum sensitivity in this assay is achieved at early time points.

Although neutralization was detected equally in both assays when measurements were made on the appropriate day, the question of why little or no nonneutralized virus was detected in the resting-cell assay during prolonged incubation still remained. Compared to PBMC blasts, BZ167 was shown previously to be 36 times less infectious in 50% tissue culture infective dose ($TCID_{50}$) assays performed in the resting-cell assay (38), making it possible that the fraction of nonneutralized virus particles infectious for resting cells was reduced to a level that requires >10 days to be detected. Lower virus infectivity is also consistent with the delay in virus production seen in resting-cell assays, where additional rounds of replication would be needed for p24 to reach detectable levels. To investigate this possibility, we performed $TCID_{50}$ assays with BZ167 and V67970 in PBMC blasts and resting cells, with the same PBMC and PHA stimulation conditions used for neutralization assays. The $TCID_{50}$ assays were incubated until no further increase in the number of wells positive for p24 antigen was detected (days 14 and 18 were equal), and the $TCID_{50}$ s were calculated by the method of Reed and Muench (12). The $TCID_{50}$ of isolate BZ167 was 2.3×10^4 in PBMC blasts and 9.0×10^2 in resting cells, while the $TCID_{50}$ of isolate V67970 was 7.0×10^5 in PBMC blasts and 2.3×10^4 in resting cells. Hence, BZ167 and V67970 had a 25-fold and a 30-fold lower $TCID_{50}$, respectively, when measured in the resting-cell assay format, and the results for BZ167 are in agreement with those obtained previously (38).

All neutralization assays performed up to this point had used the same dilution of virus for resting cells and PBMC blasts. Based on our $TCID_{50}$ measurements, the actual infectious dose that had been used was 230 versus 9 $TCID_{50}$ for BZ167 and 7,000 versus 230 $TCID_{50}$ for V67970 in the blast and resting-cell assays, respectively. Because of this inequality, neutralization assays were next performed with serum D75899 and isolates BZ167 and V67970 in PBMC blasts in which 25- and 30-fold less virus was used, respectively, to equal the infectious virus dose for resting-cell assays. The kinetics of virus production in the absence of antibody are shown in Fig. 2A along with the kinetics of virus production from the experiment depicted in Fig. 1 for comparison. Although the two experiments were performed at different times, they both used the same batch of PBMC and the same culture conditions. As one might expect, the use of a lower virus dose produced a delay in the appear-

FIG. 1. Time-dependent detection of virus neutralization in PBMC blasts and resting PBMC. Neutralization of primary isolates BZ167, V67970, H69172, and V89872 was measured on days 3 (open circles), 4 (closed circles), 5 (open triangles), and 7 (closed triangles) in PBMC blasts and on days 7 (open circles), 8 (closed circles), and 10 (open triangles) in resting cells. The dashed line corresponds to 90% reduction in p24 production. Virus production at these days for isolates BZ167 and V67970 is shown in Fig. 2.

ance of detectable p24 in the PBMC blast assay. The delay was not as great as that in the resting-cell assay, suggesting that lower infectivity is only partially responsible for the delayed replication in the resting-cell assay and that the remainder of the delay is due to the late addition of PHA and interleukin-2 (29, 35).

Neutralization was measured on days 7, 8, and 10, at which times virus production in the absence of antibody was at peak levels. In contrast to the time-dependent loss of neutralization detected with a larger virus inoculum (Fig. 1), assays per-

formed with the smaller virus inoculum produced $\geq 90\%$ neutralization at serum dilutions of $\leq 1:64$ on all three days (Fig. 2B and C). This prolonged detection of neutralization with a lower dose of virus in the PBMC blast assay paralleled the results we obtained in the resting-cell assay (Fig. 1) and indicates that neutralization can be detected equally well in both assays as long as the virus dose is adjusted so that it is equivalent on the basis of infectivity in the corresponding target cells.

It should be remembered that the primary isolates used in

FIG. 2. Virus production and time-dependent detection of neutralization in PBMC blasts with a reduced virus inoculum. (A) Kinetics of viral p24 production in the absence of antibody. Open symbols, BZ167; closed symbols, V67970; solid lines, PBMC assay at a high virus dose; dashed lines, resting-cell assay at a high virus dose; dotted lines, PBMC blast assay at a low virus dose. Values for PBMC and blast assays at high virus doses are from the experiment whose results are shown in Fig. 1. (B) Neutralization of primary isolates BZ167; (C) neutralization of primary isolate V67970. Neutralization was measured on days 7 (open circles), 8 (closed circles), and 10 (open triangles). The dashed line corresponds to 90% reduction in p24 production.

this study all had an SI phenotype. We found that 10 of 10 primary isolates with an NSI phenotype replicated poorly or not at all in the resting-cell assay and therefore could not be used for the detection of neutralizing antibodies in this assay. The inability of NSI isolates to replicate in the resting-cell assay may be explained by the recent observation that NSI isolates require the chemokine receptor CCR-5 for virus entry (11). Other members of this class of chemokine receptors are expressed on activated but not quiescent cells (16, 22, 32), and it is possible that CCR-5 exhibits a similar pattern of expression. The inability to utilize NSI isolates in the resting-cell assay is a major disadvantage for this assay, since the majority of isolates which are transmitted upon primary infection, and which are therefore to be targeted by vaccines, are of the NSI phenotype (36).

In summary, the detection of neutralizing antibodies to primary isolates was dependent on the size of the virus inoculum;

greater sensitivity was achieved by using low virus doses or by measuring neutralization before virus replication peaked in the absence of antibody. The greater detection of neutralization in resting-cell assays reported previously (38) can probably be explained by the equal volume of virus that was used in both assays, where a higher infectivity in PBMC blasts would have diminished the detection of neutralization relative to the resting-cell assay. Although those investigators also reported a reduced infectivity of primary isolates in the resting-cell assay, they did not perform experiments with sera from infected individuals with adjusted virus doses. An exception was their neutralization of BZ167 with a V3-specific monoclonal antibody, in which case greater neutralization was detected in the resting-cell assay even when the virus dose was adjusted appropriately for each assay (38). This outcome could be explained by qualitative differences in virus neutralization by monoclonal antibodies versus polyclonal antisera, and additional studies will be needed to sort out those differences. In contrast to findings with monoclonal antibodies, our results support the conclusion that when adjusted for infectious dose, the two assays are equally sensitive for detecting antibody-mediated neutralization of primary isolates with sera from infected individuals. The fact that the activated versus quiescent phenotype of PBMC had no differential effect on neutralization further suggests that antibody specificity and affinity and the susceptibility of cell-free virus particles to antibody-mediated neutralization may be more important factors in detecting the neutralization of primary HIV-1 isolates. Antibody specificity and affinity maturation are determined by epitopes on the viral envelope glycoproteins and the manner in which they are processed and presented to the immune system (5, 37). Antibody production alone may not be sufficient for neutralization, however, since there is no guarantee that the conformation of the native envelope glycoproteins will permit epitopes to be exposed for antibody binding (3, 4, 27, 30). Hence, the key to understanding why primary isolates are difficult to neutralize in vitro may lie more in the viral sequences that determine antibody production and epitope exposure on the native envelope glycoproteins and less in the improvement of the design of current assays that detect neutralization in PBMC. Because of unsurpassed sensitivity and broad applicability to SI and NSI isolates, conventional PBMC blast assays remain the best choice for detecting neutralizing antibodies to primary isolates of HIV-1.

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