J Med Primatol 1999: 28: 97–104
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JOURNAL OF MEDICAL PRIMATOLOGY
ISSN 0047-2565

Neutralizing antibody responses in Africa green monkeys naturally infected with simian immunodeficiency virus (SIVagm)

Gicheru MM, Otsyula M, Spearman P, Graham BS, Miller CJ, Robinson HL, Haigwood NL, Montefiori DC. Neutralizing antibody responses in Africa green monkeys naturally infected with simian immunodeficiency virus (SIVagm). J Med Primatol 1999; 28:97–104.

Abstract: This study assessed the magnitude and cross-reactivity of the neutralizing antibody response generated by natural SIV infection in wild-caught African green monkeys. Neutralizing antibodies of variable potency, sometimes exceeding a titer of 1:1,000, were detected in 20 of 20 SIV-seropositive African green monkeys in Kenya. Detection of those neutralizing antibodies was dependent on the strain of virus and the cells used for assay, where the most sensitive detection was made with SIVagm1532 in Sup T1 cells. Potent neutralization of SIVagm1532 was seen with contemporaneous autologous serum. Potent neutralization was also detected with laboratory-passaged SIVmac251 and SIVsmB670, but not with SIVsmE660 and two additional strains of SIVagm. Serum samples from rhesus macaques (*Macaca mulatta*) experimentally infected with either SIVmac251 or SIVsmE660 were capable of low-level neutralization of SIVagm. These results indicate that natural infection with SIV can generate strain-specific neutralizing antibodies in African green monkeys. They also indicate that some neutralization determinants of SIVagm are partially shared with SIV strains that arose in sooty mangabys and were subsequently transmitted to rhesus macaques.

Michael M. Gicheru[1], Moses Otsyula[1], Paul Spearman[2], Barney S. Graham[2], Christopher J. Miller[3], Harriet L. Robinson[4], Nancy L. Haigwood[5], David C. Montefiori[6]

[1]Institute of Primate Research, National Museums of Kenya, Nairobi, Kenya; [2]Department of Medicine, Vanderbilt University Medical Center Nashville, TN; [3]California Regional Primate Research Center, University of California, Davis, CA; [4]Yerkes Regional Primate Research Center, Emory University, Atlanta, GA; [5]Seattle Biomedical Research Institute, Seattle, WA; [6]Department of Surgery, Duke University Medical Center, Durham, NC

Key words: antibodies – neutralization – nonhuman primates – SIV

Accepted April 12, 1999.

David C. Montefiori, PhD, Department of Surgery, Box 2926, Duke University Medical Center, Durham, NC 27710. Tel.: +1-919-684-5278; fax +1-919-684-4288; e-mail: monte005@mc.duke.edu.

Introduction

African green monkeys (*Chlorocebus pygerthrus*) are a natural host for simian immunodeficiency virus (SIVagm), which is one of the oldest known primate lentiviruses [1, 21, 36]. As many as 50% of wild-caught African green monkeys are infected with SIVagm and none have been observed with immunodeficiency or other SIV-related diseases [22, 37]. This is in contrast to the acquired immunodeficiency syndrome (AIDS) that results from infection with human immunodeficiency virus type-1 (HIV-1) in humans [14] and from SIV infection in macaques [11]. In contrast to HIV-1 infection in humans and SIV infection in macaques, African green monkeys infected with SIVagm fail to make antibodies to the p28 Gag antigen [35]. Also, there is no decrease in $CD4^+$ lymphocyte numbers in infected animals compared to healthy noninfected animals, and $CD8^+$ cell numbers are unusually high as compared to other lentivirus infections in primates ([12], our own unpublished observations).

SIVagm has a similar genomic organization to HIV-1 and HIV-2 and is genetically equidistant to both types of HIV [3, 4]. The host range for established cell lines is similar to HIV, although minor differences have been observed [23, 35]. SIVagm exhibits extensive genetic variation that might even surpass that seen for HIV-1 [3, 4, 21, 26]. The amount of virus detected in the cells and plasma of SIV-infected African green monkeys is similar to that seen in HIV-1-infected, asymptomatic humans, although it does not appear to rise to levels seen in later stages of HIV-1 infection [18]. The fact that the virus can be isolated from the plasma of SIV-infected African green monkeys is additional evidence of ongoing viral replication

[18]. Titers of virus-specific antibodies, as measured by ELISA, remain stable over a long period of time, where monkeys with high virus loads have significantly higher antibody titers than monkeys with low virus loads [18].

The gp120/gp36 heterodimers of SIVagm, which are major targets for neutralizing antibodies, have a stronger association as compared to HIV-1 [2]. It is possible that this strong association adversely influences the exposure of important envelope determinants involved in antibody binding and neutralization. However, little is known about the neutralizing antibody responses to SIVagm in African green monkeys. Work of Norley et al. [35] indicated that African green monkeys infected with SIVagm generate little or no detectable neutralizing antibodies. In their study of sera from 12 SIV ELISA-positive African green monkeys, only three animals had antibodies that were capable of neutralizing SIVagm; these neutralizing antibodies were low in titer and failed to neutralize the autologous virus isolate in Molt-4 clone 8 cells. Five murine monoclonal antibodies to the SIVagm transmembane glycoprotein were able to distinguish SIVagm from other SIV strains in envelope glycoprotein-binding assays, but failed to neutralize SIVagm [39].

It is important to define the neutralizing antibody response to SIVagm in African green monkeys, and to characterize the antigenic relatedness of SIVagm to other SIV variants in terms of their neutralizing determinants, to permit a better understanding of the nature of the apathogenic infection in these animals. The present study examined neutralizing antibody responses in wild-caught African green monkeys that were seropositive for SIV. Six different SIV variants, including three SIVagm isolates, were tested for neutralization by sera from these animals. Cross-neutralization experiments were performed with sera from rhesus macaques (*Macaca mulatta*) that were experimentally infected with either SIVmac251 or SIVsmE660. We found that African green monkeys infected with SIVagm develop a neutralizing antibody response and that SIVagm is antigenically related to other SIV variants in its neutralization determinants. We also show that antibody-mediated neutralization of SIVagm is strain-specific and dependent on the cells used for detection.

Methods

Serologic reagents

Serum samples were obtained from African green monkeys (*C. pygerthrus*) that were antibody-positive for SIVagm. Animals were confirmed to be SIV antibody-positive by using HIV-2 Western blot kits as described by the manufacturer (Cambridge Biotech Corp., Rockville, MD). The wild-caught animals were housed in a semi-breeding colony at the Institute of Primate Research, Nairobi, Kenya. The animals were trapped and put in the quarantine facility for routine screening before experimentation. Animal trapping, care, and maintenance have been described [38]. Institutional animal care and use, and scientific resources and evaluation committee guide lines, were strictly followed. Additional serum samples were obtained from rhesus macaques (*M. Mulatta*) experimentally infected with either SIVmac251 or SIVsmE660. All serum samples were heat-inactivated at 56°C for 45 minutes prior to use.

Cells

CEMss [34], CEMx174 [41], Sup T1 [42], Molt-4 Clone 8 [9], PM1 [28], MT-2 [17], and H9 [40] are human $CD4^+$ lymphoblastoid cells. They were maintained in RPMI 1640 supplemented with 12% fetal bovine serum (FBS) and 50 μg/ml gentamicin and incubated at 37°C in a humidified CO_2 incubator.

Viruses

The virus strains used in the study included SIVagm1532, SIVagm774, SIVagm155, SIVmac251, SIVsmE660, and SIVsmB670. SIVagm1532 and SIVagm774 were isolated from wild-caught African green monkeys (*C. pygerthrus*) from which serum was included in the present study. These latter two viruses were isolated by cocultivation of 1 ml plasma with 5×10^6 Molt-4 clone 8 cells in a total volume of 5 ml growth medium. After overnight incubation, the cells were washed two times by low-speed centrifugation, resuspended in growth medium, and incubated until syncytium formation was observed microscopically. Syncytia were readily visible by 9 days of incubation. Virus-containing culture supernatants were made cell-free by low-speed centrifugation and filtration through 0.45-micron filters (Costar Corporation, Cambridge, MA) and stored in aliquotes at −80°C. The virus was subsequently expanded in CEMss cells for use here.

SIVagm155 was isolated from an African green monkey imported from Kenya and has been described [15, 21]. This virus was expanded in CEMss cells for our studies. SIVmac251 [8], SIVsmE660 [20], and SIVsmB670 [45] have been described previously. These latter viruses were grown in H9 cells for our studies.

Assessment of cellular tropism

Various cell cultures (20 ml containing 10^7 cells/ml) were inoculated with 5 ml cell-free virus and incubated overnight. The cells were then washed twice in growth medium, resuspended in 25 ml fresh growth medium and incubated for eight additional days. Cell densities were reduced and medium replaced on days 3 and 6 of incubation. Viral p27 in culture supernatants was quantified at a time when syncytium formation was abundant. In cases where no syncytia were observed, p27 was quantified after 9 days of incubation. Viral p27 was quantified by antigen-capture ELISA, as described by the manufacturer (Organon-Teknika/Akzo, Durham, NC).

In subsequent experiments, cells and virus were added to individual wells of 96-well microdilution plates and incubated until syncytium formation and cytopathic effects of the virus were observed. The cells were then stained for viability by using Finter's neutral red (Sigma, Immunochemicals, St. Louis, MO), as described [33].

Neutralizing antibody assays

Neutralizing antibodies were assessed in either Sup T1, Molt-4 clone 8, or CEMx174 cells, as described previously [30, 33]. Briefly, 50 µl cell-free virus (15,000 $TCID_{50}$) was added to multiple dilutions of test serum in 100 µl growth medium in triplicate wells of 96-well microtiter plates and incubated for 1 hour at 37°C. Cells (1.5×10^5) in 100 µl growth medium were added and incubated until extensive syncytium formation and nearly complete cell killing were evident microscopically. Cell densities were reduced and medium replaced after 3 days of incubation in cases where it took longer than 3 days to reach the assay end-point. Viable cells were stained with Finter's neutral red in poly-L-lysine coated plate, as described [33]. Percent protection from virus-induced cell killing was calculated by taking the difference in A_{540} values between test wells (cells + serum sample + virus) and virus control wells (cells + virus) and dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells. Neutralization titers are given as the reciprocal of the serum dilution required to protect 50% of cells from virus-induced killing. Assays were harvested when virus-induced cell killing in virus control wells was greater than 70%, but less than 100%.

Results

Cellular tropism

In order to identify a cell line that was suitable for neutralizing antibody assays with SIVagm, we screened multiple $CD4^+$ human lymphoblastoid cell lines for their susceptibility to infection and virus-induced cell killing with SIVagm1532. Ideally, we sought to identify a cell line in which the virus would cause extensive cell killing, since our measurement of virus neutralization was based the quantification of viable cells under conditions where antibody-mediated neutralization was positive. The results of our assessments with six different cell lines are shown in Table 1. Sup T1 and Molt-4 clone 8 cells proved to be highly susceptible, where nearly all cells were eventually killed by the virus by 5 days of incubation. High levels of virus replication were observed in CEMss cells, although cell killing was not as efficient as for Sup T1 and Molt-4 clone 8. Little or no infection was detected in PM1, CEMx174, and MT-2 cells. Two other viral variants, SIVagm774 and SIVagm155, induced efficient cell killing in Sup T1 cells within 5 days in the absence of syncytium formation (data not shown). Based on these results, we chose to use either Sup T1 or Molt-4 clone 8 cells for neutralization assays with SIVagm variants.

Table 1. Cellular tropism of SIVagm1532[1]

Cells	p27 production (ng/ml)	Syncytium formation	Cell killing
CEMss	918	++	++
Sup T1	441	++++	++++
Molt-4 clone 8	nt	++++	++++
PM1	2	+	nt
CEMx174	<1	-	-
MT-2	nt	-	-

[1] Cultures containing 10^7 cells were inoculated with equal volumes of virus in a total volume of 25 ml growth medium. The medium was completely removed and replaced with fresh growth medium after 1 day of incubation. Viral p27 in culture supernatants was measured at either the peak of syncytium formation or after 9 days of incubation when no cytopathic was observed. Cell viability was quantified by neutral red uptake. Syncytia were observed microscopically and are scored: +, 1–4; ++, 5–9; +++, 10–20; ++++ >20. Cell killing is scored: ++++, >70%; +++, 50–70%; ++, 30–50%; +, 20–30%; -, no cell killing; nt, not done.

Table 2. Neutralization of SIV variants by serum samples from African green monkeys naturally infected with SIV[1]

Monkey	Titer of neutralizing antibodies to SIV strain					
	agm1532	agm774	agm155	mac251	smB670	smE660
413	163	<20	132	812	6,202	54
420	225	<20	nt	nt	nt	nt
423	7,155	<20	135	nt	nt	nt
425	974	165	213	nt	nt	nt
426	35	<20	<20	<20	<20	<20
428	7,983	<20	22	nt	nt	nt
429	6,605	<20	<20	nt	nt	nt
431	86	<20	<20	nt	nt	nt
434	1,785	<20	34	nt	nt	nt
437	54	<20	<20	846	10,791	<20
438	1,625	<20	<20	nt	nt	nt
774	935	<20	<20	1,440	772	nt
1532	7,823	<20	<20	1,418	5,901	29
1718	239	<20	50	nt	nt	nt
1720	169	<20	<20	1,591	14,140	68
1723	290	<20	<20	1,224	5,352	nt
1727	560	<20	<20	47	1,762	<20
1730	258	<20	<20	108	7,997	57
1741	324	<20	566	112	7,306	32
1759	141	nt	nt	146	7,479	<20
1190[2]	<20	<20	<20	nt	nt	nt
1212[2]	<20	<20	<20	nt	nt	nt

[1] Neutralization of SIVagm1532 was measured in Sup T1 cells. Neutralization of SIVagm774 and SIVagm155 was measured in Molt-4 clone 8 cells. Neutralization of SIVmac251, SIVsmB670, and SIVsmE660 was measured in CEMx174 cells. Titers are the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing.
[2] SIV-naïve monkeys.

Neutralizing antibodies

Serum samples from 20 SIV-infected and 2 SIV-naïve African green monkeys were assessed for their ability to neutralize a variety of SIV strains in either Sup T1, Molt-4 clone 8, or CEMx174 cells. The results are shown in Table 2. Neutralizing antibodies were detected in sera from all infected animals when assessed with SIVagm1532 in Sup T1 cells. The titers of these neutralizing antibodies varied considerably between animals; where three animals had low titers ranging from 35 to 86, another 11 animals had titers in a mid-range of 141–974, and the remaining six animals had relatively high titers ranging from 1,625 to 7,983. It is worth noting that serum from animal 1532 had potent neutralizing activity against the autologous virus, although no neutralizing activity was detected against SIVagm774 and SIVagm155. In fact, sera from most infected animals had little or no neutralizing activity against these latter two agm strains. Exceptions were one serum sample that neutralized SIVagm774 (titer of 165) and seven samples that neutralized SIVagm155 (titers of 22–566). Serum samples from the two SIV-naïve animals were negative for neutralization of each agm strain of virus.

We also examined whether sera from SIV-infected African green monkeys were capable of neutralizing heterologous variants of SIV derived from either rhesus macaques or sooty mangaby monkeys. As shown in Table 2, potent neutralization was frequently observed with SIVmac251 and SIVsmB670, while little or no neutralization was detected with SIVsmE660. Titers of neutralizing antibodies measured with SIVsmB670 often exceeded 1:5,000. Titers of neutralizing antibodies measured with SIVmac251 were more variable and often lower than those generated by infection with SIVmac251 (Table 3). Titers obtained with SIVsmB670 and SIVmac251 occasionally exceeded those obtained with our most neutralization-sensitive SIVagm isolate, SIVagm1532 (see Table 2).

In reciprocal experiments, we sought to determine whether sera from animals infected with either SIVmac251 or SIVsmE660 were capable of neutralizing SIVagm. As shown in Table 3, the serum samples chosen for this analysis contained relatively high titers of neutralizing antibodies to the autologous virus. The antibodies also neutralized SIVagm1532, but not SIVagm774 or SIVagm155. Titers of neutralizing antibodies detected with SIVagm1532 were in the low to moderate range as compared to sera from

SIVagm-infected African green monkeys (see Table 2). These results are evidence that at least some neutralization determinants of SIVagm are partially shared with SIV strains that arose in sooty mangabys and were subsequently transmitted to rhesus macaques.

The above results suggest that SIVagm1532 is more sensitive to neutralization as compared with SIVagm774 and SIVagm155; however, neutralization of the former virus was measured in Sup T1 cells, whereas the latter two strains were assayed in Molt-4 clone 8 cells. In order to determine whether the differential detection of neutralization was related to the virus or the cells, neutralization assays were performed with SIVagm1532 in Molt-4 clone 8 cells, while SIVagm774 was assayed in Sup T1 cells. Results obtained with sera from seven SIV-infected African green monkeys are shown in Table 4. To our surprise, the ability to neutralize SIVagm1532 was greatly reduced in Molt-4 clone 8 cells. In contrast, the ability to neutralize SIVagm774 did not improve when assayed in Sup T1 cells (compare results to those in Table 2).

Discussion

This study utilized different strains of virus and multiple cell lines to study the neutralizing antibody response generated by natural SIV infection in wild-caught African green monkeys. The neutralization assay chosen for these assessments utilizes a reduction in virus-induced cell killing to detect and quantify virus neutralization. Although CEMx174 cells are well suited for assays with other strains of SIV [25, 30], they are not permissive to infection with SIVagm. It was therefore necessary to first identify cells in which infection with SIVagm was highly cytopathic. In this regard, Sup T1 and Molt-4 clone 8 cells proved to be highly suitable for neutralization assays with our prototypic isolate, SIVagm1532. CEMss cells were less desirable (only partial cell killing), whereas a lack of efficient infection in PM1, CEMx174, and MT-2 cells made these latter cell lines completely undesirable for use with SIVagm. These results mostly agree with those of others [1, 19, 37], with the exception of our results in MT-2 cells. Ohta et al. [37] observed readily visible giant cell formation in MT-2 cells inoculated with SIVagm-1, which is in contrast to our finding that SIVagm1532 did not infect MT-2 cells. A possible explanation for these discordant findings is that only a subset of SIVagm variants are able to infect MT-2 cells. In fact, it is unexpected that any SIVagm variants would infect MT-2 cells, since these cells lack coreceptors used by most SIV strains examined to date [7, 10, 13, 27].

SIVagm isolates also appear to exhibit different phenotypic properties of infection *in vitro*. For example, infection with SIVagm1532, SIVagm774, and SIVagm1532 was cytopathic in Sup T1 cells, but only SIVagm1532 produced visible syncytia. Although the nature of this strain-specific syncytium-inducing phenotype is unknown, possible explanations include the differential use of fusion coreceptors [7, 10, 13, 27] and, conceivably, different levels of expression of viral gp120-gp41 heterodimers on the cell surface.

Table 3. Analysis of cross-reactive neutralizing antibodies generated by infection with either SIVmac251 or SIVsmE660[1]

	Titer of neutralizing antibody to SIV strain				
Monkey	agm1532	agm774	agm155	mac251	smE660
SIVmac251-infected					
8BC	238	<20	<20	8,033	nt
8R5	91	<20	<20	4,656	nt
8R7	<20	<20	<20	1,492	nt
8RK	333	<20	<20	8,839	nt
L116	113	<20	<20	5,311	nt
L44	41	<20	<20	3,388	nt
894	80	<20	96	nt	nt
SIVsmE660-infected					
31	72	<20	<20	3,539	3,547
91	<20	<20	<20	3,309	1,212
92	206	<20	<20	4,809	4,375
96	88	<20	<20	9,441	775
98	249	<20	<20	nt	nt

[1] Neutralization of SIVmac251 and SIVsmE660 was measured in CEMx174 cells. Neutralization of SIVagm1532 was measured in Sup T1 cells. Neutralization of SIVagm774 and SIVagm155 was measured in Molt-4 clone 8 cells. Titers are the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing.

Table 4. Influence of the target cells in detecting antibody-mediated neutralization of SIVagm *in vitro*[1]

Monkey	Neutralizing antibody titer with: SIVagm1532 Molt-4 clone 8	SIVagm774 Sup T1
413	23	<20
425	<20	<20
428	<20	<20
429	<20	<20
434	<20	<20
774	nt	<20
1532	<20	<20
1741	120	<20
1212[2]	<20	<20

[1] Neutralization of SIVagm1532 was measured in Molt-4 clone 8 cells, whereas neutralization of SIVagm774 was measured in Sup T1 cells. Titers are the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing.
[2] SIV-naïve monkey.

We found that natural SIV infection in wild-caught African green monkeys in Kenya can generate high titers of neutralizing antibodies, as detected with SIVagm1532 when assayed in Sup T1 cells. Considerable variation in titers from animal to animal might be explained by differences in either the amount of virus replication or host genetics. The choice of target cells used to detect those neutralizing antibodies was critical, where little or no neutralization of SIVagm1532 was detected in Molt-4 clone 8 cells. Based on this latter observation, the inability to detect a strong neutralizing antibody response in an earlier study of SIV-infected African green monkeys [35] may have been due to the fact that neutralization was measured in Molt-4 clone 8 cells. The choice of cells used for assay has been shown to have a minor influence on the detection of neutralizing antibodies to HIV-1 [32] and primary SIVmac251 [25], but not nearly to the same extent as that seen here. Compared to HIV-1 and SIVmac251, antibody-mediated neutralization of SIVagm might be more sensitive to the relative abundance of CD4 and fusion coreceptors on the cell surface. For example, it may be more difficult to saturate the appropriate epitopes on gp120 and gp41 to inhibit infection of cells that express high amounts of receptor and coreceptor. Cell type-dependent neutralization of SIVagm may also be explained by which coreceptor is expressed on the cell surface, although this does not appear to be the case for HIV-1 [24, 31, 43].

Variable and sometimes high titer neutralizing antibodies were also detected with SIVsmB670, a virus isolate from a rhesus macaque experimentally infected with virus from a sooty mangaby [45], and a laboratory-passaged variant of SIVmac251; this latter virus was originally isolated from a rhesus macaque that was part of a chain of infections that presumably originated by transmission of SIV from a sooty mangaby to a rhesus macaque in captivity [8, 16]. These results were not surprising, since antibodies to SIVagm have been shown to cross-react with the envelope glycoproteins SIVmac251 and HIV-2, but not HIV-1 [1, 23]. At least some neutralization determinants of SIVagm would therefore appear to be partially shared with SIV strains that arose in sooty mangabys and were subsequently transmitted to rhesus macaques. The fact that little or no neutralization was detected with another SIV variant, SIVsmE660, is evidence that epitopes recognized by the neutralizing antibodies are not shared with all SIV variants.

Further evidence that the neutralizing antibodies were relatively strain-specific was found when many serum samples failed to neutralize two additional SIVagm variants, designated SIVagm774 and SIVagm155. We cannot be certain at this time, however, why the sera failed to neutralize these latter two variants. Part of the reason might be due to the fact that most assays were performed in Molt-4 clone 8 cells, where the detection of SIVagm1532 neutralization was dramatically reduced. Additional assays with SIVagm774 were performed in Sup T1 cells and also produced negative results. Failure to detect neutralization in Sup T1 cells would seem to support the notion that the antibodies did not target SIVagm774. It is also worth noting that SIVagm155 was clearly sensitive to neutralization in Molt-4 clone 8 cells by some serum samples, indicating that the virus is not entirely resistant to neutralization. Strain-specific neutralization by sera from SIVagm-infected animals is probably explained by a combination of genetic variation in relevant epitopes and differences in the native oligomeric structure of the envelope glycoproteins as they exist on the surface of different strains [5, 6, 44].

Our results define conditions that permit the sensitive detection of antibody-mediated neutralization of SIVagm. A greater magnitude of the neutralizing antibody response generated by natural SIV infection in African green monkeys was revealed under these conditions than was previously recognized [35]. However, some caveats relating to the conditions used to detect neutralization are worth mentioning. First, it is important to note that all stocks of SIVagm utilized here were isolated and subsequently expanded in established T cell lines. It is possible that this pre-selected a subpopulation of T-tropic variants

with neutralization properties that are unrelated to other variants present in the animals. In addition, multiple passage of primary HIV-1 and SIV isolates in T cell lines is known to increase the sensitivity of these viruses to neutralization *in vitro* [5, 25, 29], presumably by altering the structure of the native envelope glycoprotein complex in a way that increases the exposure of relevant epitopes [44]. In this regard, assays performed with viruses that were isolated in primary cultures of African green monkey peripheral blood mononuclear cells (PBMC) might be more predictive of antibody efficacy *in vivo* – a possibility that will need to be investigated in the future. It will also be important to determine whether neutralization of those isolates can be detected in PBMC. Finally, our results do not eliminate the possibility that neutralization was at least partially due to nonantibody mechanisms.

Acknowledgments

We wish to acknowledge the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH for providing the Molt-4 clone 8 and PM1 cells. This work was supported by NIH grants AI-35166 and 6S-1649 (DCM). Dr. Gicheru was supported by NIH grant AI-45210 (BSG).

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