Impact of host cell variation on the neutralization of HIV-1 *in vitro*

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Purpose of review

In this review we present current advances in our understanding of HIV-1 neutralization assays that employ primary cell types, as compared with those that utilize cell lines and the newer, more standardized pseudovirus assays. A commentary on the challenges of standardizing in-vitro neutralization assays using primary cells is included.

Recent findings

The data from reporter cell line neutralization assays may agree with results observed in primary cells; however, exceptions have recently been reported. Multiple variables exist in primary cell assays using peripheral blood mononuclear cells from HIV-seronegative donors; in-vitro neutralization titers can vary significantly based on the donor cells used for assay targets and for virus propagation. Thus, more research is required to achieve validated primary cell neutralization assays.

Summary

HIV-vaccine-induced antibody performance in the current neutralization assays may function as a 'gatekeeper' for HIV-1 subunit vaccine advancement. Development of standardized platforms for reproducible measurement of in-vitro neutralization is therefore a high priority. Given the considerable variation in results obtained from some widely applied HIV neutralization platforms, parallel evaluation of new antibodies using different host cells for assay targets, as well as virus propagation, is recommended until immune correlates of protection are identified.

Keywords

assay standardization, HIV neutralization, host cell, humoral immunity, vaccine assessment

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Introduction

Since the discoveries that led to the definition of antibody-mediated neutralization of animal viruses (reviewed in [1–4]), numerous approaches have been employed to measure this function *in vitro*. As multiple target cells were used to test different viruses, investigators compared models utilizing immortalized cell lines with those that employed primary cells. An important goal in vaccine development studies was to identify inexpensive, technically simple models that predicted, or correlated with, protection from infection. In early studies of polio virus [5,6], it was recognized that serum neutralization could be dependent on the virus and host cell systems used *in vitro*. In the feline immunodeficiency virus (FIV) model for HIV-1, neutralization was altered by both the passage of the virus and by host cells (cell lines versus primary cells) utilized [7]. In contrast, the effects of antibody on dengue virus infection in the $P388D_1$ mouse macrophage cell line were directly reflective of what was observed in primary human monocytes. This economical in-vitro model provided a clinically relevant surrogate for the in-vivo effects of dengue virus antibodies [8].

In studies of classical HIV neutralization (inhibition of HIV entry into target cells), the results from different host cell systems are highly variable. The challenge to develop an effective HIV immunogen has been defined by both the need to elucidate appropriate Env structure(s), and the requirement for standardized, globally transferable assays that provide meaningful assessments of the quality and potency of neutralizing antibodies (NAbs). The in-vitro measurement of HIV neutralization is a complex task, attributable to several confounding variables surrounding the virus, the antibodies and the host cells employed as targets and for viral propagation (reviewed in [9–12,13•]). Some of the critical questions center around which host cell systems are the most predictive of clinical outcomes. In this review, we focus on the performance of, and caveats associated with, the HIV neutralization assays that have been developed. The impact of the use of different host cells *in vitro* is highlighted.

T-cell line models for HIV neutralization *in vitro*

The assessment of HIV neutralization has been evolving for 25 years. In the majority of neutralization assays, virus and antibody are preincubated and then added to susceptible target cells; the cells are cultured for a specified time and an endpoint is performed to quantify virus output and neutralization. In 1985, a series of papers were published describing the virus inhibitory effects of sera from patients presumed infected with HIV-1 [then called human T-cell leukemia virus type III (HTLV-III)] [14–16]. These early neutralization assays relied on cell line infection by T-cell line-adapted (TCLA) viruses; the endpoints were either HIV-1 p24 or p17 gag proteins, or reverse transcriptase activity. In later models, additional endpoints, such as multinucleated giant cell (syncytia) formation, focus formation and cell survival were employed [17–26]. However, only TCLA viruses could be used, which was later ascribed to CXCR4 HIV-1 coreceptor usage [27,28].

Peripheral blood mononuclear cells as hosts for in-vitro HIV neutralization

The critical concern regarding the use of cell line-based models has been physiologic relevance and value as surrogates for in-vivo outcomes. Subsequent to T-cell line models, assays were developed using patient viruses (referred to as primary or clinical isolates) to infect peripheral blood mononuclear cells (PBMCs) from HIV-seronegative human donors, an approach thought to be more physiological. Freshly isolated or frozen PBMC were stimulated with mitogen [i.e. phytohemagglutinin (PHA)] and cultured with interleukin-2 (IL-2) to sustain a sufficient level of virus growth for measurement of neutralization [29–31]. Heterologous and autologous (from the same patients whose sera were tested) viruses were assessed [29,32,33]. Using PBMC assays in seminal studies, Matthews [34], along with other groups [35,36], demonstrated that sera from patients who had been immunized with Env subunit vaccines neutralized in TCLA models, but failed to neutralize primary isolates in PBMC assays. Further characterization work using primary isolates [37,38], as well as infectious molecular clones (IMCs) [39], demonstrated that passage of the virus in different cell types altered the neutralization sensitivity and envelope host cell protein composite [38] of HIV-1. It was not entirely clear whether selection of neutralization-sensitive viruses by passage in T-cell lines was epigenetic rather than genetic (as single passage often had no effect). However, passage into the H9 T-cell line did render some primary HIV-1 isolates sensitive to sera from Env subunit vaccines [40]. The use of T-cell line assays as 'gatekeepers' for vaccine assessment was, therefore, questioned.

Modifying the PBMC assay by using nonstimulated, resting cells as targets suggested that the detection of neutralization could be rendered more sensitive when the PBMCs were not preactivated with mitogen [41]. This effect seemed to diminish if the activated PBMC assay endpoint was assessed earlier, or if the infectivity of the inocula was adjusted for the target cells [42]. The PBMC assay was later adapted from an extracellular p24 endpoint to enumeration of infected cells by flow cytometric staining of intracellular p24. This endpoint eliminated variability in the cell washing steps, which were necessary to eliminate the remnants of the unbound viral inoculum as well as removing any anti-gag p24 antibodies, which would compete with the anti-p24 detection antibody and mask p24 detection at serum/plasma dilutions as high as 10^{-5} [43]. Patient or vaccine sera containing competing anti-p24 antibodies could then remain in the culture throughout the neutralization assay to more closely mimic the in-vivo microenvironment [44]. This flow cytometry-based PBMC assay was converted to single-round infection through concentrating viral stocks and via the addition of indinavir [45], thus allowing the assay to specifically assess inhibition at the stage of HIV entry in primary cells. It will be important to determine how close the results match when well standardized multiple-round and single-round infection neutralization assays are compared, both using PBMCs as target cells.

Monocyte/macrophages or dendritic cells as host cells for HIV neutralization

The use of monocyte-derived macrophages (MDMs) in antibody assays dates back to the identification of these cells as targets for HIV *in vivo* [46–48]. Early on, MDMs were used as HIV-1 neutralization assay target cells to attempt to discriminate between protective and enhancing antibodies [30]. Bispecific antibody containing whole human monoclonal antibody (mAb) against Env gp41 and the Fab' fragment of murine anti-Fc gamma receptor I antibody was found to mediate potent antibody-dependent cellular cytotoxicity as well as virus neutralization when using MDM target cells [49]. Ruppach *et al.* [50] used MDM to study the neutralization of early sequential primary HIV isolates by autologous sera. In this study, detection of NAbs in acute primary HIV infection depended on the target cells used; neutralization was detected only if primary MDMs (and not lymphocytes) were used as target cells [50]. Known HIV-1-neutralizing monoclonal antibodies (mAbs) have also been tested using MDM, or dendritic cells, as targets [51,52]. Whereas direct dendritic cell infection has remained controversial, it was reported that dendritic cell infection, as well as transfer of HIV from dendritic cells to T cells, was blocked by NAb [51].

In critically important studies, Holl *et al.* [52] demonstrated that some non-neutralizing mAbs inhibit HIV-1 infection of MDM and immature dendritic cells (iDCs) through FcR interactions. The activity of five neutralizing mAbs was

increased by 16 to 12 000-fold when MDMs or iDCs were used as target cells instead of PBMC [52]. These investigators suggested two distinct mechanisms for inhibition of HIV-1, dependent upon the in-vitro targets. The first mechanism is classic neutralization, involving the Fab portion of Ig. By the second mechanism, the Fc portion of non-NAbs interacts with the FcR on macrophages or iDCs and may lead to endocytosis and degradation of opsonized HIV particles. These types of antibodies were termed 'non-neutralizing inhibitory antibodies' or NNiAb [52]. Thus, categorizing antibodies as 'neutralizing' may be influenced by the host cells used.

Development of reporter cell systems and pseudoviruses for HIV neutralization

Primary cell assays have been proposed to more closely resemble the in-vivo host cells for HIV [53•], although mitogen-stimulated lymphoblasts in PBMC are not typical of tissue or circulating T cells. As well, viruses isolated from HIV+ patients represent a quasispecies, comprised of related, but nonidentical, sequences. With serial passage on PBMCs, perturbations in the composition of the quasispecies can occur. To circumvent some of the challenges and variability associated with primary isolates, several investigators have used cloned HIV envelope (env) genes to make viruses by cotransfection of env with an HIV backbone that has all genes intact, but bears some mutation in env such that assembly occurs using only the cotransfected env(s). The resulting virus is referred to as a 'pseudovirus' (PSV). The readout for virus infection with PSVs is typically expression of luciferase enzyme; the luciferase gene has been engineered into either the PSV backbone [54–56], or the host cells used as targets [57]. Upon infection of TZM-bl target cells, the luciferase reporter gene is under the control of the HIV-2 LTR and luciferase expression is up-regulated upon the expression of tat, which binds to the response element and activates reporter transcription [57]. These approaches have been used very successfully to assess HIV-1 neutralization, and the PSV platform has many advantages, to include: convenience for testing neutralization against sequenced primary envs from multiple clades, high reproducibility and throughput, ease and safety of reagent distribution, and facilitation of Good Laboratory Clinical Practices validation [58]. The characterization of the evolution of autologous NAb responses was facilitated using either the TZM-bl cell-based or the U87 cell-based PSV neutralization platforms [59,60]. These studies have clearly illustrated the biologic relevance and validity of this technology in predicting neutralization escape and in defining the temporal events in the development of specificity, magnitude and breadth of autologous NAbs. The ability to use single, fully sequenced envs to make PSVs for use in epitope mapping and in assessing the role of clade in neutralization 'serotypes' [61,62••,63•,64••] has also demonstrated the significant utility and benefit of the PSV platforms.

Discordant results between in-vitro HIV neutralization platforms

Specific discrepancies in the data obtained when reporter cell line-based PSV assays are compared with PBMC assays, have been reported. Binley *et al.* [61] demonstrated that the 4E10 gp41 mAb is broadly neutralizing in a PSV system, but shows only moderate activity in the PBMC assay. Conversely, the X5 gp120 mAb neutralized better in a PBMC assay [61]. A study of an antiphosphatidyl inositol phosphate (anti-PIP) mAb suggested that targeting lipids in the viral and/or host cell membrane is sufficient to neutralize HIV-1. The anti-PIP mAb neutralized HIV-1 only in a PBMC assay, and not in the TZM-bl PSV assay [65]. Similar to the anti-PIP mAb, a subset of antilipid mAbs derived from humans with antiphospholipid antibody syndrome and systemic lupus erythematosus [66], neutralize primary isolates in a PBMC assay, but not PSVs in the reporter cell platform (Haynes B, personal communication). Differences between PSV and PBMC assays were also reported using polyclonal antibodies and viruses from patients in chronic stages of HIV-1 infection. Numerous instances were noted in which, although there was no sequence difference between the env clone used for the PSV and the env sequence from the primary isolate, the same virus/antibody pairs yielded opposite results in the different assays [62••].

The contamination of certain antibody preparations with endotoxin has recently been proposed as an explanation for some of the discrepant results between TZM-bl PSV and PBMC assays. It was previously shown that the lipopolysaccharide (LPS) form of endotoxin stimulates production of C–C chemokines by MDMs, which inhibit HIV-1 infection of both MDM and T cells [67]. When antibody stocks contain a sufficient level of endotoxin, MDMs that may be present in the PBMC neutralization assay can produce chemokines (i.e. MIP-1), which then inhibit PBMC infection, giving a false indication of HIV-1 neutralization (Geonnetti A *et al.*, CROI Conference 2009, abstract #328b). This phenomenon may also be dependent upon how the PBMC are prepared for the neutralization assay, as cultures lacking in $CD14^+$ monocytic cells do not appear to be susceptible to this PBMC assay artifact (Brown B *et al.*, unpublished data; Hanson C, personal communication).

Comparison of in-vitro HIV-1 neutralization assays

The assays described above contain numerous inherent differences that are often associated with the host cells

used as targets or for viral propagation. Standardization efforts have often involved comparative studies. Early efforts to compare neutralization assays were initiated by D'Souza *et al.* [68,69] at the US NIH Division of AIDS, together with investigators from the World Health Organization (WHO). A large study (as part of the Antibody Serologic Project) was conducted in 1990–1991 by over 40 investigators from 25 organizations in seven countries to compare assays for the evaluation of HIV-1 mAbs; a second mAb study was later performed comparing PBMC assays [68,69], and variation in results was observed.

Recognizing that standardization of immunomonitoring assays will be critical in vaccine evaluation, several investigators are working on this problem. The NeutNet (Neutralization Network) Project was initiated in 2003. A phase I NeutNet methodology comparison was conducted from 2005 to 2007 and involved 18 international laboratories from 12 countries. Employing a variety of assay formats (details on www.europrise.org), study participants assessed four reagents (TRImAb, 447-52D, 4E10 and sCD4) against a panel of 11 HIV-1 isolates or their env-pseudotyped derivatives. Whereas there was some degree of consistency in certain data elements, there was also considerable variation in the results observed [70••]. The summary data shown in Table 1 demonstrate that differences in neutralization are not only dependent on the cell type but also on the inhibitory reagent used. Whereas TRImAb showed comparable results, neutralizing on average, 81.8 or 85.8% of the viruses tested in PSV or PBMC assays, respectively, the 4E10 mAb was less efficient in PBMC-based assays (average of 59.1% compared to 84.8% in the PSV assays). Soluble CD4 was also less efficient in PBMC assays (Table 1). The NeutNet phase I conclusions stated that, because in-vitro assay correlates of in-vivo protection are still unknown, evaluation of NAbs by a range of assays is currently recommended. The second phase of NeutNet will focus on the testing of polyclonal reagents against a panel of eight viruses in 17 different assays. Special relevance will be given to approaches using primary cells, as well as the definition of the appropriate calculation of inhibitory concentration. NeutNet phase II is now ongoing in 13 countries as part of the EUROPRISE Network of Excellence.

Recently, a PBMC assay has also been compared with the PSV-based platform employed at Monogram Biosciences, Inc. Nineteen patient sera were tested for neutralization against a panel of seven primary viruses from different subtypes in the PBMC assay, and against a panel of 20 PSV stocks (subtypes A–D, five virus variants per subtype), in the U87 cell line-based PSV assay. Interestingly, ranking of patient serum neutralization capacity based on the number of different subtypes that were neutralized in the PBMC assay, the number of viruses per subtype that were neutralized in the U87 assay, or by the geometric mean titers, was highly correlated between the two assays (van Gils M *et al.*, unpublished data).

In the large-scale comparison of assays by Brown *et al.* [62••], PBMC (with primary isolates) and TZM-bl (using cognate PSV) assays were systematically performed using a panel of pure clade reagents. There was 60% assay concordance in qualitative neutralization, and the remaining 40% of paired titers were positive in one assay, and negative in the other. Importantly, clade-related neutralization was detected in the PBMC assay and a unique property of the C-clade antibody pool was breadth of neutralization in both systems.

When the target cells for these two platforms were compared using four mAbs and 134 primary isolates, significant concordance in neutralization on the two cell types was observed for the 2 gp120 mAbs (IgG1 b12 and 2G12), but not for the 2F5 and 4E10 gp41 mAbs (Bunnik E *et al.*, unpublished data; Fig. 1). As the same clade B isolates were used on both PBMC and TZM-bl target cells, these data highlight the significant impact of the target cells. The mechanism of action or the target of an antibody also appears to impact neutralization in different host systems, as the gp120 mAb data correlate, whereas the mAbs to the membrane proximal external region of gp41 show no correlation using the two target

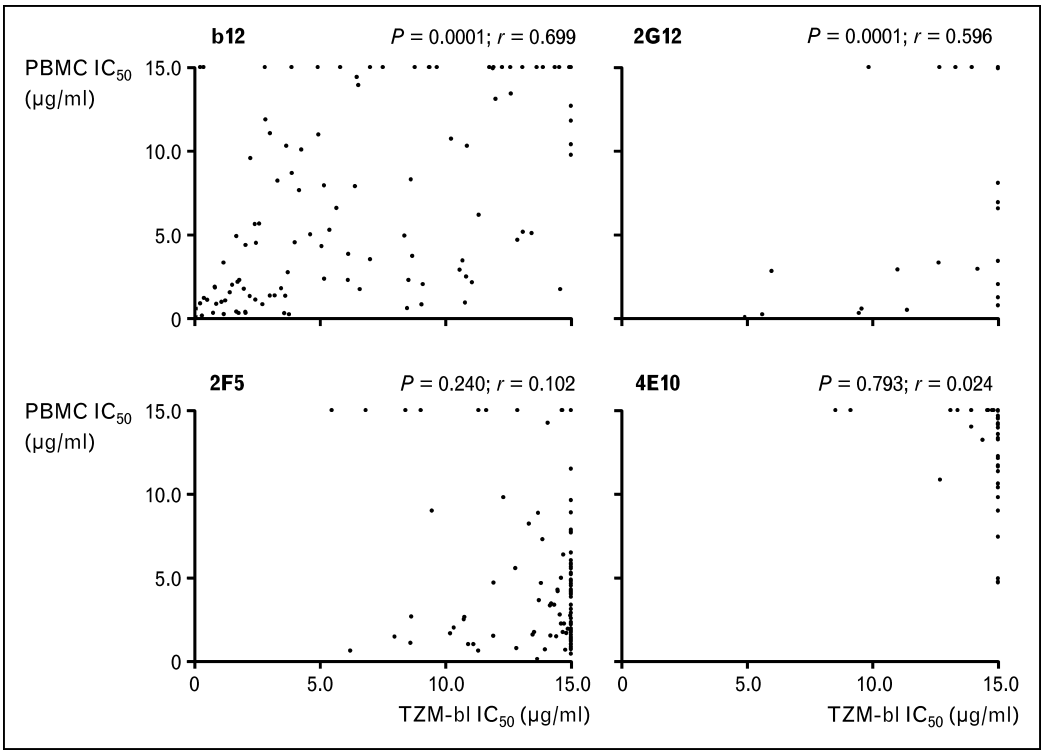
Table 1 NeutNet phase I (percentages of viruses neutralized in each laboratory)

	Plasmid pseudovirus and cell line-based assays						Virus supernatant and PBMC-based assays					
Reagent	2	5B	10	6A	1	4B	3B	6B	7	5A	8	11
TRI mAb[a]	99.9	63.6	81.8	81.8	81.8	81.8	87.5	72.7	72.7	81.8	99.9	99.9
mAb 4E10	99.9	63.6	99.9	81.8	99.9	63.6	27.3	45.5	72.7	27.3	81.8	99.9
sCD4	63.6	54.5	63.6	54.5	72.7	54.5	27.3	72.7	36.4	27.3	27.3	36.4
Mean	87.8	60.6	81.8	72.7	84.8	66.7	47.3	63.6	60.6	45.5	69.7	78.7

Assays: ▭, TZM-bl cell-based assay; ▬, extracellular p24 reduction PBMC assay. Each column represents the results obtained with one assay. Each assay was performed with a panel of 11 viruses or their env derivatives and tested with five two-fold dilutions of TriMab and 4E10 (starting at 25 μg/ml) and sCD4 (starting at 10 μg/ml). Assays are grouped on the basis of several criteria: use of plasmids or primary isolates as a source for HIV-1, either with PSV or replication competent virus; and use of cell lines or PBMCs. PBMC, peripheral blood mononuclear cell; PSV, pseudovirus.

[a] TRImAb is a cocktail of equal concentrations of the 2G12, 2F5, and IgG1 b12 mAbs.

Figure 1 Comparison of the IC_{50}s of broadly neutralizing mAbs using subtype B primary isolates ($n = 134$) and PBMC versus TZM-bl cell targets



Each dot represents one virus; a value of 15 μg/ml was arbitrarily assigned when less than 50% neutralization was observed at the top concentration (15 μg/ml) tested. To inhibit replication in the TZM-bl assay, the culture medium was supplemented with 1 μM indinavir. PBMC, peripheral blood mononuclear cell.

cell types. These data also implicate a role for virus source in neutralization assays. As shown in Fig. 1, the 4E10 mAb performs poorly against PBMC-derived primary isolates on TZM-bl cells, and yet this mAb has previously been shown (by several labs) to exhibit breadth and potency against PSV (produced in 293T cells) using cell line targets [13•,61,70••]. Thus, when using the same TZM-bl target cells, PBMC-derived primary isolates are resistant to 4E10, whereas 293T-derived PSVs are very sensitive. These intriguing observations emphasize the complex nature of the effect of both the target cells and the host cells used to propagate virus.

Additional parameters that may play a role when comparisons reveal differences in neutralization using different hosts are host cell protein composite in the virus stocks, use of reagents to facilitate virus entry (i.e. DEAE-dextran or polybrene), single versus multiple rounds of infection during the assay, preferred mode of HIV-1 entry (plasma membrane fusion versus endocytosis [71••]), stoichiometry of HIV-receptor and coreceptor interaction(s), or density of cell surface receptor/coreceptors. For example, certain cell line models such as TZM-bl, express considerably higher (2–3 logs) surface expression of CCR5 coreceptor, as compared with stimulated and unstimulated PBMCs, whereas CD4 receptor expression is similar (Rosa Borges A *et al.*, unpublished data). These membrane differences may significantly alter measurements of virus–antibody–host-cell interactions. Choudhry *et al.* [72] demonstrated that CCR5, but not CD4 cell surface concentration, had a significant effect on the inhibitory activity of CD4i antibodies and the 2F5 and 4E10 gp41 mAbs, but not on the CD4-binding site mAb, IgG1b12. The IC_{50}s for the effected mAbs were up to two orders of magnitude lower when cell lines that expressed lower, more physiological levels of CCR5 were used in neutralization assays [72]. In light of all of these potential differences, it is now becoming accepted that some antibody subpopulations may not be detected through the use of a single assay focusing exclusively on one host cell type.

Standardization of in-vitro neutralization assays for vaccine evaluation

In 2005, a standardized approach to the measurement of NAbs for vaccine trials was proposed as a result of meetings convened by the Laboratory Standardization

Subcommittee for the Global HIV/AIDS Vaccine Enterprise (GHAVE) [73]. The use of engineered cell lines and PSVs to assess sera from HIV vaccine trials was recommended as an optimized and validated approach [58]. This use is reflected in the work of the coinvestigators of the 'Comprehensive Antibody Vaccine Immune-Monitoring Consortium (CA-VIMC)', funded by The Bill and Melinda Gates Foundation in July of 2006 and centered at Duke University Medical Center. The TZM-bl assay is currently a widely applied, validated platform for HIV-1 vaccine development. However, in recognition of the differences that exist between this platform and primary cell assays, efforts are ongoing within the consortium to define and understand these differences. It should be noted here that PBMC donor variability poses a significant caveat to the direct comparison of PBMC assays to cell line-based assays, and this complication must be considered until a greater level of standardization is achieved for PBMC-based assays.

That PBMCs from different donors display differential susceptibility to HIV-1 infection has long been known [74–76]. Because of multiple variables, the interlaboratory, and even the inter-experiment variation, is problematic in PBMC neutralization assays. Recently, several laboratories have demonstrated significant variation in IC_{50}s observed when the same mAbs and virus stocks are tested using PBMCs from different donors as target cells (Wieczorek L *et al.*, unpublished data; Montefiori D, unpublished data; Hanson C, personal communication). In addition, virus stocks produced in different PBMCs also show wide variation (>1 log) in neutralization IC_{50}s when the same mAbs and target cells are used (Wieczorek L *et al.*, unpublished data). These recent studies further highlight the importance of the host cells as targets, as well as sources of virus.

These effects were further exemplified in a study showing that Env gp120 on HIV-1 produced from MDM is glycosylated with a different pattern from that produced in primary T-lymphocytes. Nearly 10-fold more serum antibody was required to neutralize macrophage-derived HIV-1 as compared with T-lymphocyte-derived virus [77], clearly demonstrating the role of host cells used for virus production. The composite of the HIV-1 envelope derived from lymphocytes versus macrophages was also shown to be quite different, using highly purified virus and proteomics [78,79]. What is striking is that host-derived membrane proteins are present in the HIV envelope in greater quantity than the actual virus-encoded Env proteins [80]. This process is not random in that specific proteins are preferentially incorporated or excluded [81]. Thus, host-acquired proteins in the envelopes of HIV-1 stocks propagated in donor PBMC with different MHC backgrounds may effect the assessment of HIV-1 neutralization.

Whereas viruses isolated using PMBC may change upon passage from host to host, the isolation of primary clonal HIV-1 variants from patient PBMC in multiple parallel micro-cocultures of donor PBMC with limiting numbers of HIV-infected patient cells was shown to prevent selection of the best fit variant in the quasispecies during culture. Using viruses cultured in this way, the neutralization profile of HIV-1 variants that coexist *in vivo* was recently obtained [82•]. Indeed, viruses isolated and propagated on PBMC are considered to most closely represent those circulating *in vivo*, whereas adaptation to cell lines alters neutralization susceptibility [37,39,83]. Furthermore, passage of 293 T-cell line-derived IMCs through PBMC can render HIV-1 more resistant to neutralization [83].

Recent efforts within the GHAVE CA-VIMC involve the production of IMC carrying the luciferase gene of *Renilla reniformis* (see chapter in this issue by C. Ochsenbauer-Jambor and J.C. Kappes). These IMC may reduce the variation observed using primary isolates, and should facilitate the standardization of approaches using primary cells. Optimizing PBMC assays to the level of reproducibility and portability that can be attained using cell line models, remains a daunting task. A greater understanding of the role of host cells in the outcomes of functional antibody assays, particularly in international settings using samples from patients with different genetic backgrounds, will facilitate detection of improvements in the NAb response to HIV immunogens.

Conclusion

Without measurable humoral immune correlates of protection from HIV-1 infection or viremic control as established in a clinical setting, it is impossible to predict which assay best quantifies protective neutralization. Evaluation of antibodies in different platforms, reflecting the biologic variation in the interaction of HIV-1 with its host cells, is an interim solution to this problem. The results from ongoing studies within NeutNet and the GHAVE CA-VIMC will be key in advancing assay standardization and in understanding differences observed in HIV neutralization using different in-vitro host cell models.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 455).

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The recent differences between assays employing PBMCs and primary isolates versus assays with PSV and cell lines are described; some potential host cell-related mechanisms behind these differences are discussed.

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