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GUEST COMMENTARY

Recommendations for the Design and Use of Standard Virus Panels To Assess Neutralizing Antibody Responses Elicited by Candidate Human Immunodeficiency Virus Type 1 Vaccines

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Laboratory measures of antigen-specific immunity are an essential component of the vaccine discovery process. For human immunodeficiency virus type 1 (HIV-1), this process will likely require iterative evaluations of vaccine immunogens to choose the most promising vaccine candidates to advance into human trials. To optimally evaluate and compare vaccine immunogens, we will need high-throughput assays that allow accurate and reproducible measurements of immune responses. In addition, vaccine sponsors and regulatory agencies appropriately require that immune assays associated with human trials be performed in laboratories that comply with guidelines for good laboratory practices (GLP). The rigorous process of assay validation associated with GLP can improve the accuracy of immune assessment assays and contribute to vaccine development by advancing our ability to distinguish incremental improvements in immune responses elicited by novel immunogens. In this commentary, we address several of these issues with regard to the measurement of anti-HIV-1 neutralizing antibodies (NAbs). We recommend the use of DNA plasmids encoding full-length functional HIV-1 envelope glycoproteins (Env); these *env* clones, when transfected with an HIV-1 *env*-defective molecular clone, produce well-characterized HIV-1 Env pseudovirions. Additionally, we recommend the establishment of standardized panels of Env-pseudotyped viruses to assess the potencies and breadths of NAbs elicited by vaccine immunogens. These virus panels would form the basis for GLP neutralization assays used to assess sera from clinical vaccine studies, and the same virus panels could be used by investigators interested in the preclinical evaluation of vaccine immunogens.

Studies of AIDS virus infection in animal models and of HIV-1 infection in humans have shown that virus-specific $CD8^+$ T cells and NAbs are two important immune responses to monitor as potential correlates of effective vaccination against HIV-1/AIDS (14, 17, 30). In particular, NAbs can confer protective immunity against lentiviral infection in animal models (7, 20, 28, 29, 31, 36). While antibodies could affect HIV-1 transmission by mechanisms that are not yet appreciated or readily measured, the ability to generate potent and broadly cross-reactive NAbs remains a major scientific obstacle and a high priority for HIV-1 vaccine development. Various assays are used to measure antibodies that neutralize HIV-1 and the related simian immunodeficiency virus and simian-human immunodeficiency virus (4, 5, 9–11, 13, 18, 22–25, 27, 33–35, 37, 41–43). These assays rely on different technologies, but all are based on the principle of measuring reductions in virus infectivity in cells that express the suitable fusion receptors for virus entry. These assays can differ with regard to the type of target cells, the methodology for detecting viral infection, the type of virus used, and whether single or multiple rounds of infection are permitted (Fig. 1). While these diverse assays can produce qualitatively similar results in terms of how each assay rank-orders neutralization potency (6, 40), there are likely to be substantial differences in accuracy and reproducibility and differences in the potential for the assays to be performed on large sample sets under GLP conditions.

Recent technological advances have provided the means to standardize several aspects of virus neutralization assays. Engineered cell lines expressing high levels of CD4, CCR5, and CXCR4 can be substituted for primary human T cells (23, 32, 33, 41), thus alleviating the requirement for individual donor cells. In addition, it is possible to utilize Env-pseudotyped lentiviral vectors expressing any HIV-1 Env that can be cloned into an appropriate DNA expression plasmid (3, 33, 41). The Env is expressed in *trans* with an *env*-deficient HIV-1, and the resulting Env-pseudotyped virions produce a single round of infection that can be monitored by reporter genes carried in the virus or the engineered cell line. A recent study by Binley et al., in which over 90 Env pseudoviruses were studied with

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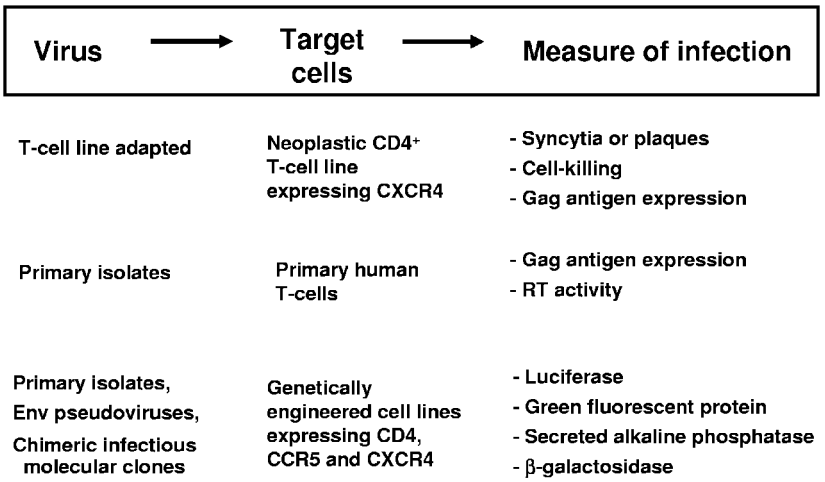

FIG. 1. Common types of assays used to measure neutralizing antibodies against HIV-1. Current assays differ with regard to the target cells used (e.g., neoplastic T-cell lines, primary human lymphocytes, or genetically engineered cell lines), the method used to measure infection (e.g., p24 antigen, reverse transcriptase, cell killing, plaque formation, or reporter gene expression), the type of virus used (e.g., uncloned PBMC-derived stocks, uncloned or molecularly cloned pseudoviruses, or replication-competent chimeric molecular clones), and whether single or multiple rounds of infection are permitted. Note that Env-pseudotyped viruses produce a single round of target cell infection. The plasmid expression vectors used to provide Env in *trans* can be clonal or can contain a quasispecies of *env* genes derived from a patient sample.

well-known antibody reagents, is a clear example of the utility of this proposed assay (1). Cloning of functional *env* genes from plasma viral RNA or from proviral DNA from primary peripheral blood mononuclear cells (PBMC) or cultured PBMC is possible. Thus, it is now feasible to consider the use of well-characterized reference strains of HIV-1 to evaluate the neutralizing antibody response elicited by HIV-1 vaccine candidates. The use of appropriate positive and negative control reagents and a rigorous program of proficiency testing can then ensure that assays performed in different laboratories generate equivalent data.

A major obstacle for effective antibody-based immunization against HIV-1 is viral diversity (8, 19, 38). To be effective, an HIV-1 vaccine will likely have to generate antibodies that neutralize a genetically and antigenically diverse set of viruses. Only by employing multiple viral strains in neutralization assays can the breadth of the NAb response be ascertained in a meaningful way. Currently, various HIV-1 strains are used by different laboratories, creating a lack of uniformity that has made it difficult to compare immunogens. Thus, there is a pressing need to establish standard panels of HIV-1 strains for wide distribution and use (26). The creation of standard virus panels would facilitate proficiency testing and GLP assay validation and would allow consistent data sets to be acquired that could be used to compare new immunogens and to prioritize the advancement of candidate vaccines. This prioritization could occur at the preclinical stage, to decide which vaccines to test in humans, and during phase I/II trials, to prioritize candidate vaccines for advanced clinical development. Standard panels would also allow refined measurements that might reveal incremental improvements in immunogen design. This would provide an increased understanding of the barriers to effective NAb induction and identify vaccine design concepts that deserve further development.

Standard virus panels will need to consist of a practical number of virus strains that represent diverse neutralization epitopes. The criteria for strain selection and related scientific issues were discussed at a workshop sponsored by HIV Vaccine Trials Network and Division of AIDS, National Institutes of Health (NIH), and attended by approximately 50 scientists (Duke University, 6 January 2004). Further discussion took place during meetings on laboratory standardization sponsored by the Global HIV/AIDS Vaccine Enterprise. There was general agreement that there is a pressing need to compare the NAb responses elicited by current vaccine immunogens and that initial virus panels should be devised as soon as possible. These panels may need to be modified in the future as new information becomes available. For the purpose of initial categorization, it was recommended that virus panels be comprised mainly of contemporary virus strains that are obtained within 3 months of sexually transmitted infection and that these viruses be grouped by genetic subtype. Recently transmitted viruses were preferred in order to avoid the potential consequences of viral genetic and antigenic drift. Sexually transmitted viruses from newly infected individuals were recommended because they most closely represent the viral strains that a vaccine will need to protect against (3, 16, 21, 39, 44–46). A further rationale for grouping isolates by genetic subtype is that over 90% of HIV-1 variants belong to genetic subtypes A, B, C, D, E (CRF01), and A/G (CRF02) (12). In the absence of definitive information about neutralization serotypes, the use of separate virus panels corresponding to each of these six major genetic subtypes makes intuitive sense.

Numerous additional criteria for selection of viruses were discussed. In order not to overestimate or underestimate the NAb response, the limited number of viruses in each panel should exhibit a distribution of neutralization phenotypes that is generally representative of most primary isolates; i.e., viruses that are exceptionally neutralization sensitive or resistant would not be included. The viruses in each panel should also be genetically and geographically diverse and represent diverse neutralization epitope specificities, as best as this can be de-

termined with known reagents such as neutralizing monoclonal antibodies and HIV-1-seropositive sera. This would provide some assurance that the panel as a whole is not biased toward a particular antibody repertoire. Additionally, there was agreement that there are substantial advantages to the use of molecularly cloned viruses, such as Env pseudoviruses. Plasmid *env* clones are stable, well-characterized reagents of known sequence that can be readily transferred between laboratories. The use of Env expression plasmids to produce Env-pseudotyped viruses provides greater assurance that genetically similar virus stocks are generated each time, thereby enhancing the accuracy and reproducibility of the assay. In addition, molecularly cloned pseudoviruses enhance the scientific value of the assay by permitting antibody specificities to be mapped in relation to a precisely known Env sequence. Mapping the NAb response generated by different vaccines should provide valuable information for future immunogen design. Finally, once a panel of functional *env* plasmids is constructed, less time and effort are needed to prepare pseudoviruses than in the process of propagating uncloned viruses in donor PBMC.

Several scientific questions were raised that need to be addressed to decide the most valid criteria for strain selection and overall panel composition. This new information would provide guidance on how the initial panels may need to be modified. Key questions include the following. (i) Is antigenic drift a concern that would require virus panels to change as the epidemic progresses? (ii) How important is it to use recently transmitted isolates rather than virus isolates obtained during chronic infection? (iii) How do the neutralization properties of clonal viruses compare to those of viral quasispecies in infected individuals? (iv) Should *env* clones be derived from cultured virus or directly cloned from plasma or PBMC of infected individuals? (v) How do Env-pseudotyped viruses compare to replication-competent virus with respect to Env incorporation and neutralization phenotype? (vi) Do Env-pseudotyped viruses produced by transfection in nonlymphoid cells differ from PBMC-derived viruses? (vii) What is the impact of using genetically engineered cell lines as target cells? (viii) Finally, what are the in vitro criteria for meaningful virus neutralization, and can sera from recent phase III vaccine trials, where protection was not observed, be used to establish such lower-limit threshold values? Several of these issues are currently being addressed, and there was a general consensus that these scientific issues should be addressed concurrently with the implementation of initial virus panels.

The number of virus isolates needed to compare vaccine immunogens is another important issue. Based on the assumption that breadth of neutralization is a key parameter of effective HIV-1 immunogenicity, the comparison of immunogens would be based on the total number of viruses neutralized. Statistical calculations can estimate the number of viruses and vaccine sera that need to be tested in order to differentiate immunogens. These sample size calculations depend on several factors and assumptions that are beyond the scope of this commentary, but the basic factors include the variability of neutralization levels among vaccine recipients and the correlation of neutralization levels among different isolates for the same vaccine recipient (i.e., is each virus an independent measurement, or are there relationships among viruses). As an example, suppose we choose to compare two candidate vaccines against a panel of 12 viruses, a feasible number for testing multiple immunogens. Depending on the statistical assumptions mentioned above, a study with between 20 and 40 vaccine recipients per immunogen would have 90% power to distinguish an antibody response that neutralized 10% of viruses from one that neutralized 30% of viruses. This type of statistical analysis will facilitate vaccine comparisons and allow us to discern incremental improvements that can be used to inform future vaccine design.

This use of virus panels described here relates mainly to preclinical and phase I/II testing of candidate vaccines. Additional issues arise when one considers phase III efficacy studies. For example, the vaccine sponsor and host countries may want to know what percentage of regional viruses are neutralized by the candidate vaccine being developed. Additional efforts are planned to establish a valid panel size by testing whether results obtained with an existing virus panel (e.g., of 12 strains from each genetic subtype) are predictive of results obtained with a much larger number of strains matched in genetic subtype to the standard panel. The results will play an important role in shaping the size of standard virus panels used in the future. Finally, critical information regarding immune correlates of protection could be derived by careful measurement of NAb responses during vaccine efficacy trials. The scientific issues related to NAb measurements in phase III trials are beyond the scope of this commentary, but the data derived by testing vaccine candidates on standard virus panels will likely play an important role in shaping the optimal measurements to be made in future vaccine efficacy trials.

To facilitate a systematic approach to the evaluation of NAb responses, we propose a three-tier algorithm for the evaluation of novel immunogens (Fig. 2). Tier 1 would represent a triage stage to identify immunogens that elicit a minimal level of virus-neutralizing antibodies. Sera from vaccine recipients would be tested against homologous virus strains represented in the vaccine and a small number of heterologous viruses that are known to be highly sensitive to antibody-mediated neutralization. Examples of the latter viruses include the primary isolate SF162 and T-cell-line-adapted viruses. This initial testing would be of interest to those involved in the immunogen design but would provide limited comparative data with other immunogens. Testing in tiers 2 and 3 would provide a greater measure of neutralization breadth for the purpose of comparing immunogens. Tier 2 would utilize the virus panels described above, i.e., panels of 12 viruses from each major genetic subtype (A, B, C, D, E, and A/G), to test neutralizing activity against viruses that are matched in genetic subtype to the vaccine strain. As an example of tier 2 testing, an Env immunogen based on a virus strain from clade C would be tested against the clade C virus panel. This immunogen could be compared to other immunogens designed to elicit clade C NAbs. To assess breadth of neutralization against viruses from other clades, a tier 3 virus panel would consist of a total of six viruses from each of the heterotypic clades (i.e., in the case of a clade C vaccine, tier 3 would include six viruses each from clades A, B, D, E, and A/G). Tier 3 testing may also include an additional set of viruses from the specific region of the world where the vaccine is to be tested. Tier 3 testing would proceed only if neutralization against tier 2 viruses was detected. Com-

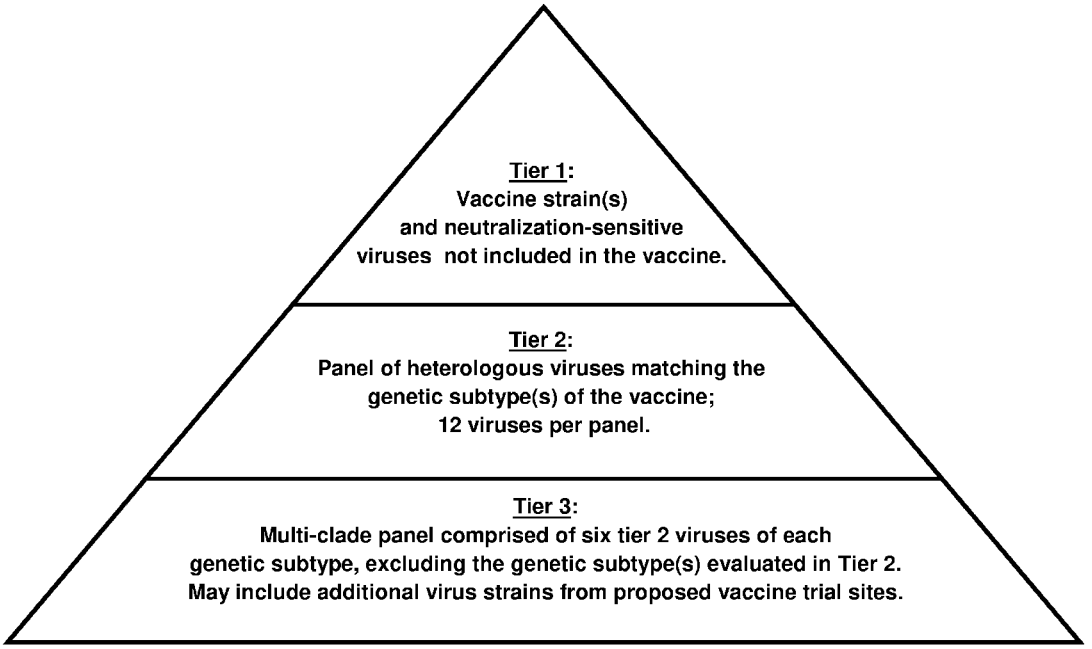

FIG. 2. Multitier approach to assessing the neutralizing antibody response generated by candidate HIV-1 vaccines.

pletion of tier 2 and 3 testing would mean that serum samples were assayed against at least 42 viruses, a number that should be sufficient for characterization of neutralization magnitude and breadth.

At the present time, only limited numbers of HIV-1 strains that meet the criteria for selection as discussed above are available as candidates for inclusion in standard panels. The genetic and phenotypic characterization of an initial panel of well-characterized molecularly cloned pseudoviruses for clade B has been completed, and the corresponding Env expression plasmids are now available through the NIH AIDS Research and Reference Reagent Program (15). However, much more work needs to be done before other panels can be assembled. Although some progress is being made, a greater effort is needed to acquire the desired isolates on a global scale and to reduce these isolates to molecular clones for characterization and eventual use. Until that time, existing non-clade B isolates that only partially meet the criteria for panel composition described above afford an immediate alternative. Examples would be the well-characterized multiclade isolates from chronically infected individuals recently described by Brown et al. (2) and other isolates that may be obtained from the NIH AIDS Research and Reference Reagent Program (www.aidsreagent.org).

In summary, we believe that the immunological assessment of current and new HIV-1 immunogens should include testing against standardized panels of pseudoviruses to allow comparisons of the potencies and breadths of elicited neutralizing antibodies. These comparisons will facilitate the prioritization of candidate vaccines in preclinical and clinical studies. Generating anti-HIV-1 neutralizing antibodies remains a major scientific challenge for vaccine development, and the improved assay accuracy associated with the use of standardized reagents and clonal viruses should allow the measurement of incremental improvements in breadth and potency of neutralization that might not otherwise be appreciated. While the virus panels described here will be important for GLP assays, the Env expression plasmids and related reagents needed to make these pseudoviruses will be available to all investigators through the NIH AIDS Research and Reference Reagent Program. A top priority is to use these virus panels to assess the NAb response generated in the recently completed phase III trial of gp120 vaccine immunogens. This is the only antibody-based vaccine candidate to be tested for efficacy in humans. The results would establish a baseline level of neutralization potency and breadth that is nonprotective, and this baseline could be used to make informed decisions about advancing future products.

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