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Brief Report

Murine Monoclonal Antibodies for Antigenic Discrimination of HIV-1 Envelope Proteins

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Abstract

In the influenza virus field, antibody reagents from research animals have been instrumental in the characterization of antigenically distinct hemagglutinin and neuraminidase membrane molecules. These small animal reagents continue to support the selection of components for inclusion in human influenza virus vaccines. Other cocktail vaccines against variant pathogens (e.g., polio virus, pneumococcus) are similarly designed to represent variant antigens, as defined by antibody reactivity patterns. However, a vaccine cocktail comprising diverse viral membrane antigens defined in this way has not yet been advanced to a clinical efficacy study in the HIV-1 field. In this study, we describe the preparation of mouse antibodies specific for HIV-1 gp140 or gp120 envelope molecules. Our experiments generated renewable reagents able to discriminate HIV-1 envelopes from one another. Monoclonals yielded more precise discriminatory capacity against their respective immunogens than did a small panel of polyclonal human sera derived from recently HIV-1-infected patients. Perhaps these and other antibody reagents will ultimately support high-throughput cartography studies with which antigenically-distinct envelope immunogens may be formulated into a successful HIV-1 envelope cocktail vaccine.

Introduction

In the influenza virus field, antibodies from small animals have long been used for characterization of the membrane molecules hemagglutinin (HA) and neuraminidase (NA). Antibodies serve as powerful reagents in that they can identify changes in protein structures, even when those changes are a consequence of a single or a few amino acid substitutions in the linear protein sequence. In the 1970s and thereafter, antibody assays served to map tertiary and quaternary structures of influenza virus membrane molecules, later confirmed as correct by crystallization (21,55).

As part of the formulation process for annual influenza virus vaccine cocktails, researchers continue to use small animal antibody–antigen reactivity patterns to characterize circulating viruses (49). Influenza viruses with novel antigenic structures that are not represented in recent vaccines are considered for representation in new vaccine formulations. The influenza virus hemagglutination inhibition (HAI) assay is often chosen as the preferred method of analysis. Enzyme-linked immunosorbent assays (ELISAs) can sometimes mimic the patterns of HAI, whereas influenza virus-specific neutralization assays will sometimes lack the sensitivity required to score fine differences in antibody–antigen interactions (7,20,51).

Other licensed cocktail vaccine formulations (e.g., papilloma virus, rotavirus, polio virus, pneumococcus) similarly benefit from antibody reactivity pattern studies. Antibodies identify clusters of antigenically distinct molecules among pathogens, representatives of which are formulated into cocktails. The strategy, while serving other vaccine fields well, has not yet been tested in a clinical efficacy study in the HIV-1 field. RV144 and HVTN 505 clinical trials tested mixed HIV-envelope vaccines, but formulations were based primarily on protein sequences (geographical clades or subtypes), rather than antigenicity (22,41).

In this study, we describe mouse immunizations with either gp140 or gp120 envelope proteins, forms of envelope that have been associated with vaccine efficacy in previous nonhuman primate studies and in the partially successful RV144 study (26,41,58). Monoclonal antibodies produced from immunized mice were tested for binding to a small panel of envelope antigens. Results demonstrate the value of antibodies from research animals for the discrimination of viral antigens. The availability of these and many other antibody reagents in the HIV-1 field provide an opportunity

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to conduct high-throughput cartography studies (48), potentially leading to the formulation of a successful, HIV-1 envelope cocktail vaccine.

Materials and Methods

Generation of monoclonal antibodies

To generate monoclonal antibodies, C57BL/6 mice were first grouped for immunizations with one of three available envelope gp140 proteins. Envelope sequences were derived from UG92005 [a clade D virus (50)], 1007 [a clade B virus (50)], and 92RW020-5 (RW, a Clade A virus, AIDS Research and Reference Reagent Repository). Immunizing vectors were described previously, including DNA recombinants, vaccinia virus recombinants, and purified recombinant proteins from transformed Chinese hamster ovary cells (11,58). At least three immunizations were performed, separated by intervals of at least 3 weeks using one or more recombinant vectors. Three days after the final injection, fusions were performed.

Additional mice were immunized with gp120 CM or MN proteins [originally described as Clade E (1,5,35) and clade B, respectively; Protein Science Corp.]. Mice were immunized thrice with intervals of at least 1 month. Doses of 5 μg protein were used per injection, first with complete Freund's adjuvant, then with incomplete Freund's adjuvant, and finally with phosphate-buffered saline (PBS) by the intraperitoneal route. Adjuvants were mixed 1:1 with soluble protein and emulsified. Final volumes were 100 μL per injection. Again, fusions were performed 3 days after the final injection.

Splenocytes from primed mice were fused with the X63-Ag8.653 myeloma cell line to produce hybridomas. To conduct fusions, splenocytes were first teased apart. Red blood cells were lysed (red blood cell lysing buffer; Sigma) and the remaining cells were washed. Splenocytes were mixed with X63-Ag8.653 cells in approximately equal numbers and pelleted. One milliliter 50% PEG 1500 (preheated to 37°C, Cat# 10783, 641001; Roche) was gently added to cell pellets over a 1-min period. Cells were maintained for an additional 1 min at room temperature. Then, 1 mL Iscove's modified Dulbecco's medium (IMDM) without sera was added gently over 1 min and another 9 mL IMDM was added over 2 min. Cells were centrifuged at 1,000 rpm (IEC Centra-8R) for 5 min. Supernatants were removed and HAT medium (Sigma) was added. In some cases, the medium was supplemented with recombinant IL-6 (human recombinant, Cat# 138600; Roche). Cells were plated in 96-well plates and incubated at 37°C, 10% CO_2. The first three digits of the monoclonal antibody name indicated the fusion experiment from which the hybridoma was derived. Fourteen cloned hybridomas with evidence of envelope discriminatory capacity in screening ELISAs were expanded for the assays described here. Antibodies that had the same binding pattern and that derived from the same fusion may have represented the same expanded B-cell clone. Antibodies from supernatants were purified by affinity chromatography on protein G columns.

Human serum samples

Human serum samples were from HIV-infected individuals between 18 and 21 years of age in Memphis, Tennessee, enrolled on an IRB-approved protocol. Patients were diagnosed with HIV-1 infections by positive ELISAs and Western blots, and had each self-reported a negative HIV-1 test conducted 2 to 15 months before their serum sampling date.

Enzyme-linked immunosorbent assays

To conduct the ELISAs, plates (Corning 96-well flat bottom) were coated with one of the purified envelope proteins, 50 μL/well at 0.5 μg/mL in PBS overnight at 4°C. After envelope coating, plates were blocked with 1% bovine serum albumin for 2 h at room temperature. Monoclonal antibodies were tested at 5 μg/mL and also with serial 10-fold dilutions. After a 1-h incubation period at room temperature, plates were washed with PBS. ELISAs were developed with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, SBA) followed by a wash and addition of p-nitrophenyl phosphate (p-NPP) at 1 mg/mL (Sigma) in diethanolamine buffer. Readings were optical density at 405 nm after 15 min of color development.

ELISAs were also performed with serially diluted human sera. For these studies, the developing reagents for ELISAs were alkaline phosphatase-conjugated anti-human IgG (SBA) followed by p-NPP. An optical density reading of 0.1 was considered positive. All titers were determined using nonlinear regression software (One site binding equation; GraphPad prism).

Additional envelopes that were not immunogens in this study, but that were included in ELISAs, were the previously described 1035 gp140 envelope protein (Clade B) (59), BaL gp120 (Clade B, #4961; NIH AIDS Research and Reference Reagent Repository), and ZM53M gp120 (Clade C, #MBS434070; mybioSource.com).

Results

Experiments were conducted to test two strategies for the analysis of HIV-1 envelope-antibody reactivity patterns by ELISAs. In the first case, mice were immunized with HIV-1 envelope recombinants to generate monoclonal antibodies for use in ELISAs. In the second case, ELISAs were performed with polyclonal human sera from patients who had been recently diagnosed with HIV-1 infections at the time of sampling. The latter samples were selected due to the expectation that recently infected individuals, having experienced a limited virus evolution, may exhibit limited envelope cross-reactivity among their virus-specific antibodies (56).

Monoclonal antibodies derived from mice primed with one of three different envelope gp140 proteins, UG92005, 1007, and RW92005. For each monoclonal, ELISAs were conducted with the original immunogen and with three other gp140 proteins. Results demonstrated that all three of the envelopes that were tested in mice were immunogenic. As shown in Table 1A, Panel 1, three monoclonals induced by UG92005 immunizations bound only to the UG92005 protein within the four-envelope panel. These antibodies were derived from two different experiments, demonstrating that the specificity was reproducibly induced by UG92005 immunizations. Five monoclonal antibodies that were induced by 1007 bound only to the 1007 protein within the four-envelope panel. One antibody derived from RW-immunized mice bound both the RW and UG92005 proteins. Patterns revealed differences in immunogenicity as well as antigenicity.

TABLE 1. MONOCLONAL AND HUMAN SERUM ANTIBODY BINDING TO GP140 (A) AND GP120 (B) ENVELOPE PROTEINS

(A) gp140

Source antibody/envelope priming	*Antibody name/time*[a]	*UG92005*	*1007*	*RW*	*1035*
Panel 1: Mouse monoclonal antibody testing					
Mouse/UG92005	106-1B5	***0.91***	*>5*	*>5*	*>5*
Mouse/UG92005	103-15C7	3.25	*>5*	*>5*	*>5*
Mouse/UG92005	103-12A3	3.36	*>5*	*>5*	*>5*
Mouse/UG92005	103-2G12	***0.87***	*>5*	*>5*	**<0.05**
Mouse/UG92005	103-10C4	**<0.05**	**0.06**	**<0.05**	5
Mouse/1007	104-9B5	*>5*	***0.9***	*>5*	*>5*
Mouse/1007	104-6D11	*>5*	**<0.05**	*>5*	*>5*
Mouse/1007	104-1B2	*>5*	**<0.05**	*>5*	*>5*
Mouse/1007	104-12D10	*>5*	**<0.05**	*>5*	*>5*
Mouse/1007	104-10G3	*>5*	**<0.05**	*>5*	*>5*
Mouse/RW	2RW12B11	***0.32***	*>5*	**<0.05**	*>5*
Panel 2: Human serum antibody testing[a]					
Patient sera	HB902/9	**>100,000**	**>100,000**	**17,685**	200
Patient sera	HB903/13	**>100,000**	**>100,000**	**31,376**	988
Patient sera	HB904/3	***3,410***	***4,890***	***1,808***	*<100*
Patient sera	HB905/8	**>100,000**	**>100,000**	**>100,000**	*<100*
Patient sera	HB906/15	**>100,000**	**>100,000**	**16,697**	*<100*
Patient sera	HB907/4	**>100,000**	**>100,000**	**22,721**	***1,116***
Patient sera	HB908/2	***1,610***	***8,500***	***1,367***	*<100*

(B) gp120

Source antibody/envelope priming	*Antibody name/time*[a]	*CM*	*MN*	*BaL*	*ZM53M*
Panel 1: Mouse monoclonal antibody testing					
Mouse/CM	119-9E1	**<0.05**	*>5*	*>5*	*>5*
Mouse/MN	120-16H6	***0.8***	***0.5***	***0.3***	*>5*
Mouse/MN	120-15D2	*>5*	**0.05**	*>5*	*>5*
Panel 2: Human serum antibody testing[a]					
Patient sera	HB902/9	***2,531***	***9,614***	**>100,000**	**21,185**
Patient sera	HB903/13	**13,736**	**>100,000**	**>100,000**	**>100,000**
Patient sera	HB904/3	*<100*	***3,417***	***3,368***	515
Patient sera	HB905/8	653	**>100,000**	**>100,000**	**12,523**
Patient sera	HB906/15	**19,133**	**>100,000**	**33,602**	***8,945***
Patient sera	HB907/4	***6,297***	**>100,000**	**>100,000**	**>100,000**
Patient sera	HB908/2	*<100*	***1,619***	***1,091***	*<100*

(A) ELISA titers are shown for mouse monoclonals (Panel 1) or human sera (Panel 2), tested against gp140 envelope proteins. Sequences were originally derived from viruses UG92005, 1007, 92RW020.5 [RW], and 1035. Sequences from UG92005, 1007, and 1035 were each shuttled through a common parent vector during cloning so that a short portion of the C-terminal region of each expressed molecule was shared.

In Panel 1, values defined the lowest antibody protein concentration (in μg) that scored positively (optical density reading 0.1 or greater) in the ELISA based on nonlinear regression analyses (GraphPad Prism). A value of ">5" indicated no detectable antibody reactivity in the ELISA with 5 μg protein. In Panel 2, values defined the highest serum dilutions that gave a positive score based on nonlinear regression analyses. A value of " <100" indicated that there was no detectable antibody reactivity in the ELISA when sera were diluted a minimum of 1:100. Relative values were coded by different formats (bold and underline = highest reactivity; bold = high reactivity; bold italics = medium reactivity; regular = low reactivity; italics = no detectable reactivity).

(B) Method descriptions and data tabulation match those in Table 1A, but with envelope gp120 instead of gp140 proteins, used both as immunogens and antigens.

[a]For human serum samples, the time in months between the day of serum collection and the patient's previous, self-reported negative HIV-1 test (based on a negative ELISA or Western Blot) is indicated.

ELISA, enzyme-linked immunosorbent assays.

The 1007 envelope, for example, elicited immune responses toward an epitope or epitopes in the 1007 envelope protein that did not exist in other tested proteins.

ELISAs were next repeated with human sera from HIV-1-infected patients who had self-reported a negative HIV-1 test conducted 2 to 15 months before their serum sampling date. Based on their self-reports, five patients were estimated to have been infected for <1 year and two of these were estimated to have been infected for only 2–3 months when samples were taken (Column 2). All seven serum samples were tested against the four target envelope proteins that had been tested with mouse monoclonal antibodies.

Table 1A, Panel 2 shows the titers of these test sera as determined using nonlinear regression analyses (GraphPad Prism software). As demonstrated, the envelope proteins could not be well discriminated based on these binding patterns. For example, the UG92005 and 1007 envelopes that had exhibited striking differences in immunogenicity

and antigenicity based on mouse monoclonal antibody binding, exhibited limited differences when tested with the different human serum samples. Sera generally bound UG92005 and 1007 best, followed by RW, and then 1035. All envelopes were bound relatively poorly by HB904 and HB908 sera, perhaps because these sera were from patients who had been HIV-1 infected for only 2–3 months at the time of sampling. The low discriminatory capacity may have been due to the small sample size and the selection of sera from a single geographical location.

In Table 1B are shown results from a second set of experiments in which gp120 proteins were used instead of gp140 proteins to immunize mice. Again, monoclonal antibodies were prepared for use as envelope-discriminating reagents in ELISAs (Table 1B, Panel 1). In this case, two different gp120 proteins, CM and MN were used for immunizations. Three antibodies were advanced for testing, one generated against the CM protein and two generated against MN. ELISAs were performed with the matched immunogens and with two additional unmatched gp120 proteins, BaL (Clade B) and ZM53M (Clade C). Results are shown in Table 1B and were similar to those described for the gp140 proteins. CM and MN gp120 proteins were each immunogenic and were well discriminated by monoclonals. Monoclonal antibody 119-9E1 bound CM and not MN protein, whereas monoclonal antibody 120-15D2 bound MN and not CM protein. Results for human sera in antibody ELISAs with gp120 proteins are shown in Table 1B, Panel 2. Some differences were observed between binding patterns among these sera (e.g., HB902 and HB906), although not as stark as for the monoclonal antibodies 119-9E1 and 120-15D2.

Discussion

Tackling diverse pathogens with human vaccines

Several fields have tackled diverse human pathogens by representing antigenically distinct membrane components in cocktail vaccines, but the strategy has never been advanced to clinical efficacy studies in the HIV-1 field. Results in previous literature suggest that this strategy may be successful. In the early 1990s, Hu *et al.* primed macaques with simian immunodeficiency virus (SIV) envelope using recombinant vaccinia virus followed by adjuvanted protein. Vaccinated animals were fully protected from a homologous SIV challenge (24). Another noteworthy success was with attenuated SIV nef-deletion mutants (16) and with passive transfers of protective antibodies from SIV infected to naive animals (52). Similarly, SHIV infections have been shown to confer protection against heterologous challenges in animal models (17,46), and healthy HIV-1-infected humans have exhibited significant protective immunity against superinfections (12,43). Perhaps individuals once infected with immunodeficiency viruses are protected against heterologous challenges due to the natural evolution of viruses and respective immune responses within the patients (40, 42,56). Ultimately, diverse lymphocytes may be primed to act in unison to block exogenous virus entry. As with other chronic infections (e.g., varicella zoster virus), a protective immune response may prevent exogenous virus infection, but may fail to clear endogenous virus, highlighting the importance of vaccinating individuals before a pathogen exposure has occurred. A multienvelope HIV vaccine approach, using dozens of envelopes, has proven protective against disease caused by a heterologous SHIV challenge in macaques (26,58) and has proven safe and immunogenic in an abbreviated clinical trial (8,25,45). Taken together, these results encourage continued testing of the strategy and simplification of cocktails to support advanced clinical studies.

Defining antigenic clusters of HIV-1 envelopes

In this study, we show that mouse monoclonal antibodies may assist the antigenic characterization of HIV-1 envelope proteins. Fourteen monoclonal antibodies are described, prepared from mice that were immunized with five different envelope proteins representing multiple clades. Although the numbers of antibodies and envelopes described in this report were small and epitopes for each antibody were not fully characterized, results demonstrated a high discriminatory capacity among the monoclonals. For example, monoclonal antibodies induced by 1007 immunizations were able to discriminate 1007 from other tested envelope proteins, including an envelope that was matched by clade (1035, clade B, <20% difference in amino acid sequence (10)). Assays with monoclonals contrasted with our serum studies, in that sera from patients who were recently HIV-1 infected were often cross-reactive and exhibited fairly similar hierarchies in their envelope binding potentials.

The mouse hybridomas are useful reagents, in that they grow constitutively in tissue culture and provide large quantities of monoclonal antibodies, easily purified if desired. The notion that HIV-1 envelopes can be categorized based on antibody reactivity patterns is not new. However, there is no consensus in the field regarding preferred assay methods and reagents. An assay that is precisely comparable to the HAI assay in the influenza virus field is not available for HIV-1 nor is there a simple, small animal model amenable to standardized virus infection. In the 1990s, Nyambi *et al.* were already immunotyping HIV-1 for the purpose of vaccine design (36,60). Results confirmed that genetic (sequence) diversity did not predict antigenic diversity (54), a finding previously demonstrated in other fields. Studies with influenza virus, for example, used escape mutant technology to show that just one or a few (often discontinuous) amino acid changes in the HA protein sequence had dramatic effects on the three-dimensional structure and respective antibody-binding sites (3,21). For HIV-1 envelope, we have found that even when a neutralizing antibody is capable of binding a linear epitope, mutation at a distant site can alter binding and mediate virus escape (15).

As a follow-up to studies by Nyambi *et al.*, Binley *et al.* used neutralization assays to categorize HIV-1 envelopes (4). These researchers noted that, ''In some assays, ''neutralization'' may result from a nonspecific cytotoxic effect of the antibody sample on the target cells. False-positive neutralization can be a significant problem when assessing serum or plasma neutralization at high concentrations.'' Indeed, certain factors in sera are known to affect HIV-1 growth and can cause virus enhancement or inhibition to confound assessment of antibody responses (9,23). The neutralization sensitivity of HIV-1 is altered by culture conditions and genetic manipulations, further complicating

assay interpretation (33). Studies in the SIV system have shown that antibodies can score negatively for neutralization *in vitro* when they are fully protective against challenge with the same virus stock in animals (52), and the RV144 study suggested that protection and neutralization were not well correlated (27,41).

The neutralization assay has provided a clear benefit to other virus fields for the purpose of developing vaccines and therapies (38). For example, it was with the neutralization assay that prophylactic antibodies against respiratory syncytial virus (e.g., Palivizumab) were titered for efficacy in cotton rats and humans (39,47). Neutralization titers toward the measles virus were also correlated with protection from disease in children (13,38). When correlations between neutralization assays and protection are not clear, scientists seek other methods of assessment. As examples, in the varicella zoster virus field, titers of antibodies to membrane antigen have been used to indicate protection, and in the rotavirus field, virus-specific serum IgA has served as a surrogate of protection (19,38). In the papillomavirus field, several assays are used, including neutralization and competitive Luminex immunoassays (6), and in the pneumococcus field, ELISAs are used to evaluate IgG levels specific for each serotype (2,53). Debates are ongoing as to the absolute merits of neutralization assays, perhaps without resolution due to the inherent complexities of the mammalian immune response (18,38).

Apart from ELISAs and neutralization assays, many other assays are available to the HIV-1 field, including CD4-binding inhibition, ADCC, and ADCVI (57), but there remains no consensus as to how assays should be conducted. Given this lack of consensus, one proposal for multi-envelope vaccine development is to: (i) initiate antigenic characterization studies with a high-throughput study (e.g., by ELISA or protein microarray analyses (51), and (ii) fine-tune vaccine cocktails with more complex assays (e.g., receptor-binding inhibition, neutralization, ADCC, and/or ADCVI). Monoclonals from research animals, humans, or genetic manipulations, may serve as important reagents in these studies. Polyclonal antibodies from single-envelope-primed animals (28,32), virus-primed animals, or HIV-1-infected patients, while inherently more complex (44), may also contribute. Targets could include standardized (5) and other stocks of HIV-1, chimeric viruses, and/or proteins of multiple forms (e.g., gp120, gp140, gp160).

An additional strategy that can assist the selection of antigenically distinct envelopes has been long discussed, and involves the assembly of longitudinal envelope escape variants from HIV-1-infected persons (34,40,42,56). Essentially, HIV-1 infected individuals may be monitored over time to capture antigenically distinct proteins as they escape contemporaneous B-cell responses (40,42,56). This strategy is regaining favor, but with a modified intent. Rather than to activate a plethora of B-cell clones with diverse antibody specificities (40), HIV vaccine researchers now strive to induce affinity maturation events in a single B-cell clone, culminating in the production of a rare, broadly neutralizing antibody (14,29,30). Whether the perceived outcome is to drive one B-cell clone or a combination of clones to recognize diverse HIV-1, the field's renewed interest in vaccination with variant HIV-1 envelope proteins by combined or successive immunizations is promising.

In summary, the idea that a successful HIV-1 vaccine may be formulated by combination of just a few antigenically distinct envelopes is regaining favor (30,36,40). Vaccine formulation may be accomplished with monoclonal and/or polyclonal antibody testing (as examined in this report) and by binding and/or neutralizing (or other) assays/strategies. Envelopes are highly diverse by sequence, but are constrained structurally because the envelope must bind human CD4 and coreceptor molecules, receptors that are well conserved among humans worldwide (37). The number of envelopes that are able to mediate infection, but that are fully mutually exclusive in antibody recognition, may be surprisingly small. The continual process of formulating and improving cocktail vaccines typifies other vaccine fields. Even when cocktails are imperfect and fail to induce immune responses with 100% pathogen coverage (31), countless lives are saved, an outcome that would be much desired in the HIV-1 vaccine field.

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Author Disclosure Statement

J.L.H. is named on patents associated with the multi-envelope vaccine strategy. No competing financial interests exist for the remaining authors.

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