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We have continued our efforts to develop an effective HIV-1 vaccine based on the V1/V2 domain of gp120. Studies with human sera and with antibodies isolated from immunized animals have shown that the V1/V2 domain contains highly conserved epitopes that can act as potent neutralizing targets for a broad range of primary HIV-1 isolates. We have prepared a prototype immunogen, based on the Case-A2 isolate, that contains the clade B V2 consensus sequence, and have shown that this antigen induces highly cross-reactive anti-V1/V2 antibodies in both rats and macaques, and that specific fractions of these antibodies possess potent cross-neutralizing activity. We are continuing to analyze this immunogen in both the rat and macaque models, and we expect to further analyze the humoral responses to immunization with this protein, including the isolation of monoclonal antibodies against various epitopes in the V1/V2 domain. Our goal during the coming year is to more fully characterize the epitopes mediating the potent neutralization, and to modify the immunogen so that those epitopes are presented more specifically and efficiently. The macaque immuncizations will provide sufficient amounts of antibodies for full characterization, and may allow challenge studies to be performed with appropriate SHIV strains.
FOREWORD

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Introduction

Whereas it is known that some human immune sera possess potent neutralizing activities for primary viruses, the identity of the target epitopes mediating this neutralization is unknown, and currently available immunogens have not been able to induce such activities. Recent evidence from our laboratory suggests that the V1/V2 domain of HIV-1 gp120 contains epitopes that are potent neutralization targets for macrophage-tropic HIV-1 isolates. The objectives of this study were to elicit V1/V2-specific antibodies by immunization with a recombinant protein that expressed the isolated V1/V2 domain and to determine the breadth and potency of HIV-1 neutralization by these antibodies.

Progress Report: Results of macaque immunization studies

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The V1/V2 domain of a clinical HIV-1 isolate (Case-A2) was expressed as a fusion glycoprotein in CHO cells. This sequence was selected since the central portion of its V2 domain was almost identical to the clade B consensus sequence of this region (Wang et al., JVI 69, 2708-15, 1995). The carrier sequence chosen was derived from the N-terminal domain of the murine leukemia virus SU protein, gp70 (Kayman et al., JVI 68, 400-410, 1994). The Case-A2 V1/V2 protein was purified by affinity chromatography on a Ni-NTA column, utilizing a His6 tag incorporated near the N-terminus of the carrier gp70 sequence. The protein was shown by SDS-PAGE to be >90% pure.

Despite the apparent purity of the V1/V2 fusion protein, a radioimmunoprecipitation analysis of the labeled protein demonstrated the presence of two conformational forms of the antigen, which differed both in their reactivity with different monoclonal antibodies and their mobility on SDS gels when analyzed under nonreducing conditions (Fig 1). Both bands were recognized by K10A11, an antibody directed against a site in the gp70-derived carrier domain, and by C9B6, a mab directed against a linear V2 epitope (not shown). The upper band was recognized by K19B3, a mab directed against a conserved V1/V2 epitope, while the lower band was recognized by SC258 and 697D, mouse human mabs directed against conformation V2 epitopes, as well as by several other mouse and human mabs directed against native conformational epitopes and by a number of human sera. This preferential recognition of lower band by the human antibodies suggested that it represented the correctly folded form, while the upper band represented an alternative, presumably nonnative conformation. The two bands coalesced after reduction of disulfide bonds with DTT, confirming that they represented distinct disulfide-bonded conformers. We were able to fractionate these forms by affinity chromatography on a column to which
monoclonal antibody SC258 was immobilized. The K19-reactive form was present in the flow through, while the native form was eluted by low pH buffer.

![Image of Western Blot](image1)

**Fig 1** Detection of two distinct conformational forms of the Case-A2 V1/V2 fusion protein. 35S-cysteine labeled Case-A2 V1/V2 fusion protein was immunoprecipitated with the indicated monoclonal antibodies, and then deglycosylated with PNGase F before SDS-PAGE analysis. The PNGase F deglycosylation was necessary to remove glycan-dependent heterogeneity which resulted in broadened bands. Two bands differing by ~2kD in apparent molecular weight were resolved upon analysis under nonreducing conditions, while only a sharp single band with lower mobility was resolved after reduction of disulfide bonds with DTT.

Three rhesus macaques (# 6876, 6876 and 7026) were immunized with purified Case-A2 V1/V2 protein in the presence of Ribi RAS triple adjuvant (Monophosphoryl Lipid A {MPL}, Trehalose Dicorynomycolate {TDM}, and Cell Wall Skeleton {CWS}) at initial doses of 25 µg/kg. Animal 6876 was boosted with the unfractonated antigen at 5 µg/kg, while the other two V1/V2-immunized animals (#7014 and #7026) were boosted with the SC238 Mab affinity-purified fraction of the V1/V2 protein. A fourth animal (#6874) was immunized with an equivalent amount of the gp70-related carrier sequence. The animals were bled prior to each immunization and at weekly intervals following each immunization. All three animals generated significant antibody titers against the fusion protein immediately after the first boost. These titers decayed after several months, and a potent anamnestic response was observed in all animals following the second boost.

Both monkeys immunized with the purified Case-A2-V1/V2 fusion protein produced antibodies that reacted with heterologous gp120s as well as with V1/V2 domains derived from env sequences of a number of unrelated HIV-1 isolates, including one Thai clade E sequence (Fig 2). These antibodies appeared to be recognizing common conserved sequences, as evidenced by the fact that almost all of the reactivity of antibodies induced by the Case-A2 V1/V2 sequence for two unrelated recombinant Env proteins (derived from the Ba-L and 451 isolates) was absorbed by

![Image of OD vs Serum Dilution](image2)

**Fig 2** Crossreactivity of V1/V2-specific macaque sera (after absorption of gp70-reactive antibodies on p621 column) of with various V1/V2 fusion proteins, demonstrating crossreactivity with two distant V1/V2 sequences, including one from a Thai clade E isolate.
the heterologous SF162 V1/V2 protein (Fig. 3). In initial assays, we found that sera of the three animals immunized with the V1/V2 protein, but not that of the control animal, were able to neutralize the macrophage-tropic NL-HX-ADA virus. These results indicate that the Case-A2 V1/V2 fusion protein was able to induce crossreactive antibodies against native gp120 epitopes.

![Graphs showing OD 405 values for BaL rgp120 and 451 rgp160](image)

1/antibody dilution

Fig. 3. Crossreactivity of V1/V2-specific macaque sera of with recombinant gp120s

To further characterize these antibodies, the V1/V2-specific IgG fraction of the immune sera was isolated by sequential immunoaffinity chromatography on a column containing the immobilized gp70-related carrier protein, followed by passage over a column containing the complete V1/V2 fusion protein (Fig. 4). This resulted in removal of >95% of all V1/V2-reactive antibodies. A portion of the V1/V2-specific antibodies bound to the second column were recovered by sequential elution with low pH buffers followed by elution with buffer containing 8M guanidine hydrochloride. After extensive buffer exchange, the isolated antibodies were quantitated and tested for HIV-1 neutralizing activities.

![Diagram showing affinity purification process](image)

Fig. 4. Fractionation of V1/V2-specific antibodies present in sera of macaques immunized with the Case-A2 V1/V2 fusion protein.
All of the eluted antibody fractions possessed neutralizing activities for a number of macrophage-tropic isolates, with the lower pH and GuHCl antibody fraction generally being more potent than that eluted at pH 2.4 (Fig. 5). In addition to NL-HX-ADA, viruses neutralized included Ba-L, a recombinant derived from NL-HX-ADA that contained the Case-A2 V1/V2 domain, and a clade C primary isolate from Malawi, 92MW965C.

**Macaque #6876 serum**

![Graphs showing neutralization of NL-HX-ADA, Ba-L, and 93MW959C](image)

**Macaque #7026 serum**

![Graphs showing neutralization of NL-HX-ADA, Ba-L, and 93MW959C](image)

Fig 5. Neutralization of macrophage-tropic isolates by V1/V2-specific macaque sera

A different result was obtained for one primary isolate, US716B (Fig. 6). Whereas the 6876 serum and V1/V2-specific antibodies isolated from this serum also neutralized this virus, the 7026 serum enhanced infectivity. This enhancing activity was removed upon absorption of the anti-V1/V2 antibodies, and was recovered in the low pH eluates of this column, demonstrating that it was antigen-specific. Interestingly, the antibodies eluted in the GuHCl fraction of this column possessed neutralizing activity for the US716 isolate. This indicated that this serum possessed both neutralizing and enhancing antibodies for the US716B virus, and that the enhancing activity dominated in the unfractionated serum.
Neutralization of 92US716B by fractionated 6876 serum  

Neutralization/enhancement of 92US716B by fractionated macaque 7026 serum

Fig 6. Neutralization/enhancement of primary HIV-1 isolate US716B by V1/V2-specific macaque sera

An unexpected result was that in contrast to the efficient neutralization of NL-HX-ADA and NL-Case-A2-ADA, these antibodies did not neutralize two related molecular recombinants that contained the identical V1/V2, but with T cell-tropic V3-V5 regions (Fig. 7). This result suggested that the neutralizing activity of these antibodies was specific for M-tropic isolates. This supports a model in which the key V1/V2 epitopes that are targeted by these antibodies function specifically in CCR5-dependent infections, either by virtue of a direct interaction with the CCR5 receptor, or as a result of a conformational structure that is specific for macrophage-tropic envelope proteins.

Fig. 7. Comparative neutralizations of macrophage-tropic and T cell-tropic HIV-1 isolates by V1/V2-specific macaque sera
These studies have demonstrated that our Case-A2 V1/V2 fusion protein is capable of inducing potent neutralizing antibodies against native epitopes in the V1/V2 domain. However, the yield of this class of antibodies in the resulting immune sera has been low, and the majority of antibodies produced appeared to be directed against epitopes that do not mediate neutralization and that may be carried on non-native forms of the immunogen. Our challenge is to learn how to modify the immunogen and/or immunization protocols so that the focus of the immune response is directed against the neutralizing targets. We are currently performing mutagenesis studies of our V1/V2 fusion protein to define the role of individual residues in determining folding, immunoreactivity and immunogenicity of this protein. Ideally, we would like to map the different classes of epitopes. This would allow us to hopefully modify the immunogen so that the deleterious epitopes are eliminated, while retaining and perhaps enhance the immunogenicity of the protective epitopes. The broad crossreactivity and potent cross-neutralizing activities we have been able to induce in rodents and macaques with our current form of the V1/V2 immunogen is very encouraging, and suggests that this vaccine, and improved versions derived from the current immunogen, may induce protective responses in humans as well.

Conclusions and Future Directions

These studies have confirmed the potency of antibodies against native epitopes in the V1/V2 domain, and have demonstrated that our Case-A2 V1/V2 fusion protein is capable of inducing such antibodies. However, the yield of this class of antibodies has been low, while the majority of the antibodies produced were directed against epitopes that do not mediate neutralization and that may be carried on non-native forms of the immunogen. In addition, we have found that a subfraction of the antibodies induced possesses enhancing activity against at least one primary isolate. Our challenge during the coming year is to define the relevant neutralizing and enhancing epitopes, and to learn how to modify the immunogen and/or immunization protocols so that the focus of the immune response is directed specifically towards the neutralizing targets.

During the coming year we shall extend these studies in the following directions.

1- We shall continue immunogenicity studies with our current V1/V2 prototype vaccine in rodents and macaque models. The rat studies will allow us to quickly evaluate different immunogen and adjuvant combinations, and to isolate additional monoclonal antibodies against specific V1/V2 epitopes. The macaque model has several advantages. First, it is important to determine whether primates exhibit a similar humoral response to this immunogen, before trials in humans can be considered. The macaque model will allow us to test this, and also allow the evaluation of the protective effects of these immune, using appropriate SHIV challenges. This animal model will allow the determination of the most effective adjuvants for inducing these antibody responses, and may also allow the determination of whether these vaccines induce CTL responses. Secondly, a difficulty in fully evaluating the activities of the immune rat sera is the limited amounts of sera that can be obtained from these animals. The macaques are much larger animals, and can therefore provide sufficient antisera for multiple analyses and fractionations, by ourselves as well as by independent laboratories.
We will repeat the studies described above in larger scale and in additional animals, to fully characterize the potency and breadth of neutralizing activity of the anti-V1/V2 antibodies induced by the Case-A2 V1/V2 immunogen, and to see how common such responses are. We will also evaluate the potency of additional V1/V2 immunogens, including other sequences isolated from macrophage-tropic clade B isolates, as well as newer constructs derived from non-clade B sequences and modified immunogens in which the gp70 sequences have been removed and replaced with appropriate T helper epitopes. Depending on the results of the epitope mapping studies designed to distinguish neutralizing from non-neutralizing epitopes, we may also be able to design and test second generation immunogens which have been optimized for the retention of neutralization epitopes.

2- Additional future directions we intend to take this study include the improvement of these V1/V2 immunogens to focus the humoral responses towards the relevant epitopes that induce the most potent neutralizing responses. This will involve evaluation of alternate strategies to either remove or modify the gp70-derived fusion sequences, to avoid the generation of antibodies against the carrier sequences. More importantly, our studies have shown that the V1/V2 domain is an immunologically complex structure. The most potently neutralizing antibodies are directed against the conserved conformational epitopes, and these are the most relevant ones for protection. A second class of antibodies are directed against the T15K V2 linear epitope. Whereas these also neutralize many viruses, they are less potent. One possibility is that mutating this sequence may increase the immunogenicity of the conformational epitopes. Previous studies with monoclonal antibodies to V1/V2 have shown that most of these have little, if any, neutralizing activity. Such antibodies, if present in excess may be deleterious, since they may block the interaction of the neutralizing antibodies, thereby inhibiting their activities. Finally, we have found in some human sera the presence of anti-V1/V2 antibodies that actually enhance the infection by some strains of HIV-1. Such antibodies could be harmful if induced in response to immunization.

We have initiated mutagenesis studies of our V1/V2 fusion protein to define the role of individual residues in determining folding, immunoreactivity and immunogenicity of this protein. Initial targets being addressed are individual N-linked glycosylation sites. We are also expressing smaller subdomains of the V1/V2 region, including proteins containing only the conserved stem of the V1/V2 domain and proteins in which the conserved stem sequences have been deleted. These studies should help us define the relevant neutralizing epitopes, and hopefully would allow us to eliminate deleterious epitopes, while retaining and perhaps enhance the immunogenicity of the protective epitopes. The broad crossreactivity and potent cross-neutralizing activities we have been able to induce in rodents and macaques with our current vaccine is very encouraging, and suggests that this vaccine, and improved versions derived from the current immunogen, may induce protective responses in humans as well.
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Meeting Abstracts

G. Tan, W.J. Honnen, S.C. Kayman and A. Pinter. Development of sensitive GFP-based infectivity and neutralization assay for macrophage-tropic HIV-1 isolates that utilizes a continuous cell line expressing the co-receptor CCR-5. Submitted for the 9th Annual Meeting of the National Cooperative Vaccine Development Groups for AIDS, May 4-7, 1997, Bethesda, MD.

A. Pinter, W.J. Honnen, S.C. Kayman, O. Troshev and Z. Wu. Production of highly crossreactive antibodies with potent neutralizing activities for a broad range of primary HIV-1 isolates upon immunization of rodents with a fusion glycoprotein containing the native V1/V2 domain of gp120. Submitted for the 9th Annual Meeting of the National Cooperative Vaccine Development Groups for AIDS, May 4-7, 1997, Bethesda, MD.


