Induction of Neutralizing Antibodies to the HIV CD4 Binding Site in Macaques by Covalent Immunization

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ABSTRACT

Structural conservation of CD4 binding site (CD4BS) in diverse HIV-1 strains permits conception of anti-CD4BS immunization as a strategy for globally effective HIV vaccination. Inducing adaptive humoral immunity to the CD4BS core (CD4BS^{core}) composed of gp120 residues 421-433 is generally prohibited because of its B cell superantigen character. This property underlies noncovalent CD4BS^{core} recognition by the framework regions of innately produced B cell receptors (BCRs), causing cellular downregulation. We hypothesized that the highly exothermic covalent reaction of electrophilic CD4BS^{core}-expressing immunogens with nucleophilic BCRs may induce B cell differentiation and amplification of the innate antibody subset directed to the native CD4BS^{core}. Immunization of mice and rabbits with electrophilic derivatives of oligomeric gp120 (EO-gp120) and synthetic peptide 416-433 (E-CD4BS^{core}) induced specific antibodies that neutralized diverse HIV-1 strains. We report here the results of macaque immunizations with the electrophilic immunogens.

Immunization with synthetic carrier protein conjugated E-CD4BS^{core} induced measurable but low-level neutralization of a primary, CCR5-dependent subtype C HIV-1 strain. Sequential E-CD4BS^{core} and full-length EO-gp120 immunization induced more robust neutralizing antibody titers up to 1:1500. EO-gp120 alone also induced neutralizing antibody production. Immunogen-binding titers were high (up to 1:500,000). The discrepant immunogen-binding and neutralization titers suggest imperfect conformational mimicry of the native, viral CD4BS^{core} by the immunogens. Full-length monomer gp120 was bound by the antibodies only at low levels, consistent with poor mimicry of the native CD4BS by this protein. The synthetic CD4BS^{core} immunogen induced neutralizing antibodies in macaques at levels lower than rabbits, potentially reflecting a species difference of the antibody repertoire. Neutralization of genetically diverse strain by the macaque antibodies will be reported. These results are the first indication that it is feasible to amplify adaptively the innate anti-CD4BS^{core} antibody repertoire to develop an HIV vaccine.

INTRODUCTION

Problem: There is no globally effective HIV vaccine.

Reasons:

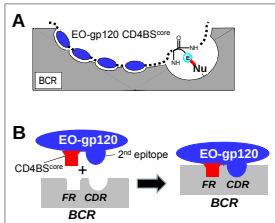
1.Mutability of the immunodominant viral epitopes

2.Poor adaptive immunological response to conserved epitopes important in virus-host cell interactions.

- ***Status of candidate vaccines that might induce broadly neutralizing antibodies:*** *No immunogen to induce such antibodies is available. gp120 and genetically engineered oligomeric gp120 does not induce broadly neutralizing antibodies to the CD4BS. The monoclonal antibody b12 expresses comparatively broad neutralizing activity. An immunogen 'reverse engineered' to present conformational complementarity to the b12 binding site did not induce neutralizing antibodies.*
- ***Currently favored explanations:*** *Affinity of vulnerable gp120 epitopes for germline B cell receptors is too low to recruit B cells into the adaptive differentiation pathway. Conversely, a very large number of V gene mutations may be needed to generate neutralizing antibodies, e.g., antibody PGT130. B cells may be intrinsically incapable of mutating the antibodies sufficiently.*

Our approach differs from the prevailing paradigm as follows:

- *We and others suggested that: (a) the CD4BS binds germline B cell receptors avidly, and (b) CD4BS immunogenicity is actively suppressed due to its superantigenic character. The primary problem appears to be defective class switching of the antibodies.[1]*
- *We have engineered a chemically activated E-vaccine to bypass the physiological restriction on synthesis of broadly neutralizing antibodies*
- *The E-vaccine recruits and improves the innate antibody subsets with CD4BS-specific reversible binding activity, catalytic activity and broad HIV neutralizing activity.[2,3] Initial feasibility of E-vaccination was shown by induction of murine monoclonal anti-CD4BS antibodies by EO-gp120 immunization.[3]*



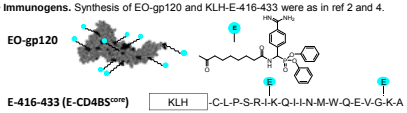
(A) COVALENT BINDING. The electrophile (E) of EO-gp120 pairs productively with naturally occurring B cell receptor (BCR) nucleophile coordinated with noncovalent binding of CD4BS^{core} (blue) to the framework regions (FR) of the BCR. The highly energetic covalent binding reaction overcomes down-regulatory effects of noncovalent binding at the FRs.

(B) BINARY BINDING. second E-vaccine region spatially proximate to CD4BS engages the complementarity determining regions (CDR), a stimulatory signal for B cells.

Panel 1. Inducing the "forbidden" anti-CD4BS response. Mouse monoclonal antibody studies indicated the stated mechanisms to improve CD4BS immunogenicity.

METHODS

• **Immunogens.** Synthesis of EO-gp120 and KLH-E-416-433 were as in ref 2 and 4.



• **Immunization:** Rhesus macaque (chinese origin), 3.5-4.9 years old , female, were immunized with KLH-E-416-433 followed by EO-gp120 or with KLH followed by EO-gp120. See Panel 2 and Panel 3 for immunization detailed. Immunization was conducted at Advance Bioscience Laboratories.

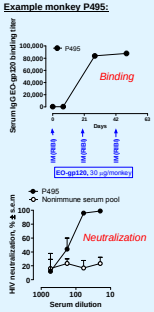
• **Binding test:** Serum IgG bound to immobilized EO-gp120 or BSA conjugated E-416-433 in ELISA plates were detected using peroxidase conjugated anti-γ chain IgG. Competitive ELISA was performed by monitoring bound IgG in absence or presence of sCD4 and control protein ovalbumin.

• **HIV neutralization:** Neutralization activity of sera was tested using primary HIV isolates and PHA/IL2 stimulated human PBMC host cells. Infected cells are monitored using p24 ELISA.[5]

RESULTS

Panel 2: EO-gp120 induces serum antibodies in monkeys that neutralize subtype C primary strain 97ZA009 in PBMC assay

Example monkey P495:



Consistent antibody induction in 3 immunized monkeys

Monkey serum ID	Immunogen	Binding IgG titer	Neutralizing titer
P495	EO-gp120	87,847	170
P495, preimmune	None	Not detectable	Not detectable
P498	EO-gp120	23,388	111
P498, preimmune	None	Not detectable	Not detectable
P499	EO-gp120	26,221	104
P499, preimmune	None	Not detectable	Not detectable
Pool, preimmune	None	Not detectable	Not detectable
Pool, nonimmune	None	Not detectable	Not detectable

Neutralizing titer, serum dilution that inhibits HIV infection of PBMCs by 50%. Human PBMC host cells, HIV subtype C 97ZA009 strain. Day 49 sera tested. The monkeys received KLH prior to immunization with EO-gp120 (not anticipated to influence antibody response to EO-gp120).

Binding titer, serum dilution displaying A490 of 0.2 by using the immunogen immobilized in ELISA plates.

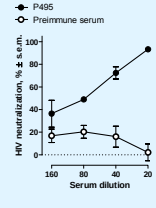
Pool, preimmune: *serum pool from the monkeys prior to immunization.*

Pool, nonimmune: *serum pool from 5 additional nonimmunized monkeys*

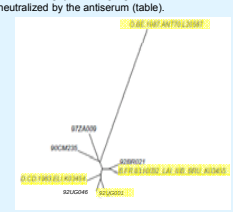
Panel 3: EO-gp120 induces broadly neutralizing serum antibodies in monkeys

Subtype, coreceptor, strain	Titer: Immune serum, P495 day 175	Titer: Preimmune serum
A, R5, 97USSN54	90	Not detectable
B, R5, 92BR021	53	Not detectable
C, R5, 97ZA009	72	Not detectable
D, X4, 92UG046	72	Not detectable
AE, R5, 90CM235	Not tested	Not detectable

Example strain subtype A, 97USSN54

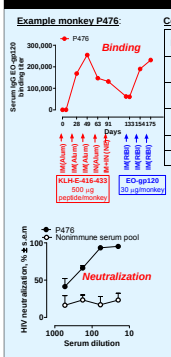


Genetically diverse strains are neutralized. Dendogram was constructed using yellow highlighted strains. Remaining strains were neutralized by the antiserum (table).



Panel 4: Sequentially administered KLH-E-416-433/EO-gp120 immunogens induce neutralizing serum antibodies

Example monkey P476:



Consistent antibody induction in 3 immunized monkeys

Monkey serum ID	Immunogen	Binding IgG titer	Neutralizing titer
P476	KLH-E-416-433 followed by EO-gp120	231,062	480
P490	KLH-E-416-433 followed by EO-gp120	156,899	137
P493	KLH-E-416-433 followed by EO-gp120	49,309	105
Preimmune	None	Not detectable	Not detectable
Nonimmune	None	Not detectable	Not detectable

See top left panel for immunization scheme.

Neutralizing titer, serum dilution that inhibits HIV infection of PBMCs by 50%. Human PBMC host cells, HIV subtype C 97ZA009 strain. Day 175 sera tested.

Binding titer, serum dilution displaying A490 of 0.2 by using the immunogen immobilized in ELISA plates.

Preimmune serum, pool from the monkeys prior to immunization. Nonimmune serum pool from 5 additional nonimmunized monkeys.

Panel 5: Sequentially administered KLH-E-416-433/EO-gp120 immunogens induce broadly neutralizing serum antibodies

Subtype, coreceptor, strain	Titer: Immune serum, P476 day175	Titer: Preimmune serum
A, R5, 97USSN54	92	Not detectable
B, R5, 92BR021	67	Not detectable
C, R5, 97ZA009	126	Not detectable
D, X4, 92UG046	44	Not detectable
AE, R5, 90CM235	42	Not detectable

See Panel 3 for dendrogram of tested strains

Panel 6: Candidate E-vaccine does not induce neutralizing antibodies to irrelevant viruses

To assess antibody specificity, neutralization of three irrelevant viruses was tested by measuring inhibition of plaques formed in infected Vero African Green Monkey kidney cells: West Nile, Dengue II and St. Louis encephalitis viruses.

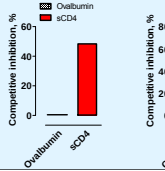
At a 1:20 dilution of the antisera from monkeys immunized with EO-gp120 alone or KLH-E-416-433+EO-gp120, no neutralization of the three irrelevant viruses was observed.

Panel 7: Inserting electrophiles into gp120 increases CD4BS immunogenicity *(gp120 devoid of electrophiles does not induce antibodies to the CD4BS)*

Antibody specificity for the CD4BS, shown by the inhibitory effect of sCD4 on antibody binding to EO-gp120

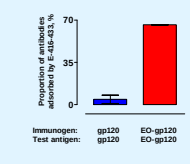
Monkeys immunized with EO-gp120 (left, P495) or E-416-433/EO-gp120 (right, P476).

Binding of immune sera to immobilized EO-gp120 was measured in presence of competitor soluble CD4 or control ovalbumin.



CD4BS is immunodominant in EO-gp120 but is poorly immunogenic in gp120 immunized mice

Serum from mice immunized with EO-gp120 or gp120 was immunoadsorbed with E-416-433, an analog of the CD4BS^{core}. This removed anti-CD4BS^{core} antibodies. Residual EO-gp120 or gp120 binding antibodies in the flow-through fraction were measured by ELISA.

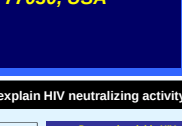


Panel 8: Endotoxin contamination does not explain HIV neutralizing activity

- Endotoxin can bind monocytes, inducing release of chemokines that may inhibit HIV infection of lymphocytes by occupying the HIV co-receptors.
- We minimize endotoxin contamination of our sera and purified antibodies (*e.g.* by using NaOH treated glassware and endotoxin-free plasticware).
- In our hands, authentic endotoxin neutralizes HIV poorly and variably depending on the PBMC host cell batch (see graph, IC50 ± 17.6 EU/mL).
- Potential endotoxin contamination of the monkey antisera cannot explain their HIV neutralizing activity. For example, at the dilution of monkey P476 antiserum showing 50% neutralization of HIV infection in *Panel 5*, the endotoxin concentration was <0.01 EU/ml (we measure endotoxin using the Endosafe-PTS system with spike recovery quality control included). This value is far lower than the minimum endotoxin concentration needed to interfere with HIV infection. Moreover, endotoxin concentrations in HIV neutralizing monkey antisera raised by EO-gp120/KLH-E-416-433 immunization were no greater than preimmune sera devoid of HIV neutralizing activity.
- Previously, we ruled out endotoxin contamination as the cause of broad HIV neutralization by our murine monoclonal antibodies and human polyclonal antibodies directed to the CD4BS^{core} (see ref 3 and 4).

Poor and variable HIV neutralization by endotoxin

LPS E.coli O111:B4 (Invivogen)
ZA009 strain
Three PBMC preps



Panel 9: Potential reason for superior induction of rabbit neutralizing antibodies by KLH-E-416-433

The CD4BS mimetic immunogen KLH-E-416-433 did not induce appreciable serum neutralizing antibodies in monkeys (not shown, day 70 serum from monkeys on *Panel 5*).

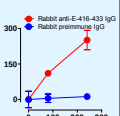
However, the same immunogen induced detectable broadly neutralizing serum antibodies in rabbits - see Table on right and ref 6. The neutralizing rabbit serum also displayed binding to whole HIV determined by p24 content of immune complexes formed by the antibodies.

Discrepant neutralizing activity in KLH-E-416-433 immunized monkeys and rabbits was evident despite robust binding responses in both species.

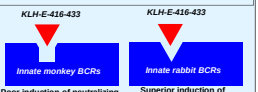
HIV neutralizing activity

Subtype	Coreceptor	Strain	Rabbit antiserum titer
A	R5	97USSN54	296
B	R5	92BR021	125
	R5	SHIV$_{SF162P3}$	NA
	X4	92HT599	50
C	R5	97ZA009	1714
	R5	98TZ017	61
	R5	98TZ013	NA
D	R5	94UG114	160
	X4	92UG001	50

Whole HIV binding



The innate B cell receptor (BCR) repertoire is structurally distinct in various species. We assume that monkey BCRs are most akin to human BCRs. Our vaccine approach relies on immunogen recognition by the innate BCR framework regions. The ability of our immunogens to induce neutralizing antibodies can be anticipated to vary in different species depending on their recognition by the innate BCRs.



CONCLUSIONS

EO-gp120 is the first candidate vaccine that induces broadly neutralizing antibodies to the CD4BS. It repairs the central impediment to antibody production, defective IgM→IgG class switching. Moreover, its CD4BS approximates the conformation of the native CD4BS expressed by the virus, evident from the neutralizing activity of anti-EO-gp120 antibodies.

We are concerned that the neutralizing antibody titers may not be sufficiently robust. However, macaque studies have indicated that neutralizing titers of ~1:40 may be sufficient to prevent HIV transmission.[7] Others have also argued that only low titers are needed to prevent transmission.[8]

REFERENCES & GRANTS

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