

Synthetic AIDS vaccine by targeting HIV receptor[☆]

Chang Yi Wang^{a,*}, Ming Shen^a, Gary Tam^b, Xin De Fang^a, John Ye^a, Fan Shen^a, Alan M. Walfield^a, James J.G. Wang^a, Ming Lie Li^a, Xuan Mao Li^a, Maria Salas^b, Michael H. Shearer^c, Ronald C. Kennedy^c, Carl V. Hanson^b

^a United Biomedical Inc., 25 Davids Drive, Hauppauge, NY 11788, USA

^b California Department of Health Services, Viral and Rickettsial Disease Laboratory, 850 Marina Bay Parkway, Richmond, CA 94804, USA

^c Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, Oklahoma, OK 73104, USA

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Abstract

A class of synthetic peptide immunogens for the cell surface HIV receptor complex has been developed to elicit antibodies that block viral entry by inhibiting gp120-CD4 interaction. These peptides extend our HIV receptor-directed approach from passive immunotherapy with mAb B4 (Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 10367) to active immunization by a synthetic peptide-based vaccine. A peptide site from CD4 was identified as a B cell epitope capable of mimicking a susceptible site on the HIV receptor complex, and then rendered immunogenic. An effective target antigenic site (B cell epitope) for the cell surface HIV receptor complex was selected by epitope mapping from among diverse CD4 and chemokine receptor peptides. It is a cyclized sequence modified from the CDR2-like domain of CD4 (AA 39–66), that was predicted to produce steric hindrance of the discontinuous recognition site of mAb B4. The immunogenicity of the targeted epitope was augmented by tandem combination with promiscuous T helper cell epitopes (Th). The antibody response to this class of immunogens attained sufficient concentrations and affinities of the correct specificity to block the interactions of HIV env glycoprotein with the cellular receptor, and prevent infection. The polyclonal antibodies generated against these fusion constructs in multiple animal species neutralized a broad array of HIV-1 primary isolates from clades A to E. Despite eliciting antibodies to the key CD4 immunomodulatory molecule, the site-specific and chemically defined immunogens displayed no overt immunotoxicity in baboons and have potential for the immunotherapy and immunoprophylaxis of HIV infection.

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1. Introduction

Blocking HIV entry by inhibiting gp120-CD4 binding is a potentially valuable strategy for HIV prophylaxis. We have been approaching this strategy by focusing on vaccines and antibodies against the host cell receptor, rather than a gp120-directed approach. The anti-host cell strategy overcomes the variable sensitivities of primary HIV isolates to neutralization by antibodies. High affinity antibodies directed against several components of the CD4 receptor/co-receptor complex for HIV on the host cell have been shown in several laboratories to affect the binding and post-binding steps of HIV infection and these anti-

bodies neutralized the infectivity of primary HIV isolates with high potency [1–4]. Monoclonal antibody 5A8 (which recognizes domain 2 of CD4) had therapeutic effect in SIV-infected macaques [5] and autoimmune anti-CCR5 and anti-CD4 antibody responses have been associated with resistance to HIV infection in exposed individuals [6,7]. Monoclonal antibody mAb B4 has a specificity for the cell membrane-associated CD4/co-receptor complex. The details of its discontinuous conformational epitope are unknown. It is a non-cytotoxic, non-immunosuppressive monoclonal antibody with neutralizing activity against HIV. We had shown previously that mAb B4 blocked in vitro replication of SIV, SHIV, HIV-2, and primary isolates of HIV-1 from multiple clades and with different co-receptor tropisms [8]. Antibody B4, given 4 h after virus exposure protected hu-PBL-SCID mice from challenge with HIV-1 AD-6. Chimpanzees infused intravenously with 5 mg/kg of B4 either 1 h before or 1 h after challenge were protected from intravenous challenge by 100 TCID₅₀ of HIV-1 DH-12 [8].

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* Corresponding author. Tel.: +1-631-273-2828; fax: +1-631-273-1717.
E-mail address: cywang@unitedbiomedical.com (C.Y. Wang).

We present here the extension of our receptor-directed approach from passive immunization by mAb B4 to active immunization by synthetic peptide immunogen. A class of synthetic peptide immunogens with a target epitope sequence modified from the CDR2-like domain of CD4 was developed by mapping sites on CD4 for functional antigenicity. That class of synthetic peptides elicited broadly neutralizing antibodies. The challenges in developing such immunogens were the identification of a B cell epitope capable of mimicking a susceptible site on the CD4 receptor complex, and then rendering it immunogenic. Effective immunogenicity required that the synthetic peptide stimulate the production of antibodies with the correct fine specificity, at levels and affinities sufficient to block the interactions of HIV env glycoprotein with the receptor, while not generating overtly immunosuppressive activities against CD4+ T cells.

The desired specificity was obtained by epitope mapping for functional immunogenicity. Peptide constructs were designed from the CD4 and co-receptor regions systematically [9], and selected for immunological cross-reactivity to the mAb B4 binding site and for their ability to elicit neutralizing antibodies. A sequence from the CDR2-like domain of CD4, at AA 39–66, was found to be the most effective target antigenic site (B cell epitope) for the synthetic immunogen. This site was predicted to produce steric hindrance of the discontinuous recognition site of mAb B4. The loop structure in the CDR2-like domain was imitated in the peptide by the insertion of cysteine residues followed by cyclization through the sulphhydryl groups [10]. The immunogenicity of the targeted CD4-CDR2 antigenic site was augmented by tandem combination with promiscuous T helper cell epitopes (Th).

Table 1
Designs of peptide immunogens

Peptide code	Design
a	Target antigenic site
b	HBsAg Th-GG-target antigenic site
c	Inv-GG-HBsAg Th-GG-target antigenic site
d	CT P11 Th-GG-HBsAg Th-GG-target antigenic site
e	Target antigenic site-GG-HBsAg Th
f	KKK-HIV-1gp41Th-GG-UBITh [®] 1-GG-target antigenic site
g	UBITh [®] 1-GG-target antigenic site
x	Target antigenic site 1-C-C-target antigenic site 2

Promiscuous Th can be provided by sequences derived from potent foreign antigens such as measles virus F protein [11], gp41 of HIV [12], and combinatorial Th peptide libraries [13–15]. Antibody responses to prototype fusion peptides of this class attained antibody concentrations and affinities sufficient to neutralize a broad array of HIV-1 primary isolates.

2. Materials and methods

2.1. Design and synthesis of CD4- and chemokine receptor peptide immunogens

Target antigenic site peptides, including those exemplified in Fig. 1, were synthesized individually by solid-phase synthesis on Applied Biosystems automated peptide synthesizers (Models 430, 431 and 433A) using Fmoc chemistry, in their corresponding “a”, “b”, “c”, “d”, “e”, “f”, “g” or “x” forms (see Table 1 for schematic descriptions of

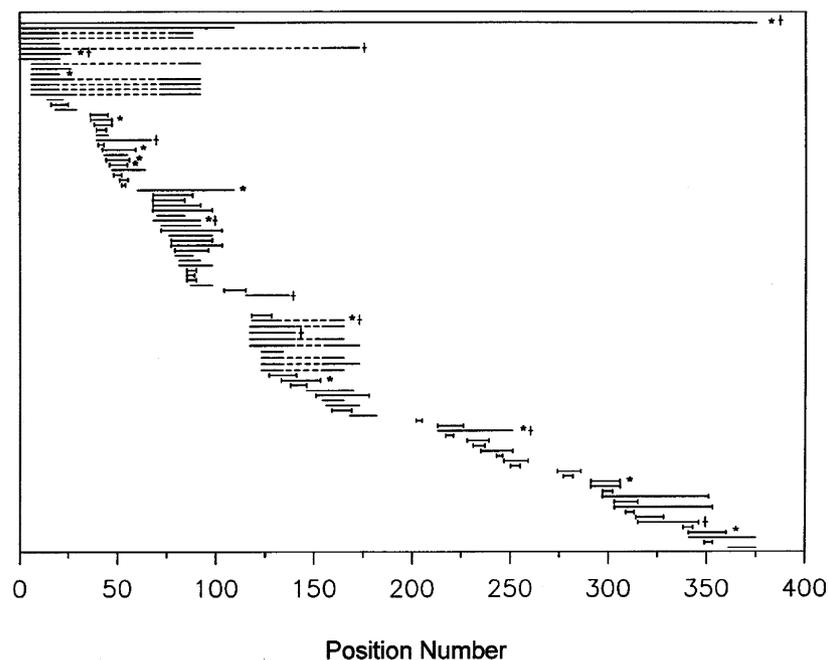


Fig. 1. Search for functionally antigenic sites on human CD4: (—) cyclized peptide; (-----) “x” peptide; (*) cross-reactive with rsCD4; (+) cross-reactive with cell surface receptor complex.

Table 2
Amino acid sequences of pathogen-derived immunostimulatory sites

Description of site	Amino acid sequence
HBs _{19–32} Th [16]	FLLTRILTIPQSLD
Invasin [14,17]	TAKSKKFPSYTATYQF
CT P11 Th [18]	TINKPKGYVVGKE
UBITh [®] 1 [15]	ISISEIKGVIVHKIETILF T RT TR
HIV-1 gp41 _{772–787} Th [12]	TDRVIEVLQRAGRAIL

peptides and Table 2 for the sequences of the tandemly attached immunostimulatory sites of the b–g peptides). Peptides designated by “a” represent a CD4 or CC chemokine receptor target antigenic site alone. These were used as the substrate antigens for peptide-based ELISAs. The “a” peptides were tandemly attached to immunostimulatory sequences to form fused peptide immunogens. Peptides marked by “b” (e.g. “b” peptides in Table 3) were synthesized as target antigenic sites in tandem with a T helper site from hepatitis B virus surface antigen, “HBsAg” [16] (Table 2) and glycine–glycine linker. Peptides marked by “c” (“c” peptides in Tables 3–5) are variants of the “b” constructs, having an invasin domain immunostimulatory peptide “Inv” [14,17] (Table 2) on the N-terminus. The peptide designated as “d”, peptide 1848d in Table 3, was

Table 3
Inhibition of mAb B4 binding to MT2 cells by immune sera to CD4 or co-receptor-derived peptides

Peptide code ^a	Description of target antigenic site	Inhibition of mAb B4 binding ^b
CD4-derived peptides ^c		
rsCD4	CD4(1–353)	–
1901b	CD4(1–26)	–
1403b	CD4(43–55)	–
1471c	CD4(39–67)	+
1848d	CD4[(68–92)-M]	–
1472c	(C)CD4[115–130 (C→S)–137](C) ^d	–
1771b	CD4 (117–140)	–
1817b	CD4[118–130(C)-159(C)-165] ^d	–
1864x	CD4(1–20) × CD4 (156–173)	–
1889b	CD4(213–251)	–
1684c	(C) CD4 (315–346)(C) ^d	–
Chemokine receptor-derived peptides ^e		
1990e	CCKR4(181–203)	–
1999e	CKR1(1–34)	–
2028b	CKR1(261–287)	–
2047b	CCR5(168–199)	–
2048b	CCR5(261–277)	–
2049x	CCR5[(88–102) × (168–199)]	–
2087b	CKR2b(100–114)	–

^a See Table 1 for explanation of letter code.

^b Indirect immunofluorescence inhibition assay.

^c Numbering of CD4 amino acid sequence from Littman et al. [30].

^d Target site is cyclized through the indicated cysteines.

^e Chemokine receptor external domain peptides, numbering system of the amino acid sequences deduced from nucleic acid sequences in LESTR or CCKR4 [28] and CC-CKR1, CC-CKR2b, CC-CKR5 [29].

a variant of the “b” constructs synthesized in tandem with a second Th peptide derived from *Chlamydia trachomatis*, “CT P11 Th” [18] (Table 2), and attached to the N-terminus through a glycine–glycine linker. Peptides marked by “e” (“e” peptides in Table 3) were synthesized as the reversal of “b” with Th sites located at the C terminus of the construct. Peptides marked by “x” in Fig. 1 and Table 3 represent peptides comprising a two-chain structure linked by an inter-disulphide bond via the naturally existing cysteine residues present on the respective chains. Peptide immunogens designated as “f” and “g”, peptides p2249f and p2249g of Tables 5 and 7, have an idealized promiscuous library Th site, UBITh[®]1, modified from measles virus fusion protein [15] (Table 2). The library Th site was synthesized by providing a mixture of alternative amino acids for coupling at each variable position. The “f” peptide, p2249f, also has an HIV gp41 helper site (Table 2) [12] attached to the N-terminus of UBITh[®]1 through a glycine–glycine linker.

After complete assembly of a desired peptide, the peptide was cleaved from the resin and protecting groups removed from amino acid side chains by standard treatment with trifluoroacetic acid. For peptide cyclization, the cleaved peptide was kept in 15% DMSO for 48 h to facilitate intrachain disulphide bond formation between cysteines. Cleaved, extracted and washed peptides were purified by HPLC and characterized by mass spectrometry and reverse phase HPLC [19].

2.2. Target antigenic site peptide ELISA or rsCD4 ELISA

ELISAs for binding to target “a” peptide (Fig. 1) or recombinant soluble CD4 (rsCD4) were done in microtitre plates as described [19] except that the plates were coated with the indicated target peptide or rsCD4 (American Bio-Technologies) at 5 and 0.25 µg/ml, respectively.

2.3. Cell binding assay

For indirect immunofluorescence (IF) staining, 0.5×10^6 CD4-expressing cells (HPB-ALL) [8] per well were washed and incubated in 50 µl (plateau concentration) mAb B4 at 10 µg/ml or diluted immune sera, and bound antibody detected by FITC-conjugated goat anti-mouse IgG or FITC-conjugated species-specific goat anti-IgG (Cappel/ICN, Costa Mesa CA). The stained cells were analyzed by fluorescence microscopy or cytofluorography (EPICS, Coulter) [8].

2.4. Indirect immunofluorescence inhibition assay

For competitive “biotinylated monoclonal antibody B4-T cell” binding inhibition assay, HPB-ALL or MT2 cells were first incubated with the competitive reagents or immune sera diluted 1:10 and washed twice before the addition of biotinylated monoclonal antibody B4 to plateau concentration.

Table 4
Ability of CD4 peptide immunogens to elicit neutralizing antibodies against HIV primary isolate

Peptide code ^a	Description of target antigenic site ^b	Amino acid sequence of target antigenic site	Target ^c	rsCD4 ^d	NA Titer ^e
rsCD4	CD4(1–353)		N/A	>5	1:10
p1471c	CD4(39–67)	NQGSFLTKGPSKLNDRADSRRLWDQGNF	>5	2.7	<1:10
p2057c	(C)CD4(27–66)(C)	(C)HWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRLWDQGN(C)	4.5	>5	1:165
p2190c	(C)CD4(27–71) [F ₆₇ → C]	(C)HWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRLWDQGN(C)PLII	>5	>5	1:15
p2189c	(C)CD4(39–71) [F ₆₇ → C]	(C)NQGSFLTKGPSKLNDRADSRRLWDQGN(C)PLII	>5	>5	1:23
p2240c	(C)CD4(39–66)(C)	(C)NQGSFLTKGPSKLNDRADSRRLWDQGN(C)	>4	>5	1:283
p1585c	(C)CD4(36–47)(C)	(C)ILGNQGSFLTKG(C)	4.3	3.0	<1:10
p1614c	(C)CD4(38–45)(C)	(C)GNQGSFLT(C)	>5	2.6	<1:10
p1586c	(C)CD4(42–59)(C)	(C)SFLTKGPSKLNDRADSRRL(C)	4.3	3.2	<1:10
p1587c	(C)CD4(44–56)(C)	(C)LTKGPSKLNDRAD(C)	4.3	3.2	<1:10
p1588c	(C)CD4(46–55)(C)	(C)KGPSKLNDRAD(C)	4.3	3.2	<1:10

^a All synthetic immunogens were “c” peptides, i.e. presented with the Inv-GG-HBsAg Th site on the N terminus.

^b All were cyclized peptides, cyclized through added cysteines, with the exception of rsCD4 and linear peptide p1471c.

^c Log₁₀ ELISA anti-target antigenic site reciprocal titre.

^d Log₁₀ ELISA anti-rsCD4 reciprocal titre.

^e Serum dilution giving 50% inhibition of infectivity of HIV-1 VL135 in MT-2 neutralization assay.

Table 5

Neutralization of HIV-1 primary isolates and TCLA isolate MN by guinea pig and swine anti-sera and monoclonal antibodies, comparisons of antibodies directed against CD4 cell surface complex and gp120^a

Source of antibodies	UG029 (clade A)	DH12 (clade B)	VL135 (clade B)	Zim748 (clade C)	UG266 (clade D)	UG046 (clade D)	TH036 (clade E)	MN (clade B)
Guinea pig anti-p2057c (15 wpi ^b)	54	34	478	418	20	ND	276	<10
Guinea pig anti-p2249b (35 wpi)	512	174	>270	322	40	135	199	<10
Swine anti-p2249f ^c (46 wpi)	193	44	593	319	11	82	282	<10
Guinea pig anti-p200 MNgp120 ^d	<10	<10	<10	<10	<10	<10	<10	203,080
mAb B4 anti-CD4 complex ^e	0.30	0.18	0.12	0.19	0.25	0.39	0.25	>50
mAb IgG1b12 anti-gp120 (μg/ml) ^f	38.5	ND	41.7	>50	>50	>50	>50	>50

^a Neutralization determinations by MT2 microplaque assay, activities of sera expressed as reciprocal titre at 50% endpoint.

^b WPI: weeks post-immunization.

^c Swine anti-p2249f (46 wpi) was further tested for neutralization by assays with PHA-stimulated PBMC (Table 6).

^d Neutralization activity of anti-peptide serum with specificity gp120 V3 domain of HIV-1 MN [32].

^e Neutralization activity of mAb B4 [8], expressed as μg/ml of monoclonal antibody.

^f Neutralization activity of mAb IgG1b12 [34], expressed as μg/ml of monoclonal antibody.

Staining of the CD4-expressing T cell lines was completed by subsequent incubation with diluted FITC-avidin, three washes, and analysis by cytofluorograph or fluorescence microscopy [8].

2.5. Virus stocks

HIV-1 stocks for neutralization are listed in Tables 5 and 6. The T cell line-adapted (TCLA) isolate MN was a gift of R.M. Hendry (California Department of Health Services, VRDL). All other viruses were primary isolates grown in PBMCs. B clade primary HIV-1 virus VL135 was isolated in 1992 from a homosexual man participating in the San Francisco Men’s Health Study [20]. Clade A isolate UG029 was acquired from the WHO Network for HIV isolation and characterization. Clade C isolate ZIM748 was a gift from D. Katzenstein, Stanford University. Clade A isolate DJ258, clade D isolates UG266 and UG046 and clade E SI isolates TH32036 and CM235 were supplied by the US Military

Table 6

Neutralization of HIV-1 primary isolates by swine anti-peptide immune sera, by PHA-stimulated PBMC assay

Swine anti-p2249f	DJ-258 (clade A)	302056 (clade B)	CM-235 (clade E)
46 wpi			
50% neut	200	350	800
90% neut	160	160	180
49 wpi			
50% neut	380	240	650
90% neut	100	125	130

HIV Research Program. Clade E isolate TH 32036 was also received as a gift from J. Bradac, NIAID. Isolate 302056 and DH-12, a patient isolate passaged in chimpanzee PBMC [21], was supplied by the NIAID AIDS Research and Reference Reagent Program. Primary isolates were expanded in PHA-stimulated PBMC. TCLA strain MN was grown in H9 cells.

Table 7
Neutralization of clade B HIV-1 primary isolate VL135 by anti-CD4 peptide guinea pig and swine sera^a

Source of antibodies	WPI ^b	Neutralization titer		
		Group 1 (n = 3)	Group 2 (n = 3)	Group 3 (n = 3)
Guinea pig anti-UBITH-receptor peptide (p2249g)	5	23	38	38
	8	161	145	200
	10	382	>270	259
Swine anti-UBITH-receptor peptide (p2249f)		Animal no.: 86	Animal no.: 87	Animal no.: 88
	5	16	99	50
	8	56	496	117

^a Neutralization determinations by MT2 microplaque neutralization assay.

^b WPI: weeks post-immunization.

2.6. Virus neutralization assays

The MT-2 microplaque assay (Tables 4, 5, 7 and 8) was carried out as described [22] except that heat-inactivated sera were serially diluted in 50% high glucose DMEM with 15% FBS, antibiotics, 2% glutamine and bicarbonate buffer, and 50% pooled, defibrinated normal human plasma. Neutralization assays on mitogen-stimulated PBMC (Table 6) were done with HIV essentially as described [23]. MAb B4 or immune sera neutralizing activity was the antibody concentration or reciprocal serum titer that provided 50% reduction in virus as compared with controls containing no antibody, except as noted otherwise. Serum dilutions or antibody concentrations for the 50% endpoints were derived by interpolation between serial serum dilutions or antibody concentrations.

2.7. Immunizations and antibodies

Guinea pig sera directed against rsCD4, CD4 receptor and co-receptor-derived target antigenic sites were obtained at the specified time points after intramuscular immunization of 4–6 week old Duncan Hartley guinea pigs with 100 µg in

0.5 ml per dose in Complete Freund's Adjuvant at week 0 and Incomplete Freund's at 3 and 6 weeks, followed by monthly boosts in Incomplete Freund's thereafter. Immune sera from swine and baboons were obtained at specified weeks after the three initial immunizations at weeks 0, 3, and 6 with 300–400 µg in 0.5 ml per dose in Montanide ISA 51 (Sep-pic, Fairfield New Jersey) water-in-oil emulsions followed by boosts administered as needed (3–4 week intervals) to maintain antibody response (Tables 5–8). Other serological reagents were obtained through previous studies, as described in Table legends.

3. Results

3.1. Identification of sites affecting mAb B4 recognition

Candidate target antigenic sites within mAb B4 recognition site for the HIV receptor/co-receptor complex were selected from domains that had been implicated in gp120 binding. This included the extracellular domains of human CD4 and the four external domains of CC chemokine receptors, including CC-CKR1, CC-CKR2b, CC-CKR3, CCKR5 and CCKR4. These peptides were modeled after structure function maps and crystal structures for the four immunoglobulin-like domains of human CD4 [4,24–27] and the overall three-dimensional model for human CD4 provided by the Protein Data Bank at <http://www.rcsb.org/pdb/>, and from the exposed domains of the chemokine receptors [28,29]. The CD4 and CC sites were mimicked as target antigenic site peptides of the “a” series and screened for their cross-reactivity with mAb B4 [9]. Peptides taken from certain CD4 sites were selected for cyclic constraint based on predictions of surface-exposed loops (<http://www.rcsb.org/pdb/>). These cyclized “a” peptides were synthesized with introduced cysteines to mimic predicted loop structures by disulphide bonds. Elsewhere, naturally occurring cysteines were substituted with serines so as to prevent the formation of loop conformations not favored by the model. For chemokine receptor-derived peptides, cross-linkage between peptides of external domains 2 and 3, was made via the existing cysteine residues found in

Table 8
Neutralization of primary isolate VL135 and TCLA isolate MN by baboon anti-peptide sera^a

WPI	Animal no.					
	9805		1988		1997	
	VL135	MN	VL135	MN	VL135	MN
6	<10	<10	<10	352	<10	176
10	<10	<10	<10	13122	29	12597
12	13	<10	<10	4900	31	17345
15	24	<10	<10	5169	21	11776
20	11	<10	<10	3107	17	11381
23	46	<10	<10	1016	19	13743
26	19	<10	<10	978	21	27679
29	32	<10			37	22124
32	46	<10			33	27579
35	20	<10			39	23502

^a Neutralizations determined by MT2 microplaque neutralization assay.

the native structures, to form “x” peptides. Representative CD4 target antigenic peptides are shown in Fig. 1, and several CD4 peptides and chemokine receptor peptides are described in Table 3.

Peptides were tested for cross-reactivities to mAb B4 by ELISA. None of the first collection of “a” peptides from the receptor/co-receptor complex reacted strongly with mAb B4 in these ELISAs. The lack of strong reactivities of mAb B4 with the continuous CD4 receptor or co-receptor peptides indicates a fully conformational nature for the mAb B4 recognition site. However, weak mAb B4 reactivities for peptides derived from various regions of CD4 (AA1–A20, AA81–92, AA60–AA109, AA118–AA165, AA235–251, AA297–AA351, or AA361–AA375) [30] were detected above background. These weak though specific reactivities were used to generate a speculative model for a conformational B4 binding site based on the folding of the cited regions into a discontinuous epitope. Our model for this presumed effector site prompted a different approach aimed at synthetic peptides from CD4 and co-receptors that will elicit high affinity antibodies reactive with continuous site(s) neighboring the conformational mAb B4 recognition site. We predicted that such peptides would evoke antibody responses effective for inhibiting both antibody B4 binding and HIV infection by steric hindrance of gp120-receptor complex interaction.

3.2. Location of potential effector sites with proximity to mAb B4 recognition site

Sequences for scattered sites on CD4 and the external domains of various co-receptors were selected as target antigenic sites and rendered as immunogenic peptides by tandem linkage to promiscuous Th epitopes (Table 2), for example HBsAg (“b” series peptides) and other related “e”, “f”, “g” and “x” peptides and the “c” series with the invasin immunostimulatory site [31] (Fig. 1, Tables 3 and 4). Other peptide immunogens had the MV_F-derived artificial Th site, UBITH[®]1 [15], and an HIV gp41 Th site [12] with KKK on the amino terminus.

The Th/target antigenic site fusions used glycine–glycine spacers for separation of the target antigenic site from the Th site, and separation of the Th from the immunostimulatory invasin or from a second Th site. The resulting peptide immunogens were screened for functional immunogenicity by testing them for the ability to induce antibodies in immunized guinea pigs with the following properties: (1) binding to the target antigenic site “a” peptide in an ELISA assay; (2) binding to rsCD4 in an ELISA assay, in the instances of CD4-derived antigenic peptides; (3) binding in an immunofluorescent assay to T cells that express the cell surface receptor/co-receptor complex comprising CD4; and (4) neutralization of an HIV primary isolate in an in vitro microplaque assay (Table 4, Fig. 1).

Most of the CD4 and chemokine receptor-derived peptide immunogens evoked guinea pig anti-peptide antibodies

with reciprocal titers in the range of 2.5 to >5 Log₁₀. Many of the CD4-derived target antigenic sites having long segments of CD4 as well as some cyclized target sites were cross-reactive with rsCD4, as shown by immunoreactivities in the anti-rsCD4 ELISA (Fig. 1, Table 4). Cross-reactivities of peptide constructs with rsCD4 were not predictable and rsCD4 cross-reactivity extended only rarely to the cell membrane-associated CD4 complex. Indirect immunofluorescence staining of cells expressing CD4, with anti-peptide immune sera, showed that only a few peptides (those marked with (+) in Fig. 1) shared strong serological reactivity with the in situ receptor complex. Interestingly, some immune sera with relatively low cross-reactivity for rsCD4 were highly reactive with the CD4 cell surface complex (Fig. 1). These were several of the sera raised to have specificities for CD4 target antigenic site peptides with a cyclized structure, for example p1472c, p1403b, and p1471c, the latter two from the CDR2-like domain of CD4 (Table 3). For the CC peptides, immune sera raised against peptides derived from domains 1, 3 or 4 of the co-receptors had cell surface reactivity (data not shown).

Immune sera having bright immunofluorescence staining patterns with CD4-expressing T cells were further evaluated for competitive inhibition of the binding by mAb B4 to CD4-expressing T cells. Among all the immune sera evaluated, only sera generated through immunization with peptide p1471c, derived from the CD4-CDR2 domain, was found to be inhibitory of mAb B4 binding (Table 3). None of the immune sera from co-receptor-derived peptides interfered with this “mAb B4-T cell” binding.

All guinea pig immune sera (6 or 8 weeks post-initial immunization) obtained for the initial peptide immunogens, whose “a” target antigenic sites are shown in Fig. 1, were also screened for their neutralizing activity against HIV-1 VL 135 by the MT-2 microplaque neutralization assay. Despite the presence of high titers of antibodies cross-reactive to rsCD4 and cell surface reactivity, none displayed significant levels of neutralizing antibodies (data not shown).

3.3. Immunogenic peptides to capture potential effector sites of the surface CD4 complex

New peptide constructs, based on the position of the p1471 target antigenic site in CD4, were designed to more accurately capture what may be an effector site on the surface CD4 complex. Peptides comprising target antigenic sites spanning the CD4-CDR2 domain, amino acid residues from 20 to 75 [30], were revisited. The new peptides covering this region were redesigned with a particular emphasis on the preservation of the 3D-structure of the CDR2-like domain by disulphide bonded loops in the range of 30–45 amino acids (Table 4), and each was immunopotiated by fusion of the N-terminus to the “Inv-GG-HBsAg Th” site (the “c” peptide format). Guinea pig immune sera were collected at 6 or 8 weeks post-initial immunization for evaluation. Target antigenic sites p2057, p2190, p2189, and p2240 were

found to elicit neutralizing antibodies against HIV-1 isolate VL135. It is worth noting that those neutralizing target antigenic sites spanned an extended area around the CDR2-like domain of CD4 and were cyclized (Table 4). The CD4 target antigenic site of p2240, cyclized CD4 positions 39–66, was the minimal “a” target for evoking effective neutralizing activity (Table 4).

3.4. Hyperimmune sera against CD4-CDR2 effector site peptides are broadly neutralizing

Hyperimmune guinea pig and swine sera against CD4-CDR2 peptide immunogen p2057c and improved immunogen homologs p2249f and p2249g had significant neutralizing antibody titers against HIV primary isolates from clades A to E in a pattern reflective of the neutralizing activity of mAb B4 (Table 5). In comparison, virus-directed antibodies with specificities for either the gp120 N-terminal V3 domain of TCLA isolate MN (guinea pig anti-MN V3 immune sera [32,33]) or a less variable conformational gp120 CD4 binding site (mAb IgG1 b12 [34]) had poor neutralizing activity against the HIV primary isolates (Table 5). Swine antisera to p2249f was further analyzed for neutralizing activity with PHA-stimulated PBMC indicator cells. The swine immune sera was found to have broadly neutralizing antibodies against isolates of clades A, B, and E at 50 and 90% endpoints (Table 6).

3.5. CD4-CDR2 peptides having promiscuous artificial Th are immunogenic in multiple species

Responses to the cyclized 39–66 CD4-CDR2 target antigenic site, more effective than responses to the original p2240c construct, were elicited by the tandem addition of two promiscuous T helper (Th) sites, the idealized library palindromic Class II site UBITH[®]1 [15] and an HIV Th site adapted from gp41_{772–787} [12] (Table 2). The HIV Th epitope should be helpful for restimulation in individuals upon HIV exposure or re-emergence. The wholly synthetic peptide immunogen, designated p2249f, is represented by the formula: “gp41_{772–787}Th-GG-UBITH[®]1-GG-cysCD4_{39–66}cys”. The domains are separated by glycine–glycine spacers and the CD4 target site is cyclized through disulphide bonds between the cysteines at the N- and C-termini of the site. p2249g, a homologous peptide without the gp41 helper site was also effective for immunizing naïve animals.

Guinea pigs and swine were immunized with peptides in Freund's Adjuvants or an ISA 51 water-in-oil emulsion at weeks 0, 3 and 6 followed by monthly boosts. Neutralizing antibodies of titers ≥ 100 were present by week eight in eleven out of twelve animals evaluated (Table 7). The neutralizing antibodies of the vaccine sera were associated with the cyclized cysCD4_{39–66}cys target antigenic site as shown by peptide ELISA and with the mAb B4 recognition site as

shown by competitive staining with antibody B4 (data not shown).

The immunogenicity of the p2249f construct was further characterized by evoking neutralizing antibody responses in baboons. Neutralizing antibodies against HIV-1 VL135, albeit with lower titers than seen in guinea pigs and swine, were consistently observed in baboons upon multiple immunizations with p2249f. (Sustained responses in the baboons required frequent boosts, approximately monthly.) In Table 8, animal no. 9805 received immunogen p2249f only, in water-in-oil emulsion. The neutralizing antibodies of this animal were effective against primary isolate VL135 while having little activity against T cell line-adapted MN. In contrast, baboon no. 1988 was given only an MN V3 peptide immunogen [32,33] and developed strongly neutralizing antibodies exclusively against MN. Baboon no. 1997 was co-immunized with both peptide immunogens and developed neutralizing antibodies effective against both the primary and the TCLA isolates. That animal showed no suppression of the anti-MN V3 neutralizing response to the second peptide immunogen despite the persistent presence of anti-CD4 neutralizing antibodies. Similar anti-MN and anti-VL135 neutralizing titers coexisted in three other baboons (nos. 1096, 1196, and 1697) that received p2249f, the MN V3 peptide, and other immunogens. The distributions of CD4, CD8, CD3 and CD20 positive B and T cells remained within normal range in the immunized baboons after persistent exposure to the site-specific anti-CD4 neutralizing antibodies.

4. Discussion

We have focused on the cell membrane-associated receptor/co-receptor complex for HIV, for the immunoprophylaxis and immunotherapy of HIV infection. This cell-directed approach offers a method to overcome the neutralization resistance of HIV-1 primary isolates and their hypermutability. As shown previously in our report on mAb B4, this approach can provide sterilizing immunity in chimpanzees by passive immunization [8]. Here we demonstrated that the anti-cell approach can also be accomplished by a vaccine for active immunization. A chemically defined immunogen has been produced that evokes a site-specific anti-cell antibody response with critical neutralization function against primary isolates of HIV-1.

For efficacy, such a synthetic immunogen must duplicate or mimic a B cell epitope, a precise site on the host cell receptor/co-receptor complex. The B cell site must resemble the targeted site with sufficient fidelity to evoke cross-inhibitory antibodies, while retaining a precise site-specificity sufficient to avoid adverse immunosuppression. Most antibodies raised by immunization with CD4 lack such useful specificities [35]. For example, high titre hyperimmune antiserum to whole rsCD4 was devoid of neutralizing activity for primary isolates of HIV (Table 4), and

antibodies with broad reactivity for extensive regions of a T cell antigen are expected to be overly immunosuppressive [36]. Here, a useful effector site on CD4 was located in the CDR2-like domain when we localized a target antigenic site (on peptide p1471c, Table 1) with immune reactivity that interfered with mAb B4 binding to CD4+ cells. The target antigenic site was refined for optimal sequence and conformation until it evoked neutralizing antibodies. Then Th sites with sufficient immunopotency to overcome the strong tolerance exhibited towards self-molecules were incorporated into prototype target antigenic site immunogens. The target site, a constrained loop corresponding to positions 39–66 is consistent with the exposed residues of CD4 between 38 and 60 that are inferred to contact gp120 [26,37].

The creation of synthetic peptide immunogens of the appropriate immunopotency and specificity was accomplished through consolidation of a collection of methods for the identification and design of synthetic peptide immunogens. These methods included: (1) a procedure for the identification of an effective high affinity target antigenic site (B cell epitope); (2) the means for stabilization of the conformational features of that target site on a synthetic peptide by the introduction of cyclic constraints, so as to maximize cross-reactivity to the native molecule; (3) the means to augment the immunogenicity of the B cell target epitope by combining it with peptides having broadly reactive promiscuous T helper cell (Th) epitopes; and (4) the means of enlarging the repertoire of Th epitopes by application of combinatorial peptide chemistry (UBITh[®]1) and thereby further accommodate the variable immune responsiveness of multiple species and outbred populations.

In an alternative method for producing synthetic immunogens targeted to the CD4 receptor complex, we devised peptide mimotopes for potential effector sites on CD4. The mimotopes were identified from a random combinatorial peptide library [38,39]. Six 8mer mimotopes that bore no resemblance to the CD4 sequence were found to bind mAb B4. Three of these were expanded into sub-libraries with enhanced recognition for mAb B4. The two best-enhanced mimotopes were incorporated into Th/mimotope fusion peptides for immunogenicity testing, but only transient, low-titer neutralizing antibodies were elicited (Wang CY and Stewart ML unpublished results). The mimotope approach to an anti-CD4 vaccine for HIV was ineffective in comparison to the approach that started with the CD4 amino acid sequence.

Tricking the immune system into launching an autoimmune response towards a critical immunoregulatory receptor complex is controversial as a means of blocking HIV infection. However, this anti-receptor approach could circumvent the resistance problems that are encountered by trying to block viral transmission and replication with antibodies and immunogens directed against the virus. That p2249f-vaccinated baboons having neutralizing antibodies at week 35 retained the ability to: (1) mount a vigorous neutralizing response against TCLA isolate MN in response to a second peptide immunogen, and (2) at week 35 retained

normal distributions of CD4, CD8, CD3 and CD20-positive T and B cells is evidence that immunotoxicity is limited when such site-specific anti-CD4 antibodies are generated. The phylogenetic proximity of humans and baboons suggests that the elicited antibodies may be largely bound to CD4 cells, thus accounting for the relatively low titer of infection inhibiting antibodies circulating in the baboon plasma. The peptide immunized baboons had antibodies with significant cross-reactivities for recombinant soluble CD4 (data not shown), so it can be argued that high titers of antibodies to CD4-CDR2 were elicited by the peptide immunogen. However, the highest quality antibodies, i.e. those responsible for entry inhibition activity (shown here as neutralizing activity), would have been those antibodies that were bound to CD4+ cells and thus were unavailable for the *in vitro* neutralizing assays. Prior to clinical application, this concept, along with any potential immunotoxic side effects could be explored by vaccinating and challenging chimpanzees.

In summary, the chemically defined immunogen generated anti-HIV receptor antibodies with a breadth of neutralizing activity similar to that of mAb B4. In addition to being a possible approach to immunoprophylaxis, immunotherapy by this approach may be an attractive orthogonal treatment with low toxicity for HIV exposure and infection, in combination with or in place of anti-retroviral drugs.

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