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AD_____

COOPERATIVE AGREEMENT NUMBER DAMD17-95-2-5017

TITLE: Support of Study Entitled, "Conformationally Restricted Synthetic Aids Vaccine"

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE
Support of Study Entitled, "Conformationally Restricted Synthetic AIDS Vaccine"

5. FUNDING NUMBERS
DAMD17-95-2-5017

6. AUTHOR(S)
Arnold C. Satterthwait, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, California 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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13. ABSTRACT *(Maximum 200*

Our proposal was directed to the identification of synthetic peptide mimetics corresponding to neutralizing epitopes recognized by IgG1b12 and MAb 58.2. Herein, we report recent results on MAb 58.2 which provides important new information regarding methods and outcomes for synthetic vaccines as well as specific information about the role that conformation plays in neutralizing immune responses to the V3 epitope.

MAb 58.2 displays broad reactivity against Clade B gp120s from primary isolates. It weakly neutralizes a cloned macrophage-tropic primary isolate, JR-CSF. We identified a constrained V-3 loop mimetic that binds >1,000 times better to MAb 58.2 than the corresponding linear peptide. Rabbit polyclonal antibodies to the constrained peptide but not the linear peptide bind rgp120 (MN). Furthermore, the polyclonal immune response to a constrained V3 peptide mimics the conformational preference and neutralizing activity of MAb 58.2. HIV-1 neutralization correlates with conformational preference and affinity for gp120. These results and others presented in the report establish beyond doubt the remarkable improvements in antigenicity and immunogenicity of constrained peptides compared to linear peptides.

14. SUBJECT TERMS
HIV-1, primary isolates, vaccine, synthetic vaccine, peptides, cyclic peptides, MAb 58.2, V3 loop, gp120.

15. NUMBER OF PAGES
40

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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A polyclonal immune response to a constrained V3 peptide mimics the conformational preference and neutralizing activity of a potent HIV-1 neutralizing MAb

Arnold C. Satterthwait

Abstract: An important issue is whether the HIV-1 neutralizing activities of MAbs can be duplicated by polyclonal responses to the epitopes recognized by these MAbs. To test this, a constrained peptide loop, [**J**HIGPGR(Aib)F(D-Ala)G**Z**]C-NH2 (Loop 5), was identified that binds about 1,000 x better than the corresponding linear peptide to a potent, broadly cross-reactive V3 MAb 58.2. A tighter binding implies a better fit. A better fit implies that the cyclic peptide mimics the fine structure and conformation of the MAb 58.2 epitope. Consequently, it should stimulate a "MAb 58.2-like polyclonal response.

To test this, the cyclic peptide and its corresponding linear peptide, acetyl-GHIGPGR(Aib)F(D-Ala)GGC-NH2 (Linear 5) were chemically linked to a "universal" tetanus toxoid T-cell epitope to form Linear 5-MAPS and Loop 5-MAPS. Rabbit antisera against the Loop 5-MAPS (3/3) but not the corresponding Linear 5-MAPS (0/3) bound baculovirus rgp120 (MN). To ascertain how close the Loop 5 antisera duplicated MAb 58.2, the conformational preference of the antisera for a series of constrained peptides was determined. A comparison of profiles identified one Loop 5 antiserum with a "MAb 58.2"-like profile and a second Loop 5 antiserum with a different profile. The "MAb 58.2"-like antiserum neutralized HIV-1 (MN) while the other antiserum did not.

These results have several implications. First, they establish a conformational requirement for a neutralizing antibody response to the V3 epitope on HIV-1. Second, they establish the importance of the cyclic peptide conformation for any AIDS vaccine based on the V3 epitope. Third, they raise the possibility that clonal selection plays a role in establishing a neutralizing response. Fourth, they indicate that conformational preference (antibody quality) rather than titer (antibody concentration) correlates with neutralizing activity. Fifth, they show that conformational scanning is an effective approach for assessing antibody quality. More generally, they verify a method and routes to achieving polyclonal responses that closely mimic the conformational preferences and neutralizing activities of specifically selected MAbs including the most potent among them.

Introduction.

A few broadly, cross-reactive MAbs, IgG b12, 2F5 and 2G12, have been identified that potently neutralize primary HIV-1 isolates.[1] Despite considerable effort by many labs including ours, it has not yet been possible to reproduce the activities of these MAbs in a polyclonal antibody response. On the other hand, weak neutralizing activity against primary isolates has been reported for several V3-loop MAbs.[2,3] Amongst the promising V3 MAbs is MAb 58.2, isolated by Repligen Corp. MAb 58.2 was the most potent, broadly neutralizing MAb among thousands of anti-V3 peptide antibodies isolated and

characterized by scientists at Repligen Corp. It potently neutralizes HIV-MN (IC_{90} = 0.39 μg/ml) and is reported to neutralize a variety of Clade B primary isolates.[3]

V3 limitations. The V3 loop epitope has long been considered an HIV-1 vaccine candidate. However, several limitations are now apparent. The most important limitation is weak neutralization of primary isolates by V3 MAbs.[2,3] Although weak activity might normally be grounds for abandoning the epitope, there are no other vaccine candidates that merit confidence at this time. It is therefore important to review the limitations carefully to determine whether any remedies might be applicable. These limitations are briefly reviewed with reference to MAb 58.2.

(1) A major limitation is the amino acid variability of the V3 epitope. Although most of the V3 sequence is hypervariable, the neutralizing epitope at the "tip" of the loop, GPGRAF, is present in about 60% of North American Clade B isolates.[4] The "tip" sequence, GPGRAF, is conserved to a much higher degree in macrophage-tropic (MT) strains than it is in T-cell tropic strains.[5] Since MT strains survive the initial infection while T-cell tropic strains do not, the MT strains are important vaccine targets. The conserved "tip" sequence in MT strains is thus an attractive target. Its conservation supports the idea that it may have a functional role. However, the relative conservation of the MT "tip" sequence suggests that the virus protects it from antibodies which is supported by further observations discussed below.

MAb 58.2 binds GPGRAF.[3] Further, in an extensive study,[6] MAb 58.2 showed broader recognition of free gp120s from North American and Brazilian Clade B primary isolates than did IgG b12. In terms of cross-reactivity with Clade B gp120s, MAb 58.2 can not be distinguished from another well considered MAb.[6]

(2) Bou-Habib et al.[7] made the important observation that although V3 MAbs bind lab adapted strains of HIV-1, they bind poorly if at all to primary isolates. This observation has often been cited as a reason to abandon V3-directed vaccines. Later studies show, however, that sCD4 induces a conformational change in gp120 which is required for interaction with the second receptor.[8] Furthermore, the interaction of gp120 with a second receptor can be blocked by V3 MAbs.[8] The V3 epitope was also exposed by adding sCD4 to two primary-like virions.[2b] Although the V3 "tip" is inaccessible on free primary isolates, it becomes accessibleupon adding sCD4. Thus the conserved MT "tipo" sequence although protected may become transiently vulnerable to V3 MAbs.

(3) Although the epitope appears to be exposed, the important issue is whether MAbs can bind the epitope and neutralize the virus. Bou-Habib et al.[7] tested selected V3 MAbs

50.1 from Repligen, two additional V3 MAbs that are not as well characterized and rabbit polyclonal antiserum raised against MT-CSF (JR-CSF) gp120 for neutralizing activity against MT-CSF. None of these antibodies neutralized MT-CSF. It was therefore concluded that the V3 epitope should be abandoned as a vaccine target. However, as discussed below, several MAbs appear to weakly neutralize MT-strains.

The negligible neutralization of MT-CSF by Bou-Habib[7] may have other origins. For example, MAb 50.1 employed by Bou-Habib et al.[7] binds CKIHIGPG in the crystal structure[8] whereas the MT-CSF sequence is SIHIGPG. White-Scharf et al.[3] show that Lys contributes to MAb 50.1 affinity. The affinity of MAb 50.1 for the MT-CSF sequence (Lys is replaced with Ser) should be suboptimal which should reduce its ability to neutralize the virus. The rabbit antiserum used by Bou-Habib et al.[7] was raised against the JR-CSF rgp120; the adjuvant was not specified. Other experiments reveal that antibodies raised against rgp120 generally form against the denatured protein.[10a] Antibodies raised against a denatured protein are not anticipated to be effective against the virus. The rabbit antiserum, however, showed weak neutralizing activity against HIV-1 (MN) (70% inhibited at 1/50 dilution) and completely neutralized T-CSF (completely inhibited, 1/50 dilution). However, T-CSF is characterized by a very unusual insert (underlined) at the "tip" of the loop, - R-I-IHIGPG which is not present in the MT-CSF sequence - SIHIGPG. An insert could disrupt the loop conformation making it more susceptible to binding by rabbit anti-denatured protein antibodies and subsequent neutralization. The fact that several V3 MAbs and relatively weak polyclonal antiserum do not neutralize MT-CSF may not be sufficient evidence to rule out the V3 epitope as a neutralizing epitope.

On the other hand, there is some evidence that indicates that V3 MAbs weakly neutralize primary isolates. Conley et al.[2a] reported that MAb 447-52D neutralize a variety of primary isolates at 100 ug/ml. Fouts et al.[2c] report that JR-Fl is weakly neutralized by MAb 447-52D (IC_{90} = 47.5 ug/ml). Stomatatos et al.[2b] report that the two primary-like macrophage-tropic HIV-1 (SF162 and SF128A) are neutralized by IC_{90} = 20 μg/ml of MAb 391-95D when grown on PBMC and 5 μg/ml when grown on macrophage. White-Scharf et al.[3] reported that MAb 58.2 inhibited several cloned primary isolates and several primary isolates at 200 ug/ml.[3] Since there is question as to whether neutralization of HIV-1 by >50 ug/ml MAb is meaningful, Drs. Meng Wang and Paul Parren retested MAb 58.2 for this study at lower concentrations against JR-CSF. As reported, MAb 58.2 neutralizes JR-CSF at IC_{90} = 20 μg/ml in a reaction that is

completely blocked by V3 peptide. This contrasts with the study carried out by Bou-Habib et al.[7] who reported that V3 antibodies do not neutralize JR-CSF.

In conclusion, it is clear that V3 MAbs neutralize lab strains far more readily that MT strains. However, V3 MAbs are capable of weakly neutralizing cloned MT-tropic primary isolates. Presumably V3 MAbs bind an epitope that becomes exposed following a conformational change induced when CD4 binds gp120. The question is, should one ignore weak neutralizing activity ?

Evaluating neutralizing MAbs. V3 MAbs have been exhaustively examined. VanCott et al.[10b] showed that the neutralization of the lab adapted strain, HIV-1(MN), by V3 MAbs correlated with the affinity of MAbs for rgp120. MAbs with fast off-rates were weaker neutralizers than MAbs with slow off-rates. Presumably, weak neutralization of primary isolates indicates fast off-rates for the MAbs. The wide ranging affinity of MAbs for gp120 (100-fold) observed by VanCott et al.[10b] raises important questions about the origins of high affinity and whether the affinity can be improved further. Since the best V3 MAbs neutralize primary isolates at about 20 μg/ml, it would take a 20-fold improvement in affinity of a V3 MAb to boost the status of the V3 epitope to that of epitopes recognized by the most potent HIV-1 neutralizing MAbs.[1]

Both intrinsic (antibody specific) and extrinsic (epitope) factors could contribute to the affinity of an antibody for an epitope.[11] Amongst factors intrinsic to the antibody are possibly (1) the size of the bound epitope - affinity might improve with an increase in the bound surface area. (2) The specific nature of the antibody combining site may determine affinity. For example, a more hydrophobic binding site may bind a hydrophobic amino acid better that a less hydrophobic pocket. (3) Conformational fit could determine affinity i.e. antibody binding pockets that mirror the shapes of neutralizing epitopes are likely to fit better and bind tighter. Amongst extrinsic factors specific to the epitope may be (1) the amino acid composition of the epitope - for example, hydrophobic amino acids might bind better and (2) epitope accessibility - specific amino acids within an epitope may be more accessible to antibody binding than others.

Since a number of complex factors determine the affinity and neutralization potency of a MAb, care should probably be taken in selecting vaccine targets on the basis of the potency of any single or several MAbs. Thus the very high potency of a MAb could be due to intrinsic factors specific to a MAb that might not be reproducible in a polyclonal response to the epitope recognized by the MAb. In like manner, weak neutralization by a MAb should not necessarily be considered grounds for abandoning an epitope since a

more potent MAb to the same epitope might emerge. The large number of chance events that must be satisfied to achieve a highly potent MAb should temper conclusions about whether or not to pursue an epitope if it is based solely on the neutralizing potency of a MAb.

Design of Antigen. It is quite clear that gp120 undergoes a conformational change upon interaction with CD4.[2b,8] It is also clear that this conformational change exposes the V3 neutralizing epitope.[2b,8] Thus it is quite possible that the "tip" of the V3 loop also undergoes a conformational change which could have a strong impact on the affinity of V3 antibodies and neutralization.[7] This could provide the virus with a superb mechanism for self-protection. By changing its shape, antibodies may no longer be able to bind. It also creates a problem for vaccine design since it may be very difficult to determine what this structure is. Since the crystal structure of a protein could display the conformation of a protected state rather than the active state, we pay particular attention to the structures bound by neutralizing antibodies.

Neutralizing antibodies provide direct information regarding the conformations of neutralizing epitopes. The conformations they recognize identify target structures that could in principle be used as vaccines to induce similar antibodies and neutralizing responses. MAb 58.2 has been co-crystallized with both a long linear peptide and a smaller cyclic peptide.[12] Although initial alanine scans identified GPGRAF as the core epitope, X-ray crystallography of MAb 58.2-peptide complexes show that the epitope bound by MAb 58.2 is bigger - RIHIGPGRAF. The crystal structure also shows that the least variable part of this epitope, GPGRAF is bound as two tandem turns. Both GPGR and RAFY are bound as Type I turns. R serves as a pivot point and occupies the β-region of conformation space. GPGRAF is thus bound as a double-headed loop, a loop structure that is found rather frequently in proteins.

While the crystal structure provides detailed information regarding the conformation of the epitope, it does not tell us how to reproduce this conformation with a peptide. Double-headed medium-sized loops are conformationally very complex. There is no precedent for the synthesis of these structures. Our approach to this problem emerged from a development of a "universal" chemical linker or covalent hydrogen bond mimic.[13] Since many protein substructures are defined by hydrogen bonds, we replace putative structure defining hydrogen bonds with the linker which provides a means for folding the peptide.[14] Since the MAb 58.2-peptide crystal structure does not reveal the necessary hydrogen bond information, we systematically varied the size of the cyclic peptide and

within each size class, the cadence of the amino acid sequence.[14b] Since most hydrogen bonds in proteins are short-ranged, it is likely that the correct conformation will be activated when the link is correctly placed.[14] The goal of our study then became a search for the smallest V3 constrained peptide that would contain the least variable part of the V3 epitope - GPGRAF - but would also fit best into the MAb 58.2 binding site. The best loop determined by this approach was [**J**HIGPGRAFYG**Z**]G-NH2.[14b] Loop fit was assessed on the basis of improved affinity for MAb 58.2 relative to the linear V3 peptide.

Two additional changes were eventually made in the cyclic peptide to give [**J**HIGPGR(Aib)F(D-Ala)G**Z**]G-NH2 (Loop 5) which binds MAb 58.2 about 1,000 x better than the corresponding linear peptide, acetyl-GHIGPGRAFGGG-NH2 from the V3 loop. Aib, α-aminoisobutyric acid (or αα-dimethylglycine) is known to favor turns and improves affinity several-fold. [14b] When D-alanine was substituted for glycine, a further gain in affinity was obtained (this study). The approximate 1,000-fold improvement in affinity is substantial and indicates that the cyclic peptide adapts a conformation that matches the MAb 58.2 binding site to a considerable degree.

An NMR study confirmed that the cyclic peptide stabilized GPGR and R(Aib)F(D-Ala) as turns.[15] but could not identify the types of turns. In part, this is due to the presence of the two glycines which lack side chains and limit the number of connectivities required for establishing NMR structures. The data, however, does indicate that the cyclic peptide populates a loop that samples different turn types in a single and/or a double-headed loop motif. Medium-sized loops are expected to be conformationally flexible. The NMR data support a picture of a dynamic equilibrium between different loop conformations but do not provide quantitative data. The argument that a significant fraction of Loop 5 samples a folded conformation is based on the NMR evidence for turns,[15] the crystal structure which shows the V3 peptide bound in turn conformations[12] and the substantial gain in affinity of Loop 5 for MAb 58.2.

Vaccine design. The cyclic peptide and corresponding linear peptide were incorporated into four-branched MAPS for vaccine tests in rabbits. To ensure immunogenicity, the peptides were covalently linked to a tetanus T-cell epitope (TT2) in a 4-branched MAPS system. Mice, rabbits and humans are known to respond to the tetanus toxoid T-cell epitope.[16] Thus immunogenicity studies in animals may be applicable to trials in humans. MAPS are often highly immunogenic.[17] MAPS are also chemically defined and modular. They are unlikely to influence the structure of the cyclic

peptide. Also, the T-cell epitope can potentially be replaced with other more effective T-cell epitopes[18] or with T-cell epitopes from HIV-1[19] for comparative studies.

Immunization. The choice of an immunization protocol introduces a whole new set of uncertainties and choices into the experiment.[20] There are few precedents to go on, since comparisons of immune responses to linear and constrained peptides have rarely been examined. There are several choices that must be made. These include the type of animal, the age and sex of an animal, the choice of an adjuvant, route(s) of immunization, dose and frequency.[20] There are at least two additional uncertainties regarding cyclic peptides. First, can the cyclic peptide survive the lengthy time of exposure to physiological conditions required to produce an immune response. Exposure to proteases is a particular concern since the cleavage of a single peptide bond in the loop would destroy its conformation. A second concern was that the adjuvant which often contains an oil base could disrupt the conformation of the cyclic peptide.

Since we had achieved a favorable result using RIBIs adjuvant in a previous study[21] with a constrained *P. falciparum* malaria peptide, we decided to continue with this adjuvant. RIBIs is a commercially available oil-in-water adjuvant.[22] Presumably the MAPS which is water soluble resides in the aqueous field of the adjuvant and thus should retain its conformation. Immunizations were carried out on young female rabbits since females reportedly produce a better quality antibody[20b] and rabbits provide sufficient serum for neutralization assays. Although anecdotal reports indicated that rabbit antiserum interferes with HIV-1 neutralization assays, this did not appear to be a problem in the neutralization assays employed in this study. Usually, quite high peptide doses (0.5 - 1 mg/rabbit) are used to immunize rabbits. However, high doses of peptides may not be appropriate for inducing high affinity antibodies.[23] High doses of immunogen can in theory activate all primed B-cells whereas lower doses may lead to the selective activation of the higher affinity B-cell receptors. Thus immunizations were initiated with a relatively low dose (0.2 mg/rabbit) which were then progressively reduced to very low values (17 ug/rabbit) during subsequent immunizations. The immunizations were carried out over an 8-month period since it has been found that affinity and neutralizing potential improves with time.[24]

Materials and Methods

(1-methylethylidene-2-Fmoc)hydrazinoacetic acid (Fmoc-Z(Act) was synthesized as previously described.[13,25]

5,5-Dimethoxy-1-oxopentanoic acid (J) was synthesized as previously described.[13,25]

Linear Peptides. Linear peptides (Table 1) were synthesized by solid phase peptide synthesis with an ACT350 multiple peptide synthesizer (Advanced ChemTech) using standard Fmoc synthesis according to the manufacturer protocol. Fmoc-Rink resin (0.5 mmol/g) was used. The peptides were acetylated on the resin with 15% acetic anhydride in DMF. Peptides were cleaved with reagent K^{24}: 6% phenol, 2% EDT, 4% thioanisole, 4% water and 84% TFA for 90 min and precipitated from the mixture with ice-cold diethyl ether. The precipitate was centrifuged, washed with cold diethyl ether, dissolved in 30% acetonitrile and lyophilized. Peptides were purified on a RP C-18 preparative column (Vydac, 201TP1022 or Cosmosil, 5C18-AR) eluting with a gradient of 0-50% acetonitrile over 30 min. Pure peptides (~40% yield) were identified by mass spectrometry and H^1NMR.

Cyclic Peptides. Two classes of cyclic peptides were prepared, those with C-terminal glycines and those ending in cysteine (Table 1). The cyclic glycine peptides were acquired from a previous study[14b] and repurified by HPLC. Both C-terminal glycine and cysteine peptides were prepared in essentially the same manner.

The cyclic cysteine peptide used for immunizations was prepared as follows. Fmoc-G**Z(Act)**C(Trt)-Resin was prepared on Rink's amide MBHA resin (100 mg, 0.064 mmol) sealed in a 2 cm^2 polypropylene mesh packets. The resin packet was placed in a 30 mL Nalgene wide mouth polyethylene bottles for wash and reaction steps. Wash solutions were added to the packet in the bottle, shaken for 1 min and poured off. Reaction solutions were shaken with the resin packet for the proscribed time and poured off. A standard coupling cycle involved the following steps: (1) The resin was swelled in its packet by shaking with 5 mL of DCM and then washed with 5 mL DMF. (2) The Fmoc protecting group was cleaved from the protected peptide-resin with 5 mL 20% piperidine in DMF for 10 min, the resin washed 4 times with 5 mL DMF, once with 5 mL 2-propanol, once with 5 mL DCM, and once with 5 mL NMP. (3) Coupling was carried out with N-α-Fmoc-L-amino acid (0.128 mmol), HOBt (17.3 mg, 0.128 mmol), PyBOP

(66.6 mg, 0.128 mmol), and DIEA (0.045 mL, 0.256 mmol) in 1 mL NMP by shaking for 30 min and then washing twice with 5 mL DCM. Fmoc-**Z(Act)** was coupled to the peptide resin using the protocol just described. Fmoc-Glycine was coupled to **Z(Act)**$C_{(Trt)}$-Resin using N-α-Fmoc-L-glycine chloride (0.128 mmol) dissolved in 1 mL of NMP, added to the resin packet and briefly shaken before adding 0.022 mL (0.128 mmol) DIEA. Each coupling step to a primary amine was checked for completion by the Kaiser ninhydrin test and if incomplete, it was repeated.

Peptide resin from the previous step was then loaded on the multiple peptide synthesizer (ACT350) to continue the synthesis, following the protocol for linear peptides. Upon completion, Fmoc-$H_{(Trt)}$IGPG$R_{(Pbf)}$AibF(D-Ala)G**Z(Act)**$C_{(Trt)}$-Resin, was transferred to a mesh packet for the following steps. After Fmoc deprotection and washing, **J** was coupled to the peptide resin using the protocol for Fmoc-amino acids described above. Following the addition of **J** to give **J**$H_{(Trt)}$IGPG$R_{(Pbf)}$AibF(D-Ala)G**Z(Act)**$C_{(Trt)}$-Resin, the packet was washed four times with 5 mL dichloromethane. The resin packet was then shaken for 15 min in a solution of 20 % trifluoroethanol in dichloromethane (20 mL) with HCl (0.032 mL 4N HCl in dioxane, 0.128 meq), the solution was poured off and the packet with [$JH_{(Trt)}$IGPG$R_{(Pbf)}$AibF(dA)CZ]$C_{(Trt)}$-Resin was washed with DCM and dried.

The resin packet from the previous step was treated with 95% trifluoroacetic acid/5% water for 60-90 min. The cyclic peptide was precipitated from the mixture with ice-cold diethyl ether. The precipitate was centrifuged, washed with cold diethyl ether, dissolved in 30% acetonitrile and lyophilized. The peptide was purified on a preparative RP C-18 column (Cosmosil, 5C18-AR) eluting with a gradient of 0-20% acetonitrile over 5 min and then 20-40% acetonitrile over 30 min to yield [JHIGPGRAibF(D-Ala)GZ]C-NH_2. A second purification on a small RP C-18 was necessary for the cyclic Cys peptides. Purity was checked by analytical HPLC and the peptide was identified by mass spectrometry and NMR spectroscopy.

MAPS Synthesis. MAPS core (Table 1) was synthesized on TGR resin (0.1 mmol/gr) with the ACT350 synthesizer (ACT350) using the standard protocol for Fmoc synthesis. Prior to the cleavage of peptide from the resin, the peptide was chloroacetylated with 15% chloroacetic anhydride in DMF. Peptide was cleaved and deprotected with TFA followed by precipitation with cold diethyl ether. After lyophilization, the MAPS core was purified by HPLC on a RP C4 column (Vydac, 214TP1022) using a gradient of 0-

20% acetonitrile over 10 min followed by 20-30% acetonitrile over 20 min. Pure MAPS core (5% yield) was identified by electrospray MS: Calcd (MH+), 9377, Obs 9374.

Linear 5/C or Loop 5/C peptides were joined to MAPS core by reacting highly purified MAPS core and peptide in 0.5 M Tris buffer pH 8.9 and 3 M guanidinium·HCl containing EDTA in N_2 atmosphere. Five times peptide excess was used to assure complete saturation of MAPS core. The reaction was monitored by analytical HPLC on RP C4 (Vydac, 214TP54) with a gradient of 10-20% acetonitrile over 5 min followed by 20-40% acetonitrile over 30 min. Purification on a preparative RP C4 (Vydac, 214TP1022) afforded pure product (50% yield). Lin5/C-MAPS was identified by electrospray MS: Calcd (MH+), 14,244, Obs 14,245 and Loop5 /C-MAPS was identified by MALDI MS: Calcd (MH+), 14,228, Obs 14,223.

Vaccine preparation and immunization. Lyophilized immunogen Linear 5/C-MAPS core or Loop 5/C-MAPS core (200 ug, 100 ug or 50 ug) were dissolved in 0.5 mL of deionized water and added to 0.5 mL of 2x RIBI's emulsion (0.5 mg MPL, 0.5 mg TDM, 0.5 mg CWS dissolved in 1.0 mL of PBS buffer) the emulsion was then vigorously mixed for 3 min. The rabbits were immunized with 1 mL of emulsion in multiple sites (SC, IP, ID, IM) as recommended by RIBI ImmunoChem Research Inc. on days 0 (200 μg), 28 (100 μg), 71 (50 μg) and 225 (50 μg for Linear 5/C-MAPS core and 17 μg for Loop 5/C-MAPScore). The rabbits were bled on days 0, 28, 38, 44, 71, 82, 89, 225 and 235. The serum was separated by centrifugation and stored at -80 °C in small aliquots.

Sera titration. Peptides containing Cys at the C-terminal were conjugated to Biotin-BMCC (Pierce) following the manufacture's protocol. Peptide-biotin (10ng/100μL PBS) was added to the ELISA wells coated with neutrAvidin and incubated for two hours at room temperature. Unbound peptide was removed by washing four times with distilled water. Sera was diluted with a solution of 1% BSA/0.05% Tween 20/PBS, added to the plate in a serial dilution and incubated for 1 hour at 37°C. After washing 10 times with distilled water, the bound antibody was determined with alkaline phosphatase-conjugated goat antirabbit IgG 100 μL/well of 1/500 dilution in 1% BSA/0.05% Tween 20/PBS. After incubation at 37° for one hour and ten times wash with distilled water, the color was developed by adding 100μL of enzyme substrate 1 mg/mL. OD was determined at 415 nm (SpectraMAX 250, Molecular Devices). Sera were also titrated with gp120 MN using

high binding ELISA wells (polystyrene 1/2 area, Costar) coated overnight with sheep Mab D7324 (0.5 μg/well in 50 uL PBS) at 4°C.

Competition assay. High binding ELISA wells (polystyrene 1/2 area, Costar) were coated overnight at 4°C with sheep MAb D7324 (0.5 μg/well in 50 μL PBS). After removal of unbound antibody by washing four times with distilled water, gp120 MN (rgp120 MN HIV-1, ImmunoDiagnostics Inc.) was added (0.025 μg/well in 50 μL of 1% BSA/0.05% Tween 20/PBS) and incubated at room temperature for two hours. Unbound gp120 was washed four times with distilled water. Rabbit antiserum or MAb 58.2 were premixed with decreasing concentrations of competing peptide and transferred to the wells (50 μL/well in 1% BSA/0.05% Tween 20/PBS). Each peptide was run in duplicate. After incubation at 37°C for one hour, the unbound antibody was removed by washing 10 times with distilled water. Quantification of the bound antibody was carried out with alkaline phosphatase-conjugated goat antirabbit IgG as described above. Background was subtracted and the values from duplicate wells were averaged.

Neutralization assays. Neutralization assays were carried out in the laboratory of Dr. Paul Parren at TSRI by Dr. Meng Wang. Assays were carried out in 96 well tissue culture plates (Costar). In a typical assay, 100 $TCID_{50}$ HIV (MN) was mixed with serially. diluted rabbit antisera or MAb and incubated for 1 hr at 37 ^{0}C. Then 100 ul was added to H9 cells in each well and incubated for 4 hr at 37 ^{0}C. After washing three times with corresponding culture medium and bringing the reaction to 100 ul with the same, incubation was continued for 7 days at 37 ^{0}C under 5% CO_2. Then 90 ul from each well was mixed with 10 ul 10% empigin and assayed for p24 in an ELISA.

For JR-CSF, 100 $TCID_{50}$ JR-CSF was mixed with serially diluted rabbit antisera or MAb to give 100 ul and incubated for 1 hr at 37 ^{0}C. Then 5 x 10^4 PHA-PBMC was added to each well and incubation continued for 4 hr at 37 ^{0}C. After washing three times with corresponding culture medium and bringing the reaction to 100 ul with the same, incubation was continued for 7-14 days at 37 ^{0}C under 5% CO_2. The time period was determined by the cell growth rate.

Neutralization titers of HIV-1 (MN/JR-CSF) are for the lowest dilution of antiserum or concentration of MAb that completely blocked p24 synthesis; these titers are reported at IC_{90} values.

Several controls were run with each assay, including no additions, the addition of pre-immunization rabbit antiserum from R27, the addition of Loop 5 (10^{-5} M) to test for antibody specific neutralization and the addition of IgG b12 (positive control). Neutralization of HIV-1 (MN) by R27 and MAb 58.2 was reproducible and passed all of the control tests. Neutralization of JR-CSF by MAb 58.2 was clearly evident in one test that passed all controls.

Results

Synthesis. The linear and cyclic peptides used in this study are listed in Table 1. The linear peptides were synthesized by standard Fmoc synthesis. The cyclic peptides were synthesized by solid-phase peptide synthesis employing the modified amino acids, **J** and **Z. J** and **Z** combine in a chemical reaction on the solid support to form a hydrazone link which serves functionally as a hydrogen bond mimic (Fig. 1). The syntheses are described in detail in the experimental section. These syntheses were straight forward and the final yields of purified loop were quite good for medium-sized peptide loops (11% for Loop 5). The most troublesome problem involved the coupling of Fmoc-Arg(Pfb) to Aib, a sterically hindered amino acid, which did not go to completion even after triple coupling. Thus each preparation of crude peptide contained significant amounts of the Arg deletion peptide. The Arg deletion peptide shares very similar chromatography properties with the Arg peptide which required careful optimization of the HPLC gradient for separation.

V3 loop mimetic. Preliminary studies identified loop peptides (Loop 4, 5) that bound about 200-fold better than the V3 linear peptide to MAb 58.2.[14b,15] Peptides used in the earlier study may have been contaminated with Arg deletion peptides. When the Arg deletion peptides were removed by careful purification, the mass spectra improved (Table 2, no evidence for Arg deletion peptide) and affinities improved (Table 1). Loop 5 showed the highest affinity. Consequently, Loop 5 and its corresponding linear peptide, Linear 5, were selected for vaccine tests in rabbits.

Conformational scanning. Linear 1-5 and Loops 1-6 (Table 1) were used to initially define the conformational preferences of MAb 58.2. Later, when rabbit anti-peptide sera became available, the same set of peptides was used to test the conformational preferences of the rabbit antibodies for comparisons with MAb 58.2. For these scans, the size of the peptide, its amino acid composition and the linker were varied. These peptides

were used in competition ELISAs to determine the relative affinities of paired sets of linear and constrained peptides for MAb 58.2 (Fig. 4, Table 3). Paired sets were required for distinguishing between the effects of amino acid substitutions and cyclization. Competition was between peptide in solution and baculovirus rgp120 (MN) on the solid surface, the same conditions used for characterizing antisera. For these experiments, baculovirus rgp120 was captured on the solid surface by adsorbed sheep polyclonal antibody which binds the C-terminal end of rgp120 and is not expected to disrupt the conformation of rgp120.

Loop 1 and 2 differ in size. The smaller Loop 1 showed lower affinity for MAb 58.2. This could be due to differences in the conformation at the "tip" and/or the constraint of the smaller loop. The larger Loops 2-5 provided a series that differ in amino acid composition. The amino acid substitutions were designed to conformationally perturb different parts of the loop, [**JHIGPXRXFXGZ**]G-NH2, at the X positions. It was intended to probe conformational preferences at different regions of the loop. However, it is quite possible that a change in conformation at one position in the loop could effect the overall conformation of the loop. A D-Ala substitution for Gly (Lin3, Loop3), normally favors a Type II turn.[26] Since MAb 58.2 binds a Type I turn,[12] a reduction in affinity is predicted. A large decrease is observed in the affinity of Loop 3 (0.5) compared to Loop 2 (167). This D-Alanine substitution leads to a significantly smaller reduction in the affinity of Linear 3 (0.3) relative to Linear 2 (1.5). This may indicate that Loop 3 locks in a Type II turn better than Linear 1. It is unlikely that the lower affinities of the D-Ala peptides is due to steric interference since an examination of the crystal structure of MAb 58.2 bound to peptides indicates that the position occupied by the D-alanine methyl group is solvent exposed. Thus it is likely that Loop 3 provides a probe for a conformational preference of V3 antibodies for a Type I turn. The substitution of Aib (Loop 4) should improve the stability of the second turn at RAFG which should improve affinity as is observed. The improvement in affinity is unlikely due to additional contact of the MAb with the extra methyl group on Aib since a crystal structure of MAb 58.2 with an Aib-peptide shows no additional contact. The substitution of Phe-(D-Ala) for Phe-Gly (Tyr in V3 sequence) (Loop 5) improves affinity further. D-Ala is not anticipated to contribute directly to affinity since it replaces Gly/Tyr which are not bound by MAb 58.2 in crystal structures. The affinity of Loop 5 is 1111-fold greater than Linear 2 which corresponds to the native V3 sequence. A disulfide loop (Loop 6) was prepared to determine whether a change in linkers would affect binding. As it turns out, the disulfide loop does not bind as

well as the hydrazone loop to MAb 58.2 indicating that the hydrazone loop forms the more complementary conformation. The range in affinity of the peptide set (Table 3) for MAb 58.2 is considerable, amounting to about 1,600-fold (Table 3, Fig. 4). Regardless of the reasons for affinity differences which can not be assessed with complete certainty, the peptide series provides an excellent way to probe and distinguish the conformational preferences of V3 antibodies.

Vaccine synthesis. In order to investigate whether Loop 5 elicits antibodies that bind gp120 (MN) and neutralizes HIV-1, Loop5/Cys was chemically ligated to a "universal" tetanus toxoid T-helper cell epitope in a four-branched MAPS core (Fig. 2). In order to measure the effect of structure on immunogenicity, a corresponding Linear 5/Cys - MAPS was also prepared. The MAPS core was difficult to obtain in pure form. This large peptide tends to aggregate and stick to HPLC columns. This reduces the yield of MAPS core considerably. Nonetheless, sufficient quantities could be obtained in nearly pure form for ligation to the linear and V3 peptides. The coupling reaction is carried out with an excess of V3 peptide in guanidinium chloride to insure complete use of the MAPS core. Following purification, 50% of the MAPS core is recovered as vaccine. Both Linear and Loop MAPS gave exact masses (Fig. 3) indicating a considerable degree of purity. These protein sized molecules are very soluble in water which makes them ideal for immunizations.

Immunological studies. Two groups of three female rabbits were immunized four times over the course of 8 months with Loop5/C-MAPScore or Lin5/C-MAPScore in RIBI's adjuvant. The dose was decreased from 200 ug/rabbit in the first immunization to 17μg loop, 50 μg linear/rabbit in the fourth immunization. The development of IgG titers was monitored by ELISA. High titers were observed on the tenth days following the third and fourth immunizations. R26 titer remained constant between the 3rd and 4th immunization while it decreased substantially for R27. The Loop-MAPS was administered at very low dose (17 μg/rabbit) for the fourth immunization. No change in titer was observed for R26 but a titer returned to its original level for R27 indicating that 17μg/rabbit is sufficient for maintaining high titer antibody.

Most of the analyses were carried out on the 10 day bleed following the third immunization. ELISA titers for these sera are reported in Fig 5. Rabbits immunized with Loop5/C-MAPScore developed antibodies that bind to gp120 (3/3) whereas the rabbits immunized with Lin5/C-MAPScore do not (0/3). Cross-reactivity was observed with Linear 5 and Loop 5 in both groups of immunized rabbits. Whereas the titer profiles were

similar for rabbits immunized with the Linear 5-MAPS, they differed considerably for rabbits immunized with the Loop 5-MAPS (Fig. 5). Rabbit 3326 (R26) showed high titer antibodies against both the linear and loop peptides, but moderate titer against rgp120. R27 showed moderate titer against the linear and loop peptides but higher titer against rgp120. R28 also showed moderate titers against the linear and loop peptides but low titer against rgp120.

The antisera were further evaluated in competition ELISA assays. In an initial screen, Linear 5 and Loop 5 alone were used to screen each of the antisera (Fig. 6). Since the R23-R25 anti-linear peptide antibodies did not bind rgp120, a C-terminal biotinylated Linear 5 peptide was adsorbed to neutrAvidin plates (Pierce) for competition ELISAs. Each of the R23-R25 anti-linear peptide sera bind the Linear 5 peptide with higher affinity than the Loop 5 (Fig. 6). A significant fraction of the R23 and R24 antibodies are capable of binding the loop while R25 shows a greater preference for linear peptide. The observation that the anti-linear sera binds Loop 5 is important since it indicates that there is a sufficient population of antibody present that is potentially capable of binding the V3 loop in rgp120. However, as noted, no binding of rgp120 binding by this sera was observed. Another feature of the anti-linear peptide sera is the shape of the competition curves which are flat for R23 and R24. Flat curves are predicted for a heterogeneous population of antibody while sigmoidal curves reflect either a narrower population of antibody or a population of antibodies with similar affinities.

Each of the rgp120 (MN) specific antibodies show high affinity for Loop 5 ($<10^{-9}$ M^{-1}, Fig. 6). The affinity is at least 10-fold higher than that shown by MAb 58.2 for the same loop ($>10^{-8}$ M^{-1}, Fig. 4). The R27 antibodies show the greatest similarity to MAb 58.2. R27 binds Loop 5 about 500 x better than Linear 5. In contrast, R26 binds Loop 5 and Linear 5 with very similar affinity. R28 rgp120 specific antibodies which form at lower titer also show a preference for Loop 5. The competition curves for the anti-loop antibodies are sigmoidal which indicate a narrower population of antibody or antibodies with very similar affinity.

The conformational preferences of R26 and R27 were compared with MAb 58.2 using full conformational scans (Table 3). Since the competition curves were approximately sigmoidal, the 50% competition point was used to compare relative affinities. R27 comes closest to duplicating the conformational preferences of MAb 58.2. The largest difference occurs for Loop 3. Whereas a substitution of D-Ala for Gly in Loop 3 decreases affinity

>300-fold for MAb 58.2, it reduces affinity only about 5-fold for R27. Thus it is possible that R27 antibodies bind GPGR in a different conformation that MAb 58.2.

The R26 antiserum shows a different set of preferences compared to R27 and MAb 58.2. The biggest difference is a strong preference for Aib containing peptides whether or not the peptide is cyclized. Aib is α,α-dimethylglycine i.e. an alanine with one more methyl group on the central carbon. The addition of this extra methyl group leads to 200-400 fold increase in the affinity of R26 antibodies for linear and cyclic peptides indicating that Aib is a core amino acid. The competition curves for R26 antibodies (Fig. 6) indicates that all of the rgp120 specific R26 antibody has a strong preference for the extra methyl group. This suggests a very narrow population of antibody in this rabbit. A heterogeneous population of antibody would not be expected to show the same strong uniform preference for this minor change in peptide composition.

The distinctly different preferences of the different anti-loop sera (Fig. 6, Table 3) suggest that each rabbit has developed a highly specific and individualized response to the loop. This is not expected of a polyclonal response where differences would be expected to average out. Instead the profiles indicate a zmuch narrower clonal response to the immunogen.

Neutralization Assays. Each of the antisera were tested for neutralization of HIV-1 (MN) (Table 4). Neutralization assays were carried out by Drs. Meng Wang and Paul Parren at TSRI (Scripps). Several controls were run concurrently with the neutralization assays. These controls included the use of pre-immunization rabbit serum and the Loop 5 peptide. Loop 5 was added to the assay to test for antibody specific neutralization. Positive controls included MAb 58.2 and IgG b12. MAb 58.2 neutralized HIV-1 MN at 1 μg/ml (IC_{90}) but not at 0.1μg/ml. This agrees with White-Scharf et al.[3] who reported IC_{90} = 0.39 μg/ml. R27 antiserum neutralized HIV-1 (MN) whereas the other sera did not. R27 neutralizing titer increased from 128-256 (IC_{90}, 10d bleed post 3rd immunization) to >512 (IC_{90}, 10d bleed post 3rd immunization). Loop 5 peptide completely blocked neutralization by MAb 58.2 and R27 antiserum. Although R26 antiserum binds rgp120 with the similar titer as R27, the R26 antiserum does not neutralize HIV-1 at 1/16 dilution. Thus HIV-1 (MN) neutralization correlates with a "58.2"-like conformational preference rather than titer to peptides and even titer to rgp120 (MN).

The potency of MAb 58.2 and R27 antibodies can be compared (Table 4). Since the concentration of rgp120 specific antibody in R27 antiserum is difficult to determine, titer

serves as a proxy. The potency of MAb 58.2 can then be expressed as rgp120 titer equivalents required for neutralization. Since neutralization was determined with 1/10 dilution of MAb 58.2, the potency ratio for MAb 58.2 is broad, between 5.8-58 rgp120 titer units/neutralization unit. A comparable ratio for the R27 antisera (10 day post third immunization bleed) based on 1/2 dilution is narrower, 47-94 (Table 4). This ratio decreases at least two-fold, to <23 for the fourth immunization bleed. Thus R27 antisera and MAb 58.2 share similar potencies against HIV-1 (MN).

Both MAb 58.2 and R27 antiserum were tested for neutralization of JR-CSF, a cloned macrophage-tropic primary isolate. MAb 58.2 neutralized JR-CSF at IC_{90} = 20 μg/ml. This neutralization was blocked by Loop 5 peptide (10^{-5} M) indicating that neutralization is antibody specific. Peptide alone did not block infection. Since the MAb 58.2 had aged for some time which reduced its titer against rgp120 by at least 2-fold, the potency of MAb 58.2 against JR-CSF could be as low as 10 μg/ml.

It takes approximately 20x more MAb 58.2 to neutralize JR-CSF as it does to neutralize MN which is in agreement with other results for V3 MAbs i.e. weaker but definite neutralization of cloned primary isolates. Assuming 20x more antibody is needed to neutralize JR-CSF than MN, 1/25 dilution of R27 antiserum should neutralize JR-CSF. In one test, R27 antisera (10 day bleed following the fourth immunization) neutralized JR-CSF at 1/16 dilution. However, this was not confirmed in a second test. Further tests will be required with concentrated R27 antibody against JR-CSF. Alternatively, a further boost of R27 might produce a more potent antisera.

Discussion

The immunized rabbits show striking differences in their antibody responses to unfolded (linear) peptides and folded (cyclic peptides)(Fig. 5, 6). This provides strong evidence that the cyclic hydrazone linked peptides survive the lengthy exposure to physiological conditions to stimulate an immune response. It validates the immunization protocol as appropriate for studies of constrained peptides. The current work further validates the general approach to improving immune responses to peptides. This includes utilizing a neutralizing MAb to identify constrained peptide mimetics of a conformational epitope, incorporation of these mimetics into MAPS outfitted with the "universal" tetanus toxoid (TT2) T-cell epitope and inoculation with decreasing doses of peptide in RIBIS adjuvant. The results provide an unprecedented look at the effect of conformation on the

antigenicity (antibody affinity) and immunogenicity (antibody induction) of a synthetic peptide. They validate Loop 5 as a V3-loop mimetic for use in any AIDS vaccine that includes the V3 epitope.

Antigenicity. Many studies show that antibodies bind peptides. Since peptides are conformationally heterogeneous in aqueous solution, this observation leaves the impression that conformation is irrelevant to the affinity of a peptide for an antibody. However, individual antibodies bind individual peptides in specific conformations determined by the shapes of the antibody binding pockets. In order for a peptide to bind an antibody, it must adapt the particular conformation that is eventually bound by the antibody. The folding of a peptide leads to a free energy cost for binding that results in lower affinity. Anfinsen estimated this cost at up to 10^4-fold.[27] The observation that Loop 5 binds MAb 58.2 about 10^3-fold tighter than the Linear 5 peptide (Fig. 4, Table 3) is in line with Anfinsen's estimates.

It is very likely that the affinity of most peptides for anti-protein antibodies can be improved by constraining them to native conformations. It is possible that the gains in affinity could be even greater that the 10^3-fold increase achieved for the cyclic V3 peptide. The linear V3 peptide is an unusual case since a fraction of this peptide forms a reverse turn at GPGR in water.[28] Thus the V3 peptide has a conformational advantage which may explain why it is often observed to bind human antibodies.

Since conformation can have a major effect on affinity, the question that can and should be asked is to what extent are antiprotein antibodies detectable with linear peptides. Most peptides that bind antiprotein antibodies show, as does the V3 peptide, a small degree of structure in water.[29] Yet the large majority of peptides show no evidence of even partial folding. Thus antiprotein antibodies detected with peptides may simply reflect the few peptides that can form a degree of structure in water. The V3 epitope was initially termed an immunodominant epitope because antibodies to this epitope could be identified with peptides. However, this epitope is probably better termed "immunodetectable" because the V3 peptide is partially folded which improves its affinity for antibodies. Conversely, many antiprotein antibodies may go undetected with peptides because the great majority of peptides are unfolded and can be expected to have low affinities for antibodies to native protein epitopes. We and others have documented three instances where non-antigenic linear peptides can be converted to antigenic peptides by folding them with the hydrazone link.[30] It is very possible that this

phenomenon is the norm rather than the exception. It is likely that many more antibodies can be detected with folded peptides than can be detected with unfolded peptides.

Immunogenicity. Despite many studies on the V3 loop, several important questions needed further clarification. Since anti-V3 peptide MAbs bind rgp120 and neutralize HIV-1, it is not clear whether conformation is a determinant of neutralizing activity. V3 MAbs, however, show a wide range of activity that has not been explained. MAb 58.2 emerged from an evaluation of approximately 3,800 MAbs directed to the V3 loop peptide.[3] Only 50 of these MAbs neutralized HIV-1 (MN).[3] MAb 58.2 was one of two MAbs with the highest potency. VanCott et al.[10] established a 100-fold range of neutralizing potencies for V3 MAbs against HIV-1 (MN) that correlated with off-rates from rgp120 (MN). Although differences in affinity and neutralization potencies can be attributed to conformational preferences, other factors could be involved.

The results presented here, however, clearly demonstrate that the V3 epitope is conformational and that the binding pockets of antibodies must complement this conformation for neutralization. Thus R23-R25 antibodies raised against the Linear 5 peptide, bind both Linear 5 and Loop 5 peptides but do not bind rgp120 MN (Fig. 5). Since the V3 epitope is exposed, and the R23-R25 anti-linear peptide antibodies are capable of binding Loop 5 (Fig. 5, 6), their inability to bind rgp120 MN (Fig. 5) strongly implies that the native gp120 MN protein restricts the V3 loop conformation to a degree that it can not readily adapt to the binding pockets of the R23-R25 anti-linear peptide antibodies. The fact that R23-R25 anti-linear peptide antibodies bind Loop 5 (Fig 5,6) very likely reflects more conformational heterogeneity in Loop 5 than in the rgp120 MN displayed V3 loop. This further implies that the native V3 loop is a stable, well-defined 3-dimensional structure.

In contrast, R26-R28 antibodies raised against Loop 5 bind rgp120 (Fig. 5). This leads to the conclusion that the binding pockets of antibodies formed against the loop complement the native conformation. This implies that Loop 5 used to raise the antibodies adapts a conformation that mimics the conformation of V3 epitope far better than the corresponding linear peptide. It further implies that Loop 5 maintained a critical conformation in RIBIS adjuvant at physiological temperature and withstood the long exposure to physiological conditions required for eliciting an immune response. Although R28 shows lower titer against rgp120 than R26 and R27, it is unlikely that this is due to physical destruction of the loop since good responses are observed in the other rabbits.

Another reason for this lowered titer against rgp120 (but not against peptide) is discussed below.

Since R26 and R27 antisera both bind rgp120 with similar titers, but only R27 antisera neutralizes HIV-1 (MN), both these sera and others were conformationally scanned in an attempt to detect differences in conformational preferences that might account for the difference in neutralization potencies of the sera. As indicated above, R27 antisera shows a conformational preference that is "MAb 58.2-like", whereas the R26 sera shows a different preference. From the competition ELISAs (Table 3), it is clear that R26 binds Aib peptides with about 200-400 x greater affinity than Ala peptides. Thus the Aib residue is a core amino acid for **all** rgp120 binding R26 antibodies. In order to compare, the conformational preference of R26 antibodies with R27 and MAb 58.2, the affinities of these antibodies for Ala peptides can be compared (Table 3, boxed data). Although the antibodies show quantitative differences in their preferences for the different Ala peptides, the order of preference does not differ suggesting that R26, R27 and MAb 58.2 have similar conformational preferences. This conformational preference can then explain why all three antibodies bind rgp120. However, R26 antibodies show a 200-400 fold lower affinity for Ala peptides compared with Aib peptides. The affinity of R26 antibodies for rgp120 may be similarly reduced. Since HIV-1 neutralization correlates with the off-rate of the antibody from gp120,[10] lower affinity could account for the lack of neutralization by R26. The vaccine might be improved by simply eliminating Aib from the loop. R27 antisera shows no apparent preference for Aib peptides (Table 3) indicating that it does not play a particularly important role in determining an effective conformational preference.

R28 shows lower titer against rpg120 than R26 and R27. On the other hand, it shows the same titer against linear and loop peptides as does R27. The lack of anti gp120 antibodies is thus not due to a poor immune response against Loop 5 but is due to a difference in the type of antibodies that are formed. It is unlikely that lower R28 titer against gp120 is due to destruction of Loop 5 in R28, since Loop 5 survived in R26 and R27 to stimulate a gp120 titer. Instead, it may reflect a very individualized response by different rabbits to Loop 5.

Conformation of the V3 epitope. Although the R26 and R27 antiserum share a similar conformational preference with MAb 58.2, there is a difference which may reflect a difference between the Loop 5 solution structure and the MAb 58.2 bound V3 peptide conformation. While the affinity of MAb 58.2 for Loop 3 decreases >300-fold relative to

Loop 2, it decreases only about 5-fold for R27 (Table 3). Since Loop 3 may stabilize GP(DAla)R as a Type II turn which MAb 58.2 finds difficult to bind, R27 which binds Loop 3 more readily may be complementing a different turn type, possibly a Type II turn at GPGR. The NMR data for Loop 5 in aqueous solution does not lead to a clear definition of the turn type at GPGR.[15] Loop 5 could form a dynamic equilibrium of Type I and Type II turns at GPGR, but the NMR data is not conclusive and another turn might be favored. The R27 immune response should reflect a response to the solution conformation, not the MAb 58.2 bound conformation. The difference in the conformational preferences of MAb 58.2 and R27 appear to reflect the differences in the solution structure of Loop 5 and the MAb 58.2 bound peptide conformation at GPGR.

Individualized antibody responses. The R26-R28 anti-loop peptide sera show very different antigenic and immunogenic profiles from one another (Figs. 5,6). First, the R26-R28 titer profiles against different antigens are clearly different (Fig. 5). Secondly, the R26 and R27 rgp120 specific antibodies show distinctly different preferences for different peptides in competition ELISAs (Table 3). Third, R27 antiserum neutralizes HIV-1 MN while the other antisera do not.

The highly individualized immune responses of R26-R28 to Loop 5 indicates either a stochastic activation[31] of B-cells or a pauciclonal response. Pauciclonal antibody responses (pauci is derived from paucity or limited) arise from genetic restrictions on the B-cell repertoire. Pauciclonal responses have been documented to both haptens in mice[32] and *Haemophilus influenza* type b in humans.[33] Whatever the basis, there appears to be a restricted clonal response to Loop 5 that differs for different animals. The case for a narrow clonal response is best made with R26 antiserum. The gp120 R26 specific antibodies show a very strong preference for the Aib peptides. The Aib peptides differ from the Ala peptides by only one methyl group which is a very small change in the loop. However, 100% of the antibodies captured by the adsorbed gp120, show a strong preference for this methyl group. A polyclonal antibody population would be expected to show a much broader preference range.

Narrow clonal responses may be a common characteristic of antibody responses to specific epitopes.[31] It is clearly an important determinant of neutralizing activity in these rabbits. Although the rabbit sample size is small in the above experiments, it appears that the immune responses to Loop 5 are more individualized than the immune responses to Linear 5 (Fig. 5, 6). This might be expected for immune responses to a constrained peptide which presents a much more limited number of structures to respond to.

Presumably, this leads to a much more selective stimulation of B-cells - stochastic or pauciclonal. Conformational scanning reveals that R27 antiserum shares very similar characteristics with MAb 58.2. It is likely that R27 responded with a narrow population of "58.2"-like antibodies. The R27 antibodies have similar potencies to MAb 58.2. Since MAb 58.2 was the most potent of several thousand antipeptide antibodies examined by White-Scharf et al.,[3] the recapitulation of similar activity in a polyclonal response is promising.

Conclusions.

1. A V3 peptide, containing the GPGRAF "tip" neutralizing epitope on gp120 was constrained to mimic the conformation of the neutralizing epitope.
2. The constrained peptide bound about 1,000 x better to an HIV-1 neutralizing Mab 58.2 indicating the large gains in antigenicity thatcan be achieved by prefolding a peptide.
2. The constrained peptide was incorportated into a MAPS outfitted with a "universal" tetanus toxoid epitope to give a completely characterized vaccine that is suitable for tests in mice, rabbits and probably humans.
3. Rabbit antibodies to the constrained peptide but not the corresponding linear peptide bound HIV-1 (MN) rgp120. This indicates that the constrained peptide mimics the conformation of the neutralizing epitope far better than the linear peptide and further that the constrained peptide can stimulate a far better quality of antibody to the V3 epitope than the linear peptide.
4. Rabbit antibodies to the constrained peptide shared similar conformational preferences with Mab 58.2. This indicates that the constrained peptide survived the immunization process and that rabbits can be induced to form antibodies with similar conformational specificities to an HIV-1 neutralizing MAb.
5. However, each of the 3 rabbits immunized with the constrained peptide, differed in aspects of their specificities for constrained peptides, indicating clonal restriction of the immune response.
6. The rabbit that responded with fine structure and conformational specificities that most closely matched that for Mab 58.2 neutralized HIV-1 (MN) with similar potency.
7. It is not clear yet whether the best rabbit antiserum can neutralize JR-CSF, a cloned, macrophage-tropic HIV-1.

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Peptide	Sequence
Lin1	Ac-GHIGPGRAFGGG-NH2
Lin2	Ac-GHIGPGRAFGGGG-NH2
Lin3	Ac-GHIGP(dA)RAFGGGG-NH2
Lin4	Ac-GHIGPGRAibFGGGG-NH2
Lin5	Ac-GHIGPGRAibF(dA)GGG-NH2
Lin5/C	Ac-GHIGPGRAibF(dA)GGC-NH2
Loop1	[JHIGPGRAFGZ]G-NH2
Loop2	[JHIGPGRAFGGZ]G-NH2
Loop3	[JHIGP(dA)RAFGGZ]G-NH2
Loop4	[JHIGPGRAibFGGZ]G-NH2
Loop5	[JHIGPGRAibF(dA)GZ]G-NH2
Loop5/C	[JHIGPGRAibF(dA)GZ]C-NH2
Loop6	Ac-[CHIGPGRAibFGGC]-NH2
MAPS core	(Cl-Ac-QYIKANSKFIGITELKKK)4(KGG)2KA-NH2
Lin5/C-MAPS	(Lin5/C-Ac-QYIKANSKFIGITELKKK)4(KGG)2KA-NH2
Loop5/C-MAPS	(Loop5/C-Ac-QYIKANSKFIGITELKKK)4(KGG)2KA-NH2

Table 1. Peptides, cyclic peptides (in brackets), MAPS core (used for vaccine synthesis), Linear 5-MAPS and Loop 5-MAPS used in this study.

Table 2

Peptide	Sequence	MH+cal	MH+obs
Lin1	Ac-GHIGPGRAFGGG-NH2	1123.5	1125[f]
Lin2	Ac-GHIGPGRAFGGGG-NH2	1180.5	1180[e]
Lin3	Ac-GHIGP(dA)RAFGGGG-NH2	1194.6	1195[e]
Lin4	Ac-GHIGPGRAibFGGGG-NH2	1194.6	1195[e]
Lin5	Ac-GHIGPGRAibF(dA)GGG-NH2	1208.6	1209[e]
Lin5/C	Ac-GHIGPGRAibF(dA)GGC-NH2	1254.6	1255[e]
Loop1	[JHIGPGRAFGZ]G-NH2	1119.6	1120[f]
Loop2	[JHIGPGRAFGGZ]G-NH2	1176.6	1177[e]
Loop3	[JHIGP(dA)RAFGGZ]G-NH2	1190.6	1191[e]
Loop4	[JHIGPGRAibFGGZ]G-NH2	1190.6	1191[e]
Loop5	[JHIGPGRAibF(dA)GZ]G-NH2	1204.6	1205[e]
Loop5/C	[JHIGPGRAibF(dA)GZ]C-NH2	1250.6	1251[e]
Loop6	Ac-[CHIGPGRAibFGGC]-NH2	1229.6	1230[e]
MAPScore	(Cl-Ac{Tcell}KKK)4(KGG)2KA-NH2	9377	9374[e]
Lin5/C-MAPScore	Lin5/dA/C-(Ac{Tcell}KKK)4(KGG)2KA-NH2	14244	14245[e]
Loop5/C-MAPScore	Loop5/C-(Ac{Tcell}KKK)4(KGG)2KA-NH2	14228	14223[m]

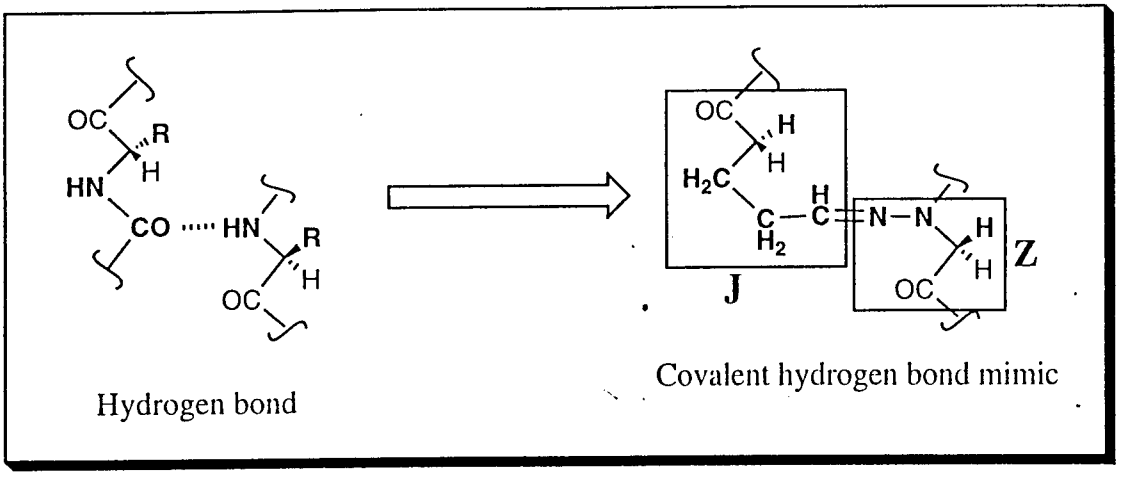
e = Electrospray
m = MALDI
f = FAB

Peptide	Sequence	Rel. Af. Mab 58.2	Rel. Af. R26	Rel. Af. R27
Lin1	Ac-GHIGPGRAFGGG-NH2	1	1	1
Lin2	Ac-GHIGPGRAFGGGG-NH2	1.5	1.2	0.6
Lin3	Ac-GHIGP(dA)RAFGGGG-NH2	0.3	0.2	0.2
Lin4	Ac-GHIGPGRAibFGGGG-NH2	13	222	1
Lin5	Ac-GHIGPGRAibF(dA)GGG-NH2	10	400	1.4
Loop1	[JHIGPGRAFGZ]G-NH2	23	9.5	6.5
Loop2	[JHIGPGRAFGGZ]G-NH2	167	20	108
Loop3	[JHIGP(dA)RAFGGZ]G-NH2	0.5	3.3	22
Loop4	[JHIGPGRAibFGGZ]G-NH2	968	370	144
Loop5	[JHIGPGRAibF(dA)GZ]G-NH2	1667	400	464
Loop6	Ac-[CHIGPGRAibFGGC]-NH2	176	167	11

Table 3. Relative affinities of Linear and Loop peptides for Mab 58.2, R26 and R27. Affinities were determined from the 50% competition point for the individual peptide (see Fig. 4 for Mab 58.2 data). Affinities are relative values, relative to data for Lin 1 in each series. The data for the Alanine peptides is boxed.

Table 4.

Titers: (reciprocal)	gp120	90%neut. HIV-1(MN)	Ratio gp120/neut
MAb 58.2, 11 mg/ml	6.4x105	$1.1x10^4$-$1.1x10^5$	5.8-58
anti-Linear			
R23	<20	<16	-
R24	<20	<16	-
R25	<20	<16	-
anti-Loop			
R26	8,000	<16	-
R27 (3rd + 10d)	12,000	128-256	47-94
R27 (4th + 10d)	12,000	>512	<23
R28	500	<16	-



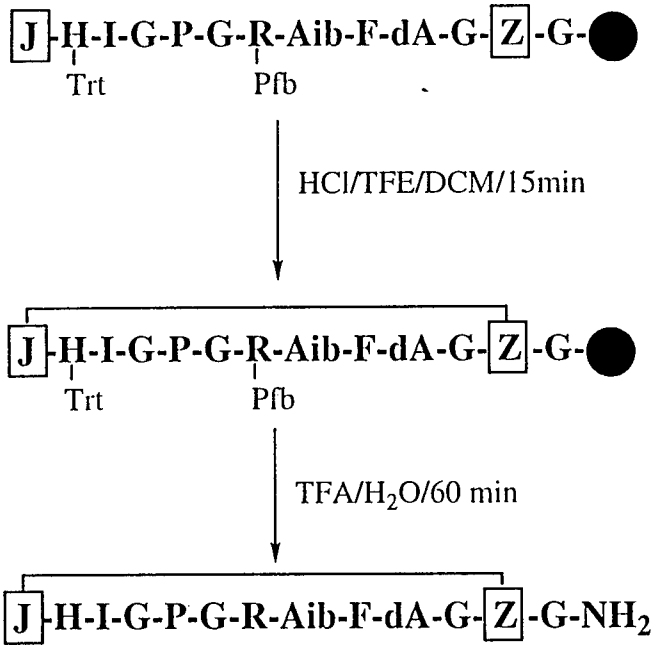
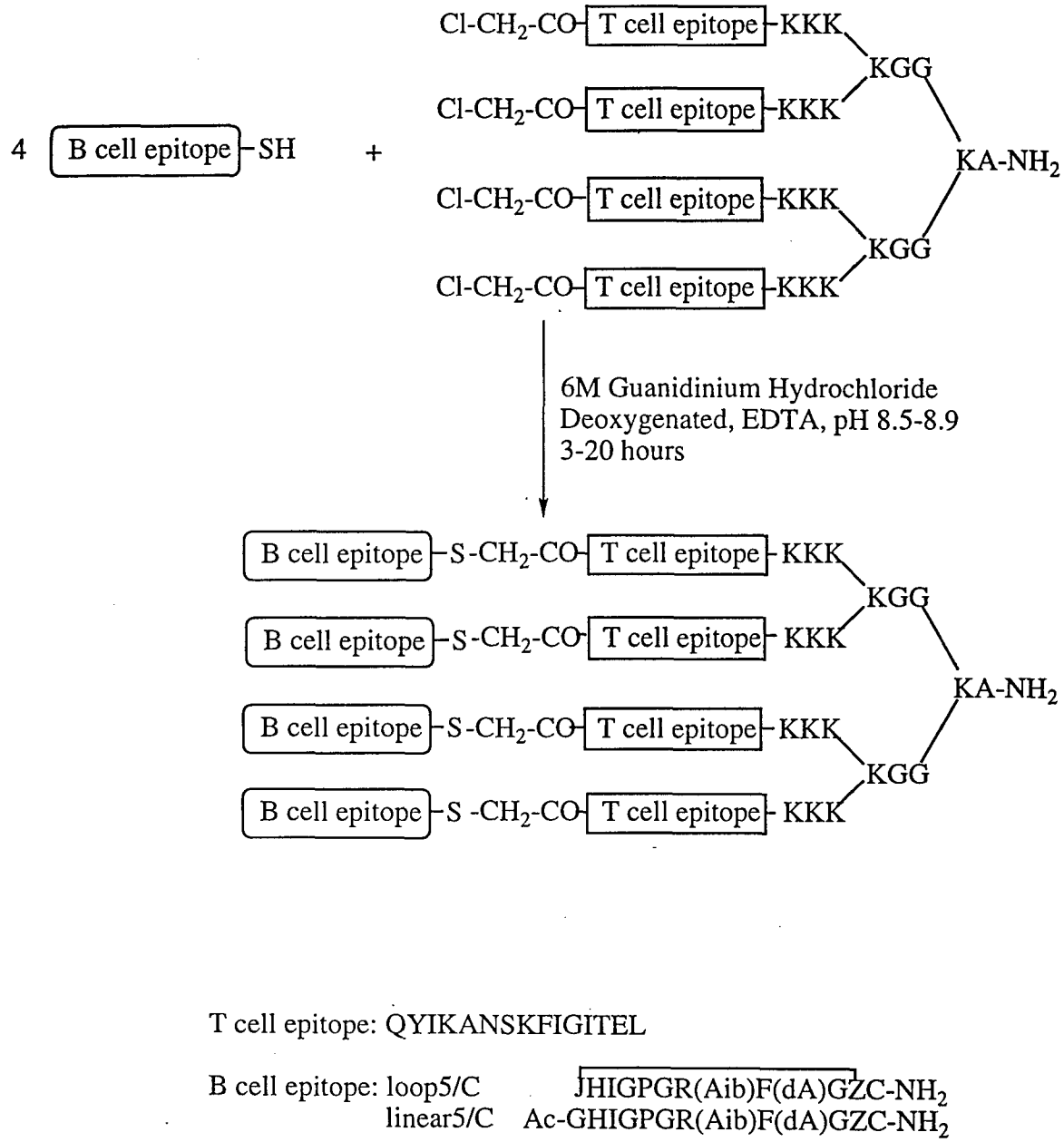


Figure 1. General scheme for the solid phase synthesis of constrained loops with a hydrogen bond mimic. Standard Fmoc chemistry is used for building up the linear sequences on the resin. Linkers **J** and **Z** enable the cyclization in the presence of acid on the solid support.



T cell epitope: QYIKANSKFIGITEL

B cell epitope: loop5/C JHIGPGR(Aib)F(dA)GZC-NH_2
linear5/C Ac-GHIGPGR(Aib)F(dA)GZC-NH_2

Figure 2. Chemical ligation of a V3 loop peptide to a universal tetanus toxoid T cell epitope to yield a four-branched multiple antigen presentation system (MAPS).

Proprietary Data

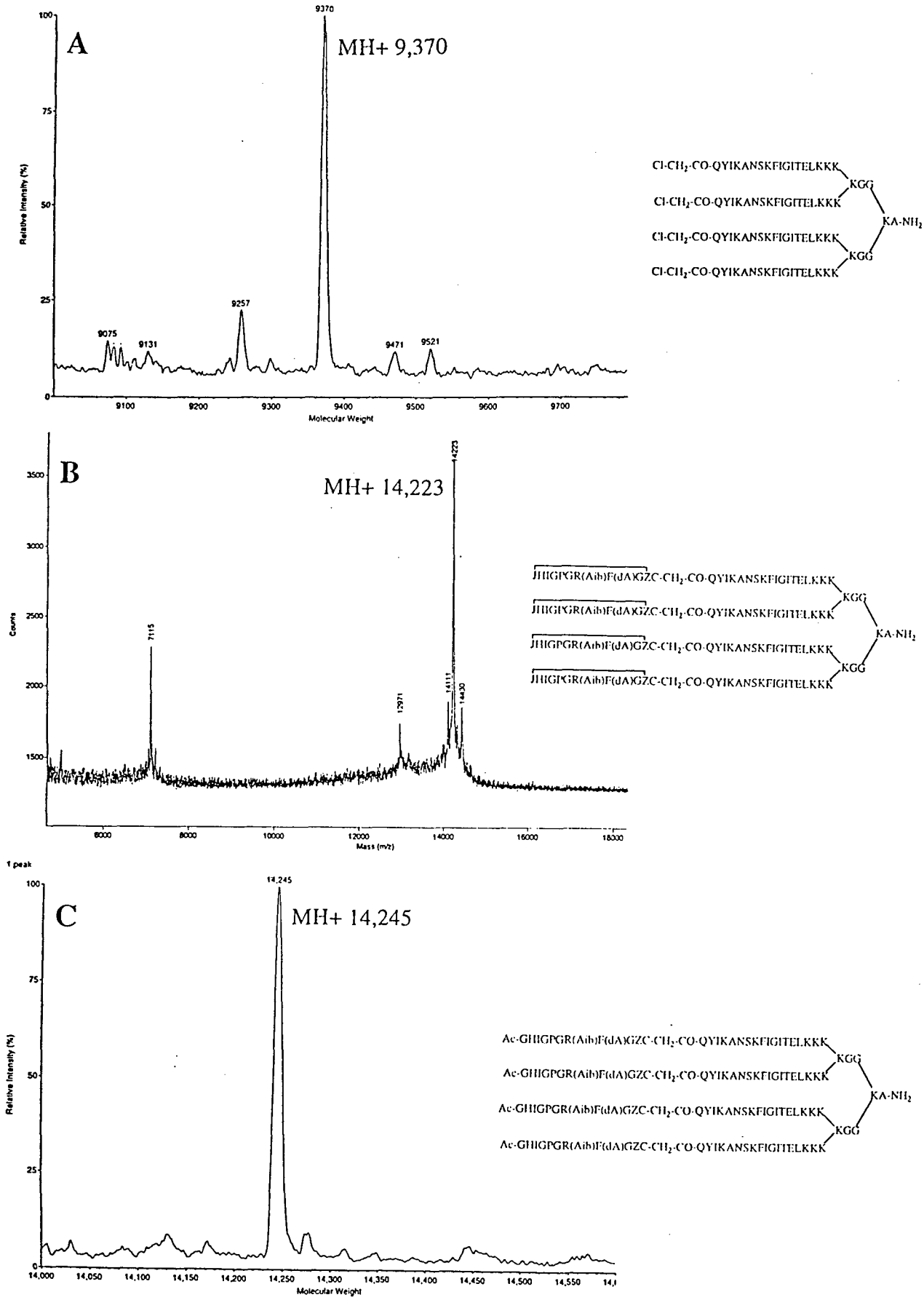


Figure 3. Characterization of MAPS core (A), Loop5/C-MAPS (B) and Linear5/C-MAPS (C) by mass spectrometry: electrospray (A and C) MALDI (B)

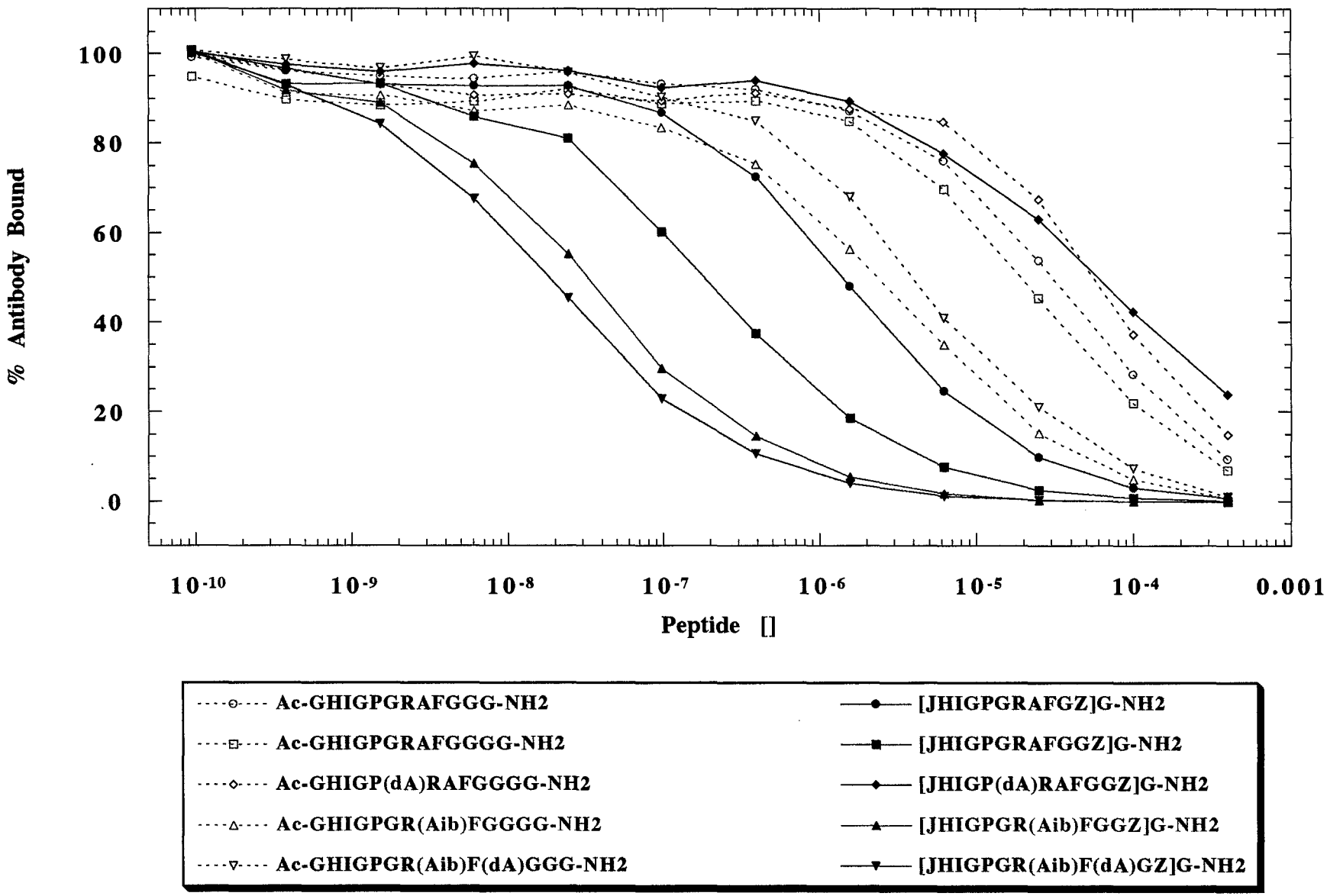


Figure 4. Competition ELISA of MAb 58.2 for gp120 MN with Linear and Loop Peptides

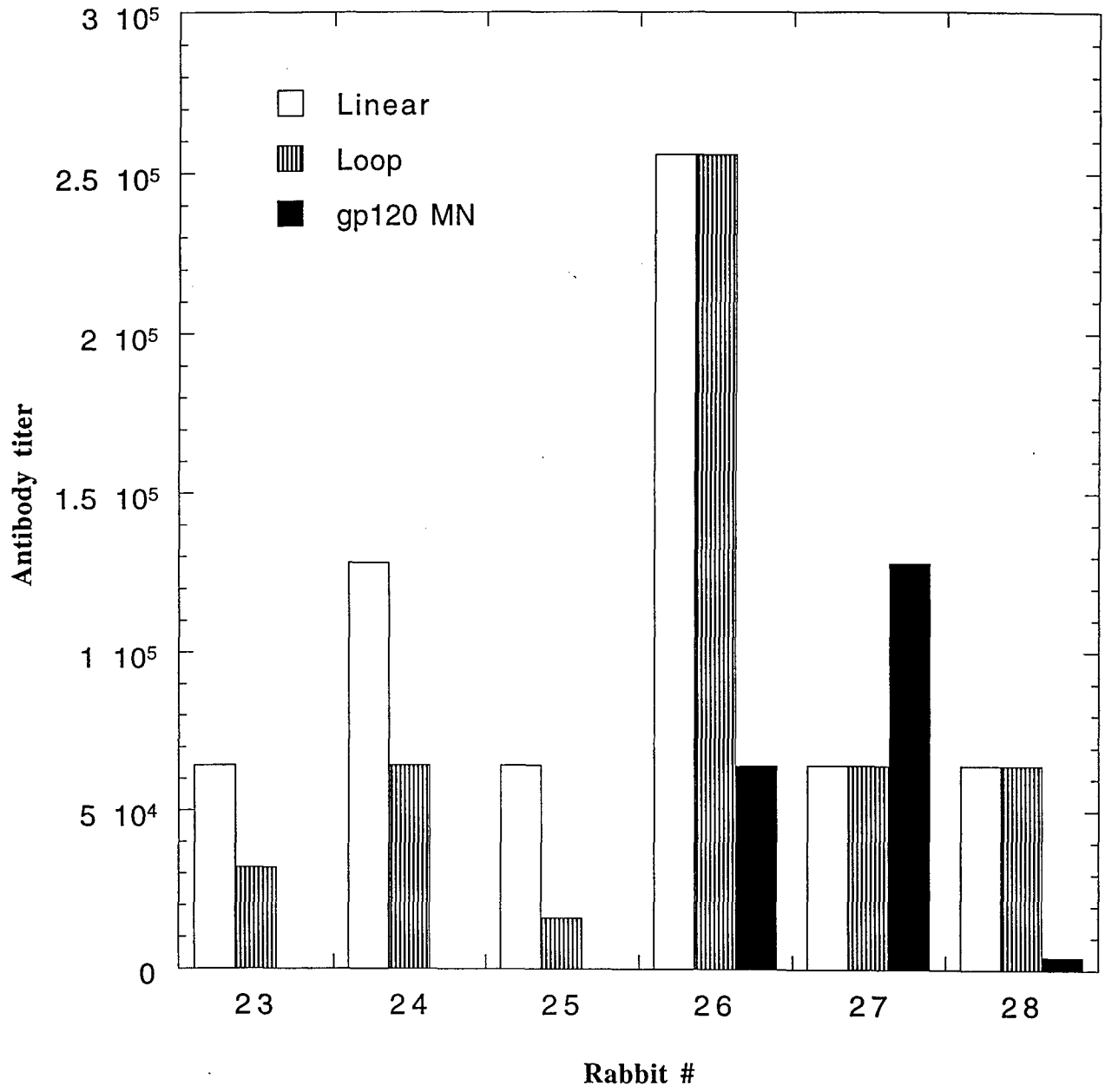


Figure 5. Antibody titers against linear5, loop5 and gp120 MN (titers were determined as the inverse of the maximal dilution giving a twice the background absorbance).

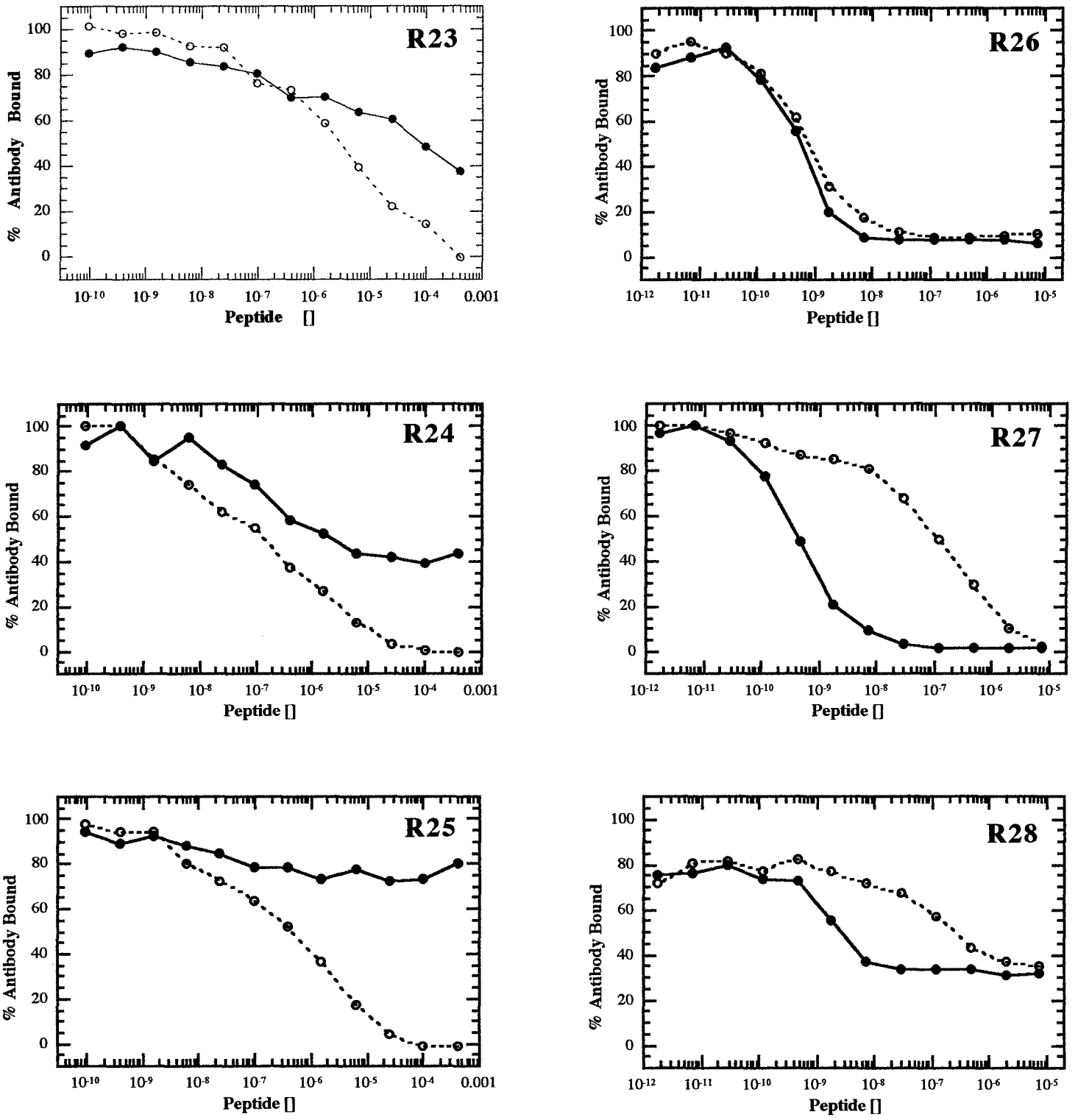


Figure 6. Competition ELISA of rabbit antisera with Linear5 (black open) and Loop5 (red closed). R23-R25 (anti-Linear5) are captured by solid phase adsorbed Linear5. R26-R28 (anti-Loop5) are captured by solid phase adsorbed gp120 MN/goat anti-C terminal gp120

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

Recd 8/16/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 9 August 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl