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Linear epitope mapping of humoral responses induced by vaccination with recombinant HIV-1 envelope protein gp160

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To enhance utility of the linear epitope mapping (Pepscan) technique for assay of humoral responses linked to vaccination, two modifications were tested. First, peptides were incubated with serum contained in baths rather than individual wells. Second, a rigorous statistical model was developed to determine which peptide/antibody-binding interactions were significant. The modifications increased the ability to detect signal in these experiments by 15- to 45-fold. These two modifications were applied to linear epitope mapping of HIV seropositive volunteers under treatment with recombinant HIV gp160 and also to rabbits immunized with the same product. Changes in fine specificity of response were observed in animal models and human vaccine recipients over the course of an immunization series with this antigen.

Keywords: HIV vaccines; HIV antibodies; gp120; gp41; Pepscan; statistical data analysis

Introduction

Vaccine development requires evaluation of assays to assess the immune responses induced by candidate immunogens, so that specific responses can be correlated with efficacy. For investigation of humoral immunity, various immunoassays ranging in specificity are available. Peptide antigens can be used to define epitopes accurately, at least for those antibodies that bind to continuous stretches of amino acids (linear epitopes) [5]. One successful application of peptides for linear epitope mapping is the Pepscan technique [11,13,28]. This technique has been used to investigate antibody reactivity to antigens from numerous pathogens including bacteria [6,7,32], parasites [10,30], fungi [19] and viruses [1,8,27].

Application of the Pepscan technique is limited by the amount of serum required for a single assay (up to several hundred microliters), rather low signal-to-noise ratio, difficulty in quantifying the responses obtained, and inability to map non-continuous (conformational) epitopes. To address these problems, we applied a technical modification of the Pepscan technique and developed an analysis method for Pepscan data. The technical modification, exposing peptides to serum in a bath format, increased signal-to-noise significantly, allowing use of serum at higher dilutions than previously possible. Antibody reactivities routinely titrated to dilutions of $1:1\times10^5$ (in HIV-infected human sera) or to $1:1\times10^6$ (in immunized rabbits). The analysis method defines statistics for distinguishing significant responses from background noise, extending utility of Pepscan for quantitation of changes in response.

These methods were applied to measure humoral responses to envelope proteins from the Human Immunodeficiency Virus (HIV-1). Infection with HIV-1 results in progressive deterioration of the immune system, eventually resulting in opportunistic infections and death for most infected patients [24,26]. Numerous products are currently being tested as possible vaccines for the prevention or treatment of HIV [2]. Linear mapping techniques have been used to describe reactivity directed against the HIV-1 envelope, gp120, and transmembrane, gp41, proteins from infected patients [14,22], and also to monitor responses directed against these proteins in vaccine studies in humans [18,23] and small animal models [16,17]. This study employed peptide libraries derived from the sequences of the HIV-1 envelope proprotein gp160 to investigate the utility of Pepscan methods in vaccine studies. Humoral responses were mapped in sera from rabbits and seropositive human volunteers immunized with candidate vaccine products containing HIV-1 envelope proteins.

Materials and methods

Synthesis

Blocks of 96 pins pre-derivatized for covalent synthesis using fmoc (9-fluoromethoxycarbonyl) chemistry were obtained from Chiron Mimotopes US (Raleigh, NC, USA). Non-cleavable supports were used. Computer-controlled hardware and appropriate software were used for generation of the peptide synthesis schedule and to guide synthesis of peptides (Craco Inc, Vienna, VA, USA) [4,29]. Peptides were synthesized using fmoc amino acids with OPfp (pentafluorophenoxy) activation (ODhbt, 3,4 dihydro-4-oxo-benzotriazine-3-oxy, derivatives for serine and

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Abbreviations used: OD, optical density; PBS, phosphate-buffered saline; RMS, root mean square; RT, room temperature.
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threonine) according to the manufacturer's instructions, with minor published modifications [3,18]. Sources for amino acids were: Novabiochem (LaJolla, CA, USA), Advanced Chemtech (Louisville, KY, USA), Bachem Bioscience (King of Prussia, PA, USA), Peninsula Laboratories (Belmont, CA, USA).

Peptides were derived from the envelope proteins of HIV-1 strains LAI and NL4-3 [20]. All peptides used in this study were 12-mers having 8 amino acid overlap; thus a given linear epitope of 4–8 amino acids was duplicated on two or three consecutive pins. In the text, peptides are named by N-terminal amino acid. Peptide sets were synthesized in duplicate, with the duplicated peptides on the same block, so that comparisons could be made between sera run simultaneously. A total of 214 peptides was required per sequence; for a typical configuration of peptides, a single dataset included peptides on five blocks. There is little variation between identical peptides synthesized on different pins [3,28]. A median coefficient of variance of 20% between data collected with such peptides was observed in this study (data not shown). Peptide 508-516, Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg, which is positive with nearly all HIV+ sera [21], was used as a positive control, and a random hydrophilic peptide, Ala-Lys-Ala-Ala-Asp-Ala-Ala-Gln-Ala-Ala-Ser-Ala, was used as a negative control.

ELISA

Two variations on the ELISA using pin-bound peptides were employed. For both methods, peptides were blocked for 1 h at RT in blocking buffer (see below), incubated with sera diluted in blocker overnight at 4°C and washed in three changes of wash solution over 15 min. They were then incubated in labeled anti-rabbit or anti-human Ig for 2 h at RT, washed and developed with the appropriate substrate, prepared according to manufacturer's instructions. Readings were taken on a microtiter plate reader using Softmax software (Molecular Devices Corp, Menlo Park, CA, USA) at 120 min. Antibodies were removed from pins by disruption immediately after use according to the manufacturer's instructions with a Blackstone (Jamestown, NY, USA) model HT-5.6 ultrasonic bath. Peptides were dried using boiling methanol in an SS-5 bath with an AZ-3000 controller (Azonic Technology Inc, San Jose, CA, USA). For the well method, pins were incubated in antibodies contained in 96-well microtiter plates as previously described [3,18]. For the bath technique, antibodies were contained in 150-ml modular reservoirs (Beckman Instruments, Fullerton, CA, USA), into which a full block of peptides could be immersed. Individual sera (for controls) were held in minitubes (Corning Glass Co, Corning, NY, USA) to separate them from the baths. The blocking buffer used to reduce high background activity observed especially with human sera was PBS containing 0.5% casein, 0.5% bovine serum albumin, 1.0% Tween 20, 2% Newborn Calf Serum, 0.2% sodium azide. Casein was dissolved by boiling in 200 ml of 0.1 N NaOH. After cooling, PBS 10× was added along with the other reagents, and the mixture was taken to pH 7.4 before diluting to the appropriate volume. Sodium azide was added as a preservative.

Test sera

Human sera were obtained with informed consent. Early-stage sera were obtained from 14 HIV-1 infected patients undergoing vaccine therapy with recombinant envelope vaccine rgp160, (NL4-3, baculovirus, MicroGeneSys) in an FDA approved phase 1 trial [25]. Sera prior to immunization were compared to those obtained after 5 years of immunization with rgp160. One control positive serum was obtained from a late-stage, HIV-1 infected volunteer. Control negative sera were obtained from seronegative laboratory workers. Sera were also obtained from rabbits immunized with HIV-1 envelope proteins in various adjuvants. Sera from rabbits immunized with rgp160 (MicroGeneSys) in complete Freund's adjuvant (FA) or muramyl dipeptide (MDP) were obtained as previously described [17]. Rabbits were immunized with the same product on alum (85 μg on days 0, 7, and 28) or encapsulated in poly(DL-lactide-co-glycolide) microspheres measuring 1–5 μm diameter (50 μg on day 0 and 30 μg on day 60) [9]. New Zealand white female rabbits were immunized subcutaneously with 200 μg of affinity purified viral gp160 (Advanced BioSciences, Kensington, MD, USA) in complete FA followed by boosts at 3 and 6 weeks with 100 μg gp160 in incomplete FA; serum analyzed in the present study was collected 8 weeks after the third immunization.

Software

Comparative epitope analysis was carried out using the 'Protean' subroutines available in DNASTAR (Lasergene, Madison, WI, USA). Standard statistical methods were applied using Statview 4.01 (Abacus, Inc, Berkeley, CA, USA).

Statistical modeling

A model was developed to characterize two properties of the sera studied: first, whether the reactivity of a test serum with any given peptide was significant compared to the expectations for controls and second, whether the reactivity of the sera from a given subject changed after immunization. The model was designed to account for two separate kinds of variation that contributed to measurement noise. First, intrinsic measurement error was characterized with repetitions on the analysis of a single serum. Variation in control reactivity was determined by repeating the analysis of a given set of peptides with multiple control sera. Second, the distribution of reactivities of a single serum to all the peptides was used to model the properties of each individual peptide, assuming that all peptide reactivities were drawn from essentially identical probability distributions.

In order to determine a cutoff for reactivity of individual peptides, the entire ensemble of responses was used to establish the background reactivity and then to determine which individual signals were significantly above background, at the desired confidence level. The measured response profile was assumed to consist of relatively few (less than 20%) significant responses distinguishable from a Gaussian noise background. The median and first quartile values were determined separately for each block of 96 peptides. The data were normalized by subtracting the

median reactivity of the set from each value in the set, and the standard deviation was calculated [15]:

standard deviation (σ) =
{(median) – (first quartile)}/0.675.

This method was used to extract the standard deviation because the positive reactivities in the data set were not censored from the calculation and variably affected determination by the more common method. The data were then divided by the calculated standard deviation and expressed as normalized reactivity (σ) compared to the median. Measurements above a cutoff of 5 σ were considered positive (see Results). This cutoff was expected to account for both measurement error and the Gaussian portion of population variation.

Results

Analysis method

Seronegative human: Seronegative was defined for the purposes of this study as not immunized with HIV antigens, and non-HIV-infected, but not necessarily unreactive. Most sera, even from seronegative donors, were reactive with one or more of the peptides used. In order to define a model for background reactivity of human sera accounting for existing reactivity, a single HIV-negative human serum was run repeatedly (n = 5) on the same peptides at a variety of dilutions (Figure 1a). These data are well modeled at low reactivities by a normal distribution, and the median and standard deviation were determined for each run (see Materials and Methods). Depending on the run, σ varied from a minimum of 0.010 up to 0.150. Figure 1b shows the results after block-by-block normalization.

The mean and standard deviation were calculated for control serum reactivity against each of the 214 peptides over five runs (Figure 2). Peptides with median reactivities below 3 σ had median standard deviation over the five runs of 0.75. This is sufficiently far from the expected value (1.0) to indicate that, in addition to measurement error, scatter included a component attributable to true reactivity. The median standard deviation rose as the reactivity increased, as expected. It was not possible to determine whether reactivities on the positive tail of the curve, with values between 3 and 5 σ (four total peptides), were truly positive with low reactivity or false positives. The two values with high reactivity, peptide 217 (13.8 σ) and peptide 721 (16.0 σ), were significant positives (discussed below).

To determine whether a given reactivity was significant or not, the base expectation for a positive reaction was calculated. The choice of cutoff was critical in this determination; this depended both on statistically determined factors and on how many repetitions of each experiment were run. With the cutoff set at 5 σ, 17 positive reactivities (median 3 per data set, range 2–5, Figure 1b) were observed out of 1070 values, for a probability of 0.0159 per peptide for a positive response. Of these, 10 resulted from reactivity of peptides 217 and 721, each of which was positive in

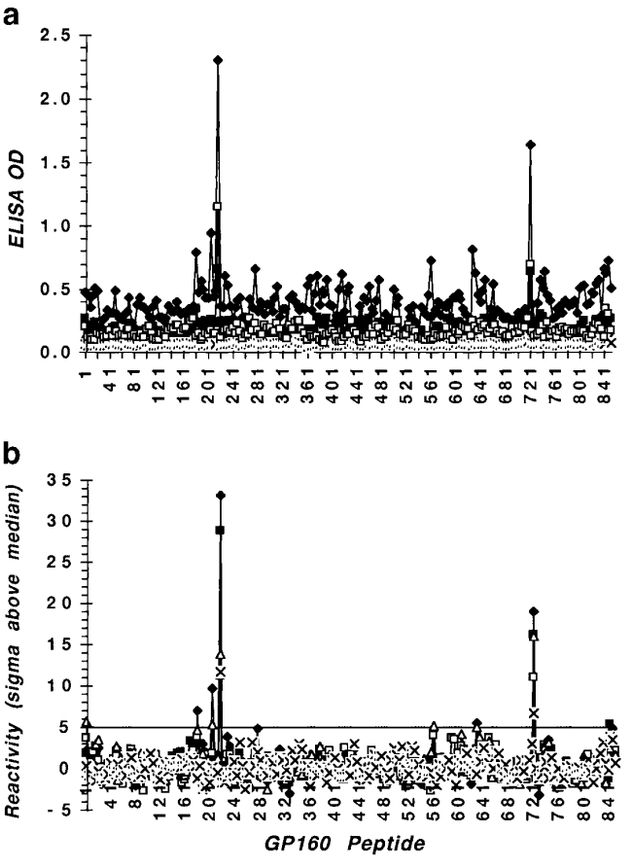

Figure 1 Pepscan experiments on sera from a single seronegative volunteer at the following dilutions: 1 : 3000 (◆); 1 : 10 000 (■), (□); 1 : 30 000 (△); 1 : 100 000 (X). (a) Raw data. (b) Conversion to reactivity (σ above median); (——) 5 σ cutoff. Connecting lines are shown for clarity and are not meant to imply continuous data.

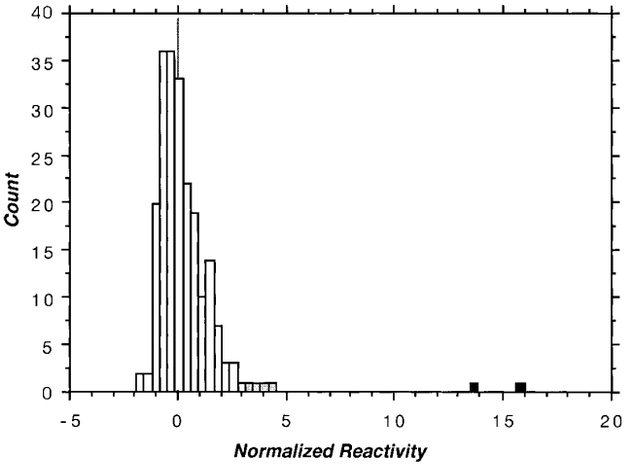

Figure 2 Frequency distribution of the signal from a seronegative volunteer. Complete combined data set after normalization; reactivity of each peptide in σ (mean of five runs). Two peptides were significant (filled) and four were positive (light shading). Vertical line represents the median (normalized to 0 σ).

all five determinations. Fitting the probability to a Poisson distribution (Table 1) showed that it was highly unlikely for any peptide to show up positive falsely five out of five times; the root-mean-square (RMS) deviation for the fit was 0.028. These reactivities were statistically significant, and so were removed from the analysis of false positives. The fit to the Poisson distribution for the five runs after the strongly positive peptides were removed was substantially better with a RMS deviation of 0.004. The calculated probability for peptide reactivity was then used to predict a false positive rate at a 5 σ cutoff. An average of three false positive reactivities was predicted for every run of 214 peptides. Repetition reduced this prediction to 0.018 duplicated falsely positive peptides, less than one occurrence in 50, assuming that the weak positives resulted from measurement error. Similar analysis with a less stringent cutoff (3.29 σ, for 99.9% confidence) yielded 32 questionably positive peptides. The fit with this cutoff yielded a calculated probability of 0.36 per peptide per run of a false positive, for an unacceptably high result of one false positive reading every three experiments, even with replication.

Examination of data from peptides with only one or two measurements above the 5 σ cutoff, however, implied the existence of an underlying distribution. These six peptides had median reactivities ranging from 1.84 to 3.94, well above the expected value of 0. Seven other peptides that never scored above 5 σ had median reactivities above 1.96. A total of 65 measurements were taken on these 13 peptides, of which only five were negative. Since the peptides were normalized to zero, the expectation was that 50% should have been negative. Some of these peptides are probably true, weak positives. Setting a high cutoff eliminated these weak positives from further analysis which was an unfortunate necessity when few replicate measurements were taken.

To estimate how well using only two replicates would handle further data, all possible pairings of two runs out of the five were made. In all cases, the two positive peptides were counted as positive (false negative rate 0%). In one case another peptide scored positive, yielding a false positive rate of one peptide in 10 runs. This method yielded an estimate of the total false positive rate including both measurement error and any non-Gaussian component of the distribution of peptide reactivity. The 5 σ cutoff was applied and two replicates were carried out on all further experiments, unless specifically noted.

Seronegative rabbit: The sera from four control rabbits were assayed to determine how frequently reactivity would be observed in seronegative subjects. Each serum was run in duplicate against the set of 214 peptides (Table 2). A median of three reactive peptides was found (range 1–5) with median reactivity 11.8 σ. This suggests that the number of positive reactivities to HIV peptides should be relatively small in the immunologically naive population. By Poisson analysis only 3% of runs are expected to have no reactive peptides, but 95% should have six or fewer. Three of these reactivities were found in two rabbits each (peptides 69, 489 and 721). The last, peptide 721, was also positive in the human negative control, strongly suggesting that reactivity to certain peptides was more likely than others in the supposedly seronegative population. Similar results were obtained when more seronegative human sera were examined (data not shown).

HIV seropositive: The serum from a late-stage HIV-positive volunteer was examined in detail (two repeats each of a titration from 1 : 3750 to 1 : 100 000, Figure 3). Peptides with all eight measurements greater than 5 σ were considered strongly positive, while those with only 1–7 measurements above the cutoff were considered weakly reactive. Nine peptides were strongly reactive, with a median reactivity of 40.5 σ (Table 3). These represent five different epitopes, assuming that consecutive peptides, such as peptide 505 and peptide 509, were both reactive due to the amino acid sequence they share. Although peptides with lower reactivity were detected above 5 σ much less frequently, at least two of these seemed physically significant. These two shared amino acids with peptides having stronger reactivities (peptide 317 was probably reacting to the same antibody population as peptide 313 and similarly peptide 501 with both peptide 505 and peptide 509).

To use this technique for monitoring responses induced by immunization, it was important to distinguish when the pattern of serum reactivity changed, either in breadth (number of reactive peptides) or intensity of existing responses. To avoid calling a reactivity 'new' when it actually represented boosting of existing reactivity, peptides reactive in post sera but also having weak reactivity in the pre-bleeds (anything greater than 3.29 σ) were called positive at both time points (ie no change in breadth). The titration showed that signals from highly reactive peaks had

Table 1 Fit to experimental Pepscan data using a Poisson distribution

Frequency of reactivity	All data		Excluding positives[a]	
	Calculated probability	Observed	Calculated probability	Observed
0 of 5	0.924	0.963	0.968	0.972
1	0.073	0.023	0.032	0.024
2	2.9×10^{-3}	4.7×10^{-3}	5.3×10^{-4}	4.7×10^{-3}
3	7.7×10^{-5}	0	5.8×10^{-6}	0
4	1.5×10^{-6}	0	4.8×10^{-8}	0
5	2.4×10^{-8}	9.4×10^{-3}	3.2×10^{-10}	0

n = Five runs of 214 peptides each.
[a]Excluding the two peptides that were positive five out of five runs.

Table 2 Reactivity of four rabbits to HIV peptides prior to any immunization

Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4	
Peptide number[a]	React.[b] (σ)	Peptide number	React. (σ)	Peptide number	React. (σ)	Peptide number	React. (σ)
		49	13.7				
69	28.1	69	27.4				
						433	10.1
		489	8.4	489	8.0		
541	35.2						
						641	10.8
721	129.6	721	8.7				
749	7.6						
		821	12.8				

[a]Peptides are 12-mers denoted by first amino acid.
[b]React., reactivity. All significant values (above 5 σ) are included.

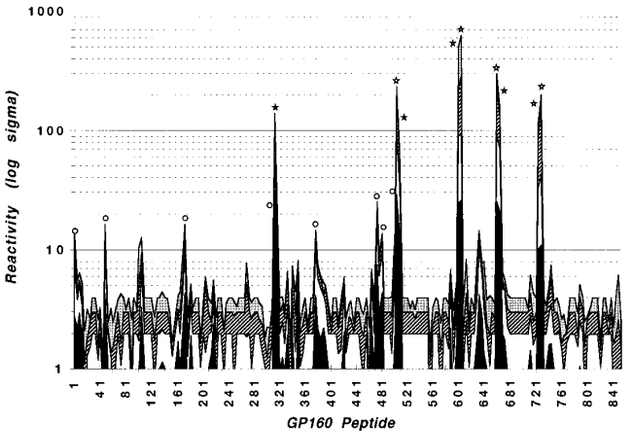

Figure 3 Pepscan experiment on serum from a late-stage HIV-positive volunteer. Dilution series including 1 : 3750; 1 : 10 000; 1 : 30 000, 1 : 100 000; as log(σ) above median. Data are shown with highest concentration at the bottom; values for lower dilutions are added successively. Strongly (☆) and weakly (○) significant signals are marked.

reproducible intensity from run-to-run. In addition, as long as a signal could be detected (the absolute absorbance was detectable by the instrument used), the associated reactivity changed relatively little over a 30-fold dilution range. Absorbance increase with higher concentration was matched by increased background.

Comparison between methods

Serum from a rabbit immunized with viral gp160 was highly reactive using both the well and the bath methods (Figure 4). This serum was tested in the dilution range from $1 : 1 \times 10^4$ to $1 : 1 \times 10^6$. The 59 reactive peptides detected using the bath method had median reactivity of 44.3 σ at 1 : 30 000 dilution; many of these reactivities (41 peptides representing 22 unrelated epitopes) were reactive past 1 : 1 million dilution. In contrast, using the well technique, 41 epitopes were detected with median reactivity of 17.8 σ at 1 : 30 000 dilution and only 17 peptides (10 different epitopes) reacted at the highest dilution. The reactivity difference over the dilution range between the two methods is illustrated for a single reactive peptide in Figure 4b. The

Table 3 Reactive peptides from a strongly seropositive serum

Strongly reacting peptides[a]		Weakly reacting peptides		
Peptide number	Median reactivity (σ)	Peptide number	Median reactivity (σ)	Percentage above cutoff[b]
313	28.9	1	3.7	25%
505	42.9	49	4.0	25%
509	14.7	173	3.5	38%
601	74.9	317	3.7	38%
605	121.9	377	4.2	50%
661	68.4	473	7.5	75%
665	40.5	481	4.1	25%
725	22.7	501	3.3	13%
729	38.9			
9 total	median 40.5 σ	8 total	median 3.8 σ	median 31%

[a]Strongly reactive peptides had all eight determinations above cutoff.
[b]Percentage (n = 8 runs) of individual runs with reactivity greater than 5 σ.

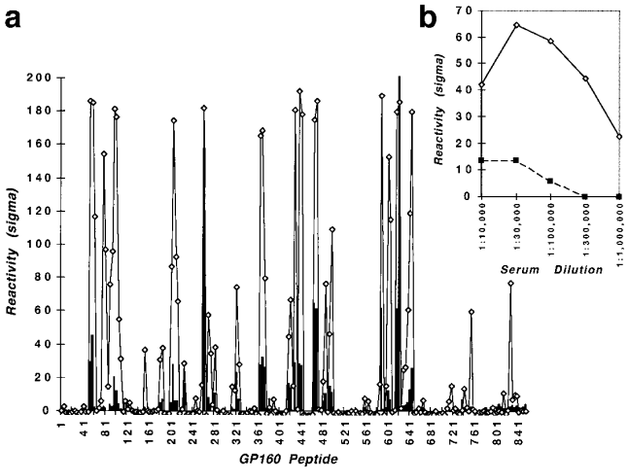

Figure 4 Pepscan experiment on serum from a rabbit immunized with viral gp160 in FA, using two methods. (a) Data at 1 : 30 000 dilution; well method (bars), and bath method (-◇-). (b) Signals determined at various dilutions using each method for the reactivity of peptide 497; well method (--■--), bath method (-◇-).

bath method shows a significant advantage for detecting reactivity at all dilutions. The reduced advantage at 1 : 10 000 dilution represents an over-estimation of σ, which is skewed by the large number of peptides detected at that dilution by the bath method (>20% of the total).

Vaccine studies: animal models

Rabbits immunized with rgp160 in various adjuvants were examined using the bath method. The Pepscan of sera from a rabbit immunized with rgp160 in FA after a three-shot priming series and after two more boosts, five shots total, is shown in Figure 5 (baseline reactivity was completely blank, not shown). After only three immunizations, there were 27 reactive peptides with a median reactivity of 24.5 σ; whereas after five shots there were 36 reactive peptides with a median reactivity of 61.9 σ. Of these, 20 were reactive in both. In seven cases reactivity decreased (median reactivity drop was 9.2-fold). On the other hand, in 16 cases reactivity was gained (median reactivity ratio of 36.4); 11 of these were new seroconversions. Similar

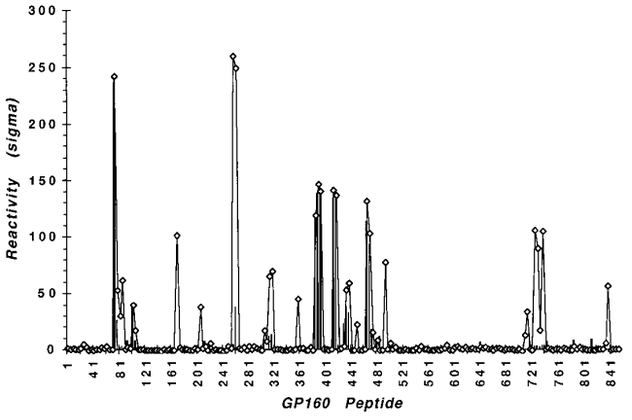

Figure 5 Pepscan of serum from a rabbit immunized with rgp160 in FA after three-immunization priming series (bar) and after two more booster immunizations, five shots total (-◇-).

results were obtained with other adjuvants (Table 4). Reactivity broadened over the course of immunization in all cases. The differences between the results obtained for the sera after boosting were significantly different from those for the sera after priming only (paired *t*-test, $P = 0.003$). This level of significance is similar to that obtained when comparing the pre-sera to that after the prime (paired *t*-test, $P = 0.0071$).

There were eight consistently reactive regions, positive with sera from greater than 80% of the rabbits, regardless of adjuvant used (Table 5). Antigenicity was compared to that predicted by various methods including hydrophilicity, antigenic index, and surface probability. All three methods predicted the epitopes at peptides 73/77, 465, 621, 717–737 and all missed the epitopes at peptides 253–261 and 317. One technique weakly predicted each of the other epitopes; peptide 101 was predicted by antigenic index and peptide 581 by surface probability.

Vaccine therapy

Reactivity of sera from 14 HIV-positive individuals enrolled in a vaccine therapy trial of rgp160 was examined using this technique. The reactivity of sera prior to aand post immunization of one of these patients who demonstrated extensive broadening after immunization is shown in Figure 6. The average pre-immunization reactivity for the cohort was 3.2 positive peptides per patient; the most common were the well-documented immunodominant in gp41 (peptides 589–601) [31] to which 12 of 14 reacted, and two others in gp41: the C terminus (peptide 849) with six of 14 reactive, and one in the cytoplasmic tail (peptides 785–797) with five of 14 reactive. After immunization, the average reactivity was 3.8 peptides per patient. The most reactive new epitope was in peptides 461 and 465 in variable region five of gp120, RDGGNNNNGSEIFRPG (amino acids common to both peptides are underlined), with four of 14 reacting; this was also a common epitope observed in the immunized rabbits. The difference between the total reactivity observed at the 5-year time point and that observed in the pre-serum was not statistically significant.

Discussion and Conclusions

The technical modification presented, incubating peptides with serum in baths rather than the standard 96-well format, allowed use of significantly less serum to acquire linear mapping data. The fact that the two techniques yielded signals differing by several orders of magnitude at the same concentration of antibody is counter-intuitive (the amount of Ab-ligand complex is defined by [AbL] = Ka[Ab][L], so the readout from the spectrophotometer would be expected to be proportional to antibody concentration). When Ab bound to the peptide on the pin, however, the antibody concentration in the bath would change to a lesser extent than it did in a well, which contained much less antibody to start. The concentration of Ab-L complex achieved with the bath method *vs* the well method is expected to be proportional to the ratio of the volumes (1000-fold higher, comparing the entire bath to a single well). The bath required about 1000-fold more serum than a single well, but could be used to incubate 90 peptides

Table 4 Evolution of humoral reactivity of rabbits immunized with rgp160 in various adjuvants

Rabbit[a]	Adjuvant	Preimmune		Primed[c]		Boosted[c]	
		Reactive peptides	Median reactivity (σ)	Reactive peptides	Median reactivity (σ)	Reactive peptides	Median reactivity (σ)
1	alum	4	31.7	12	29.5	48	57.9
2	microspheres	5	12.8	18	31.7	38	81.3
3	alum	1	8.0	44	77.2	nd	nd
4	microspheres	2	10.5	9	20.1	31	16.2
5[b]	MDP	3	10.5	20.5	31.7	38	48.8
6	CFA	0	ind	27	24.5	36	61.9

[a]Rabbit numbering corresponds to Table 2.
[b]Only one replicate.
[c]Different adjuvanting schemes call for different immunization schedules for optimal reactivity. See Materials and Methods for individual schedules.
ind = indeterminate; nd = not determined.

Table 5 Peptides consistently reactive with sera from rabbits immunized with the gp160 envelope

Peptide number(s)	Reactive sequence[b]
73,77	ACVPTDPNPQEVVLVN
101	VEQMHEDIISLW
253,257,261	THGIRPVVSTQLLLNGSLAE
317	GPGRAFVTIGKI
465	NNNNGSEIFRPG
581	LQARILAVERYL
621	NKSLEQIWNNMT
717,721,725/ 729,733,737[a]	YSPLSFQTHLPTPRGPDRPE. . . . . PRGPDRPEGIEEEGGERDRD

[a]Two or more independent epitopes.
[b]Underlined sequences indicate amino acids encompassing the specific epitope when this could be determined. One letter codes: A-Ala, C-Cys, D-Asp, E-Glu, F-Phe, G-Gly, H-His, I-Ile, K-Lys, L-Leu, M-Met, N-Asn, P-Pro, Q-Gln, R-Arg, S-Ser, T-Thr, V-Val, W-Trp, Y-Tyr.

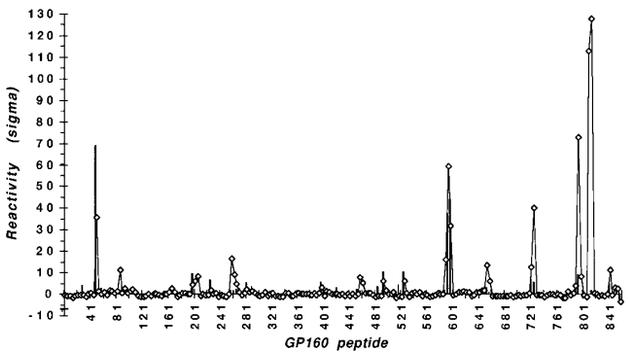

Figure 6 Pepscan reactivity of an HIV-positive volunteer prior to (bars) and post (-◇-) a 5-year immunization series with rgp160.

simultaneously. The bath, then, would theoretically yield up to 90-fold efficiency over the wells. This would be reduced by the number of peptides that bound any single antibody population (two replicates per block multiplied by 1–3 peptides containing the epitope) for a final advantage in serum usage between 15-fold and 45-fold. For example, for peptide 497 similar reactivities were observed in the bath at $1{:}1\times10^6$ dilution, and the well at $1:3\times10^4$ dilution (Figure 4b). Similar ratios were observed throughout the titration.

The technique presented for analysis of Pepscan data gives a statistical basis for determining which reactivities are significant. When sufficient replicates were run, this technique allowed weaker reactivities to be revealed with high confidence. In the analysis of the seropositive volunteer (Figure 3), seven peaks that had quite low reactivity were still determined to be truly positive. Two of these peptides (peptides 313 and 501) have amino acids in common with peptides having stronger reactivities. It follows that peptide 313, for example, contains part of the linear epitope that is completely contained by peptide 317. This raises the interesting possibility that some of these reproducible, low intensity reactivities may result from peptides that contain part of a discontinuous epitope. This possibility has been raised previously [12], but investigation requires high confidence in determining significant reactivity. The described analysis method may be helpful in resolving the difficult problem of mapping partial epitopes. When it was not possible to run many replicates, the stringency of a 5 σ cutoff was critical to eliminate false positives.

These technical modifications allowed the Pepscan technique to be used to monitor changes in seroreactivity after immunization with a vaccine, even in the presence of preexisting antibodies. Thus, broadening of antibody response was illustrated between priming and boosting immunizations in rabbits and between virally-stimulated existing immunity and that induced by vaccine therapy in some HIV-positive volunteers. In contrast with previous results [18], however, no statistically significant difference was observed in the entire seropositive cohort between reactivity before and after immunization with rgp160 as part of a vaccine therapy trial. The patients observed in this study were much further out in the immunization process than those in the previous study (5 years contrasted with 2 years or less). The possibility that the documented reactivity to linear epitopes wanes after several years will be the subject of further investigation.

Specific epitopes strongly related to immunization with this particular product were noted, including one not previously reported in variable region 5 (peptides 461 and 465) which was quite immunogenic in immunized humans as well as in animals (Figure 6, Table 5). These techniques are being applied in larger studies of both immunized rabbits and sero-

positive volunteers enrolled in various vaccine therapy trials. Mapping of linear epitopes early in such studies will allow determination of specific immunogenic regions and highlight peptides to be studied more intensively to monitor vaccine efficacy for other pathogens as well as HIV. As current methods for epitope prediction fail to predict all immunogenic regions of a protein, the Pepscan method continues to offer an advantage for this purpose.

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