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A recombinant prime, peptide boost vaccination strategy can focus the immune response on to more than one epitope even though these may not be immunodominant in the complex immunogen

David Davis*†¶, Bror Morein‡, Lennart Åkerblom‡, Karin Lövgren-Bengtsson‡, Mariëlle E. van Gils§, Willy M.J.M. Bogers§, Vera J.P. Teeuwsen§ and Jonathan L. Heeney§

Rhesus monkeys were successfully vaccinated using a strategy of priming with a candidate envelope subunit vaccine and boosting with synthetic peptides. Priming was carried out with recombinant HIV-1 SF2 envelope glycoprotein incorporated into ISCOMs, following the attachment of a lipid tail. Peptides, covalently linked to ISCOMs, representing linear sequences within the V2 and V3 regions, were used to boost functional antibodies to neutralizing epitopes in both of these regions. Injections with these peptide formulations substantially increased the titre of serum neutralizing antibodies from low or undetectable levels. In addition to completely neutralizing the homologous HIV-1 SF2 strain, these sera also neutralized the escape variant, HIV-1 SF13. However, no antibodies were boosted which could compete with human, neutralizing monoclonal antibodies recognising conformational epitopes. The peptides also boosted antibodies to a peptide whose sequence lies close to the V2 region neutralizing epitope but does not overlap with it. Importantly, the level of antibodies to an unrelated epitope associated with enhancement of HIV-1 SF13 continued to fall after the peptide boost. Successful protection against challenge with chimeric simian immunodeficiency virus expressing HIV-1 SF13 envelope glycoproteins (SHIV SF13) may be due to an increase in the ratio of neutralizing to enhancing antibodies by selectively boosting with peptides to critical neutralizing epitopes. © 1997 Elsevier Science Ltd.

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Synthetic peptides are able to focus the immune response to specific epitopes. By targeting neutralizing epitopes they may shift the balance away from those regions of a candidate vaccine with the potential to induce a deleterious immune response. They have been used in two ways. An antibody response can be primed with peptides and then boosted with whole proteins, as with inactivated poliovirus vaccines[1]. Alternatively, vaccination strategies involving peptide boosting of an existing antibody response have successfully protected primates against challenge with human immunodeficiency virus type 1 (HIV-1)[2,3] or recombinant simian immunodeficiency virus displaying HIV-1 envelope glycoproteins (SHIV)[4]. Since the level of antibodies to the external envelope glycoproteins of HIV-1 fall rapidly[5] after immunization the ratio of neutralizing to enhancing antibodies can possibly be increased by boosting with peptides representing important neutralization epitopes. In addition, antibodies to conformational epitopes may be boosted. Neurath *et al.*[6] showed that injections of peptides to the third variable region of HIV-1 gp120 boost titres of antibodies which could bind to peptides representing sequences remote from the principal neutralizing epitope in the primary

*Molecular Immunopathology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, UK. †Present address: Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium. ‡Department of Virology, The National Veterinary Institute, Biomedical Centre, Uppsala, Sweden. §Department of Virology, Biomedical Primate Research Centre, Lange Kleiweg 157, 2288 GJ Rijswijk, Netherlands. ¶To whom correspondence should be addressed. Tel.: +32 3 247 63 29; fax: +32 3 247 63 33; e-mail: cmaeckelbergh@itg.be. (Received 29 August 1996; revised version received 24 February 1997; accepted 4 March 1997)

sequence of this immunogen. Åkerblom *et al.* also demonstrated that a strong boost of homologous virus neutralizing antibodies was achieved by peptides of the V3 region following priming with a gp120 protein. Such high titres could not be obtained using an immunization schedule based solely on peptide injections[7].

The purpose of the present study was to investigate the vaccine-induced antibody response in monkeys which subsequently resisted challenge with S/HIV SF13. (HIV-1 SF13 is a later variant isolated from the individual infected with HIV-1 SF2.) Animals were first primed with recombinant HIV-1 SF2 gp120 then boosted with synthetic peptides of two independent neutralizing epitopes in an attempt to alter the immunodominance of the V3 region. Protection was indicated by a failure to detect virus by co-cultivation of their leukocytes, by PCR of DNA from circulating cells and by the absence of an anamnestic response following intravenous challenge with S/HIV SF13. We wished to confirm that peptides boosted the level of antibodies to both epitopes and also to investigate the hypothesis that successful protection was associated with an increased ratio of neutralizing to enhancing antibodies in the circulation. Investigations of these monkeys' cell-mediated immune responses, showing that peptide injections also selectively boosted HIV-1 gp120-specific Th1 responses, are presented elsewhere[4].

MATERIALS AND METHODS

Sources of immunogens

CHO-derived glycosylated HIV-1 SF2 gp120[8–10] and yeast-derived HIV-1 SF2 *gag* p24[11,12] were obtained from Dr Kathelyn Steimer and Dr Gary Ott of the Chiron Corporation. Synthetic peptides representing the HIV-1 SF2 V2-(IRDKIQKENALFRNLC) and V3-(NNNTRKSIYIGPGRAC) region neutralization epitopes were obtained from Affiniti Research Products Ltd., Exeter at 95% purity.

Preparation of ISCOMs

Recombinant gp120 and *gag* p24 were lipidated using a method modified from that of Mowat *et al.* using hydroxy-palmitic acid and succinimide ester (Sigma) dissolved in dimethylsulphoxide at a concentration of 10 mg ml^{-1} with a ratio of 40:1 over protein[13]. The protein and lipid mixture was then incubated overnight at 37°C. To form ISCOMs, cholesterol, phosphatidyl choline and Iscoprep 7.0.3 (saponin from Iscotec, Uppsala, Sweden)[14] were added to the lipidated protein in a ratio of 1:1:3.5 on a weight basis. This mixture was incubated at room temperature for 2 h before being dialysed at room temperature overnight against phosphate buffer at pH 8.6. The buffer was subsequently changed to phosphate-buffered saline (pH 7.4) and further dialysed for 48 h. The presence of ISCOMs was confirmed by electron microscopy and the preparation centrifuged through a 10–50% sucrose gradient at 39000 rev min^{-1} and 10°C for 18 h with a TST41.14 rotor in a Contron ultracentrifuge. The ISCOMs co-localised with the *gag* p24 and *env* recombinant proteins, which were detected by ELISA.

The synthetic peptides were conjugated to the envelope proteins of influenza virus incorporated into ISCOMs. The influenza ISCOMs were activated with maleimidohexanoyl-*N*-hydroxysuccinimide ester and incubated in a tenfold molar excess of peptide[15].

Injection schedules

Recombinant gp120 (30 μg) and 20 μg of *gag* p24 in ISCOMs were injected intramuscularly (i.m.) into four rhesus monkeys at 0 and 6 weeks. Fifty micrograms of each peptide ISCOM were injected into the monkeys at 6 and 16 weeks. A third i.m. *gag* injection was also included at 16 weeks. Two monkeys received three injections at 0, 6 and 16 weeks of 50 μg PR8-Flu ISCOMs as controls.

Sources of antigens and antibodies for immunoassays

The following materials were obtained through the MRC AIDS-directed programme: Recombinant HIV-1 W61D gp120 originated with Dr Claudine Bruck, SmithKline Beecham; antiserum to HIV-1 SF2 gp120 was raised in sheep by Dr Mark Page at the National Biological Standards and Control Laboratory, Potters Bar; HIV-1 *gag* p24 was produced by Agmed Inc in the Baculovirus expression system. The amino acid sequences of the peptides start at residue 41 of the external envelope glycoprotein (gp120) of the HIV-1 SF2 strain[16]. The peptides have 15 amino acids of the published sequence[9] and overlap by five amino acids. The N- and C-termini of the individual 15mer peptides are given by the formulae: $y = 10x-9$ and $y' = 10x+5$, where y and y' are respectively the N- and C-termini and x is the peptide number. Peptides were synthesised by the FMOC–polyamide method by Cambridge Research Biochemicals, Northwich, UK.

The human HIV-1 neutralizing monoclonal antibody IgG1 b12 which recognises the CD-4 binding region[17] was supplied by Dr Dennis Burton, Scripps Research Institute. Antibodies 447-52D to the V3 region[18] and 597-D to the V2 region[19] were obtained from Cellular Products, Buffalo, New York.

Radioimmunoassays

Solid phase antigens were prepared on Falcon microtest flexiplates by overnight incubation at 4°C in carbonate/bicarbonate coating buffer (pH 9.6) at 10 μg ml^{-1} for both recombinant proteins and synthetic peptides[20]. A dilution series of plasma was prepared in phosphate-buffered saline with 0.05% Tween 20 (PBS/Tween). Aliquots of the diluted plasma were incubated overnight with the solid phase at 4°C. Antibodies which remained after three washes with PBS/Tween were detected with ^{125}I-radiolabelled antibodies to human IgG[20].

Sources of cells and viruses

HUT78 cells were obtained from Dr Jay Levy, Cancer Research Institute, San Francisco. C8166 cells were obtained from the European Collection of Animal Cell Cultures, Porton Down, UK. Cells were cultured in RPMI medium with 10% foetal calf serum (FCS) in roller bottles. Medium was changed every 3–4 days.

A molecular clone of HIV-1/USA, CRI/SF-2 (ARV-2)/84 was obtained through the Medical

Research Council AIDS Directed Programme. It was cultured in HUT78 cells. Virus derived from a molecular clone of HIV-1/USA, CRI/SF-13/85 was obtained directly from Dr J.A. Levy, Cancer Research Institute, San Francisco. Both viruses were isolated from the same individual with a 5 month interval. Viral stocks were prepared as 1 ml aliquots which were snap frozen and stored in liquid nitrogen.

Neutralization assays

Virus stocks were titrated after 24 h incubation at 37°C in C8166 cells. Dilution series of monkey sera were incubated for 24 h at 37°C with virus so as to give 10 $TCID_{50}$ per 20 μl inoculum. Residual virus was detected by cultivation with 2×10^5 C8166 cells per well in 96-well plates. Plates were examined by light microscopy and when an extensive cytopathic effect was observed, 150 μl of supernatant were harvested into Triton X-100 to give a final concentration of 1%. The concentration of *gag* in the supernatants was determined by radioimmunoassay against a standard[21].

Competitive inhibition assays

A 1 in 10 dilution of plasma from each monkey was mixed with an equal volume of radiolabelled human monoclonal antibody. The mixture (50 μl) was incubated with solid phase antigens prepared on Falcon flexiplates in the combinations: IgG1 b12 versus W61D, 447-52D versus SF2 and 697-D versus W61D. After 24 h incubation at 4°C the radiolabelled antibody which remained binding to the solid phase after three washes with PBS/Tween was counted. Percent inhibition was calculated, taking the antibody bound in the absence of plasma as 100%[22].

RESULTS

Peptide ISCOMs boost antibodies to themselves and whole molecules

The titre of antibodies to recombinant HIV-1 gp120 reached its highest level at week 8 and thereafter fell in all four monkeys. Injection with synthetic peptides at week 16 reversed this decline, both to gp120 and to each peptide (*Figure 1*). Titres to recombinant HIV-1 *gag* p24 follow those to gp120 (*Figure 1*). No antibodies to any HIV-1 specific antigen were observed in monkeys injected with PR8-Flu ISCOMs (data not shown).

Injections of peptides focuses the antibody response to two epitopes

At the time of peak titres to recombinant HIV-1 gp120 each monkey was found to produce antibodies which recognised a different spectrum of peptides (*Figure 2*). All four monkeys developed titres of 1 in 500 to peptide env48 but recognised different combinations of the other peptides. Subsequently, titres decline and continue to fall even after the peptide injections. The two exceptions to this observation were monkey 9111's response to peptide env44 and monkey 9251's to peptide env28, which by week 18 had increased from undetectable levels at week 16 (*Figure 2*). This may indicate that the amino acids included in peptides env28 and 44 are sufficiently close to the V2 or V3 region in the native molecule to be able to form an epitope, such that a single antibody molecule can span sequences in both peptides. Two weeks after the third injection, antibodies to peptides env18 and/or env32 elicited the highest titres.

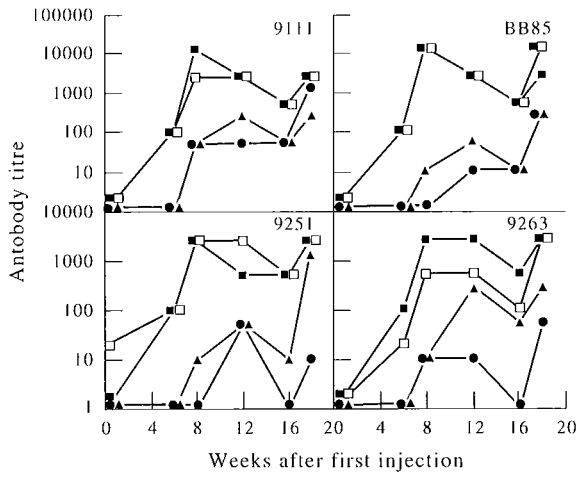

Figure 1 Changes in antibody titre over time of four monkeys to HIV-1 SF2 recombinant gp120 (■), HIV-1 *gag* p24 (□), HIV-1 SF2 V3-region peptide (▲) and HIV-1 SF2 V2-region peptide (●)

Antibodies to conformational epitopes determined by competitive inhibition assays

Only one monkey produced antibodies which could inhibit the binding of human monoclonal antibodies to solid phase recombinant gp120. Serum from monkey 9111 taken at 8 weeks inhibited the binding of radiolabelled IgG1 12b to solid phase W61D by more than 50% (*Figure 3*). No boost in the level of this antibody was observed in any monkey following injections with peptides. However, the level of antibodies able to compete with human monoclonal 447-52D (αV3) increased after injection of peptide in all four monkeys, although inhibition only reached 13–16%.

Peptide injections boost neutralizing antibodies

Only low or undetectable levels of neutralizing antibodies against the homologous virus (HIV-1 SF2) could be detected prior to the peptide boosts and these failed to completely neutralize the virus at the lowest dilution (1 in 20) used (data not shown). Subsequently, all four monkeys developed antibodies which could completely neutralize both the homologous (*Figure 4*) and the variant virus (*Figure 5*). In the latter case, shoulders, similar to those observed by Kostrikis *et al.*[23] were seen on the neutralization titration curves. Sera from monkeys injected with PR8-Flu ISCOMs failed to neutralize HIV-1 SF2 (data not shown).

Peptide injections avoid boosting enhancing antibodies, except for a single epitope

Only low levels of antibodies were induced to the six peptides which have previously been shown to induce antibodies giving the highest levels of HIV-1 SF2 enhancement in macrophage cultures[24]. Although binding of radiolabelled antibodies was increased in

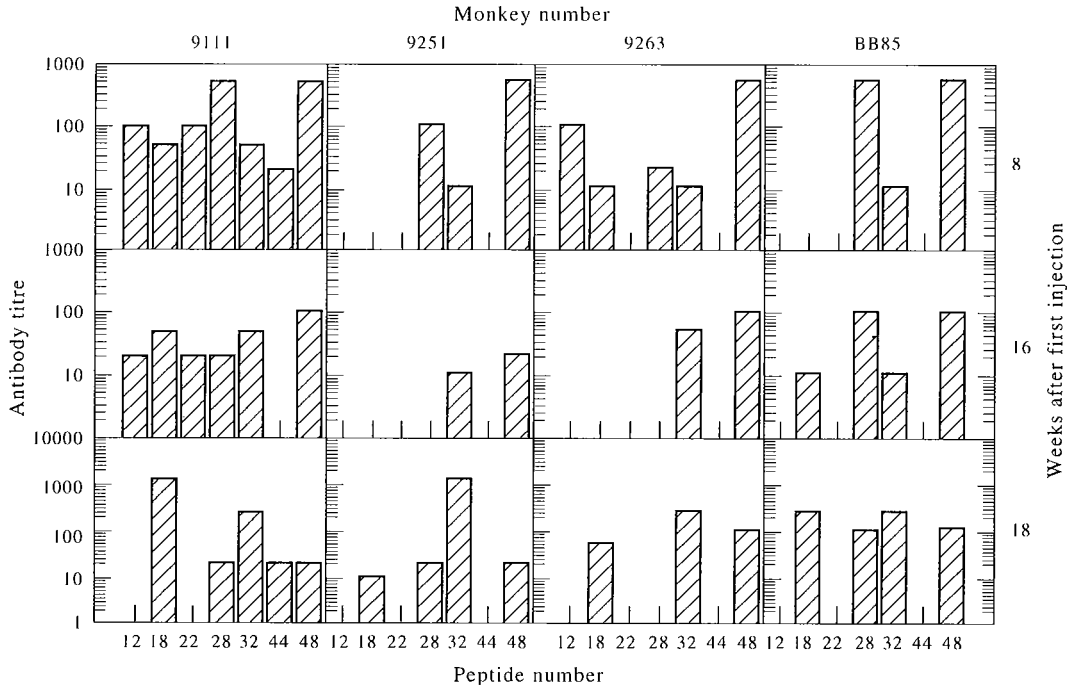

Figure 2 Changes in antibody titre over time of four monkeys to peptides previously shown to bind high levels of antibodies in serum from primates injected with HIV-1 SF2 recombinant gp120

assays using 18-week sera relative to the 16-week levels, it did not reach the 15% cut-off level (*Figure 6*).

When the peptides which induced antibodies giving the highest levels of enhancement of HIV-1 SF13 in macrophage cultures (Trischmann, personal communication) were examined, high levels of antibodies to env12 were observed in two of the monkeys. These were found after the second injection. They then began to decline and continued to fall after the final, peptide injection. In contrast, low levels of antibodies to peptide env20 were detectable after the second injection but these increased to levels greater than the 15% cut-off level after the peptide injection (*Figure 6*).

DISCUSSION

A strictly pragmatic approach to the development of an HIV vaccine would be to take a successful strategy, improve it and make it acceptable for human use. The strategy under consideration in this paper stems from the observation that boosting with V3 region peptides

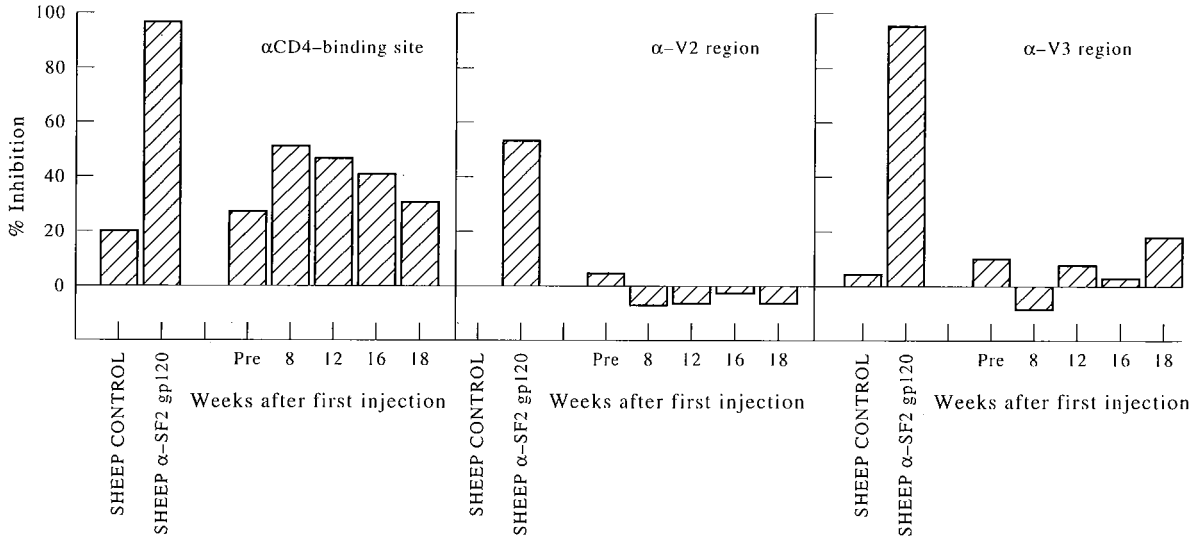

Figure 3 Changes in the capacity of plasma from monkey 9111 to inhibit the binding to their epitopes of human monoclonal antibodies recognising three different regions of HIV-1 envelope glycoprotein. Positive and negative controls are provided by sheep sera

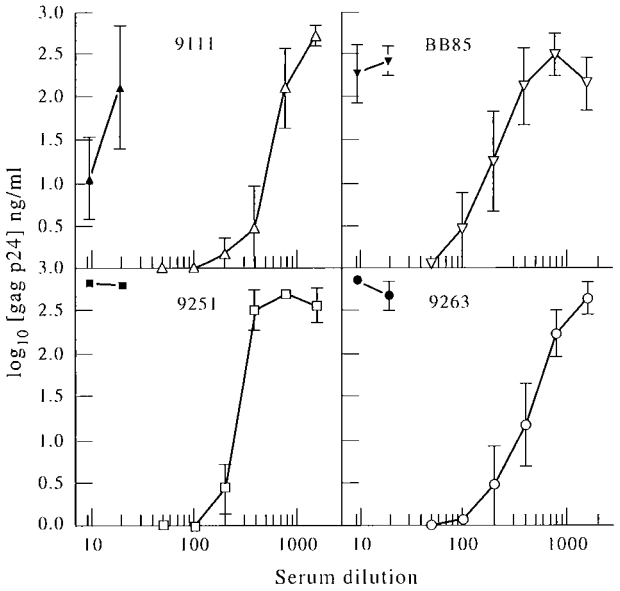

Figure 4 Neutralization of HIV-1 SF2 by sera from all four monkeys either before vaccination (closed symbols) or at 18 weeks, after the peptide booster injection (open symbols)

induced neutralizing antibodies and protected chimpanzees against challenge infection[2]. Later, it was shown that chimpanzees could be protected against heterologous challenge with a primary strain of the virus[3]. The current strategy uses an immunogen which has been used in Phase II trials and involves doses and an injection schedule which would be acceptable in humans. The adjuvant was constructed from Quil A components which lack the toxicity associated with earlier formulations involving unpurified saponin fractions[14].

The strategy was designed to induce neutralizing antibodies to multiple epitopes in an attempt to increase the dose of virus which could be included in the challenge inoculum. As the number of viruses in the inoculum increases, so does the likelihood that a variant virus, able to escape neutralization, may be included. However, if antibodies to two independent neutralizing epitopes are induced the probability that a

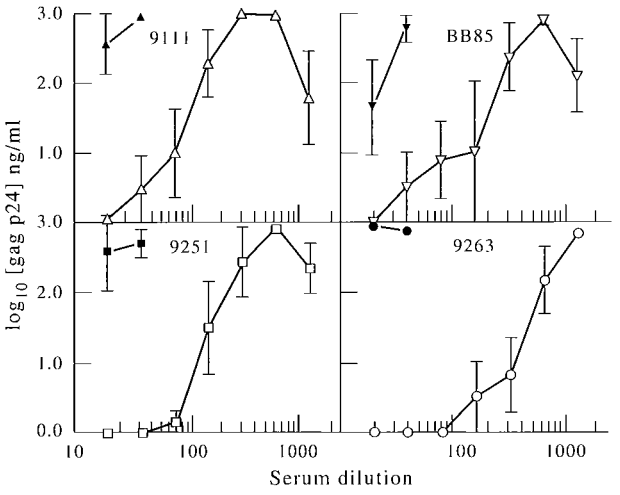

Figure 5 Neutralization of HIV-1 SF13 by sera from all four monkeys either before vaccination (closed symbols) or at 18 weeks, after the peptide booster injection (open symbols)

variant is present which can escape neutralization at both epitopes decreases in relation to the product of the mutation frequencies at each epitope. Given a mutation frequency of 1 in 10^4 at the V3 epitope[25] and assuming a similar mutation rate within the V2 epitope, the frequency of a double mutant would be 1 in 10^8. Results from the present study support this concept. HIV-1 SF13 most likely represents a variant of HIV-1 SF2 with its V2 and V3 region epitopes able to escape neutralization independently of each other. This follows from the observation that the later virus[26] is not neutralized by antibodies raised to HIV-1 SF2 V3-region peptides, but can be neutralized by antibodies to the V2 region (unpublished data). All four monkeys were protected from challenge with SHIV SF13 although neutralization titres to HIV-1 SF13 did not correlate with titres to the V2 peptide.

To be successful, such a strategy needs to overcome the natural immunodominance hierarchy of the epitopes within a complex immunogen. In this study peptide boosting focused the immune response on to the neutralizing epitopes, so that the balance of beneficial antibodies outweighed those which may be deleterious to a protective host response. The proportion of antibodies to epitopes associated with neutralization is low following the second injection of recombinant gp120. While each monkey produces antibodies to the linear neutralizing epitopes only one was found to produce antibodies to the conformational CD4-binding site. At that stage the titres of antibodies to the neutralizing epitopes are lower than those to the immunodominant epitopes. Over time antibodies to enhancing epitopes fall and for the most part continue to decline after injections with peptides which selectively boost antibodies to the neutralizing epitopes.

The strategy appears to have fulfilled its design criteria, although it was hoped that it would induce antibodies to conformational epitopes. One of the principal complaints levelled at the use of synthetic peptides for vaccination is that they appear to only induce antibodies to linear epitopes. However, if they are used to prime or boost an antibody response, they may be able to influence the titre of antibodies to conformational epitopes. If B cells were originally primed with a whole immunogen, they will be induced to divide and produce antibodies to conformational epitopes provided they display helper T-cell epitopes. To induce a booster effect, therefore, a peptide has to deliver a T-cell carrier molecule to the primed B cells. Evidence that a partial match between the peptide and the immunoglobulin receptor is sufficient for this purpose comes from the observation that injections with V3-region peptides boosted antibodies which could be detected by peptides with sequences far removed from this region in the primary sequence of the original immunogen[6]. Presumably, these regions are sufficiently close together in the native structure of the molecule that they can form an epitope which can be spanned by a single antibody combining site.

In the present study antibodies were boosted in all four monkeys to the peptide env20 which has no sequence in common with either of the peptides used as immunogens. It is unlikely that this effect is the result of stimulation with cross-reacting epitopes on the

Flu-membrane proteins used as carriers since no antibodies could be detected in control monkeys which had been injected with Flu-ISCOMs alone. Less consistently, two other monkeys showed boosting of antibody titres to peptides distant in primary sequence from the injected peptides. No monkey showed boosting of antibodies to peptide env12 whose sequence is included in the peptide used by Neurath *et al.*[6] Although these observations may be interpreted as evidence in favour of boosting of antibodies to conformational epitopes, little or no effect was observed when competitive inhibition assays were used. The tiny booster effect with the αV3 antibody may be attributed to the sharing of the linear GPGRA motif between the peptide immunogen and the target epitope of the monoclonal[27].

The successful vaccination strategy is based on the neutralization–escape hypothesis used to explain the generation and maintenance of the mixture of polymorphic variants which circulate within a chronically infected individual. However, while neutralization–escape may operate *in vivo* it may not represent the complete story of how variability within the quasispecies is maintained. The observation which leads to these doubts is that with lentiviruses, neutralization–escape variants can emerge *in vitro* in the presence of immune sera[28,29] while with many viruses the addition of a second monoclonal antibody prevents the emergence of variants[30]. Presumably this occurs because there are not sufficient viruses present in a single culture for a double mutant to emerge. One possible explanation to reconcile this observation with the escape hypothesis is that the natural antibody response to lentiviruses is functionally against a single epitope. If this were correct, it would represent a further argument in favour of inducing neutralizing antibodies to multiple epitopes. An alternative source of selection pressure may be the activity of enhancing

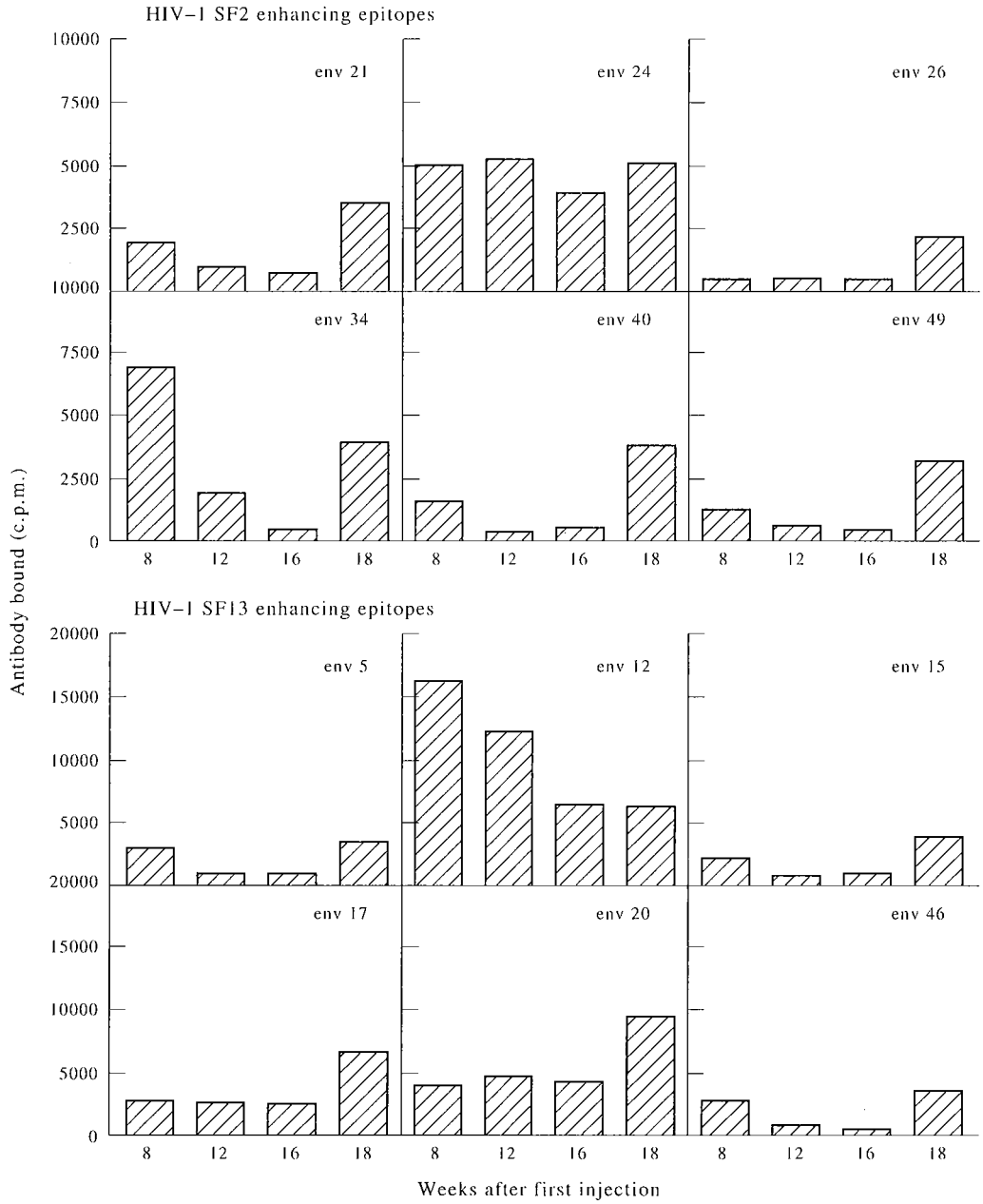

Figure 6 Changes in the levels of antibodies in plasma from monkey 9111 able to bind to solid phase peptides representing putative enhancing epitopes of HIV-1 SF2 (upper) or HIV-1 SF13 (lower)

antibodies. Enhancement has been demonstrated in the serum of HIV-1 seropositive patients taken late in infection towards their own earlier virus isolates[31]. Furthermore, the observation that the epitopes giving rise to enhancing antibodies are different between isolates of the virus[24] supports the idea that these antibodies can contribute to selection pressure.

A possible mechanism through which enhancing antibodies could exert selection pressure would be if they could rescue a neutralized virus and permit it to infect a cell which already carried a provirus. They would thereby produce the conditions for recombination. An alternative route of infection is necessary because a cell carrying a provirus will have its CD4 molecules down-regulated and enhancing antibodies will allow the virus to enter a cell via Fc or complement receptors independently of the CD4 receptor[24,32]. Recombination between viruses, each able to resist neutralization at different epitopes would increase the rate at which double mutants were generated.

The success of a peptide-boosting strategy may not merely be the result of boosting the level of neutralizing antibodies but by avoiding a simultaneous increase in the levels of enhancing antibodies. In general, the neutralizing antibody responses to HIV-1 envelope glycoproteins are isolate specific. In the present study, antibodies are induced which neutralize the homologous virus (HIV-1 SF2) with negligible levels of antibodies able to enhance its infection. These antibodies also neutralize the variant virus (HIV-1 SF13). However, antibodies are also produced which may enhance the infectivity of this virus (peptides env12 and env20) or a heterologous virus, HIV-1 IIIB (peptide env48)[24]. With HIV-1 SF13 the overall effect of the peptide booster injections was to maintain a balance in favour of neutralization while further injections with recombinant peptide might be expected to boost the levels of antibodies to putative enhancing epitopes.

The availability of modern recombinant immunogens for vaccination offers the opportunity to introduce new adjuvants and strategies. Previous immunogenicity studies with recombinant HIV-1 SF2 gp120 in primates showed a uniform response in terms of the peptides recognised by each baboon[33], although this was achieved after eight injections with immunogen formulated in an unacceptable adjuvant. In contrast, in this study the serum from individual rhesus monkeys recognises a different spectrum of peptides with two of them producing only low levels of antibodies to the V2 region epitope. Potential options for producing a more consistent response would be the use of multiple immunogenic peptides[34] or the use of pulsed-release formulations[35].

If a pragmatic approach is to be further pursued, the next step would be to develop cross-neutralizing antibodies. Previous efforts have given conflicting results. Klinman *et al.*[36] showed that injections with recombinant envelope glycoproteins from different isolates gave cross-neutralizing antibodies when they were injected sequentially but not simultaneously[36], whereas others using affinity purified gp120[37] or synthetic peptides[6] reported that original antigenic sin operates and have recommended simultaneous injections. Within the context of peptide boosting it might be better to aim at using peptides which can boost cross-neutralizing antibodies rather than mixtures of peptides representing a range of isolates. Repeated sequences of the GPGRAF motif found at the tip of the V3 loop of many isolates, may offer one such peptide[38], while the V2 region was originally selected because it was less variable than the V3 region[33]. Equally, recombinant HIV-1 SF2 gp120 can induce antibodies to the CD4-binding region[22], and mimotopes to this antibody may offer a viable option to be pursued[27].

Peptides are now increasingly being considered for vaccination. Their use as boosters offers an alternative strategy to those which have so far advanced to phase III clinical trials[39–41]. In other human trials, problems associated with hypersensitivity reactions to the T-cell carrier have arisen[42,43] and this has limited the dose of peptide which could be injected since the two were chemically coupled to each other. In the present strategy, the mass of peptide can be increased while holding constant that of the carrier since both are linked to a physical particle and so enter an antigen presenting cell together. The peptide boost strategy may be particularly relevant where multiple epitopes are available and yet immune escape remains a problem[30]. Given observed mutation rates it seems unlikely that any infectious agent can generate sufficient numbers within an individual host to escape at more than two or three independent epitopes simultaneously. A peptide boost strategy which can raise the immune response above a protective threshold at multiple epitopes may then protect individuals against escape variants but also prevent the emergence of those variants and their spread through a population.

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