Current Drug Targets, 2004, *5*, 71-88

Vaccines and Vaccine Strategies Against HIV

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Abstract: The HIV/AIDS pandemic is a global emergency and a preventive HIV vaccine is urgently needed. HIV has, however, proved a difficult pathogen to vaccinate against. This is largely because HIV has a very high mutation rate and can escape immune responses, it has a latent stage where it can rest silently integrated into host DNA, and neutralising antibodies that can neutralise diverse field strains have so far proved difficult to induce. There is however, considerable evidence now that HIV-specific CD4 and CD8 T cells can provide partial control of HIV replication and delay or prevent disease. Technologies to quantify and analyse HIV-specific T cells have advanced recently, and in particular ELISpot, intracellular cytokine staining and tetramer studies have provided clear analyses of the ability of HIV vaccines to induce T cell responses. The use of pools of overlapping HIV peptides as *in vitro* antigens has also provided a standardised reagent for accurate measurement of T cell responses. HIV protein vaccines have not induced broad neutralising antibodies or T cell responses and failed to protect humans in the only phase III efficacy trial yet completed. Viral vectors, such as canarypox, engineered to express HIV genes, have induced HIV-specific CD8 T cell responses in a minority of subjects in phase II trials and are proceeding to human efficacy trials. Currently, the most effective method of inducing CD8+ CTL immunity in non-human primates utilises priming with naked plasmid DNA and then boosting with recombinant viral vectors both encoding various parts of the HIV genome. Such vaccines have induced non-sterilising immunity to virulent Simian/Human immunodeficiency virus exposure in macaques and have entered phase I trials. Multiple other approaches are also being evaluated in what has become a global effort for a vaccine to prevent AIDS. Although an HIV vaccine is still a long way off, there is reason to be optimistic that a vaccine to prevent AIDS will eventually be developed.

INTRODUCTION – THE NEED FOR A VACCINE AGAINST HIV

The human immunodeficiency virus (HIV) first came to notice in 1981 [1]. Since then, 20 million people have died of the acquired immunodeficiency syndrome (AIDS) that it induces, whilst another 40 million people are currently living with this virus. Worldwide, HIV is spread mainly across mucosal surfaces during heterosexual contact, thus infecting people of childbearing age and their progeny and having a devastating effect, not only on the health of these people, but on the entire social and economic make-up of those areas where the epidemic is worst, namely sub-Saharan Africa and south Asia. In addition, the large pool of immunodeficient individuals has provided a huge reservoir for the emergence of old and new infectious diseases with reduced antibiotic susceptibility (e.g. tuberculosis), spawning secondary health issues of their own even among people not especially at risk of HIV itself [2].

Efforts to prevent infection, such as altering sexual practices, encouraging circumcision, aggressively treating sexually transmitted diseases, needle exchange programs and other behavioural approaches may have helped to curb HIV transmission in particular settings [3]. However, worldwide such campaigns have been difficult to implement and have so far failed to invoke a sustained impact on new HIV infections [4].

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The development of antiretroviral therapy has produced a 60% reduction in mortality from AIDS [5] but the cost of therapy is prohibitive for the majority of those infected. Furthermore, the inability of drug therapy to eliminate the virus from the host, the emergence of drug resistance and the increasing level of side-effects induced by long term therapy mean that prevention of infection is crucial in combating the HIV pandemic.

For these reasons, the development of a vaccine against HIV is one of the most pressing challenges facing modern medicine.

HIV-1: THE VIRUS

The human immunodeficiency virus belongs to the lentivirus family (summarised from ref. [6]) and is a retrovirus, based on its ability to reverse transcribe RNA into DNA, a process mediated by the enzyme reverse transcriptase. The genome is 9.5-kb in length and encodes 9 genes (Fig. **1**), namely the polymerase gene (*pol*) encoding reverse transcriptase (RT), protease (PR) and integrase (IN), 2 structural genes (*gag* and *env*) coding for the nucleocapsid and envelope proteins, respectively, 2 essential regulatory genes (*tat* and *rev*) coding for proteins involved in activation and regulation of mRNA transcripts and 4 accessory genes (*nef, vif, vpu* and *vpr*) coding proteins involved in nuclear localisation of proviral DNA and virion release and *infectivity. The genome of HIV-2 is included, as a* comparison, as it mimics SIV (simian immunodeficiency virus), which is used in many of the simian research models.

The free virus is coated by the envelope protein (*env*), also known as gp160, which is made up of two subunits

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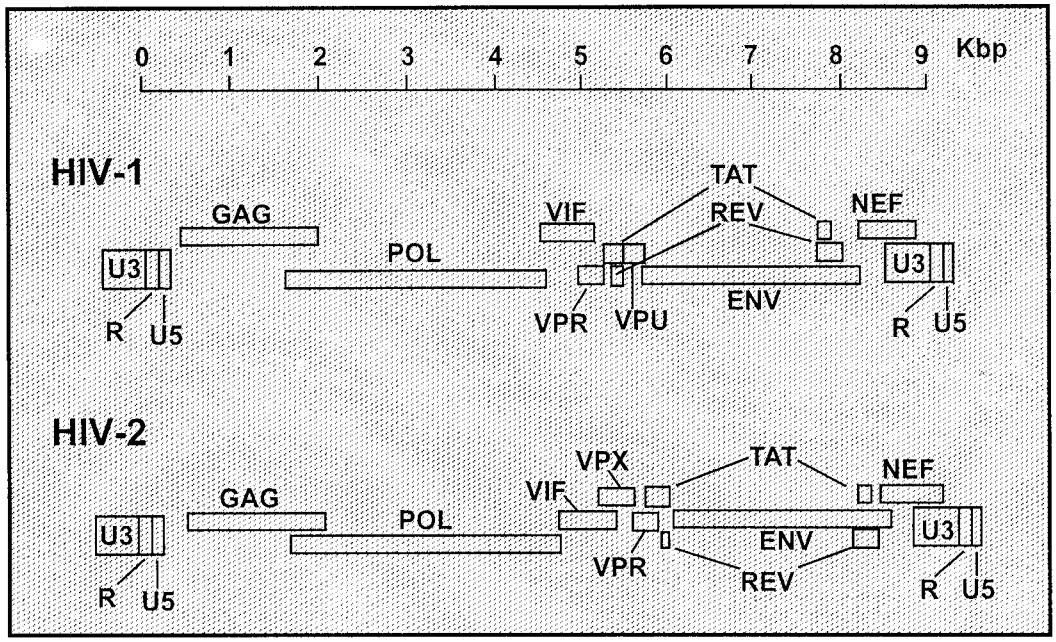

Fig. (1). The 9 kilobase genome of HIV-1, containing nine genes flanked by U3-R-U5 long terminal repeat promoters. The HIV-2 genome is also shown. HIV-2 has sequence differences compared to HIV-1 and differs by the substitution of vpu by vpx but it mimics SIV, which is used in many animal vaccine trials.

(gp120 and gp41). The virion primarily contains two copies of positive sense single stranded RNA, the *pol* enzymes and the accessory protein *vpr*, all of which is stabilised by nucleocapsid protein (*gag*).

Upon entering a new host (Fig. **2**), Gp120 binds to the CD4 receptor expressed on T-helper cells, dendritic cells and macrophages. Viral entry into the host cell is dependent on co-receptors (usually CCR5 or CXCR4), whereupon uncoating of the virus occurs in the cytoplasm. Transcription of viral ssRNA into proviral dsDNA then occurs under the influence of reverse transcriptase and the host $tRNA^{lys}$. The proviral DNA is then localised to the nucleus of the host cell under the influence of *gag* p17 and *vpr* and becomes integrated into the hosts DNA under the influence of integrase. This integrated proviral DNA is immunologically silent and remains latent within the host genome.

Transcription of the integrated proviral DNA into mRNA commences upon binding of cellular elements to the LTR. The initial mRNAs code for *tat, rev* and, less importantly, *nef*. The regulatory proteins *tat* and *rev* are critically involved in the enhanced transcription and regulation of the *gag, pol* and *env* genes, resulting in fully expressed proteins. Although *nef* is not essential for viral replication, absence of it results in dramatically reduced disease progression. The final 3 accessory proteins, *vif, vpr* and *vpu*, are expressed late in the infective cycle and are not essential for replication.

Several points about the biology of HIV-1 important for vaccine considerations need to be underscored. Firstly, the process of reverse transcription, as catalysed by the enzyme reverse transcriptase, is highly error prone including (1) the direct misincorporation of non-complementary nucleotides leading to amino acid substitutions, (2) slippage of DNA strands leading to deletions and/or additions of nucleotides, (3) frame shifts leading to misincorporation and (4) dislocation mutagenesis. The misincorporation rate for HIV-1 is 1 per 1,700 to 4,000 nucleotides, a situation that is not rectified as reverse transcriptase lacks 3'-5' exonuclease activity for proof reading. The *in vivo* error rate is thus estimated to be 1-3 misincorporations per replication cycle. Secondly, the binding of the surface subunit of the *env* protein (gp120) to the CD4 and chemokine receptors occurs via virus glycoproteins. Although *env* seems like an attractive target for a vaccine, the binding epitopes are buried among highly variable regions, which contain amino acid substitutions, deletions and insertions displaying up to 25% variability between strains. Linear and conformational determinants (including quaternary structure) of the *env* protein add to the difficulty of developing neutralising antibodies. Even if neutralising antibodies are produced, access to the conserved critical immunogenic antibody binding sites is difficult as they are masked by glycosylation and only exposed briefly at the point of binding to the CD4 and chemokine receptors. Thirdly, viral proteins are not expressed in equal amounts. For example, the expression of *pol* is approximately 20 times less than *gag* and *env*. Fourthly, different genes are expressed at different times in the life cycle of HIV-1 and have differing importance in terms of how crucial they are for both viral replication and T-cell immune responses, which might kill cells prior to new virion formation. Fifthly, the integration of the HIV genome into the host's chromosome, in the form of linear dsDNA, not only sets up an immunologically silent proviral reservoir but it occurs prior to the expression of many of the virus's genes. Vaccines targeting such gene products require the establishment of this proviral reservoir and then expression of these genes prior to activation of the immune system. In addition, the concentration of some gene products in the

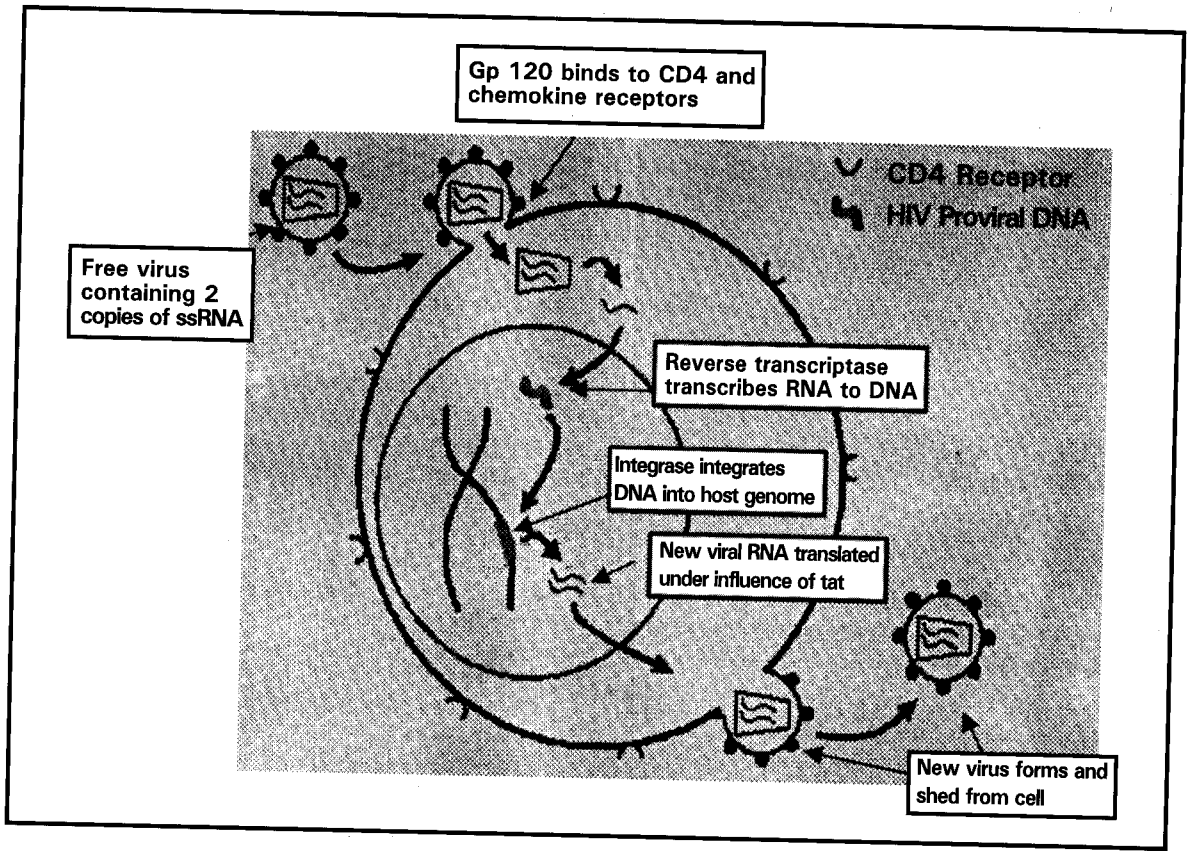

Fig. (2). Key steps in the life cycle of HIV-1.

infecting virion (in particular, polymerase enzymes and *vpr* protein) are very low and are not expressed within the host cell in any significant amount until after proviral integration has occurred.

CHALLENGES IN VACCINE DEVELOPMENT

HIV is a particularly difficult virus to vaccinate against having adapted itself to both damage and evade the immune system. There are no well-documented instances of humans clearing the virus once infection has occurred, despite vigorous humoral and cellular immune responses. The basis for this is primarily the rapid mutagenesis of the genome; about 1:2,000 amino acid substitutions occurs per replication cycle and, with up to 10^{10} viral particles released from infected CD4+ cells daily, then potentially the entire genome is substituted 10,000 times each day [7]. Clearly, some of these mutants gain an advantage in replicative fitness, surviving despite the robust immune response.

Antibodies are generated in nearly all HIV infected individuals yet replication continues via antibody escape mutants [8]. As mentioned above, evasion of antibody is accomplished by glycosylation of envelope proteins and masking of critical epitopes (eg CD4 and co-receptor binding sites) [9]. Also, HIV proteins possess high conformational activity allowing escape from antibody recognition. Highlighting this phenomenon is the observation that candidate vaccines capable of generating vigorous neutralising antibody responses have only been effective against homologous strains of HIV and have been incapable of neutralising field strains of HIV and preventing HIV infection [10]. Passive transfer studies in macaques have proved successful but achieving the sustained neutralising antibody required has not yet proved feasible [11].

HIV can similarly replicate in the presence of HIV-specific cell mediated immunity (CMI). In HIV infected individuals, up to 2% of circulating CD8+ cells recognise epitopes to HIV [12]. Mutations in HIV epitopes again explain its ability to evade recognition by T-cells [13, 14]. Evasion of CMI by HIV is also achieved by Nef-mediated reduction in CD4 and class I MHC expression and by complex gene regulation allowing latent infection in cells [15].

Vaccine development is confounded by several other problems: (1) latent, immunologically silent, integrated proviral DNA, (2) the remarkable genetic diversity of HIV both locally and worldwide, (3) the lack of a precise animal model readily infectable with primary HIV-1 strains, (4) the likely need to generate good mucosal immunity to achieve optimal protection and (5) the lack of a precise immune correlate of protection in humans from HIV-1.

Candidate HIV vaccines have been evaluated in over 70 phase I trials, in more than 3,500 humans [2], with only one concept progressing on to two phase III trials. Many more vaccines have been trialled in simian models. The most efficacious, for the induction of completely sterilising immunity in a macaque model, have been live attenuated

SIV and HIV-1 vaccines [16]. However, some healthy infant and adult macaques have developed AIDS from attenuated SIV strains [17] raising serious safety concerns. Similarly, concerns about reconstitution of deleted genetic elements, recombination of attenuated virus, reversion to virulence and oncogenic potential via insertional mutagenesis have meant that live attenuated HIV-1 vaccines are unsuitable [18, 19]. Antibody based vaccines, as stated, have only been effective against homologous strains of HIV. Other approaches using whole inactivated SIV and recombinant protein vaccines have had limited efficacy in macaques [20, 21] with poor persistence and no cross immunity to heterologous strains [22].

THE CASE FOR HOPE

Despite these factors, there is hope for a vaccine against HIV. Two cohorts of people who possess natural immunity to infection by HIV have been described. Firstly, individuals who are homozygous for a 32-base pair deletion in the CCR5 receptor remain uninfected despite multiple exposures to HIV [23]. This receptor mediates entry of most HIV infecting strains into the host cell. It is not clear whether this genetically based resistance to infection can be utilised in a vaccine strategy.

A second cohort of individuals also appears to possess immunity from infection. A few percent of African prostitutes (not possessing the CCR5 deletion) remain HIV negative despite multiple exposures. The immune correlate of protection is unknown, although it has been noted that exposed but uninfected humans mount detectable CD8+ cytotoxic T-lymphocyte (CD8+ CTL) and T-helper (Th) responses, which may protect against subsequent exposures to HIV [24, 25]. It has been suggested, though, that increased exposure correlates with increased protection, whilst lack of repeat exposure correlates with increased susceptibility to infection. This may point to an acquired mechanism of protection, which could be targeted by vaccine strategies, but it also implies that the immune correlate wanes with time and may not evoke long lasting protection [26].

A large body of additional evidence suggests that HIV-specific CD8+ CTLs play a significant role in containing HIV:

1. Strong CD8+ CTL responses generally correlate with reduced plasma RNA in HIV-1 infected people [27]
2. CD8+ CTLs exert selection pressure on HIV resulting in amino acid replacements (esp. in *nef* and *env*) in the regions to which the CD8+ CTL responses are directed, implying a role for CD8+ CTLs in maintaining immunological control of the virus [28]
3. Transfer of gag-specific CD8+ CTL clones to HIV-1 infected patients reduces the percentage of productively infected CD4+ T-cells [29]
4. Depleting SIV infected macaques of CD8+ CTLs leads to an inability to control viral replication [30]
5. CD8+ CTLs can lyse microbe-infected cells within 1-2hr of infection (i.e. hours before infectious progeny are released) [31]
6. CD8+ CTLs and CD4+ T-helper responses closely correlated with partial control of initial viraemia (and prior to development of antibody responses) [32, 33].
7. Vaccines capable of eliciting specific viral CD8+ CTL responses can control viral replication and prevent onset of disease [34, 35].

PROTECTION FROM INFECTION VS PROTECTION FROM DISEASE

1. Antibody and Sterilising Immunity

Antibodies are the only part of the adaptive immune system capable of neutralising free virus. Hence, only antibody-inducing vaccines offer the potential of completely sterilising immunity and protection from any infection. Antibodies to HIV-1 are generated in nearly all infected individuals but they are typically unable to eliminate the virus or neutralise it in its natural state. There are likely several reasons for this. First, most antibodies to HIV-1 are directed against epitopes that are not well presented on mature virions. These epitopes have been termed "viral debris" [36] and consist of parts of the envelope protein that are not exposed on the trimeric envelope protein at the surface of the virion and can only bind to shed molecules of gp120, other conformations of the envelope proteins (eg the "sprung" state of gp41) and unprocessed gp160. The phenomenon of "original antigenic sin" leads to antibodies that are raised to the more abundant, unprocessed gp160 and these bind with high affinity and act to divert antibodies from binding to more hidden epitopes, which might have greater neutralising capacity [36]. Second, the titre of antibody required to neutralise HIV-1 may be greater than that achieved by vaccination or infection. Human studies have shown that antibody titres in sera from HIV-1 infected individuals are10-100 fold below that required to effectively reduce *in vitro* viral infectivity [37]. Similarly, passive transfer of pooled human immunoglobulin preparations to chronically infected individuals have not resulted in a lowering of HIV viral load nor conferred protection to hu-PBL-SCID mice [36]. Simian studies involving passive transfer of neutralising antibodies have shown some or even complete protection but usually in the setting of very high antibody doses [36] or low dose viral challenge [37]. For these reasons, there are serious doubts as to the ability of any vaccine to elicit the quantity of antibody required to eliminate the virus and provide sterilising immunity. Recently, it has been calculated that a neutralising antibody titre of 1:38 in plasma is required to protect macaques from SHIV [38], yet it required a dose of 297mg/kg of immunoglobulin to achieve this. It is unclear if a vaccine can induce this.

Nevertheless, the argument for a vaccine that at least in part works via an antibody mechanism is compelling. Antibodies can eliminate virus in 3 ways. The so-called neutralising antibody (nAb) is the first and most extensively studied. Most nAbs discovered to date bind to highly conserved *env* epitopes that are critical for HIV binding to its receptor (CD4 molecule) and co-receptor (CCR5). Such nAbs work by blocking the virus's capacity to bind or fuse to its host target, essentially clogging up the virus-host interaction. Two such nAbs are directed against epitopes of gp120: the b12 nAb targeting the CD4 binding site and the

2G12 nAb targeting carbohydrate residues at the base of the V3 loop [36]. A third nAb (2F5) is directed against a highly conserved linear 7 amino acid sequence of gp41 [39] and binds to the gp41 coiled- coil formed during virus-cell fusion [40]. Kinetic studies, however, have determined that this fusion reaction is completed within 20 minutes [41] allowing only a small window of opportunity for this nAb to bind and neutralise.

Although these nAb are potent and neutralise a broad range of primary isolates from around the world, they are by no means universally effective (eg 2F5 epitope neutralises 72% of isolates [42] and 2G12 neutralises clades A and B, some C and D but not clade E [43]) and, to date, they have also only been isolated from a single infected donor each, suggesting that they are poorly immunogenic [36]. Furthermore, single amino acid changes in gp41 [43, 44] can allow the virus to escape recognition from nAb. Two recent studies have clearly demonstrated that serial escape from nAb occurs in HIV-1 infected people [8, 9]. At almost every time point examined during the course of infection in those studies, sera were able to neutralise virus isolated at all previous times, but not able to neutralise subsequent viral isolates.

The vast majority of the other nAbs detected *in vitro* fail to neutralise primary isolates. The explanation for this lies in the conformational differences between the CXCR4 co-receptor using T-cell line adapted (TCLA) strains of HIV, upon which most of the laboratory work has been done, and primary strains of HIV that use the CCR5 co-receptor. An example of this is the V3 loop. Although antibodies directed against the V3 loop play a major role in the neutralisation of TCLA strains, it is not an important target for primary isolates, due to decreased accessibility [45]. Secondly, TCLA strains are postulated to have a lower number of envelope oligomers per virion but with higher CD4 binding affinity [46], making them in turn more susceptible to antibody neutralisation [47]. Additionally, the gp120-gp41 complex is more open, increasing antibody accessibility to the critical CD4 binding site (CD4-bs), whilst the CD4-bs on primary isolates is closed being masked by the V1V2 hypervariable loops, is more recessed and has a quaternary structure that restricts access to antibody [37]. A further mechanism by which nAb is rendered ineffective against field strains of HIV is via steric inhibition, as typified by the 17b nAb (which is directed against a CD4-induced conformational change in gp120 that overlaps the co-receptor binding site). The proximity of the CD4 molecule to the gp120 oligomer, at the point of exposure of this critical epitope, may sterically inhibit the subsequent attachment of neutralising antibody [48]. Hence many nAbs directed against laboratory derived strains of HIV have failed to neutralise field strains of HIV. This may be one reason why a recently completed human phase III efficacy trial of gp120 protein failed to provide protection.

A second method by which antibody can produce sterilising immunity is *via* complement-mediated virolysis. The life cycle of HIV has provided it with a mechanism to avoid this. When the virion buds from its host cell, it takes host cell membrane components with it, including CD46, CD55 and CD59. It is also able to acquire factor H from plasma (which attaches to envelope glycoproteins). These host factors inhibit the full activation of the complement cascade, making HIV resistant to antibody-mediated virolysis [37].

Thirdly, antibodies may work via antibody dependent cellular cytotoxicity (ADCC). ADCC antibodies effect the interaction between HIV-infected cells expressing envelope epitopes and natural killer (NK) cells [49]. ADCC has been associated with slower progression of HIV infection and is effective at preventing cell-to-cell spread of HIV [50]. Binding affinity and accessibility of the NK cells to the FcR of the antibody, once it has bound its target, appear to be critical factors in the effectiveness of this antibody response [49].

An unfortunate footnote to the last two points is that antibodies, mediated via complement or Fc receptors, have been shown to enhance HIV infectivity (IEAs) [49]. This is of great concern when attempting to design a vaccine that elicits antibody responses.

2. Cytotoxic T-Lymphocytes and Non-Sterilising Immunity

By contrast, CTL-based vaccines require cellular entry and processing of virus prior to the generation of an immune response and hence are not able to prevent infection but they can prevent disease. HIV vaccines based on CTL responses demonstrate impressive protection from disease in highly virulent primate models, but they do not protect against a vigorous, albeit blunted, burst of initial viraemia. However, when infection of any cell with HIV or related lentiviruses occurs, there is potential to generate latent, immunologically silent infection. Even very limited infection with lentiviruses, insufficient to cause disease initially, may recrudesce at later time points. Indeed, late escape of viral control and progression to AIDS, despite good initial control of viraemia, has now been reported with chimeric SIV/HIV (SHIV) in macaque models [51]. Despite these limitations, approaches to induce high-level HIV-specific T-cell responses are likely to significantly delay disease progression as well as potentially limiting HIV spread to secondary contacts.

To this end, promising data have been generated using vaccination strategies involving priming with plasmid DNA coding for HIV-1 proteins, followed by boosting with live viral vectors genetically manipulated to also express HIV-1 antigens. Using this combined prime-boost strategy, macaques have been protected from SHIV infection [22, 52] and have100-fold reductions in HIV-1 viral loads after challenge [53]. This has been in association with strong CD8+ CTL responses. The remainder of this article will focus on this.

EVALUATION OF HIV-SPECIFIC CD8+ CTL RESPONSES

The preclinical evaluation of candidate HIV vaccines has focussed not only on their ability to protect macaques from virus challenge, but also on their capacity to induce strong CD8+ CTL responses. There has been an explosion in the technology to identify, quantify and phenotype HIV-specific T-cell immunity. Newer technologies, such as ELISpot, intracellular cytokine staining and tetramer staining can together provide very detailed analysis of T-cell immune responses. Nowadays, the evaluation of human early phase

clinical trials is heavily dependent on T-cell assays, which is novel in vaccine development. The interpretation of results requires a detailed understanding of the techniques.

The lymphoproliferative assay involves measuring proliferation of peripheral blood mononuclear cells (PBMCs), in particular CD4+ Th cells, in the presence of recall antigen, over a 7 day *in vitro* culture. Although simple to perform, the assay is insensitive and frequently suffers from high and variable background. The technique has been refined by the addition of the fluorescent dye CFSE, which allows for the tracking of the number of cell divisions and phenotyping of cells.

For many years, evaluation of CD8+ CTL responses has relied on the ^{51}chromium release assay. Whilst this assay is able to assess functional killing of radiolabelled autologous target cells, it is very time consuming, labour intensive and technically demanding, requiring the establishment of B-lymphoblastoid cell lines in outbred subjects, the use of a toxic radio-isotope and one or two weekly cycles of *in vitro* re-stimulation. The assay also suffers from poor sensitivity due to variable levels of background chromium release and cell lysis, requires a technically difficult limiting dilution assay to obtain even semi-quantitative data and is unable to differentiate the phenotype of the effector cells.

The ELISpot assay has been used extensively in evaluating the immunogenicity of candidate vaccines for HIV in recent studies. It typically detects interferon-gamma (IFNγ) liberation from PBMCs by capture onto IFNγ antibody coated plates, in response to stimulation with HIV (or SIV) antigens (Fig. **3**) and hence does not directly measure functional killing by CTLs. Whilst this is a sensitive assay (typically able to detect the production of IFNγ by as few as 5-10 cells in 200,000 PBMCs or 0.005% above background), the assay does not phenotype cells and generally underestimates the magnitude of the response. A further disadvantage is that the assay measures antigen-specific IFNγ production, which although it may be an important functional component of immunity, is not identical to functional killing.

Another method used for measuring CD8+ CTL responses is MHC class I tetramer analysis, using peptide loaded, labelled MHC molecules to detect antigen-specific cells by flow cytometry (Fig. **4**). A great advantage of this assay is that no *in-vitro* stimulation step is required to quantify responses. However the technique requires knowledge of both the CD8+ epitope and its restricting MHC class I allele and is not readily applicable in an outbred population of animals, thus requiring the researcher to focus on an inbred population containing the same MHC allele. Additionally, some alleles, in and of themselves, can confer survival advantage, irrespective of the vaccine being trialled, such as has been noted in monkeys expressing the *Mamu A*01* allele, which is commonly used to study candidate SIV vaccines in rhesus macaques [54], raising concerns as to how to compare outcomes across trials. Also, this assay cannot be used to measure CD4+ response currently, as MHC-II tetramers have proved difficult to construct [55]. CD4+ responses are of great interest, as significant data suggest such responses are beneficial in controlling acute HIV-1 infection [56]. The assay also does not provide any measure of the functionality of the CD8+ CTLs.

More recently, the intracellular cytokine-staining (ICS) assay has been developed. Cytokine production by lymphocytes is accurately quantified by flow cytometry, in response

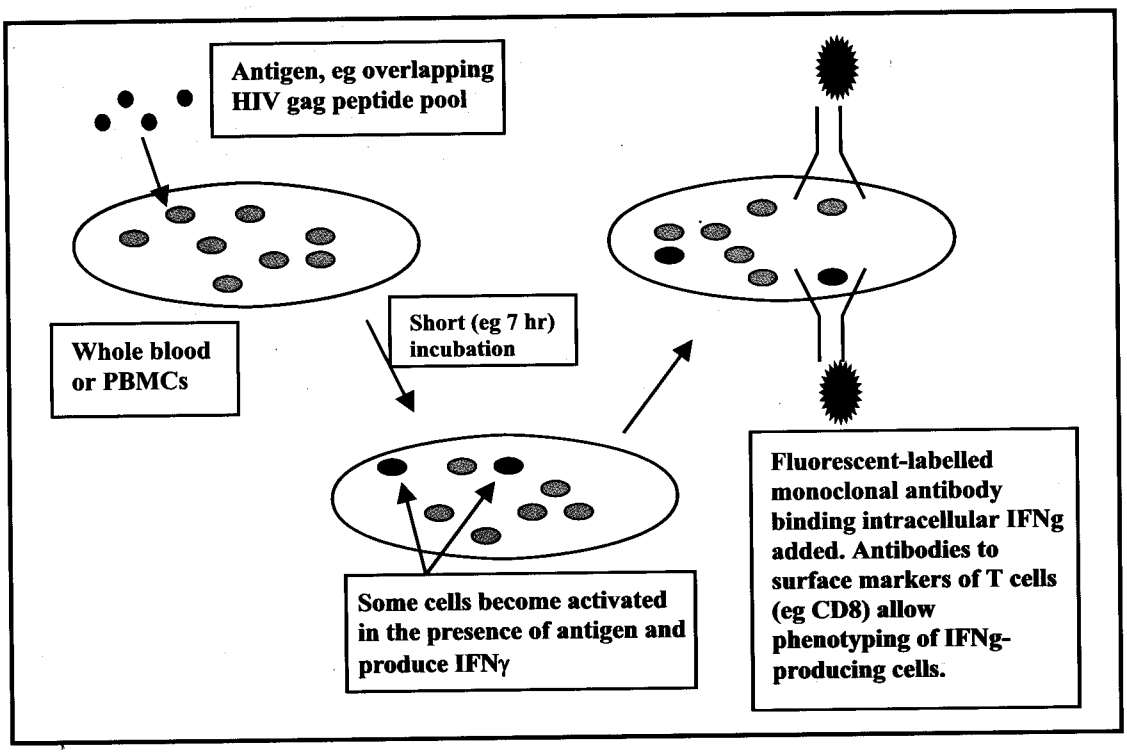

Fig. (3). Schematic representation of intracellular cytokine staining assay. The short assay allows quantification and phenotyping of T cells specific for HIV.

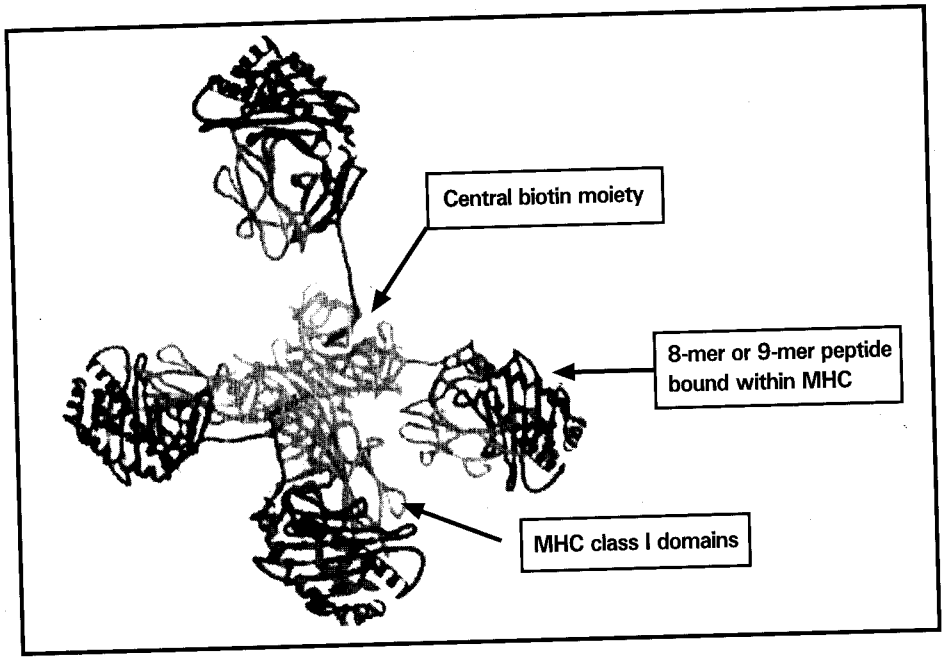

Fig (4). A tetramer molecule composed of 4 identical MHC class I domains bound to a central biotin moiety. These domains are capable of binding a specific 8-mer or 9-mer peptide. Fluorescent MHC class I tetramers can be used to quantify vaccine–induced HIV specific CD8 T cells which bind to this complex.

to stimulation with HIV/SIV antigens (typically overlapping pools of 15-mer peptides) (Fig. **5**). The advantages of this assay are that it is performed on whole blood from an outbred population of animals, it only requires a short incubation time and it allows for phenotyping of the effector cells. It is also able to accurately quantify the magnitude of the response, particularly when responses are at least 0.1% above background. The assay can also be halted at certain time points and analysis completed when convenient. Disadvantages of this assay include the need for a short *in-vitro* stimulation, which in itself can alter the phenotype of the cells being studied. It also does not directly measure T-cell killing but rather uses the liberation of certain cytokines to indirectly measure functional killing. This can be an important failing as recent work has found that CD8+ CTLs may liberate IFNγ (the most commonly studied cytokine) but be dysfunctional in their ability to kill their targets [57].

The *in-vivo* killing assay is another technique, which has recently been developed to measure functional killing. The technique has largely been performed in mice [58], although our group has recently evaluated this in macaques [59]. This involved harvesting PBMCs from a study animal and pulsing half the cells with HIV peptides for 30 minutes. The pulsed and non-pulsed cells are then labelled with different fluorescent dyes and re-infused into the same animal. Serial blood samples are then drawn over 24 hours and the survival of pulsed cells is compared with non-pulsed cells, by flow cytometry. Whilst the measurement of *in-vivo* functional killing is highly advantageous, the disadvantage of this assay is that the procedure is time intensive, invasive and untested in humans. The assays employed in evaluating CD8+ CTL responses are summarised in Table **1**.

The HIV antigens used to restimulate T-cells *in vitro* have also undergone significant refinement in recent years. Previously, vaccinia recombinants or baculovirus-produced whole proteins were used to stimulate CD8+ and CD4+ T-cell responses. Vaccinia recombinants are infectious and required vaccination of staff and baculovirus-produced

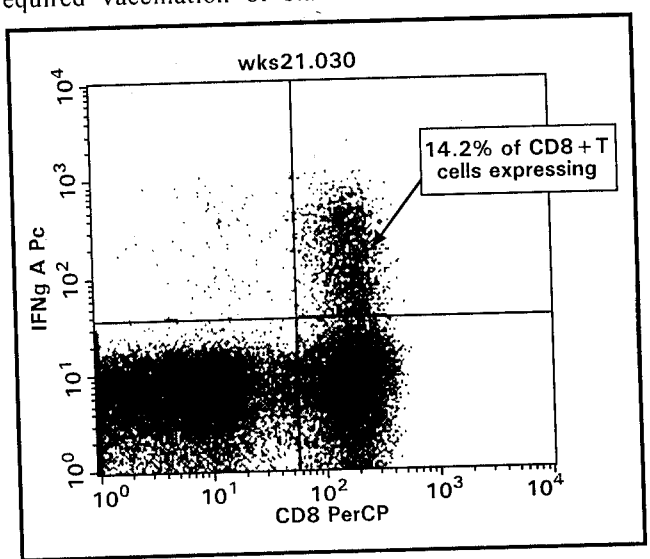

Fig. (5). Sample intracellular cytokine staining flow cytometry plot. This example shows that 14% of CD8+ T cells (upper right quadrant) in the blood of a monkey vaccinated with DNA and fowlpox virus vaccines express IFNγ in response to stimulation with a pool of 15-mer peptides spanning the length of the SIV Gag protein.

proteins are frequently poorly purified. Today, pools of overlapping 15-mer peptides or conformationally intact whole virus can provide a great deal of additional information. Table **2** summarises the antigens available for the abovementioned assays.

Table 1. Assays used to Measure CTL Responses

Assay	Advantages	Disadvantages
Lymphoproliferative assay	1. Simple 2. Measures functional proliferation	1. Cannot phenotype 2. High and variable background 3. Low sensitivity 4. Must isolate PBMCs 5. 7 day culture
CFSE assay	1. As above 2. Measures number of cell divisions 3. Can phenotype cells	1. High and variable background 2. Low sensitivity 3. Must isolate PBMCs 4. 7 day culture
^{51}Chromium release assay (CRA)	1. Directly measures functional killing	1. Labour intensive 2. Need individual cell lines 3. Uses radioactive Cr 4. High background 5. Semi-quantitative only 6. Cannot phenotype 7. Time consuming/tedious 8. Must isolate PBMCs
ELISpot	1. Extremely sensitive 2. 15 hr incubation 3. Measures breadth of response 4. Can be performed in outbred setting 5. Suitable for high throughput	1. Cannot phenotype 2. Must isolate PBMCs 3. Does not measure functional killing 4. Requires *in-vivo* stimulation 5. May underestimate responses
Tetramer staining	1. No *in-vivo* stimulation step 2. Highly sensitive 3. Can do some phenotyping 4. Can be performed on whole blood	1. Must know CD8 epitope 2. Must know MHC allele 3. Cannot measure breadth of response 4. Cannot measure CD4 response 5. Need partially inbred animals
Intracellular cytokine staining assay (ICS)	1. Performed on whole blood 2. Short (7 hr) incubation 3. Sensitive (0.05% - 0.1% above background) 4. Can phenotype response 5. Can do other phenotyping 6. Measures breadth of response 7. Can be performed in outbred setting	1. Does not measure functional killing 2. Requires *in-vivo* stimulation 3. Not used easily for high throughput 4. Determining cut-off for positive cells can be difficult 5. Specialised phenotyping may be confounded by *in-vitro* stimulation
In-vivo killing assay	1. Measures functional killing *in vivo*	1. Untested in humans 2. Concerns about toxicity 3. Technically demanding

OTHER CONSIDERATIONS IN CD8+ CTL RESPONSES

The evaluation of CD8+ CTL responses goes beyond merely quantifying the magnitude of the response. Optimal protection from HIV is likely to require not only a certain quantity of HIV-specific T-cells. Focus is also on the quality of the response including breadth of response, the number and nature of the epitopes against which the response is generated, the dynamics and kinetics of the response, the specific phenotype of the effector/memory cells that develop, the cytokine profile of the response and the durability of the response.

(i) Breadth of Response

Certain parts of the HIV genome (especially the envelope gene) are capable of high levels of mutation whilst maintaining viral fitness. Thus, vaccines that are capable of generating immune responses to numerous genes are highly desirable. The earlier on in the virus's life cycle that the vaccine targets, the greater the potential for minimising peak viral loads and mutations. Proteins such as *tat, rev* and *nef* are expressed early in the viral life cycle and T-cells responses against these proteins are likely to be more efficacious in limiting viral replication. It is important, however, to make sure that, in attempting to express high levels of multiple genes, safety is not compromised, as HIV is capable of reconstituting gene deletions with the potential for the reformation of a virulent particle [19]. Concerns about insertional mutagenesis mean that the integrase gene is usually omitted from HIV vaccines, despite it being a highly conserved and immunogenic gene. Yet, in trying to maximise the breadth of response, the efficacy of a vaccine

Table 2. Antigens Available to Induce Immune Responses *In Vitro*

Antigen	Immune response studied	Advantages	Disadvantages	Reference
Proteins (eg Baculovirus-produced)	CD4 T-cells	1. Readily purchased 2. Standardised reagent	1. High background if poorly purified 2. Proteins poorly glycosylated	[53]
Vaccinia recombinants	Generally CD8 T-cells	1. High level of expression intracellularly 2. Available from National Institute of Health (NIH) AIDS reagent repository	1. Infectious requiring vaccination of lab staff 2. Background response to vaccinia in vaccinia immunised hosts	[60]
20-mer peptides overlapping by 10aa	CD4 better than CD8	1. Available from NIH AIDS reagent repository 2. Can cover entire gene product with less peptides	1. Not ideal for CD8+CTL responses 2. Expensive 3. May require high peptide purity 4. Not ideal for epitope mapping	[61]
9-mer peptides	CD8 cells only	1. High sensitivity 2. Ideal for tetramer staining	1. Not suitable for CD4 responses 2. Need to know exact epitope and MHC allele	[61]
15-mer peptides overlapping by 11aa	CD4 and CD8	1. Available from NIH AIDS reagent repository 2. Detects CD8 and CD4 responses 3. Can be used for mapping of responses 4. More easily defines epitopes	1. Requires many peptides to cover entire gene product 2. Expensive 3. Tedious dissolution of large peptide pools 3. Issues of amount of DMSO needed to dissolve peptides	[61]
Number of peptides in each pool: 20-50	CD4 and CD8	Potentially marginally more sensitive than larger pool size	More restimulations needed, each requiring taking into account background response	[62]
Number of peptides in each pool: 120-250	CD4 and CD8	Less restimulations required compared to smaller pool size	Potentially less sensitive than smaller pool size	[62]
Whole inactivated virus	CD4 and CD8	Mimics recognition of whole virus	1. Requires processing of viral proteins 2. Not all viral proteins expressed in intact virus	[63]

can be compromised by issues of immunodominance and epitope competition (see (ii) below).

A vaccine's ability to induce responses capable of targeting a variety of field strains of HIV is likely to be of critical importance, something that antibody based vaccines, to date, have been incapable of doing. Vaccines expressing highly conserved portions of the HIV genome that are readily expressed by the virus and essential for viral fitness and that are recognised by broad HLA phenotypes are desirable. Although unattractive from a practicality viewpoint, a vaccine containing a cocktail of locally prevalent HIV strains may ultimately be necessary.

(ii) Number and Nature of the Epitopes

Treatment of HIV-1 infected people with anti-retroviral therapy has shown that a combination of at least 3 drugs is required to substantially reduce the risk of drug resistance, a process that is achieved by serial mutation of the genome providing a selective advantage. Escape from CD8+ CTL immune control has been documented in vaccinated monkeys [51] by similar genomic mutation and it is felt that the more epitopes to which the immune response is generated, the less likely it would be for the virus to escape immune control. There are certain parts of the genome that are highly conserved [6] and immune responses against these specific epitopes would be desirable [13].

Maximising the breadth of the response needs to be balanced with issues of immunodominance and epitope competition. Certain epitopes can dominate the immune response and suppress subdominant responses, a feature mediated, at least in part, by IFNγ and seen not only in natural infection but also in DNA vaccines [64]. Balanced expression of dominant and subdominant epitopes has been shown to enhance immunogenicity [65, 66]. Further to this, a murine study of hepatitis B virus found that an epitope-based vaccine induced approximately 20-fold more potent immune responses than a vaccine encoding the entire protein [67]. In addition, we have often observed greater immune responses to single epitopes than to pools of epitopes that contain the single epitope within them, perhaps suggesting that too many peptides may compete for binding sites and dampen the response (Stratov, unpublished data).

Vaccines designed to express only certain epitopes are termed polytope vaccines [68]. Such a vaccine has been

trialled for HIV in a murine model, however only 4/7 epitopes were detectable by tetramer analysis post-vaccination [69]. The use of contiguous, conjoined epitopes may have contributed to this low detection, as evidence has emerged that the insertion of junctional spacer motifs enhances immunogenicity to all epitopes [70]. The obvious disadvantage of polytope vaccines is the large number of epitopes required to cover diverse HLA types.

(iii) The Dynamics and Kinetics of the Response

The kinetics of the immune response over time is important in that animal data exist showing that the number of virus specific T-cells circulating at the time of challenge is important in the host's ability to control infection. Such a phenomenon is seen with HIV vaccines utilising recombinant viral vectors, with higher peaking of immune responses and possibly even multiple peaking [71].

The dynamics of the response in particular involves the interaction between CD4 and CD8 T-cell responses. Higher CD8+ CTL responses are likely if there is concurrent or perhaps prior priming by CD4+ Th cells. We have observed such a pattern in our studies, with an early peak in CD4 responses followed a week later with a larger peak in CD8 responses, in monkeys primed with DNA and boosted with a recombinant fowlpox virus vector (Stratov, unpublished data).

(iv) Phenotype of the Response

One of the great challenges facing HIV vaccine design is inducing an immune response that will tolerate a long latency between administration of the vaccine and any future exposure to HIV. As mentioned, human studies have suggested that naturally occurring immunity to HIV-1 wanes when a person is no longer regularly exposed to HIV, coinciding with a waning in CD8+ CTL responses [26]. The ability of the immune system to respond to antigen upon re-encountering it is a function of the memory population that is generated. Research has focussed of two types of memory cells, namely effector memory (CD28 –ve, CD62L lo, CCR7 -ve) and central memory (CD28+ve, CD62L hi, CCR7 +ve) cells [72, 73]. The effector memory population is the most desirable population to induce, being present in the peripheral tissue with a high potential for immediate killing of virus infected cells. Unfortunately, early down regulation of surface molecules (especially CD62L) during *in-vitro* stimulation means that tetramer staining (not ICS) is best suited to these analyses. Additionally, HIV is primarily transferred across mucosal surfaces, hence T-cells that carry mucosal homing molecules ($\alpha 4\beta 7$) are considered desirable [74, 75].

There are also issues of affinity/avidity when qualifying the immune response. Cells expressing the CD8β surface molecule are thought to more avidly bind epitopes [76, 77]. CD8+ CTLs expressing CD8β have been further divided into high and low expressors, the latter exclusively associated with effector memory [78].

(v) Differential Cytokine Liberation

The ELISpot and ICS assays mentioned above typically measure the production of interferon–gamma (IFN-γ). Data have emerged showing that T-cells capable of liberating IFN-γ can, however, be impaired in their functional killing ability. Researchers have looked at other cytokines such as interleukin 2 and 4 (IL-2, IL-4), tumour necrosis factor alpha (TNF-α) and perforin and some data exist showing that CD8+ CTLs may preferentially liberate one cytokine over another upon antigen recognition. Defining highly functional CD8+ CTLs is part of effective HIV vaccine design.

(vi) Durability of the Response

A great unknown in HIV vaccine design, particularly CTL based vaccines, is the durability of the response. As mentioned above (see (iv)), CTL responses wane with time, potentially with a reciprocal increase in risk of acquiring infection. Animal studies have looked at latencies up to 7 months between end of vaccination and challenge with virus. A potential downfall with CTL based vaccines is inducing immune responses that will survive long latencies. Initial vaccination campaigns may seem effective, when a large proportion of the population is infected, but as prevalence decreases and hence exposure decreases, immunity may subside and prevalence may increase. Although potentially very difficult to implement, frequent booster vaccination may be required to maintain immunity until this conundrum is resolved. In addition, the issue of re-emergence of HIV from its proviral reservoir within the human genome is of great concern given that, as mentioned above, CD8+ CTL based vaccines alone cannot completely prevent infection of any host cells with HIV. Clinical experience with CMV has shown that latent viral DNA can re-emerge and cause devastating disease when a person's cellular immunity is suppressed (eg in post-transplant or HIV-1 positive individuals). Clinical recurrences of VZV and HSV throughout a person's life are also thought to be due to fluctuations in cellular immunity often provoked by simple stressors such as surgery, pregnancy, fatigue, trauma and ageing. Re-emergence of SIV from CTL control has been demonstrated in a macaque model during rigorous CD8 depletion studies. Similar re-emergence of HIV-1 from CTL control is clearly of concern to researchers. Presumably, the greater the limitation of initial viraemia, the lesser will be the latent reservoir and less late escape will occur, emphasising the need to have robust control of initial viraemia by T-cell inducing vaccines.

VACCINES INDUCING CD8+ CTL IMMUNITY

A number of approaches have been tried to induce CD8+ CTL immunity. These include live attenuated vaccines, whole inactivated vaccines, DNA, peptide and protein vaccines and vaccines utilising recombinant viruses and bacteria. Currently, the most effective method of inducing CD8+ CTL immunity utilises priming with naked plasmid DNA and then boosting with recombinant viruses both encoding various parts of the HIV/SIV genome. A number of key trials in monkeys have been published over the last few years utilising prime-boost technology, involving DNA viruses such as fowl poxvirus (FPV), modified vaccinia Ankara (MVA) and adenovirus type 5 (Ad5), demonstrating high levels of T-cell mediated immunity. Using either modality alone, or in reverse order, is immunogenic, but to a much lesser extent [12, 22, 52]. A summary of simian prime-boost vaccine trials is found in Table **3**.

Table 3. Summary of Simian DNA Prime +/- Recombinant Viral Vector Boost Vaccine Studies

Species/no.	Vaccine design	Immunogenicity	Challenge/latency	Outcome	Comments	Ref.
Macaca mulatta/ 10 animals in 3 groups (4 controls)	DNA (id) prime. MVA (id) boost both coding SIV *gag* epitope. Compared vaccination over 9 vs 22 vs 44 wks	2xDNA prime then 2xMVA boost gp showed 40%-65% specific lysis (by CRA) and 1-5% tetramer +ve CTLs	SIV (ir, 20x MID_{50}) 1 wk post final boost	No protection afforded. Immune response peaked at vaccine levels	Small study; no protection despite short latency; 2 boosts as good as 3 but may be better than 1; 9 wk vaccination period as good as 22 wk	[12]
Macaca mulatta/ 35 animals in 9 groups (14 controls)	DNA (im) in CRL1005 vs MPL/alum vs PBS adjuvant. Boost: MVA (im) vs Ad5. All code SIV *gag*. 3 or 4 doses over 32 wks	3xDNA in CRL1005 adjuvant, followed by 1x Ad5 boost induced 20-30% tetramer +ve CD8+ CTLs; other gps were ≤5%	Highly patho-genic SHIV (iv 50xMID_{50}) 6 or 12 wks post final vaccine	DNA/Ad5 gp induced 1-2 log_{10} decrease in peak VL, 3 log decrease in set point VL and 1-2 log better CD4 counts. Similar in gp given 4xDNA in CRL1005	Only 3 monkeys per vaccine gp; vaccinees and controls poorly matched for expression of *Mamu*-A*01 allele. Ad5 better than MVA; CRL1005 better than MPL/alum	[71]
Macaca mulatta/ 32 animals in 8 groups (plus another 12 controls)	DNA (id vs gg) coding SIV and/or HIV-1 *gag/pol/env/nef*. Boost: FPV (id vs gg) encoded as per DNA or pure HIV-1 *env* protein (id). Five doses over 66 wk	Only 70/156 assays had CTL responses with 44% in 5-10% range (by CRA). Only10/24 vaccinees had >10% lysis including 4/4 in DNA(gg)/FPV gp and 2/4 in DNA(id)/FPV gp	Non-pathogenic SHIV (iv, 10x MID) 2 wks (all animals) and 45wks (10 animals) post final vaccine; 6 monkeys went on to receive highly pathogenic SHIV a further 19 wks later	3 of 4 DNA(id)/ FPV and 2/4 DNA(id)/*env* vaccinees had undetectable VL through 1st 2 challenges and survived SHIV; DNA(gg)/FPV gp did poorly	Poor generation of CTL immunity; confusing role of anti-*env* antibody in protection from challenge; protection from pathogenic SHIV challenge confounded by prior challenges with non-pathogenic SHIV	[22]
Macaca mulatta 28 animals (4 gps of 6 vaccinees plus 4 controls)	DNA x2(im vs id, 2.5mg vs 250μg) coding SIV *gag, pol, vif, vpx* and HIV *env, tat, rev*. Boost: MVAx1 coding SIV *gag, pol* and HIV *env*, given over 24 wks	Best response in DNAx2 (2.5mg id)/ MVA gp (range 0.9% - 19.0% tetramer +ve CD8+ CTLs) peaking at wk 1 post boost; responses in the 2.5mg im gp were 10 fold less and 2-3 fold less in vaccinees receiving 250μg of DNA. Peak ELISpot response in 2.5mg id gp (mean 2495 spots/10^6 PBMCs) > 2.5mg im > 250μg id > 250μg im	Highly pathogenic SHIV (ir, 20x MID) 7 months after boost	All vaccinees survived (cf 1/4 controls). One log reduction in peak and 3 log reduction in set point VL in vaccinees cf controls; mean CD4 count equally preserved across vaccinees. Mostly, peak CD8+ CTL responses boosted 2-10 fold above vaccine levels (at wk 3 post-challenge)	Simple regimen with good protection over long (7 month) latency suggests good CTL memory generated; higher dose DNA induced better CMI than low dose ($p < 0.05$); DNA id was not statistically better than DNA im.	[52]
Macaca mulatta 20 animals (3 gps of 4 vaccinees plus 8 controls); 15/20 expressed *Mamu*-A*01 allele but only 4/8 controls	DNA x4 (SIV with HIV *env*) over 40 wks +/- IL-2/ Ig x2 delivered as purified protein or via a plasmid	Response in IL-2/Ig gps: ~1.2% SIV*gag* tetramer +ve CTLs at wk 2 post 4th DNA cf <0.5% in DNA alone; HIV *env* response 2.5 fold lower. Similar results by CRA	Highly pathogenic SHIV (iv,100x MID) 6 wks post final vaccination	4/8 controls died cf 0/12 vaccinees; 8/8 IL-2/Ig vaccinees preserved CD4 counts/function cf 1/4 DNA alone and 0/8 controls. Peak VL 1 log less in vaccinees cf controls; set point 1 log less in IL-2/Ig gp cf DNA alone. CD8+ CTL *gag* response was	IL-2 gps had significant improvement in VL peak ($p=0.008$), set point ($p=0.004$) and clinical disease ($p=0.001$) cf controls; DNA alone gp had an intermediate outcome; correlation between vaccine-elicited plateau-phase CTL responses and VL peak	

(Table 3) contd....

Species/no.	Vaccine design	Immunogenicity	Challenge/latency	Outcome	Comments	Ref.
				20-30 fold above vaccine level (at wk2-3) in IL-2/Ig gps; HIV *env* response was 30 fold lower than *gag*.	and set points (p=0.007 and p=0.04). *Mamu*-A*01 genotype did not seem to bias results	[35]
Macaca nemestrina (4 controls, 4 vaccinees	DNA x2 (ed, gg), coding HIV-1 *env* and *gag* followed by boost with FPV x3 (id, gg) coding HIV-1 *gag*, *pol*, and *env* over 32 wks	Antibody detected after 2nd DNA in vaccinees. DNA prime induced CD4+ Th responses 2-4 fold above control; FPV boost induced further 6-17 fold increase includ-ing against heterologous *env*. Preferentially secreted IFNγ (Th1 cytokine) cf IL-4 (Th2). Pre-boost: 1/3 monkeys tested had *gag* and *env* responses. Post boost: responses to *gag* and *env* in all 3 (6-14% specific lysis). The FPV boost induced a 3.5-20 fold increase in precur-sor CD8+ CTLs levels	HIV-1 (iv 10^5 x $TCID_{50}$, ≥ 100x MID) 6 wks post final vaccination	4/4 controls had an acute HIV syndrome cf 0/4 vaccinees. Neither HIV-1 RNA nor proviral DNA detected in either plasma or lymph nodes, respectively cf reciprocal for controls. Only 1/36 serial PBMC samples had detect-able proviral DNA cf 36/36 in controls. Non-sterilising CTL immunity evidenced by post-challenge *nef* specific immune responses.	Study limited by non-pathogenic model. Overall, showed a 2 log reduction in VL with modest T-cell (Th1) responses. FPV boosting maximised magnitude/breadth of response; heterolo-gous *env* response highly favourable. The essentially sterilising immunity is at odds with current under-standing of CTL-based vaccines; the role of protective antibody is unclear	[53]

Note. id = intradermal; gp = group; ir = intrarectal; MID = monkey infectious dose; im = intramuscular; iv = intravenous; Ad5 = adenovirus type 5; VL = viral load; gg = gene gun; ed = epidermal

DNA vaccine studies have varied in the number and amount of DNA given, the time between each dose, the route of administration and the number of HIV/SIV genes encoded. The addition of adjuvants to DNA vaccination has been used in an attempt to augment the immune response perhaps by preferentially stimulating T-cell subsets, targeting antigen presenting cells, directing antigen into MHC I or MHC class II pathways, allowing antigen deposition with slow release or stabilisation of epitopes. These have included novel polymers (oligolysine, lipopeptides, polyactide co-polymers, CRL1005) and traditional adjuvants (aluminium phosphate, aluminium hydroxide, QS-21, MF59, monophosphoryl lipid A, mineral oil, mannose mono-oleate, incomplete Freund's adjuvant, purified protein derivative, keyhole limpet haemocyanin, bupivacaine) [2] as well as peptide linkers, bacterial toxins and macroglobulin complexes. The addition of genetic elements has been used to maximise expression (such as codon optimisation and CpG motifs [79]), as well as co-expression of cytokines [35].

With regards to the boost, the recombinant vector used also varies. Other DNA viruses have been used such as canarypox, herpes viruses, and rabies virus. Researchers have tried poliovirus in an attempt to induce mucosal immunity and work is currently progressing on alphavirus replicons because of (1) their cytoplasmic replication cycle (thus avoiding chromosomal integration of retroviral elements) and (2) the large number of replication cycles they undergo (hopefully maximising expression of HIV genes and hence immunogenicity). Bacterial vectors such as Bacillus of Calmette and Guerrin (BCG) and salmonella have also been used.

The use of recombinant human pathogens (eg vaccinia and adenovirus) is problematic in that antibodies generated as a result of prior exposure to the natural pathogen decrease the efficiency with which the viral vector can act within the individual. The prevalence of antibodies to adenovirus type 5 (Ad5) is estimated to be 40% in the USA [80] and a 100-fold increase in dose of vector is required to induce equivalent immune responses in exposed versus naïve individuals. Similar results have been found in animals previously exposed to MVA [81].

Following vaccination, trials have varied in the time delay to viral challenge, the route of administration and the pathogenicity of the challenge. The pathogenicity reflects the dose of virus given and the nature of that virus. HIV-1 is non-pathogenic in macaques, whilst SIV is very slowly pathogenic. SHIV chimers vary in their pathogenicity but

Table 4. Summary of Vaccines for HIV

Approach (examples)	Vaccine	*Main benefits*	*Main limitations*	References
Live attenuated (eg Sabin, varicella)	*Nef*–deleted live attenuated vaccine SIV	Induce both humoral and CMI. Protection even against heterologous SIV strains	Macaques developed AIDS from the vaccine; humans with *nef*-deleted HIV can progress to AIDS	[17, 18, 82, 83]
Whole inactivated virus (eg influenza, Salk polio vaccine)	Whole inactivated SIV vaccine for macaques	Full range of antigens in natural conformation; conferred good protection in macaques against homologous strains of SIV	Human immunogenicity trial showed absence of CD8+ CTLs and absence of nAb to patient isolates of HIV. Safety concerns about inadequate inactivation. Macaques not protected against even slightly divergent strains	[20, 84-86]
Recombinant subunit protein (eg hepatitis B)	Recombinant *env* glycoprotein	Safe; modest protection in chimpanzees to HIV-1 induced against a homologous strain	Human trials showed modest antibody titre with limited neutralising capacity; no CD8+ CTLs induced. No protection in human phase III trials	[10, 87, 88]
Plasmid DNA (new technology)	Plasmid DNA coding HIV-1 *env* and *rev* delivered im to humans (3 doses over 30 weeks)	Safe; relatively simple and cheap to construct; induce CD8+ CTL responses in primates and protect chimpanzees against heterologous HIV-1 challenge; can be formulated with various promoters, nucleotide motifs, adjuvants, targeting molecules and cytokines	Limited immunogenicity in humans at high doses	[35, 89, 90]
Peptide vaccines	V3 peptide	Safe	Broad nAb not induced. So far, weakly immunogenic in human trials	[91]
Recombinant viral/bacterial vectors (new technology)				
1. recombinant vaccinia virus (rVV)	rVV vaccine expressing SIV; rVV vaccine expressing HIV-1 *env* in humans	Potent CD8+ CTL response in monkeys; human data now available	HIV-1 positive individuals can develop disseminated smallpox Low magnitude and transient CD8+ CTL responses and no nAb in human trials	[92-94]
2. modified vaccinia Ankara (MVA) alone	MVA *gag/pol/env* alone vs primed with DNA vs boosted with *env* protein in simian model	Statistically similar outcome post-pathogenic SHIV challenge in terms of survival and VL for all three vaccine groups	MVA alone induced a10-fold decrease in vaccine specific CD8+ CTLs cf prior DNA priming. MVA prime boosted with *env* protein trended to a lower VL cf MVA alone, with better generation of nAb	[71, 95, 96]
3. gene deleted vaccinia (NYVAC)	NYVAC expres-ing SIV *env, pol* and *gag*	Strong CD8+ CTL induced; partial success after intrarectal challenge with pathogenic SIV	Augmentation with IL-12 did not lead to a better outcome, although better CD8+ CTL responses were detected (at the expense of antibody response)	[97]
4. recombinant canarypox (rCPV)	rCPV (*gag,pol,env nef*) +/- *env* (gp120) protein; human and simian trials	Extensive human data; non-human pathogen; safe; CD8+ CTL responses elicited to many genes. Simian data: rCPV alone induced protection post-SIV challenge equal to rCPV plus gp120; cross clade CD8+ CTL activity detected	rCPV simultaneously given with gp120 protein seemed better than rCPV alone; <50% developed any CD8+ CTL responses; only 20% of recipients had CD8+ CTL responses out to 3-6 months; cross strain nAb in only 10-15% of recipients	[54, 98, 99]
5. Fowlpox (FPV)	See table 3			
6. recombinant Herpesvirus (rHSV)	rHSV-HIV gp120 recombinants in mice	Persistent humoral and cell mediated responses detected after a single im vaccine; effective at low inocula and in the presence of prior HSV infection	Limited primate data; some safety concerns. Replication incompetent vaccines may be less effective	[100]

(Table 4) contd....

Approach (examples)	Vaccine	Main benefits	Main limitations	References
8. Alphaviruses				
(a) Sindbis Virus replicons (SIN)	SIN-HIV *gag* in mice	SIN-*gag* vaccine (pv or ir) induced protection from viral replication in mice; alphavirus (ie cyto-plasmic replication only)	Limited published primate data to date; administration of SIN-*gag* (im or in) induced higher vaginal mucosa and lymph node CD8+ CTL responses but did not prevent viral replication	[101]
(b) Semliki Forest Virus replicons (SFV)	SFV-HIV *tat* +/- *rev* then boost with MVA *tat/rev* cf DNA/ MVA *tat/rev* (in SHIV challenge model)	Equivalent immuno-genicity and post-challenge outcome across the 3 vaccine gps; novel alpha-virus vaccine exclusively using accessory genes	Peak VL reduced 2 log but only in 6/18 vaccinees	[102]
(c) Venezuelan Equine Encephalitis Virus replicons (VEE)	VEE-SIV *gag/env* x3 doses (patho-genic SIV model in macaques)	5 month latency to challenge. 6/6 and 4/6 vaccinees developed antibody and CD8+ CTL responses respectively; lower VL (peak/set point) and higher CD4 counts in vaccinees cf controls	Small study; some regulatory concerns and safety	[103]
9. Salmonella	Salmonella-SIV *gag* x3, then boost with MVA *gag*	Salmonella priming induced transient low-level CD8+ CTL; large boost post MVA; good expression of $\alpha 4\beta 7$ phenotype. Salmonella can also be used to carry DNA for mucosal delivery	No protection post challenge with SIV. Less effective than attenuated shigella	[75, 104]
10. Recombinant rabies virus (rRV)	rRV-HIV gp160 +/- boost with rgp120 protein in mice	rRV-HIV gp160 alone induced strong, long lasting CD8+ CTL response including vs heterologous gp160; protein boost induced high nAb titre (1:800)	No primate data; safety concerns as rRV vector is replication competent; trying to address this with attenuated strains without compromising immunogenicity	[105-108]
11. recombinant Poliovirus (rPV)	rPV expressing *env* gp17/gp41 (in x2, followed by iv x1) in macaques	IgA detected in rectal and vaginal secretions (4/4 and 2/4 surviving animals, respectively)	1/5 monkeys died from paralytic polio. Only 2/4 survivors developed CD8+ CTL response after all 3 doses	[109]
12. recombinant vesicular stomatitis virus (rVSV)	rVSV expressing non-pathogenic SHIV *env* (x2) followed by boosting with rVSV *env/gag*(x2) then (iv) challenge with highly pathogenic SHIV in macaques	0/7 vaccinees developed AIDS cf 7/8 controls. Peak and set point VL reduced by 1-2 log and $\geq$3 log respectively with 2 log better CD4 counts; equivalent results with challenge at 3 or 6 months post last vaccination; nAb appeared > 28d post challenge but initial control of VL seen at < 28d	Modest CD8+ CTL responses: <400 cells per 10^6 PBMCs by ELISpot; no augmentation post 2nd boost; responses increased to 1000 spots post challenge; tetramer responses of <0.1% in the 2 Mamu A*01 vaccinees. Role of nAb unclear: the best vaccinee had the lowest CD8+ CTL response post challenge; it had a large nAb recall post challenge, suggesting some homology between vaccine and challenge virus. The one control that didn't get AIDS also had high nAb	[110]
13. Bacillus Calmette and Guerrin (BCG)	BCG expressing SIV *gag* (id x2) then boost with *gag* peptide (im) followed by SIV (iv) challenge in macaques	BCG prime followed by *gag* peptide boost induced good PBMC responses (25-55% by CRL) cf 12-14% for BCG alone and 18% for *gag* peptide alone; 40-70% of CD8+ CTL subset recognised a single Mamu*A*01* epitope	Only 5 of 10 animals had Mamu A*01 phenotype thus limiting immunogenicity data to 2 animals per group. All challenged animals (no.= 6) became infected but no VL or CD4 data available to assess degree of protection	[111]

Note. IL-12 = interleukin 12; pv = per vaginal; in = intranasal

can be highly pathogenic, moderately pathogenic (following a time course more akin to natural HIV-1 infection in humans) or be non-pathogenic.

An overall summary of the approaches to HIV vaccines, with specific examples of many of the strategies, is given in table **4**.

Table 5. Current Human Trial of HIV Vaccines

Vaccine type/design	Stage/Site of trial	Preliminary results	Reference/Network
1. DNA alone			
- *gag* (clade B)	Phase I/USA	Low ELISpot responses: 21% of volunteers at 1mg dose of DNA (mean 105 spots per 10^6 PBMCs) cf 42% at 5mg	[80]
- > 25 CTL epitopes from *gag, pol, nef, env* (clade A)	Phase I & II/UK, Kenya and Uganda	12 of 18 developed CD8 and CD4 responses, which were generally low	[112]
- gag, protease, reverse transcriptase and integrase	Phase I/USA	Enrolling; no published data	VRC; NIAID
- *gag, pol, nef* (clade B) plus *env* I (clades A, B, C)	Phase I/USA	Enrolling; no published data	VRC; NIAID
- *gag, rev, tat, vpu* and RT (clade B)	Phase I/USA	Enrolling; no published data	Emory Vaccine Center, USA; HVTN
- 21 CTL epitopes from *gag, pol, env, nef, rev, vpr* formulated in homopolymer and a Th cell epitope (PADRE)	Phase I/USA and Botswana	Enrolling; no published data	HVTN
- *nef*	Phase I/Finland	Enrolling; no published data	FIT BIOTECH
2. Protein subunit			
- *env* gp120 protein (2 clade B strains)	Phase III/USA	No overall significant reduction in new HIV infection in vaccinees. Some reduction in black and Asian volunteers due to higher levels of antibodies	Vaxgen press release
- gp120 (clades B and E)	Phase III/Thailand	No published data yet	Vaxgen
- gp120,nef,tat (clade B) in QS21 and monophosphoryl lipid A adjuvant	Phase I/USA	No published data	NIAID
3. Recombinant viral vectors			
Vaccinia - rVV expressing 23 *env* genes (clades B and D)	Phase I/USA	Enrolling; no published data	St. Jude Children's Research Hospital
Canarypox - rCPV expressing gp120, gp41, *gag, nef*	Phase II/ Haiti, USA, Peru Brazil, Caribbean, France	Low CTL responses reported at meeting. HVTN decided not to proceed to phase III	HVTN/NIAID/ ANRS (France) /Aventis
Modified Vaccinia - rMVA expressing p17, p24 and >25 CTL *gag, pol, nef, env* epitopes (clade A)	Phase I & II/UK, Kenya and Uganda	rMVA alone, 4 of 5 volunteers devel-oped low CD8+ CTL responses. In DNA prime, MVA boost gp, 6/6 devel-oped responses	[112]
Adenovirus - Ad5 expressing *gag* (clade B)	Phase I/USA	67% of volunteers developed ELISpot responses (mean = 239) at 10^8 viral particles; higher dose of Ad5 required in individuals with pre-existing Ad5 antibody but with more side-effects	[80, 113]
4. Peptide			
- 3 different vaccines containing mixtures of 4, 5 or 6 clade B CD8+ CTL/CD4 Th epitopes (from gag, nef, env and/or pol) formulated as lipopeptides	Phase I/France	No published data	ANRS (France)

Note. VRC = Vaccine Research Centre; NIAID = National Institute of Allergy and Infectious Diseases; HVTN = HIV Vaccine Trials Network

HUMAN TRIALS AND SPECIFICALLY RELATED ISSUES

Many trials of candidate HIV-1 vaccines have been performed in humans but only one phase III trial of approximately 5000 subjects at high risk for HIV infection has been completed to date. Unfortunately, this bivalent envelope protein vaccine, although it induced some antibody responses, failed to provide protect mainly gay white men in North America from HIV infection (see www.vaxgen.com). A subgroup analysis of black men in the study suggested some possible benefit in that group, however, there were small numbers of black men in the study and a further study will be required to demonstrate efficacy in that group.

A number of earlier phase human trials, testing various vaccine technologies more directed towards the induction of T cell immunity, are currently underway and some preliminary data is now being presented. A summary of these trials is given in table **5**.

The issues of licensing an HIV vaccine, however, extend beyond its efficacy in clinical trials. Safety is of paramount concern when vaccines are administered to large numbers of people. A large percentage of the main target population for an HIV vaccine may already be immunodeficient. The use of recombinant human pathogens as vectors, in such people, is of particular concern, given that many currently available attenuated vaccines (eg Sabin, VZV) are not administered to immunocompromised patients. Hence, protein based vaccines will be more readily licensed, but have so far not proven efficacious.

Similarly, a vaccine requiring complicated and drawn out dosing schedules is undesirable in terms of delivery to large, scattered populations in less developed countries, as the rate of incomplete vaccination clearly increases with increasing complexity.

Finally, there are issues of cost and ease of production. DNA vaccines may ultimately be cheaper and easier to make than vaccines containing viral vectors. Similarly, the issues of patents and the difficulty they have caused in access to anti-HIV medications (especially in the third world) may plague the distribution and availability of an HIV vaccine, once it is finally produced.

CONCLUSIONS

An effective, licensed vaccine for HIV remains a long way off. Current trials of candidate vaccines have suffered from lack of standardisation of assays, use of non-comparable populations of monkeys (both number and genotype), variations in schedule and dosing of vaccine components, as well as the timing, route, dose and nature of the challenge virus and follow-up. The use of parenterally administered, high inoculum and/or highly virulent challenge viruses has been criticised as not representative of real life infection. The issue of matching the vaccine to the challenge virus is particularly relevant. In trials, the vaccine, assay antigens and challenge virus often contain consensus sequences, which are matched to each other (and often to the HLA phenotype of the animal model) and may give excellent laboratory data. However, their relevance globally or even across populations is of great concern, where people with diverse HLA phenotypes will be exposed to multiple genetic variations of HIV. It is now clear that HIV-2 may have evolved to escape T-cell responses and such viruses are being transmitted [13].

It is not surprising that this 20 year quest was described in a recent book: "Shots in the dark: the wayward search for an HIV vaccine" [114]. It is likely that the eventual HIV vaccine will involve a combination of multiple vaccine ideas and technologies and it will take a concerted effort from the scientific, commercial and political communities to develop, test, license and deliver it.

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