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Using recent advances in biological and medical sciences, a new candidate human immunodeficiency virus (HIV) vaccine has been developed and tailor-designed for a phase III clinical trial in Kenya. It has two components, DNA and MVA (an attenuated poxvirus), used in a prime-boost vaccination protocol. Both of these vaccine vehicles express a common 'chimeric' protein derived from small parts of the HIV genome. The vaccine focuses on the induction of cell-mediated immune responses.

Design and construction of an experimental HIV-1 vaccine for a year-2000 clinical trial in Kenya.

TOMAS HANKE & ANDREW J. MCMICHAEL

Development of effective HIV vaccines is one of the primary goals of the global acquired immunodeficiency syndrome (AIDS) research. Despite progress in prevention and powerful drug combinations to treat HIV infection, an estimated 15,000 people become infected every day. Over 90% of new infections occur in developing countries for which the recent medical advances are not immediately applicable or affordable. The best hope for these countries is the development of a safe, effective, accessible, preventive HIV vaccine. There is now a growing optimism among scientists that an AIDS vaccine may be possible[1].

An ideal prophylactic vaccine should induce sterilizing immunity, so that after exposure, the virus would never be detected in the body. However, this is probably an unrealistic goal. Rather, it may be aimed at a vaccine-induced immunity that results in a limited and transient virus replication, after which the virus becomes undetectable, there are no signs of disease and no transmission to other individuals. Alternatively, a potentially successful vaccine may induce immune responses that hold the virus in check at levels so low that both progression to AIDS and transmission are prevented.

To achieve protection, a prophylactic vaccine may have to induce both humoral and cell-mediated immune responses. Because HIV was isolated and sequenced, there has been a considerable effort to develop envelope-based vaccines inducing neutralizing antibodies (nAb). However, this has proved to be nearly impossible. Although some success was reported in inducing nAb against laboratory HIV strains, it has been extremely difficult to neutralize primary isolates. In phase II human trials, at least 16 test subjects immunized with recombinant gp120 subunit vaccines became infected, but did not handle the virus differently from placebo-vaccinated controls, nor were escape mutants selected. An explanation for the first 15 years of frustration has come from the crystal structure of the core gp120, which revealed several mechanisms by which HIV prevents efficient induction of nAb (ref. 2). Although some new promising approaches to induction of nAb are being developed[3,4], the emphasis of many vaccine designers has shifted to the induction of cell-mediated immune responses.

Cytotoxic T lymphocytes (CTL) are usually $CD8^+$ cells and participate in the organism's defense in at least two different ways: they kill virus-infected cells, and secrete a variety of cytokines and chemokines that directly or indirectly contribute to the suppression of virus replication. Indeed, there is growing evidence that CTL are an important component of the anti-viral responses in both HIV-infected people and the simian immunodeficiency virus (SIV)-infected macaque model of AIDS. Several observations suggested that CTL might be able to protect against HIV infection. In the laboratory, CTL killed HIV-infected cells before these produced new virions[5] and released chemokines, which inhibited HIV infection[6,7]. Therefore, *in vivo*, CTL may be able to clear the initial small number of infected cells before HIV spreads further and establishes generalized infection. This might explain detection of HIV-specific CTL responses in exposed, but uninfected commercial sex workers whose cells were fully susceptible to infection with HIV (ref. 8, 9), in uninfected infants born to HIV-infected mothers[10] and seronegative health care workers occupationally exposed to HIV-contaminated body fluids[11]. CTL-mediated protection after vaccination may depend on the levels of CTL present in the circulation and, perhaps, specificity for proteins expressed early (regulatory proteins) rather than late (structural proteins) in the replication cycle[12, 13]. More importantly, broader CTL responses against several epitopes may be required for the control of HIV replication[14].

The induction and maintenance of $CD8^+$ T-cell responses require 'help' provided predominantly by $CD4^+$ T lymphocytes[15]. In some HIV-infected individuals, high levels of HIV-specific helper responses were detected and, in particular, those specific for gag were associated with a decreased viremia in chronic HIV infection[16]. Furthermore, gag-specific $CD4^+$ T-cell responses correlated positively with levels of gag-specific CTL precursors and negatively with levels of plasma HIV-1 RNA (ref. 17). It is relevant to the development of a prophylactic vaccine that some[18,19], but not all[16], exposed seronegative individuals showed HIV-specific $CD4^+$ T-cell responses, which might have contributed to the suppression of HIV replication.

Identification of methods for induction of strong $CD8^+$ T-cell responses would provide tools for studying their role(s) in shaping the course of HIV infection and may stimulate progress towards an effective HIV vaccine. A number of approaches have been tested and some showed potential[20, 21]. We have constructed previously a prototype HIV vaccine as a string of partially overlapping epitopes recognized by murine, macaque and human CTL, which was delivered by vaccine vehicles that were safe and acceptable for use in humans, a DNA vector and modified vaccinia virus Ankara (MVA) (refs. 22, 23). The use of the mixed-species poly-epitope greatly facilitated the pre-clinical development of these vaccines[24]. In mice, the most potent protocol for induction of CTL was found to be DNA priming followed by MVA boosting[25,26]. This regimen showed an impressive protection of mice against a malaria challenge[26] and induced effectively SIV-specific CTL in rhesus macaques[27,28]. These results give

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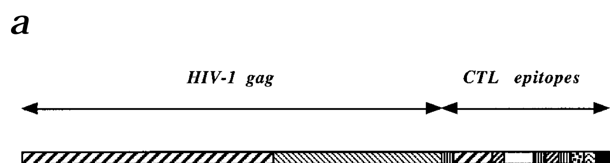

b

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  1 MPIVQNAQGQ MHQALSPRTL NAWVKVIEEK AFSPEVIPMF SALSEGATPQ  50
 51 DLNMMLNIVG GHQAAMQMLK DTINEEAAEW DRLHPVHAGP IPPGQMREPR 100
101 GSDIAGTTST LQEQIGWMTS NPPIPVGDIY KRWIILGLNK IVRMYSPVSI 150
151 LDIRQGPKEP FRDYVDRFFK TLRAEQATQE VKNWMTETLL VQNANPDCKS 200
201 ILRALGPGAT LEEMMTACQG VGGPGHKARV LGTGARASVL SGGKLDAWEK 250
251 IRLRPGGKKK YRLKHLVWAS RELERFALNP SLLETAEGCQ QIMEQLQSAL 300
301 KTSEELKSLF NTVATLYCVH QRIDVKDTKE ALDKIEEIQN KSKQKTQQAA 350
351 ADTQSSSKVS QNYALKHRAY ELEFPPIPVG EIYKRWIIFR DYVDRFYKTL 400
401 RAIFQSSMTK ITLWQRPLVE RYLKDQQLLT VYYGVPVWKR PQVPLRPMTY 450
451 KAVDLSHFLK EKGGLILKEP VHGVYHPDIV IYQYMDDLTP GPGVRYPLAC 500
501 TPYDINQMLR GPGRAFVTIP NPLLGLD                         527
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Fig. 1 A schematic representation of the HIVA protein immunogen. ***a***, The HIV-1 origin of the individual HIVA parts: ▨, p24; ▨, pol; ▥, nef; ▧, mouse epitope; ■, mAb epitope; ▧, p17; □, env; ▩, macaque epitope. ***b***, The amino acids of the immunograms. The Amino acids of the immunogen. Amino acids corresponding to the restriction endonuclease sites used to assemble the gene are shown in bold.

hope that the multi-epitope DNA and MVA vaccine approach may induce similar levels of cellular responses in humans. Here, the design rationale, construction and immunogenicity of the HIV vaccine that will enter phase I clinical trials in the United Kingdom and Kenya in year 2000 are described and discussed.

The HIVA immunogen

The vaccine focuses on the induction of cellular immune responses mediated by a concerted action of CD4$^+$ helper and CD8$^+$ effector T lymphocytes. The immunogen, designated HIVA, was tailor-designed for a phase III efficacy trial in Nairobi, Kenya. It is derived from the sequences of HIV-1 clade A, the predominant HIV clade in Nairobi[29], and consists of about 73% of the gag protein fused to a string of 25 partially overlapping CTL epitopes (Fig. 1*a*). The gag domain of HIVA contains p24 and p17 in an order reversed to the viral gag p17-p24-p15 poly-protein. This rearrangement prevents myristylation of the N-terminus of p17, which could direct the recombinant protein to the cell membrane, thus preventing efficient degradation into peptides leading to the major histocompatibility complex (MHC) class I presentation. The amino acid sequence of the gag domain (Fig. 1*b*) was derived from the protein database consensus sequence of HIV-1 clade A. In the absence of available Kenyan strain sequences, regions without a strong amino acid clade A preference were biased towards Ugandan isolates. The HIV-1 gag protein contains not only important MHC class I-, but also class II-restricted epitopes, which stimulate CD4$^+$ T-helper cells.

The C-terminus of the HIVA protein is designed as a multi-CTL epitope. Previous mouse and macaque immunization studies using polyepitope immunogens established the validity of this vaccine approach[22,23,25,27,28,30-33]. The CTL epitopes included in HIVA were identified in patients infected with HIV-1 clade A strains circulating in Kenya, are 8- to 10-amino acid long and originate from the gag, pol, nef and env proteins[9,34]. Many of these epitopes are immunodominant and relatively conserved among other HIV-1 clades (Table 1). They are presented by 17 different HLA alleles, which include frequent African alleles as well as alleles common in most ethnic populations. Optimally selected epitopes presented by the nine commonest HLA alleles may cover a general population irrespective of ethnic descent. Thus, given that the majority of HIV-infected donors make good CTL responses to gag p17/p24, each vaccinee should have the potential to respond to at least two or three CTL epitopes present in the HIVA protein.

The HIVA polyepitope contains SIV gag and HIV env epitopes recognized by macaque and murine CTL, respectively, so that the quality, reproducibility and stability of the clinical batches can be easily assessed in a mouse (or macaque if desirable) potency assay. A monoclonal antibody epitope was added to the C-terminus of HIVA for easy detection of a full-size protein and estimation of the level of expression. There is no reason to believe that the three non-HLA epitopes represent a health hazard for the vaccinated individuals.

The HIVA gene

Two strategies were employed to increase the expression of HIVA in human cells. First, to ensure an efficient initiation of translation from the first methionine codon, the HIVA open reading frame (ORF) was preceded by a 12-nucleotide-long Kozak consensus sequence. Second, most of the original HIV-1 codons were substituted with frequently used codons in highly expressed human genes. This modification increases nuclear stability and export of heterogeneous nuclear RNA (hnRNA) and may optimize translation of the mRNA. The

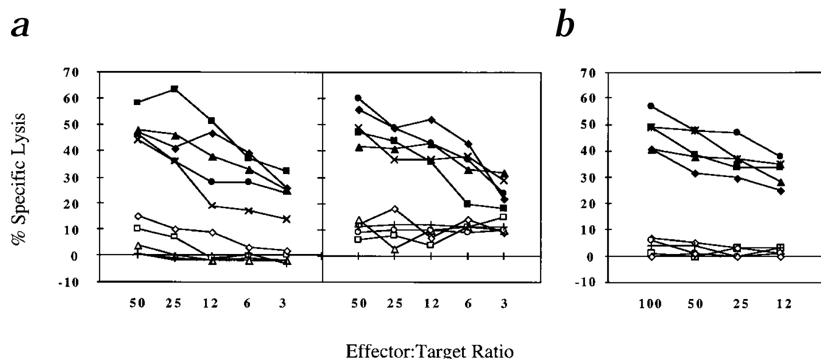

Fig. 2 Mouse potency assays. The potencies of the clinical vectors were assessed employing an epitope recognized by mouse CTL included into the polyepitope string. Mice were sacrificed 10 days after the last immunization, splenocytes from individual mice were isolated, separately peptide-restimulated *in vitro* for 5 days and tested in a ^{51}Cr-release assay against peptide pulsed (filled) or unpulsed (open) targets. Each line represents an individual mouse. ***a***, pTHr. HIVA vector delivered 1×, intramuscularly (left panel) or 2×, intradermally (right panel). ***a***, MVA.HIVA vector delivered 1×, intramuscularly.

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Table 1 CD8+ T cell epitopes included in the HIVA polyepitope region.

Epitope[a]	MHC class I restriction	Origin	HIV clade[b]
ALKHRAYEL	HLA-A*0201	nef	a
PPIPVGEIY	HLA-B35	p24	a/B/c/D/F/G
GEIYKRWII	HLA-B8	p24	a/B/c/D/F/G
KRWIILGLNK	HLA-B*2705	p24	A/B/C/D/F/G/H
FRDYVDRFYK	HLA-B18	p24	B/D (A=C/F/G/H)[c]
RDYVDRFYKTL	HLA-B44	p24	B/D (A=C/F/G/H)[c]
DRFYKTLRA	HLA-B14	p24	B/D (A=C/F/G/H)
AIFQSSMTK	HLA-A*0301, A11, A33	pol	a/B/c/D/G/H
ITLWQRPLV	HLA-A*6802	pol	a/b/C/D/F/G/H
ERYLKDQQL	HLA-B14	gp41	a/b/C/D
YLKDQQLL	HLA-A24, B8	gp41	a/b/C/D
TVYYGVPVWK	HLA-A*0301	gp120	A/B/C/D/g
RPQVPLRPMTY	HLA-B51	nef	A/b/D/E/F/G
QVPLRPMTYK	HLA-A*0301, A11	nef	A/b/D/E/F/G
VPLRPMTY	HLA-B35	nef	A/b/D/E/F/G
AVDLSHFLK	HLA-A11	nef	a/B/d/f
DLSHFLKEK	HLA-A*0301	nef	A/B/D/F
FLKEKGGL	HLA-B8	nef	A/B/C/D/E/F/G
ILKEPVHGV	HLA-A*0201	pol	A/B/C/D/G
ILKEPVHGVY	HLA-Bw62	pol	A/B/D
HPDIVIYQY	HLA-B35	pol	a
VIYQYMDDL	HLA-A*0201	pol	A/B/C/D/F/G/H
TPGPGVRYPL	HLA-B7	nef	b/c
ACTPYDINQML[d]	Mamu-A*01	p27	SIV
RGPGRAFVTI[e]	H-2D^d	gp120	HIV

[a]Epitopes are listed in the order in which they appear in the polyepitope. [b]A particular epitope sequence is present in about 50% (small letter) or 90% (capital letter) of sequenced HIV clade isolates. [c]'=' indicates that the epitopes are present in the N-terminal clade A gag domain [d]A dominant epitope derived from SIV gag p27 flanked by Ala and Leu at its N- and C-termini, respectively, recognized by rhesus macaque CTL, which can be used for potency studies in rhesus macaques.[e]A CTL epitope presented by a murine MHC class I used for the potency assay.

HIVA ORF is a part of a 1602-bp, double-stranded DNA fragment. The gene was constructed in four parts *in vitro*; each part was assembled by polymerase chain reaction (PCR) from overlapping positive- and negative-strand 70–90-mer oligodeoxynucleotides, sequenced and corrected where necessary, ligated together using unique restriction endonuclease sites and inserted into the pTHr and MVA vaccine vectors (see following section).

The pTHr vector

A new vector pTHr for direct gene transfer was designed with the aim to minimize the number of functional elements and therefore the amount of administered DNA. The construction of pTH was described previously[22]. It contains an expression efficient enhancer/promoter/intron A cassette of the human cytomegalovirus strain AD169 genome[35]. The promoter region is followed by the pRc/CMV (Invitrogen, Groningen, The Netherlands)-derived poly-linker and polyadenylation signal of the bovine growth hormone gene. The β-lactamase gene conferring ampicillin resistance to transformed bacteria and prokaryotic origin of double-stranded DNA replication ColE1 are both derived from plasmid pUC19. The pTH vector does not contain an origin for replication in mammalian cells. After insertion of the HIVA DNA into the pTH poly-linker, the β-lactamase gene fragment was removed and the resulting pTHr.HIVA was propagated using the repressor-titration system developed by Cobra Pharmaceuticals (Keele, UK), which selects plasmid-carrying bacteria without the need for a physical presence of an antibiotic-resistance gene on the plasmid[36]. Therefore, DNA vaccination does not introduce into the human organism 10^{12} copies of an antibiotic resistance gene per each 1 μg of DNA delivered.

The pTHr.HIVA was transiently transfected into 293T cells the expression of the recombinant protein was readily detected by the Pk tag-specific monoclonal antibody and visualized by immunofluorescence techniques confirming the correct assembly of the HIVA gene (not shown). The HIVA expression in immunized animals was confirmed by its immunogenicity measured in potency assays (see following sections).

The MVA vector

MVA is an attenuated vaccinia virus safe for clinical application, which has almost lost its ability to replicate in human cells[37]. The use of MVA as a vaccine[23,38,39] and its features which make it an attractive choice among the attenuated poxvirus vectors[40,41] have been described extensively. In mice, MVA appeared to be more immunogenic than the Western Reserve and NYVAC strains[38]; however, a rigorous comparison of the immunogenicities of attenuated poxvirus vectors, especially in humans, is missing. The HIVA gene has been inserted into the thymidine kinase locus of the MVA genome under the control of the P7.5 promoter.

Vaccine potency assay

The potencies of the DNA and MVA vectors were tested in groups of Balb/c mice taking advantage of the presence of the H-2D^d-restricted epitope[42]. For the pTHr.HIVA vaccine, mice were either injected once intramuscularly with 100 μg of DNA, or injected intradermally twice, two weeks apart, with a total of 2 μg of DNA per dose using the Dermal XR gene delivery device of PowderJect Vaccines (Madison, Wisconsin). The mice were killed 10 days after the last immunization, their splenocytes were isolated and peptide-restimulated *in vitro* for five days before a cytolytic assay. Both modes of the pTHr.HIVA vector delivery were highly immunogenic and induced high cytolytic activities in all immunized animals (Fig. 2*a*). Similarly, a single intramuscular injection of 10^7 plaque-forming units of MVA.HIVA elicited in all vaccinees, strong peptide-specific lytic activities measured after culture restimulation (Fig. 2*b*). These potency assays are reproducible and used routinely for testing individual vaccine batches (not shown).

Perspectives

The present vaccination approach investigates the protective roles of helper and cytotoxic T-lymphocyte responses in the absence of neutralizing antibody, as the experimental immunogen HIVA does not contain the envelope glycoprotein and does not attempt to induce antibodies neutralizing HIV.

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The requirements for an efficient generation of such responses are quite different from those for the induction of CD8+ T cells and might compromise the T-cell immunogenicity of the current vaccination approach. Therefore, nAb may be better elicited separately. In any case, a reliable method for induction of nAb against primary HIV isolates is still awaited. On the other hand, the absence of envelope in the vaccine has some advantages. Vaccination will not interfere with the anti-env antibody-based tests for HIV sero-positivity and therefore, vaccination- and infection-induced immune responses can be distinguished readily. It also offers the opportunity to add the env component to the vaccination protocol in the future without having to overcome the 'original-antigenic-sin' phenomenon, that is, antibody responses to similar but non-neutralizing epitopes, which take a long time to redirect. The Oxford vaccine contrasts with most of the current AIDS vaccine efforts. To date, over 25 different HIV vaccines have entered human trials, of which only a minority focused on the induction of CTL and mostly employed HIV clade B-derived immunogens.

The significance of the extensive genetic diversity of the HIV isolates and its implication for vaccine design have long been debated. The HIVA vaccine immunogen focuses on gag and CTL epitopes derived from HIV-1 clade A, which is now, but may not be in the future, the predominant circulating HIV strain in Nairobi[29]. Although there are some important differences between clade A and B CTL (ref. 9, 34), the capsid protein p24 is 80% conserved across HIV-1 clades due to structure/function constraints, and many of the selected CTL epitopes in the polyepitope region are shared among clades (Table 1). Therefore, the CTL responses that the vaccine aims to induce would recognize other non-A HIV clades as well. The cross-clade reactivity of immune cells from HIV-1 infected people has been observed in several instances[9,34,43–45] and some data suggested that this can be translated into a vaccination[20].

Many basic immunologic questions remain. What determines the immunodominance of CTL responses? Is the induction of 'help' desirable for a prophylactic HIV vaccine? Which T-cell subtype(s) should vaccines expand—effector, memory or both? At which site(s)—mucosal, local lymphoid organs, systemic or all three—should the responses be induced and which routes of vaccine delivery are best for that? Is the vaccine going to be equally immunogenic in Oxford and Nairobi given the differences in genetic background, nutritional status and exposure to other infections of the target populations? How can longevity/maintenance of the T-cell responses be achieved? For example, the persistence of vaccines in the body for the maintenance of immunological memory is probably desirable, but would represent currently a major obstacle for the approval of clinical trials by regulatory authorities. All these considerations affect critically the vaccine design, yet clear answers are unknown. The scheduled clinical trials of the HIVA and other vaccines should help to shed light on at least some of these questions. Last, but not least, the proof of the pudding is in the eating, that is, no animal model can substitute for a clinical assessment of an experimental vaccine.

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Whereas federal expenditure in the United States on the development of an HIV/AIDS vaccine is approximately $250 million, only $25 million is spent on research and development for a malaria vaccine. It is not malaria but tuberculosis (TB) that is the poor relation. Government funds for a vaccine to fight this disease are in single figure millions. Stefan Kaufmann of the Max Planck Institute for Infection Biology examines obstacles in addition to funding that hinder the development of a new TB vaccine.

Is the development of a new tuberculosis vaccine possible?

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"Since the time people have realised tuberculosis is preventable and since they have learned how to avoid infection, mortality rates caused by tuberculosis have declined in industrialised countries and signs are starting to appear that it can be eliminated. This is the right time to combat tuberculosis." These words were spoken a century ago by the discoverer of the tubercle bacillus, Robert Koch. "I have a very distinguished group of leaders here ... who are profoundly interested in joining forces to fight against diseases that kill people and progress in the world's poorest countries. Diseases like AIDS, tuberculosis and malaria..." With these words, United States President, Bill Clinton, opened a vaccine research meeting at the White House on 2 March this year.

Sadly, we are no closer to eliminating or even controlling tuberculosis (TB) today than we were when Koch first identified the causative agent, *Mycobacterium tuberculosis*. Every minute, more than 10 individuals develop TB, amounting to 8 million new cases annually (Fig. 1). Two to 2.5 million of these TB sufferers will die of the disease. These appalling figures put TB in the unfavourable list of the top major killers, together with AIDS and malaria[1]. The situation is worsened by the increasing incidence of multidrug resistant (MDR) strains, and the deathly combination of TB with AIDS. Co-infection with HIV and *M. tuberculosis* increases the risk of developing TB 30-fold.

The biggest burden of TB is in Southeast Asia where there are 3 million TB cases per year. Sub-Saharan Africa has nearly 1.6 million cases annually. But what we are seeing is not the re-emergence of a disease that had been controlled well in the past; rather, it is the resurgence in awareness in western countries of a problem that had always existed globally, but which had seemed to be in retreat in the industrialized world. For example, the incidence of TB increased in several parts of the United States in the early 1990s, incidence has risen sharply in several formerly socialist states[2], and the number of cases of TB in the UK has risen by 80% in the last 10 years.

Do we really need a new vaccine?

The current vaccine against TB, bacille Calmette-Guérin (BCG), was developed by the French scientists Calmette and Guérin in the first decade of the last century. BCG has been delivered for more than 70 years and has been given to more people than any other vaccine (more than 3 billion individuals and around 100 million newborns annually). Its side effects are tolerable, and it can prevent miliary and meningeal TB in young children to an appreciable degree. In striking contrast, BCG fails to protect against the most prevalent disease form, pulmonary TB in adults. In fact, data concerning the protective efficacy of BCG in adults range from 0% in South India to 80% in the UK (ref. 6). Although a meta-