NEW HOPE FOR AN AIDS VACCINE

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The twenty-first century has begun with considerable success for new AIDS vaccines in macaque models. A common feature of these vaccines is their ability to induce high-frequency $CD8^+$ T-cell responses that control, rather than prevent, infection with HIV. The new vaccines, which include DNA vaccines and live viral vectors, are based on technologies that have been developed since the start of the AIDS epidemic. The ultimate promise of these vaccines will be realized only when efficacy trials in humans are conducted.

VACCINES

ISOLATE
An isolate of HIV-1 is a population of virus that has been recovered from a patient. Isolates that have been distributed to other laboratories are sometimes called strains.

SUBTYPES OF HIV-1
These are genetically related clusters of HIV-1. Subtypes of HIV-1 are also called clades. They do not fall into specific categories based on their susceptibility to neutralizing antibody.

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DOI: 10.1038/nri776

Although HIV type 1 (HIV-1) was successfully grown in tissue culture[1,2] and completely sequenced[3] within four years of the first reports of AIDS in 1981 (REF. 4), it is only recently that vaccine strategies against this scourge of the twenty-first century have shown promise in terms of both safety and efficacy. HIV-1 has presented several unique challenges for vaccine development (BOX 1). Viral envelope (Env) glycoproteins, which mediate attachment and entry into host cells, conceal their conserved receptor- and co-receptor-binding sites in thermostable crypts that are masked further by loops of heavily glycosylated variable sequences[5,6] (FIG. 1). Antibody-producing B cells recognize the variable loops, but only infrequently recognize the conserved receptor-binding sites[7], which makes it hard to generate neutralizing antibodies with broad cross-reactivity for patient ISOLATES. HIV-1 has eluded control by vaccines through its ability to form latent proviral DNA. As an essential step in its life cycle, HIV-1 integrates into the genome of its host to form a provirus. Once integrated, the virus can hide from the immune system by its lack of protein expression[8]. Estimates of the half-life of latent proviral DNA range from 6 to 43 months, indicating that it could take up to 60 years to eradicate a reservoir of as few as 1×10^5 latently infected cells[9,10]. Finally, HIV-1 is highly variable[11,12]: HIV-1 arose from a single transmission event from chimpanzees to humans[13], but has evolved in humans to form at least 12 genetic SUBTYPES, which, in turn, have diversified further (FIG. 2). Even within a subtype, antibodies that are specific for the variable loops of isolates from one patient typically do not recognize the variable loops of isolates from other patients. As the epidemic has spread, recombinants of different subtypes have gained prominence[14]. The high error rate of reverse transcription generates at least one mutation per provirus[15], which, combined with the rapid turnover of plasma virions[16], provides a broad base of variants for selection and escape from both cellular and humoural immune responses[7,17].

Rationale for vaccine design

Because of the problems that are associated with raising neutralizing antibodies, recent vaccine approaches have focused on inducing cellular immune responses[18,19]. Cellular immune responses are mediated by white blood cells called T cells that recognize and respond to foreign peptides (epitopes) that are presented by MHC antigens.

Box 1 | **Challenges for an AIDS vaccine**

HIV is a difficult target for neutralizing antibodies:
- conserved targets are thermostably concealed
- exposed targets are highly variable
- targets are camouflaged by heavy glycosylation

HIV is able to form latent proviral DNA:
- occurs early in infection
- long half-life

HIV has high variability:
- 12 known subtypes
- intersubtype recombinants
- high error rate of reverse transcriptase

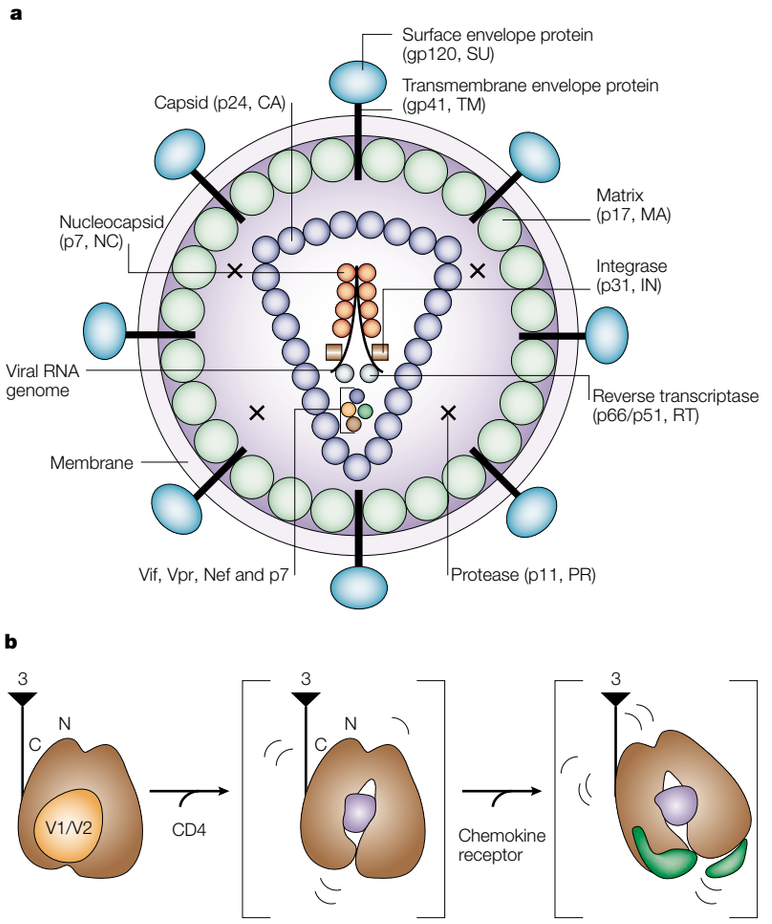

Figure 1 | **Structure of HIV-1. a** | Schematic of HIV-1 showing the envelope glycoproteins that are targets for neutralizing antibodies and the structural, as well as enzymatic, proteins that are targets for T cells. The longest three reading frames of the virus transcribe the Gag, Env and Pol polyproteins. The Gag polyprotein is processed into MA, CA, NC and p7, which make up the inner core of the viral particle. Glycoprotein 120 (gp120; SU) and gp41 (TM) are derived from the envelope (Env) polyprotein and are the outer membrane proteins of the virus. Processing of the polymerase (Pol) polyprotein yields the enzymes protease (PR), reverse transcriptase (RT) and integrase (IN), which are encapsulated in the core of the inner particle. The accessory proteins Vif, Vpr and Nef are encoded by three other reading frames in the virus. The surface envelope glycoprotein is glycosylated at 25–30 sites, and contains three variable loops that mask receptor-binding sites. Adapted, with permission, from REF. 115. © (1998) Academic Press. **b** | Schematic depicting conformational changes of the gp120 subunit of Env that expose the conserved CD4- and co-receptor-binding sites. The '3' symbolizes the 3-fold axis, from which gp41 interacts with gp120 to generate a functional trimer. The left-hand schematic depicts the form of gp120 on the surface of a virion. The CD4-binding site is partially occluded by variable loops 1 and 2 (V1/V2) — shown in orange. The middle form represents a conformational change that is depicted as an inner–outer domain shift, with the purple shape denoting the formation of the CD4-binding cavity. The right-hand schematic depicts a third conformational form, in which the gp120 'bridging sheet' and sequences in the third variable loop (shown in green) bind to the chemokine co-receptor. Adapted, with permission, from *Nature* (REF. 5) © (1998) Macmillan Magazines.

CD8$^+$ T cells recognize peptides of 8–11 amino acids presented by class I MHC, whereas CD4$^+$ T cells recognize sequences of 10–14 amino acids presented by class II MHC. These peptides originate from conserved, as well as variable, regions of HIV proteins (FIG. 1a). About 60% of the ~200 CD8$^+$ T-cell epitopes that have been defined for subtype B of HIV-1 are conserved within subtype B, whereas only about 30% of these epitopes are conserved in more distantly related subtypes, such as A or C[12,20]. So, T-cell responses have good cross-reactivity within a subtype and some cross-reactivity across subtypes. Because T-cell responses are restricted to peptides that are presented on MHC, each vaccinated individual recognizes only those peptides that are presented by his or her MHC (about 5% of the defined epitopes).

CD8$^+$ T cells (cytolytic T cells) directly combat infections, whereas CD4$^+$ T cells (helper T cells) provide growth factors and co-stimulatory molecules that support the activation and maintenance of CD8$^+$ T cells . CD8$^+$ T cells clear infections by lysing infected cells[18]. They also act by suppressing proviral expression through the release of antiviral cytokines, such as tumour-necrosis factor (TNF) and interferon-γ (IFN-γ)[21–23]. Both CD8$^+$ and CD4$^+$ T cells can block the local spread of HIV by producing chemokines that interfere with the activity of co-receptors for viral entry[22,24,25]. HIV can escape T-cell responses by mutating sequences within its target epitopes[26,27]. Studies in macaques show a direct correlation between the number of days to death and the number of CD8$^+$ T-cell epitopes that are recognized by an individual macaque[17]. So, as with antiretroviral drugs, for which multi-drug therapy is more effective than single-drug therapy, to be effective, T-cell responses need to be directed against several HIV-1 sequences. The importance of CD8$^+$ T cells for the control of viral infections has been best shown in macaques, in which the depletion of CD8$^+$ T cells results in the emergence of high levels of viraemia[28,29]. The importance of CD4$^+$ T cells is shown by the natural history of AIDS, in which patients progress to disease when they have lost >80% of their CD4$^+$ T cells.

Proteins that are produced inside cells are the best substrates for raising CD8$^+$ T cells, because these proteins have access to pathways for processing and presentation by class I MHC. In the past, the vaccines that have successfully raised specific T cells have been of the live-attenuated type. However, for immunodeficiency viruses, live-attenuated vaccines have been problematic. Vaccines with sufficient replication capacity to effectively immunize can be virulent in newborns[30] and, occasionally, revert to virulence in adults[31]. So, vaccines that consist of only a portion of a pathogen or a replication-defective mimic of the pathogen are the current preferred candidates for AIDS vaccines. For these to effectively raise CD8$^+$ T cells, they need to be expressed in cells. This has been achieved using DNA VACCINES and live viral vectors.

Promising vaccine strategies

All of the vaccines that have achieved recent success have been designed to induce cell-mediated immunity. These vaccines include interleukin-2 (IL-2)-ADJUVANTED DNA vaccines[32]; regimens consisting of DNA priming followed by recombinant modified vaccinia Ankara (rMVA)[33] or recombinant human adenovirus 5 (Ad5) boosters[34] (DNA–rMVA or DNA–Ad5 vaccines); and vaccines that use rMVA[35] (R. R. Amara *et al.*, unpublished observations) or Ad5 (REF. 34) for both priming and boosting immunizations (rMVA-only or Ad5-only

DNA VACCINE
A DNA plasmid that expresses the immunizing protein. Vaccination is accomplished by uptake and expression of the DNA by cells in the vaccinated host.

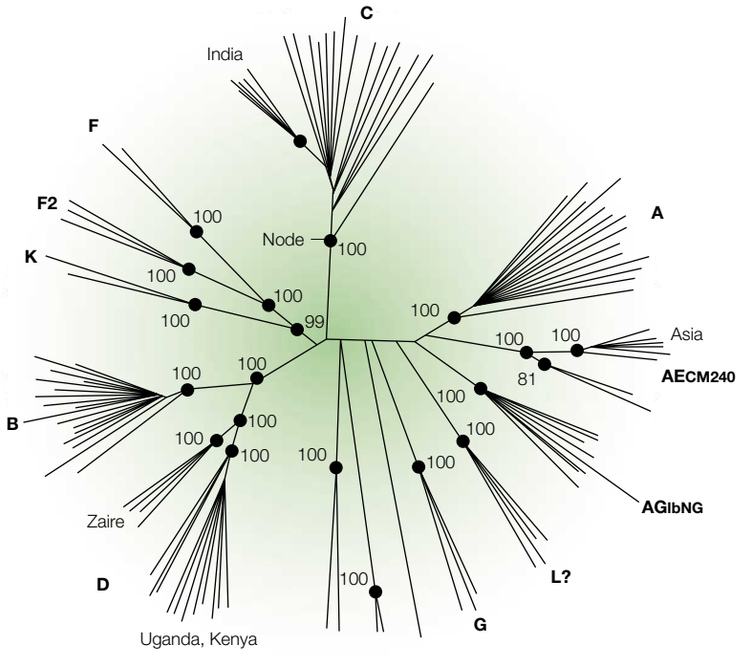

Figure 2 | **Genetic relationships of HIV-1 subtypes and circulating recombinant forms.** The schematic, which is based on full genome sequences, indicates the relationship of current subtypes of HIV-1 — indicated by the letters A, B, C and so on — and their dominant geographical distributions. The original transmission of HIV-1 from chimpanzee to human is thougth to have occurred at the starting junction for the clusters[13]. The clustal relationship was developed using a statistical method known as bootstrapping, with 100 iterations and 50% resampling of the datasets (bootstrap values are indicated at the nodes). Data are adapted, with permission, from REF. 116 © (2000) Arnold Publishers.

vaccines) (TABLE 1). All of these vaccines have been tested in a single preclinical model: immunogens have been constructed from the 89.6 or 89.6P hybrids of simian and human immunodeficiency viruses (SHIV), and the highly pathogenic SHIV-89.6P was used for the challenge[36]. SHIV-89.6P establishes steady-state levels of viraemia that are 10–100-times greater than those of HIV-1 in typical infected humans, depletes $CD4^+$ T cells within 2–3 weeks of infection[32–34,37] and causes AIDS in most infected animals within six months. In this model, 7 out of 8 animals immunized with a DNA vaccine that used IL-2 as an adjuvant[32,38]; 23 out of 24 animals given a DNA–rMVA vaccine[33]; 9 out of 10 animals given rMVA-only vaccines[35] (R. R. Amara *et al.*, unpublished observations); 3 out of 3 animals given a DNA/Ad5 vaccine; and three out of three animals given an Ad5-only vaccine[34] had reduced post-challenge viraemia to the lower limit of detection (<1000 copies of viral RNA per ml of plasma). None of the 26 control animals in these vaccine trials similarly controlled infection. Challenges were administered between six weeks[32,34,35] and seven months[33] after immunization by both intravenous[32,34,35] and mucosal routes[33,117] (R. R. Amara *et al.*, unpublished observations). The control of viraemia has been long lasting; so far, all animals with consistent control of their infection in the first six months have maintained this control. HIV-1-infected patients with <1000 copies of viral RNA per ml of plasma are LONG-TERM NON-PROGRESSORS[37] and tend not to transmit their infections[39]. So, although these vaccines do not prevent infection, they provide considerable hope for the improved survival of those who become infected and for reduced transmission of the epidemic.

ADJUVANT
Adjuvants are substances that, when added to an immunogen, increase the immune response to that immunogen. Genetic adjuvants are DNAs that encode a molecule that augments an immune response.

LONG-TERM NON-PROGRESSORS
HIV-1-infected humans who enjoy many years of productive life. Long-term non-progressors typically have levels of viral RNA in their blood of less than 1000 copies per ml.

CODON OPTIMIZATION
Changing the codons for an amino acid to those most frequently used in human cells.

MICROSPHERE
A carrier for a vaccine that facilitates immune responses by stabilizing and/or increasing the uptake of the vaccine.

New technologies and vectors

The successful vaccines have all been based on recombinant technologies that have been developed since the onset of the AIDS epidemic — DNA vaccines and live viral vectors. The first reports of the use of live viral vectors were published in 1982 (REFS 40,41) and the first reports of DNA-based immunizations came a decade later[42–44]. Both of these technologies have blossomed because of their ease of development and the practical power they bring to the development of subunit vaccines that are expressed in cells. Both, however, have had to undergo development for effective use against HIV-1 (BOX 2).

DNA vaccines for HIV-1. Several groups are developing DNA vaccines for HIV-1, and the first DNA vaccines to be tested in humans were HIV-1 vaccines[45]. The primary limitation of DNA vaccines has been the difficulty in achieving sufficient uptake and expression of DNA to induce a robust immune response. The problem of achieving high levels of gene expression has been compounded for DNA vaccines for HIV-1 because of the inefficient expression of HIV-1 messenger RNAs outside of the context of the HIV-1 genome. HIV-1 expresses mRNAs that encode structural and regulatory proteins by the subgenomic splicing of a single transcript. The HIV regulatory protein Rev regulates the splicing and movement of HIV-1 transcripts by binding to a *cis*-acting target, the Rev response element (RRE), which is present in unspliced and partially spliced HIV-1 mRNAs[46]. One way in which DNA vaccines have achieved high levels of HIV-1 protein expression is the use of Rev and the RRE to facilitate the expression of HIV-1 mRNAs. Rev-dependent subgenomic splicing can be used to express multiple HIV-1 proteins from single transcripts[33] (FIG. 3), and has the potential for expressing non-infectious virus-like particles[47]. It can also achieve high levels of gene expression. However, even higher levels of HIV-1 gene expression can be achieved by the optimization of HIV-1 genes for the codons that are used most frequently in human cells[48]. CODON-OPTIMIZED sequences typically express single gene products[32] or fusion proteins[49] (FIG. 3) and achieve exceptionally high levels of gene expression by using all transcripts for a single, efficiently translated mRNA. Most codon-optimized vaccines have focused on the group-specific antigen (Gag) protein, a primary target for $CD8^+$ T cells in long-term non-progressors[50]. At present, most DNA vaccines are used at doses of several mg of DNA, which would require the production of about one litre of bacterial culture per dose of vaccine.

Other approaches to increasing the efficiency of DNA vaccines have focused on genetic adjuvants[51], conventional adjuvants[34], MICROSPHERES[52] and *in vivo* electroporation[53]. So far, conventional adjuvants, such as alum

Table 1 | **Recent preclinical success of approaches to an AIDS vaccine**

Vaccine	Priming immunogen	Booster immunogen	SHIV-89.6P challenge	Control of challenge (controlled/total)*	Comment	Status of development for humans	References
IL-2-adjuvanted DNA	5 mg of codon-optimized Gag DNA + 5 mg of codon-optimized SHIV-89.6P Env DNA, followed 2 days later by 5 mg of IL-2–Ig DNA. Delivered i.m. at weeks 0 and 4	5 mg of codon-optimized SIV Gag DNA + 5 mg of codon-optimized SHIV-89.6P Env DNA. Delivered at weeks 8 and 40	i.v. at 6 weeks after last booster	Vaccine: 4/4 Control: 0/8	Good protection of $CD4^+$ T cells. Success also achieved in 3/4 animals by twice daily innoculations of IL-2–Ig protein for 1–14 days after DNA vaccination	Being developed for phase I trials in humans	19,26
DNA–rMVA	2.5 mg or 0.25 mg of SHIV-89.6 Gag–Pol–Env DNA. Delivered i.d. or i.m. at weeks 0 and 8	2×10^8 pfu of SHIV-89.6 Gag–Pol–Env rMVA. Delivered i.d. and i.m. at week 24	i.r. at 7 months after last booster	2.5 mg i.d. DNA prime: 13/14 0.25 mg i.d. DNA prime: 6/6 2.5. mg i.m. DNA prime: 6/6 0.25 mg i.m. DNA prime: 5/6 Control: 0/6	Good protection of $CD4^+$ T cells Control has. improved with time (at present, 1.5 years after challenge)	Gag–Pol–Env subtype B DNA and rMVA to enter separate phase I trials in 2002. Subtype AG and C DNAs and rMVAs being made for a trivalent A + B + C vaccine	20
rMVA only	1×10^8 pfu of Gag–Pol rMVA + 1×10^8 pfu of SHIV-89.6 Env rMVA. Delivered i.m. at weeks 0 and 4	1×10^8 pfu of Gag–Pol rMVA + 1×10^8 pfu of SHIV-89.6 Env rMVA. Delivered i.m. at week 21	i.v. at 6 weeks after last booster	Vaccine: 3/4 Control: 0/4	Some loss of $CD4^+$ T cells		22
rMVA only	2×10^8 pfu of SHIV-89.6 Gag–Pol–Env rMVA. Delivered i.d. and i.m. at weeks 0 and 8	2×10^8 pfu of SHIV-89.6 Gag–Pol–Env rMVA. Delivered i.d. and i.m. at week 24	i.r. at 7 months after last booster	Vaccine: 6/6 Control: 0/6	Good protection of $CD4^+$ T cells. Exceptionally tight control of the infection	HIV-1 subtype B Gag–Pol–Env rMVA to enter phase I trials in 2002. Subtype AG and C rMVAs being made for a trivalent A + B + C vaccine	23
DNA–Ad5	5 mg SIV Gag DNA in CRL1005 adjuvant. Delivered i.m. at weeks 0, 4 and 8	1×10^{11} particles of Ad5–SIV Gag. Delivered i.m. at week 32	i.v. at 6 weeks after last booster	Vaccine: 3/3 Control: 0/8	Long-lasting $CD8^+$ T-cell response. Protection with Gag alone	HIV-1 subtype B Gag DNA in phase I trials	21
Ad5 only	1×10^{11} particles of Ad5 SIV Gag. Delivered at weeks 0 and 6	1×10^{11} particles of Ad5 SIV Gag. Delivered i.m. at week 32	i.v. at 12 weeks after last booster	Vaccine: 3/3 Control: 0/6	Long-lasting acute $CD8^+$ T-cell response with >1 peak in 3/3 animals. Protection with Gag alone. Problem of pre-existing immunity in ~45% of US population	HIV-1 subtype B Gag–Ad5 in phase I trials	21

Ad5, adenovirus 5; Env, envelope protein; Gag, group-specific antigen; IL-2, interleukin-2; IL-2–Ig, IL-2 fused to the heavy chain of immunoglobulin; i.d., intradermal; i.m., intramuscular; i.r., intrarectal; i.v., intravenous; pfu, plaque-forming unit; Pol, polymerase; rMVA, recombinant modified vaccinia Ankara; SHIV–89.6P, pathogenic hybrid of SIV and HIV; SIV, simian immunodeficiency virus. *Control of challenge is defined as a reduction of the level of viral RNA to ≤1000 copies per ml of plasma.

and block copolymers[34], have not increased the efficacy of DNA-based vaccines sufficiently to induce protective immunity for immunodeficiency viruses[34]. However, a new adjuvant, IL-2, fused to the heavy chain of immunoglobulin (Ig) to increase the half-life of IL-2 activity[54], primes protective immunity both when the IL-2–Ig is delivered as a protein (twice a day for 14 days after DNA immunization) or a genetic adjuvant (single inoculation two days after DNA immunization)[32]. In mouse models, augmentation of T-cell immunity required administration of the IL-2 genetic adjuvant after, rather than with, DNA immunization. The requirement for administering the IL-2 adjuvant after the DNA-based immunization is not understood, but might be due to the ability of IL-2 to increase the number of responding T cells that enter the long-term memory pool[54].

Live vaccine vectors for HIV-1. Virtually every virus with a molecularly cloned sequence that can be used to express an infectious agent has undergone at least some development as a candidate HIV-1 vaccine. Current live viral vectors that are being developed for use against immunodeficiency viruses include several avian and mammalian poxviruses[33,35] (see http://www.iavi.org/trialsdb and http://www.hvtn.org/trials), replication-defective adenoviruses[34], alphaviruses[55], rhabdoviruses[56], herpesviruses[57], adeno-associated virus[58] and picornaviruses[59]. Bacterial vectors with the promise of easy oral delivery to facilitate worldwide administration are undergoing development[60]. Each of these vector systems is unique with respect to the size of the vaccine inserts that can be carried, the host range of cells that will be infected, the host pro-inflammatory responses

Box 2 | **New and improved technologies**

DNA vaccines:
- Rev (HIV-1 regulatory protein)-dependent subgenomic splicing
- codon optimization
- genetic adjuvants
- conventional adjuvants
- improved expression cassettes

Live viral vectors:
- extension to several families of viruses
- increased insert sizes
- development of replication-defective agents
- elimination of immune-evasion responses
- improved expression cassettes.

Heterologous prime–boost protocols
- DNA–recombinant modified vaccinia Ankara (rMVA)
- DNA–adenovirus 5 (Ad5)

stimulated and the immune-evasion strategies of the vector (TABLE 2).

The two viral vectors that have had the most preclinical success are MVA and Ad5 (TABLE 1). Both MVA and Ad5 vectors have the safety feature of being replication defective in primate cells. Replication-competent poxviruses and adenoviruses can cause disseminated disease in immunocompromised individuals. The replication of MVA in humans was attenuated by >500 passages in chick-embryo fibroblasts[61,62], during which time MVA acquired six large genomic deletions[63]. These deletions eliminated host-range genes, as well as immune-evasion genes that encode soluble receptors for IFN-γ, IFN-α/β, TNF and CC-chemokines[64], but did not compromise the ability of MVA to grow in chicken cells. Molecular biologists deleted the E1 and E3 regions of the adenovirus genome from the Ad5 vector. The E1 deletion eliminated early functions crucial to viral replication and rendered the vector replication defective, except when grown in cells that provided E1 functions in *trans*. Both the E1 and E3 deletions eliminated immune-evasion genes. E1A proteins interfere with the antiviral activity of IFNs[65] by blocking the activation of IFN response factors[66,67]. The E3 region inhibits the cytolysis of infected cells by $CD8^+$ T cells[68,69] and TNF[70,71]. The growth of MVA in chick-embryo fibroblasts poses no risk for the generation of a replication-competent virus for humans, because the host-range mutations map to more than one deletion[72]. By contrast, the growth of Ad5 in E1-expressing cells poses some risk of the acquisition of E1 sequences by recombination; these risks, however, can be minimized in the design of HELPER CELL lines[73]. MVA can be grown in chick-embryo fibroblasts to titres of 1×10^{11}–5×10^{11} plaque-forming units (pfu) per 1×10^9 cells. In preclinical trials, doses of up to 2×10^8 pfu have been used (TABLE 1). The adenovirus vectors are likely to be produced in helper cell lines, such as PRC6, which produce titres of 1×10^{13}–5×10^{13} particles (1×10^{11}–5×10^{11} pfu) per 1×10^9 cells. In preclinical trials, doses of 1×10^{11} particles have been used (TABLE 1).

Both MVA and Ad5 can express high levels of HIV-1 genes. The block in the replication of MVA occurs after the expression of the viral late genes but before virion morphogenesis[74]. The unimpaired late as well as early viral-protein synthesis, even in non-permissive human cells, accounts in part for the usefulness of MVA as a safe but efficient expression vector. The promoters that are used for HIV-1 expression in MVA at present have been designed to be active in early as well as late phases of poxvirus infections, and support high levels of continuous insert expression[75]. Because MVA produces its mRNA in the cytoplasm of cells, recombinant HIV-1 genes do not have to be engineered to overcome Rev dependence for splicing. MVA, by virtue of its size, also offers the opportunity for the expression of large inserts, and easily accommodates the expression of several HIV-1 proteins by the expression of different HIV-1 mRNAs from different promoters. In contrast to MVA, Ad5 expresses its genes in the nucleus. Transcription cassettes, which are modelled at present on those used for DNA vaccines, are placed in the position of the deleted E1 gene. Because of the nuclear expression, HIV-1 genes need to be codon-optimized to avoid Rev dependence. Size constraints over what can be placed in the E1 region limit expression to promoter-plus-insert sequences of about 5 kb, and favour the development of single-gene HIV-1 vaccines.

Both MVA and Ad5 have a broad host range for human cells, and both stimulate the production of pro-inflammatory cytokines that augment immune responses. Adenovirus vectors lead to efficient antigen presentation by infecting immature dendritic cells and causing their maturation without polarizing the T-helper response[76,77]. MVA infections mobilize innate immune responses by stimulating the production of high levels of type I interferon[64]. Adenoviruses are highly inflammatory; they stimulate the production of TNF, IL-1 and IL-6 (REF. 78). Adenoviruses can cause tumours in animal models. This, however, has not been a concern for Ad5, because of the deletion of the E1 region of the genome that supports tumour induction.

A major limitation of live-vector vaccines is the presence of pre-existing immunity to the vector. About 45% of the US population now has neutralizing antibodies that are specific for Ad5 (REF. 79). Older people, who were vaccinated against smallpox, will have pre-existing immunity for MVA; an immunity that would become universal if vaccinations for smallpox were to become routine to counter the threat of bioterrorism. However, the rMVA vaccines could serve a dual purpose — immunization against smallpox and HIV-1. This potential merits investigation, because the dual vaccine would have the practical, as well as economic, advantage of achieving two immunizations with one vaccine, and could provide a smallpox vaccine with a lower incidence of adverse effects than the current vaccine[80]. Pre-existing immunity can be overcome by higher doses of vaccine,

HELPER CELL
A cell line that provides essential viral functions for the growth of a defective viral vector.

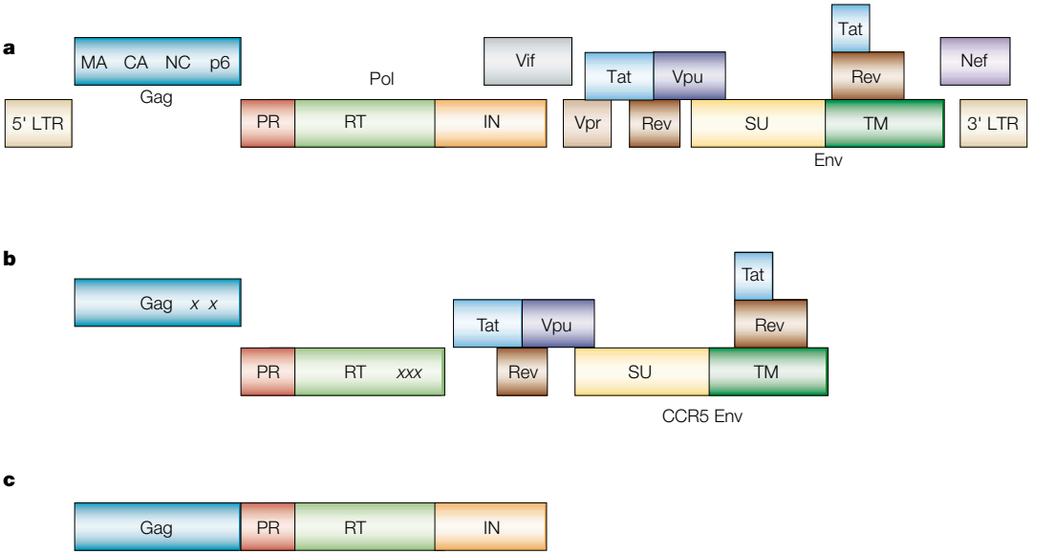

Figure 3 | **Two strategies for HIV DNA vaccine inserts. a** | Depiction of the nine open reading frames — Gag, Env, Pol, Tat, Rev, Vpu, Vif, Vpr and Nef —in the HIV-1 genome. **b** | Example of the expression of several proteins from a single DNA by HIV regulatory protein (Rev)-dependent subgenomic splicing. The construct is the HIV-1 DNA vaccine that we are advancing into human trials. '*X*' denotes a safety mutation that inactivates reverse transcriptase or the zinc fingers for packaging viral RNA. **c** | Example of the expression of a codon-optimized fusion protein. This DNA vaccine expresses the largest number of sequences among current codon-optimized vaccines, and was constructed by deleting the frameshift between the genes that encode group-specific antigen (Gag) and the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). The *PR* gene includes an inactivating mutation. The insert was developed at the National Institutes of Health Vaccine Research Center and is being advanced for phase I human trials[49]. CA, p24 capsid protein; Env, envelope protein; LTR, long terminal repeat; MA, p17 matrix protein; NC, p7 nucleocapsid protein; RT, p66/p51 reverse transcriptase; SU, gp120 surface envelope protein; TM, gp41 transmembrane envelope protein. vif, vpr, vpu and nef are viral accessory proteins; tat and rev are viral regulatory proteins.

and by heterologous PRIME–BOOST protocols. Higher doses of vaccine are a 'brute force' approach to immunizing in the presence of pre-existing immunity. Priming with an agent for which pre-existing immunity does not exist, such as DNA, establishes memory T cells that require the booster to achieve only sufficient infection to augment the primed immune response. Nevertheless, for both rMVA and Ad5 vaccines, a vector-naive population is the simplest and preferred population for vaccination.

Heterologous prime–boost protocols. A new and popular method for raising high frequencies of T cells is to combine DNA priming with live-vector boosters[81–83]. The remarkable efficiency of priming with DNA followed by boosting with a live vector is considered to be due to the DNA focusing the immune response on the vaccine antigens (as opposed to stimulating an immune response to vector, as well as vaccine, antigens). The live-vector booster then enhances this immune response by expressing larger amounts of antigen than can be achieved with DNA alone and by the stimulation of a pro-inflammatory response that augments immunity. An example of the remarkable strength of T-cell, as well as antibody, responses that can be achieved by DNA priming followed by boosting with rMVA is shown in FIG. 4. In this example, both the DNA and the rMVA encoded SHIV Gag, polymerase (Pol) and Env proteins. The frequency of Gag-specific T cells was 20 times greater for the DNA prime followed by the rMVA booster than for rMVA priming and boosting. Similar phenomena are seen when DNA priming is combined with Ad5 boosting[34]. Also, one live vector can be used to prime a second live vector[84].

PRIME–BOOST
When a single application of a vaccine is insufficient, repeated immunizations are carried out using the same vaccine preparation (homologous prime–boost) or using different vaccine preparations (heterologous prime–boost) to sequentially stimulate a better immune response.

Lessons from comparative monkey trials

Comparative vaccine trials in macaque models have been essential for the development of the current promising vaccines, and have been conducted by our own group[33,85,86], as well as the groups of Letvin[32,35] and Merck[34]. One of the most encouraging aspects of these trials is the extent to which findings in one trial have corroborated findings in other trials. Here, I summarize how our studies have pointed the way towards an AIDS vaccine, and highlight how these studies interface with those of others (BOX 3).

DNA alone does not protect. Our first trial clearly showed that DNA priming and boosting was not sufficient to induce protective immunity[85]. With the exception of one trial that used a highly avirulent HIV-1-challenge model in chimpanzee[87], DNA immunizations alone, even with codon-optimized sequences[32] with or without conventional adjuvants[34], have raised poor protective immunity.

Poxvirus versus protein boosters. In our second trial, we combined DNA priming with envelope protein or poxvirus boosters to achieve higher titre immune responses[86]. This trial showed that poxvirus boosters

Table 2 | **Examples of live viral vectors used for vaccine development**

Vector	Example	Insert size	Replication competence	Tissue tropism	Pro-inflammatory	Immune-evasion strategy	Pre-existing immune responses	Comment
Mammalian pox-virus[80,103]	MVA[33,35]	>10 kb	Replicates in chicken, but not primate, cells[72]	Broad, including dendritic cells	Type 1 IFN[103]	Soluble receptors for IFN-γ, IFN-α/β, TNF and CC-chemokines lost during attenuation[64]	Older humans and health workers who have been vaccinated against smallpox	High-level insert expression and immunogenicity owing to its expression of early, as well as late, vaccinia genes[104]. Scrambled partial Gag plus 23 CD8 epitopes for subtype A in phase I trials in England and Kenya[102]. Added CD8 epitopes include ones recognised by 17 different HLA types
Mammalian pox-virus[80,103]	NYVAC[105]	>10 kb	Replication competent[106]	Broad	Type 1 IFN	Deletion of a complement-binding protein and an inhibitor of serine proteases[106]	Older humans and health workers who have been vaccinated against smallpox	Developed by deleting 20 genes from the Copenhagen strain of vaccinia virus[106]
Avian pox-virus[107,108]	ALVAC[95,109]	>10 kb	Replicates in chicken, but not primate, cells[107]	Broad	ND	ND	No	ALVAC1521 expressing HIV-1 Gag and PR from subtype B and Env from subtype E with/without a gp120 boost to enter phase III trials in Thailand in 2002
Adeno-virus[110]	Ad5 (REF. 34)	<5 kb	Requires helper cells that express E1	Broad, including immature dendritic cells[77]	TNF, IL-1 and IL-6 (REF. 78)	Deletion of adenoviral E1 and E3 genes that inhibit IFNs and the cytolytic activity of $CD8^+$ T cells during construction	~45% of US population have neutralizing antibody owing to natural infection[79]	Unusually long acute phase for $CD8^+$ T-cell responses[34]
Alpha-virus[111]	VEE[55]	<5 kb	Live-attenuated and replication defective	Lymphoid tissue, including dendritic cells	ND	ND	VEE-vaccinated populations	Replication-defective VEE scheduled for phase I safety trials in 2002
Rhabdo-virus[112]	Vesicular stomatitis virus[56]	<5 kb	Live-attenuated	Broad, including mucosal tissues	ND	ND	Some rural workers	Can be administered intranasally[56]. Boosters use different serotypes to bypass vector-induced immunity[56]
Herpes virus[113]	Herpes simplex virus[57]	>5 kb	Live-attenuated and replication defective	Epithelial cells and ganglia	ND	None deleted	Most humans, owing to natural infections	Presumed long-term persistence of immunizing vector as a latent/re-activating infection
Adeno-associated virus[110]	AAV[58]	<5 kb	Replication defective, grown in helper cell lines	Determined by helper virus	ND	Depends on helper virus	Humans with natural adenovirus infections	Integration into host genome
Picorna-virus[59]	Poliovirus[114]	<2 kb	Live-attenuated	Mucosal tissues	ND	ND	Humans vaccinated with poliovirus	Demonstrated safety in humans

Insert sizes are approximate and depend on the nature, as well as the size, of the insert. AAV, adenovirus-associated virus; Ad5, adenovirus 5; Ag, antigen; ALVAC, a canary poxvirus; Env, envelope protein; Gag, group-specific antigen; IFN, interferon; IL, interleukin; MVA, modified vaccinia Ankara; NYVAC, New York vaccinia virus; ND, not defined; PR, protease; TNF, tumour-necrosis factor; VEE, Venezuelan equine encephalitis virus.

were superior to Env boosters. DNA priming plus poxvirus boosters controlled the challenge in the absence of detectable levels of neutralizing antibody, a finding that indicated that protection was mediated by T-cell responses. The poor ability of protein boosters to augment DNA-based immunizations has been reproduced in a study using Gag–Pol, as well as Env, protein boosters. In this trial, DNA alone provided better protection than DNA plus protein boosters[88]. However, in a third trial that used DNA plus an IL-12 genetic adjuvant followed by Env protein boosters, the Env protein increased protection[89].

Saline injection versus gene-gun inoculation. Our second trial tested whether priming could be achieved by GENE-GUN inoculation, which requires much less DNA than saline injections to raise immune responses[90]. This trial clearly showed that priming must be by saline

GENE GUN
A device that uses compressed helium to bombard DNA-coated gold beads into cells. The beads are usually 1–2 μm in diameter.

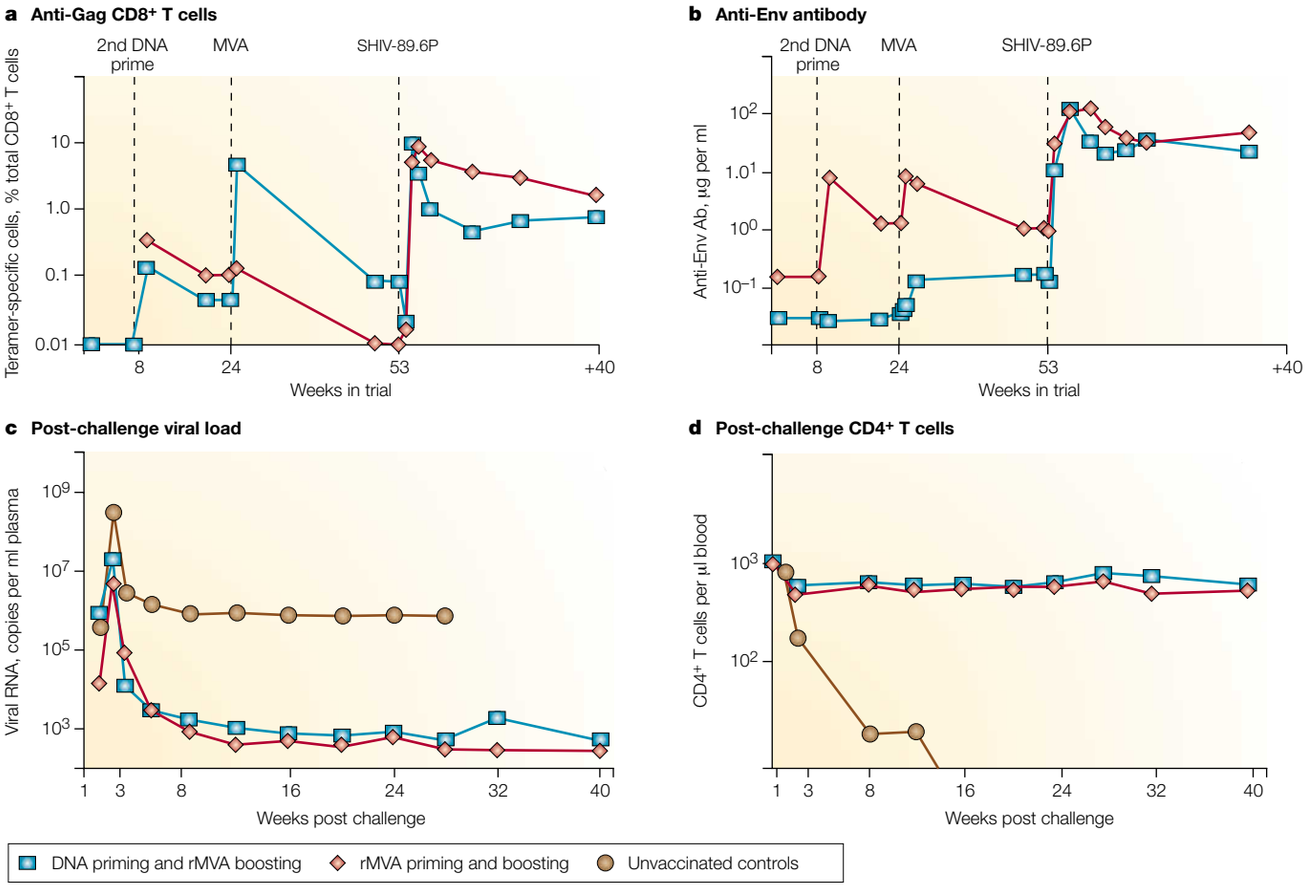

Figure 4 | **Different patterns of immune responses, but similar patterns of SHIV-89.6P control in DNA–rMVA and rMVA-only immunized animals.**
a | Group-specific antigen (Gag)-specific CD8+ T cells; **b** | anti-envelope protein (Env) antibody (Ab); **c** | post-challenge viral loads; and **d** | post-challenge CD4+ T cells in vaccinated macaques. DNA–recombinant modified vaccinia Ankara (rMVA) immunizations consisted of 2.5 mg of a Gag–Pol–Env DNA administered intradermally at weeks 0 and 8, and 2 x 10^8 plaque-forming units (pfu) of a Gag–Pol–Env-rMVA administered intradermally and intramuscularly at week 24. Recombinant MVA immunizations consisted of 2 x 10^8 pfu of a Gag–Pol–Env-rMVA administered intradermally and intramuscularly at weeks 0, 8 and 24. The simian immunodeficiency virus–HIV (SHIV-89.6P) challenge was delivered intrarectally seven months after the booster. Data are from REF. 33 and R. R. Amara *et al*., unpublished observations.

injection, not gene-gun delivery, of DNA[86]. In mouse models, saline injections of DNA tend to induce T-helper type 1 (T_H1) cells, whereas gene-gun inoculations tend to induce T_H2 cells[91]. We have proposed that the failure of gene-gun priming is due to the induction of T_H2, and not T_H1, cells, and that T_H1-supported immune responses are more effective than T_H2-supported immune responses for controlling infections with immunodeficiency viruses. The failure of gene-gun priming to raise protective immune responses, despite its ability to prime high titres of CD8+ T cells, has been shown in other experiments using SIV challenges in macaques[92], and for a DNA vaccine experiment using a *Mycobacterium tuberculosis* challenge in mice[93].

Gag–Pol–Env versus Gag–Pol immunogens. In our third trial, the ability of Gag–Pol versus Gag–Pol–Env DNA and rMVA immunogens to raise protection was compared[117]. This trial clearly showed that including Env in the immunizations increased protection. Both vaccines raised similar high frequencies of Gag-specific T cells. However, the Gag–Pol vaccine controlled the challenge in only 7 out of 12 animals, compared with 12 out of 12 animals for the Gag–Pol–Env vaccine. This result is in contrast to results of the Ad5–Gag vaccine, for which consistent control, albeit in a small group, was achieved in the absence of Env protein[34]. At the time of challenge, the Ad5-immunized animals had higher levels of specific T cells (1–25% of total CD8+ T cells) than our DNA/rMVA-immunized animals (<1% of total CD8+ T cells)[33]. This might have been the result of more prolonged expression of the Ad5 vaccine than the rMVA vaccine. Five out of six Ad5-immunized animals had two peaks in the number of Gag-specific T cells after immunization (which implies the continuing presence of the vector), in contrast to the single sharp peak in the number of Gag-specific T cells in DNA–rMVA- or rMVA-immunized animals[34]. Also, the SHIV-89.6P

T_H1/T_H2 (T-helper cells type 1 and T-helper cells type 2). These two types of T cell have distinctive patterns of lymphokine production. In mouse models, T_H1 cells produce IFN-γ (which supports the production of complement-dependent antibodies and the activation of phagocytic defences), whereas T_H2 cells produce IL-4 (which supports the production of complement-independent antibodies and the activation of eosinophils and mast cells).

Box 3 | **Findings from our path towards a human vaccine**

1994–1996 (REF. 85)
- DNA alone does not protect

1997–1999 (REF. 86)
- DNA priming followed by poxvirus boosters is superior to DNA followed by protein boosters
- Saline injections, but not gene-gun delivery, of DNA prime protective immune responses
- Protective T cells control, rather than eliminate, infections

2000–2002
- DNA priming and recombinant modified vaccinia Ankara (rMVA) boosting is able to control a virulent mucosal challenge[33]

Other results that are unpublished at present (R. R. Amara *et al.* and S. Buge, unpublished observations) include:
- rMVA priming and boosting can control a virulent mucosal challenge
- Vaccines that express envelope protein (Env) are more effective than vaccines that express only group-specific antigen (Gag) and polymerase (Pol)
- Alum-adjuvanted glycoprotein 120 (gp120) boosters do not improve protection
- Different vaccines induce different patterns of protective responses[33]

challenge in Ad5-immunized animals was administered 6–12 weeks after the final booster, much sooner than the challenge in our trials, which was given seven months after the final immunization. This difference in timing allowed less time for the long-lasting acute T-cell response in the Ad5-immunized groups to develop into a memory response. Together, these results indicate that Env is not essential for protection, but does provide an extra margin of safety. A role for Env, as well as Gag–Pol, in protective immunity against a SIV challenge has also been suggested in studies using recombinant poxviruses for priming and boosting[94].

Recombinant gp120 protein boosters. Because protein boosters induce strong antibody responses, we tested whether adding Env glycoprotein 120 (gp120) to a DNA–rMVA immunization would increase protective efficacy. Unexpectedly, protection was less consistent in the animals that received gp120 boosters than in those that received only the DNA–rMVA vaccinations. In these experiments, in collaboration with Janet McNicholl's group at the US Centers for Disease Control and Prevention, we delivered 300 μg of SHIV-89.6 gp120 in alum with the second DNA immunization and the rMVA booster (S. Buge *et al.*, unpublished observations). A poor ability of Env protein boosters to increase protective immunity has been found in trials in which 300 μg of oligomeric gp140 or monomeric gp120 in QS21 (a T_H1 adjuvant) were given as boosters for poxvirus immunizations[95,96].

Live vectors alone. Despite the fact that DNA priming followed by live-virus boosters induces much higher frequencies of T cells than priming and boosting with a live vector, both rMVA and Ad5 priming and boosting have been shown to be able to control challenge with pathogenic SHIV-89.6P[34,35] (TABLE 1). In these immunizations, control of the VIRAL SET POINT occurs in the presence of lower frequencies of antiviral $CD8^+$ T cells than are raised by the heterologous prime–boost protocols. In the case of the rMVA-only immunized animals, which expressed Gag–Pol and Env, Gag-specific T cells were present at ten times lower levels and anti-Env antibody at ten times higher levels at the time of challenge than in the DNA–rMVA-vaccinated animals (FIG. 1). So, in the rMVA-only-vaccinated animals, protection occurred in the presence of a very different balance of antibody and T-cell responses than in the DNA–rMVA-vaccinated animals; current opinion would have predicted that this balance would not be favourable for protection. Nevertheless, irrespective of current opinion, the ability to use a single-modality immunization would be beneficial for an effective campaign to control the AIDS epidemic. At present, single-modality avian poxvirus immunizations are entering phase III clinical trials (TABLE 2). In the preclinical trials that we have conducted, fowlpox vectors have not shown as much promise as MVA vectors — administration of a mixture of three fowlpox vectors expressing Gag–Pol, Env or Nef failed to control an avirulent SHIV-IIIb challenge[86].

Control rather than elimination of infection. At the end of our second vaccine trial, we transfused blood from the three macaques that had controlled a series of challenge infections to below our level of detection[86]. Remarkably, blood from one of the three monkeys transmitted the SHIV-IIIb infection that it had been exposed to six months before the transfusion, which we had never detected by reverse transcriptase (RT)-PCR (even shortly after the SHIV-IIIb challenge). This transmission of virus indicated the establishment of long-term proviral DNA in a 'protected' DNA–poxvirus-immunized animal. The presence of long-term proviral DNA means that a challenge virus that has been controlled by a cellular immune response has the potential for re-emergence, escape and the induction of immunodeficiency. Indeed, this has happened in one of the eight monkeys that originally controlled its SHIV-89.6P challenge infection after IL-2-augmented DNA immunizations[38].

Decisions for efficacy trials

The Holy Grail for the development of an AIDS vaccine has been the identification of a CORRELATE OF PROTECTION to use as a surrogate for raising protective immunity. Initially, AIDS researchers focused on neutralizing antibodies as the correlate. The pendulum then swung to the antiviral activities of $CD8^+$ T cells and the raising of protective T cells[19,97]. Our experiments that compare Gag–Pol with Gag–Pol–Env immunizations clearly show that the frequencies of Gag-specific $CD8^+$ T cells that can control the challenge in Gag–Pol–Env-immunized animals are not sufficient to control the challenge in Gag–Pol-immunized animals[117]. Both our own experiments and those of Merck show that different levels of T cells, at least those in peripheral blood, are associated with protection by homologous, as opposed to

VIRAL SET POINT
The viral set point is the steady-state level of viral RNA that is established following the acute phase of infection. At present, viral set points are the best known indicators for how rapidly a patient will progress to AIDS.

CORRELATE OF PROTECTION
An immune response that indicates that a vaccine will protect against a challenge infection.

NON-TRANSMITTERS
HIV-1-infected humans who do not transmit their infection through homosexual or heterosexual activity. These individuals typically have levels of viral RNA in their blood of <1000 copies per ml.

SUBUNIT VACCINE
Subunit vaccines encode portions of a pathogen only.

heterologous, prime–boost protocols[34] (FIG. 1). So, despite an increasing ability to accurately quantify cellular and humoural immune responses, a single correlate for protection has remained elusive, with different vaccines inducing different balances of cellular and humoural immune responses; the only true marker of protective capacity remains success in efficacy trials.

To decide which vaccines are to enter efficacy trials in humans, we will be asking how well our animal models predict protection in humans[98]. In our animal models, we use high challenge doses that infect every animal at the first exposure. By contrast, during the natural transmission of HIV-1, the low concentrations of HIV-1 in bodily fluids achieve successful transmission for only one in several hundred heterosexual acts or needle-stick exposures[99]. In our animal models, SHIV-89.6P establishes steady-state levels of viral RNA between 1×10^5 and 1×10^6 copies per ml of blood, and causes AIDS in most infected animals within six months[32–34]. In humans, most HIV-1 infections establish steady-state levels of viral RNA between 1×10^4 and 1×10^5 copies per ml of blood, and take ten or more years to develop into AIDS[37]. For HIV-1 infections in the United States, a 10–100-fold reduction in virus set-point would place the vast majority of infections in the long-term non-progressor/NON-TRANSMITTER category[37,39]. The current promising vaccines are achieving 1,000–10,000-fold reductions in viral set points (TABLE 1). If reductions in the titres of SHIV-89.6P in macaques reflect the magnitude of reductions that will be achieved by comparable HIV-1 vaccines in humans, these vaccines hold considerable hope for providing true benefit.

Timeline for human trials

Many years are required to take a vaccine from demonstrated promise in preclinical trials through safety, dosing and efficacy testing in humans. The HIV-1 vaccines that are now in phase III efficacy trials (two bivalent formulations of gp120 envelope proteins[100]) entered phase I/II human testing in 1997, and efficacy trials in the United States in 1998 and in Bangkok in 1999, which will be completed in 2002 and 2003, respectively[101]. Although many consider that these protein SUBUNIT VACCINES will not be highly successful because of their poor ability to raise $CD8^+$ T cells, the trials have been crucial to the overall development of an AIDS vaccine; by both defining the efficacy of the gp120 subunit approach and setting precedents for future trials. A candidate vaccine that is about to enter phase III efficacy trials is based on a canarypox vector in the presence or absence of gp120 protein boosters (http://www.iavi.org/trialsdb). The criteria that have been set for this vaccine to proceed to efficacy trials are the raising of specific $CD8^+$ T cells in at least 30% of volunteers at one or more time points (a cumulative cytotoxic T-lymphocyte response), lymphoproliferative responses in 60% of volunteers, and neutralizing antibodies specific for a lab-adapted strain in 70% of the volunteers. The codon-optimized Gag–DNA and Gag–Ad5 vectors of Merck's group are progressing well through phase I safety testing in humans. The International AIDS Vaccine Initiative is supporting an ongoing phase I trial for a codon-optimized CD8-epitope-based DNA–rMVA vaccine[102] in both England and Kenya, and is planning to test a vaccine that uses Venezuelan equine encephalitis virus as a vector in South Africa. The National Institutes of Health (NIH) Vaccine Research Center will have a modified and codon-optimized Gag–Pol DNA vaccine in phase I studies in 2002 (http://www.vrc.nih.gov/VRC/current-trials.htm). Our Rev-dependent Gag–Pol–Env DNA vaccine and matched rMVA developed in the laboratory of Bernard Moss at the NIH will enter safety trials in 2002. Several other products are in, or nearing, phase I trials (http://www.iavi.org/trialsdb and http://www.hvtn.org/trials). From the initiation of phase I trials to the initiation of phase III trials takes at least two years. Efficacy trails for HIV-1 vaccines typically require three years and might require longer if control, rather than prevention, of infection is the endpoint. So, in the absolutely best of circumstances, the new and promising vaccines featured here will not be ready for licensure and general distribution until 2006–2007. We can only hope that these new vaccines that raise cellular immunity will be as effective in humans as in macaques.

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Acknowledgements

I would like to thank B. Moss, H. Ertl, T. Mastro and R. Amara for critical comments. I am indebted to R. Amara for help with TABLE 2 and to J. Smith for help with FIG. 4, and I would like to thank H. Drake-Perrow for expert administrative assistance. This work was supported in part by Integrated Preclinical/Clinical AIDS Vaccine Development Program projects, the Emory/Atlanta Center for AIDS Research and Yerkes Regional Primate Research Center.

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