10
Prospects for an HIV Vaccine: Conventional Approaches and DNA Immunization

MARK A. BAUMEISTER, MICHAEL A. CHATTERGOON AND DAVID B. WEINER*

University of Pennsylvania School of Medicine, Department of Pathology and Laboratory Medicine, 505 Stellar-Chance Laboratories, 422 Curie Blvd., Philadelphia, PA 19104, USA

Introduction

The state of the HIV pandemic has reached epic proportions with an estimated 40 million infected worldwide with HIV and 22 million dead from AIDS and AIDS-related diseases. The introduction of HAART (highly active anti-retroviral therapy) in developed countries has significantly curbed the morbidity and mortality associated with HIV infection. However, infection with HIV still remains universally fatal, and the high cost of HAART makes this treatment strategy unrealistic in many of the countries devastated by this epidemic. Furthermore, the ability of the virus to integrate into the host cell genome and establish long-lasting cellular reservoirs early in the course of infection makes eradication of infection by this approach unfeasible. Therefore, the development of an effective prophylactic HIV vaccine is now more essential than ever. Despite two decades of efforts, the development of an effective vaccine has been an elusive task, and now it is clear that we will have to surmount many obstacles. Many of these obstacles are due to characteristics of the virus itself, while others involve an understanding of the host immune response. A further

*To whom correspondence may be addressed (dbweiner@mail.med.upenn.edu)

Abbreviations: AIDS, acquired immune deficiency syndrome; APC, antigen-presenting cells; HAART, highly active anti-retroviral therapy; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SHIV, SIV/HIV chimeric virus; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; TCR, T cell antigen receptor; IL-2, interleukin-2; IL-4, interleukin-4; IFN-γ, interferon-gamma; Th_1, T helper cell subtype 1; Th_2, T helper cell subtype 2; SCID, severe combined immunodeficiency mice.

Biotechnology and Genetic Engineering Reviews – Vol. 19, July 2002
0264–8725/02/19/205–242 $20.00 + $0.00 © Intercept Ltd, PO Box 716, Andover, Hampshire SP10 1YG, UK

obstacle has been availability of an appropriate animal model for studying HIV infection and disease. The further development of novel vaccine approaches, such as DNA immunization, recombinant viruses, as well as the continued optimization of conventional approaches, and possibly a combination of these approaches, is all central to the vaccine development effort.

The human immunodeficiency virus

The human immunodeficiency virus was established as the causative agent in the development of acquired immune deficiency syndrome (AIDS) by Gallo (Sarngadharan *et al.*, 1984; Zagury *et al.*, 1984) and Montagnier (Alizon *et al.*, 1984). The virus originally referred to as HTLV-III and LAV was associated with leukaemia and 'lymphadenopathy syndrome', frequently a prodrome of AIDS. Later, it was demonstrated that this virus was also the causative agent of AIDS, and was subsequently renamed HIV. More complete studies of the viral structure, life cycle and pathogenesis have not only increased our understanding of this virus, but have also facilitated the development of targetted therapies and novel vaccine designs.

HIV is a member of the genus lentivirus and belongs to the family *Retroviridae*. Two types of lentivirus are found in humans, HIV-1 and HIV-2. The greatest variation between these two lentivirus types is found in their *env* gene sequences. HIV-1, the more common type found in humans, is classified into major (M), outlier (O), or new (N) groups. The M group is further divided into the subtypes A-I, which are genetically equidistant. The O group, first isolated in Cameroon, is genetically distinct from the M group. The N group has been more recently identified as a new group within the M group subtype. HIV-1 genetic subtypes are unevenly distributed in different geographical locations. Subtype B viruses are prevalent in North America, Latin America and the Caribbean, Europe, Japan and Australia. Subtype E was initially identified in Thailand, and is also present in the Central African Republic. Subtype F was initially described in Brazil and Romania. Most recently, subtype G was described in Russia and Gabon; subtype H, in Zaire and Cameroon. As mentioned, group O viruses have been identified in Cameroon, but also in Gabon. Almost every subtype is present in sub-Saharan Africa, with subtypes A and D predominating in central and eastern Africa, and subtype C in southern Africa. Subtype C is also prevalent in India and it has been recently identified in southern Brazil. This diversity is considered a significant challenge for vaccine development. Whether we will need customized vaccines for each subtype, as in the case of the influenza virus, is an unresolved question.

Electron micrographs and computer emulations of HIV viral particles have revealed they have a spherical or, more specifically, icosadeltahedral shape (Marx *et al.*, 1988). These virions are studded with approximately 70 knobs, representing the envelope proteins that traverse the outer lipid bilayer, also called the envelope (*Figure 10.1*). The envelope protein is made up of a transmembrane subunit and an extra-viral or surface subunit that is believed to exist as a trimer. It is this structure that is the target of all neutralizing antibodies; its trimeric shape presents unique challenges for vaccine design. Cleavage of a polypeptide encoded by the *env* gene yields the two envelope protein subunits. Beneath the envelope, and moving to-

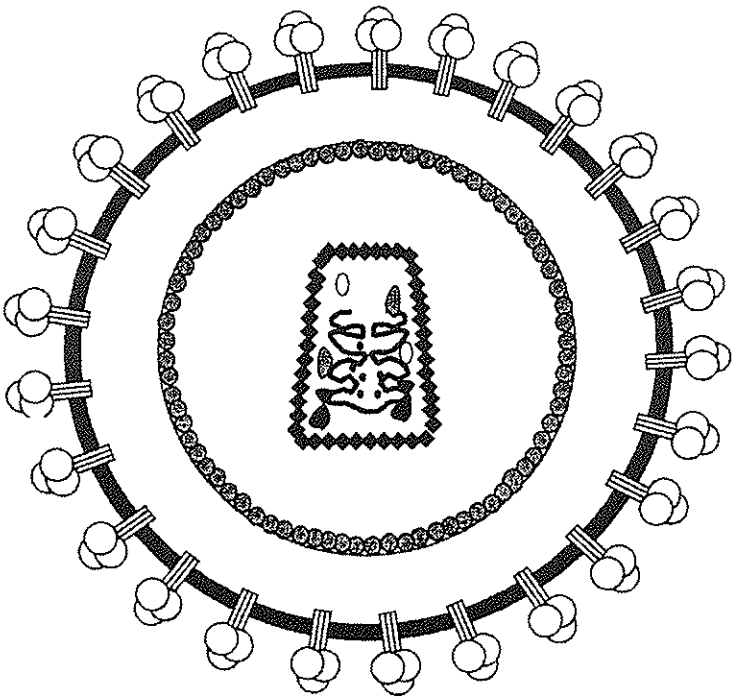
ward the viral centre, lies a matrix made up of the matrix protein (MA), followed by a cone-shaped capsid made up of the capsid protein (CA). The capsid encloses two strands of genomic RNA that are bound tightly by the nucleocapsid protein (NC). Three viral enzymes designated protease (PR), reverse transcriptase (RT), and integrase (IN), are derived by cleavage of a polypeptide precursor encoded by the *gag/pol* genes. RT and IN are enclosed by the capsid. The PR enzyme is responsible for cleavage of a polypeptide precursor encoded by the *gag* gene, giving rise to the MA, CA, NC proteins. RT contains activities necessary for the synthesis of double-stranded DNA (dsDNA) from the RNA template. IN mediates integration, by covalent linkage and insertion of the viral dsDNA, into the genomic DNA of the host cell. While only the envelope is an important target of neutralizing antibodies, all of the viral open reading frames are potential targets of the cellular immune response.

HIV replication is characterized by two phases. In the first phase, the virus attaches to its target cell via its envelope protein. The ectodomain of the viral surface subunit, gp120 binds to the CD4 receptor present on $CD4^+$ T lymphocytes and macrophages. A second receptor is also necessary for viral entry and this receptor depends on the HIV-1 strain. In general, macrophage tropic (M) strains of HIV-1 use the CCR5 chemokine receptor for viral entry. T cell tropic (T) HIV-1 strains use the CXCR4 chemokine receptor for viral entry. Fusion of the viral and target host cell membranes follows viral attachment. Following fusion, the viral capsid is released into the host cell cytoplasm. RT then reverse transcribes the viral RNA genome into dsDNA and the viral dsDNA is transported into the nucleus, where it is covalently spliced into the host cell genome by IN. The integrated viral dsDNA is then referred to as the provirus. Once integrated, the provirus is considered stable. This stable 'virus' is a significant challenge for vaccines as it suggests that viral clearance can only be accomplished by cell clearance, underscoring the importance of the cellular immune response in the clearance of HIV infection.

The later phase of replication is characterized by replication of the provirus by the host cell. The provirus is transcribed into RNA by the enzyme RNA polymerase II. The RNA transcript is then processed into mRNA transcripts corresponding to the different viral genes. This is a two-stage process: first the regulatory genes *tat, rev* and *nef* are produced from early multiply-spliced transcripts. Later, *rev* stabilizes full-length transcripts, and the structural genes are produced. Transcripts are translated and post-translationally modified, yielding the mature HIV protein constituents. Mature viral proteins are then assembled in the host cell, giving rise to the viral capsid. The capsid then fuses with the plasma membrane. Prior to fusion, mature HIV envelope proteins are transported and inserted into the plasma membrane of the host cell. Following fusion, the viral particle buds off from the host cell.

Adaptive immune response: mechanisms of lymphocyte activation and effector function

The mammalian immune system responds to infection using two systems of defence: the innate immune response, and the adaptive immune response. The innate immune response is characterized by the activation of phagocytes, such as macrophages and neutrophils, activated by the presence of cellular components present in micro-



	Gene product		Gene product
gag	Matrix (p17)	**pol**	Integrase (p32)
	Capsid (p24)		Reverse transcriptase (p66/p51)
	Nucleocapsid (p9)		Protease (p11)
env	gp120		
	gp41		

Figure 10.1. HIV virion structure. Protein products of structural genes *gag*, *pol*, and *env* are shown below the virion. Three precursor proteins encoded by these three genes are proteolytically cleaved to give the viral protein constituents. Cleavage of a gp160 precursor polypeptide encoded by the *env* gene yields the envelope protein gp41, the transmembrane subunit, and gp120, the surface subunit. The envelope gp120/gp41 complex is shown as a trimer within the viral envelope. The matrix protein is located between the viral envelope and the capsid, which encloses the viral RNA. The nucleocapsid protein binds the viral RNA. The reverse transcriptase, integrase, and protease enzymes are believed to be located within the viral capsid.

organisms, such as bacteria. These phagocytes utilize cell surface receptors that recognize general molecular motifs present on the cell walls of bacteria and yeast, such as the carbohydrate lipopolysaccharide. Binding to these motifs induces phagocytes to secrete cytokines and chemokines, which affect the behaviour of other immune system cells and can act to recruit other cells to the site of infection.

The adaptive immune response is a more specific response and is characterized by the activation of T and B lymphocytes. Lymphocytes contain highly specific 'antigen' receptors capable of recognizing small, variable protein sequences present within proteins of the pathogen. The adaptive immune response is characterized by three main mechanisms of defence, each involving the activation of naïve lymphocytes and their differentiation into effector cells. The role of the innate immune system in controlling HIV infection is still unclear.

$CD8^+$ T CELL ACTIVATION AND EFFECTOR FUNCTION

T lymphocytes can be activated by professional antigen-presenting cells (APC), such as dendritic cells or macrophages, through interactions involving cell surface molecules present on the APC surface called the major histocompatibility complex (MHC). MHC complexes exist in two types, referred to as class I or class II antigen, and participate in two different pathways involving the processing and presentation of foreign or self-antigen within the cell. T cells are classified based on cell surface molecules that are first expressed during their maturation and differentiation. T cells that express the surface molecule CD8, or $CD8^+$ T cells, recognize peptide antigen only in the context of MHC class I molecules. T cells that express the surface molecule CD4, or $CD4^+$ T cells, recognize peptide antigen only in the context of MHC class II molecules.

A naïve $CD8^+$ T cell (one that has not been previously activated by encountering antigen specific for its antigen receptor) can be activated by an APC that presents foreign peptide in the context of an MHC class I molecule. For example, a DC (dendritic cell) that is infected with HIV will present HIV peptides on its surface in the context of an MHC class I molecule. When a naïve $CD8^+$ T cell recognizes its cognate peptide antigen, in the context of an MHC class I molecule on the surface of an APC, it binds through its T cell antigen receptor, TCR (*Figure 10.2*). For the T cell to become activated and to differentiate into an effector cell, it requires a second interaction; interaction of the molecule CD80, present on the surface of the APC, with its receptor CD28, present on the T cell. These two interactions induce the T cell to produce the growth factor IL-2, which can act in a paracrine manner on the same T cell. The $CD8^+$ T cell then proliferates and differentiates into an effector T cell or CTL (cytotoxic T cell). When a CTL encounters its cognate antigen on the surface of another cell, such as a virus-infected cell, it binds and produces cytotoxins, such as perforin, a molecule that inserts into the target cell membrane, forming pores (*Figure 10.2*). This mechanism causes lysis of the target cell. CTL responses fall in the category of immune responses, referred to as cellular immunity. CTL clearance is considered the sole mechanism for removal of HIV-infected cells and is a central aspect of many vaccination approaches.

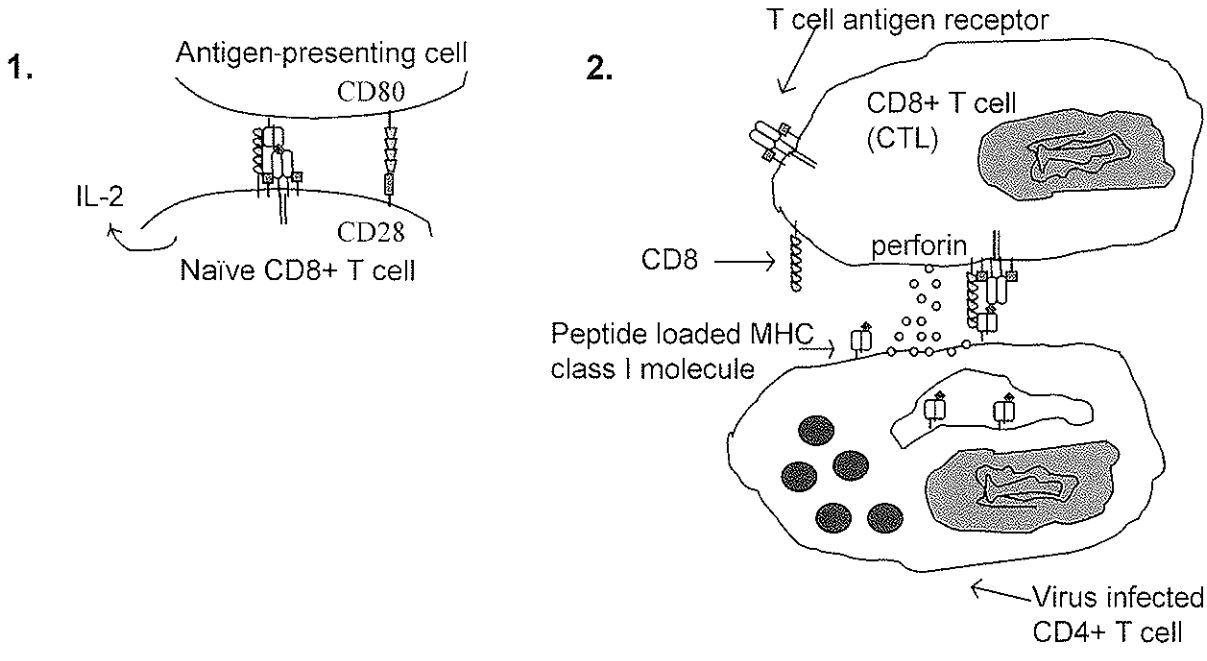


Figure 10.2. T cell activation and effector function. A naïve T cell (1) that encounters its cognate antigen in the context of an MHC class I molecule present on an antigen-presenting cell (APC), binds to the MHC class I/antigen complex via its T cell antigen receptor (TCR). A second interaction between CD80 on the APC and CD28 on the T cell induces the T cell to produce IL-2. IL-2 induces the naïve CD8+ T cell to proliferate and differentiate into an effector T cell, also called a cytotoxic T lymphocyte (CTL). When a CTL encounters, via its TCR, its cognate antigen presented in the context of an MHC class I molecule on a virus-infected cell (2), the CTL is induced to produce and secrete cytotoxins, such as perforin. Perforin is a multimeric molecule that inserts into the target cell membrane and causes lysis of the target cell.

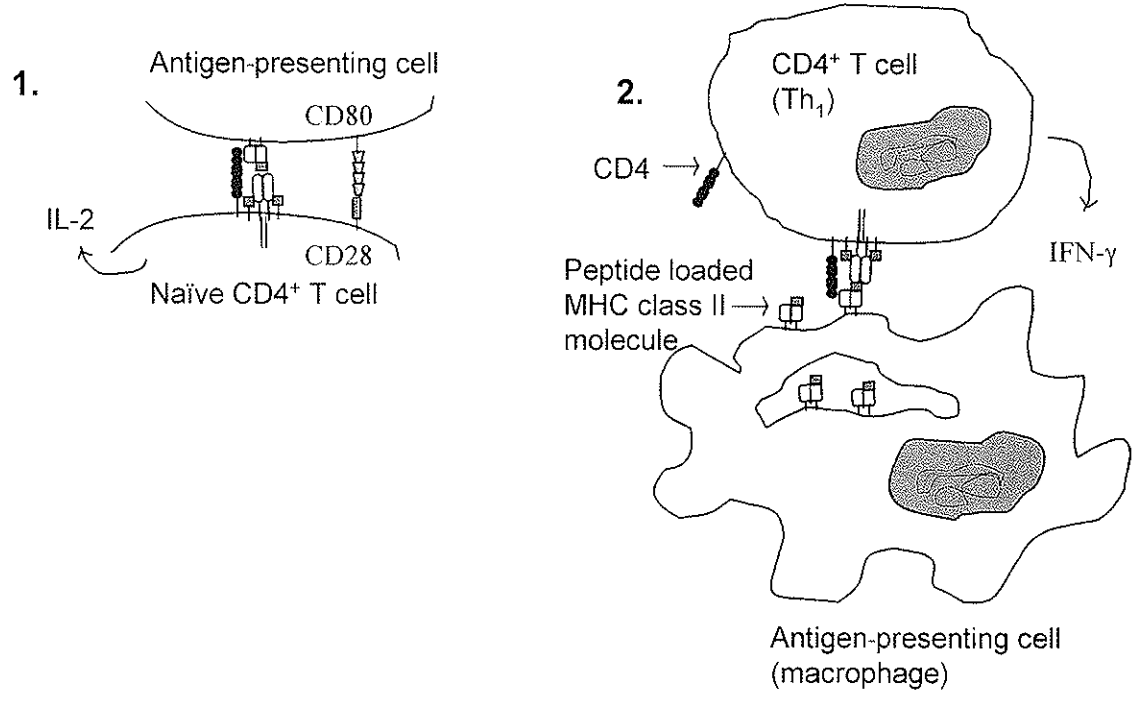


Figure 10.3. CD4+ T cell activation and effector function. A naïve CD4+ T cell (1) that encounters cognate antigen in the context of an MHC class II molecule present on an APC binds to the MHC class II/antigen complex via its TCR. A second interaction between CD80 on the APC and CD28 on the T cell induces the T cell to produce IL-2. IL-2 induces the naïve CD4+ T cell to proliferate and differentiate into an effector T helper cell (Th1 or Th2). When a Th1 T helper cell (2) encounters cognate antigen presented in the context of an MHC class II molecule, it will produce cytokines, such as IFN-γ. IFN-γ activates macrophage cells which, when activated, become very efficient at eliminating extracellular pathogens via phagocytosis.

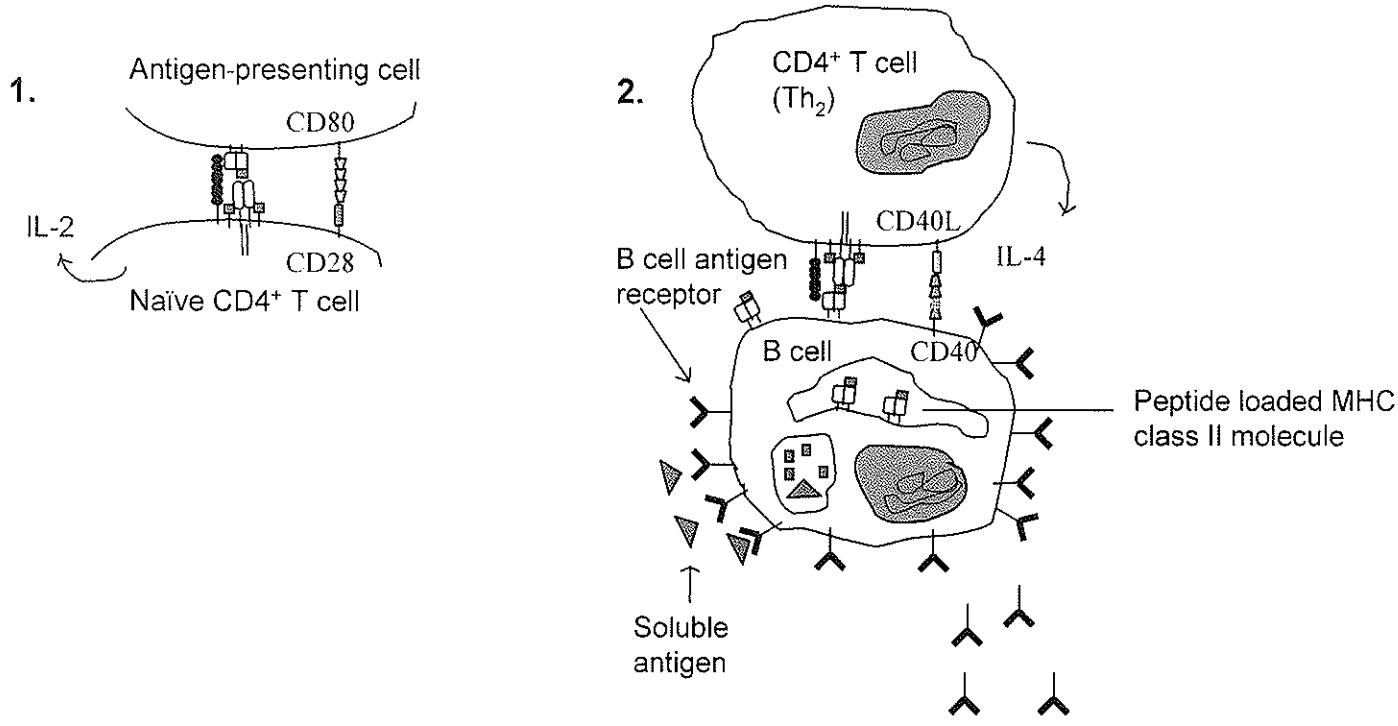


Figure 10.4. B cell activation and effector function. When a B cell (2) binds to its cognate antigen via its B cell antigen receptor (BCR), it internalizes the antigen/receptor complex and re-presents peptide antigen in the context of an MHC class II molecule. When a Th2 helper T cell recognizes cognate peptide antigen via its TCR in the context of an MHC class II molecule on the B cell surface, the Th2 cell expresses the cell surface molecule CD40L and the cytokine IL-4. CD40L on the T cell then binds to CD40 on the B cell surface. In the presence of IL-4, the B cell then proliferates and differentiates into an antibody-secreting cell.

$CD4^+$ T CELL ACTIVATION AND EFFECTOR FUNCTION

$CD4^+$ T lymphocytes can also be activated by APC. When an APC phagocytoses extracellular pathogens or secreted antigens, such as secreted HIV envelope proteins, the pathogen or antigen is processed into peptides and presented on MHC class II molecules on the surface of the APC. When a naïve $CD4^+$ T lymphocyte recognizes its cognate antigen on the surface of a DC in the context of an MHC class II molecule, it binds via its TCR. For the T cell to become activated and to differentiate into an effector T cell, it also requires the CD80/CD28 interaction. IL-2 is again produced and the T cell proliferates and differentiates into an effector cell called a T helper cell. T helper cells further differentiate into Th1 and Th2 cells, which produce distinct sets of cytokines. When a Th1 cell encounters its cognate antigen in the context of an MHC class II molecule on the surface of a DC or macrophage, the T cell produces and secretes cytokines, such as IFN-γ, a growth factor that activates macrophages (*Figure 10.3*). When activated by IFN-γ, macrophages become very efficient at eliminating extracellular pathogens. However, the contribution of IFN-γ to the clearance of virus *in vivo* remains to be fully elucidated.

B CELL ACTIVATION AND EFFECTOR FUNCTION

When a naïve B lymphocyte encounters and binds to its cognate antigen (such as a viral or bacterial antigen) via its antigen receptor, it internalizes the antigen/receptor complex, processes the antigen, and presents the foreign peptide in the context of MHC class II molecules (*Figure 10.4*). When a Th2 cell binds, via its TCR, to its cognate peptide antigen in the context of an MHC class II molecule on the B cell

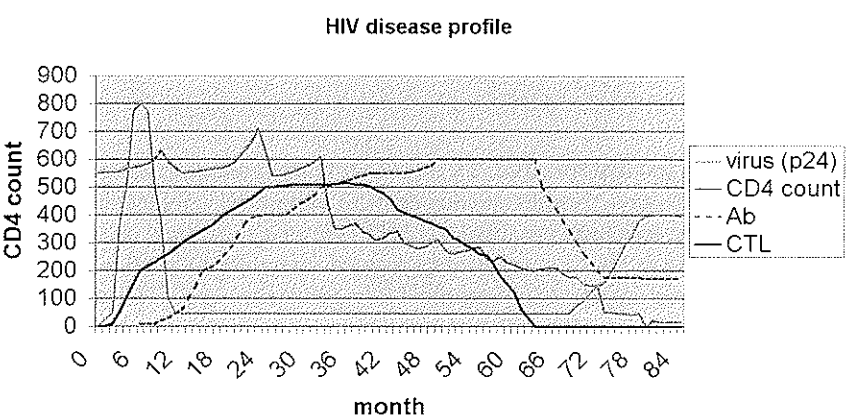


Figure 10.5. Clinical progression of HIV disease. Infection is followed by a sharp increase in viral replication within the first 6 months and drops off to a low but detectable baseline level by about 14 months. Viral replication is followed almost immediately by a virus-specific CTL response which continues to increase to about 40 months, just as the antibody response begins to plateau. The antibody response follows the CTL response within the first 6 months of infection and plateaus at about 50 months. At the peak of the CTL response, CD4+ T cell counts begin to drop, presumably due to CTL-specific killing of virus-infected CD4+ T cells. As the CD4+ T cell counts begin to drop below a critical level (~ 200 counts), and the antibody response drops off, the viral load begins to increase again. At this stage, the infected individual is diagnosed with AIDS.

surface, the T cell expresses the cell surface molecule, CD40 ligand (CD40L) and the cytokine, IL-4. A CD40 receptor present on the B cell surface then binds CD40L. When these two interactions take place in the presence of IL-4, the B cell proliferates and differentiates into a plasma cell that produces and secretes antibodies specific for the internalized antigen. These antibodies may then complex with antigens present on the pathogen. Inducing antibody responses that neutralize HIV-1 are a central goal of HIV vaccinology.

Obstacles to HIV vaccine design

CORRELATES OF PROTECTION FROM HIV

There are many characteristics unique to HIV-1, which make the development a prophylactic vaccine problematic. One of the greatest difficulties has been in determining the immune correlates for achieving protection. The typical HIV disease profile demonstrates the induction of a strong CTL response shortly after the initial increase in viral replication or viraemia (*Figure 10.5*). However, this response often does not clear the virus and as the infection progresses, a strong humoral response is observed. At the peak of the CTL response, $CD4^+$ T cell numbers begin to decline progressively, suggesting that the CTL response is important in this decline. Eventually, the $CD4^+$ T cell counts fall below a minimum level and the individual is diagnosed with AIDS. To date, there is no consensus reached on the true immune correlates for protection from HIV infection. However, CTL and neutralizing antibody responses observed in certain HIV-infected individuals suggest that control of infection involves all three arms of the adaptive immune response: the humoral (antibody) response, the CTL response, and the helper T cell response.

CTL response

Some studies suggest that specific CTL responses to HIV infection serve as a primary correlate for protection. Studies conducted with certain HIV-infected individuals, referred to as long-term non-progessors (LNTP) because they show no clinical signs of HIV disease after 10–15 years of documented infection, have shown that these individuals contain higher HIV-specific CTL precursors and CTL activity than progressor controls (i.e. HIV-infected individuals that display the typical clinical signs of HIV infection, such as progressive loss of $CD4^+$ T cell counts) (Koup *et al.*, 1994; Rowland-Jones, 1999). Furthermore, studies involving Rhesus macaques at early stages of infection with an SIV/HIV chimera virus (SHIV), have shown that a reduction in plasma viral RNA is preceded by a CTL response, and that these CTL were present before detectable neutralizing antibodies (Matano *et al.*, 1998). In more recent studies involving Rhesus macaques infected with SIV, an inverse correlation was found between viral RNA load and cellular immunity. In this case, transient increases in viral load were correlated with a decrease in cellular immunity (Jin *et al.*, 1999). Together, these findings suggest that one immune correlate for protection is the CTL response.

T helper cell response

Although evidence to suggest that the T helper cell response is a primary immune correlate for protection against HIV infection is lacking, some studies do suggest that it plays a significant role in the control of infection. Because individuals chronically infected with HIV exhibit progressive loss of CTL (Carmichael *et al.*, 1993; Kalams and Walker, 1998), this has raised the question of 'What factors control the maintenance of CTL?' (Rosenberg *et al.*, 1999). It has been suggested that virus-specific T helper cells play an essential role in maintaining CTL function (Kalams and Walker, 1998). Individuals chronically infected with HIV-1 exhibit considerable loss of virus-specific $CD4^+$ T cells from their repertoire. This is likely due to the fact that HIV-1 infects $CD4^+$ T cells and kills them by a Fas-independent mechanism (Gandhi *et al.*, 1998); several genes of HIV contribute to this cell death. However, strong CTL and T helper cell responses have been found in rare infected individuals who are able to maintain low HIV-1 viral loads (Harrer *et al.*, 1996). In addition, the administration of antiviral therapy in early stages of HIV-1 infection has been reported to lead to robust HIV-1-specific T helper cell responses and low-to-undetectable viral loads (Rosenberg *et al.*, 1999). Together, these findings suggest that the T helper cell response plays an important role in the host immune response to HIV-1 infection and may play an important role in maintaining CTL function.

Humoral response

Although some studies suggest that the CTL response is the primary immune correlate for protection, other studies suggest that the humoral immune response to HIV infection plays an important role in control of viral replication, and possibly in protection from infection. Although HIV infection has been shown to induce antibody responses to many of the major viral proteins, in most cases these antibodies show limited ability to neutralize the virus. However, studies conducted in LTNP have demonstrated that these individuals contain broadly neutralizing antibodies to HIV isolates, suggesting that neutralizing antibodies are partly responsible for the slow progression of disease (Cao *et al.*, 1995; Moore *et al.*, 1995). In another study in which macaques were immunized with a live attenuated vaccinia virus harbouring SIV genes, the monkeys exhibited neutralizing antibody titres and were able to control viral replication after challenge with SIV (Hirsch *et al.*, 1996). These findings may suggest that neutralizing antibodies to HIV represent a correlate for protection. Protection against SIV and HIV infection has been achieved in monkeys following passive transfer of antibodies from SIV and SHIV models to naïve monkeys, indirectly supporting this hypothesis (Rinaldo *et al.*, 1995; Haigwood *et al.*, 1996; Van Rompay *et al.*, 1998; Mascola *et al.*, 1999). In addition, adoptive transfer of NIH 3T3 cells transfected with a gene encoding a human monoclonal antibody (mAb), known to neutralize HIV-1, into a severe combined immunodeficiency (SCID) mouse model, reconstituted with HIV-infected human lymphocytes, has been recently shown to significantly reduce viral load in these mice (Sanhadji *et al.*, 2000).

Attempts have been made to estimate the necessary titre of neutralizing antibody to virus that will confer protection (Moore and Burton, 1999). Recently, it was shown

that passive transfer of antibodies from a chimpanzee which contained high titres of neutralizing antibodies to a primary isolate of HIV-1 into monkeys resulted in sterilizing protection from infection with a SHIV virus containing the gene-encoded envelope products of this same HIV-1 isolate (Shibata *et al.*, 1996, 1999; Igarashi *et al.*, 1999). In order to achieve 100% protection, the sera could not be diluted to greater than 1:10. However, this antibody titre has never been induced by vaccination of monkeys or humans (Moore and Burton, 1999). This may suggest that an effective HIV vaccine would have to induce both a strong humoral immune response, consisting of neutralizing antibodies, as well as a potent cellular immune response. One caveat to these studies is that the viral challenge dose used was several orders of magnitude greater than would be encountered by humans infected through normal exposure. Protection in humans, therefore, may require lower titres of neutralizing antibody than has been suggested by these studies.

Natural immunity

For certain viral infections, such as hepatitis B virus (HBV) infection, where 90% of humans infected develop neutralizing antibodies and do not become chronic carriers, natural immunity has been used as an accurate correlate for protection. However, in HIV infection, the vast majority of those infected become chronic carriers. Therefore, it is questionable whether natural immunity in HIV infection can be used to determine the true correlates for protection.

INTEGRATION

Another hindrance to HIV vaccine development is the fact that the dsDNA genome of HIV integrates into the host genome. As described, once the provirus has integrated into the host DNA, it is considered stable and further HIV replication is directed by the cellular machinery. In theory, infection with only one viral particle that is able to integrate into the host genome would pose a major obstacle to developing a vaccine that is able to induce sterilizing immunity.

GENETIC VARIABILTIY

The high genetic variability of HIV poses another obstacle to HIV vaccine development. The genetic variability found in HIV is believed to be due to the high mutation and replication rates of HIV. The rate of mutation in retroviruses is particularly high, due to the lack of exonucleolytic (proof reading) activity in their reverse transcriptase (RT) enzyme. RT, which is found in all retroviruses, has been estimated to misincorporate one in every 10^3–10^4 bases. In addition to the great geographical variability, as discussed, HIV sequences also vary within an infected individual. These variants are termed quasispecies and are believed to arise due to selective pressures, in part, supplied by the immune response. Due to the tremendous DNA sequence variability of HIV, it is a great challenge to find highly conserved amino acid sequences that can serve as targets for vaccine development.

HIV VIRAL PROTEINS

While many of the HIV viral gene products could serve as potential vaccine targets (*Figures 10.1* and *10.6A*), there is warranted concern about employing many of these proteins in an HIV vaccine. Several of the HIV proteins possess pathogenic properties against the host cell. The HIV accessory gene *vpr*, for example, when expressed in $CD4^+$ T cells has been shown to arrest infected cells in the G2 stage of the cell cycle and appears to be immunosuppressive (He *et al.*, 1995; Ayyavoo *et al.*, 1997a,b). Addition of recombinant vpr to cultures of lymphoid cell lines and primary cells infected with HIV-1 has been shown to dramatically increase HIV replication (Levy *et al.*, 1995). The gene product of the *nef* gene has also been found to interact with cellular serine (Sawai *et al.*, 1995) and tyrosine (Saksela *et al.*, 1995) kinases, suggesting that it may interfere with important signal transduction pathways of the host cell and result in killing of activated T cells (Geleziunas *et al.*, 2001). In addition, expression of the accessory gene *vpu* has been shown to cause selective degradation of CD4 in the endoplasmic reticulum (Willey *et al.*, 1992). Finally, addition of monomeric gp120 to $CD4^+$ T cell lines has been shown to induce apoptosis (Bottarel *et al.*, 1999). Consideration of the role of intact viral gene products on vaccine immunity is important.

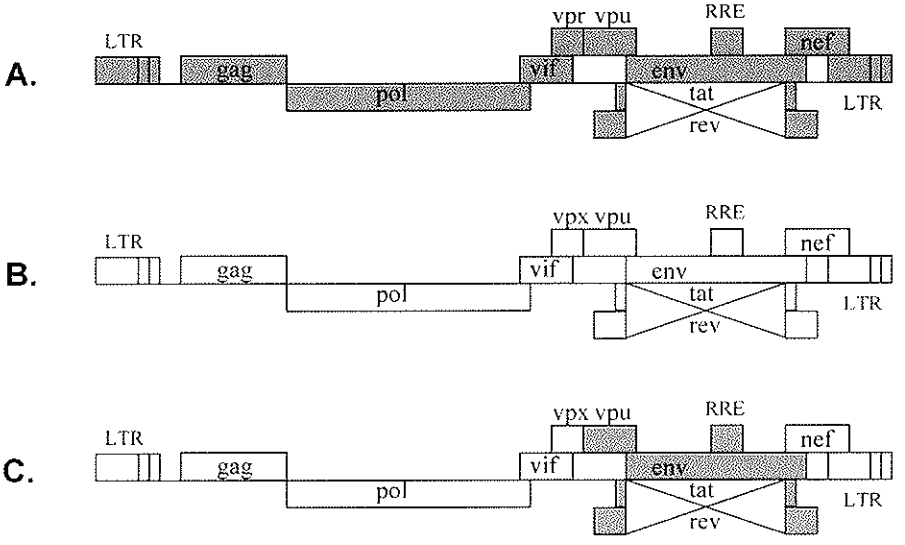


Figure 10.6. Genetic organization of HIV, SIV, and SHIV viral genomes. A) Gene structure of HIV showing the structural genes *gag, pol, env*, the regulatory genes, *rev, tat, nef*, and the accessory genes *vif, vpr, vpu*. B) Gene structure of SIV: the SIV genome contains the same structural and regulatory genes as HIV, but differs from HIV by having the accessory gene *vpx* in place of the HIV accessory gene *vpr*. C) The SHIV genome is identical to the SIV genome but contains the HIV *env, tat, rev*, and *vpu* genes.

GLYCOSYLATION OF GP120

Another obstacle to vaccine development for HIV is related to the post-translational modifications of gp120 that occur in the endoplasmic reticulum and Golgi apparatus. The mature gp120 is heavily glycosylated and this modification appears to shield important neutralizing epitopes from the humoral immune response (Reitter *et al.*, 1998).

ANIMAL MODELS

Finally, a separate, but significant, obstacle to the development of an effective vaccine for HIV is the lack of an animal model which accurately portrays the disease pathology in infected humans. Several animal models have been employed with various advantages and shortcomings when compared to infection with HIV. These are discussed below.

Mouse

Due to similarities in anatomy and physiology, non-human primate models are the preferred choice for studying human diseases. However, mice also serve as important models in vaccine development. Mice can be used to study and quantify the immune response to different immunogens in the mammalian immune system. Thus, vaccine candidates can be screened in mice to determine their immunogenicity. Another advantage to the mouse model is the ability to conduct large-scale statistical studies, as mice are abundant and easily maintained. The disadvantage of the mouse model for HIV vaccine research is that mice do not become infected with HIV-1, making them inappropriate for challenge studies. In addition, this system does not allow the therapeutic potential of an HIV vaccine candidate to be determined. Additional problems arise because mice are generally inbred and do not model the genetic diversity of the human population. However, they remain the only choice for initial screening and evaluation of candidate vaccines.

Chimpanzee

Non-human primate models routinely used in HIV vaccine research include the African green monkey, the Rhesus macaque, and the chimpanzee. However, the chimpanzee is the only model that can be used today for testing multi-component HIV immunogens (i.e. the full spectra of HIV antigens). Chimpanzees exhibit 98% identity in their DNA coding sequences with humans and it has been proposed that HIV-1 originated in the chimpanzee. This is because all known strains of HIV-1 capable of infecting man are closely related to an SIV lineage found to infect chimpanzees (Gao *et al.*, 1999). However, there are important differences between chimpanzees and humans infected with HIV. Although HIV establishes a long-term persistent infection in chimpanzees, and they develop a measurable humoral and cellular immune response, chimpanzees rarely develop AIDS. Overall, they control viral infection and exhibit lower viral load as compared to infected humans (Fultz *et al.*, 1989). Studies of chimpanzees infected for five years show that there is no

detectable decline in $CD4^+$ T cells, and that CTL responses to HIV antigens are considerably lower than those observed in humans (Ferrari *et al.*, 1993). However, an HIV-1 strain has recently emerged that is pathogenic in chimpanzees (Mwaengo and Novembre, 1998). Although the reproducibility of the pathogenesis of this strain in chimpanzees has not been currently defined, studies in chimpanzees infected with this strain could be beneficial to HIV vaccine research. In spite of this, there is considerable debate over the ethical implications of such studies since infection with this virus could be fatal to chimpanzees. Additionally, many advocates question the ethical use of these animals in medical research because of their similarities to humans and because they are an endangered species. A further hindrance to the chimpanzee model is the cost. Care for a single chimpanzee can cost greater than US$60 000. This poses a severe limitation in obtaining statistically significant results with this model. Despite differences between HIV-infected humans and chimpanzees, protection against heterologous HIV-1 challenge has been established in chimpanzees immunized with an HIV-1 DNA vaccine (Boyer *et al.*, 1997).

Rhesus macaque

Most pathogenesis and vaccine studies for HIV have been performed in the Rhesus macaque. Macaques become infected with SIV, a close phylogenetic relative of HIV. Like HIV, SIV belongs to the genus lentivirus. SIV infection and disease in the macaque is similar to HIV infection and disease in humans. SIV infection is followed by an initial viraemia, then a decrease in $CD4^+$ T cell counts, and the eventual development of AIDS-like symptoms. As with humans infected with HIV, CTL activity in macaques infected with SIV correlates with suppression of viraemia (Schmitz *et al.*, 1999). Although there are many structural similarities between HIV and SIV, such as their envelope proteins, there are important differences. While both HIV and SIV use the CD4 molecule and chemokine receptors for viral entry, SIV does not always use the same chemokine receptors as HIV. Macrophage tropic (M) HIV strains use the CCR5 receptor for viral entry, while T cell tropic (T) HIV strains use the CXCR4 receptor for viral entry. While M tropic SIV strains use the CCR5 receptor, several T tropic SIV strains do not use the CXCR4 receptor for viral entry (Edinger *et al.*, 1997). Another important difference between HIV infection in humans and SIV infection in macaques is that macaques infected with SIV show a more rapid disease course than humans infected with HIV. Additionally, SIV does not encode a single *vpr* gene product; it encodes a duplicated *vpr* gene and its copy product, *vpx*. How these changes will relate to vaccine development is still not completely understood.

The SHIV macaque model is a more recently developed model that uses an SIV–HIV chimera virus (SHIV) to study infection and disease in the macaque. This virus consists of SIV gene encoded products but has HIV envelope gene products in place of the SIV envelope (*Figure 10.6*). SHIV chimeras have *in vitro* properties similar to HIV isolates, such as replication and cytopathogenicity (Kuwata *et al.*, 1996). Infection with SHIV has been shown to cause rapid increase in viral loads, $CD4^+$ T cell depletion, and an AIDS-like disease in macaques (Cayabyab *et al.*, 1999). Despite these developments in the macaque model, a complete multi-component HIV antigen-based vaccine cannot be used in this model since HIV does dot establish a persistent

infection in macaques. However, recent studies have illustrated that plasmid vaccines, or plasmid primed with MVA booster or adenoviral vaccines, can all induce homologous type-specific immune responses (Buge *et al.*, 1999) and may prevent disease in the SHIV model.

Conventional vaccine approaches

RECOMBINANT PROTEIN/SUBUNIT VACCINES

Most candidate vaccines originally studied for HIV were subunit vaccines. Subunit vaccines usually consist of recombinant antigens formulated in an appropriate immune adjuvant. Although many HIV gene encoded proteins have been targets for vaccine development, most HIV subunit vaccines consist of recombinant HIV envelope proteins. The envelope protein is chosen because it plays a critical role in viral entry and is therefore a target for neutralizing antibodies. Recombinant envelope proteins studied include gp120, gp160, and gp140. The gp160 protein is a precursor protein that is post-translationally cleaved to give gp41, the transmembrane subunit of the envelope, and gp120, the extracellular subunit (*Figure 10.7*). The gp140 protein is a splice site deletion of gp160 and consists of gp120 and the ectodomain of gp41. This molecule is now being studied because, in theory, it mimics the oligomeric forms of the native envelope. The *gag* or *pol* gene products are also commonly used in subunit vaccines because of their ability to elicit CTL-specific responses. Recombinant proteins that are exogenously presented to the immune system, such as those used in subunit vaccines, function by inducing primarily a humoral immune response (*Table 10.1*).

Native structure of gp120

The efficacy of recombinant subunit vaccines is strongly influenced by the degree of similarity in tertiary structure between recombinant and native viral proteins. The greater this similarity, the greater the chance of inducing neutralizing antibodies to the virus and thus protection from viral entry. Most HIV-1 subunit vaccines in clinical trials have employed monomeric gp120. Although immunization with monomeric gp120 has demonstrated the induction of antibodies capable of neutralizing T cell line adapted HIV-1, these antibodies are very inefficient at neutralizing primary HIV-1 isolates. It has been suggested that HIV-infected individuals develop antibodies to viral debris, including monomeric gp120 rather than viral associated gp120 (Parren *et al.*, 1999). Antibodies that bind to monomeric gp120 but do not bind to virus-associated gp120 are unable to neutralize the virus (Parren *et al.*, 1998). This may be due to differences in monomeric gp120, such as the recombinant gp120 used in these subunit vaccines, and the native viral associated gp120. The native envelope protein is now believed to exist as a trimer within the viral envelope (Chan *et al.*, 1997; Lu and Kim, 1997). In a recent study, it was shown that gp140 trimers, which were constructed using the gene sequence from a primary isolate fused to a trimerization motif, could induce significant neutralizing antibody titres to primary HIV-1 isolates (Yang *et al.*, 2001). These findings highlight the importance of conserving the native envelope structure when employing these proteins in vaccines, with the intent of inducing antibodies capable of neutralizing the virus.

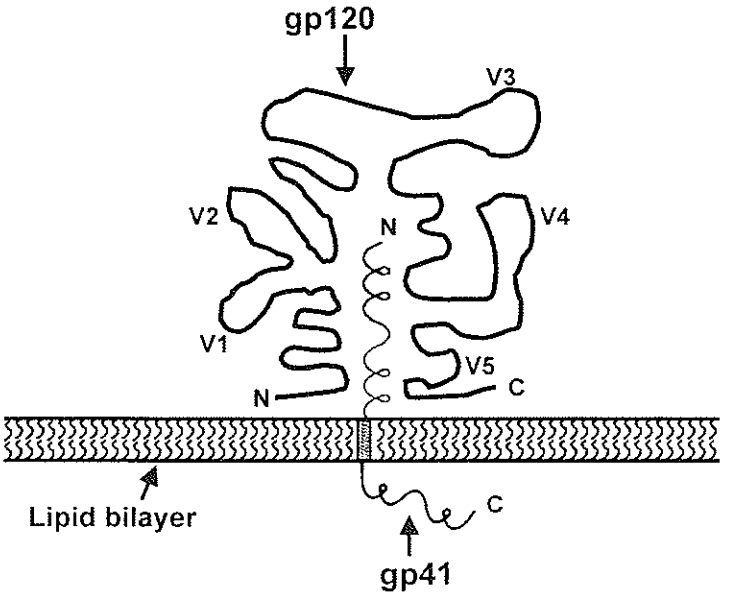


Figure 10.7. One-dimensional representation of the HIV envelope glycoprotein. The surface subunit, gp120, is about 550 amino acids in length and associates through non-covalent interactions with the ectodomain of the transmembrane subunit, gp41. The mature gp120 is heavily glycosylated, highly hydrophilic, and contains five hypervariable regions (V1–V5), and six conserved regions. The transmembrane subunit, gp41, is about 350 amino acids in length, is relatively hydrophobic, traverses the membrane, and is a type I integral membrane protein. The N-terminus of gp41 contains a short hydrophobic stretch of amino acids referred to as the 'fusion peptide' because it is implicated in membrane fusion with the target cell. C-terminal to the fusion peptide are two hydrophobic regions predicted to form an anti-parallel left-handed helix when HIV fuses with its target cell. The cytoplasmic domain of gp41 contains two alpha helices that have been implicated in cell lysis of the target cell following viral assembly.

Neutralization epitopes

Substantial evidence now indicates that neutralization can be achieved by antibody binding to the CD4 binding site (CD4bs) or the chemokine receptor binding sites (CCR5 or CXCR4) on gp120, thereby inhibiting virus attachment to the target cell (Parren *et al.*, 1999). The binding site of an mAb, 17b, that binds gp120 and has been shown to neutralize HIV-1 (Thali *et al.*, 1993) and the CCR5 binding site, have been mapped by site-directed mutagenesis studies of gp120 (Rizzuto *et al.*, 1998). In these studies, binding of mAb 17b and CCR5 to gp120 was assessed in the context of mutations introduced into a gp120 protein derived from a primary HIV-1 isolate. Using data derived from the solved crystal structure of gp120 core, in complex with a two-domain fragment of CD4 and the 17b Fab fragment (Kwong *et al.*, 1998), the predicted binding sites were mapped. The mAb 17b binding site was mapped to a region on the ectodomain of the gp120, which overlaps with the predicted CCR5 binding site (*Figure 10.8*). The binding site of another mAb, b12, that is able to neutralize 75% of clade B primary viruses (Trkola *et al.*, 1995; Burton, 1997) has been mapped to the CD4 binding site on gp120 (Saphire *et al.*, 2001). The crystal structure

Table 10.1. Vaccine strategies.

Strategy/formulation	Mechanism of action	Immunity generated	Advantages	Disadvantages
Live attenuated Recombinant virus/ bacteria may incorporate antigen	Replication in host induces immune response	Humoral and cellular	Good immunogens and long-lived immunity	Potential reversion to wild-type pathogenic organism
Inactivated Chemical inactivation	Retain immunogenicity without infectivity	Humoral	Good immunogens and safe if properly inactivated	Loss of antigenicity by denaturation. Frequent re-immunization needed
Subunit Native protein/ peptide	Elicit immune response to dormant determinants	Humoral	Safe except for rare adverse reactions	High manufacturing cost, poor antigenicity. Carrier/ adjuvant needed. Poor cellular immunity
Recombinant protein	Elicit immune response to dominant determinants	Humoral	Safer than native antigens. Some success with approach	
Synthetic peptide	Elicit immune response to dominant epitopes	Humoral	Can exclude deleterious epitopes. Inaccessible in native protein.	Lack of efficacy. Peptides limited to conserved sequences. Unrecognized by some MHC haplotypes. Lack of experience with approach. Safety under investigation.
DNA based Plasmid containing gene of interest	Immune response generated against expressed antigen and CpG motifs	Humoral and cellular	No reversion to pathogenic virus. Highly stable. Can focus on dominant epitopes.	

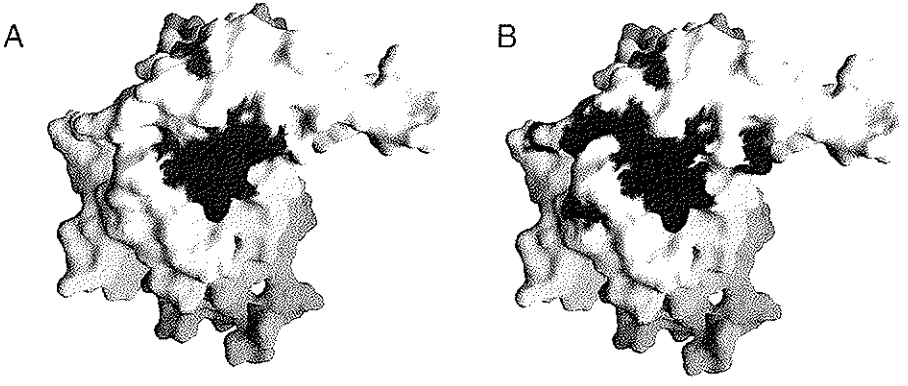


Figure 10.8. Molecular surface structure diagrams of HIV gp120 core, showing predicted CCR5 (A) and mAb 17b (B) binding sites. Amino acid changes that resulted in greater than 75% decrease in ligand binding are represented in black. The predicted binding site for mAb 17b, an antibody that possesses neutralization activity, overlaps with the predicted CCR5 binding site. Figures were generously provided by Joseph Sodroski, Department of Cancer Immunology and AIDS, DANA Farber Institute, Department of Pathology, Harvard Medical School, and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, USA.

of this antibody has recently been solved and reveals useful information about its predicted binding site on gp120 (Saphire *et al.*, 2001). These studies illustrate gp120 sites that may be critical to mimic in vaccine formulation, though this has been difficult to achieve.

Another target for viral neutralization is the envelope transmembrane subunit gp41. It is believed that a conformational change occurs in the gp120/gp41 complex during binding of the envelope to the target cell receptors, which results in exposing gp41 that was previously buried (Chan and Kim, 1998). Development of this model was based on the crystal structure of specific gp41 core peptides (Chan *et al.*, 1997). This structure consists of a six-helix bundle in which three central peptide helices, forming a coiled coil, are surrounded by three anti-parallel peptide helices. It was proposed that prior to HIV-1 fusion with its target cell, gp41 is buried beneath gp120 and is inaccessible. Upon binding of the target cell, the gp120/gp41 complex is proposed to undergo a conformational change resulting in exposure of three helices of gp41, referred to as the fusion peptide, which then insert into the target cell membrane. A second event is then proposed in which three different helices of gp41 combine with the fusion peptide to form the six-helix bundle that ultimately mediates fusion with the target cell (Chan *et al.*, 1997). An mAb to gp41 that was isolated from antibodies raised to gp41 appears to neutralize HIV-1 at a stage after HIV-1 binds its target cell (Parren *et al.*, 1999). It is believed that other, yet unidentified, gp41 neutralizing epitopes exist which are exposed during the fusogenic state of HIV-1 with the target cell membrane. Stabilizing and mimicking these transient gp41 states are critical goals for HIV vaccine development.

HIV immunotypes

Given the high diversity of HIV-1 genotypes, attempts have been made to classify HIV according to defined groups within the different HIVsubtypes that bind defined sets of antibodies. The goal is to reduce the genotype classification to one that is less diverse and can be more readily targetted by a vaccine. This classification is referred to as immunotype classification. In one study, a mathematical analysis was used to characterize peptides, corresponding to the V3 hypervariable region of different HIV genotypes, for their ability to bind several groups of mAb. Distinct immunotypes were discovered that differed from the genotype classification (Zolla-Pazner *et al.*, 1999). In a subsequent study, immunotyping of HIV-1 primary isolates was attempted, using mAb specific for epitopes within the V3, C5, and gp41 cluster I regions (Nyambi *et al.*, 2000). This study revealed fewer immunotypes than HIV-1 genotypes based on these determinants. These studies are crucial for developing a vaccine that will be capable of inducing protection against the broad genetic variability found in HIV. Such a vaccine will need to be polyvalent and contain combinations of HIV determinants that represent all the major immunotypes found in HIV.

Post-translational modification of gp120

Considerable indirect evidence now exists that suggests that a difficulty in inducing neutralizing antibodies in HIV-infected individuals is related to the post-translational modifications made to the gp120 envelope protein. The gp120 protein in HIV and SIV

contains 24 potential N-linked and up to 8 O-linked glycosylation sites, and the mature protein is heavily glycosylated (Leonard *et al.*, 1990; Bernstein *et al.*, 1994). Studies employing SIV mutant strains, generated with mutations in two or three glycosylation residues which eliminate glycosylation at these sites, have been proven to induce substantially higher neutralizing antibody titres than wild-type strains to a primary SIV isolate (Reitter *et al.*, 1998). This suggests that these carbohydrate side chains present on native gp120 shield the protein from an effective envelope-specific humoral immune response. Thus, an effective HIV-1 vaccine strategy may involve selectively eliminating glycosylation of gp120 at key residues in order to induce a potent neutralizing antibody response to HIV-1.

PEPTIDE VACCINES

Vaccines consisting of peptides only have been used with the primary goal of inducing neutralizing antibodies to key viral determinants. The V3 hypervariable region of gp120 was found to contain neutralizing activity and was a major target in the first decade of HIV vaccine development. Various studies reported that antibodies raised against peptides to the V3 sequence of TCLA HIV strains were able to neutralize TCLA HIV (Putney *et al.*, 1986; Javaherian *et al.*, 1990; dEmini *et al.*, 1992). However, it was soon found that many of these antibodies to the V3 sequence of TCLA HIV strains were unable to cross-neutralize HIV strains lacking the same V3 sequence. V3 sequences in primary HIV isolates have been found to differ significantly from those found in TCLA strains. Although the V3 sequence of a particular TCLA HIV strain, HIVMN, was later found to react with a higher percentage of primary HIV isolates, a consensus V3 sequence capable of inducing neutralizing antibodies to all the major HIV-1 strains has yet to be discovered (LaRosa *et al.*, 1990).

Peptides derived from the constant region C4 and the hypervariable region V3 of gp120 have also been used in vaccine formulations with the intention of inducing HIV-specific T helper cell and CTL responses. The C4 region contains specific T helper cell determinants, while the V3 region contains specific CTL determinants. In one study, it was shown that immunization of HIV-infected individuals with the C4 and V3 peptides of HIV gp120 induced significant lymphocyte proliferative responses, including HIV-specific CTL responses (Bartlett *et al.*, 1998). However, HIV viral RNA levels in these individuals did not differ significantly with the control group over a 52-week period, indicating that the induction of HIV-specific CTL responses by this approach did not have a significant impact on viral replication.

Another limitation inherent to peptide vaccines is that they elicit antibody responses only toward linear epitopes, while important neutralizing epitopes may be conformational and discontinuous in nature (*Table 10.1*). Overall, the future of peptide vaccines for antibody induction is uncertain.

LIVE-ATTENUATED VACCINES

One vaccine approach which has shown promise is attenuated live vector vaccines. These vectors have the advantage of reproducing in the recipient and therefore provoke both a broader and more intense range of antibodies and T lymphocyte-

associated immune responses. Furthermore, responses appear to be long-lived. Several live vectors harbouring HIV-1 proteins have been used as vaccine candidates for HIV-1. Live recombinant vaccinia and canarypox viral vectors expressing HIV gene encoded products, such as gp160 or gp120, have been shown to induce both HIV-1-specific humoral and cell-mediated immune responses (Walker *et al.*, 1994, 1995). In one study, volunteers primed with a canarypox virus, harbouring HIV gp160, and boosted with a gp120 subunit vaccine, developed HIV-specific $CD4^+$ T cell, $CD8^+$ T cell responses, and neutralizing antibody titres (Walker *et al.*, 1994, 1995). Macaques immunized with a vaccinia vector harbouring HIV proteins have also elicited HIV-specific cellular and humoral immune responses (Hu *et al.*, 1987; Shen *et al.*, 1991). Futhermore, in another study in which macaques were immunized with a vaccinia vector harbouring the SIV gene encoding gp160, followed by a boost with recombinant SIV gp160, some monkeys showed protection from challenge with primary SIV isolates (Hu *et al.*, 1992). Although these results are promising, studies in which live viral vector vaccines, such as vaccinia, had been administered to immunosuppressed individuals resulted in some individuals developing life-threatening viral infections (Redfield *et al.*, 1987). For this reason, there is reluctance to use these vector vaccines in large-scale human trials. Interest has instead moved to other viral vectors that are more attenuated, such as avian poxviruses. These viruses have limited *in vivo* replicative capacity in humans. However, in preliminary studies with avian poxviruses harbouring HIV genes, 10-fold higher doses were required to induce similar HIV-specific antibody and CTL responses, compared with those induced by vaccinia vectors (Pialoux *et al.*, 1995). In general, it has been observed that the lower the replicative capacity of a virus, the lower the immunogenicity, while the higher the replicative capacity, the higher the risk of reversion to wild-type pathogenic virus, clearly a conundrum to this approach.

Another class of viral vectors now being studied as potential HIV-1 vaccine vectors is the alpha viruses. These viruses contain an mRNA promoter for their viral structural proteins, which transcribe the structural proteins at levels 10-fold greater than the host genome and would therefore be expected to induce substantially greater immune responses. An attenuated strain of an alpha virus, the Venezuelan equine encephalitis virus (VEE), has been studied as a potential HIV-1 viral vaccine vector (Caley *et al.*, 1997). In one study, immunization of mice with a VEE vector harbouring HIV-1 *gag* resulted in humoral and cellular immune responses specific for HIV-1 matrix and capsid proteins. More recently, immunization of macaques with a VEE vector harbouring SIV *gag* and *env* genes induced limited replication control against challenge with a pathogenic strain of SIV (Davis *et al.*, 2000). In a recent study, genes delivered by replication-incompetent adenovirus vectors induced strong CTL lowered viral load and protected CD4 T cells in an SHIV 89.6 challenge study (Shiver *et al.*, 2002).

Other potential viral vaccine vectors for HIV now being investigated are the adeno-associated viruses (AAV) (Johnson *et al.*, 2001). The wild-type AAV virus is non-pathogenic in humans and has been shown to mediate long-term expression of the transgene in non-human primates. Because these vectors lack any AAV genes and can be packaged with only the gene of interest and the appropriate expression elements, there is a great deal of interest in AAV as a vaccine delivery system. In a recent study, macaques immunized with an AAV vector, harbouring SIV genes,

exhibited antigen-specific CD8+ T cell responses, as well as persistent neutralizing antibody titres (Johnson *et al.*, 2001). Preliminary challenge studies in these macaques suggested that immunized monkeys had significantly lower virus burdens than controls.

Other live vaccine vectors now being considered for use as potential HIV vaccines are of bacterial origin. One bacterial vaccine vector candidate is bacille Calmette-Guerin (BCG). Immunization of macaques with recombinant BCG, harbouring SIV genes, has been shown to elicit SIV-specific CTL activity (Yasutomi *et al.*, 1993). Other bacterial vectors, such as *Salmonella* and *Listeria*, are also being studied as potential HIV vaccine vectors. In one study, a *Salmonella* vector, harbouring HIV gp120, was shown to induce significant gp120-specific antibody and CD4+ T cell responses in mice (Wu *et al.*, 1997). In other studies, in which *Listeria monocytogenes* was used as a vector to express the HIV Gag protein, it was found that mice immunized with this vector developed strong Gag-specific CTL, and CD4+ T cell responses (Frankel *et al.*, 1995; Mata and Paterson, 1999; Mata *et al.*, 2001).

As described above, there are many difficulties in developing an HIV vaccine using current approaches. There are advantages and disadvantages to each approach (*Table 10.1*). An effective HIV subunit vaccine must likely induce both broadly neutralizing antibodies and cell-mediated immunity to primary HIV isolates. To induce neutralizing antibodies, the native structure of the envelope protein must be conserved and presented to the immune system in an immunogenic form. Subunit vaccines primarily induce humoral immune responses, and most potential HIV subunit vaccines have not proven effective at inducing broadly neutralizing antibodies against primary HIV-1 isolates. Alternatively, live-attenuated virus vectors are capable of inducing both humoral and cell-mediated immunity because they replicate in the host cells of the recipient. In addition, viral particles are assembled by the host cell machinery, and therefore have the advantage of presenting viral proteins that more likely resemble their native structure. Nevertheless, some of these viral vectors have the possibility of reversion to wild-type pathogenic strains and could pose a serious threat to immunocompromised individuals.

Overall, several promising avenues for live-attenuated vector vaccines are beginning to emerge. One limitation of these approaches is the development of anti-vector host immunity. These responses may limit effectiveness in the clinic. Other approaches that provide the cellular immune benefits of live approaches without this host immunity, such as DNA vaccines, may circumvent this limitation.

DNA immunization

In recent years, considerable attention has shifted to the technology of DNA immunization. In this approach, genes encoding antigens from a particular pathogen are cloned into an expression vector. The DNA is then administered to the vaccine recipient, and is taken up by host cells. Antigens are subsequently expressed by the host cell, processed, and presented to the immune system. As with live viral infection, immunization with DNA results in the expression of viral proteins by the host cell machinery. Again, because DNA-encoded viral proteins are assembled by the host cell machinery, their structure would be expected to resemble their native viral counterparts. Thus, DNA immunization confers many of the same broad immuno-

logical advantages of live vectors, but lacks the safety issue associated with them (*Table 10.1*).

MECHANISM(S) OF ANTIGEN PRESENTATION

Although DNA immunization is known to induce both antigen-specific humoral and cellular immune responses, the mechanism(s) of antigen presentation to the immune system following immunization is not fully understood. Antigen presentation, following DNA immunization, has been proposed to occur by three possible routes: 1) direct priming of lymphocytes by transfected somatic cells, such as keratinocytes, or myocytes; 2) direct transfection of professional antigen-presenting cells (e.g. dendritic cells); 3) cross-presentation, whereby directly transfected somatic cells or antigen-presenting cells secrete the gene encoded antigen. The antigen can then be endocytosed by antigen-presenting cells, processed and presented to the immune system via the MHC class I or MHC class II pathways. Thus, in this manner, DNA vaccines mimic the broad immune responses which characterize live-attenuated vaccine approaches.

Direct transfection of somatic cells

It was originally proposed that somatic cells, such as myocytes or keratinocytes transfected with DNA vectors, could function as antigen-presenting cells and could directly activate $CD8^+$ T cells, since they constitutively express MHC class I molecules (*Figure 10.9*). However, one difficulty with this hypothesis is that myocytes lack cell surface molecules called co-stimulatory molecules, such as CD80 and CD86, which play a critical role in activating $CD8^+$ T cells. A number of studies have been conducted to investigate this hypothesis. In one study, bone marrow chimeric mice were employed to determine the contribution of non-bone marrow-derived (e.g. monocytes) and bone marrow-derived (e.g. dendritic cells) cells to the activation of $CD8^+$ T cells. This study reported that only when non-bone marrow-derived cells where transfected with CD86 could they function to induce CTL responses (Agadjanyan *et al.*, 1999). In another study, it was reported that removal of muscle tissue immediately after immunization did not affect the immune response (Torres *et al.*, 1997). Together, these studies provide compelling evidence against the hypothesis that transfected somatic cells can directly activate CTL.

Direct transfection of antigen-presenting cells

Another mechanism that has been proposed is that a small number of professional antigen-presenting cells (dendritic cells or macrophages) residing at the site of immunization are directly transfected (Pardoll and Beckerleg, 1995) (*Figure 10.10*). These cells would then traffic via the afferent lymph to regional lymphoid tissue, where they could activate $CD8^+$ T cells, $CD4^+$ T cells, and B cells (Pardoll and Beckerleg, 1995). Because Langerhans' cells and interstitial dendritic cells (two types of antigen-presenting cells) are present in the skin, this mechanism seems plausible with epidermal immunization (Banchereau and Steinman, 1998). Evidence for this mechanism is suggested by a number of studies. In one study, plasmid DNA

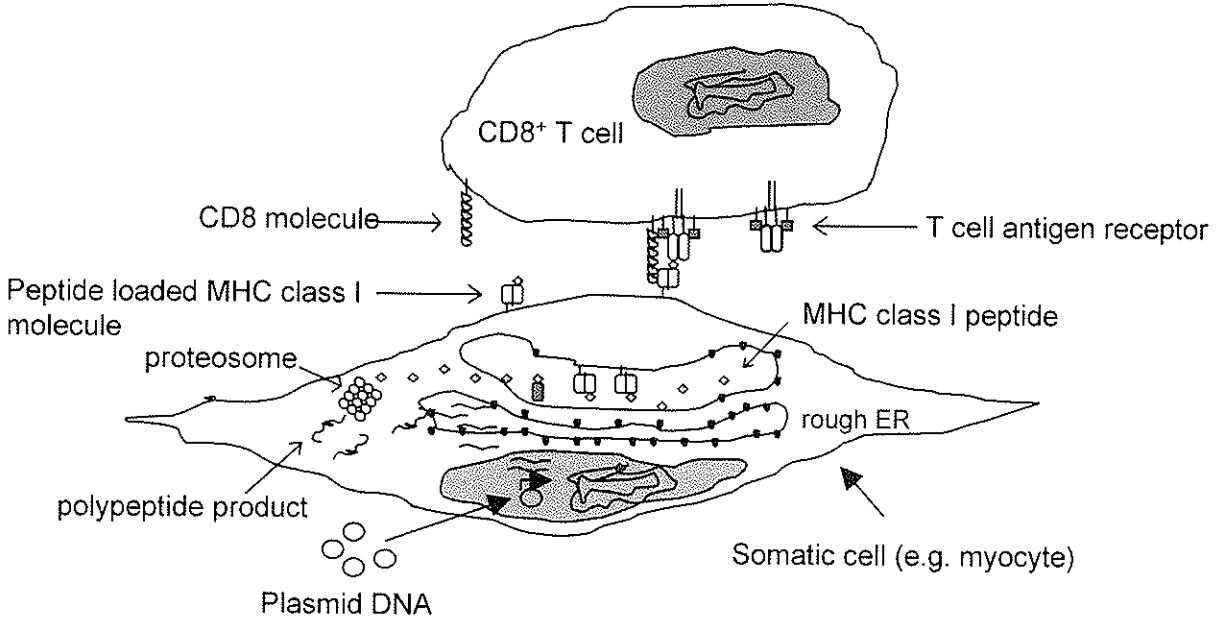


Figure 10.9. Model for direct transfection of somatic cell by genetic immunization. Somatic cells, such as myocytes or keratinocytes, are directly transfected. The gene-encoded antigen is expressed by the somatic cell, processed via the MHC class I pathway, and presented on MHC class I molecules. A CD8+ T cell that recognizes its cognate peptide antigen in the context of an MHC class I/peptide complex on the somatic cell surface binds via its TCR and is activated.

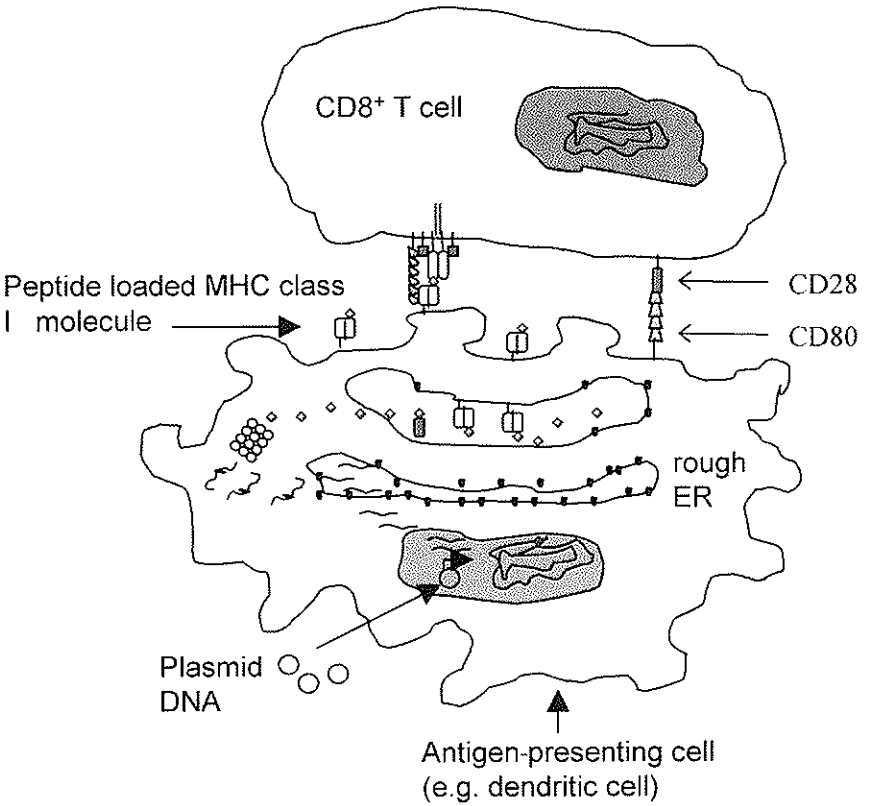


Figure 10.10. Model for direct transfection of antigen-presenting cell. An APC, such as a Lagerhans cell, that is present near the site of immunization, is directly transfected. The plasmid-encoded antigen is expressed and processed via the MHC class I pathway and presented on MHC class I molecules. The APC also expresses CD80 on its surface. A CD8+ T cell that recognizes its cognate peptide antigen in the context of an MHC class I molecule/peptide complex binds via its TCR. The T cell also binds to CD80 on the APC surface via its CD28 surface molecule. The CD8+ T cell is stimulated through both the TCR/MHC class I/peptide interaction and the CD28/CD80 interaction and is activated to evolve into an effector cell.

was isolated from dendritic cells located in the skin and lymph nodes following intramuscular DNA immunization (Casares *et al.*, 1997). In another study, following 'gene gun' administration of a plasmid or encoding green fluorescent protein (GFP), Langerhans cells expressing GFP were isolated from the draining lymph nodes (Condon *et al.*, 1996).

Cross-presentation

A third proposed mechanism of antigen processing and presentation following genetic immunization is that antigen or MHC class I or class II peptides, processed by

transfected myocytes or keratinocytes, are transferred after being secreted, shed from the surface, or released from dying cells to bone marrow derived, antigen-presenting cells, which have migrated to the site of immunization (*Figure 10.11*). Transferred antigen or peptides may then enter either the MHC class I or class II antigen processing and presentation pathways. Peptide antigen that enters the class I pathway would get loaded onto MHC class I molecules and be used to prime CTL. Antigen or peptide that enters the MHC class II processing pathway would get loaded onto MHC class II molecules and be used to prime $CD4^+$ T cells. Although this hypothesis contradicts the dogma that only endogenously produced antigen can enter the class I processing pathway, it is consistent with reports that exogenously produced protein can be taken up by antigen-presenting cells and re-presented via the MHC class I pathway (Carbone and Bevan, 1990; Huang *et al.*, 1994; Kovacsovics-Bankowski and Rock, 1995; Pardoll and Beckerleg, 1995; Suto and Srivastava, 1995; Albert *et al.*, 1998a,b). Other studies have also supported this hypothesis. In one study, dendritic cells, when cultured with monocytes infected with influenza, were able to activate influenza specific CTL (Albert *et al.*, 1998b). In another study, this same group reported that dendritic cells could phagocytose influenza-infected monocytes undergoing apoptosis and could subsequently activate influenza-specific CTL (Albert *et al.*, 1998a).

IMMUNOSTIMULATORY CpG MOTIFS

In addition to an immune response generated against specific gene encoded products following DNA immunization, injection of bacterial derived DNA is also known to induce a non-specific immune response (Krieg, 1996). The basis for this immunostimulatory property of bacterially derived DNA is believed to be due to the high frequency of unmethylated cytosine-phosphate-guanine (CpG) dinucleotides present in bacterial DNA. In vertebrate DNA, the cytosine in these dinucleotides is usually methylated. DNA containing unmethylated CpG motifs has been shown to activate murine macrophages and dendritic cells. Studies have shown that CpG motifs preferentially induce a type 1 helper T lymphocyte (Th1) response and induce the production of IL-2, IFN-γ, and IL-12 (Sato *et al.*, 1996; Klinman *et al.*, 1997; Leclerc *et al.*, 1997; Roman *et al.*, 1997). Recently, a member of the Toll-like receptor family, TLR9 was proposed to be the receptor in eukaryotic cells that recognizes bacterial CpG motifs, as macrophages and dendritic cells from mice, which contain a mutation in this gene, are unable to respond to stimulation by unmethylated CpG DNA (Hemmi *et al.*, 2000). However, the role of CpG motifs for plasmid vaccines studied in primates has not been very encouraging, and more study in this area is needed.

ROUTE OF IMMUNIZATION

Some of the first studies with DNA vaccination showed that antibody, T helper cell, and CTL responses could all be generated and that the route of immunization (e.g. intramuscular, epidermal, and mucosal) influenced the direction of the response (Fynan *et al.*, 1993; Ulmer *et al.*, 1993; Davis *et al.*, 1994). Most DNA vaccines studied to date induce predominantly a Th1 response when injected intradermally. Conversely, intramuscular or epidermal gene gun delivery has been shown to induce

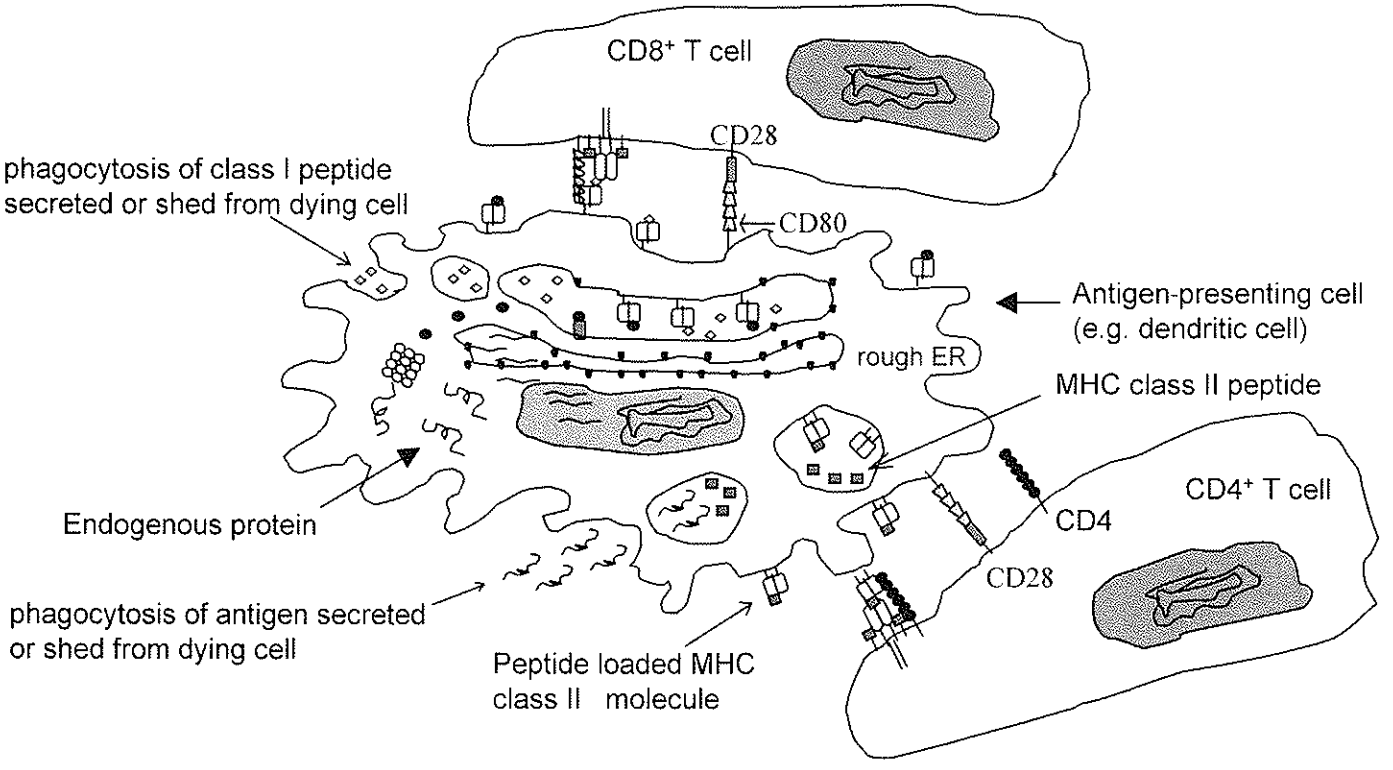


Figure 10.11. Model for cross-presentation of antigen. Antigen or processed MHC class I or class II peptides that are processed by transfected somatic cells, is transferred to APC after being secreted, or released from dying cells. MHC class I peptide antigens may then enter the MHC class I pathway and get loaded onto MHC class I molecules. These MHC class I/peptide complexes present on the APC surface can be recognized by antigen-specific CD8+ T cells. MHC class II peptide or whole antigen could be phagocytosed by APC, processed, and presented on MHC class II molecules. These MHC class II/peptide complexes on the APC surface can then be recognized by antigen-specific CD4+ T cells. A CD4+ T cell or a CD8+ T cell that recognizes its cognate antigen on the surface of the APC in conjunction with CD80 can then be activated to mature into an effector cell.

primarily a Th2 response (Feltquate *et al.*, 1997). The reason for this difference is not understood; however, it is believed that needle injection delivers DNA to the extracellular space, where it is then taken up by cells, while gene gun delivery results in direct transfection of target cells. This may account for the different responses.

DOSE AND REGIMEN

The dose of DNA delivered and the immunization regimen can also affect the immune response. Depending on the gene-encoded antigen, 10–100 μg of DNA is usually required to induce an immune response when administered intradermally or intramuscularly. However, gene gun immunizations usually require 10-fold less DNA to induce comparable antibody or CTL responses. Studies of the humoral response to immunization with a gene-encoded influenza antigen have shown a dose response increase in antibody titre that plateaus at an optimal dose. However, the optimal antibody response in these studies did not seem to differ whether the optimal dose was given in one injection or multiple injections (Deck *et al.*, 1997; Robinson *et al.*, 1997).

Although the frequency of immunization may not affect the optimal immune response, immunization frequency has been shown to affect the type of response. In one study, in which mice were immunized intradermally with an HIV-1 envelope DNA vaccine, it was shown that immunization with the first three doses induced a strong CTL response but weak antibody response, while a fourth immunization induced a strong antibody response and a drop in the CTL response (Fuller and Haynes, 1994).

SAFETY

Although DNA immunization appears to be a safe procedure, there are certain safety concerns that have been raised with this approach. One concern is that immunization with plasmid DNA could result in plasmid DNA integration into the host cell genome, potentially disrupting endogenous genes. In one study, this potential hazard was tested (Nichols *et al.*, 1995). Mice were injected intramuscularly with 100 μg of plasmid DNA. Tissue samples were taken at different time points from the sites of injection and from 12 other sites. Genomic DNA was purified from plasmid DNA and examined by polymerase chain reaction (PCR) for integration of plasmid DNA. The test sensitivity was apparently capable of detecting as low as one integration per 1.5×10^5 nuclei, a sensitivity that was determined to be three times lower than the spontaneous mutation frequency. No integration events were detected. However, in a more recent study, it was found that the methods used for separating genomic DNA from plasmid DNA in combination with the high sensitivity of PCR make it very difficult to determine integration due to the inevitable contamination of genomic DNA with plasmid DNA (Martin *et al.*, 1999). Further studies are necessary to precisely quantify integration frequencies; however, they appear to occur at a very low frequency.

Another concern with injecting bacterial-derived DNA into humans is the possibility of inducing an autoimmune response against human DNA. To investigate this possibility, one study used Lupus model mice, mice that are predisposed to developing

anti-DNA antibodies (Katsumi *et al.*, 1994). Mice were immunized with bacterial-derived plasmid DNA. Anti-DNA-specific B cells were then quantified by enzyme-linked immuno spot (ELIspot) assays. Anti-DNA antibody titres were measured. It was determined that immunized mice contained two to three times higher titres of anti-DNA-specific B cells than Lupus control mice. Although this raised some concern, it was later determined that this increase in DNA-specific B cells and anti-DNA antibody titres did not accelerate disease in the Lupus model mice (Xiang *et al.*, 1995; Mor *et al.*, 1997). Whether this effect could cause adverse effects in humans with pre-existing autoimmune disorders has yet to be determined. Importantly, in human studies of several hundred volunteers, no evidence for the induction of anti-DNA antibodies has been observed to date.

HIV DNA VACCINES

DNA vaccination with plasmids encoding HIV-1 antigens has shown promising results in non-human primates. DNA immunization studies of macaques using multi-component HIV-1 encoded antigens revealed that monkeys could be protected from challenge with a chimeric SHIV virus (Kim *et al.*, 2001). Immunization of Rhesus monkeys with a DNA vaccine containing the gene for HIV-1 gp160 followed by boosting with recombinant gp160 resulted in high neutralizing antibody titres, HIV-1 envelope-specific CTL activity, and protection from viral challenge with a chimeric SHIV virus (Letvin *et al.*, 1997). Immunization of chimpanzees with a DNA vaccine encoding *env*, *rev*, and *gag/pol* genes was also shown to induce cellular and humoral immune responses and protection from challenge with a heterologous HIV-1 strain (Boyer *et al.*, 1997). Clinical trials using HIV DNA vaccines are currently under way. To date, DNA vaccines for SIV or SHIV in primates have elicited protection from SIV, SHIV, or HIV viral challenge in more cases than with any other vaccine type.

Novel improvements to DNA immunization

Several new methods are now being used to improve expression of antigens encoded by DNA vaccines, such as the use of stronger promoters, or the use of episomal vectors. Other manipulations involve employing targetting sequences to selectively deliver antigens to the Class I or Class II antigen presentation pathways. Another technique now being used is called codon engineering. This technique involves the conversion of codons usually used by the virus to those more commonly used by eukaryotes in order to optimize expression in mammalian cells. This is important as *rev* limits the expression of HIV structural genes by preventing the splicing of mRNA transcripts. Changing codon usage removes this *rev* dependence and facilitates higher gene expression. Finally, plasmids encoding cytokines or co-stimulatory molecules are now being used in conjunction with plasmids encoding antigen to augment a particular immune response, as described below.

CYTOKINE ADJUVANTS

Many laboratories have reported that co-injection of a plasmid encoding antigen with

plasmids encoding cytokines can influence the magnitude and direction of the immune response. Recently, it was demonstrated that a plasmid encoding hepatitis B virus, HBsAg, and IL-2 induced marked increases in antibody responses and T cell proliferation over a plasmid encoding HBsAg alone (Chow *et al.*, 1997). Immunization of Rhesus monkeys with a plasmid encoding SIV *gag* and a plasmid encoding IL-2/Ig (an IL-2/immunoglobulin fusion protein) showed marked increases in SIV gag-specific humoral and CTL responses (Barouch *et al.*, 2000). Co-injection of mice with a plasmid encoding an HCV antigen and a plasmid encoding IL-4 resulted in a marked decrease in CTL responses and an increase in the antibody response, compared to injection with the HCV antigen-encoded plasmid alone (Geissler *et al.*, 1997). In the HIV system, Kim and co-workers found that co-delivery of IL-12 expression cassettes with DNA vaccines for HIV-1 in mice resulted in splenomegaly, as well as a Th1 shift in the specific immune responses induced (Kim *et al.*, 1997a). This result was later confirmed in macaques and chimpanzees where the co-immunization of genes expressing IL-12 and IL-18 increased the magnitude of Th1-type immune responses (Kim *et al.*, 1999; Boyer *et al.*, 2000). Most recently, Barouch and colleagues have demonstrated that the delivery of a fusion molecule, IL-2/Ig, augments the vaccine-elicited humoral and cellular immune responses in higher primates (Barouch *et al.*, 2000). In these studies, monkeys receiving the fusion molecule develop 30-fold higher envelope-specific antibody and higher antigen-specific CD8+ T cell numbers.

CO-STIMULATORY MOLECULES

Another mode of augmenting the immune response has been through co-delivery of a plasmid encoding an immunogen with a plasmid encoding a co-stimulatory molecule, such as CD86, or CD40. The rationale behind this strategy is that cells co-transfected with both the plasmid encoding the immunogen and the plasmid encoding a co-stimulatory molecule will exhibit enhanced antigen presentation capabilities over cells transfected with plasmid encoding the immunogen alone. As described, CD40 and CD86 play important roles in activation of T cells by antigen-presenting cells. Studies of mice co-injected intramuscularly with a plasmid encoding HIV-1 antigens and a plasmid encoding CD86 resulted in a marked increase in the CTL response over injection with the HIV-1 antigen-encoded plasmid alone (Kim *et al.*, 1997b).

SUBUNIT VACCINE BOOST

Because DNA immunization is known to induce a strong CTL response, combining a DNA vaccine with a recombinant subunit vaccine boost could elicit both a Th1 and Th2 profile, and therefore induce greater protection. As described, this approach has been demonstrated in macaques immunized with an HIV DNA vaccine and boosted with recombinant gp160. Monkeys exhibited HIV-specific CTL responses and high titres of HIV specific neutralizing antibody (Letvin *et al.*, 1997).

Conclusion

Although the development of a prophylactic HIV-1 vaccine has not been achieved to date, there are promising indications from new vaccination methods that suggest a

prophylactic vaccine may be possible. The fact that protection from different isolates of SIV, SHIV, or HIV-1 in macaques and chimpanzees has been achieved through DNA vaccination is promising. In addition, there are many new strategies or combinations of new and conventional strategies that have yet to be tried. Ultimately, a competent HIV-1 vaccine may involve a combination of DNA immunization followed by subunit vaccine boosts, or a cocktail of DNA-encoded antigens with plasmids encoding cytokines or co-stimulatory molecules. Finally, with the many different live viral vectors now being studied, a safe and successful vaccine may appear within our grasp.

References

AGADJANYAN, M.G., KIM, J.J., TRIVEDI, N., WILSON, D.M., MONZAVI-KARBASSI, B., MORRISON, L.D., NOTTINGHAM, L.K., DENTCHEV, T., TSAI, A., DANG, K., CHALIAN, A.A., MALDONADO, M.A., WILLIAMS, W.V. AND WEINER, D.B. (1999). CD86 (B7-2) can function to drive MHC-restricted antigen-specific CTL responses *in vivo*. *Journal of Immunology* **162**, 3417–3427.

ALBERT, M.L., PEARCE, S.F., FRANCISCO, L.M., SAUTER, B., ROY, P., SILVERSTEIN, R.L. AND BHARDWAJ, N. (1998a). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *Journal of Experimental Medicine* **188**, 1359–1368.

ALBERT, M.L., SAUTER, B. AND BHARDWAJ, N. (1998b). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86–89.

ALIZON, M., SONIGO, P., BARRE-SINOUSSI, F., CHERMANN, J.C., TIOLLAIS, P., MONTAGNIER, L. AND WAIN-HOBSON, S. (1984). Molecular cloning of lymphadenopathy-associated virus. *Nature* **312**, 757–760.

AYYAVOO, V., MAHALINGAM, S., RAFAELI, Y., KUDCHODKAR, S., CHANG, D., NAGASHUNMUGAM, T., WILLIAMS, W.V. AND WEINER, D.B. (1997a). HIV-1 viral protein R (Vpr) regulates viral replication and cellular proliferation in T cells and monocytoid cells *in vitro*. *Journal of Leukocyte Biology* **62**, 93–99.

AYYAVOO, V., MAHBOUBI, A., MAHALINGAM, S., RAMALINGAM, R., KUDCHODKAR, S., WILLIAMS, W.V., GREEN, D.R. AND WEINER, D.B. (1997b). HIV-1 Vpr suppresses immune activation and apoptosis through regulation of nuclear factor kappa B. *Nature Medicine* **3**, 1117–1123.

BANCHEREAU, J. AND STEINMAN, R.M. (1998). Dendritic cells and the control of immunity. *Nature* **392**, 245–252.

BAROUCH, D.H., CRAIU, A., KURODA, M.J., SCHMITZ, J.E., ZHENG, X.X., SANTRA, S., FROST, J.D., KRIVULKA, G.R., LIFTON, M.A., CRABBS, C.L., HEIDECKER, G., PERRY, H.C., DAVIES, M.E., XIE, H., NICKERSON, C.E., STEENBEKE, T.D., LORD, C.I., MONTEFIORI, D.C., STROM, T.B., SHIVER, J.W., LEWIS, M.G. AND LETVIN, N.L. (2000). Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 4192–4197.

BARTLETT, J.A., WASSERMAN, S.S., HICKS, C.B., DODGE, R.T., WEINHOLD, K.J., TACKET, C.O., KETTER, N., WITTEK, A.E., PALKER, T.J. AND HAYNES, B.F. (1998). Safety and immunogenicity of an HLA-based HIV envelope polyvalent synthetic peptide immunogen. DATRI 010 Study Group. Division of AIDS Treatment Research Initiative. *AIDS* **12**, 1291–1300.

BERNSTEIN, H.B., TUCKER, S.P., HUNTER, E., SCHUTZBACH, J.S. AND COMPANS, R.W. (1994). Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. *Journal of Virology* **68**, 463–468.

BOTTAREL, F., FEITO, M.J., BRAGARDO, M., BONISSONI, S., BUONFIGLIO, D., DEFRANCO, S., MALAVASI, F., BENSI, T., RAMENGHI, U. AND DIANZANI, U. (1999). The cell death-inducing ability of glycoprotein 120 from different HIV strains correlates with their ability

to induce CD4 lateral association with CD95 on CD4+ T cells. *AIDS Research and Human Retroviruses* **15**, 1255–1263.

BOYER, J.D., UGEN, K.E., WANG, B., AGADJANYAN, M., GILBERT, L., BAGARAZZI, M.L., CHATTERGOON, M., FROST, P., JAVADIAN, A., WILLIAMS, W.V., REFAELI, Y., CICCARELLI, R.B., MCCALLUS, D., CONEY, L. AND WEINER, D.B. (1997). Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination [see comments]. *Nature Medicine* **3**, 526–532.

BOYER, J.D., COHEN, A.D., UGEN, K.E., EDGEWORTH, R.L., BENNETT, M., SHAH, A., SCHUMANN, K., NATH, B., JAVADIAN, A., BAGARAZZI, M.L., KIM, J. AND WEINER, D.B. (2000). Therapeutic immunization of HIV-infected chimpanzees using HIV-1 plasmid antigens and interleukin-12 expressing plasmids. *AIDS* **14**, 1515–1522.

BUGE, S.L., MURTY, L., ARORA, K., KALYANARAMAN, V.S., MARKHAM, P.D., RICHARDSON, E.S., ALDRICH, K., PATTERSON, L.J., MILLER, C.J., CHENG, S.M. AND ROBERT-GUROFF, M. (1999). Factors associated with slow disease progression in macaques immunized with an adenovirus-simian immunodeficiency virus (SIV) envelope priming-gp120 boosting regimen and challenged vaginally with SIVmac251. *Journal of Virology* **73**, 7430–7440.

BURTON, D.R. (1997). A vaccine for HIV type 1: the antibody perspective. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10018–10023.

CALEY, I.J., BETTS, M.R., IRLBECK, D.M., DAVIS, N.L., SWANSTROM, R., FRELINGER, J.A. AND JOHNSTON, R.E. (1997). Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. *Journal of Virology* **71**, 3031–3038.

CAO, Y., QIN, L., ZHANG, L., SAFRIT, J. AND HO, D.D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection [see comments]. *New England Journal of Medicine* **332**, 201–208.

CARBONE, F.R. AND BEVAN, M.J. (1990). Class I-restricted processing and presentation of exogenous cell-associated antigen *in vivo*. *Journal of Experimental Medicine* **171**, 377–387.

CARMICHAEL, A., JIN, X., SISSONS, P. AND BORYSIEWICZ, L. (1993). Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *Journal of Experimental Medicine* **177**, 249–256.

CASARES, S., INABA, K., BRUMEANU, T.D., STEINMAN, R.M. AND BONA, C.A. (1997). Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *Journal of Experimental Medicine* **186**, 1481–1486.

CAYABYAB, M., KARLSSON, G.B., ETEMAD-MOGHADAM, B.A., HOFMANN, W., STEENBEKE, T., HALLORAN, M., FANTON, J.W., AXTHELM, M.K., LETVIN, N.L. AND SODROSKI, J.G. (1999). Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). *Journal of Virology* **73**, 976–984.

CHAN, D.C. AND KIM, P.S. (1998). HIV entry and its inhibition. *Cell* **93**, 681–684.

CHAN, D.C., FASS, D., BERGER, J.M. AND KIM, P.S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–273.

CHOW, Y.H., HUANG, W.L., CHI, W.K., CHU, Y.D. AND TAO, M.H. (1997). Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. *Journal of Virology* **71**, 169–178.

CONDON, C., WATKINS, S.C., CELLUZZI, C.M., THOMPSON, K. AND FALO, L.D. JR. (1996). DNA-based immunization by *in vivo* transfection of dendritic cells. *Nature Medicine* **2**, 1122–1128.

DAVIS, H.L., MICHEL, M.L., MANCINI, M., SCHLEEF, M. AND WHALEN, R.G. (1994). Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. *Vaccine* **12**, 1503–1509.

DAVIS, N.L., CALEY, I.J., BROWN, K.W., BETTS, M.R., IRLBECK, D.M., MCGRATH, K.M., CONNELL, M.J., MONTEFIORI, D.C., FRELINGER, J.A., SWANSTROM, R., JOHNSON, P.R. AND JOHNSTON, R.E. (2000). Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles [erratum

appears in *Journal of Virology* 2000 Apr;**74**(7):3430]. *Journal of Virology* **74**, 371–378.

DECK, R.R., DEWITT, C.M., DONNELLY, J.J., LIU, M.A. AND ULMER, J.B. (1997). Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* **15**, 71–78.

DEMINI, E.A., SCHLEIF, W.A., NUNBERG, J.H., CONLEY, A.J., EDA, Y., TOKIYOSHI, S., PUTNEY, S.D., MATSUSHITA, S., COBB, K.E. AND JETT, C.M. (1992). Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* **355**, 728–730.

EDINGER, A.L., AMEDEE, A., MILLER, K., DORANZ, B.J., ENDRES, M., SHARRON, M., SAMSON, M., LU, Z.H., CLEMENTS, J.E., MURPHEY-CORB, M., PEIPER, S.C., PARMENTIER, M., BRODER, C.C. AND DOMS, R.W. (1997). Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 4005–4010.

FELTQUATE, D.M., HEANEY, S., WEBSTER, R.G. AND ROBINSON, H.L. (1997). Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *Journal of Immunology* **158**, 2278–2284.

FERRARI, G., OTTINGER, J., PLACE, C., NIGIDA, S.M., ARTHUR, L.O. AND WEINHOLD, K.J. (1993). The impact of HIV-1 infection on phenotypic and functional parameters of cellular immunity in chimpanzees. *AIDS Research and Human Retroviruses* **9**, 647–656.

FRANKEL, F.R., HEGDE, S., LIEBERMAN, J. AND PATERSON, Y. (1995). Induction of cell-mediated immune responses to human immunodeficiency virus type 1 Gag protein by using Listeria monocytogenes as a live vaccine vector. *Journal of Immunology* **155**, 4775–4782.

FULLER, D.H. AND HAYNES, J.R. (1994). A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. *AIDS Research and Human Retroviruses* **10**, 1433–1441.

FULTZ, P.N., MCCLURE, H.M., SWENSON, R.B. AND ANDERSON, D.C. (1989). HIV infection of chimpanzees as a model for testing chemotherapeutics. *Intervirology* **30** Suppl 1, 51–58.

FYNAN, E.F., WEBSTER, R.G., FULLER, D.H., HAYNES, J.R., SANTORO, J.C. AND ROBINSON, H.L. (1993). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 11478–11482.

GANDHI, R.T., CHEN, B.K., STRAUS, S.E., DALE, J.K., LENARDO, M.J. AND BALTIMORE, D. (1998). HIV-1 directly kills CD4+ T cells by a Fas-independent mechanism. *Journal of Experimental Medicine* **187**, 1113–1122.

GAO, F., BAILES, E., ROBERTSON, D.L., CHEN, Y., RODENBURG, C.M., MICHAEL, S.F., CUMMINS, L.B., ARTHUR, L.O., PEETERS, M., SHAW, G.M., SHARP, P.M. AND HAHN, B.H. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes [see comments]. *Nature* **397**, 436–441.

GEISSLER, M., GESIEN, A., TOKUSHIGE, K. AND WANDS, J.R. (1997). Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *Journal of Immunology* **158**, 1231–1237.

GELEZIUNAS, R., XU, W., TAKEDA, K., ICHIJO, H. AND GREENE, W.C. (2001). HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* **410**, 834–838.

HAIGWOOD, N.L., WATSON, A., SUTTON, W.F., MCCLURE, J., LEWIS, A., RANCHALIS, J., TRAVIS, B., VOSS, G., LETVIN, N.L., HU, S.L., HIRSCH, V.M. AND JOHNSON, P.R. (1996). Passive immune globulin therapy in the SIV/macaque model: early intervention can alter disease profile. *Immunology Letters* **51**, 107–114.

HARRER, T., HARRER, E., KALAMS, S.A., ELBEIK, T., STAPRANS, S.I., FEINBERG, M.B., CAO, Y., HO, D.D., YILMA, T., CALIENDO, A.M., JOHNSON, R.P., BUCHBINDER, S.P. AND WALKER, B.D. (1996). Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Research and Human Retroviruses* **12**, 585–592.

HE, J., CHOE, S., WALKER, R., DI MARZIO, P., MORGAN, D.O. AND LANDAU, N.R. (1995). Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *Journal of Virology* **69**, 6705–6711.

HEMMI, H., TAKEUCHI, O., KAWAI, T., KAISHO, T., SATO, S., SANJO, H., MATSUMOTO, M., HOSHINO, K., WAGNER, H., TAKEDA, K. AND AKIRA, S. (2000). A toll-like receptor recognizes bacterial DNA [see comments]. *Nature* **408**, 740–745.

HIRSCH, V.M., FUERST, T.R., SUTTER, G., CARROLL, M.W., YANG, L.C., GOLDSTEIN, S., PIATAK, M., ELKINS, W.R., ALVORD, W.G., MONTEFIORI, D.C., MOSS, B. AND LIFSON, J.D. (1996). Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *Journal of Virology* **70**, 3741–3752.

HU, S.L., FULTZ, P.N., MCCLURE, H.M., EICHBERG, J.W., THOMAS, E.K., ZARLING, J., SINGHAL, M.C., KOSOWSKI, S.G., SWENSON, R.B. AND ANDERSON, D.C. (1987). Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. *Nature* **328**, 721–723.

HU, S.L., ABRAMS, K., BARBER, G.N., MORAN, P., ZARLING, J.M., LANGLOIS, A.J., KULLER, L., MORTON, W.R. AND BENVENISTE, R.E. (1992). Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* **255**, 456–459.

HUANG, A.Y., GOLUMBEK, P., AHMADZADEH, M., JAFFEE, E., PARDOLL, D. AND LEVITSKY, H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* **264**, 961–965.

IGARASHI, T., BROWN, C., AZADEGAN, A., HAIGWOOD, N., DIMITROV, D., MARTIN, M.A. AND SHIBATA, R. (1999). Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma [see comments]. *Nature Medicine* **5**, 211–216.

JAVAHERIAN, K., LANGLOIS, A.J., LAROSA, G.J., PROFY, A.T., BOLOGNESI, D.P., HERLIHY, W.C., PUTNEY, S.D. AND MATTHEWS, T.J. (1990). Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1 [erratum appears in *Science* 1991 Jan 4;**251**(4989):13]. *Science* **250**, 1590–1593.

JIN, X., BAUER, D.E., TUTTLETON, S.E., LEWIN, S., GETTIE, A., BLANCHARD, J., IRWIN, C.E., SAFRIT, J.T., MITTLER, J., WEINBERGER, L., KOSTRIKIS, L.G., ZHANG, L., PERELSON, A.S. AND HO, D.D. (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *Journal of Experimental Medicine* **189**, 991–998.

JOHNSON, P., SCHNEPP, B., MONTEFIORI, D., LETVIN, N. AND CLARK, R. (2001). *SIV vaccines based on adeno-associated virus vectors*. Paper presented at: AIDS Vaccine 2001 (Philadelphia PA).

KALAMS, S.A. AND WALKER, B.D. (1998). The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *Journal of Experimental Medicine* **188**, 2199–2204.

KATSUMI, A., EMI, N., ABE, A., HASEGAWA, Y., ITO, M. AND SAITO, H. (1994). Humoral and cellular immunity to an encoded protein induced by direct DNA injection. *Human Gene Therapy* **5**, 1335–1339.

KIM, J.J., AYYAVOO, V., BAGARAZZI, M.L., CHATTERGOON, M.A., DANG, K., WANG, B., BOYER, J.D. AND WEINER, D.B. (1997a). *In vivo* engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. *Journal of Immunology* **158**, 816–826.

KIM, J.J., BAGARAZZI, M.L., TRIVEDI, N., HU, Y., KAZAHAYA, K., WILSON, D.M., CICCARELLI, R., CHATTERGOON, M.A., DANG, K., MAHALINGAM, S., CHALIAN, A.A., AGADJANYAN, M.G., BOYER, J.D., WANG, B. AND WEINER, D.B. (1997b). Engineering of *in vivo* immune responses to DNA immunization via codelivery of costimulatory molecule genes. *Nature Biotechnology* **15**, 641–646.

KIM, J.J., NOTTINGHAM, L.K., TSAI, A., LEE, D.J., MAGUIRE, H.C., OH, J., DENTCHEV, T., MANSON, K.H., WYAND, M.S., AGADJANYAN, M.G., UGEN, K.E. AND WEINER, D.B. (1999). Antigen-specific humoral and cellular immune responses can be modulated in rhesus macaques through the use of IFN-gamma, IL-12, or IL-18 gene adjuvants. *Journal of Medical Primatology* **28**, 214–223.

KIM, J.J., YANG, J.S., NOTTINGHAM, L.K., LEE, D.J., LEE, M., MANSON, K.H., WYAND, M.S., BOYER, J.D., UGEN, K.E. AND WEINER, D.B. (2001). Protection from immunodeficiency

virus challenges in rhesus macaques by multicomponent DNA immunization. *Virology* **285**, 204–217.

KLINMAN, D.M., YAMSHCHIKOV, G. AND ISHIGATSUBO, Y. (1997). Contribution of CpG motifs to the immunogenicity of DNA vaccines. *Journal of Immunology* **158**, 3635–3639.

KOUP, R.A., SAFRIT, J.T., CAO, Y., ANDREWS, C.A., MCLEOD, G., BORKOWSKY, W., FARTHING, C. AND HO, D.D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *Journal of Virology* **68**, 4650–4655.

KOVACSOVICS-BANKOWSKI, M. AND ROCK, K.L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**, 243–246.

KRIEG, A.M. (1996). An innate immune defense mechanism based on the recognition of CpG motifs in microbial DNA. *Journal of Laboratory and Clinical Medicine* **128**, 128–133.

KUWATA, T., SHIODA, T., IGARASHI, T., IDO, E., IBUKI, K., ENOSE, Y., STAHL-HENNIG, C., HUNSMANN, G., MIURA, T. AND HAYAMI, M. (1996). Chimeric viruses between SIVmac and various HIV-1 isolates have biological properties that are similar to those of the parental HIV-1. *AIDS* **10**, 1331–1337.

KWONG, P.D., WYATT, R., ROBINSON, J., SWEET, R.W., SODROSKI, J. AND HENDRICKSON, W.A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659.

LAROSA, G.J., DAVIDE, J.P., WEINHOLD, K., WATERBURY, J.A., PROFY, A.T., LEWIS, J.A., LANGLOIS, A.J., DREESMAN, G.R., BOSWELL, R.N. AND SHADDUCK, P. (1990). Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant [erratum appears in *Science* 1991 Feb 15;**251**(4995):811]. *Science* **249**, 932–935.

LECLERC, C., DERIAUD, E., ROJAS, M. AND WHALEN, R.G. (1997). The preferential induction of a Th1 immune response by DNA-based immunization is mediated by the immunostimulatory effect of plasmid DNA. *Cellular Immunology* **179**, 97–106.

LEONARD, C.K., SPELLMAN, M.W., RIDDLE, L., HARRIS, R.J., THOMAS, J.N. AND GREGORY, T.J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *Journal of Biological Chemistry* **265**, 10373–10382.

LETVIN, N.L., MONTEFIORI, D.C., YASUTOMI, Y., PERRY, H.C., DAVIES, M.E., LEKUTIS, C., ALROY, M., FREED, D.C., LORD, C.I., HANDT, L.K., LIU, M.A. AND SHIVER, J.W. (1997). Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 9378–9383.

LEVY, D.N., REFAELI, Y. AND WEINER, D.B. (1995). Extracellular Vpr protein increases cellular permissiveness to human immunodeficiency virus replication and reactivates virus from latency. *Journal of Virology* **69**, 1243–1252.

LU, M. AND KIM, P.S. (1997). A trimeric structural subdomain of the HIV-1 transmembrane glycoprotein. *Journal of Biomolecular Structure & Dynamics* **15**, 465–471.

MARTIN, T., PARKER, S.E., HEDSTROM, R., LE, T., HOFFMAN, S.L., NORMAN, J., HOBART, P. AND LEW, D. (1999). Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. *Human Gene Therapy* **10**, 759–768.

MARX, P.A., MUNN, R.J. AND JOY, K.I. (1988). Computer emulation of thin section electron microscopy predicts an envelope-associated icosadeltahedral capsid for human immunodeficiency virus. *Laboratory Investigation* **58**, 112–118.

MASCOLA, J.R., LEWIS, M.G., STIEGLER, G., HARRIS, D., VANCOTT, T.C., HAYES, D., LOUDER, M.K., BROWN, C.R., SAPAN, C.V., FRANKEL, S.S., LU, Y., ROBB, M.L., KATINGER, H. AND BIRX, D.L. (1999). Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *Journal of Virology* **73**, 4009–4018.

MATA, M. AND PATERSON, Y. (1999). Th1 T cell responses to HIV-1 Gag protein delivered by a Listeria monocytogenes vaccine are similar to those induced by endogenous listerial antigens. *Journal of Immunology* **163**, 1449–1456.

MATA, M., YAO, Z.J., ZUBAIR, A., SYRES, K. AND PATERSON, Y. (2001). Evaluation of a

recombinant Listeria monocytogenes expressing an HIV protein that protects mice against viral challenge. *Vaccine* **19**, 1435–1445.

MATANO, T., SHIBATA, R., SIEMON, C., CONNORS, M., LANE, H.C. AND MARTIN, M.A. (1998). Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *Journal of Virology* **72**, 164–169.

MOORE, J.P. AND BURTON, D.R. (1999). HIV-1 neutralizing antibodies: how full is the bottle? [letter; comment]. *Nature Medicine* **5**, 142–144.

MOORE, J.P., CAO, Y., QING, L., SATTENTAU, Q.J., PYATI, J., KODURI, R., ROBINSON, J., BARBAS, C.F., BURTON, D.R. AND HO, D.D. (1995). Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. *Journal of Virology* **69**, 101–109.

MOR, G., SINGLA, M., STEINBERG, A.D., HOFFMAN, S.L., OKUDA, K. AND KLINMAN, D.M. (1997). Do DNA vaccines induce autoimmune disease? *Human Gene Therapy* **8**, 293–300.

MWAENGO, D.M. AND NOVEMBRE, F.J. (1998). Molecular cloning and characterization of viruses isolated from chimpanzees with pathogenic human immunodeficiency virus type 1 infections. *Journal of Virology* **72**, 8976–8987.

NICHOLS, W.W., LEDWITH, B.J., MANAM, S.V. AND TROILO, P.J. (1995). Potential DNA vaccine integration into host cell genome. *New York Academy of Sciences. Annals* **772**, 30–39.

NYAMBI, P.N., MBAH, H.A., BURDA, S., WILLIAMS, C., GORNY, M.K., NADAS, A. AND ZOLLA-PAZNER, S. (2000). Conserved and exposed epitopes on intact, native, primary human immunodeficiency virus type 1 virions of group M. *Journal of Virology* **74**, 7096–7107.

PARDOLL, D.M. AND BECKERLEG, A.M. (1995). Exposing the immunology of naked DNA vaccines. *Immunity* **3**, 165–169.

PARREN, P.W., MONDOR, I., NANICHE, D., DITZEL, H.J., KLASSE, P.J., BURTON, D.R. AND SATTENTAU, Q.J. (1998). Neutralization of human immunodeficiency virus type 1 by antibody to gp120 is determined primarily by occupancy of sites on the virion irrespective of epitope specificity. *Journal of Virology* **72**, 3512–3519.

PARREN, P.W., MOORE, J.P., BURTON, D.R. AND SATTENTAU, Q.J. (1999). The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. *AIDS* **13** Suppl A, S137–162.

PIALOUX, G., EXCLER, J.L., RIVIERE, Y., GONZALEZ-CANALI, G., FEUILLIE, V., COULAUD, P., GLUCKMAN, J.C., MATTHEWS, T.J., MEIGNIER, B., KIENY, M.P., GONNET, P., DIAZ, I., MERIC, C., PAOLETTI, E., TARTAGLIA, J., SALOMON, H., PLOTKIN, S., THE AGIS GROUP AND L'AGENCE NATIONALE DE RECHERCHE SUR LE SIDA. (1995). A prime-boost approach to HIV preventive vaccine using a recombinant canarypox virus expressing glycoprotein 160 (MN) followed by a recombinant glycoprotein 160 (MN/LAI). The AGIS Group, and l'Agence Nationale de Recherche sur le SIDA. *AIDS Research and Human Retroviruses* **11**, 373–381.

PUTNEY, S.D., MATTHEWS, T.J., ROBEY, W.G., LYNN, D.L., ROBERT-GUROFF, M., MUELLER, W.T., LANGLOIS, A.J., GHRAYEB, J., PETTEWAY, S.R. JR., WEINHOLD, K.J., FISCHINGER, P.J., WONG-STALL, F., GALLO, R.C. AND BOLOGNESI, D.P. (1986). HTLV-III/LAV-neutralizing antibodies to an *E. coli*-produced fragment of the virus envelope. *Science* **234**, 1392–1395.

REDFIELD, R.R., WRIGHT, D.C., JAMES, W.D., JONES, T.S., BROWN, C. AND BURKE, D.S. (1987). Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *New England Journal of Medicine* **316**, 673–676.

REITTER, J.N., MEANS, R.E. AND DESROSIERS, R.C. (1998). A role for carbohydrates in immune evasion in AIDS. *Nature Medicine* **4**, 679–684.

RINALDO, C., HUANG, X.L., FAN, Z.F., DING, M., BELTZ, L., LOGAR, A., PANICALI, D., MAZZARA, G., LIEBMANN, J., COTTRILL, M. AND GUPTA, P. (1995). High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *Journal of Virology* **69**, 5838–5842.

RIZZUTO, C.D., WYATT, R., HERNANDEZ-RAMOS, N., SUN, Y., KWONG, P.D., HENDRICKSON, W.A. AND SODROSKI, J. (1998). A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**, 1949–1953.

ROBINSON, H.L., BOYLE, C.A., FELTQUATE, D.M., MORIN, M.J., SANTORO, J.C. AND WEBSTER, R.G. (1997). DNA immunization for influenza virus: studies using hemagglutinin- and nucleoprotein-expressing DNAs. *Journal of Infectious Diseases* **176** Suppl 1, S50–55.

ROMAN, M., MARTIN-OROZCO, E., GOODMAN, J.S., NGUYEN, M.D., SATO, Y., RONAGHY, A., KORNBLUTH, R.S., RICHMAN, D.D., CARSON, D.A. AND RAZ, E. (1997). Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nature Medicine* **3**, 849–854.

ROSENBERG, E.S., LAROSA, L., FLYNN, T., ROBBINS, G. AND WALKER, B.D. (1999). Characterization of HIV-1-specific T-helper cells in acute and chronic infection. *Immunology Letters* **66**, 89–93.

ROWLAND-JONES, S. (1999). Long-term non-progression in HIV infection: clinico pathological issues. *Journal of Infection* **38**, 67–70.

SAKSELA, K., CHENG, G. AND BALTIMORE, D. (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO Journal* **14**, 484–491.

SANHADJI, K., GRAVE, L., TOURAINE, J.L., LEISSNER, P., ROUZIOUX, C., FIROUZI, R., KEHRLI, L., TARDY, J.C. AND MEHTALI, M. (2000). Gene transfer of anti-gp41 antibody and CD4 immunoadhesin strongly reduces the HIV-1 load in humanized severe combined immunodeficient mice. *AIDS* **14**, 2813–2822.

SAPHIRE, E.O., PARREN, P.W., PANTOPHLET, R., ZWICK, M.B., MORRIS, G.M., RUDD, P.M., DWEK, R.A., STANFIELD, R.L., BURTON, D.R. AND WILSON, I.A. (2001). Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design. *Science* **293**, 1155–1159.

SARNGADHARAN, M.G., DEVICO, A.L., BRUCH, L., SCHUPBACH, J. AND GALLO, R.C. (1984). HTLV-III: the etiologic agent of AIDS. *Princess Takamatsu Symposium* **15**, 301–308.

SATO, Y., ROMAN, M., TIGHE, H., LEE, D., CORR, M., NGUYEN, M.D., SILVERMAN, G.J., LOTZ, M., CARSON, D.A. AND RAZ, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.

SAWAI, E.T., BAUR, A.S., PETERLIN, B.M., LEVY, J.A. AND CHENG-MAYER, C. (1995). A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity. *Journal of Biological Chemistry* **270**, 15307–15314.

SCHMITZ, J.E., KURODA, M.J., SANTRA, S., SASSEVILLE, V.G., SIMON, M.A., LIFTON, M.A., RACZ, P., TENNER-RACZ, K., DALESANDRO, M., SCALLON, B.J., GRAYEB, J., FORMAN, M.A., MONTEFIORI, D.C., RIEBER, E.P., LETVIN, N.L. AND REIMANN, K.A. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**, 857–860.

SHEN, L., CHEN, Z.W., MILLER, M.D., STALLARD, V., MAZZARA, G.P., PANICALI, D.L. AND LETVIN, N.L. (1991). Recombinant virus vaccine-induced SIV-specific CD8+ cytotoxic T lymphocytes. *Science* **252**, 440–443.

SHIBATA, R., SIEMON, C., CHO, M.W., ARTHUR, L.O., NIGIDA, S.M. JR., MATTHEWS, T., SAWYER, L.A., SCHULTZ, A., MURTHY, K.K., ISRAEL, Z., JAVADIAN, A., FROST, P., KENNEDY, R.C., LANE, H.C. AND MARTIN, M.A. (1996). Resistance of previously infected chimpanzees to successive challenges with a heterologous intraclade B strain of human immunodeficiency virus type 1. *Journal of Virology* **70**, 4361–4369.

SHIBATA, R., IGARASHI, T., HAIGWOOD, N., BUCKLER-WHITE, A., OGERT, R., ROSS, W., WILLEY, R., CHO, M.W. AND MARTIN, M.A. (1999). Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nature Medicine* **5**, 204–210.

SHIVER, J.W., FU, T.M., CHEN, L., CASIMIRO, D.R., DAVIES, M.E., EVANS, R.K., ZHANG, Z.Q., SIMON, A.J., TRIGONA, W.L., DUBEY, S.A., HUANG, L., HARRIS, V.A., LONG, R.S., LIANG, X., HANDT, L., SCHLEIF, W.A., ZHU, L., FREED, D.C., PERSAUD, N.V., GUAN, L., PUNT, K.S., TANG, A., CHEN, M., WILSON, K.A., COLLINS, K.B., HEIDECKER, G.J., FERNANDEZ, V.R., PERRY, H.C., JOYCE, J.G., GRIMM, K.M., COOK, J.C., KELLER, P.M., KRESOCK,

D.S., MACH, H., TROUTMAN, R.D., ISOPI, L.A., WILLIAMS, D.M., XU, Z., BOHANNON, K.E., VOLKIN, D.B., MONTEFIORI, D.C., MIURA, A., KRIVULKA, G.R., LIFTON, M.A., KURODA, M.J., SCHMITZ, J.E., LETVIN, N.L., CAULFIELD, M.J., BETT, A.J., YOUIL, R., KASLOW, D.C. AND EMINI, E.A. (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331–335.

SUTO, R. AND SRIVASTAVA, P.K. (1995). A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* **269**, 1585–1588.

THALI, M., MOORE, J.P., FURMAN, C., CHARLES, M., HO, D.D., ROBINSON, J. AND SODROSKI, J. (1993). Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *Journal of Virology* **67**, 3978–3988.

TORRES, C.A., IWASAKI, A., BARBER, B.H. AND ROBINSON, H.L. (1997). Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *Journal of Immunology* **158**, 4529–4532.

TRKOLA, A., POMALES, A.B., YUAN, H., KORBER, B., MADDON, P.J., ALLAWAY, G.P., KATINGER, H., BARBAS, C.F. 3RD, BURTON, D.R., HO, D.D. AND MOORE, J.P. (1995). Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. *Journal of Virology* **69**, 6609–6617.

ULMER, J.B., DONNELLY, J.J., PARKER, S.E., RHODES, G.H., FELGNER, P.L., DWARKI, V.J., GROMKOWSKI, S.H., DECK, R.R., DEWITT, C.M., FRIEDMAN, A., HAWE, L.A., LEANDER, K.R., MARTINEZ, D., PERRY, H.C., SHIVER, J.W., MONTGOMERY, D.L. AND LIU, M.A. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745–1749.

VAN ROMPAY, K.K., BERARDI, C.J., DILLARD-TELM, S., TARARA, R.P., CANFIELD, D.R., VALVERDE, C.R., MONTEFIORI, D.C., COLE, K.S., MONTELARO, R.C., MILLER, C.J. AND MARTHAS, M.L. (1998). Passive immunization of newborn rhesus macaques prevents oral simian immunodeficiency virus infection. *Journal of Infectious Diseases* **177**, 1247–1259.

WALKER, M.C., WALKER, B.D., MESTECKY, J. AND MATHIESON, B.J. (1994). Conference on advances in AIDS vaccine development – 1993. Summary: cytotoxic T-cell immunity workshop. *AIDS Research and Human Retroviruses* **10** Suppl 2, S177–179.

WALKER, M.C., FAST, P.E., GRAHAM, B., BELSHE, R. AND DOLIN, R. (1995). *Phase I/II preventative vaccine trials*. Paper presented at: AIDS Research and Human Retroviruses.

WILLEY, R.L., MALDARELLI, F., MARTIN, M.A. AND STREBEL, K. (1992). Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. *Journal of Virology* **66**, 226–234.

WU, S., PASCUAL, D.W., LEWIS, G.K. AND HONE, D.M. (1997). Induction of mucosal and systemic responses against human immunodeficiency virus type 1 glycoprotein 120 in mice after oral immunization with a single dose of a Salmonella-HIV vector. *AIDS Research and Human Retroviruses* **13**, 1187–1194.

XIANG, Z.Q., SPITALNIK, S.L., CHENG, J., ERIKSON, J., WOJCZYK, B. AND ERTL, H.C. (1995). Immune responses to nucleic acid vaccines to rabies virus. *Virology* **209**, 569–579.

YANG, X., WYATT, R. AND SODROSKI, J. (2001). Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. *Journal of Virology* **75**, 1165–1171.

YASUTOMI, Y., KOENIG, S., HAUN, S.S., STOVER, C.K., JACKSON, R.K., CONARD, P., CONLEY, A.J., EMINI, E.A., FUERST, T.R. AND LETVIN, N.L. (1993). Immunization with recombinant BCG-SIV elicits SIV-specific cytotoxic T lymphocytes in rhesus monkeys. *Journal of Immunology* **150**, 3101–3107.

ZAGURY, D., BERNARD, J., LEIBOWITCH, J., SAFAI, B., GROOPMAN, J.E., FELDMAN, M., SARNGADHARAN, M.G. AND GALLO, R.C. (1984). HTLV-III in cells cultured from semen of two patients with AIDS. *Science* **226**, 449–451.

ZOLLA-PAZNER, S., GORNY, M.K., NYAMBI, P.N., VANCOTT, T.C. AND NADAS, A. (1999). Immunotyping of human immunodeficiency virus type 1 (HIV): an approach to immunologic classification of HIV. *Journal of Virology* **73**, 4042–4051.