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Novel Strategies Toward the Development of an Effective Vaccine to Prevent Human Immunodeficiency Virus Infection or Acquired Immunodeficiency Virus

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The inexorable spread of the human immunodeficiency virus (HIV) pandemic and the increasing deaths caused by acquired immunodeficiency syndrome (AIDS) in developing countries underscore the urgency for an effective, safe, and inexpensive vaccine against HIV. Although many attempts have been made, a candidate vaccine of proven efficacy and safety in nonhuman primate models is not yet available. This is mostly due to HIV envelope (Env) variability and to the difficulty of eliciting high titers of long-lasting neutralizing antibodies capable of blocking entry of different virus strains. Nevertheless, new strategies have been developed and new information is now available that can lead to new concepts and open new avenues to obtain an effective vaccine against HIV infection.

I. HOW AIDS VACCINE STRATEGIES HAVE EVOLVED FROM STERILIZING IMMUNITY TO THE CONTROL OF INFECTION

Over the last 15 years, most efforts in HIV vaccine development have been based on the envelope protein (Env) of HIV that is responsible for the binding
and entry of the virus, with the rationale of generating neutralizing antibodies (NA) capable of inducing sterilizing immunity and protection. However, results from studies in nonhuman primate models have been largely disappointing because of the inability of such vaccines to elicit NA at titers necessary to block infection with homologous viruses or to elicit NA against viruses isolated from infected individuals (primary isolates). The reasons are several (Table 1) but are mostly related to the Env variability (1), which hampers recognition by NA that are generally directed against conformational epitopes (reviewed in Reference 2).

Moreover, partly due to the heavy glycosylation of the key envelope protein (gp120), neutralizing Env epitopes are poorly exposed even on the oligomeric form of the viral envelope (a recombinant form, conformationally indistinguishable from the spikes present on native virions). In fact, even in the course of the natural infection, titers of NA are relatively low, indicating that the relevant epitopes are poorly exposed or weakly immunogenic. However, a sterilizing immunity is conceptually possible. In this respect, experiments of passive immunization in nonhuman primates have demonstrated that NA generated during natural infection are protective (when transferred in a naive animal); however, this occurs only at very high antibody titers (3) that are approximately 1000-fold higher than those present in sera from most HIV-1-infected humans or simian/human immunodeficiency virus (SHIV)-infected monkeys (4–6). In addition, these antibodies are poorly cross-reactive (7) and therefore effective only against infection with homologous virus.

Sterilizing immunity has been obtained upon passive immunization of adult and newborn macaques with a mixture of three human monoclonal antibodies (MAb) directed against neutralizing epitopes of Env. These animals were challenged intravenously (adults) or orally (newborns) with a homologous SHIV (SHIV-vpu+) encoding the env gene of HIV-1Lai-B (8). Of note, the synergy among the MAb s allowed neutralization at antibody concent-

ations significantly lower than those needed with each MAb alone (9). The importance of infusing a mixture of antibodies also comes from another study in which protection against a mucosal challenge with the pathogenic SHIV89.6PD was greater in the group that received 3 different antibody preparations (4 out of 5 monkeys) than in the group treated with 2 (2/5) or 1 (2/4) (10). Moreover, the passively immunized monkeys that were not protected experienced a milder infection and did not develop the severe loss of CD4 cells observed in the control animals, indicating a partial containment of the infection. Notably, the level of protection was higher than that obtained after intravenous challenge (11), suggesting that antibodies are more effective when a physical barrier (i.e., the vaginal mucosa) separates the virus from target cells. Exactly where and how these antibodies neutralize the virus is presently unknown (12). Thus, sterilizing immunity is achievable, even against mucosal challenge and in the absence of antiviral secretory IgA.

Nevertheless, the identification of broadly represented neutralizing epitopes and/or the induction of high titers, long-lasting NA are still major obstacles to the development of an effective sterilizing vaccine based on Env (13).

Hope comes from a new approach (14) utilizing a modified (fusinocent)-competent) version of the native Env that, at least in mice, has induced broadly cross-reactive NA, suggesting that sterilizing immunity could be achieved with novel Env-based vaccine strategies.

The most compelling evidence that it is possible to generate a protective immune response against different HIV strains comes from studies in nonhuman primates vaccinated with live attenuated SIV and protected from heterologous challenges with highly pathogenic strains (15–18). However, ethical concerns and the appearance of revertant pathogenic viruses in vaccines (19–21) presently hamper their use in humans (Table 1). Nevertheless, this approach provides a model to study the correlates and mechanisms of protection and supports the concept for strategies in which the gene for one or more viral antigens is expressed in the host. These include naked DNA or live vectors expressing Env alone or associated with other viral genes.

Examples of some of these strategies include modified vaccinia virus Ankara (MVA), canarypox, fowlpox, adenoviruses, and alphaviruses that are being investigated with different modalities of immunization (i.e., combined prime-boost regimens) in nonhuman primate models with SIV or SHIV (reviewed in Reference 22). These approaches have a variety of advantages, including better delivery and potency of immunization. For example, most of these vectors, as well as naked DNA, contain unmethylated CpG sequences that can also boost the innate immunity favoring a Th1-type of specific immune response and CD8-mediated antiviral activity (CAF), including beta

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chemokines production. This type of response appears to contribute to protection both after vaccination or during natural infection (23, 24). Moreover, live vectors can be utilized to induce a mucosal immunization, a strategy particularly important to block virus transmission.

Results from these approaches have provided key information for vaccine development. Although in most cases no sterilizing immunity was obtained with homologous or heterologous virus challenge, lower plasma viremia levels were observed after high-dose virus challenge. Thus, the control of infection can be achieved in the absence of NA. In most cases, protection correlated with a Th-1 type of immune response, including cytotoxic T lymphocytes (CTLs), whose relevance for protection has progressively increased as opposed to NA (25–27). Thus, new promising strategies have been formulated based on the construction of a single “minigene” encoding multiple viral major histocompatibility complex (MHC) class I restricted epitopes capable of inducing CTLs (termed polypeptide or polytope CTL) (28).

Because sterilizing immunity against different virus strains has not yet been achieved with Env-based vaccines, secondary endpoints in HIV vaccine development are acquiring more importance and have led to the concept that control of viral infection and blocking disease onset may be a more achievable goal of AIDS vaccine strategies.

In this respect, recent approaches aimed at eliciting immunity against the HIV regulatory gene products Tat, Rev, and Nef have provided exciting results. In particular, Tat (29, 30) or Tat and Rev (31) vaccination appear capable of controlling primary infection with highly pathogenic viruses, providing the first evidence of cross-protection in nonhuman primates. Similarly, immunization of macaques with a recombinant vaccinia expressing SIV-Tat generated enough cytolytic (CTLs) and nonlytic (CAF) antiviral activities to control subsequent infection with a homologous strain (32). Vaccination with Tat, Rev, and Nef has also been investigated in mice (33) and in HIV-1 infected humans (34), providing evidence of safety, immunogenicity of both protein and DNA vaccination.

Such strategies target regulatory proteins that are the first to be expressed after infection and are essential for viral replication, infectivity, and pathogenesis. In particular, because of its release upon infection and its extracellular functions (35–38), Tat may represent an optimal candidate for such a strategy. Tat and Rev also have the major advantage of being highly conserved throughout viral clades (1). In addition, these strategies can be used for both preventive and therapeutic vaccination.

Another concept involves natural immunity boosted by the sole vector. This appears to contribute to protection achieved with different vaccine strategies (live attenuated, fowlpox, DNA, Tat protein; see below). Of note, the same type of immunity and antiviral activity is induced by allogeneicization (reviewed in Reference 39) that although nonvirus-specific, can strongly oppose HIV, resulting in lower susceptibility of the host to infection (40) or in a better control once the infection is established. Thus, although further studies are needed, it appears that boosting of natural immunity represents an additional and valuable tool for fighting HIV that must be explored in a greater detail.

Although many vaccine strategies are under study, this review focuses only on some of these most recent approaches.

II. LIVE ATTENUATED VACCINES: CONCEPTS TO LEARN

The best protective results against both homologous and heterologous virus challenge have been obtained in macaques with a live attenuated SIV that has the nef gene deleted. In these animals, persistent infection is established, but the virus replicates poorly, does not cause disease (15), and confers protection against challenge with pathogenic SIV or SHIV (16, 17, 41–43). Despite the attenuation, increased by additional deletions in vpr and in the negative regulatory elements, these viruses still cause disease in newborn macaques upon mucosal exposure (20) or intravenous challenge and, more importantly, in some of the vaccinated adult macaques (44, 45). In the latter, progression to disease is delayed, stressing the tight relationship between replication rate and pathogenicity. On the other hand, poorly replicating viruses such as some SHIV appear to be inefficient in conferring protection. This suggests that the replication rate affects pathogenicity but also the development of a protective immune response (46–48).

Therefore, more attenuated viruses are being generated with the rationale of determining the minimal requirements to confer protection. Of note, the progression to disease observed in a few of the vaccinated animals underscores the importance of host factors in pathogenesis and the key effect of infectious virus threshold required for progression to diseases in different individuals. However, the possibility that the virus may revert to a more pathogenic geno/phenotype is among the major concerns that prevent the use of live attenuated vaccines in humans.

Nevertheless, this approach in the animal model may help identify mechanisms by which superinfection with pathogenic strains is blocked, a very valuable aim given the poor understanding of protective immunity against HIV. In particular, experiments of passive transfer of serum from macaque vaccinated with an attenuated SIV and protected from superinfection by the
wild-type viral strain does not confer protection to naive animals, ruling out a role for antibodies (49). On the other hand, neither partial depletion of CD8+ cells nor a more profound depletion of most T cells abrogate protection from superinfection (50). Thus, mechanisms other than antibodies or CD8+ CTLs are responsible for protection. The potency of such mechanism(s) in the protected animals is underscored by the lack of reactivation, upon stimulation with a recall antigen, of either the attenuated strain used for vaccination or the pathogenic strain used for challenge (51). A growing body of evidence indicates that natural immunity may be key to protection possibly through the induction of CAF, beta chemokines, and interleukin-16 secretion by αβCD8+ T cells and γδT cells (52–56).

III. NOVEL STRATEGIES

A. Fusion-Competent Env-Based Vaccine

The possible solution to the problems related to Env variability and induction of sterilizing immunity comes from a recent major breakthrough in the HIV research that further demonstrates the importance of basic science for vaccine development.

The definition of the crystal structure of the HIV-1 envelope has revealed new functional aspects concerning its binding to CD4 and the conformational changes it undergoes to engage the CCR5 and CXCR4 coreceptors and to subsequently fuse to the cell membrane. It is during these transient conformational changes that new cryptic epitopes are exposed. Thus, it is conceivable that the recombinant gp160 or gp120 oligomers utilized so far for immunization, although identical to the native envelope spikes, may not expose these functional epitopes and therefore will not elicit antibodies against them.

From these considerations stemmed a completely new approach to generate broadly cross-reactive NA. LaCasse et al. (14) showed thatforming fixation of COS-7 cells expressing a dual tropic molecularly cloned Env of the B clade at the time they were starting to fuse with the human U937 glioma cells (expressing both CD4 and CCR5) results in a whole cell preparation, termed fusion-competent, in which those cryptic epitopes are exposed. Indeed, in a mouse model (transgenic for human CD4 and CCR5 to avoid the generation of an immune response against these molecules), vaccination with this fusion-competent immunogen induced antibodies that were strongly neutralizing and broadly cross-reactive, indicating that critical, and presently unknown, neutralizing epitopes are exposed only upon the transient confor-

mational changes necessary for fusion to occur. Moreover, the capability of these antibodies to neutralize strains from several clades indicates that they recognize highly conserved epitopes, likely because of their functional role in the fusion event. Thus, these data formally prove that it is possible, at least in the mouse model, to induce antibodies that are strongly neutralizing in vitro. Of course, confirmation of these results in more suitable preclinical models such as nonhuman primates and in vivo evidence of protection are needed before considering any potential clinical application.

B. Vaccines Based on Naked DNA and Live Vectors

Expressing Env Alone or Associated with Other Viral Genes (MVA, Canarypox, Fowlpox, Adenovirus, Alphaviruses)

Vectors that express viral genes may represent an alternative to live attenuated virus vaccines. In fact, expression of viral genes in the host mimics the infection with attenuated strains without their risk and should elicit a comparable immune response and efficacy. There are additional advantages of the DNA-based approaches, such as the ease of preparation, the reproducibility, the stability at room temperature, the possibility of mucosal immunization, the low cost, and the fewer inoculations needed, features that are extremely appealing when thinking of large-scale vaccination, particularly in developing countries. Moreover, it is possible to insert genes coding for molecules, such as cytokines or chemokines, in the vector which could help the immune system to mount a specific response against the nominal antigen. In addition, naked DNA and most vectors contain unmethylated CpG sequences that are commonly present in prokariotic but not eukariotic DNA and elicit strong natural immunity. This in turn promotes the adaptive immune response, particularly of the Th-1 type. In fact, another substantial advantage of the DNA-based approaches relies on their capability to elicit strong MHC class I restricted CTL responses, a feature almost exclusively limited to vaccines in which de novo synthesis of the immunogen occurs. This is very relevant for vaccine development because an increasing body of evidence indicates an important protective role for CTLs both after vaccination and in the course of natural infection (reviewed in Reference 57). In addition, recombinant vectors often provide priming, so that subsequent boosts with a subunit protein results in a humoral immune response of higher titer and longer duration. Moreover, the vector itself, due to its own tropism, can deliver immunogen to cells or tissues of interest. Several live viral and bacterial vectors are be-
ing used in recent protocols. Examples of strategies with MVA, canarypox fowlpox, adenovirus, and alphaviruses are reported below.

1. Vaccinia

Vaccinia and related avipox viruses such as canarypox have been widely used for HIV vaccines. MVA has the advantage of having been safely tested in over 120,000 humans (58), allowing insertion of several genes and eliciting an immune response similar to that observed during the natural infection with the pathogen. Preexisting immunity to vaccinia because of smallpox vaccination is an issue, and boosting such a reactivity may hamper the repeated use of the vector because of immediate clearance (59–61). There are also some concerns about the potential pathogenicity of such a vector in immunocompromised hosts.

A large study conducted within a European Collaborative vaccine program with nonhuman primates has shown that intramuscular immunization of macaques with recombinant MVA vectors expressing Env and other SIV proteins (Gag/Pol, Tat, Rev, and Nef) does not confer protection upon intravenous challenge with 50 MID$_{50}$ of the homologous pathogenic virus SIVmac251, although in some cases a reduction in viral load and a less pronounced CD4+ T-cell decline indicated a beneficial effect of the vaccination (62). These data are consistent with results from previous works (63, 64). Protection has been reported with the MVA vector alone (23), likely due to the boost of innate immunity with antiviral activity. Thus, it is conceivable that even in vaccinia-naive animals, priming with MVA may result in a strong response against the vector but not against HIV/SIV gene products. Thus, if the dominant immunogenicity of the MVA vector is confirmed, then its use for boosting rather than priming should be recommended. In fact, the strong immune response against vaccinia might be beneficial and boost antigen-specific response. Studies in monkeys are under way to address this issue, but results in the mouse model already indicate better MHC class I restricted peptide-specific T-cell induction when the animals are primed with DNA and boosted with MVA (65).

The MVA vector is also suitable for mucosal immunization, as indicated by studies in BALB/c mice immunized intrarectally with recombinant vaccinia expressing HIV gp160 (66). The immunized animals developed specific serum antibody and strong HIV-specific CTL responses. Of note, mucosal immunization generated a systemic response, overcoming the block represented by the preexisting systemic immunity to vaccinia. In addition, boosting with the same vector and by the same route was effective. The similarity of vaccinia immunity between mouse and humans suggests that a similar response should occur in humans.

2. Canarypox

Canarypox is a harmless avipox (referred to as ALVAC) used to express HIV antigens. ALVAC has already been used for several human vaccines, such as measles (virus hemagglutinin and fusion proteins), rabies virus (glycoprotein) and, more recently, to deliver the HIV-1 envelope glycoprotein (67). Like MVA, ALVAC can accommodate large amounts of foreign DNA in its genome, infect mammalian cells, and it is stable at room temperature. In contrast to MVA, ALVAC is host range restricted and does not produce infectious progeny virus in mammalian cells (67), an important safety factor especially for therapeutic vaccination in immunocompromised hosts. However, this very same feature may be responsible for the apparent lesser immunogenicity of ALVAC as compared with MVA (68). Of note, an ALVAC–Env-based vaccine is the only one currently tested in phase III trials in a canarypox/protein prime/boost regimen (reviewed in Reference 69). Although this regimen has been proved to elicit a good cellular and humoral response in humans (68, 70), its protective efficacy against infection with viruses from different clades is still an open question, hopefully to be answered by the ongoing trials.

3. Fowlpox

An attenuated fowlpox virus has been widely used in poultry to protect from infection with the wild-type pathogenic virus and recently used as a vector in vaccine studies (67). Although a recombinant fowlpox virus is 100 times less efficient than canarypox in generating protective immunity (71), recent results in macaques suggest that the use of a fowlpox vector in a prime/boost regimen may provide better immunization and protection than other approaches (72). In this study, eight different immunization protocols were compared for their ability to protect against pathogenic SHIV89.6P in rhesus macaques. The animals were primed either by intradermal injections or by gene gun delivery with DNA coding for the HIV-env and the SIV-nef, -gag, and -pol genes. Boosters were either identical to the primings or consisted in either purified Env protein or recombinant fowlpox viruses carrying HIV-env and SIV-nef, -gag, and -pol genes. The combination of intradermal DNA priming followed by recombinant fowlpox virus boosting gave the best protection against the heterologous intravenous challenge with the SHIV89.6P. However, this challenge had been preceded by two homologous challenges with nonpathogenic SHIV-IIIb, whose impact on the subsequent heterologous
challenge is yet undetermined. Of note, protection against the first of the two homologous challenges was observed also in the control group that had been primed with the empty plasmid vector and boosted with the recombinant Env protein. These animals developed the highest anti-IIIB-Env antibody titers and neutralizing activity, suggesting that antibodies were probably sufficient to protect from the first homologous challenge but not from the second one, performed 43 weeks later, when the antibody titers had declined. It is therefore conceivable that priming with the plasmid vector might have boosted the innate immunity and enhanced the response to the Env protein. It is also possible that stimulation of native immunity might have generated a first line of antiviral defense through the induction of CAF and chemokines responsible for the protection. As mentioned, however, protection against identical challenge was not observed 43 weeks later, suggesting that regardless of the mechanism, long-lasting effective immunity even against a homologous challenge is still a major problem. It will be of interest in future protocols to address the impact of the natural immunity on protection per se or on the development of an adaptive protective response after immunization.

4. Adenovirus

Adenovirus (Ad) vectors are highly promising HIV vaccine vehicles not only because they elicit good cell-mediated immunity and prime high-titered humoral immune responses, but also because the vector replicates in the epithelium of the upper respiratory tract and gut, resulting in induction of mucosal immune responses. Wild-type Ad4 and Ad7 vaccines have been used for over 25 years in the military and have proven safe and effective at preventing acute respiratory disease in recruits (73). These vaccines are easy to administer as enteric-coated capsules and are inexpensive and stable. Although preexisting immunity or induction of immunity to the vector after immunization is a concern, Ad4 and Ad7 vectors are reasonable choices for future development of HIV recombinants. Although prevalence in humans of Ad5 is significant, a recent report indicated that after cessation of the Ad4 and Ad7 military vaccine program in 1996, 66% and 73% of new military recruits lacked protective antibodies against Ad4 and Ad7, respectively. In addition, vectors of other serotypes could be developed from the 49 Ad serotypes currently identified (74).

Recent studies have demonstrated the ability of Ad4-, Ad5-, and Ad7-expressing-HIV gp160 (after boosting with the Env protein) to elicit humoral immunity and prime high-tiered neutralizing antibody responses in a dog model (75). Further studies indicated that a prime boost approach could elicit humoral, cellular, and mucosal Env-specific immune responses in chim-

panzees (76, 77) and long-lasting immunity and protective efficacy against both homologous (78, 79) and, to a lesser extent, a high-dose heterologous (minimally passaged) non-syncytium-inducing HIV primary isolate (80).

Priming with Ad5 host range mutant SIVEnv recombinant, followed by boosting with native SIV Env in rhesus macaques, induced viral-specific humoral, cellular, and mucosal immune responses (81) but not sterilizing immunity with a highly pathogenic SIV vaginal challenge. However, a reduced viral load was observed during the acute infection in immunized macaques (82). Notably, reduced viral burdens and slow progressor status resulted from a vaccine based only on Env. The use of multicomponent Ad recombinants should elicit even better protective efficacy, and construction of these is in the progress (83).

5. Alphaviruses

Alphaviruses are RNA-viruses recently used to deliver heterologous genes for vaccine and gene therapy applications. The advantages of such vectors are their high levels of replication and gene expression, their ability to infect a variety of cell types, and the ability to manipulate cDNA clones from which infectious viral RNA can be transcribed (for review, see References 84-86). The general strategy for construction of alphavirus-based expression vectors is to substitute the genes encoding the structural proteins with a heterologous gene(s), maintaining the transcriptional control via the highly active subgenomic RNA promoter (84, 85, 87). Vector replicon RNA can be transcribed in vitro and used directly. Alternatively, the replicon RNA can be packaged into infectious vector particles by cotransfection of cultured cells with a complementing defective helper RNA, which provides the virion structural proteins in trans. After infection of the host, large quantities of the protein(s) are made, yet the alphavirus itself cannot replicate because it no longer contains the sequences for the structural proteins (85, 88). Sindbis virus, Venezuelan equine encephalitis virus (VEE), and Semliki Forest virus (SFV) are among the alphaviruses being exploited by using such approaches (87, 89-91).

VEE is an alphavirus of particular interest because of its tropism for the follicular dendritic cells in the lymph node, cells that are key for long-term antigen sequestration and presentation to B cells (92, 93). In addition, VEE replicon particles (91, 94) can express high levels of both glycosylated and nonglycosylated proteins. Protein expression is centered in the lymph node of the vaccinated animal, where dendritic cells are targeted for single-cycle infection either by the parental envelope proteins or by mutant envelope proteins at higher vaccine doses (95). This strategy may be important for
inducing a strong immune response to HIV proteins with low intrinsic immunogenicity. In addition, mice immunized parenterally with VEE replicons are protected against influenza mucosal challenge, a feature highly desirable in sexually transmitted infections such as HIV. Immunogenicity studies in nonhuman primates showed that vaccination (six inoculations: two subcutaneous, two intravenous, two subcutaneous) with VEE replicons expressing the Gag and Env of a molecular clone (termed SIVsm H-4i) of the highly pathogenic SIVsm E660 swarm (96) induced humoral and cellular (CTL) response in three and two of the four vaccinated animals, respectively (97). When challenged intravenously with 50 MID50 of the pathogenic SIVsm E660, all animals became infected. However, the acute phase of infection was milder than that observed in the control animals as indicated by lower viral loads and delayed disease progression. Of note, the containment of infection correlated with the presence of both antibody titers and CTLs. These, however, were detected only in two of the four vaccinated animals, indicating that further studies are needed to optimize the immunogenicity. Not surprisingly, sterilizing immunity was not achieved despite immunization with Env, additional evidence of the poor cross-reactivity. In fact, the monkeys had been immunized with Env from a molecular clone and challenged with the virus swarm, a situation close to an heterologous challenge.

SFV represents another delivery system that allows efficient expression with only one round of infection. This approach is also suitable for mucosal immunization (98, 99). Experiments in the mouse model indicate that immunization with SFV replicons induces both a humoral and cellular immune response of the Th-1 type (99). Immunization of mice with vectors encoding influenza antigen is protective against challenge with influenza virus (99). SFV vectors expressing HIV-1 Env have been utilized for vaccination in macaques (100, 101). Although both humoral and cellular immunity were induced, sterilizing immunity upon challenge with low or highly pathogenic SHIVs was not achieved (100, 101). However, compared with nonimmunized animals, the infection was milder, as indicated by lower plasma viremia (101) and by the survival of the vaccinated animals to a lethal challenge with SHIV-PBj14 (100). For reasons already discussed, it is conceivable that immunization with HIV-1 antigens other than Env, such as the regulatory (and early) genes tat and rev, may improve the effectiveness of this approach. Indeed, priming of macaques with SFV vector expressing the SIV Tat and Rev followed by boosting with the same antigens expressed by an MVA vector resulted in an efficient control of pathogenic SIV upon intravenous challenge (31) (see following).

C. Polypeptide Vaccines

The appreciation of the importance of CD8+ CTL response in protection against HIV or SIV infection has provided the rationale for a vaccine approach based on a synthetic gene coding for partially overlapping CTL epitopes (or polypeptide) of one or more viral proteins (102–104). Among the advantages of this approach are the possibility to lower the amount of the protein to administer and to drive the immune response against the most relevant epitopes. Moreover, the inclusion of several epitopes restricted by different MHC alleles allows the elicitation of an effective CTL response in most people. In fact, eight to nine selected MHC class I-restricted epitopes would ensure the immunization of the general population if one epitope is sufficient to confer protection in each individual (105, 106).

An HIV polypeptide, termed H, that included 20 human epitopes restricted by 12 different human leukocyte antigen (HLA) alleles, 3 macaque epitopes and 1 murine epitope to permit testing for immunogenicity in all these recipients (65) has been shown to be immunogenic in mice (107), with the best CTL induction obtained by priming with naked DNA constructs followed by MVA boosting (65). Vaccination of mice with a construct coding for both HIV and malaria epitopes, and including two mouse epitopes with distinct MHC restriction, generated CTL against both the pathogens. This demonstrates the feasibility of contemporarily generating an immune response against two unrelated pathogens and to two murine epitopes with a different MHC class I restriction (108). The immunogenicity of the polypeptide H has been further confirmed in the macaque model with a DNA-prime MVA boost vaccination regimen (108). However, no protection was observed upon mucosal challenge, indicating that the selected Gag epitopes were not protective. Thus epitope(s) selection appears to be the most critical issue for this very promising vaccine approach. Of note, the polypeptide immunogen does not elicit an antibody response. It would be of relevance to evaluate the immunogenicity and efficacy of vaccines that also elicit specific antibodies, because epitope interference or beneficial synergy between the cellular and humoral responses may both occur (109–114).

D. Vaccine Based on Regulatory Genes: Tat, Rev, Nef

The results from Env-based vaccine strategies have suggested that blocking virus replication and disease onset in the lack of a sterilizing immunity may represent at present a more attainable goal of AIDS vaccine development. The control of virus replication may modify the virus–host interaction favoring
the host immune response providing protection and may be used for both preventive and therapeutic vaccine strategies.

Such a strategy should therefore use viral products that exert key functions in the early virus life-cycle, infectivity or pathogenicity, that are capable of inducing a broad immunity and that are conserved among the HIV clades. These includes the regulatory genes tat, rev, and nef.

1. Tat

The Tat protein of HIV possesses all the characteristics mentioned above and is the most studied system both in pathogenesis and vaccine development. Tat is produced early after HIV infection and is essential for virus replication and infectivity (115–117). Moreover, the Tat protein is released in the extracellular milieu and is taken up by the neighbor cells where it trans-activates virus gene expression and replication (35, 36, 38, 118, 119). Extracellular Tat also induces the expression of the chemokines receptors CCR5 and CXCR4, thus favoring the transmission of both macrophage-tropic and T lymphocyte-tropic HIV-1 strains to uninfected cells (120, 121). Tat is also involved in AIDS pathogenesis and in AIDS-associated malignancies, such as the Kaposi’s sarcoma (34, 37, 119, 122–131).

In vivo infection, the presence of a humoral immune response against Tat correlates with the control of disease progression (129, 132–134) by the inhibition of the uptake and therefore of the effect of extracellular Tat on both HIV replication (36, 134) and T-cell immunosuppression (129). Similarly, the presence of an early anti-Tat CTL response inversely correlates with the progression to the symptomatic stage of the infection (135–137). Further soluble Tat can induce CD8+ T-cell-mediated CTL responses by entering the MHC class I pathway (138) due to its capability of being taken up by cells (36, 38, 118) and very efficiently by antigen presenting cells (APC through integrin receptors, which also direct cell migration to Tat (123, 130, 131; B. Ensoli, unpublished data, 1999).

Finally, Tat has well-conserved immunogenic epitopes among the different HIV-1 clades, with the exception of the O subtype (1; S. Butto, unpublished data). Thus, although it cannot block virus entry, a Tat-based vaccine may control virus replication.

This hypothesis has been confirmed by recent studies in cynomolgus monkeys vaccinated with a biologically active HIV-1 Tat protein (29). Six monkeys were immunized subcutaneously with 10 μg of Tat in RIBI (three monkeys) or alum (three monkeys) and one animal with Tat (6 μg), intradermally (ID), in the absence of adjuvants. During the following 36 weeks the animals received seven to eight boosts. No toxicity (acute or chronic; local or systemic) was ever detected in the vaccinated animals throughout the immunization period. The six monkeys inoculated with Tat and RIBI or alum developed very high titers anti-Tat antibodies capable of neutralizing Tat activity and blocking virus replication in vitro. In contrast, the animal given Tat ID developed low and transient anti-Tat antibody titers. The anti-Tat vaccine also elicited cellular immune responses, including DTH and T-helper proliferative response to Tat and anti-Tat CD8+ T CTL secreting tumor necrosis factor alpha (TNF-α) upon antigen-specific stimulation.

At week 50 after immunization (14–18 weeks after the last boost), all animals were challenged with the SHIV89.6P, a chimeric virus that contains the tat gene of HIV-1 and is highly pathogenic in macaques (139). The virus stock used for the challenge was derived from a cynomolgus macaque inoculated with the original SHIV89.6P from rhesus monkeys, obtained from Dr. N. Letvin. To determine virus pathogenicity in cynomolgus and the monkey infectious doses (MID50), the original virus stock obtained from the rhesus and the virus stock obtained from the cynomolgus macaques were inoculated into six and eight monkeys, respectively. High levels of viral replication, including p27 antigenemia, plasma viremia, proviral DNA, anti-SIV antibody titers, and a profound and persistent decrease in CD4 T-cell counts, were observed in all monkeys independent of the virus stock utilized, and no differences were found when the data obtained were compared with those already published by Dr. Letvin’s lab, including the rate of animal death (29, 139, 140; 140a). Therefore, all vaccinated and control macaques were challenged intravenously with 10 MID50 of SHIV89.6P. At this time a naïve control monkey was included in the protocol and inoculated with a dose threefold lower (2.8 MID50) as an additional control of the virus inoculum.

After challenge, all the controls but only two of the seven Tat protein-vaccinated monkeys (one given Tat and RIBI and one Tat and alum) were infected, as indicated by the presence of high levels of p27 antigen (detected by ELISA) and viral RNA (detected by branched-DNA and quantitative-competitive RNA-polymerase chain reaction) in plasma, proviral DNA copies, cytophemia, or positive virus isolation. In contrast, all these parameters were always negative for all the other five vaccinees up to 68 weeks postchallenge, with the exception of SIV proviral DNA, which was only sporadically and barely detected (<10 copies/μg of DNA) in a few animals. This and the presence of low and transient anti-SIV (or anti-HIV Env) antibody titers in these protected animals indicated that infection occurred but was blocked by the immune response induced by the vaccination.

Of note, anti-SIV or anti-HIV Env antibodies correlated with the level of infection. They were very high in all the control monkeys, followed by
the two infected and vaccinated animals that had at least 2 logs lower titers and a delayed increase as compared with the control macaques, whereas they were very low and transient in the five protected monkeys. Consistent with the data from the virological assays, the number of CD4+ T cells remained in the normal range after the viral challenge and during all the follow-up period in the five protected monkeys, whereas it decreased considerably in all the controls and in the two vaccinated and infected macaques (29). Notably, protection correlated (100%) with the presence, before challenge, of anti-Tat-specific CTLs and with TNF-α production by CD8+ T cells upon Tat stimulation but not with the presence of anti-Tat antibodies. Nevertheless, the two infected and vaccinated monkeys (that had high anti-Tat antibody titers but not CTLs) developed lower (1 log) and delayed anti-SIV antibody titers as compared with the controls, suggesting that they were exposed to a lower antigen load. Of interest, a potent and stable CAF activity was detected early after challenge in all the vaccinated and protected monkeys but not in the infected animals, including the two vaccinated macaques (D. Goletti, unpublished data, 1999). In contrast, no correlation of protection was observed with the production of beta-chemokines at postchallenge time (RANTES, MIP1α, MIP1β) (D. Goletti, unpublished data, 1999).

Thus, immunization with a biologically active Tat was safe and induced a broad immune response capable of blocking virus replication to undetectable levels, preventing the CD4 T-cell decline and disease onset. Protection correlated (100%) with a CD8+ T-cell-mediated activity that includes both antigen-specific CTLs and a Th-1 type cytokine production and nonantigen-specific immune responses such as CAF, suggesting that Tat vaccine can also potentiate innate immunity. The same protection data and correlates of protection have been recently obtained by a different protocol utilizing Tat DNA (B. Ensoli, unpublished data, 1999). Thus, although the Tat strategy does not block virus entry, it can efficiently control virus replication and render the natural infection “abortive.” This is likely to occur against different virus strains as suggested by studies in HIV-infected Ugandan patients that although infected by different virus strains, have antibodies recognizing Tat from the clade B used in the vaccination studies (S. Butò, unpublished data, 1999).

Although Tat protein or DNA inoculation was safe both in naïve (29; B. Ensoli, unpublished data, 1999) and SHIV-infected monkeys (B. Ensoli, unpublished data, 1999), the possibility to use transdominant negative mutants of HIV-1 Tat lacking the transactivating activity but preserving the immunogenicity of the wild-type Tat was also investigated (141). Intramuscular immunization of mice with plasmids encoding two transdominant negative mutants (Tat22 and Tat22/37) (142) elicited an immune response to wild-type Tat protein that was comparable with that induced by inoculation of wild-type Tat DNA. In particular, the humoral response was comparable to wild-type Tat protein in terms of IgG subclasses, antibody titers, epitope specificity and neutralization of the biological activities of Tat, and by the proliferation of splenocytes from the immunized mice in response (141). These mutants are currently being investigated in monkey trials.

A modified Tat, inactivated by carboxymethylation, has also been recently utilized as a vaccine. Studies in mice, rabbits, monkeys, and seronegative and seropositive humans indicated that the inactivated Tat, termed Tat toxoid, is safe and immunogenic, as assessed by measurements of antibody titers and proliferative responses (143, 144). To evaluate its efficacy, initial experiments have been performed in rhesus macaques that were vaccinated with 20-100 μg of the Tat toxoid and then challenged intrarectally with 2500-5000 tissue culture infectious dose (TCID50) of the pathogenic SHIV 89.6PD (30). In comparison with control animals, an attenuated infection was observed in vaccinated monkeys, as indicated by the reduction of p27 antigenemia and plasma viremia. In addition, the monkeys that controlled the infection (plasma viremia < 2500 copies/mL) exhibited both humoral and proliferative responses to Tat at prechallenge, suggesting that both arms of the immunity contributed to controlling disease. No information is presently available on other aspects of the cellular immune response (such as CTLs and CD8+ cell-mediated antiviral activity) to more precisely compare the Tat toxoid approach with the native Tat protein vaccine that, utilizing a biologically active Tat protein, also exploits the unique capability of Tat to be taken up by APC, to enter the MHC class I pathway of presentation, and to generate CD8+ CTLs (138). This is of importance because CTLs, but not anti-Tat antibodies, correlated with protection after vaccination with a biologically active Tat or Tat DNA in monkey trials (29; B. Ensoli, unpublished data, 1999). Nevertheless, it appears that systemic vaccination with the Tat toxoid attenuates disease upon mucosal challenge, in the apparent absence of mucosal immunity.

2. Tat and Rev

Rev is another regulatory gene expressed early after infection and essential to virus replication. As for Tat, an early immune response to Rev has been shown to correlate with long-term nonprogressor status (137). The efficacy of immunization against both tat and rev regulatory genes has been demonstrated in macaques vaccinated twice with SFV and twice with the recombinant MVA, both expressing the SIVmac32H Rev and Tat, and challenge intravenously with 50 MID50 of SIVmac 32H (pJ5). As expected from this
nonsterilizing approach, infection occurred as indicated by plasma viremia. However, plasma viremia was transient and significantly lower than in the controls, and no cell-associated viremia could be detected in the vaccinated macaques, whereas productively infected cells were easily detected in the control animals (31). However, no information is available from these initial experiments on the type of immune response elicited or on the correlates of protection. Further studies are ongoing to clarify these issues and the impact of immunization with Rev in this vaccine strategy.

3. Tat, Rev, Nef

Vaccination against all three HIV regulatory genes tat, rev, and nef, has also been investigated in mice and humans. Hinkula et al. (33) were the first to demonstrate that both humoral and cellular immune responses are induced in mice (four different strains) upon immunization with cDNA plasmids encoding these HIV-1 regulatory genes with no interference being detected among these plasmids, even when plasmids encoding for the structural proteins Env and Gag were associated in the vaccination protocol. The epitope specificities of the humoral response was comparable with that obtained by vaccinating with the corresponding protein. In addition, epitope-specific T-cell responses and a Th-1 pattern of cytokine secretion were detected. The efficacy of a vaccine based on all three regulatory gene products together and in the absence of other viral genes (i.e., env, gag) has not yet been tested in nonhuman primates. Studies in HIV-infected patients have confirmed that DNA vaccination with either tat, rev, or nef is safe (no evidence of increased viral load) and induces B- and T-cell responses (including CD8+ CTLs) that were absent or low before immunization (34), demonstrating the feasibility of genetic immunization to induce new immune responses in HIV-infected patients. A more recent study (145) in which the same immunization approach was combined with highly active antiretroviral treatment (HAART) confirmed the safety and immunogenicity of the vaccine, whereas the reduction of the viral load was dependent on HAART, indicating that this combination may improve the capacity of the immune system to deal with the HIV infection.

E. Alloimmunization for Anti-HIV Vaccine

A substantial body of evidence (reviewed in Reference 39) suggests that alloimmunization generates an immune response with antiviral activity that may strongly suppress HIV replication, resulting in lower susceptibility of the host to infection (40) or in better control once the infection is established. A recent study in which women were alloimmunized with their partners’ mononuclear leukocytes to prevent spontaneous recurrent abortions showed a strong and long-lasting CD8-T-cell activation, with increased production of beta chemokines and soluble antiviral suppressor factors and decreased expression of the CCR5 coreceptors in both activated lymphocytes and monocytes, the major targets of HIV-1 infection (146). In vitro experiments demonstrated that all these factors contributed to the decreased susceptibility to HIV infection with both lab-adapted and primary strains. Alloimmune recognition belongs to the natural immunity (147) and has been invoked as one of the factors responsible for reduced risk of HIV vertical transmission due to increased HLA disparity (148). This immune response is directed against the human HLA antigens that are exposed on the viral membrane and not against the virus, circumventing the problem of the virus variability.

A further advantage is the large number of alloreactive T cells that would respond, a proportion comprised between 1 and 10% of all T lymphocytes. Although naturally present, this alloimmunity can, in fact, be further potentiated by immunization with HLA-mismatched leukocytes (146, 149). Finally, alloimmunization is safe (150, 151). Although no evidence of in vivo protection is yet available, protection was achieved in macaques when the challenge virus was grown on xenogeneic human cells (152–156), suggesting that this may also apply to the allogeneic model in humans. The very same monkeys were not protected when rechallenged with SIV grown on allogeneic macaque cells (155, 156). However, these monkeys had not been alloimmunized. Thus, the unprimed natural alloreactivity is not sufficient per se to confer protection, as also indicated by the relative ease with which both HIV and SIV are transmitted in human and nonhuman primates, respectively. Further studies are needed to develop a protective vaccine based on the alloimmunization. However, the lessons learned with this strategy may be key to understanding the role of innate immunity in eliciting a protective response from different vaccination strategies.

IV. CONCLUSIONS AND PERSPECTIVES

A considerable amount of data have been generated in the past 15 years clarifying several aspects of HIV pathogenesis, shedding light on the inefficacy of most past vaccine approaches, and promoting the development of novel immunization strategies (Table 1). Past experience has definitively proved that regardless of the approach, HIV vaccination is safe, with the only exclusion being live attenuated viruses. Paradoxically, this is an important exclusion because attenuated viruses have been shown to provide the best protection, indicating that achievement and maintenance of effective immunity may require prolonged presentation to the immune system of multiple, native, viral
Table 2  Role of Nonadaptive (Natural) and Adaptive (Antigen-Specific) Immune Responses Against HIV/SIV

<table>
<thead>
<tr>
<th>Response</th>
<th>Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevention</td>
</tr>
<tr>
<td>Nonadaptive</td>
<td>+ (+)</td>
</tr>
<tr>
<td>Adaptive</td>
<td></td>
</tr>
<tr>
<td>Antibody</td>
<td>+ + +</td>
</tr>
<tr>
<td>CD4⁺ T-helper cell</td>
<td>+ +</td>
</tr>
<tr>
<td>CD8⁺ CTLs</td>
<td>- + (+)</td>
</tr>
</tbody>
</table>

*Including CAF, beta chemokines, and antiviral activities mediated by natural killer T cells.

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