The effect of Mucosal, Synthetic Peptides, Live Vectors, Infectious DNA, Inactivated Virus, Secretory antibodies, and Broad Neutralizing Antibodies Vaccines on Humeral immunity and Cellular immunity as potential Invasion Blocker vaccines (Part Nine)

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Abstract

Most infectious agents enter the body at mucosal surfaces and therefore mucosal immune responses function as a first line of defence. Despite recent advances in our understanding of HIV-1 pathogenesis and immunology, however, major scientific obstacles remain.

The development of a safe and effective HIV-1 vaccine is a critically important global health priority.

Several new vaccine vector approaches offer promise in exquisite control of acute infection and in improving the breadth of T cell responses.

Critical to staying on the path toward development of an efficacious vaccine is utilizing information from previous human and non-human primate studies in concert with new discoveries of basic HIV-1 host-virus interactions.

Blockade of HMBG1 has been found to restore natural-killer-cell-mediated killing of infected dendritic cells, normally suppressed by HIV-1.

A renewed and coordinated commitment to basic discovery research, preclinical studies, and clinical trials will therefore be required to overcome the hurdles currently facing the field.

In this article, I discuss HIV-1 and invasion blocker vaccines, Virologic and Immunologic Challenges, HIV-1-specific humoral immunity, HIV-1-specific cellular immunity, Developing a mucosal vaccine for HIV, Secretory antibodies and protection against HIV, Broad Neutralizing Antibodies: Understanding Targets, Host Control, and Maturation Pathways, HIV-1 Vaccine Efficacy Trials and New Generation Vaccines

Key Words: HIV-1, Cellular immunity, Humoral immunity, Mucosal vaccine, Secretory antibodies, Invasion blocker vaccines

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1. Introduction

The adaptive immune system is designed to distinguish antigens, pathogens and vaccines that enter the body through mucosal surfaces from those that are introduced directly into tissues or the bloodstream by injection or injury. Mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, whereas injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces (1),(2). The extraordinary diversity of HIV-1, the capacity of the virus to evade adaptive immune responses, the inability to induce broadly reactive antibody responses, the early establishment of latent viral reservoirs, and the lack of clear immune correlates of protection represent unprecedented challenges for vaccine development. An ideal vaccine would completely block infection and provide sterilizing immunity. Recent insights of ways vaccines can potentially stimulate protective T and B cell immunity, the identification of new targets for broad neutralizing antibodies (BnAbs), and the discovery of new mechanisms of host control of HIV-1 BnAb induction offer renewed hope for the development of a safe and effective preventive HIV-1 vaccine. Approaches that aim either to improve upon current findings from a partially efficacious vaccine strategy or approaches toward the generation of broadly neutralizing antibodies by rational immunogen design strategies are likely to be fast-tracked through careful probing of the intersections of immune correlate analyses within and among human vaccine clinical trials. Clues about potentially protective immune responses can be discerned, and of equal importance, potentially detrimental or distracting immune responses can be identified. Vaccines based on live, weakened HIV-1 can mutate and regain their pathogenicity after introduction to the human bloodstream (3).New and innovative approaches are necessary to address the spread of this virus. The major advantage of preventing the virus from replicating is the decreased chance of generating viral escape mutants (4). Recent findings with respect to innate-mediated immune responses against HIV-1 could lead to the development of potent microbicides/antiviral agents that can successfully prevent viral acquisition, replication and dissemination as well as valuable insights into possible adjuvants that can shape innate immune responses to enhance the effect of future vaccines.

2. Virologic and Immunologic Challenges

The challenges in the development of a prophylactic HIV-1 vaccine are unprecedented. The extraordinary worldwide diversity of HIV-1 presents perhaps the greatest hurdle (5). Driven by the error-prone reverse transcriptase, the HIV-1 M group has diversified into nine divergent clades as well as multiple circulating recombinant forms. Amino acid sequences of Env can differ up to 20% within a particular clade and over 35% between clades (5),(6). A vaccine immunogen will therefore need to contend with a remarkably high degree of viral diversity,

and vaccine protection will necessarily be dependent on the capacity of immune responses to cross-react with highly heterologous viruses. Although cross-reactive humoral and cellular immune responses against conserved regions of the virus have been reported, it is reasonable to assume that protective efficacy will diminish substantially with increasing divergence between vaccine antigens and infecting viruses (6). Suggestive evidence regarding immune correlates of protection might be obtained from viral challenge studies in nonhuman primates and from studies of HIV-1-infected individuals who spontaneously control viral replication to very low levels. However, definitive immune correlates of protection will likely only emerge in the context of successful vaccine efficacy studies in humans (5),(6).

3. HIV-1-specific humoral immunity

Virus-specific NAb titers represent a key immune correlate for most licensed viral vaccines, and thus early studies focused on developing HIV-1 Env subunit immunogens. Advances in our understanding of Env structure and function have begun to elucidate why generating broadly reactive NAbs to HIV-1 by vaccination may be so difficult (7). The HIV-1 Env glycoprotein is a trimer on the virion surface with extensive N-linked glycosylation that effectively shields many conserved epitopes from antibody recognition (8),(9). Highly immunogenic variable loops also elicit type-specific antibodies that may redirect humoral responses away from conserved regions. In addition, key conserved regions, such as the chemokine coreceptor binding site, are only formed after Env binds its cellular receptor CD4 and undergoes an extensive conformational change (10). The development of mutations in N-linked glycans has also been shown to lead to rapid evasion of host NAb responses (11),(12). Nevertheless, broadly reactive NAb activity has been identified in a small number of HIV-1- infected subjects, and this reactivity appears largely directed against conserved regions of the Env glycoprotein such as the CD4 binding site (13). The broadly reactive mAb b12 also binds to the CD4 binding site, suggesting that this region of Env may represent a critical point of vulnerability that is potentially amenable to neutralization (14). Another conserved region is the membrane proximal external region (MPER) of gp41, which represents the target of the broadly reactive mAbs 2F5 and 4E10. However, MPER-specific NAbs may be difficult to elicit by vaccination for multiple reasons, including tolerance control and immunoregulation (15). sequestration of the epitope in the lipid membrane (16), exposure of the epitope only transiently during viral entry (17), , or possibly a combination of multiple factors. The development of immunogens that induce broadly reactive NAbs is perhaps the most important priority for the HIV-1 vaccine field (7). Proof-of-concept passive transfer studies in nonhuman primates have shown that administration of high doses of broadly reactive mAbs can afford sterilizing protection from infection, thus demonstrating the potential of virusspecific humoral immunity (18),(19). However, it has not been possible to induce such broadly reactive NAbs by vaccination to date. Although there has been substantial progress in our understanding of Env structure and function, there are currently no vaccine candidates that are aimed at eliciting broadly reactive Env-specific NAbs in clinical trials. It is likely that next generation Env immunogens will need to be engineered antigens.Strategies that are being pursued include generating biochemically stabilized Env trimers, constraining Env immunogens in structurally defined conformations, scaffolding conserved neutralization epitopes onto foreign proteins, developing methods to circumvent immunoregulation, and designing immunogens to target specific regions such as the CD4 binding site, the MPER region, and structurally conserved elements of the V3 loop. The relevance of non-neutralizing

antibodies that mediate other effector functions such as antibody-dependent cell-mediated virus inhibition, complement activation, and phagocytosis is also being investigated.

4. HIV-1-specific cellular immunity

Virus-specific T lymphocyte responses are believed to play a critical role in controlling HIV-1 replication and are therefore being actively explored in vaccine development strategies. Early studies showed that virus-specific CD8+ T lymphocyte responses emerge during acute infection coincident with initial control of primary viremia (20),(21),(22).. Potent cellular immune responses have also been reported in long-term nonprogressors (23), and specific HLA alleles and the breadth of Gag-specific T lymphocyte responses have been correlated with control of viral replication in HIV-1-infected individuals (24),(25).. These data indicate the potential importance of cellular immune responses in immune control of HIV-1. Concordant with these observations, experimental depletion of CD8+ T lymphocytes has been shown to abrogate immune control of simian immunodeficiency virus (SIV) replication in rhesus monkeys (26),(27).. It is therefore likely that the breadth of epitope-specific T lymphocyte responses will prove critical for an HIV-1 vaccine, not only to maximize immunologic coverage of HIV-1 diversity but also to minimize the potential for viral escape from recognition by T lymphocytes. Recent advances in the characterization of T lymphocyte responses by multiparameter flow cytometry have highlighted the functional diversity of virus-specific T lymphocytes in terms of cytokine secretion, degranulation, proliferation, and other effector functions in various subpopulations of effector and memory T lymphocytes. It is likely that the complex functionality of T lymphocytes may ultimately prove more relevant than IFN-γ secretion as measured by ELISPOT assays for the evaluation of vaccine-elicited cellular immune responses. Polyfunctional T lymphocytes capable of performing multiple functions have been reported in long-term nonprogressors (28), in recipients of effective vaccines such as vaccinia (29), and in certain preclinical challenge studies (30). These considerations suggest that the breadth (31), and quality (32), of T lymphocyte responses may prove critical in addition to the magnitude of these responses. As a result, vaccine-induced T lymphocyte responses will presumably be unable to prevent lifelong infection, since the virus rapidly establishes latent reservoirs (33),(34). HIV-1 preferentially infects HIV-1-specific CD4+ T lymphocytes (35), and rapidly depletes the majority of memory CD4+ T lymphocytes in gut-associated lymphoid tissue (GALT) within the first 4–10 days of infection (36),(37),(38). This sets the stage for progressive immunodeficiency as well as for chronic immune activation, which likely results at least in part from microbial translocation across damaged gastrointestinal mucosa (39).

5. Triggering an Immune Reaction against HIV-1

The host cell then produces copies of the virus and, upon death, ejects these copies into its environment. In most organisms, the presence of foreign bodies, such as viruses, triggers an immune response, a reaction in which the organism's immune system can target and destroy alien particles. While this reaction is sufficient to combat the majority of pathogens, HIV-1 has proven to be an exception. Lentiviruses such as HIV-1 target the immune system directly, crippling the host organism's ability to create the necessary antibodies to combat the infection.

The host organism's immune system may recognize HIV-1 as a threat and attempt to repel the virus, but the increased CD4 T-cell concentrations from this response only provide additional host cells for viral replication. The natural response to HIV infection neither controls viral replication nor prevents superinfection, and as a result no protective immune response against the virus has ever been observed (40). In order to compensate for the inability to naturally produce these protective antibodies, artificial antibodies can be engineered and introduced into the host organism (41). These antibodies are capable of protecting CD4 T-cells against HIV-1 infection under controlled conditions, but face the enormous roadblock of enormous HIV-1 diversity (42). Antibodies neutralize the virus by binding to the surface gp120 and trans-membrane gp41 envelope glycoproteins, blocking HIV-1 entry into susceptible cells. However, the virus exploits several mechanisms to shield itself against antibody recognition, including a dense outer coating of sugar molecules and extreme variation of the aforementioned surface proteins (43). The outer coating protects the viral RNA from direct attacks, making indirect methods such as neutralizing the protein binding sites, a more feasible option of prevention. There are four major approaches for dealing with HIV-1 diversity. The first is the development of an antibody based on a single clade, or variant, of the virus similar enough to all other clades to produce a crossreactive immune response (41). By selecting the clade with the closest sequence to all others, an immune response could be generated capable of offering protection against all similar clades. This approach has been proven effective in test models, but only after antibody concentrations have been raised to high enough levels to make practical application impossible (41). The second approach is to derive vaccine immunogens from centralized sequences of viral RNA. Despite the variability of HIV-1, all clades are similar enough that a common ancestral sequence could be extrapolated from currently circulating viruses (41). These centralized sequences are designed to minimize the difference between a vaccine immunogen and circulating viruses, but may not be able to offer optimal protection from all variants. The third approach is to deliver a vaccine consisting of a cocktail of immunogens derived from different clades (44). Initial results with this technique have been encouraging, but doubts remain due to the possibility of interference between closely related peptide sequences in the vaccine, which may limit responses to some antigens (41). The final major approach is the use of "mosaic" immunogens designed to optimize coverage of CD4 T-cell epitopes (45). This technique involves assembling a polyvalent vaccine candidate capable of targeting multiple variants of HIV-1. Potential problems with this technique are similar to those of other cocktail techniques, such as immune interference (41).

6. HIV-1 Resistance and CCR5-Δ32

Individuals were found to have a homozygous defect in the Chemokine Receptor-Five (CCR5) allele resulting in a truncated protein incapable of serving as a receptor for HIV-1. The defective allele, called Delta-32 (Δ32) appears to have no significant negative effect on cellular function. Other chemokine receptors, such as CCR1, compensate for the deletion of CCR5, but do not act as receptors for HIV-1(46),(47). The theory surrounding the development of a CCR5-based vaccine is relatively simple: since HIV-1 enters the host cells by binding to a CD4 receptor and then interacting with a chemokine receptor, primarily CCR5 (48),(49), (49)disabling or sufficiently downregulating the protein could significantly hamper or even prevent viral replication (46),(49)., The preventative potential of CCR5-Δ32 was demonstrated practically by Hütter et al. in 2007, when a patient suffering from both HIV-1 infection and acute myeloid leukemia received an allogeneic stemcell transplantation with stem cells from an unrelated

donor who had been screened for homozygocity for the CCR5-Δ32 allele (50). The patient achieved complete chimerism, and his blood monocytes displayed a homozygous CCR5-Δ32 genotype (49). While this case clearly emphasizes the importance of CCR5-targetted treatment strategies, uncertainty remains over whether a cure for HIV-1 infection has been achieved in this patient (50). Mutation into a strain capable of entering host CD4 cells through other chemokine receptors, such as CXCR4, could result in a relapse. Despite the proven effectiveness of allogeneic stem-cell transplants, the rarity of the CCR5-Δ32 homozygous genotype is extremely rare, occurring almost exclusively in populations of western European heritage, and only in 1% of those populations (46). The rarity of this genotype, combined with the expense of stem-cell transplants would make this therapy prohibitively expensive for general use. A plausible alternative is the use of gene therapy to disable the production of the CCR5 protein (51). The expression of the CCR5 gene can be disabled by introducing small interfering RNA (siRNA) to interfere with the production of the associated protein (51). Several highly potent siRNAs have already been derived and have proven capable of almost completely disabling CCR5 expression. If these siRNAs can be mass-produced, they may represent a realistic vaccine against HIV-1.

7. Challenges in mucosal vaccine design

Mucosal vaccines that are given orally or deposited directly on mucosal surfaces face the same gauntlet of host defences as do microbial pathogens: they are diluted in mucosal secretions, captured in mucus gels, attacked by proteases and nucleases, and excluded by epithelial barriers. So, relatively large doses of vaccine are required and it is impossible to determine exactly what dose actually crosses the mucosa. Soluble, non-adherent antigens are taken up at low levels, if at all, and in the intestine, such antigens generally induce immune tolerance (52).. The vaccine formulations and delivery strategies that have been used to address these challenges have been reviewed elsewhere(53). In general, mucosal vaccines are likely to be most effective when they mimic successful mucosal pathogens in key respects: they would ideally be multimeric and/or particulate, adhere to mucosal surfaces (or even better, adhere selectively to M cells), efficiently stimulate innate responses, and evoke adaptive immune responses that are appropriate for the target pathogen.

8. Breaching the epithelial barrier

The effectiveness of live pathogens as mucosal vaccines and vaccine vectors is partly a result of their adaptation to survive in luminal environments and to efficiently invade organized mucosal lymphoid tissues. Indeed, two of the most effective oral vaccines, live attenuated poliovirus3 and live attenuated *S. typhi*1, are derived from pathogens that preferentially adhere to M cells and exploit M-cell transport to invade organized mucosal lymphoid tissues in the intestine (54),(55), The efficiency of non-living mucosal vaccines is unlikely to be comparable to these successful invaders, but uptake into the mucosa can be significantly increased. Protein, peptide and DNA vaccines as well as live vaccines can be partially protected from degradation by oral delivery in enteric-coated gelatin capsules1 or by inclusion in copolymeric micro-particles (56), liposomes (57), or proteasomes (58).. The retention of vaccine antigens on mucosal surfaces by delivery in adherent gel-forming polymers, such as chitosan, has been shown to increase antigen uptake and immune responses (59). The coupling of antigen with proteins that themselves are adherent to epithelial surfaces has also enhanced mucosal immune responses,

presumably by promoting adherence and entry into epithelial-cell transport pathways (60). Particulate vaccines have several theoretical advantages for mucosal delivery. M cells are particularly accessible to microparticles and actively transport them into Peyer's patches. Microparticles that are are small (up to 1 μm diameter) and adherent to M cells are taken up most efficiently (60),(61, 62), Ligands and antigens can therefore be targeted to Peyer's patches by association with microparticles. Particulate vaccines that enter mucosal inductive sites have the additional advantage of being readily taken up by mucosal DCs and providing antigen depots. Virus-like particles (63),(64), and small vesicles derived from bacterial outer-membrane components (58),(65), are particularly promising as mucosal vaccines because their size is appropriate for uptake by M cells and DCs, their surface structures mimic those of mucosal pathogens, and they can activate an innate immune response. In practice, however, large amounts are needed for mucosal immunization because microparticles tend to be trapped in mucus and only a small fraction of the administered dose is likely to enter mucosal inductive sites.

9. Alerting the mucosal immune system

Non-living macromolecules, protein-subunit antigens and non-microbial particles generally evoke weak or undetectable adaptive immune responses when applied mucosally. To be distinguished from harmless substances and nutrients, mucosal vaccines must raise alarms in the mucosa by including substances that activate innate signaling pathways in epithelial cells and/or in the underlying antigen-presenting cells. The best-known mucosal adjuvants are the secreted enterotoxins of *V. cholera* and *E. coli*, cholera toxin and *E. coli* heat-labile enterotoxin (66). As microgram oral doses of these toxins induce severe diarrhoea in humans, several genetically modified forms have been engineered to reduce or eliminate the toxicity associated with the enzymatic A subunits of these toxins (66).. Interest in mutated toxins as adjuvants for nasal vaccines has been dampened by the observation of B-subunit-dependent retrograde transport to the brain by olfactory nerves in experimental animals (67), and association with adverse neurological effects in humans (68).. Recently, alternative chimeric toxins that couple an enzymatic A subunit with more restricted cell-targeting molecules show promise for safe nasal use in humans (69). Meanwhile, new information about the functions of immune-modulatory cytokines in mucosal immunity and the discovery of TLRs have provided promising new alternatives. For example, there is evidence that mucosal immune responses to vaccines can be increased by the incorporation of cytokines such as IL-12, granulocyte/ macrophage colony-stimulating factor (GM-CSF) or a combination of both (70). Various TLR ligands including CpG-containing oligonucleotides (71),, flagellin (72), and bacterial porins68 have shown adjuvant activity when administered mucosally together with antigens. TLR ligands and toxins function as mucosal adjuvants, presumably because they activate key innate signalling pathways and stimulate the appropriate mucosal DCs that in turn orchestrate adaptive immune responses that are designed for defence against live pathogens. Many live attenuated mucosal vaccine vectors, including poliovirus, adenovirus and enteric bacteria, are currently under development and have been extensively reviewed1,(73). The superiority of live attenuated pathogens as mucosal vaccines and vaccine vectors is due in part to their ability to activate multiple innate responses, and the importance of innate immunity in the development of adaptive immune responses is becoming increasingly clear. Nevertheless, some live vaccines present safety and acceptability issues that might reflect innate immune responses and inflammation, such as mild enteritis-like symptoms in the case of oral administration of certain live attenuated bacteria1. An additional

concern for live nasal vaccines is the possibility of retrograde transport to the brain through olfactory nerves, as has been found with live attenuated adenovirus (74).

10. Developing a mucosal vaccine for HIV

10.1. The role of mucosal immunity in protection against HIV

Mucosal transmission of simian immunodeficiency virus (SIV) in non-human primates, and presumably of HIV in humans, can occur without epithelial-cell damage of the oral, rectal and genital mucosae (75),(76).. HIV presents a daunting challenge to vaccinologists. It seems to exploit mucosal antigen-sampling mechanisms at these sites, including vesicular transepithelial transport pathways of M cells and uptake by intraepithelial DCs (77),(78). The mucosal tissues of the rectum and tonsils both contain abundant mucosal lymphoid follicles and associated M cells (79),(80), and M cells provide a short and rapid pathway across the epithelial barrier (80). This could explain the observed transmission of HIV to adults through infected semen, or to babies through infected milk. Epithelial cells themselves are not productively infected by HIV, but they serve as gateways for the delivery of infectious HIV parasites to antigen-presenting DCs and macrophages. As mucosal antigen-presenting cells interact with local CD4+ T cells, they unwittingly infect and ultimately disable the very cells that are needed to mount an effective immune response. Infection of local target cells can occur rapidly after deposition of virus on mucosal surfaces86. However, dissemination of virus to regional lymph nodes and other tissues might be delayed for up to several days (81),(76), providing a window of opportunity for local control of the infection by mucosal immune effectors. In any case, whether transmitted mucosally or injected, HIV and SIV replicate preferentially in mucosal tissues, such as the intestinal mucosa, that are rich in CD4+ T cells (82),(83). Therefore, the ultimate goals of anti-HIV vaccines should be first to interrupt mucosal transmission at its earliest stages, before the virus has crossed the epithelial barrier and infected its first target cell, and then to prevent the establishment of viral reservoirs in mucosal tissues. To achieve these goals, HIV-specific vaccines must generate multiple immune effectors, including HIV envelope- specific antibodies in mucosal secretions, and CTLs and neutralizing HIV-envelope-specific antibodies in the mucosa and circulation. Given what we know about the induction of mucosal immune responses, it is unlikely that injected HIV vaccines alone will induce the mucosal responses that are required (84),(85). The challenge is to identify the key effectors that are required and then to design a vaccination strategy that induces them. Many candidate mucosal vaccines, adjuvants and delivery strategies have been tested in mice and found to induce mucosal (as well as systemic) HIV-specific humoral and cell-mediated immune responses (73). Priming of macaques by nasal administration of DNA encoding non-replicating SHIV (77)..6P particles, followed by boosting by nasal administration of modified vaccinia virus Ankara (MVA) expressing SHIV proteins, induced greater percentages of SHIV-specific CD8+ T cells in the rectum than in the blood and afforded significant protection against rectal challenge with SHIV(77).6P (86) In other studies, DNA-vaccine-mediated induction of SIV-specific CTLs (and antibodies) in the rectal mucosa was associated with protection against rectal challenge with heterologous virus (87) Until now, the greatest success in preventing mucosal transmission of immunodeficiency viruses has been achieved through the vaccination of macaques with live

attenuated SIV (88),(89), which induces antiviral CTLs in the rectal and genital tract mucosa because of its ability to proliferate at these sites (90). Live attenuated HIV is generally considered to be too risky for use as a human vaccine, but studies in monkeys have shown the advantages of using other live vectors for stimulating mucosal immunity. For example, mucosally administered, recombinant SIV-expressing adenoviruses have been effective in preventing rectal transmission of highly pathogenic SIV in macaques, perhaps because adenovirus can replicate in intestinal tissues (73). Nasal administration of vaccines, such as live non-pathogenic SHIVs or poliovirus that expresses SIV proteins in macaques, provided significant protection against mucosal SHIV or SIV transmission (91),(92).

10.2. Secretory antibodies and protection against HIV

Prevention of HIV infection will clearly require specific antibodies (93). HIV-specific serum antibodies might neutralize virus that has entered mucosal tissues by blocking the attachment and/or entry of virus to target cells (93),(94). On mucosal surfaces, secreted HIV-specific antibodies could provide an additional layer of protection by preventing virus from contacting mucosal surfaces, adhering to epithelial cells or crossing the epithelial barrier (95).. Indeed, large doses of vaginally administered gp120-specific monoclonal antibodies (denoted as b12) prevented vaginal SHIV transmission in macaques (96). A few mucosally administered vaccines have elicited mucosal IgA antibodies in macaques. However, the potential role of vaccine-induced secretory antibodies in protection against HIV or SIV has yet to be adequately tested. Meanwhile, studies using cultured monolayers of polarized epithelial cells have provided information about the interactions of HIV and HIV infected cells with epithelial barriers and the specific antibodies capable of blocking these interactions. For adhering to mucosal surfaces, HIV uses receptors on epithelial cells that are distinct from the CD4 receptor– chemokine co-receptor pairs that are required for the infection of target mononuclear cells. Although apical surfaces of epithelial cells in the rectum and female genital tract might contain the chemokine co-receptors CCR5 and CXC-chemokine receptor 4 (CXCR4) that might facilitate HIV transport (97). they are CD4 negative (98). However, epithelial-cell membranes contain galactosylceramide, a glycolipid that is recognized by a conserved region in the tip of the V3 loop of HIV gp120 (99), and by a highly conserved region (amino acids 650–668) in the gp41 ectodomain (100). Transepithelial transport of HIV across cultured epithelia was inhibited by gp120- specific or gp41-specific antibodies that prevented the HIV–galactosylceramide interaction, but not by CD4- specific antibodies (101),(102),(103). Clinical studies have sought to identify correlates of mucosal protection in humans (104). Although a few HIV-specific vaccines have been administered mucosally in human trials, so far none of these vaccines has resulted in measurable concentrations of HIV-specific secretory IgA (105).

10.3. Broad Neutralizing Antibodies: Understanding Targets, Host Control, and Maturation Pathways

Recently, the HIV-1 vaccine field has extensively embraced recombinant human antibody cloning for production of human BnAbs from chronically HIV-1-infected subjects (106),(107),(108). Improved recombinant antibody technology has combined with new methods for isolating HIV-1 Env-reactive memory B cells from antigen-specific B cell sorts (109),(110),(111), from plasma cell sorts (108),(112),(113), and from clonal memory B cell cultures (114),(115),(116). As a result, a large number of human BnAbs have been identified that target 1

of 4 major conserved areas in the HIV-1 envelope, including 1) the gp120 CD4 binding site (CD4bs) region (117),(118),(119), 2) the membrane proximal external region (MPER) of gp41 (111),(120), and 3) two new gp120 BnAb peptide-glycan epitopes, one in the Env gp120 V1V2 loop (115),(116),(121); and the other in the V3 Region (122),(123),(124). The latter BnAb group is especially potent, eliciting NHP protection from SHIV infection in passive immunoprophylaxis studies at plasma levels as low as 2 ug/ml (125). Nevertheless, a critical issue in HIV-1 vaccine development is that current vaccines do not induce BnAbs. They arise after many years of HIV-1 infection in only ~20% of subjects (126),(127),(128), and typically have more than one BnAb lineage in a given subject (129). Early observations of two first generation BnAbs (MPER antibodies 2F5 and 4E10) revealed long heavy chain (H) third complementarity determining regions (CDR3s) and autoreactivity with non-HIV-1 antigens (15). These findings led to the hypothesis that host tolerance mechanisms may prevent BnAb induction (15),(130). Using 2F5 MPER BnAb homologous recombinant mice, Verkoczy et al. (131),(132), demonstrated that indeed most mAb 2F5-bearing B cells are deleted in the bone marrow and a minor cell population (~5%) survive in the periphery as anergic B cells (132). Similar observations have been made with MPER BnAb 4E10 knock-in mice by Nemazee et al (133), and L. Verkoczy and B. Haynes (personal communication). Kelsoe et al have recently identified kynureninase (KYNU) and splicing factor 3b subunit 3 (SF3B3) as the primary high affinity autoantigens recognized by 2F5 and 4E10 BnAbs, respectively (134). Thus, unusual antibodies undergoing complex maturation pathways are required to achieve binding to the four conserved areas of HIV-1 Env to which BnAbs bind (117),(119). Similarly, mAb 4E10 and Cap206- CH12 both bind to the same MPER site and use VH1-69 and Vκ3-20 even though they were derived from two separate individuals (111),(135). Thus, despite the vast redundancy in the human B cell repertoire, for some of the BnAb sites, only a few VH and VL pairs will suffice. Moreover, these findings strongly suggest that one reason BnAbs are not readily made is that their unusual traits predispose their precursors to be limited in development, and even when allowed to develop, are in limited numbers such that the BnAb response is always subdominant to other non-neutralizing Env responses. The full BnAb maturation pathways are being unraveled by isolation of mature antibodies from more distal BnAb clonal lineages and inferring their intermediate ancestor antibodies and the unmutated common ancestor (UCA, the putative naive B cell receptor) using computational analysis and pyrosequencing (112),(136). One proposed reason that BnAbs are difficult to induce with immunization is that there are “holes” in the B cell germline repertoire and the BnAb UCA antibodies (naive B cell receptors) are not available to bind Envs to drive the BnAb lineages (137),(138), (139). However, Env constructs have been recently found to bind to VIV2 UCAs and gp41 MPER BnAbs (140). Sequential virus envelopes isolated by BnAbs from SHIV-infected rhesus macaques and used as immunogens have been proposed to recreate viral evolution pathways (141). A recent study has taken a new approach to Env immunogen design by mapping both BnAb and virus evolution from the time of transmission to development of plasma BnAb activity in the same HIV-1-infected subject (142). The transmitted/founder virus bound well to the BnAb lineage UCA, as well as to all descendants of the BnAb clonal lineage. Moreover, the trajectory of virus evolution was mapped and a series of Env mutations were identified that developed concomitant with antibody evolution, revealing the pattern of simultaneous Env-BnAb evolution (142). A strategy has been proposed to use such data to drive otherwise unfavorable subdominant BnAb lineages to be dominant responses, termed B cell lineage immunogen design (143), by administering sequential or swarms of immunogens designed to bind optimally to each stage of the BnAb maturation pathway and to selectively induce affinity maturation in BnAb and not other non-

BnAb lineages. Another proposed strategy is based on optimization of immunogens in the form of the native trimer (144).

11. HIV-1 Vaccine Efficacy Trials

Due to the uniqueness of each efficacy trial and outcome, each study can provide information regarding the minimum bar to overcome to achieve HIV-1 vaccine efficacy. Even when there is no overall vaccine efficacy, the heterogeneity of immune responses among vaccines has enabled follow-up studies to identify associations of immune responses with HIV-1 infection risk in subsets of vaccines. Furthermore, an integral part of understanding immune correlates of protection is to also define patterns of immune responses that associate with non-protective vaccines. One of the major goals for the HIV-1 vaccine field is to define correlates of protection from HIV-1 infection, such that the field has biomarkers that will clearly predict vaccine outcome in HIV-1 vaccine efficacy trials. There have been six HIV-1 vaccine efficacy studies to date (145),(146),(147),(148), each testing either a different vaccine strategy, or different populations with different risk factors and geographic locations. HIV-1 vaccine efficacy studies have been analyzed for correlates of infection risk; however, a correlate of protection for an HIV-1 vaccine has not yet been identified. Both the non-efficacious vaccines (149),(150),(151), and the one partially efficacious HIV-1 vaccine (152),(153),(154),(155), trial have yielded several correlates of infection risk; although more weight is given to those findings from trials where there was overall vaccine efficacy. In total, three of these studies (VAX004, Step, and RV144) found significant correlations with HIV-1 infection risk/incidence and two of these studies (Step and RV144) identified potential sites of immune pressure on the virus (virus sieve).

12. Cellular Immunity and HIV-1 Vaccine Efficacy Trials

HIV-1 specific CD8+ T cells are associated with control of HIV-1 replication as demonstrated in acute HIV-1 infection (21),(156),(157). (McMichael *et al*.(158), and in studies of those rare individuals who can naturally control HIV-1 infection long term (28),(159),(160). Moreover, studies in NHP have demonstrated, as proof of concept, that vaccine induced CD8+ T cells can be protective (161),(162),(163). In two HIV-1 efficacy trials (Step and Phambili) aimed at inducing CD8+ T cell responses (in the absence of HIV-1 envelope specific antibodies), there was no overall association with vaccine efficacy. However, these studies indicated that there were vaccine-elicited CD8+ T cells that could impact virus replication; although not sufficient enough to provide overall decreased risk of infection in the trial (either due to low magnitude/breadth/function of T cell responses and/or pre-existing vector immunity *etc*.). HVTN 505, also designed to target the induction of CD8+ T cell responses (as well as HIV-1 envelope specific antibodies), was discontinued and unblinded due to lack of efficacy. Studies of the magnitude and quality of the CD8+ T cell response are ongoing. Since there was evidence of increased infections in two of the Ad5 vector based vaccine studies, Step and Phambili (164), but not in HVTN 505 (148), there is a greater emphasis on understanding immune responses to vaccine vectors and impact on subsequent immunity (165). An alternate interpretation is that perhaps many other types of HIV-1 vaccines induce some level of CD4+ T cell activation; however, the key is to have sufficient overall HIV-1 specific immunity to tip the balance toward

protection. Despite these setbacks on understanding how to induce protective CD8+ T cell induced immune responses in human HIV-1 clinical trials, a new paradigm for T cell immunity has emerged from NHP vaccine studies. Picker and colleagues report on a CMV based vaccine regimen that protects NHPs from infection and induces a novel subset of CD8+ T cells that can broadly recognize HIV-1 epitopes through class II restriction (161). The series of papers by Picker and colleagues provide new hypotheses to test for inducing effective cellular immunity, both in terms of both new immunogen vector design and new subsets of CD8+ T cells to target with vaccine approaches.

13. Humoral Immunity and HIV-1 Vaccine Efficacy Trials

Four of the HIV-1 vaccine efficacy trials induce HIV-1 Env-specific antibody responses by three diverse strategies: recombinant HIV-1 gp120 immunogen alone (VAX003 and VAX004); vector prime with gp120 boost (RV144); and DNA prime, vector boost (HVTN 505). Although VAX003 and VAX004 efficacy trials each tested gp120 protein only as an immunogen strategy to induce humoral responses, these trials were distinct in that they were conducted in different risk populations (injection drug users (IDU) *vs*. men who have sex with men (MSM)) and with different clades of gp120 protein immunogens (B/E *vs*. B/B). Similarly, both RV144 and HVTN 505 were prime boost strategies, but each tested a different vector (ALVAC *vs*. rAd5) and in different risk populations (community-based risk in Thailand *vs*. MSM in the U.S.). Thus, there are four major differences across these HIV-1 vaccine efficacy trials that need to be considered when comparing studies: (1) differences in the HIV-1 gp120 sequence content; (2) differences in the approach (prime/boost and vector *vs*. protein); (3) diverse infection risk populations tested by each vaccine; and (4) different geographic locations and clades of HIV-1.

13.1. Antigenicity of Immunogens

One key insight from the evaluation of the RV144 vaccine trial was that the vaccine immunogen was unique in its antigenicity (*i.e.*, exposure of specific epitopes) compared to other envelope proteins (114),(166). Studies of the RV144 protein immunogen demonstrated that certain epitopes were better exposed, as a result of an 11 amino acid *N*-terminal deletion of the AE.A244 gp120 envelope, thus leading to the induction of dominant V1V2 antibody epitope specificities that were well exposed on the vaccine immunogen (166). In particular, the A244 gp120 used in RV144 was antigenic for both linear V2 epitopes bound by strain-specific V2 antibodies, as well as for conformational V1V2-glycan epitopes bound by V1V2 broad neutralizing antibodies (BnAbs) (114),(166). Thus, 8,000 individuals have been immunized with an antigen expressing a BnAb epitope, yet only non-glycan-dependent nAbs were induced (114),(167),(168). Thus, in addition to defining correlates of protection in RV144, a second critical reason to study vaccine responses in RV144 is to understand why BnAbs were not induced by an antigenic immunogen. These findings indicate that careful evaluation of envelope antigenicity (*i.e.*, determining what epitopes are exposed for immune recognition) can provide insights into the types of antibodies that may be elicited by vaccination.

13.2. Multiple Antibody Specificities and Function: Vaccine Efficacy

Due to the natural redundancy of the immune system, HIV-1 vaccines may induce multiple potentially protective immune responses. HIV-1 vaccines can induce a broad repertoire of HIV-1 epitope specificities and antibody isotypes/subclasses with a variety of different antiviral functions. Even in RV144, where V1/V2 Env IgG responses were identified as an immune correlate of risk of HIV-1, there was broad heterogeneity among vaccines in their immune responses with a diverse repertoire of antibody forms, specificities and functions (169). Some of these diverse immune measurements were not tested in the immune correlates analysis, due to inherent assay limitations, sample limitations and the requirement of minimizing test variables in order to maintain statistical power. The interaction models from RV144, as well as several RV144 follow-up studies that examine several antibody specificities and/or function (170), support the hypothesis that multiple antibody specificities with different functions may act in concert. Evaluating multiple antibody specificities and antiviral functions, in addition to identifying single correlates of infection risk, will be central to identifying mechanistic correlates of protection for HIV-1 toward the goal of identifying an efficacious vaccine strategy.

14. New Generation Vaccines: Implications for Safety

Immune Goals Drive Vaccine Design and Enlarge Potential for Risk the immune determinants of protection against HIV infection remain undefined. The unique ability of HIV to evade immune controls in natural disease and in experimental systems suggests that all avenues of immune containment should remain on the research agenda. Based on classical theory, three elements may be required to prevent infection: 1) neutralization of free virus would be more effective with a more vigorous, broadly strain-reactive, sustained antibody response; 2) destruction of infected cells requires induction of cytotoxic T lymphocytes that recognize multiple HIV epitopes; and 3) protection against sexual transmission of HIV requires an antibody and cellular response at genital and rectal mucosal surfaces (171). Each vaccine formulation or variation on a formulation is regarded as a new product by the FDA, and separate evaluations of each are required. New approaches may carry special risks, some unique to that system. of vaccine *in vitro* laboratory studies and in animal models can be poor predictors, particularly of infrequent or late events. The major types of experimental vaccines in development are addressed below, along with implications for their safety

14.1. Synthetic Peptides

Specific B and T lymphocyte epitopes selected to stimulate antibody and cytotoxic T lymphocytes may be combined. Vaccines directed at multiple epitopes (multivalent vaccines) have been prepared containing subtypes of HIV that are endemic in diverse regions of the globe. Immune responses have been improved by arranging peptides into complex structural forms, as well as by adding new adjuvants or carrier molecules. Peptide-based vaccines have induced cytotoxic T lymphocyte responses in the SIV/macaque model.

14.2. Live Vectors Carrying Genes Coding for Immunizing Antigens

A *vector* is a living virus or bacterium used as a carrier to express one or more "foreign" genes encoding desired antigens. Vectors under study include canarypox virus (a relative of vaccinia virus), adenovirus (a cause of respiratory disease), BCG (an attenuated bovine tuberculosis organism), Salmonella or Shigella (typhoid-like bacteria), and attenuated poliovirus. Canarypox can be altered to express HIV antigens, but canarypox does not itself multiply in the human (172),(173),(174),(175),(176),(177). Live vectors have important advantages in inducing protective responses. First, protein antigen synthesized in a vector can induce cytotoxic T lymphocyte responses not expected with antigen administered as inert protein. Second, vectors carrying multiple *env*, *gag*, and *pol* genes but not RNA or other sequences essential for viral replication can assemble into a viral configuration, or *pseudovirion (178).* The nonreplicating structure of the pseudovirion is designed to duplicate advantages of a whole inactivated vaccine but eliminate its risks. Vaccines using *virus-like particles* (VLP) have also been produced without use of live vectors (179). Third, vectors that grow on body surfaces, such as adenovirus or Salmonella, can induce HIV local mucosal immune responses. The vector must be: 1) stably attenuated and unable to produce the natural human disease caused by the vector, 2) safe from unwanted spread to contacts and community at large, and 3) safe for individuals with impaired immunity. The safety problems that have occurred in licensed smallpox (vaccinia virus) vaccines allow us to predict potential safety problems with vaccines using live vaccinia virus vectors. These may include severe skin and mucous membrane infections, invasive and neurological diseases, and even death in susceptible immunosuppressed individuals (75).

14.3. Infectious DNA

The development of vaccines composed of pure viral genetic material, infectious or "naked" DNA, is a novel departure from traditional vaccines. Viral DNA coding for a single or multiple genes, injected directly into the muscle or skin, provides the genetic code for synthesizing new protein, which in turn behaves as a potent antigen. Persistent antibody and cytotoxic T lymphocyte responses have been induced in laboratory animals (180),(181). Mechanisms leading to the potent immune responses are not understood.

14.4. Inactivated Whole Virus Vaccine

Development of inactivated as well as live attenuated HIV vaccines, using classical approaches, were seriously considered in early deliberations.

15. Stage of development Vaccine design

Phase I/II Trials Envelope proteins (gp160, gp120) Vaccinia vector/gp160 Currently entering Synthetic peptides trials Live vectors/multiple proteins Virus-like particles Pseudovirions Immune modulators/delivery systems Preclinical research Infectious DNA Inactivated whole virus Live attenuated virus SOURCE Off Ice of Technology Assessment, 1995 Preparation of a safe inactivated whole-virus vaccine, exemplified by the Salk-type of inactivated polio vaccine, requires inactivation of a high-titered preparation of live virus using gentle physical- chemical means to preserve full immunogenicity, yet ensuring inactivation of all live viruses. The process must guarantee absence of even a single infectious dose in large volumes (hundreds of thousands of patient units) of vaccine. The safety problem was resolved by simple refinements

in the inactivation process. There has also been theoretical concern regarding residual reactive viral DNA in the product. In addition, the safety of the "lymphoblastoid" cell lines used to prepare the virus is unknown. "Adventitious agents," that is, unwanted agents growing silently in the cell cultures used to prepare vaccine stock, have posed safety problems in the past (182). The sfety of an inactivated whole-virus vaccine for HIV was reviewed at a workshop in 1990. It was the consensus that a safe product is technically feasible but that product development should proceed with caution (183).

16. New Approaches to Improve Vaccine Performance

16.1. Mucosal Immunity

No vaccine has yet provided an immune barrier at the mucosal membranes of the rectum, vagina, and urethra—the sites of sexual transmission of HIV (184),(185, 186). The mucosal administration of vaccine vectors that grow on mucosal surfaces may provide a critical tool for the prevention of HIV transmission by sexual routes. Antigen uptake from mucosal surfaces is poor compared with injection. New strategies to improve the uptake of antigens from mucosal surfaces involve use of biodegradable microspheres, cholera toxin B, liposomes (phospholipid droplets), and immunostimulating complexes (iscoms) to enhance passage of antigen through cell membranes for more efficient processing (187).

16.2. New Adjuvants and Delivery Vehicles

Adjuvants are nonviral materials incorporated into vaccine formulations to augment the magnitude or spectrum of immune responses to vaccines (188). Adjuvants have been discovered largely empirically, and are commonly derivatives of bacteria or plants. The introduction of new adjuvants into clinical practice has been slowed by concerns about the adjuvant's toxicity. Significant transient toxicity was shown in comparative trials of experimental adjuvants. Exploration of adjuvants is currently undergoing a renaissance in an effort to selectively enhance HIV antibody, cytotoxic T lymphocyte, or mucosal immune responses (189),(187),(190). The microsphere particle size and polymer composition can be altered to target a single dose of antigen to specific tissue sites such as mucous membranes, and to release the antigen in pulses, obviating the need for a multiple dose vaccination schedule.

16.3. Cytokines

Cytokines comprise a family of soluble substances (e.g., 1L-2, 1L-4, interferons, etc.) that mediate functions of immune cells. Cytokines can play a significant role in providing protective immune responses following vaccination (191). Any of the above approaches to improve vaccine performance may have unexpected side effects. So far, several new adjuvants have caused early transient difficulties and have been withdrawn from use.

17. Conclusion and Remarks

New technologies and approaches have allowed the field to cast a broader net in evaluating vaccine strategies. New approaches towards understanding systems biology contributes to a better understanding of the interplay between the vaccine-induced host immune response and

virus. Analysis of host genetics, viral genetics and immune responses (192),(193), have already provided numerous insights into both innate and adaptive responses of virus control and disease progression in HIV-1 infected individuals. A highly effective HIV-1 vaccine will likely need to harness T cell and B cell immunity to protect against both virions and virus-infected cells. Progress has been made in using replicating vectors for induction of T cells that can exert early control of virus replication in non-human primates and in defining the immune correlates of infection risk in the ALVAC/ AIDSVAX B/ER vaccine efficacy trial. Novel antigen concepts, such as centralized consensus (194),(195), and mosaic (196), immunogens, may also result in increased breadth of cellular immune responses and improved coverage of viral diversity. As a result, development of improved T cell based and antibody based vaccine strategies should be pursued in parallel. Available data indicate that mucosal HIV vaccines should be particulate or live vectored, include components that alert the innate immune system, and include immunogenic, conserved forms of the envelope protein gp41 as well as gp120. Mucosal HIV vaccines would ideally be administered as part of a prime–boost strategy that induces both mucosal and systemic immunity. Genetic, hormonal and inflammatory conditions further increase the individual variations complicating all types of prophylactic trials. Moreover, new data implies that the mucosal milieu contributes to the differentiation of effector-like cells and that this differs from responses previously studied in blood. Epithelial cells lining the mucosal tissue can also be primed by microbial contact and stimulate and direct both the innate and adaptive immunity in a significant manner. The extraordinary diversity of HIV-1, the capacity of the virus to evade adaptive immune responses, the inability to induce broadly reactive antibody responses, the early establishment of latent viral reservoirs and the lack of clear immune correlates of protection represent unprecedented challenges for vaccine development. The major advantage of preventing the virus from replicating is the decreased chance of generating viral escape mutants capable of avoiding the treatment (51). Since each replication cycle increases the chance both of generating an escape mutant and of developing latent reservoirs, viral replication must be prevented long enough for the host's body to purge the infection. Down regulating the CCR5 gene could result in inactive CCR5 proteins, preventing HIV-1 from infecting host cells and reproducing (51),(49), Despite these concerns, down regulating the CCR5 gene remains the most plausible solution to the issue of HIV-1 vaccination. However, this therapy must be combined with additional measures in order to prevent intense selection of CXCR4-tropic strains; the emergence of which would negate the effectiveness of the treatment (51).

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