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Nonneutralizing Functional Antibodies: a New "Old" Paradigm for HIV Vaccines

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Animal and human data from various viral infections and vaccine studies suggest that nonneutralizing antibodies (nNAb) without neutralizing activity *in vitro* may play an important role in protection against viral infection *in vivo*. This was illustrated by the recent human immunodeficiency virus (HIV) RV144 vaccine efficacy trial, which demonstrated that HIV-specific IgG-mediated nNAb directed against the V2 loop of HIV type 1 envelope (Env) were inversely correlated with risk for HIV acquisition, while Env-specific plasma IgA-mediated antibodies were directly correlated with risk. However, tier 1 NAb in the subset of responders with a low level of plasma Env-specific IgA correlated with decreased risk. Nonhuman primate simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus (SHIV) challenge studies suggest that Env-mediated antibodies are essential and sufficient for protection. A comparison of immune responses generated in human efficacy trials reveals subtle differences in the fine specificities of the antibody responses, in particular in HIV-specific IgG subclasses. The underlying mechanisms that may have contributed to protection against HIV acquisition in humans, although not fully understood, are possibly mediated by antibody-dependent cell-mediated cytotoxicity (ADCC) and/or other nonneutralizing humoral effector functions, such as antibody-mediated phagocytosis. The presence of such functional nNAb in mucosal tissues and cervico-vaginal and rectal secretions challenges the paradigm that NAb are the predominant immune response conferring protection, although this does not negate the desirability of evoking neutralizing antibodies through vaccination. Instead, NAb and nNAb should be looked upon as complementary or synergistic humoral effector functions. Several HIV vaccine clinical trials to study these antibody responses in various prime-boost modalities in the systemic and mucosal compartments are ongoing. The induction of high-frequency HIV-specific functional nNAb at high titers may represent an attractive hypothesis-testing strategy in future HIV vaccine efficacy trials.

The identification of immune markers that correlate with protection against infection or disease after vaccination (or natural infection) remains the "holy grail" for vaccine scientists, as these immune markers have the potential to expedite vaccine development and provide unique insights into the pathogenesis of infection. For most of the vaccines available today, a functional antibody response is the identified correlate of protection (1). In the cases of candidate preventive human immunodeficiency virus (HIV) vaccines and experimental vaccines against the closely related retrovirus simian immunodeficiency virus (SIV), recent studies have suggested an antibody correlate of risk or protection. However, these studies suggested an association with antibodies that did not mediate broad or potent neutralization (2–5).

Transmission of HIV type 1 (HIV-1) occurs through homosexual or heterosexual intercourse, through injection of blood or blood-derived products, and from mother to child during pregnancy, at delivery, or through breastfeeding. Although several nonexclusive mechanisms may contribute to protection from HIV-1 acquisition (6), given the confounding variables of risk, route, and intensity of exposure, thinking about the HIV-1 immune correlates of protection has been dominated by the paradigm that the induction of neutralizing antibodies (NAb), in particular broadly neutralizing antibodies (bNAb) covering 50 to 90% of transmitted viruses, should be the goal of vaccine development efforts (7, 8). With regard to HIV-1, the Thai RV144 trial showed that vaccine-induced IgG antibody against the first and second variable domains (V1V2) of the surface glycoprotein envelope (Env) was associated with lower risk of HIV infection but that IgA against Env was directly associated with risk of infection (2, 9, 10). While these V1V2 antibodies are not broadly neutralizing, they do appear to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and limited, tier 1 NAb (11–14), and in the setting of a low level of Env-specific IgA, they are inversely correlated with the risk of infection. In addition, several groups have described correlates of protection against the acquisition of SIV (4, 5, 15, 16) exclusive of bNAb. These findings prompted this review of the potential functions of vaccine-induced antibody responses, aside from *in vitro* neutralization, that may contribute to protection against HIV-1 acquisition (17).

We review the vaccine-induced immune mechanisms of protection that are antibody mediated for viral diseases, along with the latest findings of the RV144 correlate-of-risk analysis suggesting that nonneutralizing antibodies (nNAb) can be functional and indeed neutralize pathogens *in vivo*, including HIV-1.

LESSONS LEARNED FROM NON-HIV VACCINES

Neutralization of viruses has long been considered the main mechanism of protection, and this may well be true, but it also must be admitted that neutralization is usually measured by prevention of infection of cultured cells (18). Thus, these *in vitro* measurements may or may not relate to the protection afforded by prior infection or vaccination.

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A recent and comprehensive review on nNAb defines nNAb as functional antibodies with low or no neutralization activity *in vitro* (19). Functional nNAb have long been recognized as contributing to protection against non-HIV infections, such as, for example, alphaviruses (20–22), flaviviruses (23–27), influenza virus (28–32), respiratory syncytial virus (33, 34), vesicular stomatitis virus (35), Ebola virus (36, 37), mouse hepatitis virus (38), cytomegalovirus (39–41), lymphocytic choriomeningitis virus (42), and vaccinia virus (43). Vaccines against feline leukemia virus protect by a mechanism that is not neutralization and may be either nonneutralizing antibody or cytotoxic T lymphocytes (44). nNAb function in multiple different ways, including ADCC, complement-mediated cytotoxicity, steric inhibition of proteins important in viral replication, and antibody-dependent cell-mediated phagocytosis (ADCP) (19, 45, 46). Plasma samples obtained from convalescent H1N1-infected human subjects showed high ADCC activity against pandemic H1N1 influenza virus. E1 and E2 ADCC epitopes overlapped immunodominant epitopes of hemagglutinin (47). Although high titers and the avidity of nNAb for influenza vaccine antigen have also been associated with severe influenza (48), nNAb induced by influenza vaccines that fail to prevent influenza infection may nevertheless have collateral benefit, protecting against secondary bacterial diseases like *Streptococcus pneumoniae* and *Klebsiella pneumoniae* when a neutralizing-antibody-inducing vaccine is not available (49). Moreover, nNAb act together with $CD8^+$ T cells to confer heterosubtypic immunity (31). Although nNAb may not prevent infection, they may modify disease. nNAb to Sindbis virus in mice lyse virus-infected cells and prevent encephalitis (20). nNAb also can reduce the neurovirulence of flavivirus infection (23) and play an alternative role either in protection or in enhancement of disease (26, 27). Although protection against rotavirus is usually mediated by neutralizing antibodies directed against the vp4 or vp7 attachment protein, nonneutralizing IgA antibodies directed against the vp6 capsid protein also protect by inducing a conformational change in the trimer (50). In the case of lymphocytic choriomeningitis virus, chronic infection can be prevented in mice if nonneutralizing, binding antibody that interacts with T cells is present at the time of challenge (40, 42). The ubiquity of these phenomena argue that functional nNAb are important in protection against viral infection without negating in any way the desirability of evoking neutralizing antibodies through vaccination (51).

NONNEUTRALIZING ANTIBODIES AND CANCER THERAPY

HIV and cancer share interesting similarities; in both, it is presumed that in order to be effective, all affected cells (productively infected and neoplastic, respectively) must be eliminated. Like cancer cells, HIV has evolved mechanisms to evade or suborn immune surveillance. Monoclonal antibody (MAb) treatment of solid-tumor malignancies is associated with the development of resistance due to a broad array of potential genetic alterations (52). In cancer, numerous proteins are expressed at the surfaces of tumor cells, representing potential targets for therapeutic and, by definition, nonneutralizing MAb. The killing of tumor cells is accomplished via several mechanisms, including direct action of the antibody through receptor blockade or agonist activity, induction of apoptosis, delivery of a drug or cytotoxic agent, complement-dependent cytotoxicity, ADCC, regulation of T-cell function, and specific effects of an antibody on tumor vasculature and stroma (reviewed in references 53 and 54). Several MAb, such as rituximab, an anti-CD20 antibody with good activity in patients with chronic lymphocytic leukemia (CLL), have now been licensed for clinical use. During clinical CLL, there are millions of transformed cells per milliliter of blood. In contrast, the target of an HIV-specific nNAb are the few cells infected (with a single transmitted/founder virus [9]) during inchoate HIV infection.

HIV-1 VACCINE EFFICACY TRIALS USING RECOMBINANT ENVELOPE PROTEINS

Data from three studies of nonhuman primate (NHPs) (4, 5, 55) have suggested that Env is a necessary component for successful protection from SIV acquisition. The failure of the Merck recombinant adenovirus type 5 (rAd5) Gag-Pol-Nef vaccine seems to support this finding (56, 57). Monomeric gp120 HIV-1 envelope proteins alone failed to protect high-risk volunteers in two efficacy trials, Vax003 (58) and Vax004 (59–61), while the RV144 prime-boost regimen using a recombinant canarypox vector prime and the same alum-adjuvanted envelope protein boost used in Vax003 conferred modest protection against HIV acquisition (62). Another efficacy trial (HVTN 505) tested a regimen with a DNA vaccine prime composed of DNA plasmids encoding Gag, Pol, and Nef from HIV-1 subtype B and Env from subtypes A, B, and C and a replication-defective-rAd5–HIV-1 vaccine boost containing a mixture of four rAd5 vectors encoding the HIV-1 subtype B Gag-Pol and Env matching the DNA Env components. The phase IIB trial was recently stopped for futility, showing no efficacy and no statistically significant effect on viral load and a nonsignificant excess of HIV infection in the vaccinated group (63). These findings allowed for a comparison of the vaccine-induced immune antibody responses between these three trials, which may shed some light on our still-limited understanding of the potential mechanisms of protection.

Vax003 AND Vax004 MONOMERIC HIV-1 ENVELOPE PROTEIN EFFICACY TRIALS

Vax004 tested AIDSVAX B/B, a bivalent recombinant HIV-1 subtype B (GNE8 and MN) gp120 envelope glycoprotein subunit vaccine in U.S. men who have sex with men (MSM) and women at high risk for heterosexual transmission of HIV-1 (59). The vaccine did not prevent HIV-1 acquisition, nor did it affect HIV-1 disease progression (61). High NAb levels against the easy-to-neutralize MN strain were, however, significantly inversely correlated with HIV-1 incidence, while low levels against more-difficult-to-neutralize viruses did not, suggesting that the level and breadth of elicited NAb were not sufficient for protection (60). Interestingly, the level of vaccine-induced antibody-dependent cellular virus inhibition (ADCVI) activity correlated inversely with the rate of acquiring HIV-1 infection following vaccination. Moreover, ADCVI activity correlated poorly with NAb or CD4-gp120-blocking antibody activity measured against laboratory strains and was modulated by Fc receptor (FcR) polymorphisms (64).

Vax003 tested AIDSVAX B/E, a bivalent recombinant HIV-1 subtype B/E (A244 CRF01_AE and MN subtype B) gp120 envelope glycoprotein subunit vaccine in injection drug users in Bangkok, Thailand. The vaccine did not prevent HIV-1 acquisition, nor did it affect HIV-1 disease progression. Prior to infection, the levels of (i) antibodies to gp120, A244 V2, and A244 V3, (ii) blocking of A244 binding to CD4, and (iii) MN neutralization were not significantly different between the HIV-infected and uninfected

vaccine recipients and did not correlate with the rate of HIV infection (58).

RV144 PRIME-BOOST EFFICACY TRIAL

Vaccine efficacy. RV144, consisting of an ALVAC HIV vaccine (vCP1521) prime and an AIDSVAX gp120 B/E boost, provided the first evidence that an HIV-1 vaccine can provide protective efficacy against HIV-1 acquisition. A modified intent-to-treat analysis showed estimated efficacies of 31.2% after 42 months (62, 65), 60% at 12 months, and 44% at 18 months after the first vaccination, suggesting an early, but nondurable, vaccine effect (66). Vaccination did not affect the clinical course of HIV-1 disease after infection, though there was evidence of reduction in seminal fluid viral load (67).

Vaccine-induced immune responses. Gamma interferon (IFN-γ) enzyme-linked immunosorbent spot assay (ELISPOT)-positive responses were detected in 41% of the vaccinees and were predominantly $CD4^+$ T-cell mediated. Responses were targeted within the HIV-1 Env region, with 60% of vaccinees recognizing peptides derived from the Env V2 region, which includes the α4β7 integrin binding site (68, 69). Intracellular cytokine staining confirmed that Env responses predominated and were mediated by polyfunctional effector memory $CD4^+$ T cells. All Env-specific $CD4^+$ T-cell lines displayed a cytolytic activity despite the absence of $CD8^+$ T cells (70).

Binding antibody against the MN and A244 Env vaccine antigens was nearly uniformly present but dropped 15-fold after 6 months; p24 responses were less frequent. Antibody-dependent cell-mediated cytotoxicity (ADCC) in vaccine recipients and MAb from vaccine recipients mediating ADCC were described previously (13, 71). Whereas NAb to tier 1 viruses were detected in both RV144 and Vax003, the tier 1 NAb titers were higher after the RV144 regimen (ALVAC HIV vaccine prime and two-protein boosts) than after two gp120 protein administrations alone, confirming a priming effect for the ALVAC HIV vaccine (11). Further analysis suggested that the lack of a clade-specific HIV-1 NAb response was associated with the presence of certain HLA class II alleles and heterodimers in Southeast Asians (72).

Correlates of protection. The RV144 trial provided a unique opportunity to perform a case-control study of correlates of risk for HIV acquisition. Plasma IgG binding antibody to scaffolded gp70 V1V2 CaseA2 envelope protein (HIV-1 subtype B) correlated inversely with risk, while Env plasma IgA correlated directly with risk, raising the hypotheses that IgA responses directed against Env and IgG responses directed against V1V2 may be mechanistically associated with protection but in opposite directions. Neither low levels of V1V2 antibodies nor high levels of Env-specific IgA antibodies were associated with higher rates of infection than in the placebo group. In vaccinees with low levels of Env-specific IgA antibodies, levels of IgG avidity, ADCC, NAb, and Env-specific $CD4^+$ T cells were inversely correlated with risk of infection (2, 3, 73). A subsequent analysis showed that RV144 antibodies to subtype A, C, and CRF01_AE gp70 V1V2 scaffolded proteins also correlated inversely with risk (74), suggesting that the RV144 regimen might protect against heterosexual transmission of HIV strains heterologous (A and C) to the vaccine components. Two weeks after the last vaccination, 97% of RV144-studied plasma samples from vaccine recipients contained antibodies to V2 region synthetic peptides, falling to 19% at 48 weeks, suggesting that waning vaccine efficacy may be correlated to waning V2 antibody response.

Interestingly, gp70 V1V2 antibodies were lower in HVTN 505 than in RV144 (75). The response to V3 CRF01_AE also inversely correlated with the risk of HIV infection in vaccine recipients with lower levels of Env-specific plasma IgA and neutralizing antibodies. In Vax003 and Vax004 (no protection), serum IgG responses targeted the same epitopes as in RV144, with the exception of an additional C1 reactivity in Vax003 and infrequent V2 reactivity in Vax004. These results along with a recent sieve analysis (76) generate the hypothesis that IgG to linear epitopes in the V2 and V3 regions of gp120 are part of a complex interplay of immune responses that contributed to protection in RV144 (77).

Approximately 90% of the incident infections in RV144 were infections with CRF01_AE, the predominant circulating strain in much of Southeast Asia (78, 79). A sieve analysis identified two vaccine-associated genetic signatures in V2 corresponding to sites 169 and 181, further supporting the hypothesis that vaccination-induced immune responses directed against the V2 loop were associated with protection (9). Monoclonal antibodies from RV144 vaccine recipients contact the V2 K169 residue, providing additional evidence that vaccine-induced antibodies correspond to the observed sieve effect. These V2-specific antibodies can mediate ADCC, neutralization, and low-level virus capture (12, 80). These findings generate the hypothesis that V2 IgG plays a role in protection against HIV-1 acquisition but do not distinguish between a mechanistic or nonmechanistic means of protection (81).

In sera from HIV-1 CRF01_AE-infected blood donors, nine ADCC epitopes have been localized to the C1 and V2 region of gp120 (82). Sequences in gp70 V1V2 antigens other than V2, such as C1 and V1, may significantly contribute to the binding responses. It is suggested that nNAb mediating ADCC that can block HIV infection are directed against viral entry epitopes, including the CD4-inducible A32 epitope of a highly conserved region of gp120 at the gp120-gp41 interface (83), and there is evidence of cooperativity between the anti-C1 and anti-V2 activities, with increased anti-V1V2 binding in the presence of vaccine-induced anti-C1 antibody (84). This epitope was a target of ADCC-mediating MAb isolated from RV144 vaccinees (13). Some light has recently been shed on the role of plasma IgA in RV144. In the presence of low anti-Env IgA, both ADCC and NAb responses correlated with decreased risk of infection. ADCC responses were predominantly directed to the C1 conformational region of gp120 (13, 85, 86). These ADCC antibodies mediated cross-clade target cell killing. ADCC-mediating antibodies exhibited low levels (0.5% to 1.5%) of somatic mutations of the V heavy (VH) chain and disproportionately utilized VH1 family genes (74%) (13), similar to what occurred with CD4 binding site bNAb; however, these antibodies clearly differed from bNAb in both degree of somatic hypermutation and neutralization (87, 88). IgA antibodies elicited by RV144 block C1 region-specific IgG-mediated ADCC (10). Whether V2 antibodies might block the gp120-α4β7 interaction and contribute at least partially to the protective effect against HIV-1 sexual transmission remains to be demonstrated (89–91). In future trials, assessing IgG and IgA to V1V2 binding antibody immune responses in the mucosal compartments will be key.

In previous clinical studies, monomeric gp120 induced high levels of Env-specific IgG4 antibodies (92), while an ALVAC (vCP1452) prime and an MN gp120 in alum boost elicited lower

levels of IgG4 than of IgG1 and IgG3 antibodies (93). Antigen-specific IgG3 antibodies are associated with long-term control of *Plasmodium falciparum* (94) and monocyte-mediated cellular inhibition of parasite growth *in vitro* (95). Similarly, an early appearance of chikungunya virus-specific neutralizing IgG3 antibodies is associated with clearance of the virus and long-term clinical protection (96). Conversely, an IgG4 response has been associated with progression to AIDS (97). IgG3 can fix the complement and has a high affinity for FcγR (98). In RV144, an Env IgG3 response was correlated with decreased risk of HIV infection, a response that declined rapidly compared to overall IgG responses (99). A recent comparison of RV144 and Vax003 showed that Env-specific IgG3 and V1/V2 IgG3 response rates were higher in RV144 vaccinees than in Vax003 vaccinees and, conversely, that IgG4 responses were considerably lower in RV144. V1/V2 IgG3 responses and IgG3 responses specific for V1/V2 169K correlated with decreased risk of HIV-1 infection after IgA adjustment (100). Chung et al. recently showed that the RV144 regimen elicited highly coordinated Fc-mediated effector responses, with the selective induction of highly functional IgG3 antibodies. In contrast, Vax003 elicited monofunctional antibody responses influenced by IgG4 selection. Moreover, only RV144 induced IgG1 and IgG3 antibodies targeting the crown of the HIV envelope V2 loop, although with low coverage of breakthrough viral sequences (101). It is speculated that ALVAC priming, due to its unique proinflammatory cytokine and chemokine response following vaccination in rhesus monkeys and infection in human peripheral blood mononuclear cells (PBMC), may shift the IgG subclass response to IgG3 in humans after vector priming and envelope protein boosting compared with envelope vaccination alone (102). The contribution of Fc-FcγR interaction-mediated functions through mechanisms such as ADCC, antibody-dependent cell-mediated viral inhibition (ADCVI), and ADCP antibodies remains to be explored (103, 104). A recent *post hoc* analysis of RV144 showed an association between the FcγRIIC polymorphism, vaccine efficacy, and correlates of risk, emphasizing the potential role of FcR genetics in predicting vaccine efficacy (105).

NONHUMAN PRIMATE CHALLENGE STUDIES

Several nonhuman primate (NHP) challenge studies support the RV144 findings. Vaccination with ALVAC SIV alone conferred protection from infection in neonate macaques exposed to repeated oral low-dose challenge. However, neither binding antibody, NAb, nor cell-mediated immune responses correlated with protection (106). Recently, an immunization regimen recapitulating the RV144 regimen protected against mucosal challenge of SIV_{mac251} acquisition in 30% of the vaccinated animals. Protected animals had high-avidity antibodies to gp120 that recognized the V2 variable envelope region and reduced SIV_{mac251} infectivity in cells expressing a high level of α4β7, suggesting a functional role for V2 antibodies (16). In another experiment where macaques were immunized with ALVAC SIV and SIV gp120 formulated either in alum or MF59, only the alum group showed a significantly reduced rate of SIV acquisition compared to that of unvaccinated controls. The frequency of plasmablasts expressing α4β7 and CXCR4 (hematopoietic homing marker) was higher in the alum group, while there was a trend for a higher frequency of plasmablasts expressing CXCR3 (inflammatory site homing marker) in the MF59 group (107). The significance of these findings remains unclear.

An Ad26 prime and modified vaccinia virus Ankara (MVA) boost regimen using vaccines expressing *gag-pol* and *env* from SIV_{sme543} conferred 80 to 83% reduction in the per-exposure probability of infection against repeated low-dose intrarectal inoculations of heterologous, neutralization-resistant SIV_{mac251}. Protection against SIV acquisition correlated with Env- and V2-specific binding and tier 1-strain-neutralizing antibodies. Monkeys vaccinated with an Ad35/Ad26 prime-boost regimen expressing either Gag-Pol and Env or only Gag-Pol showed protection only when Env was present, suggesting that Env-specific responses are critical in preventing virus acquisition. Immunological correlates of protection were consistent with the prior experiment (4). A recent NHP intrarectal SIV_{smE660} challenge study in which animals were vaccinated with a DNA prime and an Ad5 boost expressing either mosaic Gag, mosaic heterologous Env, or heterologous Env based on a natural SIV_{mac239} sequence confirmed that Env-elicited immune responses are necessary and sufficient to provide protection from acquisition (69%). Plasma IgG binding to gp120 Env at the time of challenge did not correlate significantly with time to infection, but excluding animals who were infected with neutralization-resistant viruses resulted in a strong correlation of protection with IgG binding to gp120. Correlation with a delayed time to infection was also observed for plasma antibody avidity, CD4 binding site activity, and neutralization of some viral strains. Antibody to SIV V1V2 predicted protection against infection. However, the sieve analysis showed that selection against V1V2 sequences by the SIV vaccines was limited to neutralization-sensitive viruses, suggesting that selection is vaccine sequence specific and not broad. As in the human RV144 trial, there was no association between protection from infection and protection from pathogenesis, suggesting that humoral responses that effectively block acquisition are not necessarily correlated with cellular responses that control pathogenesis. The authors suggest that the primary mechanism of protection is by lowering the effective infectious dose, that is, *in vivo* neutralization (55), and that the moderate RV144 efficacy was due to antibodies that could neutralize some viruses circulating in that cohort; these sensitive viruses were susceptible to vaccine-matched V1V2, leading to sieving being an alternative hypothesis with regard to the possible role of nNAb in protection against HIV acquisition in RV144.

An emerging understanding of the early events in mucosal SIV, simian-human immunodeficiency virus (SHIV), and HIV-1 infections was recently presented (108), justifying the need to develop vaccines inducing mucosal immune responses. A relatively small number of immune effectors at the mucosal site of entry might be at the right place at the right time to be "enough and soon enough" to clear infection. Interestingly, human dimeric IgA1 MAb-treated rhesus macaques remained free of virus after intrarectal SHIV challenge, while treatment with dimeric IgA2 was much less effective. Protection was correlated with virus capture and inhibition of transcytosis of cell-free virus (109). Another approach using gp41 protein and derived peptide administered by mixed intramuscular and intranasal modalities was capable of protecting immunized monkeys against SHIV challenge (110) and of eliciting systemic and mucosal antibodies that inhibited HIV transcytosis in the absence of neutralizing antibodies in humans (111).

Recently, it was demonstrated that Ad26-MVA and Ad26-Ad35 vector-based vaccines expressing HIV-1 mosaic Env, Gag, and Pol

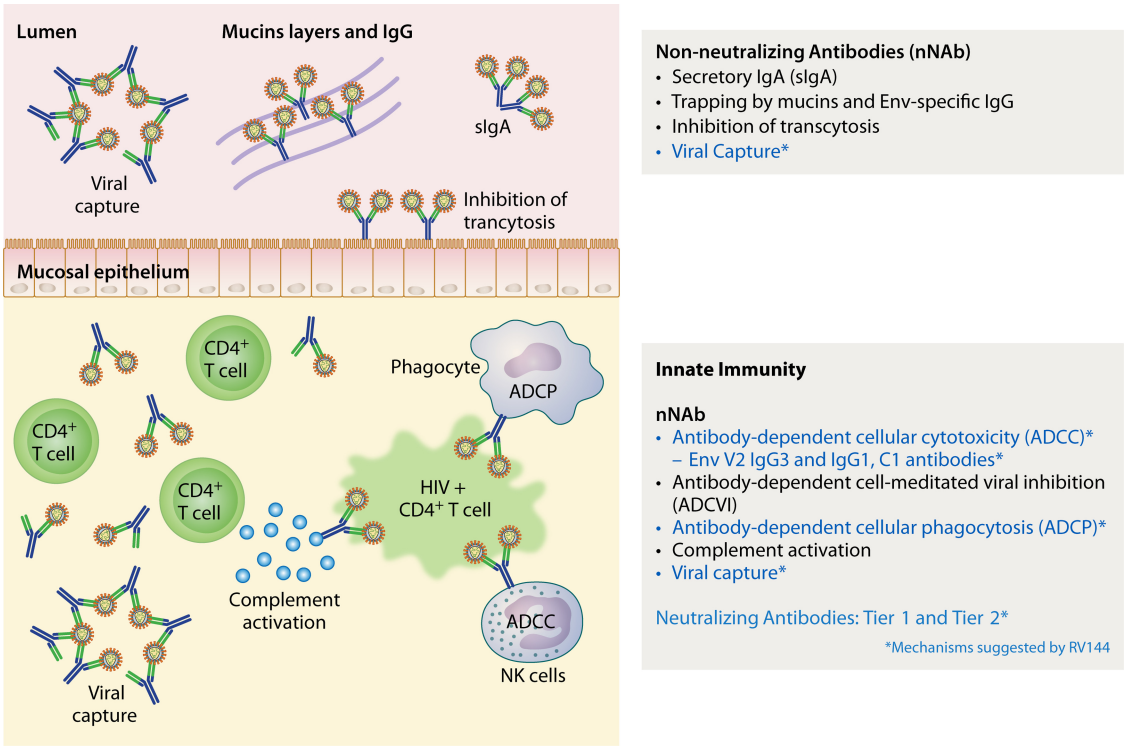

FIG 1 Possible vaccine-induced immune mechanisms of protection against HIV-1 acquisition in humans. To date, viral capture has been demonstrated in plasma but not in mucosal secretions, as mucosal secretions were not collected in RV144. Whether this potential mechanism occurs in mucosal secretions remains to be demonstrated in ongoing clinical trials where mucosal secretions are collected.

delivered in a prime-boost regimen resulted in a significant reduction in the per-exposure acquisition risk following repetitive, intrarectal SHIV-SF162P3 challenge, a difficult-to-neutralize virus. Protection was most strongly correlated with enzyme-linked immunosorbent assay (ELISA) binding antibody titers against the homologous Mos1 Env immunogen and to a lesser extent with ELISA binding antibody titers against other Env immunogens. In addition, protection was significantly correlated with NAb titers against SF162, which is a neutralization-sensitive virus related to the challenge virus SHIV-SF162P3. NAb titers against other tier 1 viruses also showed trends toward correlation with protection. Interestingly, protection was also correlated with a functional nNAb phagocytic score (ADCP), and a trend was observed with antibody-dependent complement deposition (ADCD) involving C3b complement. However, no significant correlation of protection with surface plasmon resonance binding to cyclic V2 peptides or with gp70-V1V2 scaffolds nor any measure of $CD8^+$ T lymphocyte responses was detected. These key findings suggest that multiple antibody functions may contribute to protection against acquisition of difficult-to-neutralize viruses (5).

A panel of bNAb and nNAb screened for their high ability to block HIV acquisition and replication *in vitro* in an independent or FcγR-dependent manner were formulated for topical vaginal application in a microbicide gel and tested for their antiviral activity against SHIV-SF162P3 vaginal challenge in nonhuman primates. While a combination of 2G12, 2F5, and 4E10 fully prevented vaginal transmission in 10 of 15 treated monkeys, a combination of 246-D and 4B3 nNAb monoclonal antibodies (112) had no impact on HIV acquisition but reduced plasma viral load. The results of this challenge study did not recapitulate the *in vitro* findings with 246-D and 4B3, which recognize the principal immuno-dominant (PID) domain of gp41, a relatively conserved epitope among HIV-1 subtypes and easily accessible on virions (113), and can efficiently capture a broad range of HIV strains (114). A similar observation was made with another anti-PID antibody, F240, whose vaginal application was able to protect two macaques against neutralization-sensitive SHIV-SF162P4 and decreased viral load in two of the five animals (115). These results indicate that antibodies with distinct neutralization and inhibitory functions differentially affect *in vivo* HIV acquisition and replication by interfering with early viral replication and dissemination (116).

Two V2-specific MAb, CH58 and CH59, were isolated from B cells of RV144 vaccine recipients (12). The epitope mapping suggested that CH58 and CH59, although not bNAb, bind at or near the site in V2 to which the HIV-1 V1V2 bNAb (e.g., PG9 and CH01) bind (117, 118). These MAb inhibit the interaction of V2 peptide with $\alpha 4\beta 7$ and capture infectious CRF01_AE 92TH023 virions (12). The availability of pathogenic SHIV constructs with HIV-1 E, C, or B envelopes are critically needed for assessing the efficacy of the infusion of these two MAb as well as of passive HIV-1-specific immunoglobulins isolated from vaccine recipients.

PLAUSIBLE NONNEUTRALIZING ANTIBODY MECHANISMS OF PROTECTION

Figure 1 displays the possible vaccine-induced immune mechanisms mediated by antibodies that might be involved in protection against HIV acquisition. It remains difficult to attribute a predominant role to one given mechanism over the others, as it is

likely that several mechanisms are involved simultaneously, synergistically, or even perhaps antagonistically. Mechanistic and nonmechanistic (81) functions involved in protection against acquisition may differ by body compartment and be redundant.

HIV-infected women with gp120-specific IgG-mediated ADCC activity in their cervico-vaginal secretions had lower genital viral loads than did women with serum ADCC activity only (119). In another study, HIV-infected women had ADCC activity in their cervico-vaginal secretions without serum ADCC, suggesting that ADCC antibodies were produced locally (120). Breast milk IgG ADCC responses to gp120 but not to virus neutralization correlated with reduced perinatal transmission of HIV-1 (121). In the cervico-vaginal and rectal secretions present in the lumen, one could speculate that nNAb act by inhibiting transcytosis, mostly mediated by secretory IgA (sIgA), as suggested by several animal (109, 110, 122, 123) and human (111, 124) studies. It must be stressed, however, that IgA may be a two-faced antibody. In RV144, levels of Env-specific plasma (monomeric) IgA antibodies were inversely correlated with risk of HIV infection (2) by blocking C1 region-specific IgG-mediated ADCC (10). Conversely, secretory IgA antibodies play a crucial role in protection in the mucosal compartment. Whether HIV vaccines can induce plasma IgG antibodies without IgA antibodies ("excision of the spine on the rose") or only sIgA (preferentially IgA1) in mucosal tissues remains speculative. The specific mechanisms of inhibition of transcytosis involved have recently been reviewed (104). Whether IgG can also contribute to this inhibition is not entirely clear. Other mechanisms evoked include the possible hindrance of HIV mobility trapping of viruses linked to IgG and IgA within mucin layers outside the cervico-vaginal epithelium (125, 126) and viral capture by sIgA and IgG.

It is speculated that the nNAb detected in the lumen are also present in the mucosal tissues that contain cells capable of effector functions. ADCC and ADCVI have been less explored in rectal secretions and gut tissues in both animal and human studies. These mechanisms may play a critical role for protection against HIV acquisition in MSM populations, with rectal transmission, the highest-risk mode of sexual HIV transmission, at 1:20 to 1:300 infections per exposure (127). Two distinct subsets of NK cells exist in the gut, one localized to intraepithelial spaces (intraepithelial lymphocytes [IEL]) and the other to the lamina propria (LP). The frequency of both subsets of NK cells is reduced in chronic infection, whereas IEL NK cells remained stable in spontaneous controllers with protective killer immunoglobulin-like receptor/human leukocyte antigen genotypes (128). The main mechanism is represented by NK cell-mediated ADCC (reviewed in references 103 and 104), while monocytes might also play a significant role (129, 130). Antibody-dependent cell phagocytosis (ADCP) is generally mediated by the expression of FcγRII on the surfaces of macrophages, immature dendritic cells, and neutrophils, leading to opsonized virus being taken up into endosomes and degradation (131). Mostly described in HIV-infected subjects, the protective role of vaccine-induced ADCP remains to be explored in future vaccine studies (103). Antibody-dependent cell-mediated virus inhibition (ADCVI) is the measure of the net antiviral activity resulting from ADCC, production of β chemokines, and phagocytosis (132). Vaccine-elicited ADCVI-inducing antibodies correlated with protection in SIV or SHIV challenge studies (122, 123, 133, 134). In Vax004, ADCVI activity was associated with lower rates of infection (64). The inhibition of virus replication in mucosal tissues *ex vivo* along with HIV-specific nNAb in mucosal secretions is currently being explored in RV144 follow-up studies (RV305, RV306, RV328) (135–137).

However, ADCC may be modulated by several factors, including antibody Fc glycosylation, fucosylation of antibody glycans, and IgG subclasses (138). Similarly, acidity and Env-specific IgG were found to enhance virus transcytosis across epithelial cells via FcRn and may facilitate translocation of virus to susceptible target cells following sexual exposure (139). In HIV-infected individuals, ADCC has been shown to exert immune pressure on the virus, leading to escape mutants (140). Whether this phenomenon may apply in the context of vaccine-induced ADCC remains to be shown.

While the main function of HIV-specific NAb is to block viral entry into host cells, nNAb and NAb functions are not exclusive of each other and may act as allies (141, 142). ADCC activity has been described for several bNAb, including b12 (143–145), 2G12 (146), and 2F5 (147). The ability of bNAb to protect macaques against SHIV challenge was mediated by the Fc receptor but not by complement binding alone, suggesting that interaction of Fc-receptor-bearing effector cells with antibody-complexed infected cells is important in reducing virus yield from infected cells (134).

IMPROVING THE INDUCTION OF HIV-SPECIFIC NONNEUTRALIZING ANTIBODIES

The RV144 correlates-of-risk analysis suggests that an increase in the magnitude, affinity, breadth, and, importantly, frequency and durability of V2- and V3-specific antibodies of IgG3 and IgG1 subclasses may confer a higher and more durable rate of protection against HIV-1 infection than other antibodies. Perhaps the first approach to this end is to improve the antigen design of the HIV-1 envelope based on the RV144 results.

ANTIGEN DESIGN

The analysis of A244 gp120 used in RV144 showed that the antigenicity of the gp120 C1 region and the V2 conformational epitopes may be enhanced by the deletion of 11 N terminus amino acids of gp120 (Δ11). This enhanced antigenicity is specific to the CRF01_AE gp120 A244 antigen and may not necessarily apply to other gp120 proteins from the same strain or from different HIV-1 strains. Conformational V1/V2 MAbs gave significantly higher levels of blocking of plasma IgG from A244 Δ11 gp120-immunized animals than that of IgG from animals immunized with unmodified A244 gp120 (148). This improved envelope design is now proposed for the next generation of CRF01_AE- and B-based HIV vaccines in Thailand. The priming agent may also significantly contribute to the shaping of the antibody response to the envelope protein boost, as suggested earlier (100, 102, 149). Head-to-head comparisons of different pox vectors and DNA primings with the same adjuvant-formulated envelope protein boost might help to elucidate the quality of the antibody response, in particular the IgG and IgA subclasses and cytokine profiles induced by the protein boost and the early gene activations that lead to these responses.

MAGNITUDE, FREQUENCY, AND DURABILITY OF ANTIBODIES

The vaccine-induced HIV-1 Env-specific antibody response was not durable in vaccinated HIV-uninfected (66) or HIV-infected (61) humans or NHP (150). Several causes have been evoked, including poorly elicited Tfh responses (151–153), poor Env B-

cell receptor signaling strength (154), poor B-cell memory (155), Env-antibody complex killing of FcγRIIb plasma cells before they reach bone marrow (156), and altered catabolism of Ig in plasma cells (157).

HIV vaccine developers have included more-potent adjuvants than the universally used aluminum hydroxide in vaccine candidates to enhance the efficacy of envelope protein antigens, to induce appropriate immune responses not sufficiently generated in the absence of adjuvant, to enhance and/or to shape antigen-specific immune responses, to expand B-cell diversity, and to contribute to dose sparing of the antigen (158, 159). Several adjuvants have been tested in mice (149), NHP, and humans (160), showing a significant benefit of envelope proteins formulated with either MF59 (161), AS02 (162), AS01 (163, 164), or virosomes (111). In AVEG 015, a trial comparing an HIV-1 envelope protein formulated with different adjuvants, the formulation of HIV-1 gp120 with L(MPLA) and alum induced significantly higher levels of neutralizing antibodies and T-cell lymphoproliferation than alum, MF59, or MPLA alone (165). A recent *post hoc* analysis of the AVEG 015 plasma samples revealed very high titers of cyclic V2 and gp70 V1V2 CaseA2 (subtype B) antibodies as early as after the second protein administration. The frequency of the antibody response to gp70 V1V2 CaseA2 was 100% as early as 2 weeks after the second vaccination and 10 months after the fourth vaccination in the L(MPLA) adjuvant-plus-alum group, while the frequencies were 85% and 100%, respectively, in the alum group. These antibody titers were 5- to 10-fold higher than those observed in the group who received the antigen formulated with alum, were detected at high levels 40 weeks after the fourth vaccination, and were higher than those observed in RV144. Moreover, antibodies were cross-reactive with gp70 scaffolds AE and C (166).

Experiments with nanoparticle malaria vaccines suggest that an increased antigen deposition/retention locally in the tissue drives B-cell responses, enhancing dendritic cell antigen presentation over that obtained with soluble protein immunizations (167). Prolonged antigen presentation mediated by nanoparticle vaccines (168) may also have contributed to the formation of germinal centers (169) with enhanced development of $CD4^+$ Tfh cells (170), which provide critical cytokines and signals required to initiate somatic hypermutation and affinity maturation for effective B-cell memory (171).

The RV144 regimen administered ALVAC HIV and AIDSVAX BE in two different arms. Coimmunization of a DNA prime and an SIV Env protein boost administered in the same muscle was able to significantly augment the magnitude of the Env-specific antibody titers in NHP (172, 173) and rabbits (174). One hypothesis might be that the coadministerion in the same muscle targets antigen-presenting cells (in particular dendritic cells and macrophages) that would reach the same draining lymph nodes, acting synergistically.

CONCLUSIONS

Animal and human data from various viral infections and vaccine studies suggest that functional nNAb that are not neutralizing *in vitro* are important in protection against viral infection *in vivo*, without negating in any way the desirability of evoking neutralizing antibodies through vaccination. This was illustrated by the recent RV144 HIV vaccine efficacy trial, which demonstrated that IgG-mediated nNAb were correlated inversely with risk for HIV acquisition while IgA-mediated antibodies were directly correlated with risk. The underlying mechanisms that may have contributed to protection, although not fully understood, are likely mediated by ADCC, tier 1-strain neutralization, viral capture, and ADCP. Whether ADCVI and inhibition of transcytosis may have played a role remains to be explored. Taken together, correlated data from RV144, sieve analysis of RV144 breakthrough viruses, and low-dose intrarectal challenge studies of NHP combined with the presence of these nNAb in mucosal tissues, cervico-vaginal secretions, and rectal secretions challenges the hypothesis that bNAb are the only immune response that might confer protection. The induction of a high frequency and high titers of HIV-specific functional nNAb may represent an attractive hypothesis-testing strategy in future HIV vaccine efficacy trials.

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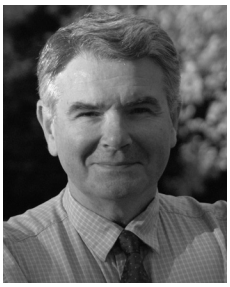

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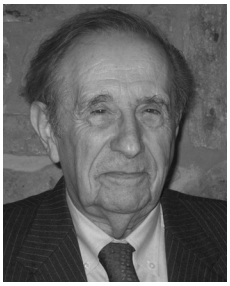

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