HIV-1 sensitivity to neutralization: biological and molecular studies
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Humoral immunity in HIV disease and vaccine development

Virus neutralization is the result of an interaction between an antibody (Ab) and a virion, rendering the virus non-infectious for permissive cells. The mechanism behind neutralization is still largely unknown. Since 1990 it has become clear that the majority of Abs produced by HIV infected individuals are not effective against primary isolates, while they do recognize and neutralize T-cell-line-adapted (TCLA) HIV variants (1-5). Primary HIV-1 variants are considered to be optimally adapted to replication in the presence of Abs in vivo that are directed against epitopes expressed on the free monomeric or immature envelope glycoproteins gp120 and gp41. It is assumed that in the oligomeric structure of primary HIV most neutralizing epitopes are masked. However, the continuous emergence in vivo of escape mutants suggests that the neutralization resistance of primary HIV is far from absolute. (6-13). The neutralization sensitivity of TCLA isolates is assumed to be determined by their more open oligomeric envelope configuration which may render them sensitive to neutralization by patient sera, soluble CD4 (sCD4), and Abs recognizing epitopes close to the CD4-binding site and the V3-loop (14-25). Differences in antibody binding to the oligomeric envelope complex was reported to correlate with differences in neutralization sensitivity between TCLA and primary isolates (26-31). This correlation could not be confirmed in several other studies which may be explained by the complexity of the technique used for measuring Ab binding (32-36).

Protection from HIV transmission and disease progression by humoral immunity

Despite the neutralization resistance of primary HIV variants, passive transfer studies have provided evidence that Abs can protect against infection. Partial protection against infection and elevated clearance of virus particles after intravenous or mucosal challenge with pathogenic HIV strains by neutralizing MAbs or sera in rhesus monkeys has been achieved (37-43). One general restriction of these studies is the use of broadly neutralizing Abs (such as IgG1b12, 2G12 or 2F5). These MAbs were carefully selected from vaccinated mice or isolated from patient cDNA libraries and are not commonly produced in infected individuals (see table 1, chapter 1) (44-46). Although the search for and production of this type of broadly neutralizing Abs may be very laborious, they may be very useful for understanding primary HIV neutralization and may have therapeutic applications.

Other evidence for protection from infection by Ab comes from vertical transmission studies where high titer neutralizing Ab in the mother correlated with reduced transmission to the child (47;48). Although the exact moment of vertical transmission is still not known, treatment with antiretroviral therapy around the moment of birth has strongly reduced the vertical transmission rate. Therapeutic application of neutralizing antibodies in this setting should therefore be considered.

Finally, individuals classified as long term asymptomatic (LTA) in general have high titers of neutralizing Abs late in infection. Although, Ab responses during acute infection did not differ between LTA and rapid progressors, therapeutic application of Ab with potent neutralizing activity against primary HIV isolates may also be considered as additional therapy during chronic infection (49-55).
Dichotomy in antibody production during HIV infection

For long it has been assumed that during acute HIV infection, only non-neutralizing antibodies are elicited with specificity for monomeric gp120 that originated from lysis of infected cells. By using highly sensitive precipitation techniques we were able to show, in chapter 12, that during acute infection first a humoral immune response to native mature protein is generated while later in infection Abs emerge that recognize monomeric gp120, preceding the capacity to neutralize TCLA isolates. This observation may be explained by the initial proper presentation of oligomeric gp120 exposed on the surface of infected cells. Later during acute infection, the number of available target cells may become limiting due to massive CD4+ T cell depletion. In this phase of infection, it may take too long for a virion to encounter a potential target cell. Due to subsequent viral degradation the amount of free monomeric gp120 and membrane bound gp41 may increase and trigger B cells for the production of Ab directed against these degraded envelope proteins. These Abs may have a high affinity for gp120, but lack the capacity to neutralize primary HIV. By binding to gp120, these antibodies cannot only interfere with the binding of already existing neutralizing antibodies but may mask epitopes and thereby prevent the generation of new neutralizing antibodies.

Development of neutralization-resistant virus escape variants from humoral immunity

Although unable to neutralize primary HIV, Abs directed against monomeric gp120 have been demonstrated to have high neutralizing potential for TCLA HIV. On the basis of this knowledge we expected efficient humoral immune control in a laboratory worker who was accidentally infected with the TCLA IIIB variant. However, this individual had a progressive disease course which coincided with the development of neutralization resistant HIV. Most remarkable, development of disease coincided with resistance to sCD4 and MAb IG1b12, which both distinguishes TCLA from primary HIV isolates. This conversion from a TCLA neutralization sensitive towards a neutralization resistant phenotype in vivo is the first direct evidence that neutralization resistance of primary HIV is an escape mechanism from humoral immunity (chapter 3).

Increased neutralization resistance was also observed after sequential in vivo passage of non-pathogenic SIV, SIV containing HIV envelope (SHIV) and feline immunodeficiency virus (FIV) through monkeys or cats, respectively (56-59). In addition to the changes observed in the laboratory worker, passages of a HIV-IIIB containing SHIV isolate through rhesus monkeys resulted in a progeny with increased resistance to sCD4 and IgG1b12, that was highly cytopathic in rhesus monkeys. Kimata et al (56) demonstrated that the increased resistance to neutralization was accompanied by changes in the SIV structural genes that enhanced viral replicative and cytopathic abilities. This increased cytopathicity was also observed for the viruses isolated from the laboratory worker when tested in the SCID-hu Thy/Liv mice model (60-62).

The emergence of neutralization resistant HIV and SIVcpz escape variants has also been reported in both natural and experimental chimpanzee infection, which is in agreement with virus replication in the presence of humoral immunity (63-66). In a recent report, the development of an AIDS-like syndrome in a chimpanzee, which is an extremely rare event, was associated with the emergence of neutralization resistant virus variants (67;68). The selection of neutralization resistant escape variants in this species seems to be an extremely slow process (chapter 5). Since evolution of a viral quasispecies is highly dependent on the viral population size, this selection process could be related to the very low level of replication, as reflected by viral load levels below the limit of detection. This would simply implicate that it takes much longer to
accumulate the required mutations for a neutralization resistant phenotype (69).

Molecular determinants for escape from humoral immunity

In human, high level virus production and the error prone activity of the HIV reverse transcriptase (70-72) support the rapid generation of mutant viruses from which neutralization resistant escape variants can be selected. This process is facilitated by the remarkable intrinsic flexibility of the HIV envelope (73-75). Indeed, the in vivo reversal of the TCLA HIV-IIIB isolate towards a neutralization resistant variant in the accidentally infected laboratory worker was accompanied by a Glutamate (Glu) to an Alanine (Ala) substitution at position 370 in gp120 (chapter 14). This mutation was unexpected as the Glu370 is highly conserved among HIV-1, HIV-2 and SIV isolates and directly involved in CD4 binding (75). This was also demonstrated by (76;77) mutating Glu370 in the background of either X4 or R5 HIV isolates which resulted in a reduced CD4 binding capacity (78-82).

In the neutralization resistant laboratory worker virus, the Glu370 mutation together with mutations in the variable loops 1 and 2 determined resistance to CD4-binding site directed neutralization. The Glu to Ala mutation apparently was of major importance for escape from humoral immunity and was probably allowed in the context of compensatory mutations (83). These mutations are probably not essential for the neutralization resistant phenotype itself but rather indispensable for the evolutionary process, creating a background in which the substitution of residue 370 is allowed.

Mechanism of selection of pre-existing virus variants

As discussed above, it is most likely that the humoral immune response drives HIV evolution towards a neutralization resistant phenotype. However, during in vitro passage in primary cells, the neutralization resistant phenotype seems to be maintained in the absence of antibodies. A viral envelope configuration best adapted to replication in primary cells could coincide with a neutralization resistant phenotype. In chapter 6 we propose that in primary PBMC cultures a diversity of virus variants can emerge, including virus variants with increased neutralization sensitivity. Their coexistence in PBMC cultures suggests an equal fitness under these conditions. The heterogeneity of cells in PBMC may provide distinct cellular niches for neutralization sensitive and resistant HIV. However, in the presence of antibodies in vivo, these neutralization sensitive variants are negatively selected (6-13;83-86). In that respect, it may be interesting to study the neutralization sensitivity of HIV-1 biological clones obtained from end-stage AIDS patients in whom humoral immunity is deteriorated, to see whether in the absence of neutralizing antibodies neutralization sensitive HIV variants indeed emerge and persist in vivo.

HIV passage through chimpanzee cells and T cell lines selects for the virus variants with increased neutralization sensitivity (chapter 5 and 6). The underlying mechanism for this phenomenon is unknown. Adaptation to growth in T cell lines reflects adaptation to usage of low level CD4 (87). Selection of viruses with a neutralization sensitive envelope configuration therefore most likely occurs during entry and may be determined by the membrane expression patterns of CD4 and the HIV coreceptors CCR5 and CXCR4. How these proteins form membrane complexes when they interact with the HIV envelope is currently attracting attention. CD4 and CCR5 have been shown to intrinsically colocalize on human cells, whereas CXCR4 can only be found in complex with CD4 after addition of gp120 (88-94). It is tempting to speculate that the make-up or density of the CD4-coreceptor complex on chimpanzee cells is different from human cells and selects for an HIV envelope configuration which coincides with increased neutralization sensitivity. The emergence of an envelope with a configuration that can efficiently interact with the chimpanzee CD4-coreceptor complex and resists
Ab binding may require an extremely long time which could be an alternative explanation for the long time required to obtain neutralization resistant HIV variants in chimpanzee.

**Potentiation of humoral immunity**

As several studies have demonstrated a beneficial effect of neutralizing antibodies on the clinical course of HIV infection, strategies to potentiate or preserve the humoral immune response should be considered. For HIV, such strategies should also aim for the exposure of relevant epitopes in sufficient quantities and preservation or restoration of B cell help by CD4+ T cells. During highly active antiretroviral therapy (HAART) reconstitution of immunity by increasing CD4+ cell numbers should also restore B cell help. It has been hypothesized however that early in HIV infection, HIV specific CD4+ cells are selectively depleted (95). As a consequence of their activated phenotype in response to HIV, these cells are also the ideal target cells for viral replication. It has indeed been demonstrated that initiation of HAART during acute infection preserves CD4+ helper T-cell activity but that HAART in chronically infected individuals does not restore CD4+ T-cell help (96). Improved humoral immunity has been observed in individuals who started HAART during acute infection and not during chronic infection, but only when structured treatment interruptions (STI) were applied suggesting that repeated exposure to antigen is an absolute requirement for maintenance of HIV specific B cell activation (97-101). Persistence of this initial immune response correlates with high CD4+ T-cell numbers (102), and delayed disease progression in LTA (49-53;103). As an alternative for STI as source for antigen stimulation, therapeutic vaccination may be beneficial but again only in individuals who started HAART during acute infection. This strategy may reactivate memory B cells that were involved in the earliest humoral immune response and produce antibodies with specificity for the native mature (oligomeric) envelop gp120 which would be most beneficial to the host (chapter 2). Several promising approaches to increase the exposure of relevant epitopes have been developed and will be discussed below. However, although these new approaches may lead to the production of neutralizing antibodies, it remains to be established whether these, even when present in sufficient quantities, will be able to neutralize primary HIV on which the relevant epitopes will still be masked or buried.

**Humoral and cellular immunity induced by HIV-vaccines**

In recent DNA vaccine studies, (104;105) prime-boost regimes with HIV gag, pol or env containing expression plasmids in rhesus macaques resulted in controlled viral replication after challenge with pathogenic SHIV strains in all animals (106). In one animal, with undetectable levels of viral RNA in plasma, functional CD4+ and CD8+ T lymphocyte responses and high neutralizing antibody titers, an escape variant emerged resulting in loss of immune control and development of disease (107).

Antibody responses were not observed after vaccination but could be demonstrated after challenge (104;106). It cannot be excluded however that the absence of Ab after vaccination is due to the use of monomeric gp120 in the ELISA for the detection of Ab (chapter 2). During the period of low to undetectable virus RNA levels, gp120 Ab binding and autologous neutralizing Ab levels remained unchanged or declined slowly. This can be explained by the absence of free antigen during the chronic phase. It remains to be established how an envelope protein transduced by DNA vaccination is properly presented to the immune system.

As discussed above, in the presence of abundant monomeric or immature gp120 envelope proteins, an effective neutralizing Ab response is not elicited. Therefore it is not likely that the AIDSVAX monomeric gp120 vaccine will induce protective humoral immunity (108).
Novel HIV vaccine strategies and non-Ab entry inhibitors
The very low level or complete absence of Abs with neutralizing activity against oligomeric primary HIV in vivo may be related to the relatively non-immunogenic structure of oligomeric gp120 as compared to monomeric and immature forms of gp120. We have shown in chapter 2 that in acute infection, Abs are present that only recognize mature, properly folded gp120. It might be feasible to construct envelope complexes that are immunogenic and express neutralizing epitopes that are exposed on primary oligomeric gp120/gp41 either before or after CD4 binding. Efforts on the creation and testing of such modified protein conformations are being made (109-119). These studies include attempts to create pure soluble or immobilized oligomeric forms of env, either by deletion of the gp120/gp41 cleavage-site to obtain gp140 proteins or by introduction of linkers between the gp41 ectodomains. Increasing Ab specificity may also be accomplished by deletion of variable loops like V3 and V2 to increase exposure of relevant epitopes. This could also be achieved by chemical components that may induce stable conformational changes in gp120, thereby reducing the flexibility of the antigenic structure or by trapping of the envelope fusion intermediate, by chemical fixation during fusion of gp120 expressing cells with cells containing CD4 and the coreceptor (120).

Some of these complexes have been used for immunization of mice or for the screening of phage-display libraries to select for new neutralizing MAbs for therapeutic applications (121). Furthermore attempts were made to screen for new Abs on precipitates of the gp120-CD4-CCR5 complex (115). Although these studies are not finished yet, the difficulty of obtaining good Ab responses is still a major problem for HIV vaccine development. In addition to Abs neutralization of HIV-1 infection, multiple stages of the entry process are considered as target for the development of therapeutic agents. Focus is especially directed towards development of gp41 fusion inhibitors (122-124) and chemokine antagonists (such as AMD3100, APO- and NNY-RANTES, T22, TAK-779, refs (125-129)). At present, several clinical trials are conducted to evaluate the safety and efficacy of some of these compounds (130).

Concluding remarks
The efficacy of humoral immunity seems to be determined by the quality and quantity of the antibody response and the neutralization sensitivity of HIV. Although humoral immunity against HIV does not seem to be very efficient, its magnitude correlates with protection. Since all antiretroviral drugs currently available are still insufficient to achieve complete eradication of HIV, additional or alternative approaches for inhibiting viral replication should be explored. The generation and therapeutic application of broadly neutralizing Abs should be considered. Therefore a major task for the near future is to further develop therapeutic vaccines, to increase efficacy of STI and develop new vaccine strategies to induce neutralizing Abs with high affinity for the oligomeric envelope structure of primary isolates.

References
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