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Towards an AIDS vaccine

The transmembrane envelope protein as target for broadly neutralizing antibodies

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Key words: AIDS, neutralizing antibodies, transmembrane envelope proteins, retroviruses

Abbrevations: AIDS, acquired immunodeficiency syndrome; FeLV, feline leukemia virus; FPPR, fusion peptide proximal region; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; MPER, membrane proximal external region; PBMCs, peripheral blood mononuclear cells; PERV, porcine endogenous retroviruses; TM protein, transmembrane envelope protein

Although the development of an effective vaccine is the main goal in the fight against AIDS, all attempts by numerous laboratories to develop a vaccine have failed so far. In addition, it is still unclear whether cytotoxic T cells or neutralizing antibodies or both should be induced. The major advantage of neutralizing antibodies is their ability to prevent infection and subsequent integration of the provirus into the cellular genome where it may persist in a form invisible to the immune system. Broadly neutralizing antibodies have been found in HIV infected individuals, including antibodies directed against a highly conserved region in the membrane proximal external region (MPER) of the transmembrane envelope (TM) protein gp41 of HIV-1. We successfully induced neutralizing antibodies against different gammaretroviruses by immunization with their respective TM proteins. These antibodies recognized epitopes not only in the MPER but also in the fusion peptide proximal region of the TM protein. In the case of feline leukaemia virus (FeLV), these antibodies protected cats from antigenemia following challenge. To understand the mechanism of neutralization, the interactions between neutralizing antibodies and their corresponding epitopes in the TM protein of gammaretroviruses and HIV-1 were analyzed. These data may help to design antigens able to induce specific broadly neutralizing antibodies.

Introduction

In 2008, 33.4 million people lived with an infection by the human immunodeficiency virus (HIV),—which is the cause of the acquired immunodeficiency syndrome (AIDS), 2.7 million got newly infected, and an estimated 2 million people were killed by AIDS, among them 300,000 children. Although the therapy has improved significantly in recent years, AIDS still cannot be cured, and the combination therapy or HAART (highly active antiretroviral therapy) is associated with severe side effects. Therefore a prophylactic vaccination is the best means to prevent further distribution of the HIV pandemic. However, all attempts to design effective vaccines have failed so far.

Since HIV-1, like all retroviruses, integrates its genetic information into the genome of the infected cells where it may persist undetected from the immune system, induction of neutralizing antibodies preventing infection and integration seems to be the best vaccination strategy. Neutralizing antibodies usually interact with the surface and/or the TM proteins involved in the infection process and may thus prevent infection. Both proteins are exposed on the virus surface as trimeric envelope spikes (**Fig. 1**).

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Function of the TM Protein during Infection

The structure of the envelope proteins and the mechanism of infection are very similar for all retroviruses (**Fig. 1**). The surface envelope protein, gp120 in the case of HIV-1 and gp70 in the case of gammaretroviruses, interacts with the receptor molecule(s) on the cell surface. The CD4 molecule and the chemokine receptors CXCR5 or CCR4 are the receptors for gp120 of HIV-1; different transporter molecules are the receptors for gp70 of gammaretroviruses.[1,2] After interaction of the surface envelope proteins with the receptors, the glycine-rich fusion peptide at the N-terminus of the TM protein intercalates into the cellular membrane and conformational changes in the TM protein, including an interaction of the C-helical and N-helical regions of the TM proteins, bring the membrane proximal external region (MPER) close to the fusion peptide proximal region (FPPR). This promotes fusion between viral and cellular membranes and internalization of the virus into the target cell.[1,3]

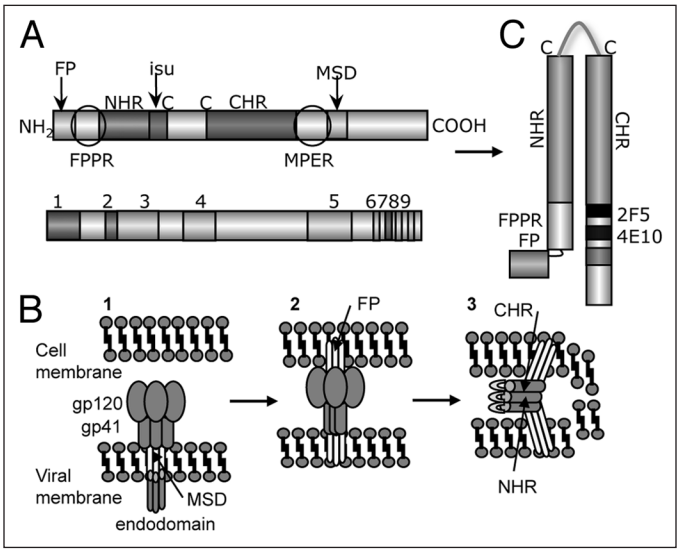

Figure 1. (A) Main functional domains of the retroviral TM protein (FP, fusion peptide; FPPR, fusion peptide proximal region; NHR, N-terminal helical region; isu, immunosuppressive domain; C-C, Cys-Cys-loop; CHR, C-terminal helical region; MPER, membrane proximal external region; MSD, membrane spanning domain). Below the membranotropic domains are indicated, the darker the shade of grey, the greater the effect[45] (1) corresponds to the FP, (2 and 3) to a part of NHR, (4) to the Cys-Cys-loop, (5) to the MPER and MSD, (6 to 9) are located in the endodomain of gp41. (B) Schematic presentation of the conformational changes during infection. (1) localization of the viral knob consisting of the surface envelope protein gp120 and the TM protein gp41 anchored by the MSD in the viral membrane, (2) invasion of the fusion peptide into the cellular membrane, prehairpin intermediate and (3) hairpin or six-helix-bundle formation by the CHR and NHR. (C) Localization of the epitopes recognized by the broadly neutralizing antibodies 2F5 and 4E10 in the MPER of gp41 of HIV-1.

Neutralizing Antibodies Specific for the TM Protein in AIDS Patients

Low amounts of neutralizing antibodies were commonly found in HIV-infected individuals, most of them directed against the SU protein.[4-9] Antibodies against the TM protein are mainly non-neutralizing and directed against the immunodominant Cys-Cys loop.[10,11] Usually patients develop antibodies against the immunodominant region (called cluster I), the C-terminal helical region (cluster II), the MPER (cluster IV), but not against the N-terminal region of gp41 including the fusion peptide, the FPPR or polar region and the N-terminal helical region.[12,13] The humoral immune response to gp41 during HIV infection is strong with titers up to 625-fold higher than anti-gp120 titers[14] and precedes the response against gp120.[15] In addition, broadly neutralizing antibodies such as 2F5 and 4E10, directed at the MPER of gp41 (**Fig. 1**) and neutralizing up to 95% of all HIV strains, were isolated from infected individuals.[16-22] Passive infusion of mixtures of 2F5, 4E10 and two neutralizing antibodies directed at gp120, 1b12 and 2G12, into rhesus monkeys protects against SHIV infection. If these antibodies could be induced in man, a preventive HIV-1 vaccine might be possible.[23,24] Also, these antibodies reduced the virus load in humans after cessation of the antiretroviral therapy.[25,26] In addition, when 2F5 and 4E10 were characterized, uncommon properties were observed: (1) they are characterized by an uncommonly long hydrophobic CDR3 region;[27,28] (2) 4E10, but not 2F5, reacts with cardiolipin.[29-31] Previously it was thought that 2F5/4E10-like antibodies are rare in HIV-1 infected individuals. Meanwhile, however, there is evidence that such antibodies are more common.[12,32-34] Most importantly, broadly neutralizing antibodies reacting with the MPER may recognize epitopes different from those of 2F5 and 4E10.[34]

Attempts to Induce Neutralizing Antibodies Specific for the TM Protein of HIV-1

Several novel immunogen design strategies were developed in the past: (1) preserving the native structure of functional Env trimers or stabilization of the trimers by cross-linking; (2) unmasking of epitopes; (3) stabilization of intermediated epitopes; and (4) using structural analogs of conserved regions such as the MPER (reviewed in refs. 35 and 36). All attempts to generate broadly neutralizing antibodies such as 2F5 and 4E10 failed.[37-42] Conformational constraints such as enhancement of the alpha-helicity resulted in increased antigenicity, but no neutralizing response.[39,40] Binding of 2F5 and 4E10 to their epitopes requires surprisingly few crucial residues in their epitope domains (LEL DKW ANL, the epitope of 2F5 and NWF N(D)I SNW LW, the epitope of 4E10, amino acid changes to produce resistance to the corresponding antibody occurred only in the residues shown in bold),[43] indicating that not the sequence, but the conformation of epitopes is of great importance. Different reasons, why it is difficult to induce 2F5/4E10-like antibodies were discussed: (1) they represent autoimmune antibodies;[29-31] (2) other antibodies against gp41 may mask the epitopes in the MPER.[44] The most likely explanation is that it is difficult to reconstruct the conformation of the epitope at a particular time during the conformational changes of gp41 in the infection process. In addition, a strong interaction of different domains of the TM protein of HIV with lipids was observed (**Fig. 1**).[45] When the interaction between the mAb 4E10 and its epitope in the lipid membrane was studied, it was shown that the MPER undergoes unexpected conformational changes upon binding of the antibody,[46] showing the complexity and flexibility of the interaction between epitope and antibody.

Only a few publications reported immunization studies resulting in weakly neutralizing antibodies. For example, stable mimetics of the gp41 pre-hairpin intermediate were used to induce D5-like antibodies.[38] D5 is a monoclonal reacting with the NHR of the pre-hairpin. The concentration of the neutralizing antibodies was very low. When the MPER of gp41 was presented in hepatitis B surface antigen (HBsAG) S1 protein that forms nanoparticles in the presence of lipids, no neutralizing antibodies were induced. However prime boost immunization with proteoliposomes containing HBsAG-MPER and the HIV envelope protein did raise neutralizing antibodies.[47] A trimeric form of a chimeric protein composed of the triple stranded coiled-coil region from the influenza hemagglutinin subunit 2, HIV gp41 CHR and MPER also did not induce neutralizing antibodies.[37] In contrast to these mostly unsuccessful attempts to

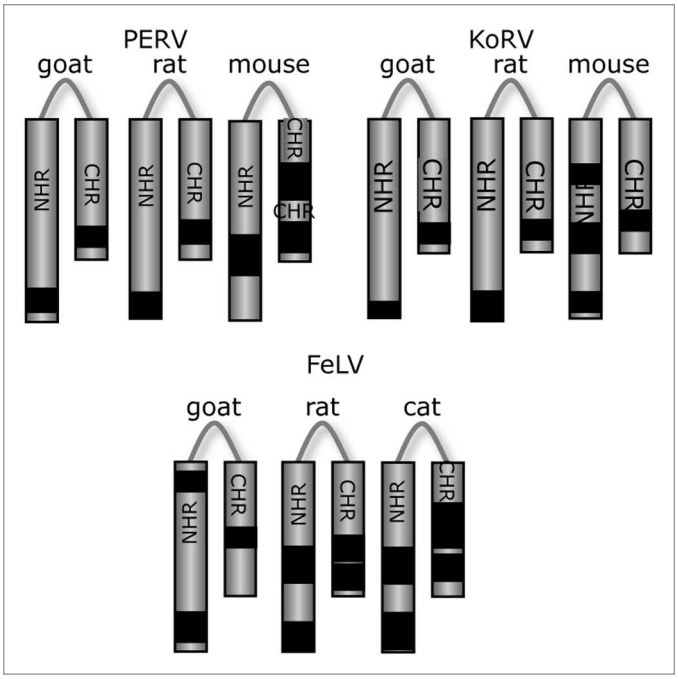

Figure 2. Localization of the epitopes (black) recognized by neutralizing antisera induced in different species (goat, rat, mouse, cat) after immunization with the TM proteins p15E of PERV, FeLV and KoRV. The fusion peptide and the membrane spanning domain are not shown.

induce broadly neutralizing antibodies recognizing the MPER of gp41 of HIV, immunization studies with the TM proteins of different gammaretroviruses reproducibly resulted in neutralizing antibodies (see below).

Successful Induction of Antibodies Neutralizing Gammaretroviruses by Immunization with their TM Protein

Corresponding immunization studies were first performed with the TM protein of porcine endogenous retroviruses (PERVs), in connection with virus safety in xenotransplantation using pig cells or organs. Transplantation of pig cells or organs into human recipients is under development to help overcome the lack of human donor organs. Pigs, like all mammals, carry endogenous retroviruses in their genome. These porcine endogenous retroviruses (PERVs) were shown to be released from normal pig cells and to infect human cells, representing a special risk in xenotransplantation. The development of a PERV-specific vaccine may help to prevent transmission of the virus to xenotransplant recipients. To generate such a vaccine, goats, rats and mice were immunized with the TM protein p15E of PERV.[48] In all immunization studies neutralizing antibodies were obtained and, when the epitopes of the corresponding sera were analyzed, one epitope was found in the FPPR of p15E, designated E1, another in the MPER, designated E2 (**Fig. 2**). Surprisingly the localization of the epitope in the MPER was similar to the localization of the epitopes of 2F5 and 4E10 in the TM protein gp41 if HIV-1. Despite the evolutionary difference between

1 AAIGALFLGFLGAAGSTMGAASMTLTVQARQLLSDIVQQQ
2 AAIGALFLGFLGAAGSTMGAASMTLTVQARQLLSDIVQQQ
3 AAIGALFLGFLGAAGSTMGAASMTLTVQARQLLSDIVQQQ
4 AAIGALFLGFLGAAGSTMGAASMTLTVQARQLLSDIVQQQ
5 AAIGALFLGFLGAAGSTMGAASMTLTVQARQLLSDIVQQQ
6 NEQELLELDKWASLWNWFNITNWLWY

Figure 3. Domains in the FPPR and MPER interacting with each other and with the monoclonal antibodies 2F5 and 4E10. (1 and 5) represent the sequence Env512-552 in the FPPR, (6) is the sequence Env656-681 in the MPER. (1) a synthetic peptide corresponding to the underlined sequence, enhances the binding of 2F5 to the peptide NEQ ELL ELD KWA SLW containing the epitope of 2F5 (the epitope is underlined),[54,55] (2) the underlined sequence was reported to be recognized by 4E10 with the boxed sequence as core epitope,[59] (3) the underlined sequence enhances binding of 2F5 to its epitope,[60] (4) the underlined sequence was found to interact with the FPPR,[56] (5) a peptide corresponding to the underlined sequence supports formation of a stable six-helix bundle when added to the NHR peptide number (6) and (6) sequence Env656-681, a peptide corresponding to the underlined sequence supports formation of a stable six-helix bundle when added to the peptide number (5), the epitopes of 2F5 and 4E10 are boxed.[57]

the lentivirus HIV-1 and the gammaretrovirus PERV a limited sequence homology of the epitopes was observed. The epitope FEG**WFN** in p15E corresponds in localization and sequence to the epitope N**WFN**(D)IT in gp41 of HIV-1 recognized by the broadly neutralizing antibody 4E10 (identical amino acids in bold). Whereas immunizations of goats with p15E of PERV resulted in sera recognizing only two epitopes,[48] immunization of rats and mice resulted in sera recognizing up to two epitopes in the FPPR and two additional in the MPER (designated E1a and E1b as well as E2a and E2b, unpublished data). The epitopes E2a and E2b in p15E of PERV correspond to the epitopes of 2F5 and 4E10 in gp41 of HIV-1. Preliminary data indicate that only the antibodies directed against the epitopes in the MPER/E2 region are involved in neutralization; however the function of the E1 region is still unclear, and we cannot exclude the presence of additional antibodies which do not recognize linear peptides used for epitope mapping.

Since there is no animal model to analyze the neutralizing activity of sera against the TM protein p15E of PERV, similar immunization studies were performed with the TM protein p15E of FeLV. FeLV induces leukemias and immunodeficiencies in cats. Immunization studies in goats, rats and cats resulted in strongly neutralizing antibodies and the sera recognized epitopes localized in the FPPR and MPER[49-52] (**Fig. 2**). The epitope FEGWFN is identical in FeLV and PERV and corresponds in localization and sequence to the epitope NWFN(D)IT in gp41 of HIV-1 recognized by the broadly neutralizing antibody 4E10 (identical epitopes in bold). Despite the fact that cats carry the sequence of the epitopes in p15E in the form of endogenous feline retroviruses in their genome, cats also produce antibodies against these domains.[51] When cats were immunized with p15E of FeLV and challenged with infectious virus, protection from

Table 1. Criteria for the characterization of neutralizing antibodies

Neutralizing in two different neutralization assays, e.g., based on measurement of provirus integration by real time PCR and TZM-bl cell based assay
Neutralizing activity is dose-dependent and can be diluted out
Neutralizing antibodies bind to the antigen used for immunization in Western blot assays, ELISA or immunoprecipitation
Epitope was identified
Neutralizing activity confirmed in an independent laboratory

antigenemia was observed in three out of six immunized cats (50%), whereas all three non-immunized cats got infected and died from leukemia.[50] These data indicate that immunization with the TM protein of a retrovirus induces protective immunity. In parallel, cats were immunized with the recombinant surface envelope protein gp70 of FeLV (which is the commercial vaccine Leucogen). Immunized with gp70, all cats were protected. Moreover, when cats were immunized with a combination of gp70 and p15E, higher titres of neutralizing antibodies were observed (unpublished data). The same results were obtained when rats were immunized simultaneously with gp70 and p15E of FeLV[52] or PERV (unpublished data). In addition, when goats, rats and mice were immunized with p15E of the KoRV, neutralizing antibodies were induced[53] and similar epitopes were observed as in the case of FeLV and PERV (unpublished data).

Interaction between the MPER and FPPR

When the epitopes of the monoclonal antibodies 2F5 and 4E10 were mapped, using the same method as used for the epitope mapping of goat, rat, cat and mice sera after immunization with the TM proteins of different gammaretroviruses such as PERV, FeLV and KoRV, only one epitope was recognized by each monoclonal antibody which was localized in the MPER.[54] In order to analyze whether a domain in the FPPR may be involved in the mechanism of neutralization by 2F5 and 4E10, overlapping peptides corresponding to the entire surface envelope protein gp120 and the TM protein gp41 of HIV-1 were used in order to study which of these peptides enhance the binding of 2F5 and 4E10 to a peptide containing their epitopes. One peptide was identified, enhancing the binding of 2F5 and 4E10 to their epitopes, located in the FPPR just opposite the MPER[54,55] (**Fig. 1**). Although neither 2F5 nor 4E10 did bind to this FPPR-derived peptide, the presence of this peptide increased binding of the antibodies to their epitopes three to four times, indicating that interaction between the FPPR-derived and the MPER-derived peptides results in a conformation allowing better binding of 2F5 or 4E10. A direct interaction between the FPPR-derived and the MPER-derived peptides was confirmed by surface plasmon resonance (SPR).[54] This interaction suggests that, for the induction of neutralizing antibodies of the type 2F5/4E10, simultaneous use of both domains may be essential.[55] Meanwhile, other publications also support the interaction of domains in the FPPR and the MPER during the conformational changes occurring during infection of HIV-1.[56-58] (**Fig. 3**) It should be mentioned that, besides the interaction between 4E10 and 2F5 and their epitopes in the MPER, binding of both antibodies to regions in the FPPR have been reported,[59,60] although not distinctly characterized. Interestingly, no interaction was found by SPR analysis between peptides corresponding to the E1 domain and the E2 domain in the MPER of p15E of PERV (unpublished data).

False Positive Results in Neutralization Assays

The problem of false positive results in neutralization assays is a still largely underestimated topic. Analyses of sera from animals immunized with TM proteins of different gammaretroviruses as well as with gp41-derived proteins or peptides often show false positive results. False positive results were also observed when preimmune sera were analyzed. Isolation of the immunoglobulins sometimes, but not always, eliminated the false positive effect. There are only speculations on how such false positive effects may be generated. For example, uncompleted coagulation processes, opsonizing antibodies, cross-reacting antibodies, cytokines induced by infections of the immunized animals with other microorganisms may cause inhibition of virus infection in neutralization assays. The influence of endotoxin on the results of neutralization assays was well studied.[61] Endotoxin in the form of lipopolysaccharides may be released into sera by contaminating bacteria and can induce beta-chemokines in blood cell-based neutralization assays inhibiting HIV-1 infection as shown by.[61] In addition, interferon gamma released from blood cells after interaction with endotoxin may give false positive results in TZM-bl cell-based neutralizing assays. Therefore, preparation, handling, transport, freeze-thawing and storage of preimmune and immune sera are of great importance when their neutralizing activity is investigated. As mentioned above, in some cases the use of purified immunoglobulins in neutralization assays together with epitope mapping confirming the binding to the TM protein may prove the specificity of the neutralizing antibodies. Neutralizing antibodies should be thoroughly characterized using strong criteria (**Table 1**).

Influence of the Immunosuppressive Domain

The immunosuppressive (isu) domain of the TM proteins of retroviruses is located in the C-terminal part of the N-terminal helical region of the protein (**Fig. 1**). The isu domain is highly conserved among all retroviruses, and peptides corresponding to this domain were shown to inhibit lymphocyte proliferation, to modulate cytokine release from normal peripheral blood mononuclear cells (PBMCs) and to modulate gene expression in normal PBMCs (reviewed in refs. 62–66). Mutations in the isu domain abrogated the immunosuppressive activity of the TM proteins, and immunization with TM proteins containing the mutated isu domain resulted in increased antibody responses (reviewed in refs. 67 and 68, and our own results), indicating that the isu domain should be inactivated in order to achieve an effective immunization against retroviral TM proteins.

Outlook

Retroviral TM proteins undergo complex conformational changes during the infection process (**Fig. 1**). Intermediated structures, which are short-lived, may represent the required targets for neutralizing antibodies. To gain insight into antigenicity, immunogenicity and function of different domains of the TM proteins during infection, additional studies should be performed. Only if these complex conformations are characterized, corresponding antigens can be designed and neutralizing antibodies preventing retroviral infections including infections with HIV-1 can be induced.

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