Induction of a MHC Class I-Restricted, CD8 Positive Cytolytic T-Cell Response by Chimeric HIV-1 Virus-Like Particles in Vivo: Implications on HIV Vaccine Development

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Summary

New insights into HIV-pathogenesis suggest that the cell mediated immune response might play a crucial role in controlling HIV infection by suppressing HIV-replication in CD4-positive cells by a lymphokine-like soluble factor and by killing HIV-infected cells via classical CTL mediated lysis. This type of a cellular immune response rather than an antibody response seems to be most promising to protect if not from infection, so at least from disease. Therefore rationally designed candidate vaccines should be capable of inducing a cell mediated immunity in addition to a humoral immune response. In order to avoid adverse side effects upon immunization, carefully selected antigens and epitopes should be presented in a favourable manner to the immune system. In previous experiments, we could demonstrate that the gag-polyprotein precursor, known to include a series of T-helper and CTL epitopes, assembles to highly immunogenic, complete noninfectious HIV-1 virus-like particles (VLP). Based on these VLP we developed a novel antigen presentation system, which allows the presentation of selected epitopes derived from HIV reading frames other than gag to the immune system. Alternatively complete derivatives of the HIV-1 external glycoprotein can be presented by the VLP. Immunological analysis of different VLP preparations in a BALB/c mouse model revealed the induction of a strong CTL response. The significance of these observations for future vaccine strategies is discussed.

Key-Words: HIV, vaccine, cell mediated immune response.

Introduction

The paradigm of a rational vaccine design is the development of the recombinant hepatitis-B-virus vaccine by inducing high titers of virus neutralizing antibodies towards structures exposed on the surface of the virion. In analogy, HIV vaccine development focused for the last decade primarily on the induction of high titers of neutralizing antibodies to a recombinant HIV-1 envelope glycoprotein (rgp120) in order to achieve a broad sterilizing immunity. However, large-scale phase III field trials for testing of two different rgp120 candidate vaccines have been judged by the ARAC (AIDS Research Advisory Committee) not to be very promising and therefore have been put on ice (Macilwain, 1994). One of the reasons for the failure of this approach is certainly the low complexity and high variability of these vaccine preparations in addition to their inability to induce a strong CTL response.

Although the precise role of HIV-specific CTLs in infected individuals is not completely clear to date, there is ample evidence that a shift from a Th1-predominant pattern of cytokine production (IL-2; IFN-γ) stimulating the cell mediated immune response to a Th2-predominant phenotype (IL-4, IL-10) clearly correlates with disease progression towards AIDS (Amadori et al., 1992; Clerici et al., 1993a, b;
Sher et al., 1992). This hypothesis is consistent with recent observations demonstrating high levels of Th1 cells and an intact CTL response in long term survivors and confirms data obtained for a series of individuals at risk who have been exposed to HIV without showing any signs of infection (Rowland et al., 1992). Dr. Levy and his group demonstrated in a series of excellent experiments, that CD8 positive cells with previous contact to HIV antigen secrete a soluble lymphokine-like factor that suppresses HIV replication in infected cells. According to very recent data, this effect seems to be especially strong within the group of long term non progressors (Walker et al., 1986, 1989a, b). In African green monkeys (Agm), the natural host of simian immunodeficiency virus (SIV), a suppressive factor exhibiting identical properties has been reported by Dr. Ennen and coworkers, which is supposed to be responsible for the control of SIV in Agm (Ennen et al., 1994). In addition, long term non progressors seem to recognize a variety of different CTL epitopes already early during HIV infection whereas individuals progressing to disease demonstrated a very weak CTL response needing extensive in vitro restimulation (Dr. T. Harrer, Erlangen, pers. communication).

Therefore one of the key issues in the developing future HIV-candidate vaccines is to induce a strong cell mediated in addition to a humoral immune response towards a complex and, if possible, conserved antigen “cocktail”. In order to meet these requirements novel antigen presentation systems need to be designed. A series of different formulations have been devised in the past in order to achieve favourable antigen presentation ranging from replicating vectors, lipopeptides to particulate carrier molecules. Induction of a strong immune response in absence of adjuvants by particulate structures has been suggested by several groups including ourselves. Based on the hepatitis B virus 22 nm lipoprotein particles, the HBV core particles or by the yeast transposon TY-A gene a number of heterologous antigen presentation systems have been designed allowing the induction of humoral and cellular immune responses to short “foreign” peptides presented by the particulate carrier (Michel et al., 1990; Ulrich et al., 1992; Schlienger et al., 1992; Kingsman and Kingsman, 1988). However, in order to induce protective immune responses to highly variable viruses, the presentation of complex antigens containing a series of conserved B- and T-cell epitopes will be required to restrict immunological escape and to achieve synergistic effects upon immunization.

Results and Discussion

As reported previously, the HIV group specific antigen Pr55\textsuperscript{ag} includes all requirements for the formation of immature HIV virions (Gheysen et al., 1989; Wagner et al., 1992a). The Pr55\textsuperscript{ag} molecules are synthesized on free ribosomes and accumulate at the inner side of the plasma membrane where they form the immature viral particle that is released from the cell by budding. However, the HIV-1 Pr55\textsuperscript{ag} polyprotein precursor is not only the catalyst of viral assembly but seems to play a key role in the control of disease progression by including a number of T-helper and cytotoxic T-cell (CTL) epitopes. In the context of recent studies on long term survivors there is increasing evidence, that cell mediated immunity towards conserved epitopes and reading frames including the gag-gene product might correlate with an extended asymptomatic phase or even complete control of the disease. These considerations prompted us to investigate the possibility of constructing a novel, noninfectious and per se highly immunogenic antigen presentation system on the basis of Pr55\textsuperscript{ag} virus-like particles (VLP) (Wagner et al., 1991). However, to extend the immunogenic potential of the autologous gag-VLPs, two different concepts have been elaborated (Fig. 1). Construction of these VLPs and immunogenicity studies have been performed as follows:
**“Foreign” epitopes inserted into chimeric VLPs**

This concept follows the construction of Pr55\(_{\text{gag}}\) expression cassettes allowing the insertion of carefully selected epitopes from HIV reading frames other than gag. Resulting chimeric proteins should assemble into premature VLP when expressed in eucaryotic cells which results in the presentation of additional immunologically relevant epitopes. This concept also allows to exclude epitopes suggested to be associated with adverse side effects such as induction of graft versus host-like diseases (Habeshaw et al., 1992), antibodies enhancing the infection of CD4\(^+\) cells by HIV (Robinson et al., 1990) or gp120 mediated apoptosis (Gougeon et al., 1993). Considering previous data on the characterization of Pr55\(_{\text{gag}}\) assembly domains (v. Poblotzki et al., 1993; Niedrig et al., 1994) careful deletion analysis within Pr55\(_{\text{gag}}\) revealed two domains located within p24CA (aa 211–241) and within the p6L1 moiety (aa 436–471), which are dispensable for the assembly of the mutants to premature VLP (Wagner et al., 1994). Consequently we inserted the gp120 principal neutralizing determinant V3 as a model epitope into these susceptible sites of the Pr55\(_{\text{gag}}\)-deletion constructs or fused this domain to the carboxyl-terminus of the complete precursor protein (Fig. 2). For initial immunological studies, these constructs have been expressed by re-
A: HIV-1.A1 Pr55.gag

B: QCTRPNNTLRIRIGPGRAFVTIGKIGNMROAHCN

Fig. 2: Schematic drawing of the mutant Pr55.gag polypeptides. (A) Deleted amino acids are indicated by open boxes. The associated numbers represent the stretch of deleted amino acids relative to the sequence of the complete HIV-1.A1 Pr55.gag precursor. A carboxy-terminal fusion peptide is indicated as a black box. The wt-Pr55.gag precursor is shown as a reference. The total number of amino acids of the mutant Pr55.gag polypeptides are given in italic letters. The correct designation of the mutant vaccinia viruses (V-) is given at the right. (B) Three domains derived from reading frames other than Pr55.gag were either inserted into the mutant gag polypeptides (Pr55Δ1-Δ3) to replace the deleted sequences or fused to mutant Pr55F4 to generate carboxy-terminal fusionproteins. V3c: composite sequence based on a subset of sequences of the HIV-1 gp120 major neutralizing epitope V3. The complete amino acid sequence of the V3 epitope is shown at the left side of the figure, respectively.

combinant vaccinia viruses in mammalian cells. For large scale purification of chimeric VLP, recombinant baculoviruses were established. After infection of insect cells with the recombinant baculoviruses, chimeric VLP resembling immature virions could be readily rescued and purified from the cell culture supernatants in good purity and yields ranging from 5–20 mg/l depending on the position and epitope integrated (Table 1) (Wolf et al., 1994).

Table 1 Formation of chimeric HIV-1 Pr55.gag/V3 VLP

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Recombinant vaccinia viruses expressing Pr55.gag/V3 chimeric polypeptides induce V3-specific CTLs in vivo

The HIV-1 Pr55.gag precursor itself, which has been described above as a basic component for the presentation of immunologically relevant cell epitopes from recombinant VLPs has been repeatedly demonstrated to be an important target of the cell mediated immune response. However, the suggested importance of a CTL response prompted us to investigate, whether Pr55.gag chimeric antigens could also induce CTL responses to epitopes inserted into or fused to the gag-precursor. One critical aspect in the induction of class I restricted, CD8+ CTLs by chimeric antigens is, whether altered flanking sequences or changes in the local conformation at an antigenic site might codetermine processing and presentation of a translocated epitope. In order to adress this question BALB/c mice were immunized with recombinant vaccinia viruses expressing the Pr55.gag/V3 chimeric polypeptides described above,
whereas a consensus sequence of the V3-domain is located at different positions within the Pr55\textsuperscript{gag}-precursor (v-Pr55\textsuperscript{gag}/V3-1, -2, -3; 10⁸ PFU/mouse) (Wagner et al., 1992b). The ability of the V3 domain to generate CTLs, not only in humans but in BALB/c mice allowed a rapid evaluation of Pr55\textsuperscript{gag}/V3 chimeric antigens in a convenient animal model (Takahashi et al., 1988). Syngenic P815 target cells were used for a 5 days in vitro restimulation of the isolated splenocytes and as targets in a 3 h cytolytic assay. In a first set of experiments (Fig. 3A) splenocytes of BALB/c mice (H-2\textsuperscript{d}) were restimulated in vitro by syngenic targets treated with a 16mer V3 peptide (V3c-16; RIRIGPGRAFVTIGKI). Restimulated effector cells were tested for cytolytic activity against P815 cells pulsed with the indicated peptides. Lysis of target cells was observed irrelevant of whether the homologous 16mer V3c-peptide (V3c-16; RIRIGPGRAFVTIGKI) or the heterologous V3\textsubscript{LAI}-peptide (V3\textsubscript{LAI}-16; RIQRGPRAFVTIGKI) was used for the precoating of target cells (1 μM). Splenocytes, which were not restimulated in vitro did not recognize target cells following incubation with peptide V3c-16, with peptide V3\textsubscript{LAI}-16 or a control peptide (not shown).

In order to compare processing and presentation of the V3-antigenic peptide from the above described Pr55\textsuperscript{gag}/V3 chimeric proteins to that of the original gp120\textsubscript{LAI} external glycoprotein, splenocytes of in vivo primed BALB/c mice were stimulated in vitro by P815 cells pulsed with peptide V3c-16 and an irrelevant peptide nef-16 (Fig. 3B). Target cells were infected with the chimeric Pr55\textsuperscript{gag}/V3 vaccinia viruses, with v-gp120\textsubscript{LAI} and, for control, with v-Pr55\textsuperscript{gag} and wild type vaccinia virus (v-WT). Placing the V3 epitope in different positions in the chimeric proteins in this study did not lead to significant differences in the efficiency of recognition of the V3-epitope by specific CTLs if compared to authentic gp120\textsubscript{LAI}. Treatment of the CTL effector cells with antilyt 2 monoclonal antibody plus rabbit comple-

ment, but not anti-L3T4 antibody plus complement or complement alone led to a loss of killing activity in all cases tested confirming previous data (Takahashi et al., 1988). This demonstrates, that the effector cells recognizing and killing Pr55/V3-1, -2, or -3 and gp120 expressing targets as well as V3-peptide pulsed target cells are conventional Lyt2\textsuperscript{+}L3T4	extsuperscript{-} (CD8\textsuperscript{+}CD4\textsuperscript{-}) CTL (data not shown).

These results demonstrate that a selected domain (V3), known to include a CTL epitope, can replace different regions within an antigenic carrier protein (Pr55\textsuperscript{gag}) without significant loss of biological activity (Wagner et al., 1993).

**Induction of a strong CTL response by chimeric VLPs**

According to new results it seems to be possible to induce CD8\textsuperscript{+} CTLs by exogenously applied lipoprotein particles, lipoproteines or liposome mediated protein transfer. Therefore we tested the capacity of chimeric Pr55\textsuperscript{gag}/V3 VLP to induce a V3-specific CD8\textsuperscript{+} CTL response. Therefore different VLP preparations were injected either intraperitoneally (IP), subcutaneously (SC) and intravenously (IV) into BALB/c mice. Five days post immunization, spleen cells from primed mice were transferred into culture and restimulated with V3 peptide-labelled syngenic P815 cells in a 5 day mixed lymphocyte-tumor cell culture (MLTC). After the 5 days in vitro restimula-

tion, effector cells were tested for specific cytotoxic activity. Target cells in the standard ⁵¹Cr release assay were again syngeneous A20 or P815 cells labelled with a 16mer V3 consensus peptide (RIRIGPGRAFVTIGKI) previously demonstrated to be recognized by V3 specific CTL (Wagner et al., 1992b). The induction of V3-specific CTLs strictly depended on the dose of administered antigen ranging from 20 μg to 100 ng, which was still considered positive. The route of administration whether IP, SC or IV did not influence the CTL reacti-
Altered flanking does not influence the induction of V3-specific CTL

Fig. 3: (A) Induction of V3LAI- and V3c specific cytolytic T-lymphocytes after immunization of BALB/c mice with the indicated recombinant vaccinia viruses. Splenocytes were isolated 2 months after immunization with 10⁸ PFU/mouse of the indicated recombinant vaccinia viruses and restimulated for 1 week in vitro using syngenic target cells precoated with peptide V3c-16 (1 µM; right panel). Percent specific lysis using different ratios effector:target cells (E:T) was determined in a 3 h cytolytic assay after pretreatment of the targets with peptides V3c-16 (RIIRGPGRAFVTIGKI) and V3LAI-16 (RIQRGPGRAFVTIGKI). For control target cells were treated by irrelevant peptides as shown here for peptide nef-16. (B): V3-specific splenocytes were generated by in vivo immunization of BALB/c mice with the indicated vaccinia viruses. The effector cells were restimulated in vitro by syngenic target cells pulsed with peptide V3c-16 and tested in a 3 h cytolytic assay. For this purpose, P815 target cells were infected by the recombinant vaccinia viruses v-Pr55V3c-1, v-Pr55V3c-2, and v-Pr55V3c-3 and for control by v-gpl20, v-Pr55g and wild type vaccinia virus (vWT). The effector:target ratios (E:T) are indicated. Symbols representing synthetic peptides or recombinant vaccinia viruses are given at the left side of the figure in the legends. The percent of specific ⁵¹Cr release achieved for the indicated E:T ratios was calculated as 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release). Maximum release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was determined from target cells prepared as described above and incubated without added effector cells. Spontaneous release was below 10% in all experiments. Standard errors of the means of triplicate cultures were always less than 4% of the mean.

V3 specific CTLs were not only found in splenocytes, but in lymphnodes when tested. Immunization of BALB/c mice with naked Pr55g/V3 VLP efficiently primed the CTL response in absence of adjuvant or replicating vector (69% specific lysis). In contrast VLP adsorbed to alum or emulsfied in IFA only weakly stimulated a CTL response (24%, 37% specific lysis) (Fig. 4). As demonstrated above for different types of Pr55g/V3 recombinant vaccinia viruses, the position of the V3-domain within different variants of chimeric VLP (Pr55g/V3-3, Pr55g/V3-4, Pr55g/V3-5) did not influence the induction of a V3-specific CTL response. In comparison only weak priming of CTL was detected for in vivo priming with recombinant gp160. Immunisation of Pr55g VLP or V3-16mer peptide was not sufficient for priming a specific CTL response (Fig. 5). These data clearly demonstrate that recombinant chimeric VLP represent useful tools for inducing a strong, specific CD8+/CTL response in vivo in addition to a humoral immunity.
VLP presenting various derivatives of the gp120 external glycoprotein

Recently the induction of antibodies has been proven for HIV patients as well as for immunized chimpanzees neutralizing a variety of different HIV strains by recognizing conserved conformations within the gp120 external glycoprotein (Steimer et al., 1991). In order to be capable of inducing this antibody population we established a novel approach, which allows stable and covalent anchoring of gp120 or derivatives thereof on the surface of the recombinant HIV-1 virus-like particles by a heterologous transmembrane (TM)-region. Using this strategy well documented side effects associated with the gp41 transmembrane protein can be excluded. To allow stable presentation of HIV-1 gp120 epitopes in a correct, immunologically relevant conformation, we constructed recombinant baculoviruses expressing chimeric gp120 derivatives, which are covalently linked via their COOH-termini to a heterologous type-1 transmembrane moiety (TM) of the Epstein-Barr-virus (EBV) gp250/350. Both domains are separated by a flexible (gly/ser), hinge region to allow independent folding of both domains. To avoid either un-
Fig. 5: BALB/cJ mice (H-2^d) were either not primed (A) or primed by a single injection of either 50 μg of V3-16mer peptide (B), or 6 μg of the indicated chimeric VPL (Pr55^K5; Pr55^W3-3 to V3-5) (C-F), in absence of adjuvants. Lymphoid cells were prepared from immunised mice 6 days post immunisation and cocultivated with syngenic V3-16mer peptide labelled syngenic P815 cells, irradiated with 20000rad. A control group included unprimed BALB/c cells stimulated in vitro with V3-16mer peptide labelled P815 cells. Cytotoxic effector cell populations were harvested after 5 days of in vitro culture. The cytotoxic response was determined against the syngenic target cell line A20 pulsed for 1h with 10^{-6}M V3-16mer peptide (closed symbols). Negative controls were not pulsed target A20 cells in a standard ^{51}Cr release test (open symbols).
specific cleavage of the gp120 at carboxyterminal cleavage sites or incorrect folding of the env-chimeras, we additionally established derivatives truncated from the COOH-terminus by either five (gp120/5') or 20 amino acids (gp120/20'), linked to the TM. The chimeric proteins were produced in insect cells using the baculovirus expression system. A recombinant baculovirus expressing the complete gp160 HIV-1 envelope protein was constructed as a positive control (Fig. 6). Surface expression of the produced HIV-1 gp120/TM chimeric glycoproteins on infected insect cells has been proven by APAAP-immunostaining. High affinity recognition by a panel of different conformation dependent monoclonal antibodies (Mab) revealed a correct folding of all proteins presented on the cell surface. In order to produce HIV-1 Pr55<sup>gag</sup> VLP carrying stably anchored gp120 on their surface we coinfected insect cells with Pr55<sup>gag</sup> and gp160 or various gp120/TM recombinant baculoviruses. Ultrathin sections of insect cells coexpressing Pr55<sup>gag</sup> and the gp120 derivatives (gp120/TM, gp120/5'<sup>-</sup>TM, gp120/20'<sup>-</sup>TM, gp160) revealed efficient budding of recombinant VLP. Harvested cell culture supernatants were analyzed by sucrose sedimentation gradients revealing almost identical yields of recombinant VLP for all coinfections tested. The antigenic peaks were detected at the expected sucrose density of 1.15–1.18 g/cm<sup>3</sup>. The peak fractions were analyzed by immunoblotting using a panel of different Pr55<sup>gag</sup>, gp120 or gp41 specific monoclonal antibodies recognizing the particulate carrier in addition to the presented gp120 or 160 glycoproteins. All tested peak fractions demonstrated the Pr55<sup>gag</sup> carrier as well as the expected glycoprotein derivative (will be published elsewhere).

To study the capability of the chimeric gag/env VLP to induce cytotoxic T-lymphocytes in vivo, BALB/c mice were immunized with the different gag/env hybrid VLP, again in complete absence of adjuvants or replicating vector (Fig. 7). For control BALB/c mice were injected with V3-peptide (RIQRGPGRAFVTIG-KI), purified gp160 or HIV Pr55<sup>gag</sup> VLP only.

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**Fig. 6:** Construction of chimeric glycoprotein mutants. The wild-type glycoprotein precursor gp160 is diagrammed at the top of the figure. The HIV-1 gp41 transmembrane domain of the gp160 was replaced by a heterologous Epstein Barr Virus (EBV) type 1 transmembrane domain (TM) (53aa), which was linked to the COOH-terminal end of the gp120 by a flexible gly/ser (6aa) stretch (gp120TM). Additionally we evaluated two truncated derivatives lacking 5 (gp120/5'<sup>-</sup>TM) or 20 amino acids (gp120/20'<sup>-</sup>TM) at the COOH-terminus of the gp120TM construct, respectively.
As described above, neither the synthetic V3-peptide nor Pr55<sup>gag</sup> VLP were sufficient to prime an adequate V3-specific CTL response. A comparably weak CTL response could be demonstrated after administration of purified gp160 (Fig. 7). These data clearly indicate, that anchoring of gp160 or derivatives thereoff on the surface of recombinant Pr55<sup>gag</sup> VLPs results in a favourable antigen presentation, which is capable of inducing a highly efficient CTL response to the presented membrane proteins.
Chimeric HIV-1 Virus-Like Particles as a Candidate Vaccine

Conclusion

With respect to a rational vaccine design there is ample evidence that cell mediated immunity rather than humoral immunity plays a crucial role in the control of virus replication and the progression to the symptomatic state of disease. We have constructed a novel per se highly immunogenic vaccine system allowing the presentation of (i) either selected, immunologically relevant epitopes of the complete external glycoprotein or (ii) of derivatives of the complete external glycoprotein by noninfectious, recombinant Pr55\(^{ag}\) VLP. Immunological analysis revealed both, the induction of a humoral (Wolf et al., 1994) in addition to a cell mediated immunity in complete absence of adjuvants. Studies analyzing the safety, immunogenicity and efficacy of the presented candidate vaccines in primates are in progress.

References


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