Neutralizing antibodies to autologous virus are elicited immediately during acute human immunodeficiency virus type-1 infection

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It has been suggested that primary infection with human immunodeficiency virus type-1 (HIV-1) induces only few antibodies that recognize native HIV-1 envelope proteins but mostly gp120 specific antibodies directed against immature and degraded forms of the envelope proteins. We here show however that the antibody response during primary HIV-1 infection is only directed against mature properly folded gp120. The presence of these antibodies in patient serum coincided with the capacity to neutralize autologous virus and a primary NSI/R5 HIV-1 isolate, but not the T-cell-line-adapted (TCLA) IIIB virus. After three months, antibodies reactive with gp120 folding intermediates, proteolytically cleaved and degraded envelope proteins developed which preceded the appearance of antibodies able to neutralize the TCLA IIIB virus.

Introduction

The envelope protein complex of HIV-1 controls the key process of viral entry. The envelope protein is initially produced as a precursor, gp160, which is extensively glycosylated and proteolytically cleaved into two subunits by a cellular convertase (1,2). The resulting surface protein (gp120) and transmembrane protein (gp41) remain non-covalently associated and exist as trimers on the surface of the virions, infected cells, and HIV-1 envelope expressing cells (3-5). The finding that the HIV-1 envelope protein complex mediates the binding to CD4 and to a co-receptor (a seven-transmembrane protein of the chemokine family) (6-11), prompted the search for new strategies to inhibit viral entry and inspired the design of new types of HIV-1 vaccines. Most HIV vaccines are composed of recombinant forms of gp120, gp140 or gp160 (12-14). These antigens have been shown to elicit antibodies which only block infectivity of T-cell line adapted (TCLA) viruses or autologous virus from which the antigen was derived (13,15-17).

In contrast, HIV-1 infected long-term non-progressors who tolerated infection more than 8 years, generate antibody responses that are able to neutralize heterologous HIV-1 isolates (18-22). However, the impact of humoral immunity on the clinical course of HIV-1 infection remains unclear and other factors such as cytotoxic T-cell activity, coreceptor polymorphism, and the presence of replication-deficient virus variants may all contribute to disease progression.

It has been proposed that the antibody response does not inhibit viral replication in vivo because it is mainly directed to non-native, immature envelope proteins released from infected cells or virions (23,24). In this hypothesis, only a minority of the antibodies, if any, are directed at neutralizing epitopes that are present on functional envelope proteins as expressed on HIV-1. Such low titers could be due to a lower immunogenicity of the neutralizing epitopes. As a potent anti-HIV neutralizing antibody response remains one of the principal goals in vaccine development, we re-evaluated the humoral response shortly after primary infection with HIV-1 and during asymptomatic follow-up.

Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy blood donors by Ficoll-Isopaque density gradient centrifugation. Cells were stimulated for 3 days in Iscoves modified Dulbecco’s medium (IMDM) supplemented with 10% fetal calf serum (FCS), penicillin (100U/ml), streptomycin (100µg/ml), and phytohaemagglutinin (PHA) (5µg/ml) and subsequently cultured in the absence of PHA. HeLa cells were grown in Minimal Essential Medium (MEM) with 10% FCS.
Viruses

From patient W a biological clone was isolated four days after the patient seroconverted for HIV-1 antibodies. Virus isolation and virus stock preparation was performed on PHA stimulated PBMC according to standard procedures (25;26). The ACH.172B-al was obtained from a broncho alveolar lavage from an HIV-1 infected individual in the Academic Medical Center in Amsterdam. Stocks of ACH172Bα-1 and HIV-1 IIBB were also prepared on human PBMC. Each week, virus production in the supernatant was monitored in an in-house p24 antigen capture ELISA. If sufficient virus production was demonstrated, 50% tissue culture infectious dose (TCID50) was determined by end point dilution. For expression of recombinant proteins Vaccina virus was used (27).

Sera

The rabbit polyclonal serum 40336 (R40336) was raised against purified HIV-1 IIB gp160 expressed in baculo virus-transfected insect cells (28). It recognizes gp160 and gp120 folding intermediates, denatured, as well as native mature env protein. From four patients who seroconverted for HIV-1 antibodies, serum or plasma samples were collected and tested by ELISA for the presence of antibodies against HIV-1 p24. In addition HIV-1 RNA was also determined as described previously (29). Serum samples were obtained from patient W 1 day before SC and 3, 5, 13, 20, 53 and 90 days after SC and one and three years after SC. As control sera we used HIV1g, a preparation of purified polyclonal Ig derived from HIV-infected donors (50) and Amsρ, pooled sera of 34 patients from the Amsterdam Cohort.

Neutralization assay

Serum samples of patient W, HIV1g and Amsρ were tested for neutralizing capacity (29;31). From each virus isolate, a final inoculum of 10 TCID50 in a volume of 100 μl was incubated for 1 hour at 37°C with increasing concentrations of serum. Subsequently, the virus serum mixtures were added to 105 3-days PHA stimulated PBMC in 96 wells microtiter plates. The following day, cells were washed. On days 7 and 14 virus production in culture supernatants was analyzed by an in-house p24 antigen capture ELISA. Means of triplicate experiments of each serum sample were plotted. Percent neutralization was calculated by determining the reduction in p24 production in the presence of the serum sample to cultures with virus only. The 90% inhibitor concentration (IC90) was determined by linear regression.

Metabolic labeling and immunoprecipitation

Pulse chase experiments were performed as described by Braakman et al. (32). In brief, recombinant Vaccina virus infected cells that expressed HIV-LAI gp120 were preincubated in medium lacking methionine and cysteine. The cells were pulse-labeled with 50 μCi [35S]-labeling mix (Amersham Pharmacia Biotech AB), and chased for various times. Cycloheximide was included in the chase to stop elongation of nascent chains. Cells were transferred to ice and washed with ice-cold HBBS containing 20 mM iodoacetamide to alkylate free sulphhydryl groups in the labeled proteins and subsequently lysed in 0.5% Triton X-100 in MNT containing 20 mM iodoacetamide and protease inhibitors (chymostatin, leupeptin, antipain, pepstatin, PMSF and EDTA).

Before immunoprecipitation, protein A Sepharose beads (Amersham Pharmacia Biotech AB) were incubated with R40336 (3 μl) or serum from patient W. Lysates of the pulse chased cells were added and incubated for 15 h at 4°C. The immunoprecipitates were washed twice and pellets were resuspended in 10 mM Tris- HCl pH 6.8 or 0.2% SDS in 100 mM sodium acetate pH 5.5, and heated for 5 min. at 95°C. The latter sample was diluted with an equal volume of 100 mM sodium acetate pH 5.5, followed by addition of protease inhibitors and 0.025 U of endo H (Boehnnger Mannheim GmbH). The endoH treated mixtures were incubated for 2 h at 37°C, before sample buffer was added and heated to 95°C for 5 min. Samples were analyzed by both nonreducing and reducing SDS-PAGE.

Biochemical modification HIV-1 envelope proteins

To study recognition of degraded env-proteins in vivo by patient sera, the circular V3 loop peptide, gp120 (MN) and gp160 (IIBB) were treated with thrombin (EC 3.4.21.5) which activity was stopped by the addition of an excess of hirudin (Sigma Chemicals, St. Louis MO) (28,33). Furthermore, recognition of degraded proteins was studied by treatment with plasmin (10-50 nM) and EndoH (1 U/ml) (Boehnnger Mannheim). After 2h at 37°C, plasmin activity was stopped by an excess of aprotinin and PMSF. The HIV env proteins were prepared in 100 mM ammonium acetate pH 5.5, before EndoH was added and incubated for 16 h at 4°C. The removal of the glycans was checked by SDS-PAGE (data not shown). Proteins and V3 peptide were radiolabeled as described before (28). It was found that 20 μg of peptide or 5 μg of protein were labeled with Na125I using chloramine T for 30 seconds. Preparations were purified by dialysis or gel filtration, and subsequently aliquoted in a final concentration of 0.05% bovine serum albumin (fraction V, Sigma) and stored until use at ~20°C.

HIV-1 envelope protein detection

Env gp120 and gp160 were captured by a lectin, Galanthus nivalis agglutinin (GNA, Boehnnger Mannheim) on ELISA plates prior to detection by antibodies present in serum of patient W. Bound antibodies were detected by alkaline
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Figure 1
Immunoprecipitation of metabolically labeled biosynthetic forms of gp120 (LAI) by patient W sera and a control rabbit serum (R40336). Five hours after infection with the recombinant Vaccinia virus, HeLa cells were pulse-labeled for 10 min and chased for 0, ½, 1, 2, or 4 hours (32). Cells lysates were precipitated for gp120 with R40336 (A), or with sera from patient W (B), obtained 13, 20, 53, or 90 days, or 3 years after SC. On the reduced gel (lanes 6-10), Ru represents uncleaved-immature protein, still containing its signal-sequence and Rc denotes cleaved-mature protein with a higher electrophoretic mobility and lacking the signal-sequence. This cleavage is late and completely post-translational (A.Land et al. in prep.) (37). On the nonreduced gel (lanes 1-5), IT represents immature-incompletely oxidized folding intermediates and NT denotes native, completely oxidized, compact protein in which all disulfide bonds have been formed and the signal-sequence has been removed. The asterisk designates a prominent Vaccinia virus background band. Experiments were repeated with longitudinal serum samples from four other patients who seroconverted for HIV-1 antibodies, showing the same gp120 immunoprecipitation profiles (data not shown).

Results
HIV envelope protein recognition by patient sera during primary infection
HIV-1 envelope recognition of sera from patient W was tested by immuno-precipitation of radiolabeled newly synthesized protein. During biosynthesis, gp120 and gp160 are translocated into the ER where folding, N-linked glycosylation, and the formation of disulfide bonds takes place. Correct folding (and gp160 trimerization) is a prerequisite for further transport to the Golgi complex and finally the plasma membrane. A previously developed pulse-chase assay based on the difference in electrophoretic mobility between fully and partially oxidized gp120 was used (34) (A.Land et al. in prep.). Lysates of HeLa cells infected with a recombinant vaccinia virus carrying the LAI gp120 cDNA (27) were precipitated in parallel with R40336 (Figure 1a) and sera from patient W (Figure 1b). The immunoprecipitates were deglycosylated before analysis by nonreducing and reducing SDS-PAGE (35). Serum R40336 precipitated all forms of gp120 present during biosynthesis. Within 4 h after the chase, precipitation with R40336 revealed a shift...
from immature-uncleaved folding-intermediates (band RU, and IT, in the reduced and nonreduced gels, respectively) to cleaved-native protein (RC and NT in the reduced and nonreduced gels, respectively) (Figure 1a). The same cell lysates were used for immunoprecipitation with sequential serum samples from patient W. In contrast to R40336, the cleaved NT form of gp120 was virtually the only form of gp120 that was recognized by patient sera under nonreducing conditions. Only after 90 days and 3 years after SC a faint band of uncleaved gp120 was detected (Figure 1b). In addition, these two serum samples also bound uncleaved gp120 in the reduced gel (designated RU in Figure 1b). Although the earlier folding intermediates of gp120 that were recognize by R40336 in the nonreduced gel remained unrecognized by the patient sera, the humoral response to gp120 appeared to broaden, as it originally was only directed against mature gp120.

The early humoral response to HIV-1 envelope proteins was also analyzed by ELISA using lectine captured purified recombinant gp120 (MN) and gp160 (IIIB) (Figure 2). Shortly after seroconversion, a weak but specific response to both rgp120 and rgp160 appeared, which increased after SC. This confirms our findings that an antibody response against native mature gp120 was mounted after HIV-1 infection, with serum titers gradually increasing.

HIV-1 neutralization with longitudinally obtained serum samples

The capacity of longitudinal serum samples of patient W to neutralize an autologous virus isolated 4 days after SC (W/IV), the primary isolate ACH.172Ba-1 (R5/NSI) and the TCLA IIIB (X4/SI) was determined. All viruses were efficiently neutralized by the two pooled control sera HIV1g and Ams<sup>hp</sup> (Figure 3a and 3b). The serum sample taken before SC did not neutralize any of the viruses. The autologous virus W/IV was neutralized by the three patient sera obtained from SC to day 53 post-SC, but only at the highest concentration (1:20). Interestingly, a serum sample taken 3 years after SC did neutralize the autologous virus W/IV much more efficient (Figure 3a). In contrast to IIIB, which was only neutralized by serum obtained 3 years after SC, the virus isolate ACH.172Ba-1 was neutralized already by serum obtained 13 days after SC and this ability increased over time (Figure 3b).

Antibody recognition of V3 and gp120 after protease treatment

Serum samples of patient W obtained around SC indeed contained antibodies which are able to neutralize primary isolates. Serum obtained 3 years after SC could efficiently neutralize early autologous virus but also the TCLA IIIB isolate. Neutralization of IIIB by this serum sample may be explained by a switched recognition of envelope epitopes by the patient W serum. For
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Figure 3
Neutralization sensitivity determined on human PHA-stimulated PBMC of autologous virus W/IV, isolated 4 days after SC (A), or the heterologous viruses IIIB and ACH.172Ba.l (B) which were incubated with increasing dilutions of serum samples taken from patient W (2 days before SC, at SC, 7 and 53 days and 3 years after SC) and the control sera HIVIg and Ams<sup>hps</sup>. P24 production was measured and mean OD values were calculated from triplicate cultures. Percentage neutralization was calculated by determining the reduction in p24 production in the presence of the serum samples relative to control cultures lacking these sera. IC<sub>90</sub> values were calculated by linear regression.

that reason, we studied the antibody recognition patterns in longitudinal serum samples with envelope proteins treated with proteolytic enzymes in vitro. Digestion of gp120 by proteins involved in coagulation and fibrinolysis that are present in the blood may occur. The circular V3 loop peptide of ACH.172Ba.l and gp120 (MN) were treated with two serine proteases, thrombin and plasmin, or proteins were deglycosylated by endoH treatment. Binding of antibodies present in sera obtained 3 and 90 days and 1 year after SC to the endoH and plasmin treated V3-loop, but not after digestion by thrombin increased over time, suggesting that antibodies are elicited by
degraded envelope proteins (Figure 4a). This was confirmed by the increased recognition of endoH and trypsin, but not plasmin treated gp120 by the patient W sera. Since plasmin is known to cleave gp120 at the carboxyterminal end which is not recognized by Abs present in serum, the binding to plasmin treated V3 suggests that these Abs recognized linear V3 which is not present on gp120. Altogether these data suggest that the early humoral response is directed towards mature properly folded gp120 rather than towards intracellular gp120 folding intermediates prominently present in cellular debris. The ability of serum obtained later after SC to block infectivity of TCLA virus may reflect the gradual broadening of the antibody response against protease cleaved HIV envelope protein, which epitopes are also expressed on TCLA viruses.

Discussion
We here report on the repertoire of the antibody response during primary HIV-1 infection and its capacity to neutralize autologous and heterologous HIV-1. First we studied in detail the antibody recognition pattern to native HIV-1 envelope gp120 and its folding intermediates, using pulse-chase experiments (32). Despite the use of highly purified gp160 for immunization, the R40336 serum recognized all protein

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Figure 4
Recognition of serum samples obtained from patient W 3 and 90 days, and 1 year after SC to 125Iodinelabeled circular V3 peptide from ACH.172Ba-l (CTPRNNTRKSHIGPRTFYTTGEIIGDIRQAHC) (28) (A) or MN derived gp120 (B). The radiolabeled antigens were presented in fluid phase before and after treatment with thrombin, plasmin and endoH. The binding of the antibody is expressed in percent bound radiolabeled antigens calculated from the input value. Values are corrected for nonspecific binding. The same results were obtained with rgp160 IIB (data not shown).
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conformations. These different protein conformations may indeed have been present as during immunization the antigen may have been prone to proteolysis (2,36-38). In contrast, only the mature, properly folded form of gp120 was recognized by sera obtained from 13 up to 53 days after SC of patient W. Only three months after SC, sera could precipitate uncleaved, immature forms of gp120. Our data demonstrate that the primary immune response against HIV-1 gp120 is strictly conformation dependent, with no cross-reactivity against folding intermediates of gp120. Although we can only speculate on the nature of the conformational epitopes, the early immune response seems to be directed against membrane-exposed gp120 that has traversed the Golgi complex for which a correct folding of the envelope protein is a prerequisite. Our results are thus in disagreement with the suggestion that the early antibody response would primarily be elicited by cell-derived folding intermediates of gp120 released by lysis of infected cells or shedded from the viral surface (15,39-41).

The reactivity of sera from patient W with lectin-captured native gp120 (MN) and gp160 (IIIB) was demonstrated by ELISA, and could be observed 3 days after SC onwards, pointing to a higher sensitivity of this assay as compared to the immunoprecipitation assay. Antibody reactivity to gp120 and gp160 was identical, however from 13 days after SC, the antibody response to gp120 rapidly increased. Pulse-chase experiments with metabolically labeled envelope of gp160 of ACH.172.Ba-l or LAI and the sera of patient W obtained at day 13 after SC revealed a band of gp160 and traces of uncleaved gp160 under reducing conditions (data not shown). Since gp120 is only recognized as mature native gp120 we conclude that the recognition of uncleaved gp160 is most likely mediated by antibodies directed against gp41 (12).

There is no direct unequivocal evidence for a role of neutralizing antibodies in the control or prevention of HIV-1 infection, although so called long term survivors reportedly have higher titres of neutralizing antibodies than individuals with a progressive disease course (18-22;42). In addition, there is some evidence for a correlation between the presence of maternal neutralizing antibodies and reduced HIV-1 transmission to the fetus (43;44). At the highest concentration tested, serum samples obtained 7 and 53 days after SC were able to neutralize the autologous virus of patient W obtained at day 4 after SC and the ACH172.Ba-L isolate. The absent neutralization at higher serum dilutions may point to high absorption of otherwise neutralizing antibodies to free virions during the viremia of primary infection. The neutralizing activity in high dilutions of the serum sample obtained 3 years after SC against the autologous virus may point to cross-reactivation of memory B cells that were first activated at the time of primary infection. Alternatively, neutralizing epitopes may not have changed over time, continuously activating the same B cell repertoire.

In contrast to the low autologous neutralizing capacity, an increasing neutralizing activity against primary HIV-1 isolate ACH172.Ba-l was observed in serum obtained from 13 days after SC onwards. This increase coincided with the increase of antibody reactivity against native mature gp120 in the pulse-chase experiment. The differential neutralization of autologous virus and the ACH172.Ba-l variant may lie in a better exposure of the relevant epitopes on the ACH172.Ba-l virus which may be a consequence of long term propagation in PBMC in vitro.

The early serum samples were unable to neutralize the TCLA IIIB variant, suggesting the absence of V3 loop directed antibodies generally involved in the neutralization of TCLA viruses. Indeed, both in the early and late serum samples, antibodies with reactivity against the ACH172.Ba-l derived V3 loop peptide lacked reactivity with the IIIB derived V3 loop peptide (data not shown). The appearance of antibody reactivity against folding intermediates of gp120 in later serum samples coincided with neutralizing activity against the TCLA IIIB variant.
In conclusion, we here show that during primary infection antibodies are elicited against mature, properly folded native gp120 and that these antibodies have neutralizing activity against autologous HIV-1. Subsequently, antibodies directed against folding intermediates emerged which most likely lack neutralizing capacity against primary isolates. As these antibodies may have high affinity for gp120, they may even interfere with neutralizing antibodies by competition and steric hindrance. This observation may provide new directions for future vaccine development as vaccination also elicits non-neutralizing antibodies that may reduce the neutralizing antibody efficacy. Not only should an HIV vaccine be composed of an envelope protein complex in which membrane associated conformational epitopes are exposed within the oligomeric structure of gp120, it also seems to be important to avoid the deterioration of the vaccine protein complex into antigenic preparations in which highly immuno-dominant epitopes become accessible to the immune system, thereby preventing the generation of non-neutralizing, or even interfering, antibodies.

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