Changes in HIV-specific antibody responses and neutralization titers in patients under ART

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TABLE OF CONTENTS

1. ABSTRACT

In this study, we tested for antibody reactivities against gp120 and gp41-derived peptides, recombinant gp160, gp41 and tat in HIV-positive sera under antiretroviral therapy (ART) and determined their neutralization capacity. As a baseline, sera from patients in stage A, B and C of the disease, long term non-progressors (LNPs) and HIV-negative individuals were included. Compared to LNPs or sera from patients in group A, the reactivity of sera in stage B or C against gp120-derived peptides was reduced parallel to disease progression. Reactivity of these samples was compared with sera of patients under ART. Parallel to the decrease of viral load, the reactivity against gp120 and gp41-derived epitopes, recombinant gp160 and gp41 or the native gp120/41 complex was significantly reduced. Antibody-mediated neutralization of HIV-1 was detectable prior to ART but revealed substantial decreases coupled with progression of therapy. Responses to recombinant tat dropped after three months of therapy, increased however at later time points to initial levels. These data indicate that in parallel to the decrease in viral load and antibodies against gp120, the neutralization capacity of sera under ART is reduced, and can not be compensated by an increase in tat-specific antibodies.

2. INTRODUCTION

Infection with immunodeficiency virus (HIV) results in massive impairment of the immune response. T cells, the main target of HIV, are severely affected and permanent activation of B cell results in hypergammaglobulinemia due to production of HIV-specific antibodies (Ab) and polyclonal B cell stimulation (1-12), which is enhanced by IL-15 (13).

Beside Ab against gag and viral accessory gene products, such as tat, a substantial amount of HIV-specific Abs is directed against gp120 and gp41, the envelope glycoproteins (14, 15). There is a modest decrease in over all amount of Ab against the env proteins during the progression of infection, and reactivity changes against certain epitopes are observed (16, 17). Neutralizing Ab titer response to the env proteins in HIV-1 infected individuals against autologous and heterologous viral isolates are usually low and are absent in the first few months post infection (18, 19). A correlation of Ab titers against the viral envelope proteins between long-term nonprogressors or rapid progressors is controversial (16, 17, 20, 21) and it is also not clear, whether Ab titers against tat are an indicator for a slow disease progression (22, 23). However, a decline of gag Ab seem to be a good prognostic marker for progression to AIDS (24, 25).

Table 1. Amino acid sequence of gp120 and gp41-derived peptides used in the study

HIV-1 gp 120 Peptides	Sequence
Peptide 7	NFDMWKNDMVEQMHEDIISL
Peptide 8	EQMHEDIISLWDQSLKPCVK
Peptide 9	WDQSLKPCVKLTPLCVSLK
Peptide 22	VSTVQCTHGIRPVVSTQLLL
Peptide 28	NTRKRIRIQRGPGRAFVTIG
Peptide 29	RGPGRAFVTIGKIGNMRQA
Peptide 30	KIGNMRQAHCNISRAKWNNT
Peptide 35	GEFFYCNSTQLFNS
Peptide 38	TEGSNNTEGSDTITLPCRI
Peptide 40	KQIINMWQKVGKAMYAPPIS
Peptide 41	KAMYAPPISGQIRCSSNITG
Peptide 47	EPLGVAPTKAKRRVVQREKR
HIV-1 gp 41 Peptides	
Peptide 54	GAAGSTMGAVSLTLTC
Peptide 56	LLSGIVQQQNNLLRAC
Peptide 58	HLLQLTVWGIKQLQAC
Peptide 59	KQLQARVLAVERYLRC
Peptide 60	ERYLRDQQLLGIWGC
Peptide 61	GIWGCSGKLICTTAVPWNAS
Peptide 62	CTTAVPWNASWSNKS
Peptide 63	WSNKSLEDIWDNMTWC
Peptide 64	DNMTWMQWEREIDNY
Peptide 66	TLLEESQNQQEKNEQC
Peptide 67	EKNEQELLELDKWASC
Peptide 69	SITNWLWYIKIFIMIC
Peptide 66a	NYTSLIHSLIEESQNQQEKNEQELLELDKWAS

Due to antiretroviral therapy (ART) various immunological parameters of HIV-infected patients improve significantly. CD4 cell counts are raising, neopterin concentrations decrease and opportunistic infections such as candidiasis are hardly observable (for reviews see 25, 26). T cell activation is reduced and the improvement of antigen-specific and non-specific T cell functions are well documented (25-32). However, only limited data are available, which focus on the effect of ART on B cell reactivity (5-12). A significant reduction in the Ab response against gp120 or p24 and a normalization of hypergammaglobulinemia is reported. Additionally, production of neutralizing Ab in a limited number of patients under therapy was investigated (10). In this study we examined the Ab responses of sera derived from chronically HIV-infected individuals with and without receiving ART against certain epitopes in the envelope glycoproteins. In addition, we investigated the reactivity of sera under therapy against the native form of the gp120/41 complex, possible changes in neutralizing capacity and responses against the viral tat protein.

3. MATERIALS AND METHODS

3.1. Antibodies, HIV-1 synthetic peptides and proteins

For detection of antibodies various peptides derived from gp41 and gp120 (listed in Table 1) were obtained through the Medical Research Council (MRC) AIDS Reagent Project (Herts, UK) and the NIH AIDS Research and Reference Program. The soluble recombinant glycoprotein sgp160 was a gift from Baxter (Vienna, Austria), sgp41 was from Biotest (Dreieich, Germany). The

tat-protein was generously distributed by F. Embl (University of Heidelberg, Germany). The peptides 66a and A_2 were gifts from A. Clivio (Milano, Italy). Peroxidase-conjugated rabbit anti-human IgG was from DAKO, Glostrup, Denmark. The broadly neutralizing human monoclonal antibody 2F5 directed against the conserved ELDKW epitope on gp41 was kindly provided by H. Katinger (Polymun, Vienna, Austria).

3.2. Patient population

Blood samples were obtained from HIV-1 seropositive patients attending the University Hospital of Innsbruck, Austria. Sera were heat inactivated (56 °C, 30 min) and stored at –80 °C. In total, serum samples from 122 individuals were screened. 86 samples were from HIV-1 infected patients with untreated HIV infection at different stages of disease, 9 persons were regarded as long-term non progressors and as a control 27 seronegative individuals were included. To evaluate possible changes in the antibody reactivity during therapy a panel of 40 serum samples from 10 HIV-infected individuals was examined. The first batch was collected before therapy was initiated, the following samples 3, 6 and more then 9 months after receiving antiretroviral drugs. All patients received two NRTIs (D4T in combination with 3TC, Retrovir or Videx; mean 43.6 months) and PI (IDV or RTV; mean 29.2 months), five got additionally an NNRTI (EFV; mean 26 months). The serum samples from the latest time point tested (more than 9 months) contained 2 NRTIs and 1 PI. One patient received in addition a second PI, and one patient an NNRTI. A summary of immunological and virological parameters are given in table 3 Patients of this cohort were all chronically infected and at least for two years HIV-1 positive. HIV-infection was monitored by quantification of viral load, urine neopterin and CD4^{+}T cell counts from these ten patients in periodical intervals. Presence or absence of candidiasis was also determined. HIV-1 RNA was measured by a commercially available reverse transcription-polymerase chain reaction assay (Amplicor HIV Monitor test, Roche Diagnostic Systems, Branchburg, N.J., USA). For the measurement of CD4^{+}T cells fluorescein-isothiocyanate- or phycoerythrin-labelled monoclonal antibodies from the Leu series (Becton-Dickinson, Mountain View, Calif., USA) and a lysed whole-blood technique were used. Flow-cytometric analysis was performed with FACScan (Becton-Dickinson). Urine neopterin was measured by radioimmunoassay (Henning, Berlin, Germany).

3.3. Serum antibody assays

Anti-HIV serum reactivity to 12 peptides of gp41, or gp120, to recombinant soluble gp41 (sgp41) and gp160 (sgp160) was determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates (Greiner, Kremsmünster, Austria) were coated with 50 µl of the peptide solution in TRIS coating buffer (pH 9.6) at following concentrations: peptides derived from gp41 and gp120 with 500 ng per well, sgp41 with 200 ng per well and sgp160 with 150 ng per well. Experiments were performed in triplicates. After incubation overnight at 4 °C, the plates were washed three times with washing buffer [0,1 % (v/v) Tween 20 in phosphate-buffered saline (PBS)].

The wells were then blocked with 3 % (w/v) skim milk (Difko Laboratories, Detroit, USA) in PBS for 45 min at room temperature. The plates were washed again three times and diluted serum samples (1:50 in PBS, 50 µl per well) were added and incubated for 1 h at room temperature. The plates were washed with washing buffer and bound antibodies were detected by addition of 50 µl of rabbit anti-human IgG labeled with horseradish peroxidase (DAKO, Glostrup, Denmark), diluted 1:6000 in 0.1 % (w/v) skim milk in PBS for 1 h at room temperature. Wells were washed again and the substrate (0.1 M Sodium acetate pH 5.5, TMB diluted 1:100, 30 % H_2O_2 diluted 1:2000, 100 µl per well) was added. The reaction was stopped with 100 µl of 2 M H_2SO_4 and analyzed for absorbance at 450 nm with the reference wavelength at 620 nm. In order to determine non-specific binding, an unspecific control-peptide (K1) was used. The relative OD $_{450\ nm/620\ nm}$ of all samples was calculated by the following formula:

$$rel.OD = \frac{OD(sample)}{OD(K1)}$$

3.4. Reactivity against tat

To determine the Ab-reactivity of patient sera under therapy to the tat-protein, an ELISA was performed similar as described above. Following modifications were introduced: the concentration of tat was 100 ng per well. The assay was standardized with sera from HIV negative individuals. The median obtained with these sera was set at 1 adsorption unit. HIV positive sera were scored as positive, when the value of adsorption unit was doubled compared to the control group.

3.5. FACS Analysis

For FACS analysis, M8166 cells were infected with HIV$_{IIIB}$. After washing with PBS supplemented with 1% BSA and 0.1% NaN_3 (washing buffer), cells (5 x 10^5/ analysis) were incubated with 50µl of patient sera (1:50 or 1:500) for 30 min on ice followed by further washing steps. Samples were then incubated with anti-human IgG-FITC (Dako) in a 1:50 dilution, washed again and fixed with 200µl PBS/1% Formalin/0.1% PBS. As negative control, a pool of NHS was used. Samples were analyzed by a FACScan flow cytometer and Lysis II software (Becton Dickinson).

3.6. Purification of IgG from serum

Purification of IgG from serum was performed using of protein A-sepharose (Pharmacia Biotech). Protein A-sepharose was washed three times with binding buffer (0.1 M sodium acetate, pH 5.0). Thereafter, serum and binding buffer (1:1) were added to the protein A-sepharose for 30 min. Protein A-sepharose was washed four times with binding buffer. Bound IgG antibodies were eluted with 100 mM glycine-buffer, pH 2.5 by gentle shaking followed by centrifugation. The pH of the eluate, which contained the IgG-fraction was subsequently raised to neutral by addition of 0.5 M Tris buffer, pH 7.6.

3.7. HIV neutralization assay

To determine the activity of serum antibodies for their potential inhibition of HIV$_{IIIB}$ replication, purified

IgGs of sera isolated from ten patients with HIV-1 infection before treatment and after receiving antiretroviral drugs were tested. Sera were diluted serially in cell culture medium using tenfold dilutions, starting from the 1:50 and 1:100 dilution, respectively, in a 96-well plate (Costar) to neutralize a fixed concentration (1:5000 final dilution) of a HIV$_{IIIB}$ virus-stock containing 0.7 µg/ml of p24 antigen. HIV$_{IIIB}$ used for infection has been prepared as a cell-free supernatant of infected M8166 cells. The virus-antiserum mixture was then co-cultured with 2.5 x 10^4 M8166 cells in a total volume of 200 µl per well. Experiments were performed in duplicates, and the average values are reported. As a negative control various dilutions of virus (100 µl/well; "input virus") were applied to several wells without adding antibodies. As a positive control the neutralizing monoclonal antibody 2F5 was used (33). Two wells which only contained virus in appropriate dilution (1:5000) in complete medium and two wells with uninfected cells without antibodies were included as additional controls. The relative extent of replication of HIV$_{IIIB}$ incubated in the different sera was determined by using HIV p24 antigen assay as described previously (34). Neutralization titers were calculated using the ID$_{50}$ software available from the National Center for Biotechnology Information (National Institute of Health, author J.L. Spouge). The program follows the sequence Poisson model, descriptive logistic fit, Spearman-Karber calculation, with omission of error estimation for the Spearman-Karber fit.

3.8. Statistical analysis

Differences between control values and patient sera were statistically analysed by SPSS 11.0. Correlation coefficients were obtained from medians by unpaired students t test or Mann-Witney U test for continuous variables; p-values lower than 0.05 (p < 0.05) were scored as significant.

4. RESULTS

4.1. Reactivity of sera from patients without treatment to peptides of gp120 and gp41

Serum reactivity to 12 gp120-derived peptides from 9 persons who were regarded as long-term non progressors (LNPs), from 41 patients in stage "A" of HIV (according to the 1987 definition of the Centres for Disease Control), from 17 patients in stage "B" and from 28 patients in stage "C" were tested by ELISA to evaluate differences in the antibody-levels during different stages of infection. Furthermore, 27 sera of HIV-negative persons were included. Compared to uninfected individuals, serum samples from patients with untreated HIV-1 infection contained considerable amounts of antibodies against peptide #9, a region at aa 111-129 in gp120, against peptides #28 (aa 305-324) and #30 (aa 326-345), which are parts of the V3-loop and against peptide #47 (aa 494-513) at the C-terminal end of gp120. The progress of disease was coupled with a substantial decrease in serum reactivity to all four peptides mentioned above from stage "A" to stage "C" (Table 2). The samples were also screened for the presence of antibodies directed against 12 peptides derived from gp41. All sera from patients in stage "A", "B"

Table 2. Clinical parameters defining the group of 10 patients under antiretroviral therapy (mean values   range)

Time	viral load, log 10 [copies/ml]	urine neopterin [µmol/mol creatinin]	CD4 cells/µl	Candidiasis
Before therapy	5.67 3.85-6.24	731 160-1616	92 1-178	6/10
3 months	2.36 2.04-3.01	214.5 93-613	188.5 25-394	0/10
6 months	2.305 2.16-3.17	157 102-241	216.5 94-490	0/10
11 to 38 months	1.91 1.70-2.24	129 53-455	420.5 104-917	0/10
Standard values	0	<137	410-1590	0

Table 3. Serum reactivity (relative O.D.) of HIV positive and negative individuals against different peptides (#9, #28, #30, #47, #60, #61, #66, #67) derived from gp120 and gp41.

	# 9	# 28	# 30	# 47	# 60	# 61	#66	# 67
LNP (n=9)	1.564	1.540	1.681	2.238	4.384	3.912	1.309	1.137
A (n=41)	1.667	2.362	1.876	2.500	4.296	3.338	1.673	1.552
B (n=17)	1.463	1.940	1.926	1.623	2.336	1.796	2.226	1.802
C (n=28)	1.336	1.752	1.703	1.374	4.969	2.104	1.585	1.884
Neg. (n=27)	0.987	0.929	1.049	0.979	1.123	0.889	1.146	1.121

or "C" were found to be positive for antibodies against peptides #60 (aa 585-599), #61 (aa 595-614), #66 (aa 645-660) and #67 (aa 655-670). In case of peptide #60, the median serum antibody-concentration in stage "A" of HIV-infection (4.296) was higher than in stage "B" (2.336) but increased again in stage "C" (4.969). The median amount of antibodies to #61 was significantly higher in stage "A" (3.338) than in stage "B" (1.796). Compared to stage "B", stage "C" showed slightly higher levels of antibodies (2.104) but lower levels than in stage "A". Moderate increased levels of antibodies directed against peptide #66 were expressed in stage "B" (2.226) compared to stages "A" (1.673) and "C" (1.585). Only low levels of antibodies directed against peptide #67 were expressed in stage "A" of infection (1.552) which remained more or less constant as infection progressed (1.884 in stage "C"). LNPs exhibit a similar pattern as patients in stage "A".

4.2. Reactivity of sera from patients under ART to peptides of gp120 and gp41

Sera from different time points of ten HIV-infected patients treated with ART were examined. HIV-infection was monitored by quantification of viral load, urine neopterin and numbers of $CD4^+T$ cells at the entry of therapy and after several months of ART (median values are summarized in Table 3). Presence or absence of candidiasis was also determined. Before therapy a chronic $CD4^+T$ cell loss had to be ascertained. After three months of therapy $CD4^+T$ cell counts were doubled (92 $CD4^+T$ cells /µl versus 188.5 $CD4^+T$ cells/µl). 11 to 38 months after induction of ART normal $CD4^+T$ cell numbers were observed (420.5 $CD4^+T$ cells /µl). All patients were found to be negative for candidiasis after 3 months of ART, although six of ten patients had suffered from candidiasis when they had been naïve to antiretroviral therapy. The mean HIV RNA copy number prior to treatment was 5.67 log 10 HIV RNA copies/ml. During the course of therapy a significant drop of viral load could be observed (1.91 log 10 HIV RNA copies/ml after 11 to 38 months of therapy). Furthermore, each of the HIV-positive patients under ART experienced progressive reductions in urine neopterin values over the observation period (mean values decreased from 731 to 129 µmol urine neopterin / mol creatinin).

Sera were screened for their serum reactivity to 24 peptides derived from gp41 and gp120 by ELISA. In all serum samples tested, antibodies directed against peptides #28, #30, #47, #61, #66 and #66a could be detected. As shown in Figure 1, during the course of therapy serum reactivity to these peptides clearly decreased. The median serum reactivity to peptide #28 dropped from 1.419 before therapy to 1.008 11 to 37 months after set-up of ART. Antibody level to peptide #30 decreased from 2.025 to 1.501. The median serum reactivity to peptide #47 obtained before therapy was 2.146 and dropped to 1.122 at the end of the observation period. Median antibody-levels to peptide #66 decreased from 2.492 to 1.306, and serum reactivity to peptide #66a decreased from 2.354 at the time before therapy to 1.294 to study end. In general, the amount of antibodies showed the highest levels before therapy. Only the antibody level to peptide #61 peaked at 3 months after the induction of ART (3.597) and decreased thereafter again to 1.312.

4.3. Reactivity of sera from patients under ART to sgp41 and sgp160

The levels of antibodies directed against the transmembrane envelope glycoprotein sgp41 and the precursor protein sgp160 were examined. Sera were diluted as indicated starting from a 1:100 and 1:500 dilution, respectively. A serum pool of ten HIV-negative sera was used as a control. As described for gp120 and gp41, all serum samples tested contained antibodies to sgp41 and sgp160 and nine of ten patients exhibited decreases in serum reactivity during the course of therapy. As a representative example the results of patient #3 are presented (Figure 2). The exception was patient number #6 who showed a moderately but not significantly increased serum reactivity to sgp160 (not shown).

4.4. FACS analysis of sera under ART with HIV-1$_{IIIB}$-infected cells

To further evaluate whether Ab titers against conformational epitopes are changing during therapy FACS assays were performed. M8166 cells infected with HIV$_{IIIB}$ were incubated with patient-sera collected before and at different intervals during therapy (Figure 3). In contrast to the results obtained by ELISA experiments, which showed a clear drop in HIV-specific Ab titers already after three
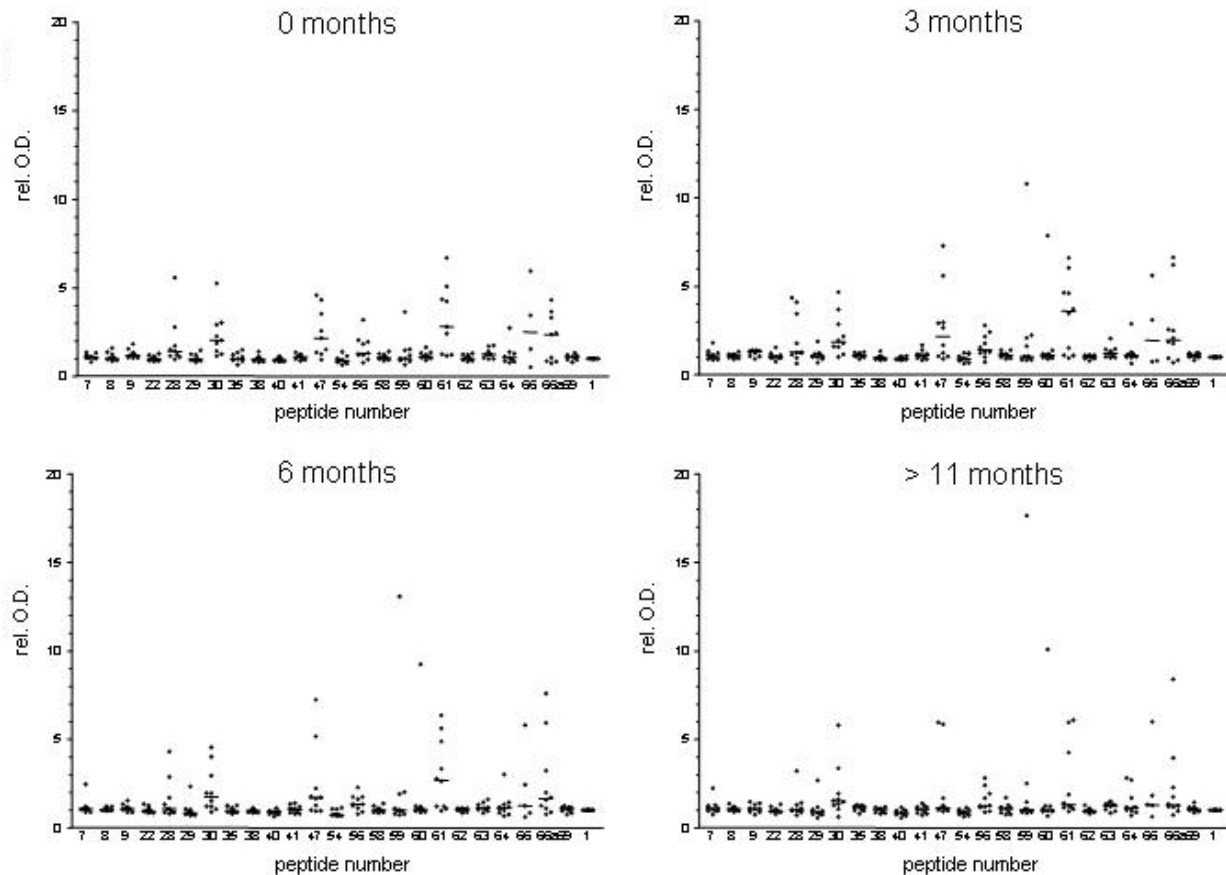
Figure 1. Reactivity of patient sera before and during antiretroviral therapy against gp160-derived peptides in an ELISA. Each serum, obtained at four different time points (as indicated in the graph) was tested in duplicates. The dots represent the serum of an individual patient. Reactivity of the sera against gp160-derived peptides was calculated as described in the material and method section, the bars indicate the median of the relative optical densities.

months of ART, the reactivity of the sera against the native gp120/gp41 complex remained unchanged during the first 6 months of therapy with a median of 15.075 before the ART was initiated compared to a median of 14.6 after 3 months and 15.175 after six months of therapy (Figure 3). After 11 months however, the Ab concentration against native gp120/gp41 decreased significantly and dropped to a median of 9.75 (Figure 3). This trend was observable in all patients with the exception of patient #2, who showed constant antibody levels over the whole observation period (data not shown). A 1:500 dilution of all samples confirmed the results obtained with the 1:50 serum dilution (data not shown).

4.5. Reactivity of sera from patient under ART to tat

To test Ab responses against tat as a representative of HIV regulatory proteins, an ELISA was performed. The assay was normalized by determining the reactivity of ten sera of HIV negative individuals against tat and by defining the median obtained by this control group as 1 adsorption unit. The tat protein, fixed to a microtiter plate was recognized by all HIV[+]-sera before therapy (median 2.95) except two individuals, who remained below 2 adsorpton units, the double value given by the control group (Figure 4). The antibody titer against tat dropped

after therapy was initialised and reached after three months nearly background level (median 1.4). Surprisingly, after 6 months of therapy, the antibody response against tat increased and at the last time point determined, all except one patient reacted with the tat protein again with a median of 2.96, which was comparable to levels before therapy was started.

4.6. Effect of ART on antibody-mediated neutralization of HIV-1

We next sought to determine whether highly active antiretroviral therapy might affect the titer of neutralizing antibodies. To detect also minor changes in neutralization capacities, a lab adapted strain (HIV$_{IIIB}$) was chosen, which are known to be more susceptible to neutralization compared to primary isolates. To exclude any inhibition of HIV-1 replication due to the presence of antiretroviral drugs in the sera of treated patients, IgGs were purified as described in the material and Method section. Isolated IgG were incubated with HIV-1$_{IIIB}$ and M8166 cells and the anti-HIV humoral response was measured for each of the patients prior to and at various times under ART. The results of these neutralization assays were monitored by HIV p24 ELISA (Table 4).
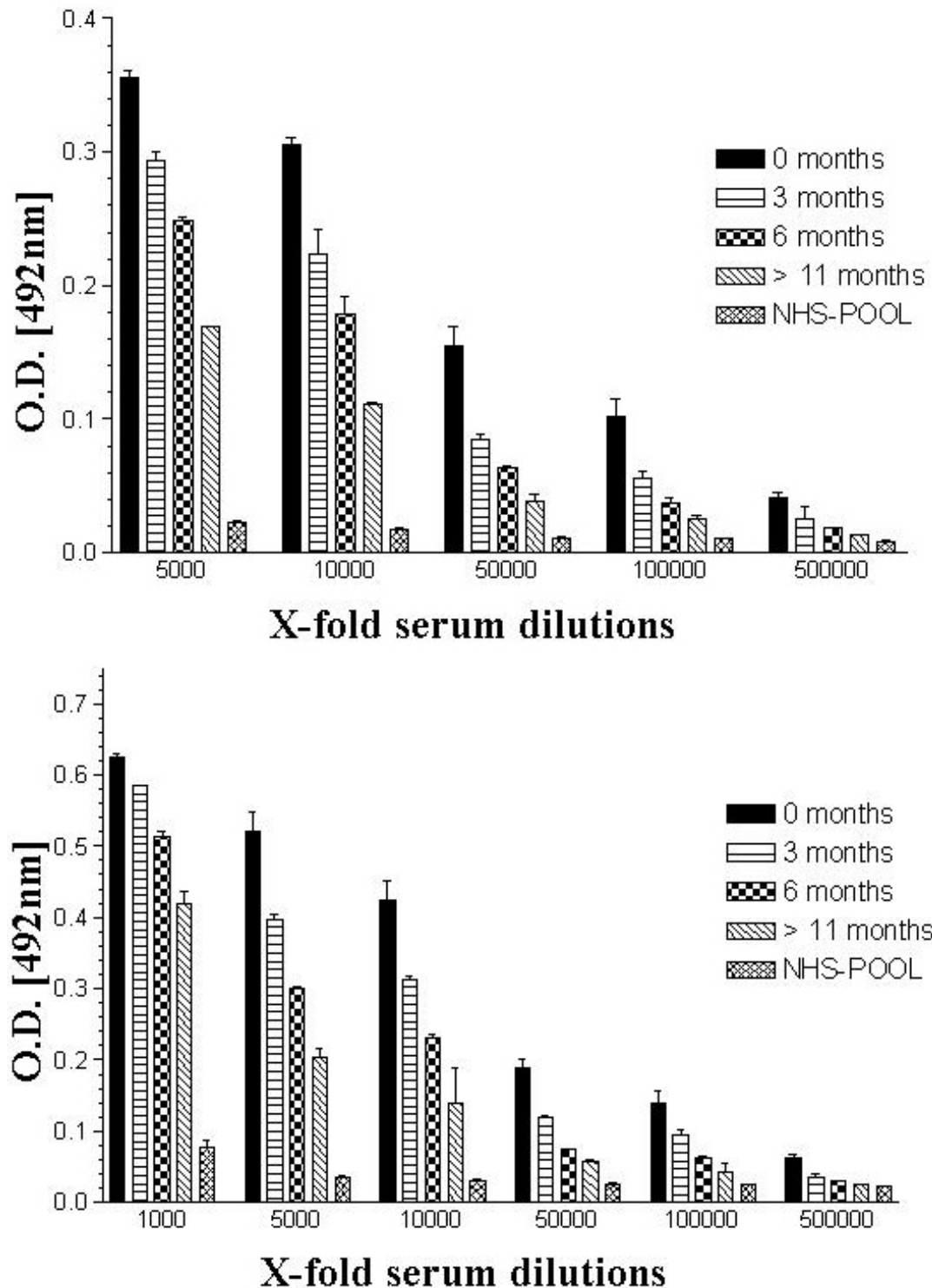
Figure 2. Reactivity of patient sera before and during antiretroviral therapy against recombinant gp41 (upper graph) and recombinant gp160 (lower graph) in an ELISA. Each serum, obtained at four different time points (as indicated in the graph) was tested in duplicates at various dilutions. The bars represent the optical densities obtained by ELISA of an individual representative patient (patient #3). During therapy, the antibody titers decreased from time point "0 month" to "11 months" at all dilutions tested. All other patients behaved similar, except patient #6, who showed a slight but not significant increase in the serum reactivity against both recombinant proteins. For comparison, a pool of normal human sera (NHS) is included.

Table 4. Neutralization titers of sera of HIV-infected individuals obtained at different time points of therapy

	Neutralizing anti-HIV antibody-titers			
	prior to therapy	3 months of ART	6 months of ART	11 to 38 months of ART
Patient 1	5000	5000	500-1000	500
Patient 2	500	500	1000	500
Patient 3	100	< 50	< 50	< 50
Patient 4	1000-5000	500	1000	500
Patient 5	1000	500	500	< 50
Patient 6	< 50	< 50	< 50	< 50
Patient 7	500	500	100	100
Patient 8	< 50	< 50	500	500
Patient 9	100	< 50	< 50	< 50
Patient 10	Not determined	500	500	50-100
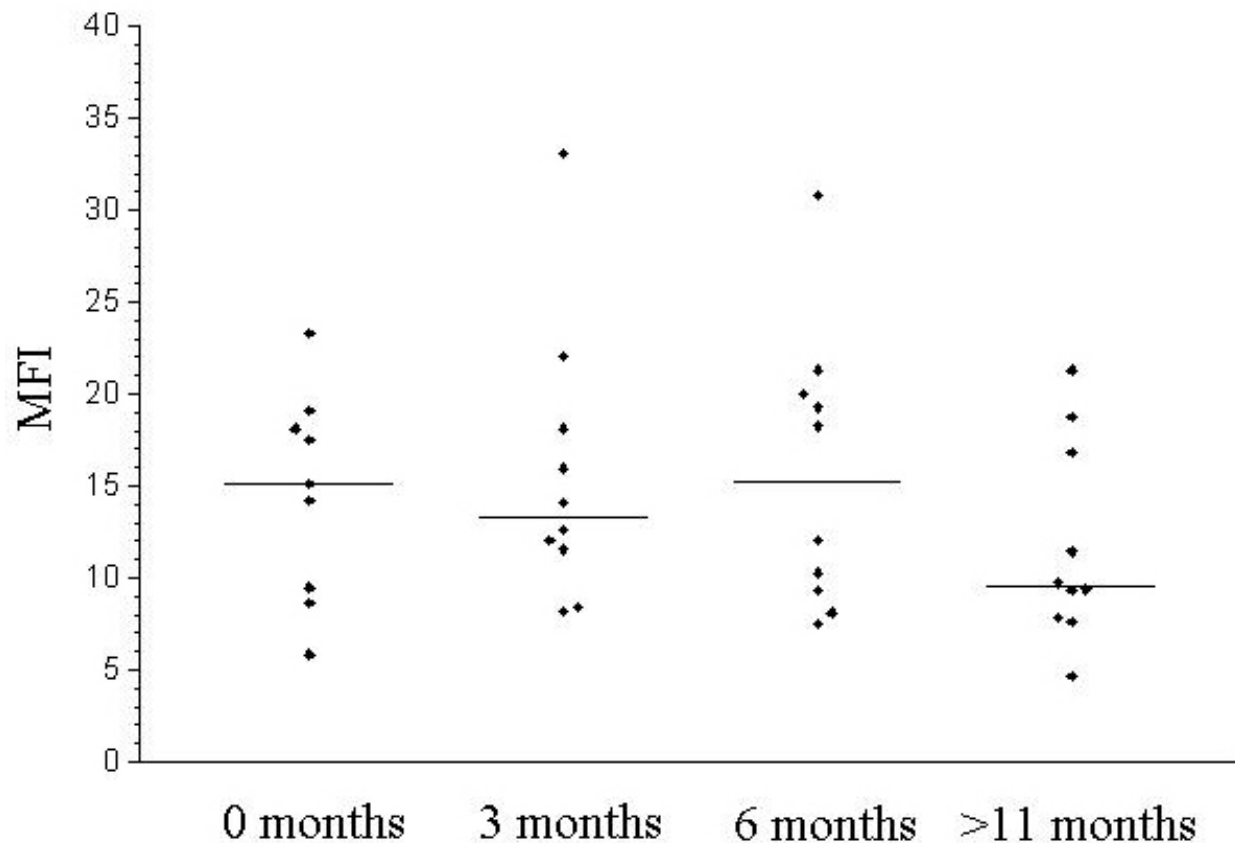
Figure 3. FACS analysis of sera before and during antiretroviral therapy using HIV_{IIIB}-infected M8166 cells. Each serum, obtained at four different time points (as indicated in the graph) was tested in duplicates at a 1:50 dilution and a 1:500 dilution (not shown). The dots represent the serum of an individual patient, the bars indicate the median of the measured median fluorescense intensities. As negative control, a pool of NHS was used.

Three of ten patients had no detectable changes in their ability to neutralize HIV under ART. For patient number #2, #6 and #8, the ELISA showed constantly low anti-HIV antibody-titers during the course of therapy. Patient #6 had a weaker neutralization efficiency (1:50) than patients #2 and #8 (1:500). Contrary to these observations, the majority of sera (70 %) revealed substantial decreases in neutralizing titers coupled with progressive course of ART. Compared to patients #9 and #3 (Table 4), patients #1 and #4 expressed high titers of neutralizing antibodies against HIV-1 before therapy

(1:5000). At 11 to 38 months of antiretroviral therapy, a significantly impaired neutralization capacity was observed (1:500). Similarly, patients #5 and #7 were positive for neutralizing antibodies to HIV before therapy and exhibited declining antibody-titers during ART. Unfortunately, serum from patient #10 before therapy was no more available for this assay. During therapy levels of neutralizing antibodies decreased in patient #10. As a reference antibody, the broadly neutralizing human MAb 2F5 (anti-gp41) was used (33, 34). 2F5 inhibited HIV_{IIIB} infection of M8166 cells to a dilution of 20 ng/ml.
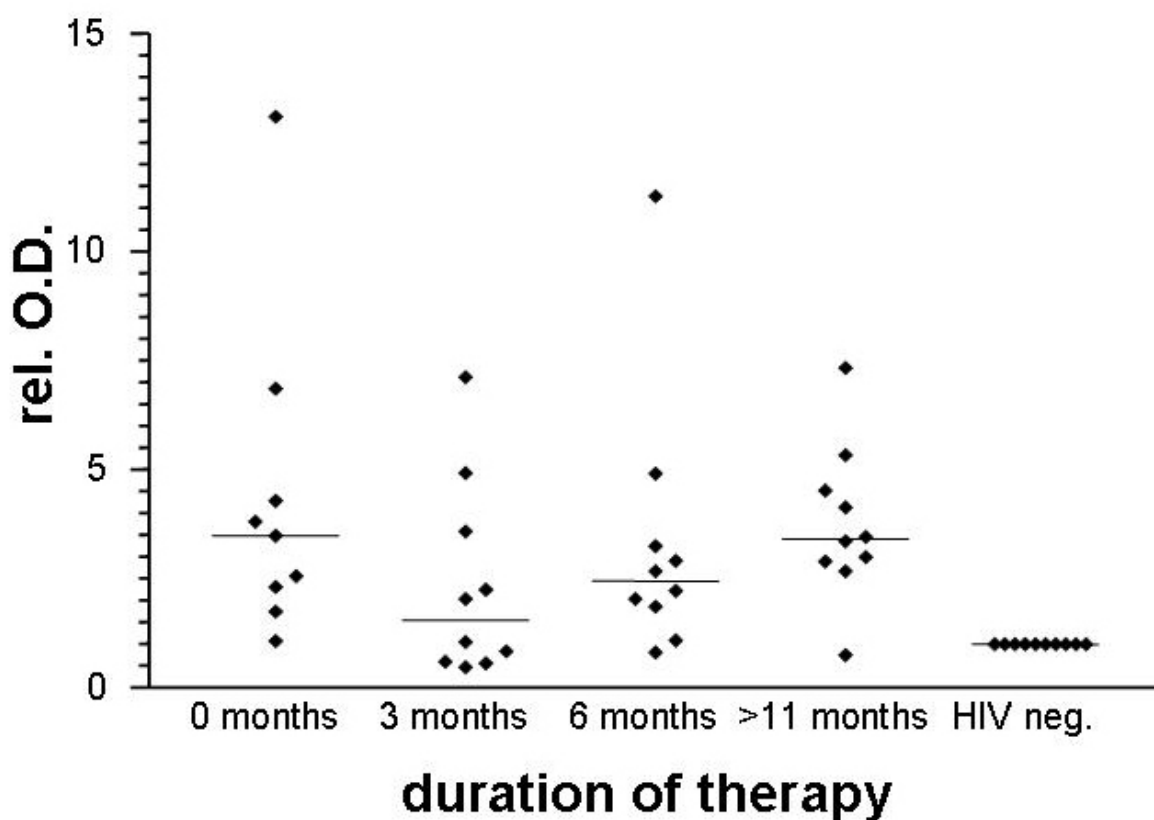
Figure 4. Reactivity of patient sera against recombinant tat before and during antiretroviral therapy. Each serum, obtained at four different time points (as indicated in the graph) was tested in duplicates. The dots represent the serum of an individual patient, the bars indicate the median of the relative optical densities. Reactivity of the sera against recombinant tat was compared with the mean of ten HIV negative individuals, which was normalized to 1 absorption unit.

5. DISCUSSION

In this retrospective study we analysed changes in antibody reactivity of HIV-infected individuals against certain gp120 and gp41 derived peptides at different stages of the disease and compared these data to antibody recognition patterns of patients before and after initiating antiretroviral therapy. In this latter group we further analysed the antibody response to recombinant gp160, native gp120/41 complex and recombinant tat. The response to therapy was characterized by decrease in viral load, urine neopterin levels, increase in CD4 counts and the disappearance of oral Candida infections (Table 2). In addition, we investigated the neutralization capacity of Ig isolated from sera of patients successfully treated with ART.

During all stages of the disease antibody reactivity to four gp120-derived peptides was observed, although during disease progression the antibody titer against peptides #9, #28 and #30 decreased moderately. In contrast, the antibody reactivity to peptide #47, which was comparable between patients in stage A and long term non-progressors, dropped significantly and reached the lowest level in stage C. This is in line with observation of Loomis-

Price *et al.*, who identified this decrease as prognostic marker for HIV infection (16). In accordance to others (15), peptides #47 (constant region 5 in gp120), #60 and #61 (immunodominant loop of gp41) were preferentially recognized by sera in early stages of infection. Interestingly, LNPs which are discussed to interact with the 2F5-epitope (35) showed no significant enhanced interaction with peptide #67. The discrepancy to a previous report (35) may be due to differences in the peptides used and the low amount of LNPs in the studies. In our assay the reactivity against peptide #66 and its overlapping counterpart peptide #67 was similar. Thus, we concluded that the antibodies of the few patients tested recognized a common epitope and not specifically the ELDKW-motif which is present in peptide #67 only.

The antibody reactivity against most of the envelope protein derived peptides and recombinant gp160 or gp41 decreased over time in 9 of 10 patients under therapy. This is in accordance with the observations of different other groups, which all reported a decline in HIV-specific antibody response and a normalization of hypergammaglobulinemia in most patients successfully treated with ART (5-12). Similarly, the titers against the native gp120/41 complex, which is expressed on HIV-

infected cells, was reduced. In line with this findings, also the titer of neutralizing antibodies against HIV decreased in 7 out of 10 patients. In contrast to our observations, Binley *et al.* reported of little or no changes in neutralizing antibody titers in most chronically infected individuals which received ART (10). The reasons for this discrepancy may be due to the limited amounts of patients in both studies (only 10 patients in both sets of experiments) and differences not only in the virus isloates but also in the antibody preparations used in the neutralization assays. While others have used the patients' sera directly in their assays, we have purified the IgG with protein G sepharose to exclude the presence of antiviral drugs in plasma, which may affect the neutralization assays by diminishing virus replication after entry (36). The reduced titers of neutralizing and non-neutralizing envelope specific antibodies in most patients under therapy support previous findings which indicate that the induction of antibodies against the gp120/41 complex is mainly dependent on the amount of antigen and independent of T-helper cells (37). Since all patients in this study were successfully treated and the viral load (and therefore the amount on viral antigen) was strongly decreased, a reduction in envelope specific antibodies was likely. However, the antibody responses to the viral envelope protein developed not homogeneous. In one of the tested sera no neutralizing antibodies against HIV$_{IIIB}$ were detected already before therapy (patient #6), while 2 out of 10 patients (patient #2 and #8) had an increase of neutralizing antibodies. The absence of neutralizing antibodies against HIV$_{IIIB}$ in patient #6 may be due to sequence variations between the patient isolates and the strain tested in the *in vitro* assay. However in ELISA and in FACS analysis the serum of patient #6 clearly cross-reacted with HIV$_{IIIB}$ sequences.

In contrast to the reduced reactivity against HIV envelope proteins in most sera of patients under ART, recombinant tat protein exhibited a different pattern. Following a significant drop after 3 months of therapy, the antibody response against tat recovered and reached after the latest time point tested the inital level again which was deterimed before the start of therapy. A possible T cell dependence for the antibody respsonse against tat is therefore likely, similar as already reported for gag another intraviral and non-glycosylated protein (37). The initial drop of antibody titers at beginning of therapy was accompanied by a rapid decline of HIV but only by a moderate increase of CD4+ T cells compared to healthy individuals. Thus, only few viral particles were presented to a limited amount of T helper cells. Although at later time points the plasma viral load remained at low levels, the CD4+ helper cell counts raised significantly, which increased the probability that a specific helper cell got in contact with antigen presentig cells. Augmentation in IL-2 and IFN-gamma pattern induced by ART (38) may additionally favour the clonal expansion of T helper cells and provide a further explanation for the raise in anti-tat antibodies during therapy. In addition, antigen-antibody complexes trapped in the germinal center may be released and therefore increase the amount of plasma antibodies against tat in patients under therapy.

In this study we have shown that during time, the reactivity of antibodies against different gp120 and gp41-derived peptides in treated and untreated patients changes and the neutralizing capacity of these gp120 and gp41 reacting antibodies decreases in most individuals. The reduced recognition of certain gp120 and gp41-derived epitopes in untreated patients during disease progression from stage A to stage C is probably due to an exhausted immune system of specific B cell clones, while the decreased antibody reactivity against the envelope proteins in treated individuals may be the result of reduced viral loads, i.e. lower amounts of antigen. The immune response to the envelope proteins supposed to be relatively independent on T cell help (37). By contrast, the antibody response to tat seems to be more dependent on T-helper cells (37). However, inspite of increased antibody responses against tat, which is discussed as candidate vaccine for clinical trails (39), the neutralizing capacity of most of the sera tested decreased. Whether exogenous vaccination with viral antigens or structured treatment interruptions can boost antibody responses and support together with CTL responses antiretroviral therapies (reviewed in 40) to induce long lasting suppression of HIV remains to be determined.

6. ACKNOWLEDGMENT

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