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In vitro anti-HIV-1 antibody production in subjects in different stages of HIV-1 infection

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SUMMARY

We evaluated the *in vitro* antibody production from peripheral blood mononuclear cells (PBMC) against HIV-1 proteins in infected adults. Fifty-four HIV-1 infected patients (four recent seroconverters, 15 asymptomatics with a CD4 count higher than 500/μl, 27 asymptomatics with a CD4 count between 200 and 500/μl and eight symptomatic patients) were tested. PBMC were incubated in the presence or absence of 1% pokeweed mitogen (PWM) at 37°C for 8 days. Western blot assay, p24 antigen ELISA and anti-p24 antibody ELISA were performed on serum and culture supernatants. Spontaneous production of anti-*env* antibody in culture supernatants was evidenced in all subjects. All the positive supernatants for anti-core antibodies (18/54) were derived from asymptomatic patients. PBMC from recent seroconverters and from symptomatic patients did not produce any anti-core antibody. Antibody production decreased after stimulation with PWM. The concentration of p24 antigen did not significantly increase in p24 positive supernatants following acidification ($P = 0{\cdot}1$), suggesting that the inability to detect p24 antibody was not due to the anti-p24 antibody complexed to p24 antigen in culture supernatants. *In vitro* production of anti-p24 antibodies was significantly more frequent in asymptomatic subjects with high $CD4^+$ cell counts ($P = 0{\cdot}02$) and was absent in recent seroconverters. This last finding suggests that during the initial phases of the infection, anti-p24 antibody production may be restricted to cells residing in lymphoid organs. In addition, the lower percentage of anti-core antibody in people with low $CD4^+$ cell counts is not merely a consequence of the binding of the antibody to an increased amount of antigen, but probably reflects an impaired production or a sequestration of producing cells in lymphoid tissue during the late stages of the infection.

Keywords HIV-1 IVAP immunoglobulins

INTRODUCTION

Infection with HIV-1 results in a general dysregulation of the immune system and in particular has a number of effects on B cell function [1–3]. HIV-1-infected individuals often have polyclonal B cell activation with hypergammaglobulinaemia [3–5] and lymphoid hyperplasia [3,6]. The B cell hyperactivity may be secondary to a polyclonal activating effect of HIV-1 [7,8]. B cell functional defects are also reflected *in vitro* by a reduction of B cell proliferation in response to mitogens and antigens [9,10], a reduction of immunoglobulin-secreting cells in response to mitogens [11–14], and a lack of response to HIV-1 or to viral antigens [15].

It remains unclear to what extent the specific anti-HIV-1 antibody production is affected by the general impairment of the humoral response. It is known that the profile of antibodies directed to viral proteins is progressively modified and that the detectable antibody response to core proteins disappears as HIV-1 infection proceeds to AIDS [16]. It has also been shown [17,18] that much of the HIV-1 p24 antigen in the serum of HIV-1-infected individuals is complexed with anti-p24 antibody.

A recent study revealed that over a 6-year period subjects who lacked detectable anti-p24 antibodies in their serum progressed more rapidly to AIDS [19]. However, it remains unclear whether the reduction in anti-p24 antibody levels is a consequence of binding to increasing amounts of p24 antigen as virus replication increases, or is due to an actual decrease in antibody production. It is also known that the presence of p24 antigen in serum is a strong predictor of AIDS [20,21]. Other studies have shown that peripheral blood mononuclear cells (PBMC) from seropositive subjects when placed in culture spontaneously produce antibodies directed against HIV-1-specific proteins [22,23]. Moreover, both PBMC and B

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lymphocytes when cultured with HIV-1 proteins produce antibodies predominantly directed against *env* proteins [24,25].

In vitro production of anti-p24 antibody has not been extensively investigated during different phases of HIV-1 infection.

In this study we address the question as to whether the pattern of anti-HIV-1 antibody production from cultured PBMC correlates with the disease progression. We compared subjects in different stages of HIV-1 infection including individuals who have recently seroconverted.

MATERIALS AND METHODS

Study population

The study was conducted on a consecutive basis on 54 HIV-1-infected subjects who volunteered to participate within the cohort followed at the Clinica delle Malattie Infettive of the University of Milan. Four of them were recent seroconverters who had been infected within 6 months of enrollment (RS), 15 were asymptomatic with CD4 counts greater than 500/μl who had presumably been infected less than 4 years before the study began (recent asymptomatics, RA), 27 were asymptomatic after more than 4 years from the presumptive date of infection (old asymptomatics, OA) with CD4 counts between 200 and 500/μl, and eight of them were symptomatic with a CD4 count lower than 250/μl. Ten healthy HIV-1$^-$ volunteers served as a control group. CD4$^+$ cell count and serum HIV-1 p24 antigen levels were performed on inclusion in the study. CD4$^+$ cell counts are expressed as mean $\pm$ s.d.

Thirteen of 27 (48%) of the OA and 7/8 (87%) of the symptomatic patients had been receiving Zidovudine (500–600 mg/day) at the time of the study, from a mean duration of 4 months (range 2–11).

Cell cultures

PBMC were obtained by Ficoll–Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of 30 ml of heparinized blood, and resuspended at 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% non-essential aminoacids, 50 mg/ml gentamicin, and 5 mg/ml phytohaemagglutinin (PHA). PBMC were cultured in the presence or absence of 1% pokeweed mitogen (PWM, GIBCO Laboratories, Grand Island, NY) in 24-well plates (Costar, Cambridge, MA) at 37°C for 8 days in a 5% CO_2 atmosphere. Cell free supernatants were recovered by low speed centrifugation and stored at −20°C.

Assays

All sera and supernatants were tested: (1) for anti-HIV-1 antibody production via a Western Blot (WB) assay (New LAV Blot I, Diagnostic Pasteur, Paris, France), (2) for anti-HIV-1 p24 antibodies by a commercial ELISA (Wellcozyme HIV-1 anti-p24, Wellcome, Beckenham, UK), and (3) for HIV-1 p24 antigen using a commercial ELISA (HIVAG-1 Monoclonal, Abbott, North Chicago, IL). The acidification of the supernatants was performed by adding 50 μl of 0·5 M HCl to 100 μl of the sample and incubating for 1 h. The acid was neutralized and pH restored to a physiological level by adding 50 μl of 0·5 M NaOH.

IgG supernatant concentration was evaluated by laser immunonephelometry.

Rationale of the assays

Western blot assay was performed to identify different patterns of the spontaneous production of anti-HIV-1 antibodies. Anti-p24 antibodies were also measured in supernatants via ELISA in order to confirm the data obtained with WB as the ELISA is a more sensitive method. A p24 antigen ELISA assay was performed prior to and after the acidification of the supernatants in order to evaluate the possible interference of antigen–antibody complexes in anti-p-24 detection. IgG production was measured as a confirmatory test in order to evaluate the presence of spontaneous production of antibodies in supernatants.

Statistical analysis

Data were compared using a χ^2 for linear trend test, Fisher's exact test and Student's *t*-test for paired data (EPI Info version 5.0 software, CDC, 1990).

RESULTS

Assays on patients' blood

As shown in Table 1, the mean CD4 cell count differed in the four groups of patients. The p24 serum antigen was present in 4/27 (15%) of OA, 1/8 (13%) in symptomatic subjects, whereas it was negative in the other two groups. Serum WB were positive either for *env* and core proteins in all the patients. Table 1 also shows the laboratory features of the study population at time of the enrollment into the study.

Detection of anti-HIV-1 antibodies in culture supernatants of PBMC

All 54 patients produced antibodies directed to the *env* region. We did not note any statistical difference in antibody production by PBMC from the different groups of patients. PWM stimulation of cultured lymphocytes decreased antibody production compared with the supernatant from unstimulated PBMC derived from the same patient. Of interest, antibody

Table 1. Characteristics of the study population at the enrollment

Clinical stage	*n*	Mean CD4$^+$/μl $\pm$ s.d	HIV-1 p24$^+$ (%)	Serum anti-env Abs (%)	Serum anti-core Abs (%)	Zidovudine treatment (%)
Recent seroconverter	4	895 ± 267	0 (—)	4 (100)	4 (100)	0 (—)
Recent asymptomatic	15	788 ± 219	0 (—)	15 (100)	15 (100)	0 (—)
Old asymptomatic	27	352 ± 89	4 (15)	27 (100)	27 (100)	13 (48)
Symptomatic	8	115 ± 103	1 (13)	8 (100)	8 (100)	7 (87)

Table 2. Pattern of *in vitro* antibody production in HIV-1-infected patients divided by clinical stage

Clinical stage	*n*	SN anti-env Abs (% positive)	SN anti-core Abs (% positive)
Recent seroconverter	4	4 (100)	0 (—)
Recent asymptomatic $\geqslant 500\,CD4^+/\mu l$	15	15 (100)	9 (60)
Old asymptomatic $\geqslant 200-<500\,CD4^+/\mu l$	27	27 (100)	9 (33)
Symptomatic	8	8 (100)	0 (—)
P value		NS	0.02

SN, culture supernatant; NS, not significant.

production was abolished in 18% of spontaneous producers, and in another 41% we observed a loss of one or more specific antibodies (e.g. anti-gp120) as assessed by WB. We did not detect any HIV-1-specific bands using the supernatants prepared from PBMC from the normal controls.

HIV-1 specificity of in vitro *produced antibodies*

Specific anti-gp160 or anti-gp120 antibodies were detected by WB in all supernatants from HIV-1-infected subjects. Table 2 shows the pattern of anti-HIV-1 antibody production. Among the patients who secreted specific antibodies we saw a difference within groups. The production of anti-p24 antibodies detected by WB and ELISA (there was an absolute concordance between the tests), was present in 60% of RA and 33% of OA, and was absent in RS and symptomatic subjects (Fisher's exact test; $P = 0{\cdot}02$).

Detection of HIV-1 p24 antigen in PBMC supernatants

Supernatants derived from the PBMC cultures from 18 HIV-1-infected subjects including the five subjects with p24 antigen detectable in their serum were tested for p24 antigen. Among these patients, 15 were classified as asymptomatic (six RA and nine OA) and three as symptomatic.

Detectable p24 antigen was present in supernatants of 11 subjects (one RA, seven OA and three symptomatic) out of 18 (61·1%) with a mean p24 antigen concentration of 44·7 pg/ml (range 12·5–250 pg/ml). Ten out of these 11 supernatants had undetectable levels of anti-p24 antibody. The mean concentration of p24 antigen did not significantly increase after acidification of supernatants (42·2 pg/ml, range 12·5–248 pg/ml, Student's *t*-test for paired data; $P = 0{\cdot}1$). No p24 antigen positivity emerged following acidification of the seven p24 antigen negative supernatants.

Detection of total IgG in PBMC supernatants

Spontaneously produced IgG in the supernatants showed a broad range of values (2–148 mg/*l*). The levels of spontaneous IgG were statistically unrelated to the $CD4^+$ cell count or the clinical stage of infection. The mean concentration of IgG in supernatants of anti-core negative patients was lower than in anti-core positive patients (17 ± 8 *versus* $40{\cdot}7 \pm 43{\cdot}6$ mg/*l*), but this difference was not statistically significant.

DISCUSSION

Our results showed that the production of anti-core antibodies by PBMC seems to vary significantly during the different stages of HIV-1 infection in those patients with detectable anti-p24 antibodies in their serum. The majority of subjects with more than $500/\mu l$ $CD4^+$ cells had a detectable anti-p24 response with the exception of recent seroconverters whose PBMC failed to produce anti-p24 antibodies *in vitro*. Symptomatic patients failed to produce *in vitro* anti-p24 antibody. Asymptomatic individuals with a $CD4^+$ cell count less than $500/\mu l$ had a demonstratable *in vitro* anti-p24 antibody response in one-third of the cases.

Those patients with evidence of disease progression generally had low levels of p24 antigen in their serum. Zidovudine administration to these patients may have reduced antigen production by inhibiting HIV-1 replication. Consistent with these data, cell culture supernatants from several of these patients contained p24 antigen, probably as a consequence of HIV-1 replication during a medium-term culture in the absence of Zidovudine. The antigen was more likely to be present in supernatants negative for anti-p24 antibody (76% in antigen positive patients *versus* 20% in antigen negative patients). This finding suggested a possible interference of immune complexes in anti-p24 antibody detection, but acidification of the supernatant prior to the p24 antigen assay failed to increase p24 antigen levels. This result is indirect evidence that the absence of detectable anti-p24 antibody is not secondary to its complexing with antigen obscuring its detection.

In our study anti-p24 antibodies were not produced by the PBMC of 36/54 of the enrolled subjects, despite the presence of anti-p24 antibodies in all the sera. Other authors [23] reported WB profiles of the supernatants that did not always correspond with serum pattern, while another study [26] correlated spontaneous secretion of anti-p24 IgG antibodies with its presence in serum. The finding of a different distribution of spontaneous *in vitro* production of anti-core antibodies in various phases of the disease suggests that this phenomenon may be due to a different selection of patients.

In vitro production of anti-core antibodies was absent in the majority of subjects with laboratory or clinical evidence of disease progression and also in a proportion of asymptomatic subjects with more than $500/\mu l$ $CD4^+$ cells. It is possible that the majority of the anti-core antibody production occurs in

lymphoid tissues where HIV-1 replication takes place and that those B cells fail to circulate. Perhaps consistent with this hypothesis is the absence of circulating anti-p24 producing cells in recent seroconverters. Both recent seroconverters and patients with advanced infection frequently have active viral replication [27,28]. Several observations suggest that recent seroconverters have an activated immune system probably due to an attempt to counteract viral replication and the subsequent spread of the infection [29–31]. Circulating anti-p24 secreting cells may appear in subsequent phases of the infection when virus replication is lower. In individuals who progress toward AIDS the level of anti-core antibodies progressively falls [32,33]. In addition, longitudinal studies of the immune response in HIV-1-infected patients have demonstrated that the reduction in antibody reactivity to HIV-1 p24 and p17 is an early predictor of disease progression [34–36]. The disappearance of anti-core antibody producing cells from peripheral blood during the latest phases of the disease could be an expression of the general deregulation of the immune system, with a decrease of T helper lymphocytes function, but also to a further sequestration of secreting cells at the sites of active viral replication. An investigation of antibody production profiles in supernatants prepared from lymphoid tissue would be an interesting follow up to the present study.

The testing of IgG spontaneous production was used to confirm antibody production in supernatants. Several anti-p24 antibody producers showed a relatively high IgG spontaneous production. The difference in the mean concentration between this group and the non-producers did not reach statistical significance and the level of IgG production failed to correlate with clinical stage or $CD4^+$ cell count.

As previously reported [12], mitogen stimulation with PWM of cultured lymphocytes led to a decrease in the anti-HIV-1 antibody response in a high percentage of patients. The reason for this inhibition is still unclear, but may reflect the immune deregulation caused by HIV-1 infection.

In conclusion, a prospective evaluation of *in vitro* anti-core antibody production by PBMC from HIV-1-infected patients. will be needed to determine the usefulness of this test as a predictive marker of the progression of HIV-1 infection. Such a study would also clarify: (i) when and under what circumstances anti-p24 producing B lymphocytes are detectable in peripheral blood; (ii) when and why these cells decrease or disappear from the circulation; (iii) if more active viral replication contributes to a distribution into different compartments of the anti-p24 secreting cells. Understanding these points should lead to a better knowledge of the humoral response to HIV-1 and better define the possible value of this test as a predictive assay for the monitoring of HIV-1-infected patients.

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