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HIV-specific lymphoproliferative responses in asymptomatic HIV-infected individuals

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SUMMARY

In vitro lymphoproliferative responses to HIV-1 recombinant antigens (gp160, p24, and Rev protein) were studied in 83 patients with asymptomatic HIV-1 infection (CDC groups II and III) and circulating CD4 lymphocyte numbers $>400/mm^3$. Significant response to at least one of the three antigens was detected in 52·4% of the subjects, but the responses were weak, and concordance of the response to the three antigens was rare, the frequency of individuals responding to each antigen not exceeding 22·4%. Increasing frequencies of response were observed when recall antigens (tetanus toxoid and *Candida albicans* glycomannoprotein) (65·5%) and anti-CD3 MoAb (76·6%) were used as stimuli. Although a significant association between lymphocyte response to p24, but not gp160, and steadiness of CD4 lymphocyte numbers before the assay was observed, no predictive value for lack of CD4 cell decrease was confirmed for either antigen, and fluctuation of the responses to HIV antigens was seen during subsequent follow up. The panel of T cell assays used could be regarded as appropriate for monitoring both HIV-specific responses and T lymphocyte function during immunotherapy with soluble HIV antigens.

Keywords HIV lymphocyte proliferation gp160 p24

INTRODUCTION

Depletion of CD4 lymphocytes is the central feature of HIV-1 infection and can be reasonably considered the main cause of immunodeficiency [1]. However, functional abnormalities of CD4 T cells, even during the asymptomatic phase of the disease when CD4 cell numbers are relatively elevated, have been described. In particular, a progressive loss of T cell functions, in the area of decreased responses to recall antigens, alloantigens and mitogens, has been reported in groups of asymptomatic HIV-infected patients who, in spite of numbers of circulating CD4 lymphocytes comparable to those of healthy uninfected individuals, were displaying the full range of functional abnormalities [2]. A significant association with disease progression was demonstrated for lymphocyte reactivity to MoAb to CD3, which was virtually lost 12 months before the diagnosis of AIDS in a group of asymptomatic patients with an average of 360 CD4 cells/mm^3 [3].

Since T cell abnormalities can be demonstrated before a significant depletion occurs, a particularly important issue is the development of an HIV-specific CD4 lymphocyte response. The impairment or early inactivation, conceivably through direct infection or altered signaling, of this crucial component of the antiviral response could well be the primary event leading to a progressively inefficient control of the virus. An early report indicated that an *in vitro* lymphoproliferative response to whole HIV virion antigen was detectable only in 46% of fully asymptomatic patients and in 16% of patients with lymphadenopathy, despite relatively high numbers of circulating CD4 cells and conserved responses to other viruses [4]. When IL-2 release after *in vitro* lymphocyte stimulation with HIV-1 synthetic peptides was measured, 82% of asymptomatic patients with conserved response to influenza virus antigen showed a significant response, but only 29% of them showed positive in a lymphoproliferative assay [5].

The study of *in vitro* responses to single HIV proteins could result in a higher sensitivity than using whole viral antigen extracts, and could provide more information about the specificity of the response itself. Furthermore, the current effort in evaluating the possibility of altering the natural history of the disease by active immunization of asymptomatic patients with soluble HIV antigen makes the study of *in vitro* lymphocyte responses to those antigens mandatory [6]. We therefore evaluated the lymphoproliferative response to HIV

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recombinant proteins, chosen among structural and regulatory gene products, in asymptomatic HIV-infected subjects. In parallel, lymphocyte function expressed by responsiveness to recall antigen and mitogens was assessed in the same patients. The combination of HIV-specific and polyclonally induced proliferative responses has not been extensively studied in the past, and the panel of antigenic and mitogenic stimuli used in this study is proposed for cell-mediated immunity evaluation during trials of post-exposure immunization with soluble HIV proteins. The relationship between specific responses and the behaviour of CD4 lymphocyte numbers is investigated to assess whether any of these parameters could be regarded as disease progression markers.

PATIENTS AND METHODS

Patients

Eighty-three HIV-1-infected asymptomatic adults (aged between 18 and 50 years), included in groups II and III of the Centres for Disease Control classification [7] and with CD4 cell numbers > 400/mm^3, were studied. According to sex they were divided into: 27 females (32·5%), 56 males (67·5%). CD4 lymphocyte numbers were 596 ± 160/mm^3 (mean ± s.d.), range 401–1198. According to the risk factor for infection they were grouped as follows: 26 male homosexuals, 31 intravenous drug users, one who received infected blood transfusion, and 25 who were likely to have been infected through heterosexual contact. Fourteen age- and sex-matched HIV-1$^-$ healthy individuals were included in the study as controls.

Serodiagnosis of HIV-1 infection was made by standard ELISA assay and confirmed by Western blot (Cambridge Biotech Inc., Rockville, MD). The time between the first known positive test for anti-HIV-1 antibody and the lymphocyte proliferative response assessment varied from 1 to 97 months (median 41 months).

HIV-1 antigens

Three recombinant (r) HIV-1 antigens derived from the HIV-1$_{IIIB}$ sequence were selected for lymphocyte stimulation assays (p24, gp160, and Rev protein) as representative of two structural (core and envelope) and one regulatory proteins. Baculovirus-expressed rgp160 was obtained from MicroGeneSys Inc. (Meriden, CT); baculovirus-expressed rp24 and *Escherichia coli*-expressed rRev protein were purchased from American Bio-Technologies Inc. (Cambridge, MA).

To assess the binding of gp160 to CD4, competition with MoAb Leu-3a (Becton Dickinson, Mountain View, CA), known to bind CD4 in the same region as HIV-gp120 [8], was assessed by direct immunofluorescence and flow cytometry. Five-hundred thousand peripheral blood mononuclear cells (PBMC) from healthy HIV$^-$ donors, obtained as described below, were incubated in 200 μl RPMI 1640 with 10% fetal calf serum (FCS) in the presence of 50 μg/ml gp160 or baculovirus-expressed rgp120 (American Bio-Technologies) for 45 min on ice. After two washes with Hanks' balanced salt solution containing 0·1% NaN_3 and 2% FCS (HBSS–Az), the cells were incubated with FITC-conjugated Leu-3a or OKT4 (Ortho Diagnostic Systems, Raritan, NJ) according to standard procedures. Bound fluorescence was visualized by an Ortho Cytoron (Ortho Diagnostic Systems) flow cytometer.

Lymphoproliferative response to antigens

PBMC were isolated by density gradient centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway) at 800 *g* for 30 min. Separated PBMC (1×10^6 cells/ml) were cultured in 200 μl of RPMI 1640 with 10% AB human serum negative for anti-HIV-1 antibodies (HS) (N.A.B.I., Miami, FL) in 96-well flat-bottomed cell culture plates (Falcon, Lincoln Park, NY) in the presence of gp160, p24 or Rev protein, respectively, all at the concentration of 2 μg/ml. Baculovirus control protein, kindly provided by MicroGeneSys, was included in some assays at the same concentration. To test proliferative responses to recall antigen, glycomannoprotein (GMP) of *Candida albicans* [9] (kind gift of Professor A. Cassone, Ist. Superiore di Sanita, Rome, Italy) and tetanus toxoid (TT; Wyeth Labs., Marietta, PA), respectively, at the concentration of 50 μg/ml and 1 Lf/ml were added to the cultures. Cultures were set up in triplicate.

To test the ability of Env antigen to inhibit TT-induced proliferation, a TT-specific T cell line was restimulated with autologous irradiated PBMC and TT in the presence or absence of gp160 (MicroGeneSys) or chinese hamster ovary (CHO) cell-derived rgp120 (Celltech Ltd., Slough, UK), as described elsewhere [10].

Lymphoproliferative response to mitogens

Mitogen-driven cultures were set up essentially as described above for antigen cultures with the following differences: 1×10^5 PBMC were added in each well in 100 μl RPMI 1640 with 10% FCS. Anti-CD3 MoAb OKT3 (Ortho Diagnostic Systems) or purified phytohaemagglutinin (PHA; Murex Diagnostics, Dartford, UK), respectively, at concentrations of 25 ng/ml and 1 μg/ml, were added.

Specific stimulation index calculation

After 6 days of culture at 37°C in humidified 5% CO_2 atmosphere (3 days for cultures with mitogens), 0·5 μCi of tritiated thymidine (^{3}H-TdR; specific activity 25 Ci/mmol; Amersham, Aylesbury, UK) was added to each well, and after a further 24 h (4 h for cultures with mitogens) cells were harvested onto glassfibre filters by a cell harvester and incorporated radioactivity was measured in a scintillation counter and expressed as ct/min. Stimulation index (SI) was calculated by dividing ct/min of stimulated cultures by ct/min of unstimulated cultures. Lymphoproliferative responses to antigen were considered significant for SI > 2. This cut-off value was chosen considering that the mean + 2 s.d. of SI obtained in cultures of PBMC from HIV$^-$ controls for each of the three HIV antigens tested was always < 2. The same cut-off has been employed by other authors in similar assays with HIV antigen [11].

Statistical analysis

Groups of SI data and CD4 cell numbers were compared by Student's *t*-test. Correlation between variables was assessed by linear regression analysis. The slope of the line representing variation of CD4 cell number *versus* time was calculated by the least squares method. Contingency tables were constructed and the χ^2 test was performed to assess the significance of the association between the presence (or absence) of lymphocyte responses and CD4 cell decline (or steadiness/increase) expressed by the calculated slopes.

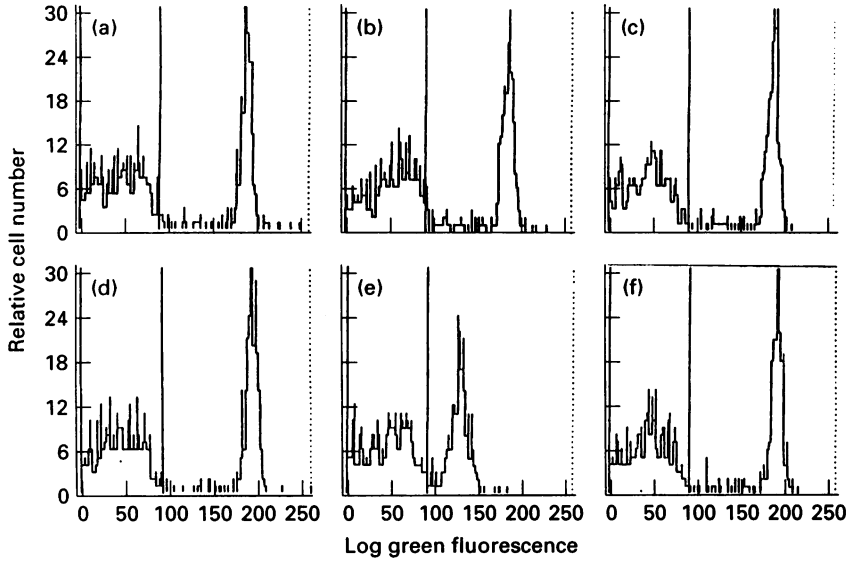

Fig. 1. Binding of anti-CD4 MoAbs OKT4 (top panels) and Leu-3a (bottom panels) to peripheral blood mononuclear cells (PBMC) in the presence of the following inhibitors: (a,d) no inhibitor; (b,e) baculovirus-expressed rgp120 (American Bio-Technologies); (c,f) baculovirus-expressed rgp160 (MicroGeneSys). Vertical markers represent the 99% upper limit of fluorescence displayed by PBMC stained with an irrelevant FITC-conjugated MoAb. Note the reduction of fluorescence bound when Leu-3a, but not OKT4, was incubated after rgp120 but not rgp160.

RESULTS

Binding of gp160 to CD4

In a previous report [10] we described the absence of lymphoproliferative response to gp120 in asymptomatic HIV-1-infected individuals. To explain it, the hypothesis of an inhibition of antigen-specific T lymphocytes through binding of gp120 to CD4 was made. Therefore, in order to explore further the lymphocyte responses to Env antigen, we selected a gp160 which did not show a significant binding to CD4. As shown in Fig. 1, gp160 from MicroGeneSys did not show any reduction of binding of Leu-3a, which competes with gp120 for binding to CD4, on PBMC. A significant competition with Leu-3a for binding to PBMC was seen when gp120 from American Bio-Technologies was used as inhibitor. As expected, no competition with OKT4, known to bind CD4 in a different site [8], was observed with either protein.

When gp160 was added to a TT-specific T cell line at the same time as autologous antigen-presenting cells (APC) and TT, no significant inhibition of restimulation was observed. In contrast, CHO-derived gp120 induced a 75% inhibition of ^{3}H-TdR uptake by TT-specific cells at the concentration of 2 μg/ml (Fig. 2).

HIV-specific proliferative responses

SIs obtained in HIV antigen-containing cultures of PBMC from infected patients and healthy controls are depicted in Fig. 3. No significant response to p24 and Rev was observed in 13 (10 tested for response to Rev) healthy controls (maximum

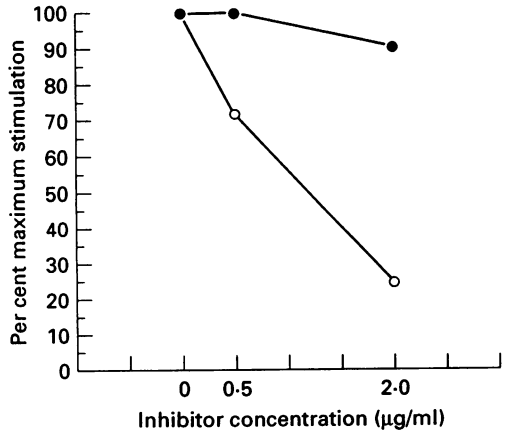

Fig. 2. Inhibition of restimulation of a tetanus toxoid (TT)-specific T cell line in the presence of chinese hamster ovary (CHO) rgp120 (Celltech) (○) or baculovirus-expressed rgp160 (MicroGeneSys) (●). No significant inhibition was seen with the concentration of gp160 used in the proliferative assays.

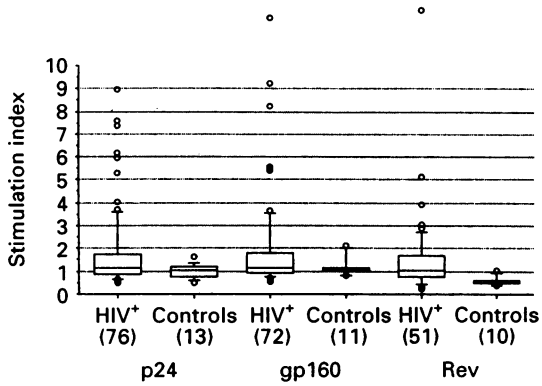

Fig. 3. Lymphocyte proliferative response to HIV-1 recombinant antigens. Results are expressed as SI and depicted as box plots. Numbers of subjects included in each group are shown in parentheses.

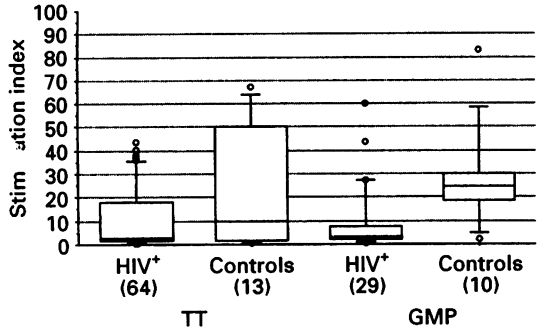

Fig. 4. Lymphocyte proliferation induced by recall antigens tetanus toxoid (TT) and *Candida albicans* glycomannoprotein (GMP). Explanation as in Fig. 3.

SI 1·64 and 1·02, respectively). Of 11 control subjects tested, one showed a significant response to gp160 (SI 2·84); however, similar proliferation was seen in response to baculovirus protein extract in the same sample (SI 3·65). No proliferation in response to either gp160 or baculovirus protein extract was observed in the other control subjects (SI ranging from 0·80 to 1·98). Lymphoproliferative responses to HIV antigen in HIV-infected asymptomatic patients were not significantly different from those of controls. However, 17 out of 76 (22·4%) of them showed SI $\geqslant$ 2 in p24-stimulated cultures, 16 of 72 (22·2%) after gp160 stimulation, and nine of 51 (17·6%) in Rev-containing cultures. Maximum SIs were 8·94, 12·06, and 12·43 in cultures stimulated with p24, gp160, and Rev, respectively. Of 42 subjects tested with all three antigens, 22 (52·6%) showed a response to at least one of them. This latter group had numbers of circulating CD4 lymphocytes significantly higher than those of patients showing no response to HIV antigen ($663 \pm 197/mm^3$ *versus* $574 \pm 105/mm^3$; $P < 0{\cdot}05$). Of those 22 subjects, 15 responded to one antigen, six showed a response to two antigens, and only one responded to all the HIV antigens tested. The presence of lymphoproliferative responses was not correlated to the estimated duration of the infection, at least according to the time from the first known positive antibody test. Similarly, no significant differences of responses to recall antigens and mitogens (see below) between the group of subjects responding to at least one HIV antigen and those responding to none were seen.

Fourteen subjects were re-tested for lymphoproliferative responses to p24 and gp160 after a period varying from 5 to 18 months (median 15 months). Of six patients who scored positive (SI $\geqslant$ 2) for response to p24, only two were confirmed positive at the second evaluation. Of the remaining eight subjects, two were found positive at the second test. In the same group of patients tested at two time points, two out of four subjects with proliferative response to gp160 at the first assay were positive in the second assay, and only one of those who scored negative initially was found positive later.

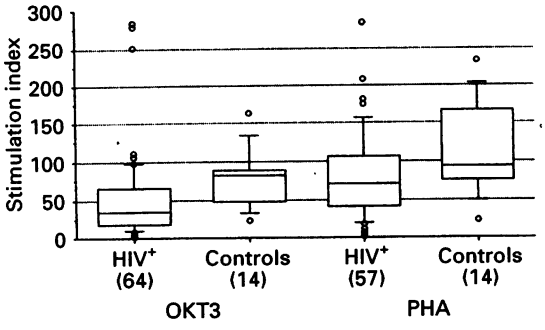

Fig. 5. Lymphocyte proliferation induced by mitogens OKT3 and phytohaemagglutinin (PHA). Explanation as in Fig. 3.

Lymphoproliferative response to recall antigens

Significant proliferation (SI $\geqslant$ 2) in response to TT was found in 35/64 patients (54·7%) and in 9/13 controls (69·2%) (Fig. 4). The difference of SI between the two groups was not statistically significant. Samples from 29 patients were tested also for lymphoproliferative response to GMP (Fig. 4), and their SIs ranged from 0·6 to 63·2 (mean 9·05). Significant response to GMP was found in all healthy control subjects ($n = 10$; SI range 2·2–83·3; mean SI 27·4), confirming a previous report [9]. Seventeen patients (58·6%) showed a significant response to GMP, and 12 of them (41·4%) to both GMP and TT. Nineteen out of the 29 patients tested with both GMP and TT (65·5%) showed a significant response to either one of the two recall antigens. Comparing the CD4 cell numbers of those patients responding to at least one recall antigen with those of patients responding to none, no significant difference was found (554 ± 150 and 567 ± 74 (mean $\pm$ s.d.), respectively).

Mitogen-driven lymphocyte proliferation

The overall T lymphocyte function was evaluated by mitogen stimulation of PBMC. In Fig. 5, proliferation induced by OKT3 expressed as SI is reported. A significantly lower proliferation was seen in the group of infected patients compared with healthy controls ($P < 0{\cdot}05$). Mean SI obtained with OKT3 stimulation were 50·0 (64 determinations) for HIV-infected patients and 78·2 (14 determinations) for controls. Fifteen HIV-infected patients (23·4%) showed OKT3-induced stimulation < 20% of the mean SI of seronegative controls (SI = 15·6).

No significant difference in the proliferative response induced by PHA measured as SI (Fig. 5) was found in asymptomatic HIV-infected patients compared with HIV^- healthy controls. Mean SI obtained with PHA stimulation were 84·8 (57 determinations) for HIV-infected patients and 109·6 (14 determinations) for controls. Only six HIV-infected patients (11·5%) showed SI lower than 20% of the mean SI of seronegative controls (SI = 21·5). ^{3}H-TdR incorporation in unstimulated 3-day cultures was low in both patients and controls (ranges 114–2856 and 196–1512 ct/min, respectively), even though seven HIV-infected patients showed high background values (ct/min $\geqslant$ 1500).

Proliferative responses and CD4 lymphocyte numbers

No significant correlation was observed between the proliferative response to OKT3 and CD4 cell numbers. On the other hand, PHA-induced proliferation was correlated with the number of circulating CD4 lymphocytes ($r = 0{\cdot}35$; $P < 0{\cdot}01$). Similarly, the magnitude of the lymphoproliferative response to GMP was significantly correlated with the number of CD4 cells ($r = 0{\cdot}43$; $P < 0{\cdot}05$), confirming previously reported results from a different group of patients [9]. No significant correlation was found comparing SI obtained with the other antigens tested and CD4 cell numbers.

At least three determinations of CD4 cell numbers (up to six) over a period of time ranging from 12 to 24 months both before and after the lymphoproliferative response evaluation were available in 22 patients. The slope of CD4 cell numbers *versus* time was therefore calculated before and after the time of testing, and the patients were defined as non-decliners for slope values higher than −15 CD4 cells/year. According to these criteria, the analysis of CD4 cell number behaviour in the time preceding the assay revealed that seven of the eight patients with positive responses to p24 were classified as non-decliners, as well as six of the 14 subjects with no response to p24. The association of response to p24 with a lack of CD4 cell decline was significant ($P < 0{\cdot}05$). However, such an association did not hold when the slopes calculated in the period following the test were considered. Six of the eight patients with positive p24 response appeared to be decliners, and only two of them maintained steady or increasing CD4 cell numbers. Similarly, 13 of the 14 patients with no response to p24 were decliners in the period following the test. Responses to gp160 were found in both decliners (4/6) and non-decliners (3/11) as defined by the pre-assay slopes. No significant association between response to gp160 and CD4 cell slope before and after the assay was observed.

DISCUSSION

The detection of a lymphocyte proliferative response to specific viral antigen is a common finding during a viral infection and is associated with protection against reinfection [12]. Early reports indicated, in contrast to lymphocyte responses to herpes viruses, that *in vitro* proliferation induced by HIV antigen in seropositive individuals is infrequent and weak [4,13,14]. We reconsidered this issue with two major aims: (i) to test in a relatively large population whether a panel of HIV recombinant antigens, rather than a whole virion antigen preparation, could be more sensitive in detecting specific proliferative responses; and (ii) whether, even if relatively not frequent, the presence of specific responses has a clinical correlate, taking CD4 cell numbers as surrogate marker. The choice of antigens was made in order to study the response to both structural antigens, one from the envelope and one from the core, and a regulatory protein (Rev). We clearly show that lymphoproliferative responses to HIV recombinant antigen are present in a significant proportion of asymptomatic HIV-infected patients, but the concordance of the responses to the different antigens is rare. The response was not associated with the risk category, nor sex, age and duration of known seropositivity (the actual time of seroconversion was not known in most patients). The higher numbers of circulating CD4 lymphocytes found in the responder patients suggest that HIV-specific proliferative responses are associated with the earlier stages of infection, even though, from the functional point of view, responders and non-responders had similar responses to recall antigens and mitogens.

Considering the frequency of responding patients, the use of a panel of recombinant antigens rather than whole virion antigen appears to render the assay relatively more sensitive; Wahren *et al.* [4], using whole HIV, reported a percentage of 28·5% (8/28) significant responses in a group comparable to ours (fully asymptomatic and LAS patients). A prevalence of approximately 50% positive proliferative responses in asymptomatic HIV-infected patients was reported also by Reddy & Grieco [15], who employed two recombinant proteins representing large portions of *env* and *gag* products. The number of asymptomatic patients studied, however, was very small in this latter report. It must be emphasized that in our study most patients showed a response to only one HIV antigen. This observation, together with the relatively low magnitude of the response itself, suggests that the HIV-specific response is incomplete and conceivably scarcely functional.

We previously reported that the detection of a significant proliferation in response to p24 is never associated with a decrease of CD4 cell numbers in the 2 years preceding the assay [16]. This finding suggested that the detection of a significant proliferative response to p24 could be a marker for a non-progressive state. The extension of that observation, which is confirmed by the data reported here, with the analysis of CD4 cell numbers in the period following the proliferation assessment suggests that the presence of a proliferative response to p24 is not predictive of a subsequent non-decliner state. Concerning gp160, no significant association with maintenance of CD4 cell numbers was observed. Furthermore, the presence of significant proliferative responses was not confirmed upon retesting within a 18-month period in several patients. Fluctuation of proliferative responses to peptides of gp120 has been recently reported by Geretti *et al.* [17], who showed both loss and appearance of significant responses in two determinations separated by a 1-year period. It can be hypothesized that the periodical appearance of HIV-specific T lymphocytes is somewhat associated with a certain degree of protection, but mechanisms such as direct infection, inactivation, and viral escape render these cells useless for maintainance of steady CD4 cell numbers.

The question now arises of whether this defective lymphocyte response is the result of a limited immunogenicity of the virus or of a loss of the specific response during the course of disease. Much evidence allows one to rule out the first hypothesis: (i) HIV-specific T lymphocytes are present in the repertoire of healthy HIV^- individuals, and can be readily activated *in vitro* by repeated stimulations with antigen-pulsed APC [18]; (ii) proliferative response to gp160 is easily detected in healthy volunteers immunized with gp160 itself [19,20]; (iii) HIV-specific T helper responses, detected both as IL-2-specific release and proliferation, have been reported in HIV^- individuals exposed to HIV and possibly protected from full infection by the development of such a response [21–24]; (iv) *in vitro* proliferative responses to HIV peptides can be restored by IL-12 [25]. Taken together, these findings suggest that HIV-specific T helper cells undergo early inactivation (functional anergy rather than depletion), possibly through a preferential exposure to virus contained in specific APC. Such an hypothesis is suggested by the *in vitro* finding that antigen-specific T cell clones become unresponsive to a second stimulation with appropriately presented antigen, in the absence of detectable cell death, when in a previous stimulation antigen processing was performed in the presence of HIV [26].

According to the above hypothesis, the HIV-specific T helper cell defect can be seen as the first event of the already mentioned sequence of T cell function deterioration, preceding the impairment of the responses to recall antigens, alloantigens, and mitogens. The mechanism responsible for this progression of T cell abnormality could well be the same, and be related to

virus-dependent altered signaling, delivered through infected APC, which progressively involves more and more cells, regardless of direct CD4 lymphocyte infection and actual depletion.

Even though we can conclude that the measurement of HIV-specific proliferative responses is of limited clinical value, we think that the panel of lymphoproliferation assays presented here is a valid approach for evaluation of immunotherapy protocols employing HIV antigen preparations in infected patients. As seen in a phase II trial of immunization with gp160 that we are currently conducting, and in similar experiences from other groups [6,27], the active immunization of HIV^+ individuals with gp160 yields significant specific proliferative responses, essentially as in immunized seronegative individuals. We do not know whether the response to HIV antigen is qualitatively different when the antigen is given as a soluble glycoprotein, or is seen in the context of the whole infectious virus, therefore we cannot rule out the possibility that most of the response to the soluble immunogen is actually a new response rather than a boost or a rescue of existing memory cells. The possibility of the reversal of a previously given inactivation signal, suggested also by the reported effect of IL-12 [25], supports the therapeutic approach of active immunization in infected patients. It will be interesting to see whether the detection of proliferative responses to the viral antigen given for immunotherapy, or other unrelated viral antigen, has any correlation with maintainance (or deterioration) of the asymptomatic stage.

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