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Research Article

Peripheral blood lymphocytes (PBL) were obtained from HIV-1-infected patients at different stages of disease. The absolute number of IgM-, IgG-, and IgA-producing lymphocytes per 10(6) PBL was increased 2.8-, 3.4-, and 1.9-fold, respectively, compared with normal controls. 2-17% of IgG-secreting patient cells reacted with the gp160 envelope glycoprotein of HIV-1 (a 737-fold increase over background), while 1-9% reacted with p24 (140-fold over background). In addition to this HIV-specific B cell activation, the number of lymphocytes reactive with nonviral antigens such as DNA, myosin, actin, trinitrophenylated keyhole limpet hemocyanin, and ovalbumin was increased by a mean of 17.9-fold. Evidence suggests that the latter changes reflect an HIV-induced polyclonal B cell activation unrelated to the production of anti-HIV antibodies. For example, the proportion of IgG anti-gp160- and anti-p24-secreting lymphocytes declined in patients with advanced disease, whereas the number of B cells producing antibodies to non-HIV antigens rose. Moreover, CD4 cell count and T4/T8 ratio showed a significant inverse correlation with the degree of polyclonal activation but not with anti-HIV responsiveness. These observations demonstrate that both quantitative and qualitative changes in B cell activation accompany (and may be predictive of) disease progression in HIV-infected individuals.

Human Immunodeficiency Virus Infection Induces Both Polyclonal and Virus-specific B Cell Activation

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Abstract

Peripheral blood lymphocytes (PBL) were obtained from HIV-1-infected patients at different stages of disease. The absolute number of IgM-, IgG-, and IgA-producing lymphocytes per 10^6 PBL was increased 2.8-, 3.4-, and 1.9-fold, respectively, compared with normal controls. 2–17% of IgG-secreting patient cells reacted with the gp160 envelope glycoprotein of HIV-1 (a 737-fold increase over background), while 1–9% reacted with p24 (140-fold over background). In addition to this HIV-specific B cell activation, the number of lymphocytes reactive with nonviral antigens such as DNA, myosin, actin, trinitrophenylated keyhole limpet hemocyanin, and ovalbumin was increased by a mean of 17.9-fold. Evidence suggests that the latter changes reflect an HIV-induced polyclonal B cell activation unrelated to the production of anti-HIV antibodies. For example, the proportion of IgG anti-gp160- and anti-p24-secreting lymphocytes declined in patients with advanced disease, whereas the number of B cells producing antibodies to non-HIV antigens rose. Moreover, CD4 cell count and T4/T8 ratio showed a significant inverse correlation with the degree of polyclonal activation but not with anti-HIV responsiveness. These observations demonstrate that both quantitative and qualitative changes in B cell activation accompany (and may be predictive of) disease progression in HIV-infected individuals. (*J. Clin. Invest.* 1992. 89:561–566.) Key words: HIV • AIDS • B cells • polyclonal activation • gp 160

Introduction

Infection with HIV-1 induces profound abnormalities in the host's immune system, including a loss of CD_4^+ T cells (1–5), suppression of responses to T-dependent antigens (6–9), and abnormal activation of B lymphocytes (9, 10). In the first year after infection, many patients develop hypergammaglobulinemia (11–13), characterized by increased levels of serum antibodies reactive with self antigens (such as T cells and nuclear determinants; foreign antigens such as trinitrophenyl (TNP)[1] and sheep red blood cells; and HIV viral proteins, including gp160 and p24 [14–22]).

This B cell activation was initially believed to be "polyclonal" in nature since: (*a*) it included antibodies reactive with a large number of different antigens (9); (*b*) components of the HIV virus (especially gp41) were reported to induce normal B cells to polyclonally proliferate and secrete Ig in vitro (23–26); and (*c*) the number of Ig-secreting lymphocytes increased in HIV-infected patients, whereas the total number and proportion of peripheral blood B cells did not (3, 9, 27, 28). Yet more recent studies find little evidence of polyclonal memory B cell activation in HIV^+ patients (29, 30) and the stimulatory effects of gp41 are now regarded primarily as a reflection of in vitro culture conditions (31, 32). It has recently been proposed that the B cell activation induced by HIV-1 infection is primarily directed against the virus itself (30, 32). For example, limiting dilution analysis of PBL from AIDS patients cultured in vitro showed that 20–100% of these B cells produced anti-HIV antibodies (30, 33).

The humoral immune response mounted by retrovirus-infected individuals may be important in regulating HIV replication/spread in vivo (34–36), preventing superinfection by other pathogens, and/or mediating autoimmune damage to host tissues (37–41). It is therefore important to determine: (*a*) the processes involved in regulating B cell activation in HIV-1-infected patients, and (*b*) whether changes in these processes are associated with disease progression. In addition, abnormal B cell activation is a feature of other infectious diseases (e.g., EBV, cytomegalovirus, and parasitic infections [42–45]) such that studies of HIV-1 might yield insights relevant to other diseases as well.

To examine the nature of the B cell activation present in HIV^+ individuals, investigators have monitored changes in the concentration of serum antibodies and the activation state of lymphocytes cultured in vitro (6, 29, 30, 33, 46, 47). While both of these approaches have value, it is now possible to directly monitor the number and antigenic specificity of B cells activated as a consequence of HIV infection. This can be accomplished by using an ELISA spot assay which is sensitive enough to detect the Ig produced by single freshly isolated in vivo activated peripheral blood lymphocytes (PBL; 48–51).

We used the ELISA spot technique to study large numbers of HIV-infected patients with Center for Disease Control (CDC) class II, III, and IV disease. Results indicate that HIV infection induced both virus-specific and polyclonal B cell activation. These two types of B cell activation appeared to be regulated independently, in that (*a*) HIV-specific responses declined as a function of disease severity while polyclonal responses increased, and (*b*) only polyclonal responses correlated significantly with CD_4 cell number and CD_4/CD_8 ratio.

Methods

Subjects. A total of 86 HIV-positive patients (23 with CDC class II disease, 55 with class III, and 8 with class IV disease) were studied. All were followed as outpatients in the Department of Transfusion Medicine of the National Institutes of Health (NIH) Clinical Center. 20 of

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1. *Abbreviations used in this paper:* ASC, antibody-secreting cell; PBL, peripheral blood leukocytes; TNP, trinitrophenyl; TNP-KLH, trinitrophenylated keyhole limpet hemocyanin.

these patients were being treated with zidovudine. 51 seronegative donors were used as controls.

ELISA spot assay of human cells. Flat-bottomed Immulon I microtiter plates (Dynatech Labs, Inc., Chantilly, VA) were coated with goat anti-human Ig (Boerhinger Mannheim Corp., Indianapolis, IN), or soluble Ag (including ssDNA, myosin, actin, ovalbumin, trinitrophenylated keyhole limpet hemocyanin (TNP-KLH), the p24 Gag of HIV-1 [hereafter p24, kind gift of Keith Higgings, Chiron Corporation, Emeryville, CA], or glycosylated recombinant GP160 [MicroGeneSys, Inc., Meriden, CT]) and then blocked with 1% PBS as previously described (50, 52).

Peripheral blood mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). White blood cells recovered from the interface were washed six times in medium consisting of RPMI 1640 supplemented with 2.5% FCS. These cells were resuspended at a concentration of 10×10^6/ml. Serial dilutions of freshly isolated PBL, starting with 10^6 cells/well, were incubated on Ag-coated plates for 9–10 h at 37°C in a 5% CO^2 in air incubator. The cells were then washed away with PBS/0.05% Tween 20 and the plates overlaid with phosphatase-conjugated antibodies to human IgG, IgM, and IgA (Boerhinger Mannheim Corp.). The antibodies produced by individual B cells that bound to antigen on the plate were visualized by addition of a 5-bromo-3-chloro-4-indolyl phosphatase solution (Sigma Chemical Co., St. Louis, MO) in a low melt agarose kept at 56°C. Phosphatase acts on this substrate to produce a blue spot that cannot diffuse through the agarose once it solidifies at room temperature (48). That dilution of cells producing ~ 20–30 spots/well was used to calculate the total number of antibody-secreting cells (ASC) for each antigen per 10^6 PBL.

The presence of BSA in the FCS used to conduct these assays completely inhibited the binding of BSA-specific antibodies to BSA-blocked plates, giving the assays zero background. The sensitivity and specificity of this assay has been documented in antigen inhibition tests and in studies involving antigen-specific hybridoma cell lines (48–50).

Statistical analysis. Correlation coefficients were determined using Pearson's procedure based on linear regression analysis. Statistical significance was determined used a two-tailed t test unless otherwise described.

Results

Number and specificity of Ig-secreting cells in HIV-1-infected patients. The frequency of PBL secreting IgG, IgM, or IgA antibodies in 86 HIV$^+$ individuals was compared with that of 51 normal donors. As seen in Table I, the number of cells secreting Ig of each isotype was increased 1.9–3.4-fold in the patient group. An antigen-specific ELISA spot assay sensitive enough to detect one Ab-secreting cell among 10^6 PBL was used to determine the specificity of the antibodies produced by these cells. This assay was selected on the basis of its (*a*) > 20-fold higher sensitivity for spontaneously secreted Ig compared with conventional ELISAs (48–50, 53, and data not shown), (*b*) freedom from artifacts induced by prolonged in vitro culture and/or mitogenic stimulation (54), and (*c*) ability to provide an accurate measure of B cell activation in vivo (48, 50, 53, 55).

Among HIV-infected individuals, the repertoire of IgG-secreting cells was significantly skewed toward reactivity against HIV-associated antigens. Antibody from 7.1% of all IgG-producing cells specifically bound to recombinant purified HIV_{IIIB} gp160, a 737-fold increase over control background ($P < 0.0001$, Table I). Antibody from an additional 3% of IgG-secreting cells bound to the p24 antigen of HIV (140-fold over background, $P < 0.001$).

By comparison, the repertoire of IgM- and IgA-secreting cells was not skewed toward the production of gp160 or p24 reactive antibodies. These PBL exhibited a general and proportional increase in reactivity against a diverse panel of autoantigens (DNA, myosin, actin) and conventional antigens (TNP-KLH, ovalbumin), consistent with a process of polyclonal B cell activation (Table I). The number of cells secreting IgG antibodies against members of this antigen panel was increased 9–25-fold compared with controls (mean increase, 17.9-fold). This indicated either that cross-reactive B cells were being poly-

Table I. Number of B Cells Secreting Ab Reactive with Each Antigen

		Total	gp160	p24	DNA	TNP	MYO	ACT	OVA
IgG									
HIV$^+$	Mean	10,722	737	329	82.8	70.4	15.2	31.8	43.7
	SE	491	93	94	7.9	9.4	2.2	4.6	5.2
Normal	Mean	3,118	1.0	2.3	3.3	2.8	1.7	1.6	4.0
	SE	179	0.2	0.7	0.4	0.5	0.5	0.3	0.4
Ratio HIV$^+$/normal		3.4	737*	140*	25.1‡	24.8‡	9.1§	19.4‡	10.9‡
IgM									
HIV$^+$	Mean	327	11.4	29.8	15.8	13.6	14.9	16.1	8.1
	SE	47	3.7	7.4	3.3	3.6	6.3	6.0	2.6
Normal	Mean	116	3.3	14.5	3.9	2.6	2.7	2.3	2.1
	SE	13	0.5	4.5	1.0	0.9	0.8	1.6	0.5
Ratio HIV$^+$/normal		2.8	3.5	2.1	4.0	5.3	5.5	6.9	3.8
IgA									
HIV$^+$	Mean	718	1.6		1.2	1.7			1.2
	SE	161	0.4		0.6	0.9			0.6
Normal	Mean	374	0.8		0.3	0.6			0.3
	SE	70	0.3		0.2	0.3			0.2
Ratio HIV$^+$/normal		1.9	2.0		3.5	3.0			3.5

The number of cells secreting antibody reactive with the HIV_{IIIB} gp160 and p24, the autoantigens DNA, actin (ACT), and myosin (MYO), and the conventional antigens ovalbumin (OVA) and TNP-KLH were measured using the ELISA spot assay. Results represent the mean (and SE) of Ig-secreting cells/10^6 PBL from up to 86 HIV-infected donors and 51 normal controls. * $P < 0.005$; ‡ $P < 0.01$; § $P < 0.05$.

clonally activated or that HIV-reactive lymphocytes were producing cross-reactive antibodies.

Relationship between HIV-specific and polyclonally activated B cells. We investigated the possibility that the IgG antibodies reactive with members of the antigen panel were induced by stimulation of, or reflected cross-reactive binding by, HIV-specific B cells. As seen in Fig. 1 and Table II, the number of B cells reactive with DNA, TNP, actin, ovalbumin, and myosin showed strong positive correlations with one another and with the total number of IgG-secreting cells present in HIV-infected patients. In contrast, the number of anti-gp160- and anti-p24-secreting cells were positively correlated with one another but not with cells producing non-HIV binding antibodies. These findings suggest that polyclonal IgG production in HIV^+ individuals was independent of HIV-specific B cell activation.

Relationship between disease progression, T cell abnormalities, and B cell activation in HIV^+ individuals. We investigated whether the number of antigen-specific IgG-secreting cells in the peripheral blood correlated with disease progression. Results indicate that the proportion of IgG anti-HIV-secreting cells was significantly lower among patients with CDC class IV than class II or III disease ($P < 0.01$, two-sample t test; Fig. 2). Conversely, the degree of polyclonal B cell activation was significantly increased among patients with CDC class IV disease ($P < 0.005$).

Another interesting correlation was noted between CD_4^+ count, CD_4/CD_8 ratio, and the degree of polyclonal activation. As seen in Table III, the proportion of cells secreting IgG antibodies against non-HIV antigens showed a significant inverse correlation with these parameters, unlike those reactive with HIV. In addition, the total number of IgG- and IgA-producing cells, and serum IgM and IgA concentration, showed significant inverse correlations with CD4 count and/or CD4/CD8 ratio (Table IV).

Discussion

Hypergammaglobulinemia and increased B cell activation are common early features of HIV infection (9–13). This work documents that both virus-specific IgG and polyclonal IgM and IgA responses are present in HIV-infected individuals. These two forms of B cell activation appear to be independently regulated, since (*a*) the extent of gp160- and p24-specific B cell activation did not correlate with that of polyclonal B cell stimulation, (*b*) polyclonal but not HIV-specific B cell activation varied as a function of CD_4 count and CD_4/CD_8 ratio, and (*c*) HIV-specific B cell activation decreased while polyclonal B cell activation increased among patients with advanced disease.

This work established the number of lymphocytes secreting anti-gp160 antibodies in different patient populations. We found that 2–17% (mean 7.1%) of IgG-producing cells in HIV-infected individuals reacted with recombinant purified gp160, while 1–9% reacted with p24 (Table I). These frequencies are consistent both with the number of anti-HIV-secreting cells induced in mice immunized and boosted with these antigens (56) and with the frequency of antigen-specific B cells found in other diseases involving chronic immunostimulation (51).

Table II. Correlation Matrix among B Cells Secreting IgG Antibodies Reactive with Different Antigens

	p24	DNA	TNP	MYO	ACT	OVA
gp160	0.51*	−0.06	0.10	−0.06	−0.10	−0.04
DNA			0.81*	0.68*	0.72*	0.65*
TNP				0.77*	0.77*	0.65*
MYO					0.61*	0.68*
ACT						0.66*

Data represent Sperman's rank correlation coefficients based on 20–59 determinants for each pair of variables. Statistical significance was determined using a nonparametric procedure. MYO, myosin; ACT, actin; OVA, ovalbumin.
* $P < 0.005$.

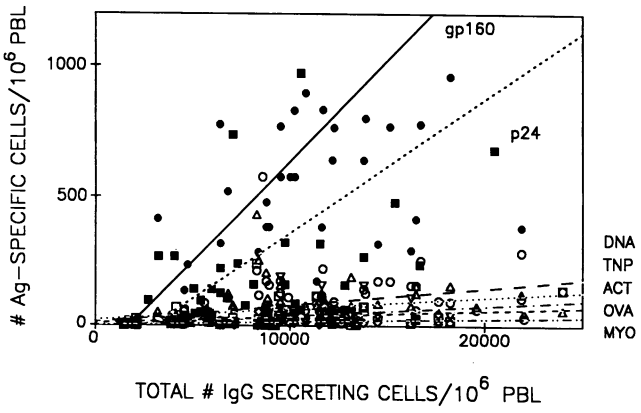

Figure 1. The number of cells secreting IgG antibodies reactive with each antigen gp160 (●), p24 (■), DNA (○), TNP-KLH (△), actin (□), ovalbumin (▽), and myosin (◇) was plotted as a function of the total number of IgG-secreting cells/10^6 PBL from each patient. For all plots, the correlation coefficient of Ig-secreting versus Ag-specific cells had an $r \geq 0.28$ and a $P < 0.05$.

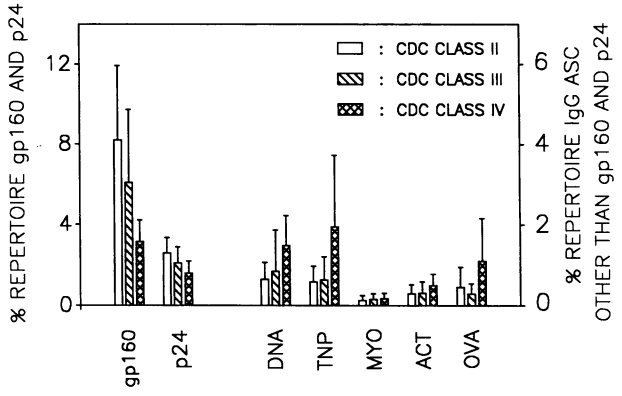

Figure 2. Percent of Ig-secreting cells vs stage of disease. The percent of cells secreting IgG antibodies against each antigen was plotted as a function of CDC disease stage. There was a significant decline ($P < 0.01$) in the number of B cells secreting anti-gp160 antibodies among patients with CDC stage IV disease, as opposed to a significant increase ($P < .005$) in the frequency of cells polyclonally activated against other antigens. This trend was maintained when only patients being treated with zidovudine were analyzed ($n = 4$, CDC stage II; $n = 5$, CDC stage III; $n = 4$, CDC stage IV). The total number of IgG-secreting cells/10^6 PBL expressed by patients was 9,100±4,500 (stage II), 10,300±5,100 (stage III), and 11,800±5,100 (stage IV).

Table III. Correlation between the Frequency of Cells Secreting Antigen-specific IgG Antibodies and T Cell Parameters

Antigen	T4	T4/T8
gp160	−0.06	0.10
p24	0.07	0.05
DNA	0.28*	−0.34‡
TNP	−0.29*	−0.21
MYO	−0.41*	−0.45‡
ACT	−0.38*	−0.47‡
OVA	−0.28*	−0.34*

Data represent Pearson's coefficient of correlation based on 35–68 determinants for each pair of variables. MYO, myosin; ACT, actin; OVA, ovalbumin.
* $P < 0.05$, ‡ $P < 0.01$.

They also support the observation that gp160 represents the immunodominant antigen expressed by HIV.

Of interest, the frequency of B cells producing antibodies against viral antigens was significantly lower in patients with CDC stage IV than stage II or III disease (Fig. 2). This finding is consistent with evidence from other investigators showing that disease progression is associated with a decrease in Ab titer against HIV proteins (57–60) and suggests that an active humoral response against HIV may delay disease progression (or alternatively, that the ability to maintain such a response degrades as immune status declines). A possible confounding influence on these studies was the more frequent use of zidovudine by patients with advanced disease. To control for this variable, all patients receiving zidovudine therapy were analyzed independently. Again, those with the most advanced disease expressed the lowest numbers of anti-gp160- and anti-p24-secreting B cells (Fig. 2, legend).

Amadori et al. have reported that 20–100% of B cells from the peripheral blood of AIDS and AIDS-related complex patients produce anti-HIV antibodies (30). Our estimate of p24- and gp160-specific B cells is somewhat lower, a difference we attribute to our use of the ELISA spot assay to study in vivo activated lymphocytes versus Amadori's analysis of in vitro cultured cells. As we and others have shown, in vitro culture and mitogen stimulation can alter the repertoire of B cells induced to secrete Ig (54). Moreover, it seems unlikely that a majority of an individual's expressed repertoire would be directed against a single pathogen, since the humoral immune system is constantly being stimulated by numerous exogenous antigens. Ongoing studies of sera and mitogen-stimulated PBL from patients conducted in our lab also find that HIV-specific antibodies constitute < 30% of the total immune response (data not shown).

Table IV. Correlation between the Number of Ig-secreting Cells and T Cell Parameters

	T4	T4/T8
IgG-ASC	−0.26*	−0.33‡
Serum IgG	−0.10	−0.20
IgM-ASC	−0.13	−0.11
Serum IgM	−0.15	−0.33‡
IgA-ASC	−0.32*	−0.20
Serum IgA	−0.52§	−0.40§

Correlation coefficients determined as described in Table III. The correlation between serum Ig level and number of Ig-secreting cells was 0.46 for IgG, 0.13 for IgM, and 0.34 for IgA.
* $P < 0.05$; ‡ $P < 0.01$; § $P < 0.005$.

While technically more difficult than conventional studies of serum Ig, the ELISA spot assay provided data on the state of B cell activation in vivo unobtainable by other methods. In addition, the ELISA spot assay is unaffected by factors such as the rate of Ig catabolism or the presence of Ag, variables that influence serum Ab levels. The ELISA spot assay has been compared to conventional ELISAs designed to detect Ig spontaneously secreted by B cells cultured in vitro. While these two techniques yield concordant results ($r^2 = 0.63$–0.87; reference 61 and data not shown), the ELIspot assay has repeatedly been found to be considerably more sensitive (48–50). A limitation inherent to our study was that only B cells from the peripheral circulation could be analyzed. Since large numbers of activated B cells may be present in the spleen and lymph nodes, our results might not reflect the state of B cell activation in the patient as a whole. Indeed, this limitation may account for the differences noted between the level of certain serum Ig isotypes and numbers of Ig-secreting cells detected in this work (Table IV).

Polyclonal activation appeared to be the primary process responsible for the stimulation of IgM- and IgA-secreting lymphocytes in HIV-infected individuals (Table I). Leitman et al. have shown that increased serum IgA levels are strongly predictive of disease progression (62). While the cause of this polyclonal activation is unclear, it may reflect direct immunostimulation by one or more components of the virus (such as gp41 [23–26]), bystander stimulation due to lymphokines released by HIV-specific T cells (63), and/or abnormalities in immune regulation resulting from a loss of T cell function. In the case of IgG-secreting cells, there was an unexpected 9–25-fold increase in the number of non–HIV reactive lymphocytes despite an increase of only 3.4-fold in the total number of IgG-producing cells (Table I). While indicative of polyclonal activation, this result further suggests that a subpopulation of highly cross-reactive B cells might be specifically stimulated in HIV^+ individuals. This possibility is currently under investigation.

There was a significant inverse correlation between the number of IgG-secreting B cells among patients and their CD4 cell count, suggesting that these two processes may be causally linked (Table IV). Indeed, an association between polyclonal B cell activation and defective T_4 function has been reported in other disease states, such as experimentally induced graft-versus-host disease, murine lupus, and murine AIDS (41, 64–68).

The level of B cell activation found in HIV-infected individuals is comparable to that detected in patients with autoimmune diseases such as systemic lupus erythematosus (69). This polyclonal activation is associated both with the production of pathogenic autoantibodies (such as IgG anti-DNA) and the suppression of immune responses to foreign antigens. It is possible that pathogenic organisms (e.g., HIV, EBV, cytomegalovirus) "intentionally" induce polyclonal responses in an effort to suppress the host's capacity to mount a specific response against the pathogen. In this context, it is interesting to note the inverse correlation between polyclonal activation and HIV-specific responses manifest by patients with CDC class II and

III versus IV disease (Fig. 2). Ongoing research may elucidate the mechanism(s) by which HIV and other pathogens abnormally activate host B cells and determine the effect of this process on disease progression.

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