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Serial study of CD5$^+$ and CD5$^-$ B cell subpopulations in 335 HIV seropositive patients

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SUMMARY

B cell subpopulations, as defined by double-labelling techniques with CD5 and CD19 monoclonal antibodies (MoAbs), were serially studied in 335 HIV-1 seropositive patients. At the time of the first consultation, no important modifications in either CD5$^+$ or CD5$^-$ subpopulations were observed, whatever the stage of the disease. However, in 18 out of the 335 patients (5·37%), a sharp increase in B cells exceeding 20% and 300/mm^3 was observed. This increase in B cells was mainly accounted for CD5$^-$ CD19$^+$ B cell subpopulations and was associated with: (i) evolution of the disease, since only four patients presented it at their first consultation (one lymphadenopathy-associated syndrome (LAS) and three AIDS); (ii) advanced stages of disease since, at the time of B cell augmentation, two patients were staged as LAS, four as ARC and 12 as AIDS; (iii) a high incidence of non-Hodgkin's lymphomas (NHL) since three out of the 18 patients presented a histologically confirmed NHL and three others a clinical pattern compatible with this diagnosis. However, in three patients with B hyperlymphocytosis, polymerase chain reaction (PCR) studies of immunoglobulin gene rearrangement revealed the existence of a polyclonal expansion of B cells. These results justify inclusion of a *pan*-B cell marker in routine phenotypic studies of HIV-infected individuals, as well as the search for NHL among patients presenting this abnormality.

Keywords HIV infection CD5$^+$CD5$^-$ B cells

INTRODUCTION

AIDS is a disease caused by HIV (Barré-Sinoussi *et al.*, 1983), and is characterized by marked depletion of CD4 T cells and strong depression of cellular immunity (Ammann *et al.*, 1983; Cavaillé-Coll *et al.*, 1984; Aiuti *et al.*, 1989; Edelman & Zolla-Pazner, 1989). In addition to these alterations in cellular immunity, polyclonal B cell stimulation frequently occurs and is associated with elevated levels of serum immunoglobulin, circulating immune complexes, numerous autoimmune phenomena and poor response of B cells to the usual inducers of B cell proliferation (Katz *et al.*, 1986; Kekow *et al.*, 1986; Kopelman & Zolla-Pazner, 1988; Mayer-Siuta, Keil & De Bari, 1988).

In recent years several reports (Hayakawa *et al.*, 1984; Hardy & Hayakawa, 1986; Casali & Notkins, 1989) have suggested that mouse Ly1-B and their human counterpart CD5$^+$ B cells could correspond to a distinct B cell subset mainly involved in the production of autoantibodies. In the present retrospective work we examined B cell subpopulations, using double-colour fluorescence techniques to detect CD5$^+$ and CD5$^-$ B cells along with CD4 and CD8 subpopulations in 335 HIV$^+$ patients.

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SUBJECTS AND METHODS

Patients

We have studied 335 HIV-1$^+$ patients (ELISA with confirmation by Western blot) followed at Pasteur Hospital, Paris. Most were homosexual men. These patients were initially classified according to the Centers for Disease Control (1986) criteria, as follows: 201 seropositive asymptomatic carriers (SPC); 78 lymphadenopathy-associated syndrome (LAS); 31 ARC and 25 AIDS. Follow up, including phenotypic determination, was performed two to three times a year for patients without complications so that the majority of patients included in the present study had at least five follow-up studies. The control group included 47 healthy heterosexual donors from the blood bank of the Pasteur Institute.

Cytofluorometric analysis

Peripheral blood (50 μl) was incubated with two different monoclonal antibodies (MoAbs) coupled either to FITC or to PE. Those employed for all patients were CD4PE-CD8 FITC (Coultronics, Hialeah, FL) or CD5 FITC-CD19 PE (Becton

Table 1. T and B lymphocyte subpopulations (first consultation)

	$CD4^+$		$CD8^+$		$CD5^+CD19^-$		$CD5^-CD19^+$		$CD5^+CD19^+$	
Subjects	%	AN/mm^3	%	AN/mm^3	%	AN/mm^3	%	AN/mm^3	%	AN/mm^3
Controls ($n=47$)	41·4±7·1	941·4±316·2	29·1±5·7	669·3±228·9	72·2±7·4	1284·2±382·7	7·5±3·1	161·1±99·9	1·6±0·6	32·4±16·7
SPC ($n=201$)	27·0±9·9	578·0±264·6	42·1±7·2	881·1±262·2	68·4±9·7	1426·7±60·1	5·2±3·1	107·0±80·0	0·9±0·7	19·9±15·7
LAS ($n=78$)	26·3±9·7	582·4±291·5	43·2±11·1	974·7±382·5	68·0±8·6	1531·6±616·6	5·7±3·7	123·9±82·7	0·9±0·6	20·6±13·4
ARC ($n=31$)	19·3±9·4	339·0±235·2	47·4±11·4	852·3±418·1	64·2±11·1	1101·7±540·2	5·4±3·1	86·6±50·4	0·9±0·5	13·8±9·9
AIDS ($n=25$)	8·19±7·8	163±165	52·1±12·0	900·4±423·6	58·6±12·1	994·8±495·4	5·8±4·6	92·4±68·3	1·2±0·9	18·1±10·9

AN/mm^3, absolute number per mm^3; SPC, seropositive asymptomatic carrier; LAS, lymphadenopathy-associated syndrome.

Dickinson, Mountain View, CA). After 30 min of incubation at 4°C, cells were washed twice and erythrocytes were lysed (Immunolyse, Coultronics).

Ficoll–Hypaque density gradient isolated peripheral blood lymphocytes (PBL) were stained by the direct immunofluorescence technique with rabbit $F(ab')_2$ anti-human κ and λ polyclonal antibodies conjugated to PE and fluorescein, respectively (Dakopatts, Glostrup, Denmark).

Analysis of cells was carried out on 10000 lymphocytes, gated using both forward and right angle scatters in an EPICS-752 cell sorter (Coultronics).

Detection of V_H rearrangement by polymerase chain reaction (PCR) amplification

PCR primers. The 5′ primer was given by the highly conserved stretch of 20 bp located near the 3′ end of framework 3 (FW3) region, corresponding to codons 88–95. The degenerated sense primer employed (5′ACACGGCIITGTATTACTG3′) was designed to anneal to all 17 published V_H segment sequences. As there is important homology of the six germline J_H-segments, at the 3′ end of FW4 regions between codons 101 and 112, the 3′ primer was designed to match perfectly five of the six sequences and to anneal despite one mismatch to the J_H2 sequence (5′CCTGAGGAGACGGTGACC3′).

PCR fast procedure. Fast PCR was performed according to Trainor *et al.* (1990) and modified by Vandenvelde, Verstraete & Van Beers (1990). Briefly, PBL were previously isolated by Ficoll–Hypaque gradient, and after washings suspended in phosphate-buffered saline (PBS); 50 μl of this suspension were covered with 70 μl light mineral oil (Sigma Chemical Co., St Louis, MO) and heated to 99°C for 15 min. The 50 μl reaction mixture contained 50 mM NaCl, 10 mM Tris-HCl (pH 8·3 at 25°C), 3 mM $MgCl_2$, 200 μM each of adenosine 5′triphosphate, cytidine 5′-triphosphate, guanosine 5′-triphosphate and thymidine 5′-triphosphate, 30 pmol of each primer, 1 unit of Taq polymerase (Perkin, Norwalk, CT), 100 μg/ml of gelatin, 5% v/v of dimethylsulfoxide and 10 μl ($2·5 \times 10^5$) of boiled PBL. After 40 cycles of amplification, DNA was analysed by electrophoresis on a 3% Nu Sieve-GG (FMC Co., ME) agarose gel in TBE buffer (89 mM tris base, 8·9 mM H3BO4, 2 mM EDTA, pH 8) stained using 3·33 μg/ml ethidium bromide, visualized and photographed using short wavelength u.v. light. PCR products' sizes were evaluated by comparison with a pBR322DNA-*Hae*III molecular weight marker (MWM) (Boehringer Mannheim, Mannheim, Germany).

RESULTS

Table 1 summarizes results observed in 47 uninfected controls and 335 HIV^+ patients at the time of their first consultation. The well-known decrease in the CD4 subset related to disease progression (Cavaillé-Coll *et al.*, 1984; Stites *et al.*, 1986; Vuillier, Lapresle & Dighiero, 1988; Aiuti *et al.*, 1989), as well as the increase in CD8 subpopulations are shown. For total B cells, no important modifications could be evidenced. The decrease in absolute numbers observed for ARC and AIDS patients was related to lymphopenia observed in these patients (data not shown). In addition, when B cells were segregated into $CD5^-$ and $CD5^+CD19^+$ B cell subpopulations, they did not appear to undergo significant modifications. Although no important modifications could be observed in the 335 HIV^+ patients, consistent increases in B cells, which reached 300/mm^3 with relative values exceeding 20%, were observed in 18 patients (5·37%). These patients included 16 men and two women, from the following risk groups: 12 homosexual men; three drug addicts; one heterosexual woman with a seropositive partner; one transfusion recipient; and one from an African endemic region (Table 2). When these patients were seen for the first time, they were staged as: SPC, four; LAS, seven; ARC, one and AIDS, six. Four (one LAS and three AIDS) out of 16 patients for whom data were available presented increased values of total B cells at the time of the first study. As three out of the four patients in the study were at the AIDS stage, it cannot be excluded that they could have presented lower levels of $CD19^+$ cells in early stages. For the others, increased B cell counts appeared during evolution. Interestingly, at the time high B cell values were observed, all patients except two were staged as ARC (four) or AIDS (12). However, the increase did not necessarily correlate with progression of disease.

Patients were divided into two groups according to the extent of B cell increase. Group 1 included 11 patients (1–11, Table 2) and was defined by sustained absolute values exceeding 300/mm^3 and relative B cell values $\geq 30\%$. Group 2 comprised the remaining seven patients (patients 12–18) whose relative values were $\geq 20\%$ $< 30\%$ and whose absolute values only occasionally exceeded 300/mm^3.

Since these results could correspond to a leukaemic phase of a malignant lymphoma, we retrospectively assessed this possibi-

Table 2. Clinical and phenotypic characteristics of HIV patients displaying increased values of total B cells

Patient no.	Age (years)	Sex	Risk* factor	Initial status				After CD19 ≥ 300 mm³					Associated lymphoma
				Date	Stage	CD4 values	CD19 values	Date of first CD19 ≥ 300 mm³	CD4 values at this time	Stage at this time	Highest value of CD19 (date)	Treatment at this time AZT	
1	38	F	2	88·06	AIDS	153	314	88·06	153	AIDS	777 (89·09)	Yes	Yes†
2	34	M	1	86·09	LAS	362	597	86·09	362	LAS	597 (86·09)	No	No
3	32	M	1	87·10	LAS	450	N.D.	88·06	67	ARC	512 (89·05)	Yes	No
4	45	F	4	87·09	ARC	184	130	90·01	100	AIDS	465 (90·01)	Yes	Yes†
5	21	M	1	85·12	LAS	757	N.D.	89·07	18	AIDS	436 (87·07)	Yes	No
6	32	M	1	87·12	AIDS	16	585	87·12	16	AIDS	585 (87·12)	Yes	Yes
7	46	M	1	89·07	AIDS	63	144	90·01	40	AIDS	488 (90·01)	Yes	Yes†
8	42	M	5	88·12	LAS	6	232	89·06	12	ARC	554 (89·06)	Yes	No
9	29	M	1	89·05	AIDS	40	232	89·08	74	AIDS	491 (89·09)	Yes	No
10	41	M	4	87·02	LAS	351	90	89·08	13	AIDS	361 (89·08)	Yes	Yes
11	44	M	1	85·12	AIDS	420	588	85·12	420	AIDS	588 (85·12)	Yes	Yes
12	44	M	1	89·07	SPC	11	186	89·11	34	ARC	313 (89·11)	Yes	No
13	34	M	1	88·09	SPC	43	50	89·05	216	AIDS	384 (89·11)	Yes	No
14	53	M	3	87·06	SPC	95	27	89·12	48	AIDS	473 (89·12)	Yes	No
15	33	M	2	88·03	SPC	62	86	89·11	21	AIDS	450 (89·11)	Yes	No
16	38	M	1	88·03	AIDS	180	153	89·07	21	AIDS	399 (89·07)	Yes	No
17	36	M	2	87·12	LAS	225	267	88·08	166	ARC	305 (88·08)	Yes	No
18	37	M	1	87·04	LAS	360	279	88·03	385	LAS	328 (88·03)	No	No

* 1, homosexual; 2, drug addiction; 3, transfusion, 4; seropositive partner; 5, African endemic region.
† Suspected on clinical data but non-histologically confirmed.
SPC, seropositive asymptomatic carrier; LAS, lymphadenopathy-associated syndrome.

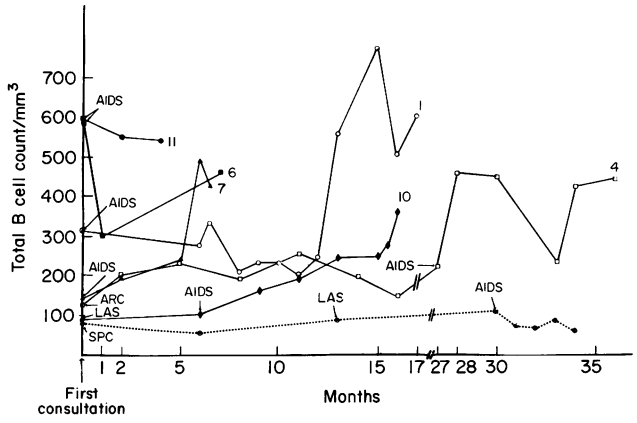

Fig. 1. Evolution of total CD19⁺ cells over time in the six patients in whom lymphoma was either confirmed or suspected. Numbers 1, 4, 6, 7, 10 and 11 correspond to patients' numbers in Table 2. For each patient the stage of the disease is mentioned for the first consultation and during the evolution of the disease. Discontinuous line shows the evolution of total B cell count for a representative HIV⁺ patient during similar disease progression but without rise in B pool. SPC, seropositive asymptomatic carrier; LAS, lymphadenopathy-associated syndrome.

lity. Three of the 11 patients from group 1 had histologically confirmed non-Hodgkin's lymphoma (NHL) diagnosed either before death or during post-mortem examination (patients 6, 10 and 11 in Table 2), whereas two other patients presented cerebral scans compatible with the diagnosis of cerebral lymphoma (patients 4 and 7); and one (patient 1 in Table 2) presented marked hepato-splenomegaly and deep abdominal enlarged lymph nodes of unexplained origin. For the remaining five patients from group 1 and the seven patients from group 2, no evidence of NHL could be detected. In addition, the three patients staged as AIDS and with absolute value of total B cells $> 300/mm^3$ at the first consultation appeared to develop NHL during their evolution (patients 1, 6 and 11 in Table 2).

For 16 of 18 patients described in Table 2, initial immunoglobulin levels in sera were in a range of $19{\cdot}09 \pm 5{\cdot}78$ g/l for IgG, $4{\cdot}31 \pm 1{\cdot}97$ g/l for IgA and $1{\cdot}65 \pm 0{\cdot}92$ g/l for IgM. At the time of highest B cell count, no significant variations were observed, since immunoglobulin levels accounted for $18{\cdot}44 \pm 4{\cdot}1$, $3{\cdot}32 \pm 1{\cdot}92$, $1{\cdot}36 \pm 0{\cdot}75$ g/l for IgG, IgM and IgA respectively. However, patient 1 displayed 28 g/l IgG and 9 g/l IgA in initial status compared with 16 g/l IgG and 3 g/l IgA at the time of B hyperlymphocytosis.

Figure 1 shows the evolution of total CD19⁺ cells over the period in the six patients who presented a suspected or confirmed diagnosis of lymphoma. The increase of B cell population was persistent despite some fluctuations between individual values.

In order to obtain further information on clonality of HIV-related B hyperlymphocytosis the PCR technique was performed in three patients (numbers 6, 14 and 15, Table 2) (Fig. 2). At this time, total B cell counts were 471, 433 and $340/mm^3$ respectively. As shown in Fig. 2, PCR profiles failed to show any clonal rearrangement and were identical to that of the normal control. Moreover, none of these patients displayed a κ or λ excess in flow cytometry (data not shown).

Taking into account the strong variations observed in uninfected individuals in the percentage of CD5⁺CD19⁺ cells

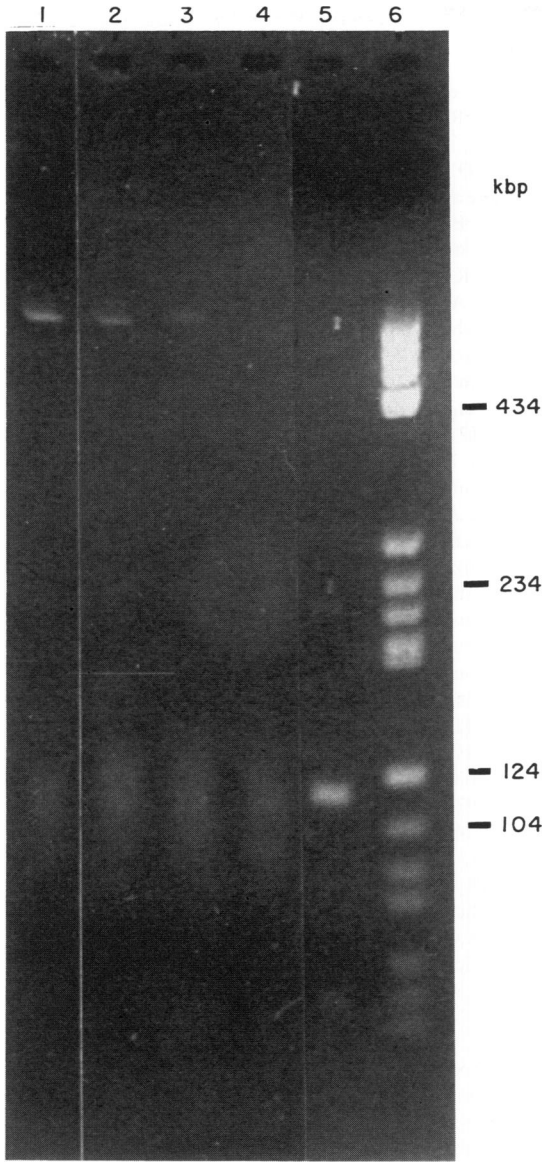

Fig. 2. Study of V_H gene rearrangement by PCR analysis of peripheral mononuclear cells from three patients with high levels of total B cells. Lanes 1, 2, 3, HIV-infected patients with high values of total B cells (471, 433, 340/mm^3, respectively); lane 4, uninfected subject (negative control); lane 5, patient with chronic lymphocytic leukaemia (positive control). Molecular size standards (kb) are represented on lane 6. No monoclonal band was detected for the three HIV$^+$ patients (lanes 1, 2 and 3).

within B cells (Kipps, Herzenberg & Vaughan, 1986; Vuillier, Scott-Algara & Dighiero, 1991), we considered the CD5$^+$CD19$^+$ subpopulation as significantly increased if it exceeded 2·8% of total lymphocytes, absolute values ≥ 66/mm^3, and CD5$^+$CD19$^+$/total CD19$^+$ ratio $> 25\%$ (normal values + 3 s.d.). In these conditions, only seven among 335 patients studied fulfilled these requirements. Four of them belonged to the above mentioned group displaying increased CD19 levels of total B cells, with only one of them presenting a CD5$^+$CD19$^+$/total CD19$^+$ ratio exceeding 40%. The three other patients, who had never increased total B cell count, displayed CD5$^+$CD19$^+$ ratios of 23%, 43% and 48%.

DISCUSSION

In the present work we examined CD5$^+$ and CD5$^-$ B cell subpopulations in 335 HIV-1$^+$ individuals. Our results confirm previous reports indicating that there are no major alterations in absolute and relative values of total B cells during the different stages of HIV disease, although our mean values for normal controls and for HIV$^+$ subjects were slightly lower (Stites *et al.*, 1986; Martinez-Maza *et al.*, 1987). Interestingly, CD5$^+$ B cells, considered to be mainly involved in autoantibody production (Hayakawa *et al.*, 1984; Hardy & Hayakawa, 1986; Casali & Notkins, 1989), do not appear to increase during HIV-1 infection despite the presence of numerous autoimmune phenomena related to this disease. The increased CD5$^+$CD19$^+$ values observed in four patients who did not display increased total B cells could be explained by heterogeneity in the proportion of CD19$^+$CD5$^+$ cells reported among normal subjects (Kipps *et al.*, 1986). In contrast to reports of other investigators (Volk *et al.*, 1988), CD5$^+$CD19$^+$ cell increase was detected at transient times in some patients, without any established relationship with an infection stage, a B cell increase or B lymphoma development. This increase was not persistent.

However, our results indicate that in a small number of patients (18 out of 335, 5·37%), an important increase in total B cells, with absolute values exceeding 300/mm^3 and with relative values exceeding 20%, can be observed. This is generally observed during evolution and, with some fluctuations, appears to persist. Interestingly, it seems to be associated with evolution of disease, since 16 out of 18 patients presenting an increase in total B cells were already staged as ARC or AIDS. Despite B lymphocyte augmentation, no modification in serum immunoglobulin levels was observed, except for one patient in whom the B lymphocyte increase parallelled a decrease in immunoglobulin.

The B cell increase in our patients could correspond to a T-independent B cell stimulation by cytomegalovirus (CMV), Epstein–Barr virus (EBV) and hepatitis B virus co-infection, as previously reported (Gritti *et al.*, 1987). CMV and EBV co-infection can particularly induce an increase in B proliferation and coactivation of transformant genes with oncogenic potential (Hiddemann, 1989). Unfortunately, information concerning EBV and CMV co-infection is not available in our series. Nevertheless, Joab *et al.* (1991) recently studied the incidence of antibodies against ZEBRA protein (*Bam*HI Z EBV replication activator) which is known to be expressed by EBV when it switches from latent to productive cycle. Their results indicated a high incidence of these antibodies in HIV-infected patients compared with uninfected controls, suggesting that EBV is frequently reactivated in these patients. However, in 10 patients with B hyperlymphocytosis for whom this study was performed, the incidence of antiZEBRA antibodies did not differ from data on the total HIV-infected population.

Another important finding revealed here was the potential association of B cell hyperlymphocytosis to the occurrence of NHL (six out of 18 patients). These data obtained in a small series of patients should be compared with the incidence of NHL among AIDS patients in our Institution (32 NHL among 219 AIDS, 14·8%).

It could be postulated that this B hyperlymphocytosis could correspond at least for some patients to a leukaemic phase of lymphoma. However, our study on three patients failed to demonstrate a clonal origin for this hyperlymphocytosis. Moreover, patient 4 in whom a presumptive diagnosis of cerebral lymphoma was made, based on cerebral scans, displayed a polyclonal pattern in PCR and flow cytometry. Thus, it remains to determine whether this polyclonal B cell expansion in blood could be considered as a transient polyclonal state which precedes malignant transformation, as postulated by some authors who reported oligoclonal B cell expansion in hyperplasic lymph nodes as a prerequisite for NHL development (Knowles *et al.*, 1988). In that case we can consider that the patients in our study are at an early stage of B cell polyclonal hyperactivation due to different factors such as HIV proteins, EBV or CMV reactivation or abnormal lymphokine production (Edelman & Zolla-Pazner, 1989). This polyclonal B cell activation could create favourable conditions for the occurrence of mutations and oncogene activation. However, in our retrospective study, a B lymphocyte activation state could not be determined.

As most of the patients experiencing B cell hyperlymphocytosis were receiving zidovudine, and as the impression exists that there is an increasing frequency of lymphomas among AIDS patients receiving zidovudine, the relationship between zidovudine and elevated levels of B cells can be raised. However, it is important to stress that only a small fraction of patients under zidovudine treatment experienced significant augmentation of B cells.

In conclusion, although most HIV^+ patients do not experience major alterations in B cell subpopulations, about 5% of patients present a marked increase, particularly when they evolve to advanced stages of the disease. These results justify the inclusion of a *pan*-B cell marker in routine phenotypic studies for HIV-1-infected individuals. An increase in B cell populations should prompt the search for NHL and the study of clonal abnormality among B cells (κ/λ clonal excess and rearrangement of immunoglobulin genes).

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