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Serum IgD elevation is an early marker of B cell activation during infection with the human immunodeficiency viruses

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SUMMARY

Serum IgD levels in individuals infected with the human immunodeficiency viruses (HIV) were studied as a means of monitoring the character and timing of B cell activation in individuals with this infection. Significantly increased levels of IgD were characteristic of homosexual men who were HIV seropositive but asymptomatic or mildly symptomatic. The hyper IgD globulinaemia became progressively more pronounced in patients with increasingly severe infection and reached its most marked level in patients with AIDS-related complex (ARC). In ARC patients, IgD levels were increased 8·8-fold above normal which was disproportionately greater than the 2·4-fold increase in IgG, the 1·8-fold increase in IgA and the 1·6-fold increase in IgM. IgD levels declined in AIDS patients (although remained elevated compared to controls). The data suggest that an unusual type of B cell activation is responsible for the unique pattern of hypergammaglobulinaemia seen in this disease and that the B cell activation occurs early in the pathogenesis of HIV infection, often before development of symptoms, and continues throughout the course of infection.

Keywords AIDS HIV IgD polyclonal B cell activation B cells

INTRODUCTION

Polyclonal B cell activation is a common manifestation of infection with the human immunodeficiency viruses (HIV: Coffin *et al.*, 1986), previously known as the lymphadenopathy-associated virus and human T lymphotropic virus, type III (Baree-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984). A prevalent and often early reflection of this phenomenon is hypergammaglobulinaemia which is present in >90% of AIDS patients (Friedman-Kien *et al.*, 1982; Stahl *et al.*, 1982) and is a common abnormality noted in patients with AIDS-related complex (Zolla-Pazner *et al.*, 1984). While IgG and IgA are the most frequently elevated isotypes, increased in 49% and 65% of AIDS patients respectively, elevated IgM levels have been noted in 24% of patients (El-Sadr *et al.*, 1984). Within the IgG isotype, it is the IgG_1 and IgG_3 subclasses that are preferentially elevated (Aucouturier *et al.*, 1986).

Levels of IgD have been much less thoroughly studied. Chess *et al.* (1984) included IgD measurements in their studies of hyperglobulinaemia in patients with AIDS and reported that serum IgD is elevated; Papadopoulos & Frieri (1984) also noted apparently elevated IgD levels in eight of eight patients with AIDS, although quantitative information was not available in their

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study. No previous study, however, has investigated the stage of HIV infection at which increased levels of IgD first appear.

Polyclonal hypergammaglobulinaemia is common to many disorders. Although there is not always a balanced involvement of all immunoglobulin classes in this process (for example, IgA is disproportionately increased in alcohol-induced hepatic disease (Taylor & Thomas, 1984) elevated IgD levels in disease are only rarely observed (Leslie *et al.* 1979). In Hodgkin's disease, however, where increases in IgD have been reported, levels of IgD can vary from normal to 45 times normal (Corte *et al.*, 1977; 1978).

Because increases in serum IgD are not common and because IgD levels are quite strikingly elevated in patients with AIDS, we considered the possibility that these observations might reflect an unorthodox type of B cell activation in HIV infection. We therefore undertook an investigation to describe more fully the increased IgD levels in patients infected with HIV, to explore its relationship to other immunoglobulin isotypes, to determine when, in the natural history of this infection, the rise in IgD begins and to detail the relationship (or lack of one) of IgD levels to serological responses to other infectious agents.

MATERIALS AND METHODS

Subjects. Eighteen patients with AIDS, diagnosed at the New York Veterans Administration Medical Center in accordance with the definition of the Centers for Disease Control (Centers for Disease Control, 1985) were studied. Fifteen had one or more opportunistic infections including *Pneumocystis carinii* pneumonia (nine patients), disseminated *Mycobacterium avium-intracellulare* infection (two patients), oesophageal candidiasis (three patients), tuberculosis (one patient), toxoplasmosis (one patient) and cryptococcal meningitis (one patient). One patient had both Kaposi's sarcoma and *Pneumocystis* pneumonia, another patient had Kaposi's sarcoma only, and another had both Burkitt's lymphoma and tuberculous meningitis.

Patients with AIDS-related complex (ARC) were identified according to criteria previously established (Zolla-Pazner *et al.*, 1984). Briefly, individuals with ARC met the following two criteria:

(I) At least one of the following signs or symptoms:

(a) Unexplained lymphadenopathy of greater than 1 cm in diameter involving at least two non-inguinal groups of lymph nodes and lasting for at least three months, (b) oral thrush in the absence of antibiotic administration, (c) malaise, fatigue and significant weight loss.

(II) At least two of the following immunological abnormalities:

(a) Leukopenia of $<4{,}000$ white blood cells on at least two separate determinations taken at least 1 month apart, (b) cutaneous anergy using four or more common skin-test antgens, (c) a helper/suppressor ratio $<1{\cdot}0$ and (d) elevation of at least one serum immunoglobulin isotype. Eight patients with ARC were studied; all patients with ARC were seropositive for HIV by Western blot (Schupback *et al.*, 1984).

'High risk patients' were outpatients seen in the Infectious Disease Clinic at the New York Veterans Administration Medical Center who met neither the definitions for AIDS nor ARC but who were either homosexual males or intravenous narcotic abusers who self-referred to the Clinic because of symptoms such as weakness, lymphadenopathy and anxiety concerning exposure to HIV. All 10 members of this group were positive for HIV antibody by Western blotting.

'Homosexual controls' were drawn from a group of 62 men originally selected in 1981 as healthy homosexuals who agreed to serve as controls in a case-control study of Kaposi's sarcoma (Marmor *et al.*, 1984; Jaffe *et al.*, 1983). Serum from each of these subjects was tested for HIV antibody at the National Cancer Institute. Those who were seronegative as of 1985 by an enzyme linked immunoassay (ELISA) (Weiss *et al.*, 1985) were categorized as 'seronegative homosexual controls' ($n=33$); those who had antibody by 1985 by ELISA and Western blot tests were defined as 'seropositive homosexual controls' ($n=29$).

Twenty-five blood specimens from 21 hospital employees were used to provide data for the 'heterosexual controls'.

Serum samples. Blood samples were obtained from the individuals in each group under sterile

conditions and centrifuged to obtain the serum. Immediately after centrifugation, each sample was brought to 10 mM with ε-amino caproic acid and to 0·001 mM with phenylmethylsulphonyl-fluoride to minimize proteolysis of IgD. Each sample was aliquoted and stored at −20°C, or in liquid nitrogen. Serum IgD was measured by a sandwich micro-ELISA assay using two different polyclonal rabbit antisera to human IgD. The level of sensitivity of the assay was 1–5 ng/ml. Serum levels of IgG, IgM, IgA were measured by a radial immunodiffuson assay using kits from Meloy Labs (Springfield, VA). For measurement of antibody titres to Epstein-Barr virus (EBV), an immunofluorescent assay was performed using kits from Litton Bionetics (Charleston, SC). Antibodies to cytomegalovirus (CMV) were measured by complement fixation (CMV-CF), using a kit from M.A. Bioproducts (Walkersville, MD), and IgG anti-CMV was measured using an automated fluorescent technique (CMV-IFA) from International Diagnostics Technologies, (San Jose, CA). The ELISA for IgG antibody to HIV was performed using the method described by Weiss *et al.* (1985) and/or by Western blot analysis using the method of Schupback *et al.* (1984).

Lymphocyte typing. One million mononuclear cells were obtained from heparinized blood after centrifugation through Ficoll-Hypaque (Böyum, 1968). After washing, 10^6 cells were stained using monoclonal antibodies directed against cell surface antigens of mature human T lymphocytes (OKT-3), helper/inducer T lymphocytes (OKT-4) or suppressor/cytotoxic lymphocytes (OKT-8) (Ortho-Mune (Raritan, NJ)). The monoclonal antibody Leu 10 (Becton-Dickinson (Mountain View, CA)) was used to enumerate B lymphocytes. Fluorescein-labelled goat anti-mouse immunoglobulin from Ortho-Mune was used as the secondary antibody. Samples were analysed by flow cytometry using a Cytofluorograf II (Ortho, Westwood, MA). Lymphocyte populations were distinguished from monocytes and granulocytes by correlated analysis using forward- and wide-angle light scatter.

Statistical analyses. Analyses for Pearson's correlation coefficient, the Mann-Whitney *U*-test and the Kruskal-Wallis tests were performed using software for the IBM-PC. For analysis of antibody titres, the log of the antibody titre plus one was used.

RESULTS

Serum levels of IgD appeared to be elevated in all the patient groups—high risk, ARC, AIDS—when compared to levels determined using 25 specimens from heterosexual controls (Fig. 1). It had previously been shown, however, that levels of serum IgD are not distributed normally in the general population, but fall into a bimodal or trimodal distribution (Litwin *et al.*, 1985; Dunnette *et al.*, 1977). Since the IgD level of any given individual in our study, before HIV infection, was not known, it was impossible to determine if, for example, a 'normal' value for IgD reflected an elevated IgD level in an individual who previously showed a low level. Similarly, one could not determine if a high level of IgD was the result of HIV infection or a reflection of a normally high IgD phenotype.

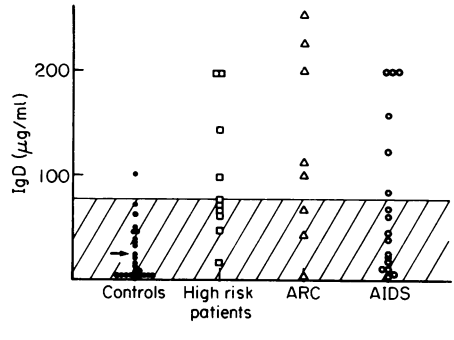

Fig. 1. Serum levels of IgD in heterosexual controls and in patients at various stages of HIV infection. Hatched area represents mean ± 2 s.d. derived from heterosexual controls; arrow represents the normal mean (24 μg/ml).

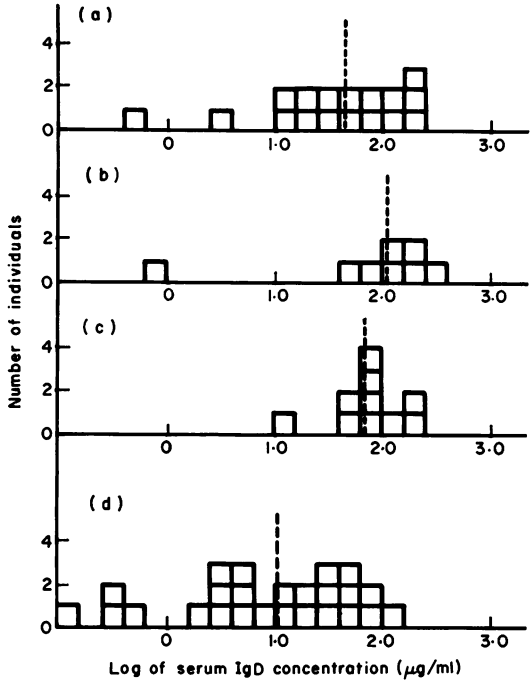

Fig. 2. Distribution of serum IgD levels amongst heterosexual controls and patients at various stages of HIV infection. The vertical dashed lines represent the median for each group. (a) AIDS, $n=17$; (b) ARC, $n=8$; (c) high risk seropositive patients, $n=10$; (d) heterosexual controls, $n=25$.

To analyse this aspect of the data, the difference in IgD levels amongst heterosexual controls and patients was analysed on the basis of population distribution. Figure 2 shows the distribution of IgD levels in 25 serum specimens from heterosexual controls. The distribution is similar to that reported by Dunnette *et al.* (1977) and Litwin *et al.* (1985); our median value of 12 μg/ml, with a mean of 24 μg/ml, compares well with the values published by these authors. Groups of high risk patients, of patients with ARC and patients with AIDS all showed a shift to higher levels of serum IgD with median values of 75·0, 105·9 and 44·7 μg/ml, respectively (Table 1).

The marked shift to the right in patients with minimal clinical disease (the high risk patients)

Table 1. Mean and median levels of serum immunoglobulins at various stages of HIV infection

	Heterosexual controls	High risk patients	ARC	AIDS
Mean IgG levels* (mg%)	968 ± 65 (24)	1869 ± 236 (10)	2369 ± 268 (8)	2031 ± 197 (18)
Mean IgA levels* (mg%)	159 ± 11 (24)	289 ± 51 (10)	281 ± 50 (8)	431 ± 67 (18)
Mean IgM levels* (mg%)	187 ± 15 (24)	261 ± 55 (10)	308 ± 46 (8)	284 ± 43 (18)
Median IgD levels (μg/ml)	12 (25)	75 (10)	106 (8)	45 (17)

* Mean ± s.e. (number tested).

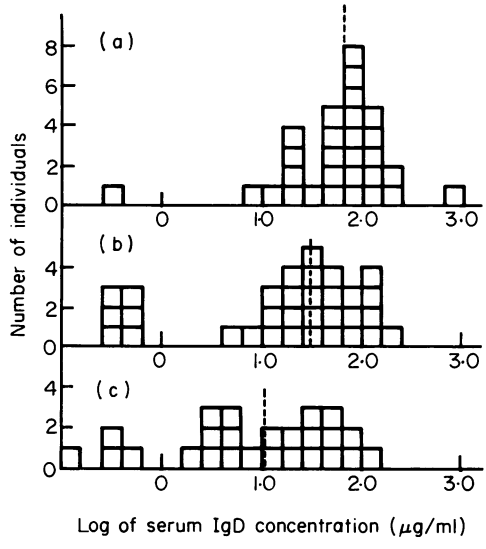

Fig. 3. Distribution of serum IgD levels amongst heterosexual controls and seronegative and seropositive homosexual controls. The vertical dashed lines represent the median for each group. (a) Homsexual seropositive controls, $n=29$; (b) homosexual seronegative controls, $n=32$; (c) heterosexual controls, $n=25$.

suggested that B cell activation, as reflected by IgD elevation, was an early sign of disease. To study this point more fully, the serum IgD levels of seronegative and seropositive homosexual controls were quantified. Figure 3 shows a consistent rise in the median levels of serum IgD from 'heterosexual controls' to seronegative homosexual controls to seropositive homosexual controls (Krunskal-Wallis test statistic = 15·83, $P<0·001$). Subsidiary tests showed the difference between the *first two* groups to be of borderline significance (Mann-Whitney U-test statistic = 522, $P=0·09$) but the differences between seronegative and seropositive homosexual controls and between heterosexual controls and seropositive homosexual controls were highly significant (Mann-Whitney U-test statistic = 298, ($P=0·01$) and Mann-Whitney U-test statistic = 584 ($P=0·0001$), respectively).

The data show that, when compared to serum IgD levels in adult heterosexuals (median = 12·0 μg/ml), IgD incrementally increases in seronegative homosexual controls (26·6 μg/ml), in seropositive homosexual controls (63·1 μg/ml), in high risk patients (75·0 μg/ml) and peaks in patients with ARC (105·9 μg/ml) at serum IgD levels nearly 9-fold higher than normal levels. Patients with AIDS have a lower median level of IgD (44·7 μg/ml) than do patients with ARC but the IgD levels in patients with confirmed AIDS are generally high.

Analysis of the other immunoglobulin isotypes is shown in Table 1. Mean levels of IgG are shown to peak in the patients with ARC in a pattern similar to that observed with IgD; and levels of IgM remain relatively constant throughout the various stages of disease. In contrast, the mean level of serum IgA increases to maximal levels in patients wth AIDS. This table also illustrates the point that *relative* increases in IgD substantially exceed those of the other isotypes with IgD increasing nearly 9-fold above the level in heterosexual controls whereas IgG, IgA, and IgM increase no more than 3-fold.

The mechanism underlying the B cell activation in HIV infection has not been defined. Some workers have suggested that B cell activation could be secondary to EBV or CMV infection or reactivation (Lane *et al.*, 1983; Groopman & Gottlieb, 1983). To investigate whether this hypothesis could explain the IgD data, antibody titres to EBV and CMV were measured and compared to immunoglobulin levels. While studies of IgD were our primary focus, comparisons were made between levels of IgD, IgG, IgA and IgM and antibody titres to EBV and CMV. Table 2 shows that no significant correlation exists between any isotype and any of the three antibody titres measured

Table 2. Pearson correlation matrix of serum immunoglobulin levels and serum antibody titres to EBV and CMV*

	EBV titre	CMV-IFA titre	CMV-CF titre
EBV titre	1.000		
CMV-IFA titre	−0·020	1·000	
CMV-CF titre	−0·011	0·839†	1·000
IgG	0·019	0·147	0·311
IgA	−0·140	−0·103	0·065
IgM	−0·149	0·196	0·363
IgD	−0·352	−0·135	0·007

* Values represent r (Pearson correlation coefficient) calculated for each pair of variables using data from high risk patients ($n=10$) and patients with ARC and AIDS ($n=18$); for analysis of antibody titres, the log of the titre plus one was used.

† $P<0·001$

for EBV, CMV-IFA or CMV-CF. The only significant correlation which was found was between titres of CMV-IFA and CMV-CF ($P=0·001$), a correlation which is expected since they both measure antibodies to the same infectious agent.

To determine whether increased serum IgD levels were associated with any of the imbalance in lymphocyte subsets known to exist in HIV-infected patients, a correlation matrix was constructed to compare levels of the four immunoglobulin isotypes with the percentage of circulating T helper, T suppressor and B lymphocytes (Table 3). Increases in IgD significantly correlated with increases in IgG, IgA and the percentage of B cells and with a decrease in the percentage of the T helper cells. In addition, the frequency of circulating T helper cells was also negatively associated with the levels of IgG and IgA isotypes. No significant correlation was found between serum IgD levels and the percentage of T suppressor cells or the level of serum IgM.

Table 3. Pearson correlation matrix of serum immunoglobulin levels and lymphocyte variables*

	IgD	IgG	IgA	IgM	%TH	%TS	%B
IgD	1						
IgG	0·309†	1					
IgA	0·427‡	0·484§	1				
IgM	0·214	0·671§	0·481§	1			
%TH	−0·274†	−0·435‡	−0·373‡	−0·216	1		
%TS	0·128	0·308†	0·336†	0·186	−0·734§	1	
%B	0·294†	0·079	0·116	−0·047	−0·206	0·079	1

* Values represent r (Pearson correlation coefficient) calculated for each pair of variables using data from heterosexual controls ($n=19$), high risk patients ($n=10$) and patients with ARC or AIDS ($n=25$).

† $P<0·05$.

‡ $P<0·01$.

§ $P<0·001$.

DISCUSSION

Patients with mild to severe clinical manifestations of HIV-associated disease display elevated levels of IgD in their sera. These levels are highest in persons with ARC and somewhat lower (but still significantly above normal) in patients with confirmed AIDS. Serum IgD levels are also significantly elevated in seropositive homosexual controls indicating that activation of B cells and production of IgD in these individuals occur early in the disease process.

While IgD levels in seronegative homosexual controls are higher than those in heterosexual controls, the difference between these groups is of borderline significance ($P=0{\cdot}09$). This tendency toward increased serum IgD levels in seronegative homosexual men is consistent with previously published findings which suggest that many sexually active homosexual men show evidence of immune stimulation (Stahl *et al.*, 1982; Handzel *et al.*, 1983; Tung *et al.*, 1985). Similar findings of immune stimulation have been reported amongst intravenous drug users (Cushman, 1973; Sapira, 1968). The activation of the immune system in individuals in these and other groups at risk for AIDS is thought to be a consequence of exposure to multiple infectious agents and/or to antigenic cells and substances from allogeneic semen and blood. The indviduals in the groups at risk for AIDS may, as a consequence, be particularly susceptible to infection with the aetiological virus of AIDS. In addition, because of continued stimulation of their lymphocytes by factors mentioned above, HIV gene expression and virus production may be promoted, leading to more rapid and severe development of disease (Zagury *et al.*, 1986).

Peak IgD levels, found in patients with ARC, drop to lower (although still elevated levels) in patients with AIDS, paralleling the pattern of the rise in IgG and IgM isotypes (Table 1 and Aucouturier *et al.*, 1986). These findings may reflect the sequential changes in lymphoid histology in this disease, typified by follicular hyperplasia in individuals with 'lymphadenopathy syndrome' and ARC and the 'B cell burn-out' in many patients with fulminant AIDS (Zolla-Pazner & Sidhu, 1983; Modlin *et al.*, 1983). The IgA isotype alone departs from this pattern, rising consistently and peaking in patients with AIDS.

This pattern of hypergammaglobulinaemia in HIV infection is distinct from patterns observed in other infectious diseases. For example, in EBV-mononucleosis, IgM and IgE isotypes are most strikingly elevated in the first weeks or months after disease onset with IgD being generally the least elevated of the five immunoglobulin isotypes; however, none of the immunoglobulin isotypes are elevated more than 3-fold (Bahma, Heiner & Horwitz, 1984). In bacterial and *Mycoplasma* pneumonias, immunoglobulins are rarely elevated above normal levels (Nardbring, Hagman & Johanson, 1969). In patients with tuberculosis, where IgM levels are often high, IgD is present at normal or sub-normal levels (Buckley & Trayer, (1972) and in patients with other bacterial and fungal infections, IgD may be elevated, although no consistent correlation exists between increased IgD and chronic infection (Buckley & Fiscus, 1975). The particular pattern of hyperglobulinaemia found in individuals infected with HIV appears to be unique and suggests that the B cell activation in this disease is independent of EBV infection or reactivation or the result of other secondary infections. This conclusion is further supported by the absence of a significant correlation between levels of the various immunoglobulin isotypes and EBV or CMV antibody titres (Table 2) and by studies in the literature which suggest that the cells spontaneously secreting immunoglobulin in HIV-infected individuals are not EBV-infected (Crawford *et al.*, 1984). Entirely consistent with this conclusion are studies of paediatric and transfusion-related AIDS patients which show that hypergammaglobulinaemia can occur in the absence of EBV or CMV infection (Groopman *et al.*, 1984; Rubenstein *et al.*, 1983; A. Rubenstein, personal communication).

While the unusually elevated levels of IgD appear to be independent of EBV and CMV infection, there are a number of other possible explanations to be considered for the unusual pattern of B cell activation in HIV infection:

(a) Production of IgD antibody could constitute an unusual response to this particular infectious agent. While production of specific IgD antibody has been reported infrequently in infection (found in hepatitis (Brzoksa *et al.*, 1975) and infections caused by *Hemophilus influenzae* and *S. pneumonia* (S. Litwin & B. Zehr, unpublished data)), IgD antibodies to autoantigens have

been observed in a number of studies (reviewed in Leslie & Martin, 1979). The possibility exists therefore, that the observed IgD might contain a high level of specific antibodies to the aetiologic virus of AIDS or autoantibodies. Indeed several types of autoantibodies have been reported in AIDS patients (Zolla-Pazner, 1984; Williams, Masur & Spira, 1984; Dorsett *et al.*, 1985).

(b) Direct infection of B cells with HIV could trigger the B cell activation, IgD overproduction and hyperglobulinaemia. Indeed, there are reports in the literature which suggest that patients' B cells may be infected (Casareale *et al.*, 1984), and that, *in vitro*, B cell lines can be infected (Montagnier *et al.*, 1986; Termsette *et al.*, 1985).

(c) Viral proteins could serve to stimulate B cells in the absence of B cell infection, or, alternatively, lymphokines produced by other infected cells could activate B cells. The work of Pahwa *et al.* (1985) showing that HIV viral products can stimulate human B cells supports this hypothesis.

(d) Overproduction of immunoglobulin could result from defective regulation of T suppressor cells. While T suppressor cells have been shown to be functional *in vitro* in the presence of pokeweed mitogen (Lane *et al.*, 1983; Beneviste *et al.*, 1983), other studies have demonstrated T suppressor cell defects which allow the outgrowth of EBV-infected B cell lines from the blood of AIDS patients (Mawle, Schepper-Campbell & McDougal, 1985; Birx, Redfield & Tosato, 1986). Defective function of suppressor T cells could therefore permit uncontrolled immunoglobulin production by B cells.

(e) Stimulation of B cells to produce IgD by any of the proposed mechanisms could result in the induction of T cells bearing Fc receptors for IgD which, in a mouse model system, have been shown to augment humoral responses (Xue *et al.*, 1984; Coico *et al.*, 1985). The elevated IgD levels could, therefore, perpetuate and enhance the immunoregulatory aberrations of this disease.

Finally, the differential increase in the immunoglobulin classes at various stages of HIV infection suggests that attribution of the hyperglobulinaemia to global B cell activation may be an oversimplification. Indeed, multiple mechanisms may be responsible for the observed changes, different B cell subsets and/or B cells at various stages of maturation may be differentially affected and changes in T suppressor cell function might be of critical importance. Further studies of the B cell activation in HIV infection are, therefore, required to elucidate the mechanisms underlying this phenomenon. Such studies are of practical importance as well as theoretical interest since B cell activation may result in a failure of the B cells to respond to and to produce specific antibody (Lane *et al.*, 1985; Pahwa *et al.*, 1984) with a consequent predisposition to severe bacterial infections (Simberkoff *et al.*, 1984).

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