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B-cell activation in HIV infection: relationship of spontaneous immunoglobulin secretion to various immunological parameters

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SUMMARY

Peripheral blood mononuclear cells from HIV-infected individuals spontaneously secrete elevated levels of IgG, IgM and IgD. This increased level of synthesis and secretion is similar in HIV-infected subjects with no or few symptoms, in ARC patients and in AIDS patients. Thus, abnormal B-cell activation is characteristic of patients with mild as well as severe manifestations of HIV infection. The level of spontaneous cellular secretion of IgG, IgM and IgD correlates with serum levels of these isotypes. Levels of spontaneous cellular secretion of IgG and IgM correlate negatively with the *percentage* but not with the absolute number of T4-positive cells and correlate positively with the *percentage* but not with the absolute number of T8-positive cells. The data suggest that the proportional distribution of these T-cell subsets is a critical factor in the B-cell dysregulation leading to overproduction of IgG and IgM. On the other hand, spontaneous IgD secretion correlates with neither the percent nor the absolute number of T4 or T8 cells suggesting that the increase of IgD-secretion by B cells is independent of the T-cell defects. The data imply that more than one mechanism underlies the B-cell activation in HIV-infected individuals.

Keywords AIDS polyclonal B-cell activation hyperglobulinaemia IgD.

INTRODUCTION

Polyclonal B-cell activation, as reflected by hypergammaglobulinaemia, was documented in early descriptions of patients with AIDS (Gottlieb *et al.*, 1981; Friedman-Kien *et al.*, 1982) and is as ubiquitous a feature of this disease as are the T-cell abnormalities (Zolla-Pazner *et al.*, 1984). Indeed, the activation of B cells, reflected in increased serum immunoglobulin (Ig) levels, has been demonstrated in individuals infected with the human immunodeficiency virus (HIV) at all stages of infection, including stages prior to development of significant symptoms or clinical disease (Stahl *et al.*, 1982; Zolla-Pazner *et al.*, 1984; Mizuma *et al.*, 1987).

While producing elevated levels of Ig, the B cells of HIV-infected individuals appear to be refractory to stimulation by mitogens, to polysaccharide antigens and to newly encountered protein antigens (Stahl *et al.*, 1982; Lane *et al.*, 1983; Ammann *et al.*, 1984; Pahwa *et al.*, 1984). The resulting inability to produce the antibodies required for protective responses to many bacterial pathogens plays an important role in predisposing these patients to the development of bacterial sepsis and pneumonia which are found in approximately 10% of AIDS patients (Simberkoff *et al.*, 1984).

Several other pathological consequences of B-cell activation occur in HIV-infected patients:

(1) The polyclonal activation results in the production of autoantibodies and circulating immune complexes which are associated with significant morbidity (reviewed in Zolla-Pazner, 1984).

(2) A positive direct antiglobulin test has been noted in some AIDS patients, a finding that may be associated with clinical haemolysis (Schreiber *et al.*, 1983).

(3) Positive tests for antinuclear antibodies as well as clinical presentations consistent with systemic lupus erythematosus have been described in patients with ARC and AIDS (Zolla-Pazner, 1984, and Kopelman & Zolla-Pazner, submitted for publication).

(4) An increased frequency of B-cell malignancies occurs in AIDS patients and HIV-infected individuals (Ziegler *et al.*, 1984).

In addition, the B-cell activation may, itself, amplify the immunoregulatory abnormalities in this disease. Thus, it has been shown in model systems that (a) activated B cells can

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induce autoreactive T cells and suppressor T cells (L'age-Stehr *et al.*, 1980), (b) mitogen-activated B cells can serve to mediate suppressor functions (Gilbert & Hoffman, 1983) and (c) overproduction of IgD, which occurs in HIV-infected individuals (Chess *et al.*, 1984; Mizuma *et al.*, 1987), can perturb T cell immunoregulatory circuits (Xue *et al.*, 1984). The activated B cells in HIV-infected hosts could therefore augment the immune deregulation which is initiated by the aetiological agent itself.

All of these phenomena underscore the importance of understanding the mechanism or mechanisms that induce the B-cell activation associated with HIV infection. In an effort to illuminate these mechanisms, the level of spontaneous synthesis of various Ig isotypes was studied at the cellular level to monitor the activated B cells and their products.

MATERIALS AND METHODS

Subjects

Fourteen patients with AIDS met the definition of the disease specified by the Centers for Disease Control (Centers for Disease Control, 1985). Patients with AIDS-related complex (ARC) was identified according to criteria previously established (Zolla-Pazner et al., 1984). High-risk subjects were patients at the New York Veterans Administration Medical Center who met neither of the definitions for AIDS or ARC but who were either homosexual males or intravenous narcotic users who self-referred to the clinic because of some symptoms or anxiety concerning exposure to HIV. All patients with AIDS or ARC and all of the high-risk subjects were seropositive for HIV by Western blot or by ELISA (see below). Control blood samples were drawn from healthy hospital employees.

Serum samples

Blood samples were obtained by venipuncture under sterile conditions. Immediately after centrifugation, each serum sample was brought to 10 mM with ε-amino caproic acid (EACA) and 0·06 mM with phenylmethyl sulphonylfluoride (PMSF) to minimize proteolysis of IgD. Each sample was aliquoted and stored at −20°C until used. Serum IgD was measured by a sandwich micro-ELISA assay using two different polyclonal rabbit antisera to human IgD (Litwin & Zehr, 1987b). The lower limit of sensitivity of the assay was approximately 1–5 ng/ml. Serum levels of IgG, IgM and IgA were measured by a radial immunodiffusion assay using kits from Meloy Laboratories (Springfield, VA). Antibodies to HIV were measured using a commercial ELISA (Electronucleonics, Columbia, MD) and/or Western blot analysis according to the method of Schupack and co-worker (Schupback *et al.*, 1984).

Lymphocyte typing

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood after centrifugation through Ficoll–Hypaque. After washing, 4×10^6 PBMC were used for phenotypic analysis as previously described (Friedman-Kien *et al.*, 1982) 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). To enumerate B lymphocytes, the monoclonal antibody Leu-10 (Becton-Dickinson, Mountain View, CA) was used. Fluorescein-labelled goat anti-mouse immunoglobulin (Ortho-Mune) was used as the secondary antibody. Fluorescence intensity of cells was measured by flow cytometry using a Cytofluorograf II interfaced with a 2140 computer (Ortho, Westwood, MA). Lymphocyte populations were distinguished from monocytes and granulocytes by correlated analysis using forward- and wide-angle light scatter.

Culture conditions and Ig production

For quantitation of IgD and IgG production, PBMC were washed in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD), and suspended at a concentration of 5×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell suspensions were dispensed into 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) in 200 μl/well ($1{\cdot}0 \times 10^6$ cells/well). Cultures were performed in a humidified atmosphere containing 7% CO_2 at 37°C for 24 h. At the end of the culture period, supernatants were collected; EACA and PMSF were added in the same concentration as for serum samples, and supernatants were frozen at −20°C until used. IgD and IgG in these supernatants were measured by the ELISA method described by Litwin and Zehr (Litwin & Zehr, 1987b).

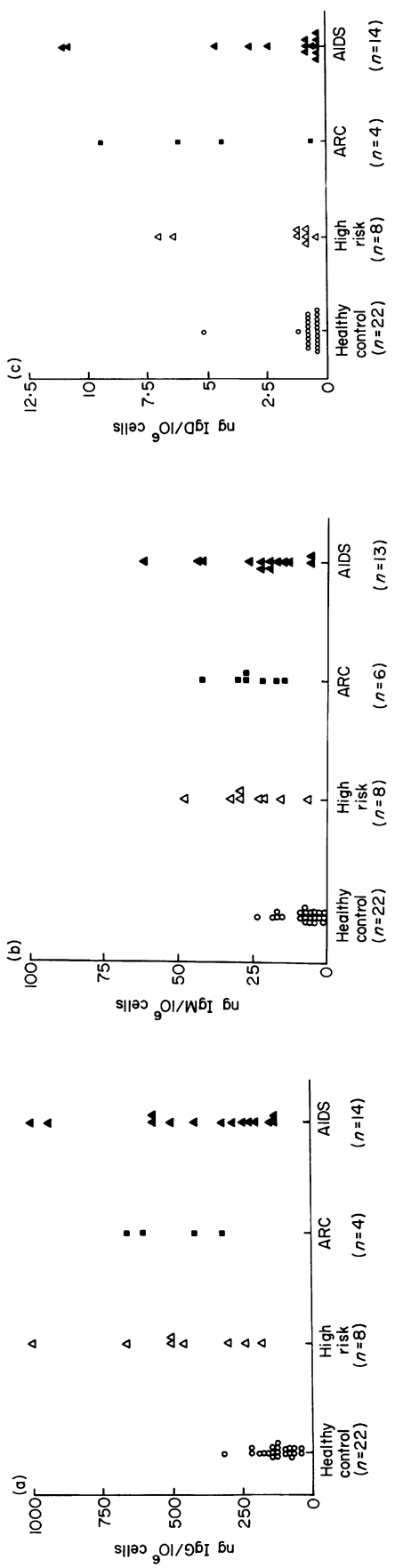
For quantitation of IgM production, the culture technique of Kelly, Levy & Sikora (1979) was modified. PBMC were cultured in Immunlon II flat-bottomed microtitre plates (Dynatech, Alexandria, VA) which had been precoated with 200 μl/well of an IgG fraction of goat anti-human IgM (μ-chain specific) (Cappel Labs, Cochranville, PA). All coating and washing steps were done under sterile conditions. PBMC were suspended in RPMI-1640 medium (M.A. Bioproducts) supplemented with 5% fetal calf serum (Reheis Chemical Co., Kankakee, IL), 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Two hundred microlitres of a suspension of PBMC, at a concentration of 1×10^6/well or human IgM standards at a concentration of 10–500 ng/ml, were added to each well of a precoated plate and incubated for 24 h at 37°C. At the end of the incubation, the quantity of IgM produced and bound was assayed using alkaline phosphatase-conjugated goat anti-human IgM (Cappel Labs) and the colour developed with *p*-nitrophenylphosphate. Absorbance of each wall was measured at 405 nm using an automated ELISA reader (Dynatech).

RESULTS

Spontaneous secretion of IgG, IgM and IgD

As compared with normal controls, most high-risk patients and patients with ARC or AIDS spontaneously secreted increased levels of IgG, IgM and IgD (Fig. 1a, 1b and 1c). For IgG secretion in 24 h cultures, all three patient groups showed a 3 to 3·7-fold increase in the level of IgG compared to levels of IgG secretion from cells of normal controls ($P < 0{\cdot}001$ for each patient group compared to normals). Mean levels (±s.e.m.) of IgG secretion by PBMC from AIDS, ARC, high-risk patients and controls were $402{\cdot}9 \pm 75{\cdot}8$, $500{\cdot}0 \pm 78{\cdot}7$, $480{\cdot}0 \pm 93{\cdot}0$ and $133{\cdot}2 \pm 14{\cdot}1$ ng/10^6 cells/24 h (Fig. 1a).

Similarly, when levels of spontaneous IgM secretion were compared, cells from each patient group had approximately a 3-fold higher level of IgM secretion than did cells from normal controls ($P < 0{\cdot}001$ for each patient group compared to normals). There was no significant difference between patient



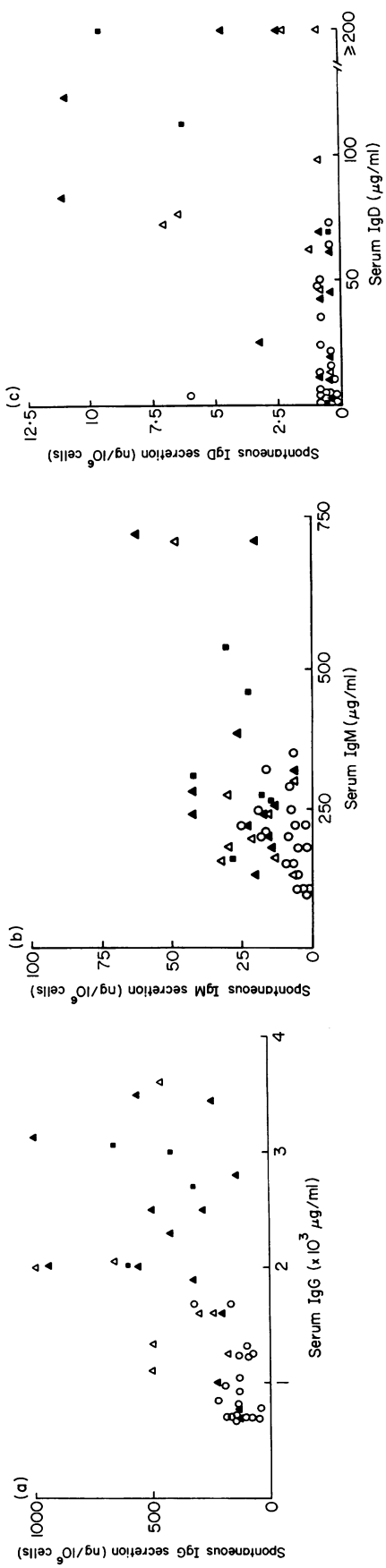


Fig. 1. Levels of Ig production by peripheral blood mononuclear cells from controls (○) HIV seropositive high-risk subjects (△), patients with ARC (■), and AIDS (▲). Secretion of IgG (a), IgM (b) and IgD (c) into culture supernatants during 24 h of culture is expressed as ng/10^6 cells cultured.

Fig. 2. Correlation between serum levels of a given Ig isotype and the secretion of that isotype by the subjects' peripheral blood mononuclear cells in culture for 24 h. Data are shown for normal controls (○), HIV seropositive high-risk subjects (△), patients with ARC (■) and patients with AIDS (▲). (a) AIDS, $n = 14$; ARC, $n = 4$; risk, $n = 8$; control, $n = 19$. The correlation coefficient (r^2) between levels of serum IgG and spontaneously secreted IgG is 0·601 ($P < 0{\cdot}001$). (b) AIDS, $n = 13$: ARC, $n = 6$; risk, $n = 8$; control, $n = 20$. The correlation coefficient (r^2) between levels of serum IgM and spontaneously secreted IgM is 0·580 ($P < 0{\cdot}001$). (c) AIDS, $n = 13$; ARC, $n = 4$; risk, $n = 8$; control, $n = 20$. The correlation coefficient (r^2) between levels of serum IgD and spontaneously secreted IgD is 0·554 ($P < 0{\cdot}005$).

Table 1. Pearson correlation matrix comparing levels of serum immunoglobulins with levels of spontaneous cellular immunoglobulin secretion*

	log IgM serum	log IgG serum	log IgD serum	log IgA serum	log IgM in supernatant	log IgG in supernatant	log IgD in supernatant
log serum IgM	1·00						
log serum IgG	0·62***	1·00					
log serum IgD	0·18	0·29*	1·00				
log serum IgA	0·48*	0·57***	0·46***	1·00			
log IgM in supernatant	0·56***	0·55***	0·39**	0·43***	1·00		
log IgG in supernatant	0·38*	0·71***	0·23	0·37*	0·73***	1·00	
log IgD in supernatant	0·49***	0·57***	0·55***	0·40**	0·55***	0·49***	1·00

* Data represent correlation coefficients based on 44–59 determinations for each pair of values. PBMC were cultured for 24 h in culture medium without antigenic or mitogenic stimulation.
* $P<0·05$. ** $P<0·01$. *** $P<0·005$.

Table 2. Pearson correlation matrix comparing levels of spontaneous Ig secretion with the number or percent of T4 and T8 cells*

	T4 cells		T8 cells	
	Absolute No.	Percent	Absolute No.	Percent
log IgM in supernatant	0·16	−0·47***	0·06	0·39**
log IgG in supernatant	−0·01	−0·52***	−0·05	0·35*
log IgD in supernatant	0·18	−0·29	−0·10	0·18

* Data represents correlation coefficients based on 23–46 determinations for each pair of variables. Culture conditions as in Table 1.
* $P<0·05$, ** $P<0·01$, *** $P<0·005$.

groups. Mean levels (±s.e.m.) for IgM secretion by PBMC from AIDS, ARC, high-risk patients and controls were 23·5±4·5, 25·8±4·1, 24·5±4·7 and 8·4±1·3 ng/10^6 cells/24 h, respectively (Fig. 1b).

Because our previous studies (Mizuma *et al.*, 1987) and other studies (Chess *et al.*, 1984) had shown serum levels of IgD in HIV-infected patients to be increased, spontaneous IgD secretion was evaluated. Figure 1c shows the distribution of spontaneous secretion from each group. These data show that cells from only 1/22 specimens from normal donors secreted IgD (> 2·0 ng/10^6 cells/24 h). However, cells from 10/26 (38%) HIV-infected individuals secreted measurable levels of IgD (2·4–11 ng IgD/10^6 cells/24 h). IgD in the supernatants showed an elevated ratio of IgDλ/IgDk (data not shown) which is consistent with the presence of secreted IgD (Litwin & Zehr, 1987a). Because controls secreted essentially no measurable IgD, statistical analysis of the data was based on ability to secrete or not secrete IgD using Fisher's Exact Test. These analyses showed that when controls were compared to all HIV-infected patients, to AIDS patients or to ARC patients, there was a significant difference in ability to secrete IgD ($P=0·005$, $P=0·024$ and $P=0·006$, respectively). There was no significant statistical difference between the ability of cells from normal controls and cells from high-risk individuals to secrete IgD ($P=0·166$). Since, however, cells from two of eight high-risk patients secreted high levels of IgD, it is possible that, while less frequent, IgD secretion does characterize a subset of high-risk HIV-infected subjects.

Correlation of serum Ig with spontaneously secreted Ig

Both synthetic and catabolic rates control serum Ig levels and, at least in the case of IgD, these may be affected by genetic factors influencing serum IgD levels (Litwin *et al.*, 1985). In order to assess the contribution of the documented increase in Ig synthesis on serum Ig levels, the correlation between these two parameters was measured for IgG, IgM and IgD. Fig. 2a, b, and c show the relationship of serum IgG, IgM and IgD levels with the spontaneous cellular secretion of each of these isotypes. With all three isotypes there was a positive correlation seen (for IgG, $r^2=0·601$, $P<0·001$; for IgM, $r^2=0·580$, $P<0·001$; for IgD, $r^2=0·554$, $P<0·005$).

When comparisons were made between levels of serum isotypes (IgM, IgG, IgD and IgA) and levels of spontaneous secretion of IgM, IgG and IgD, it was found that significant correlations existed between most combinations (Table 1). This suggests that the B-cell activation, with resultant spontaneous Ig synthesis and hypergammaglobulinaemia, is a global event affecting Ig-producing cells regardless of their maturation stage (reflected by the isotype they produce).

Correlation of Ig secretion with the number or percent of T4 or T8 cells

When levels of spontaneous secretion of IgM and IgG were compared to various lymphocyte parameters, significant negative correlations were found with the *percentage* of $T4^+$ cells and significant positive correlations were found with the *percentage*

of $T8^+$ cells (Table 2). However, no correlation existed between spontaneous secretion of IgM or IgG and the *absolute number* of $T4^+$ or $T8^+$ cells. These data suggest that the proportional representation of $T4^+$ and $T8^+$ cells is more critical to B-cell activity in these patients than is the absolute number of either $T4^+$ or $T8^+$ cells. Levels of IgD secretion, on the other hand, did not correlate with either the percent or the number of $T4^+$ or $T8^+$ cells, suggesting that increased IgD synthesis and secretion is T-cell independent.

DISCUSSION

In normal subjects, there is no correlation between the serum level of IgD and the levels of IgG, IgM or IgA despite the fact that the levels of the latter classes correlate with each other; the same situation holds true for the level of spontaneously secreted IgD which does not correlate quantitatively with the levels of spontaneously secreted IgG, IgM or IgA in healthy subjects (S. Litwin, unpublished data). However, in HIV-infected individuals, serum IgD *is* correlated with serum IgG and IgA and spontaneously secreted IgD levels correlate with levels of spontaneously secreted IgG and IgM (Table 1). These data argue for the presence of a common 'stimulus' in AIDS which evokes IgD, IgG, IgM and IgA responses. However, additional factors contribute to the hypergammaglobulinaemia of HIV infection since elevated levels of spontaneously secreted IgM and IgG (but not IgD) are associated with alterations in the percentages of circulating lymphocyte subsets (Table 2). The inference is that there is a minimum of two factors involved in the B-cell activation of AIDS: one related to non-specific stimulation of all Ig classes and the other to IgM and IgG regulation.

Hypergammaglobulinaemia in AIDS is usually described in terms of elevated levels of IgG and IgA (Seligmann *et al.*, 1984; Schnittman *et al.*, 1986). Increased levels of serum IgD have also been reported in 40–50% of HIV-infected individuals (Chess *et al.*, 1984; Mizuma *et al.*, 1987) and IgM is increased in approximately 25% of AIDS patients (Zolla-Pazner, 1984). Average increases in these isotypes relative to normal levels range from 8·8-fold above normal in the case of IgD to 1·6-fold above normal for IgM (Mizuma *et al.*, 1987). This apparent disproportional increase in the serum levels of the different Ig isotypes probably reflects both polyclonal B-cell activation and alterations in the various homeostatic mechanisms that control serum Ig levels. Indeed, the unusually elevated levels of IgD in these patients might be grounds to invoke a third mechanism involved in B-cell activation.

When spontaneous secretion of Ig is measured rather than serum levels of the various isotypes, parallel data are obtained, i.e., levels of IgM and IgG synthesis are increased but IgD synthesis and secretion is the most marked. The studies described above do not, however, address whether Ig secretion is increased a small amount in all B cells or whether increased Ig secretion characterizes a small percentage of B cells. Studies by Lane and co-workers (Lane *et al.*, 1983) would appear to support the latter hypothesis. In those experiments, about 1–10% of B cells from patients appeared to be activated compared to 0–1% in controls. The features that distinguish the activated from the unactivated B cells remain unclear. That these cells do not appear to be infected by EBV is implied by the studies of Crawford and co-workers, which showed that B cells from HIV-infected individuals which were actively producing Ig did not simultaneously express EBV antigens (Crawford *et al.*, 1984). However, B cells of HIV-infected individuals do display phenotypic changes associated with activation and immaturity (Martinez-Maza *et al.*, 1987), although these characteristics were ascribed to the entire circulating B-cell pool of HIV-seropositive patients and not necessarily to the cells actively secreting Ig.

Increased polyclonal Ig synthesis is not unique to HIV infection. It has been documented in a multitude of diseases, e.g., EBV-induced mononucleosis (van Furth *et al.*, 1966), cytomegalovirus infection (Hutt-Fletcher, Balachandron & Elkins, 1983), systemic lupus erythematosus (Budman *et al.*, 1977) and Hodgkin's disease (Romagnani *et al.*, 1983). The relevant issue to the present study is what mechanism(s) underlies the B-cell activation in HIV infection. Several direct and indirect possibilities exist:

Three putative mechanisms could account for *direct* activation of B cells in HIV-infected individuals. The first is the activation of B cells by infection with HIV. The relevance of this hypothesis is supported by the findings of several authors who have shown that normal and EBV-transformed B cells can be infected with HIV (F. Ruscetti, personal communication; Montagnier *et al.*, 1984; Popovic, Bead-Connoie & Gallo, 1984; Levy *et al.*, 1985). No evidence exists, however, showing that B cells of HIV-infected individuals are infected with the virus or that, once infected, Ig synthesis and secretion are enhanced. A second direct mechanism for HIV activation of B cells is suggested by data showing polyclonal activation of B cells by HIV viral particles and viral proteins (Pahwa *et al.*, 1985; Gurney *et al.*, 1986; Schnittman *et al.*, 1986; Yarchoan, Redfield & Broder, 1986). Finally, the virus may activate B cells in the form of a continual antigenic stimulus.

Indirect mechanisms could also account for the B-cell activation of HIV infection. Thus, HIV-infected T cells or macrophages might produce B-cell stimulatory lymphokines. Alternatively, the loss of T-cell-mediated immunity could result in the reactivation of EBV and cytomegalovirus with resultant waves of infection and activation of B cells (Friedman-Kien *et al.*, 1982; Quinn *et al.*, 1984; Yarchoan *et al.*, 1986).

The data derived from this study indicate that both T-dependent and T-independent mechanisms are involved. The IgD stimulation, in particular, appears to be a direct induction of IgD-secreting B cells, independent of T cell defects (Table 2). Whether this represents a novel HIV-induced B-cell differentiation pathway, a unique response to HIV antigens and mitogens or an abnormal deregulation of molecular processing at the level of transcription or translation of the IgM-IgD message remains to be elucidated.

IgG and IgM stimulation on the other hand correlate with alterations in the frequency of T-cell subsets. However, the correlations are (a) between increased IgG and IgM synthesis and *reduced* percentages of T4 cells and (b) between increased IgG and IgM synthesis and *increased* percentages of T8 cells. Thus, the correlations are in the opposite direction from those originally described by Rheinherz and co-workers (Rheinherz *et al.*, 1980) in which increases in IgG secretion in an *in vitro* pokeweed mitogen-driven system were associated with *increases* in T4 cells or with *decreases* in T8 cells. The paradoxical associations between IgG and IgM secretion and lymphocyte subsets remain enigmatic. They could be explained by idiosyncratic responses of B cells to aberrant or disproportional

immunoregulatory signals and/or to other non-T-cell mediated mechanisms which regulate Ig homeostasis.

Finally, the correlation of IgG and IgM secretion with the altered percentages rather than absolute numbers of T-cell subsets suggests that the proportional representation of these cells may be a critical element in the immunoregulation of B cells, and more important than the level at which these cells circulate. On the other hand, the lack of correlation of IgD secretion with either the percentage or level of circulating T-cell subsets suggests that this phenomenon is not T-cell-mediated, and that regulation of B-cell activity in this infection is under the influence of multiple controls.

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