

# Mechanisms of B Cell Activation in Patients with Acquired Immunodeficiency Syndrome and Related Disorders

## Contribution of Antibody-producing B Cells, of Epstein-Barr Virus-infected B cells, and of Immunoglobulin Production Induced by Human T Cell Lymphotropic Virus, Type III/Lymphadenopathy-associated Virus

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### Abstract

Patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) have hyperimmunoglobulinemia and increased numbers of circulating immunoglobulin-secreting cells. In this paper, we studied the basis for this B cell hyperactivity. Limiting dilution studies of B cells from seven patients with ARC and four with AIDS revealed that some B cells spontaneously produced antibodies to human T cell lymphotropic virus, type III/lymphadenopathy-associated virus (HTLV-III/LAV) ( $39:10^6$  and  $7:10^6$  B cells, respectively), suggesting that chronic antigenic stimulation by HTLV-III/LAV was one contributing factor. The patients also had an increased number of spontaneously outgrowing B cells than did normals ( $6:10^6$  vs.  $<2:10^6$  B cells), suggesting that they had an increased number of Epstein-Barr virus (EBV)-infected B cells. However, fewer B cells from patients were immortalized by exogenously added EBV than were B cells from normals. In additional studies, HTLV-III/LAV induced immunoglobulin secretion (mean 2,860 ng/ml) by peripheral blood mononuclear cells from normals; this HTLV-III/LAV-induced immunoglobulin secretion required the presence of both B and T cells. Thus, antigenic stimulation by HTLV-III/LAV, increased numbers of EBV-infected B cells, and HTLV-III/LAV-induced T cell-dependent B cell activation all contribute to the B cell hyperactivity in patients with HTLV-III/LAV disease.

### Introduction

Acquired immunodeficiency syndrome (AIDS)<sup>1</sup> was first reported in 1981 as a progressive immunodeficiency disorder

characterized by opportunistic infections and the development of Kaposi's sarcoma (1-3). It has since been learned that this disorder is caused by infection with a newly discovered human retrovirus, human T cell lymphotropic virus, type III (HTLV-III), also known as lymphadenopathy-associated virus (LAV), which preferentially infects and destroys T cells bearing the OKT4 antigen (4-6). It is now apparent that the scope of disease caused by HTLV-III infection includes other manifestations in addition to AIDS (7, 8). In particular, patients with HTLV-III infection have been reported to develop lymphadenopathy, night sweats, diarrhea, and oral candidiasis; this symptom grouping has been termed AIDS-related complex (ARC). In addition, they have an increased incidence of non-Hodgkin's lymphoma, usually of B cell origin, including Burkitt's lymphoma, in which the tumor cells contain Epstein-Barr virus (EBV)-related antigens (9, 10). Recently, the clinical definition of AIDS has been expanded to include this group of patients, even in the absence of Kaposi's sarcoma or opportunistic infections (11).

Initial immunological evaluation of patients with AIDS suggested that while they had a profound defect of cellular immunity (especially helper/inducer T cell-mediated functions), humoral immunity was relatively spared. Subsequent studies, however, have revealed a variety of alterations in B cell functions. While the serum immunoglobulin (Ig) levels are usually high in patients with AIDS (12-15), they have decreased titers of serum antibodies to a variety of antigens as compared with normals (12, 15) and produce little or no antibody in response to immunization with neoantigens (12, 13). These results suggested that these patients had defects in specific antibody production in the face of hypergammaglobulinemia.

In vitro studies have further defined the alterations in B cell function in these patients. In accordance with their hypergammaglobulinemia, from 0.1 to 1% of the lymphocytes in patients with AIDS have been found to be B cells spontaneously producing Ig (13). Stimulation of their peripheral blood mononuclear cells (PBMC) with mitogens, however, has been shown to result in lower Ig production than stimulation of PBMC from normals (13, 16). The mechanisms responsible for these alterations in B cell function are unclear at this time. In regard to the spontaneous Ig production, it is known that patients with AIDS are often infected with EBV and/or cytomegalovirus (15), and it has been hypothesized that the infection by these viruses might be responsible for their spontaneous Ig production (13).

The present studies were undertaken to explore further the function of B cells from patients with AIDS. In particular, we examined specific antibody production and spontaneous transformation (usually an indication of EBV infection of B cells). Our results indicate that these patients have high numbers of B cells that spontaneously produce anti-HTLV-III antibody in vi-

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1. *Abbreviations used in this paper:* AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; EBV, Epstein-Barr virus; FCS, fetal calf serum; HTLV-I and HTLV-III, human T cell lymphotropic virus, types I and 3; IFN, interferon; LAV, lymphadenopathy-associated virus; PBMC, peripheral blood mononuclear cells; PBS-Tween, phosphate-buffered saline containing 0.05% Tween 20 and 0.02% sodium azide; PWM, pokeweed mitogen.

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tro, and in addition have B cells that spontaneously produce antibodies of other specificities. We also demonstrate that patients with HTLV-III infection have higher numbers of spontaneously immortalizing B cells than do normal controls, but that these cells do not account for all the B cells that spontaneously produce Ig. Finally, we demonstrate that HTLV-III can induce polyclonal Ig production by normal B cells in a T cell-dependent fashion, and thus may directly be responsible for some of the B cell activation in patients with AIDS or ARC.

## Methods

**Patients.** Seven patients with ARC (either oral candidiasis or generalized lymphadenopathy with persistently decreased helper/inducer T cells) and four patients with AIDS (11) were studied. Two of the patients with AIDS had Kaposi's sarcoma, and two had presented with *Pneumocystis carinii* pneumonia. One patient with ARC was 13-yr-old; all of the other patients were from 20 to 35 yr old. Each of the patients had risk factors that made them more likely to be infected with HTLV-III, and each had antibodies to HTLV-III (Table I). None of the patients had received chemotherapeutic drugs or immunomodulatory agents except for one patient with AIDS who had received a course of alpha interferon (IFN), which ended 2 wk before the study. Six healthy laboratory workers or nurses, ages 26–35, who had antibodies to EBV were used as controls; none of them were known to be in AIDS risk groups and all lacked antibodies to HTLV-III.

**Serum Ig and antibody determinations.** Serum IgM, IgG, and IgA levels were determined by radial immunodiffusion using plates obtained from Meloy Laboratories, Springfield, VA. Serum antibodies to HTLV-III and to A/Aichi influenza virus (A/Aichi/2/68 MN25241 [H3N2]) were determined by enzyme-linked immunosorbent assay (ELISA) using methods modified slightly from those previously described (17–19). Briefly, individual wells of microtiter plates (Immunolon I; Dynatech Corp., Alexandria, VA) were coated overnight with 100  $\mu$ l of carbonate buffer pH 9.6 containing either 3  $\mu$ g/ml doubly banded disrupted HTLV-III virus (a gift of Marjorie Robert-Guroff, Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD) or 12.5 hemagglutinin U/ml of zonally purified formaldehyde-inactivated A/Aichi influenza virus (a gift of Brian R. Murphy, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The wells were washed twice with deionized water, blocked by incubation for 20 min with 100  $\mu$ l of 2% (vol/vol) bovine serum albumin in carbonate buffer, washed twice with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) and 0.02% sodium azide (PBS-Tween), and filled with a 100- $\mu$ l aliquot of sera diluted in PBS-Tween with 1% fetal calf serum (FCS). Each serum sample was run at six successive 1:3 dilutions starting at 1:100. Each plate also contained six threefold dilutions of a reference serum containing a high titer of antibodies to the appropriate virus. After a 2-h incubation, the plates were washed three times with PBS-Tween, incubated for 3 h with alkaline phosphatase-conjugated goat anti-human Ig (Cappel Laboratories, Cochranville, PA), washed four times with PBS-Tween, and developed with 1  $\mu$ g/ml *p*-nitrophenol-phosphate as described (19). The optical density at 405 nm was read after 3 h. The amount of specific antibody in a serum sample was calculated by comparison of its optical density to a curve of absorption vs. concentration obtained with the reference serum; this was done using linear regression analysis of log-logit transformed data (19). Results were expressed in arbitrarily defined units of antibody.

As previously determined (19), 1 U of anti-A/Aichi antibody was estimated to be 2.3 ng of antibody. Using a similar technique, 1 U of anti-HTLV-III antibody was estimated to be 2.6 ng. Briefly, wells of a microtiter plate were coated with either HTLV-III virus or with goat anti-human Ig antibody (gift of David L. Nelson, National Cancer Institute, Bethesda, MD). After washing, the wells were filled with dilutions of either the reference serum for anti-HTLV-III antibody or with a known Ig standard (Meloy Laboratories), respectively. After a second washing, the wells were incubated with alkaline phosphatase-conjugated goat anti-

human Ig (Cappel Laboratories) and developed with *p*-nitrophenol phosphate. The amount of specific anti-HTLV-III antibody in the reference serum was then estimated by comparing the two curves as previously described (19).

Total Ig produced in the culture supernatants was assayed by ELISA as previously described (19). Briefly, the ladder of reagents from the solid phase up consisted of goat anti-human Ig, the supernatant being tested, and alkaline phosphatase-conjugated goat anti-human Ig; *p*-nitrophenol phosphate was used as the developer.

**Lymphocyte preparation and culture conditions.** PBMC were obtained by Ficoll-Hypaque centrifugation of heparinized peripheral blood, washed in RPMI 1640 medium (Gibco, Grand Island, NY), and incubated for 1 h at 37°C in RPMI 1640 medium supplemented with 10% FCS to partially deplete the cells of cytophilic Ig. PBMC from patients or controls were then separated into E-rosette-enriched and E-rosette-depleted fractions by rosetting with 2-aminoethylisothionium bromide-treated sheep erythrocytes followed by separation of these cells on Ficoll-Hypaque gradients (20). E-rosette-depleted cells, E-rosette-enriched cells, and unseparated cells were then further depleted of cytophilic Ig by two centrifugations through FCS. Cells were suspended in media and counted with Trypan blue.

The fraction of B cells in the E-rosette-depleted population was determined as previously described (21). Briefly, the cells were incubated at 37°C with latex beads (Sigma Chemical Co.), washed through FCS, and then stained at 4°C with fluorescein-conjugated goat anti-human Ig (Cappel Laboratories). Fluorescent cells that did not ingest latex beads were considered B cells; these comprised 18–35% of the E-rosette-depleted populations from patients or controls. Cells that ingested latex beads (monocytes) comprised 48–55% of these populations, and E-rosetting cells (21) comprised <3%.

**Precursor frequency determinations and in vitro antibody production.** The precursor frequencies of B cells producing anti-HTLV-III or anti-A/Aichi influenza virus antibodies or forming immortalized cell lines were determined by limiting dilution cultures. Multiple wells of 96-well microtiter plates (Costar, Cambridge, MA) were established with varying numbers of B cells from patients or normals and  $1 \times 10^5$  irradiated (2,500 rad) normal allogeneic PBMC as a feeder/helper population. The cells were cultured in 0.2 ml of RPMI 1640 medium supplemented with 4 mM L-glutamine, 10% FCS, and antibiotics (all from Gibco). In general, 16 wells were established per B cell number, and an additional 16 with no B cells as controls. Some wells were stimulated with pokeweed mitogen (PWM) (Gibco) 1:100 vol:vol or with the B95-8 strain of EBV (1:10 dilution of the supernatant of the B95-8 marmoset line containing  $10^6$  infectious particles per milliliter, a gift of Giovanna Tosato, Food and Drug Administration, Bethesda, MD). Some wells contained 15  $\mu$ g/ml cycloheximide as a control for cytophilic Ig release.

After 14 d incubation at 37°C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>, 0.1 ml of each supernatant was harvested and replaced with fresh media. The harvested supernatants were assayed at a 1:3 dilution for antibodies to HTLV-III and A/Aichi influenza virus. All wells were scored as positive or negative for the presence of antibodies to each virus. Wells that had an optical density at 405 nm greater than the mean plus 3 SD of control wells (no B cells) were scored as positive. The cutoff for positive wells was generally  $\sim 1$  ng/ml for antibodies to either virus.

The plates were cultured for an additional 4 wk (total 6 wk) and at that time were assessed for outgrowth/immortalization by microscopic examination by phase microscopy. A microculture was considered to be immortalized when both large single cells and clumps or large cells were recognized (22). It has previously been shown that the frequency of wells with outgrowth is not affected by feeding during the 6-wk culture period (23). In some cases, outgrowth was assessed in the presence of sheep anti-alpha IFN antibody (24) (a generous gift of Kathryn Zoon, Center of Drugs and Biologics, Food and Drug Administration) sufficient to neutralize  $10^2$  U of alpha IFN per milliliter.

**Calculations of precursor frequencies.** Precursor frequencies were calculated by Poisson analysis using both the maximum likelihood and minimum chi-square methods as described (21). In each case, the good-

ness of fit test yielded a  $P$  value of  $>0.05$ , and there was a  $<15\%$  difference between the two methods. Results shown are those obtained with the minimum chi-square method. The precursor frequency was in each case corrected for the fraction of B cells in the E-rosette-depleted population.

**Ig production by normal PBMC.** In additional studies, PBMC, E-rosette-depleted cells, and E-rosette-enriched cells from normals who were seronegative for HTLV-III were tested for Ig production in response to HTLV-III or PWM.  $2 \times 10^6$  million PBMC,  $0.5 \times 10^6$  E-rosette-depleted cells, and/or  $1.5 \times 10^6$  E-rosette-enriched cells were cultured in round-bottomed tubes (Falcon 3033 tubes, Falcon Labware, Div. of Becton-Dickinson & Co., Cockeysville, MD) in 2 ml of the culture media described above. To some tubes were added PWM (1:100 vol/vol) or live pelleted HTLV-III (prepared from the supernatant of infected H9 cells as described in reference 4),  $2 \times 10^9$  viral particles per tube. Supernatant collected at 14 d in culture was assayed for total Ig production.

**Statistical calculations.** Geometric means and SEM were calculated for the precursor frequencies and antibody concentrations using conventional formulas. The precursor frequencies and concentrations of antibody were compared by the Wilcoxon rank sum test.

## Results

**Serum Ig and antibody levels.** As compared with the normal controls, the patients with ARC or AIDS had increased serum levels of IgM, IgG, and IgA (Table I). In addition, each of the patients, but none of the normals, had antibodies to HTLV-III virus detectable by ELISA (Table I). Based on the estimation that 1 U of anti-HTLV-III antibody was approximately equal to 2.6 ng, the antibody to HTLV-III in these patients was a significant fraction of their total Ig: a mean of  $\sim 3\%$  in the ARC patients and 1% in the AIDS patients. Each of the patients and normals in addition had antibodies to A/Aichi influenza virus; however, the patients' antibody titers were approximately one-third that of the normals ( $P < 0.05$ ) (Table I). A/Aichi (H3N2) influenza virus first appeared in 1968, and cross-reacting H3N2 strains have circulated since that time. Both patients and normals

would therefore be expected to have had similar antigenic exposure to these viruses by prior infection or immunization. Thus, the patients had hypergammaglobulinemia, but somewhat decreased titers of antibody to influenza virus as compared with normals.

**Anti-HTLV-III antibody produced by cultures of PBMC from patients with ARC or AIDS.** Preliminary studies demonstrated that washed PBMC from patients with ARC or AIDS spontaneously produced antibodies to HTLV-III when placed into culture. To investigate this phenomenon at the cellular level, B cell-enriched populations of cells from the patients were placed into limiting dilution microcultures with  $10^5$  irradiated (2,500 rad) allogeneic normal PBMC as a feeder/helper population. Supernatants were harvested at 14 d and assayed for antibodies to HTLV-III. When the fraction of wells without detectable antibody was plotted against the number of cultured B cells, a straight line was obtained (Fig. 1). This suggested that one cell was limiting in these cultures and that the antibody produced by a single precursor could be measured in the assay system. By Poisson statistics, where this line crossed 0.37 there was a mean of one precursor per well, and the precursor frequency of B cells that spontaneously produce anti-HTLV-III antibodies could be determined.

Using this technique, we determined the frequency of precursors that produced anti-HTLV-III antibodies in each of the patients with ARC or AIDS (Fig. 2). In the absence of PWM or EBV, the patients with ARC had a mean of  $39.2 \times / \div 1.67$  (geometric mean  $\times / \div$  SEM) precursors that spontaneously produced anti-HTLV-III antibodies per  $10^6$  B cells, while the patients with AIDS had  $5.93 \times / \div 2.04$  precursors/ $10^6$  B cells. The addition of either PWM or EBV to these cultures did not result in an increase in the precursor frequencies, which indicated that these agents did not recruit any additional precursors to produce anti-HTLV-III antibody (Fig. 2). In addition, when the total amount of anti-HTLV-III antibody produced in the wells was examined, there

Table I. Immunoglobulin and Antibody Levels in Patients and Normal Controls

Diagnosis	IgM	IgG	IgA	Anti-HTLV-III Antibody	Anti-A/Aichi Antibody
	mg/ml	mg/ml	mg/ml	U/ml $\times 10^{-3}$	U/ml $\times 10^{-3}$
ARC	1.6	15.3	1.4	219	74
ARC	1.8	20.7	1.8	193	61
ARC	3.9	24.9	2.4	220	19
ARC	3.5	40.3	4.1	2,760	47
ARC	3.1	48.4	2.7	222	24
ARC	4.3	20.7	4.3	530	35
ARC	1.3	15.8	0.8	144	8
AIDS	1.9	17.3	1.7	38	30
AIDS	1.2	14.9	1.3	293	26
AIDS	2.5	16.4	1.3	166	87
AIDS	1.9	22.1	9.6	46	9
Normal control	1.9	8.6	1.0	$<0.2$	41
Normal control	0.9	13.4	1.7	$<0.2$	240
Normal control	1.2	11.0	2.6	$<0.2$	45
Normal control	1.4	17.2	1.6	$<0.2$	140
Normal control	1.0	9.2	1.1	$<0.2$	72
Normal control	1.2	12.5	1.3	$<0.2$	197
Mean normals ( $\times / \div$ SEM)	1.2 (1.11)	11.7 (1.11)	1.4 (1.16)	$<0.2$	98 (1.36)

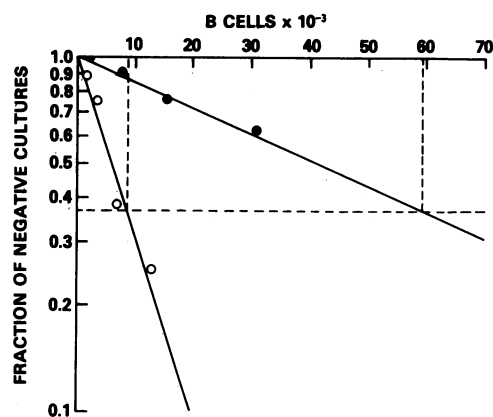


Figure 1. Representative limiting dilution analyses to determine the precursor frequencies of cells producing anti-HTLV-III antibody in cultures with media alone. Multiple (16 per dilution) replicate cultures were established with each of various numbers of B cells from patients with ARC and  $10^5$  irradiated (2,500 rad) normal allogeneic PBMC. After 14 d in culture, the supernatants were harvested and assayed for anti-HTLV-III antibody. The fraction of cultures negative for anti-body was plotted against the number of added B cells, and the precursor frequencies calculated as described in the text. The number of B cells added was calculated as the number of B-enriched cells cultured times the fraction of B cells in this population. Results obtained with two separate donors are shown. Calculated precursor frequencies were  $1/8,810$  B cells ( $114/10^6$  B cells) for one donor (○) and  $1/59,500$  B cells ( $16.8/10^6$  B cells) for the other donor (●).

was no increase either with the addition of PWM or with EBV (Fig. 3). Thus, PWM and EBV both failed to recruit additional precursors in cultures of B cells from these patients, and in addition did not increase the amount of antibody produced by the

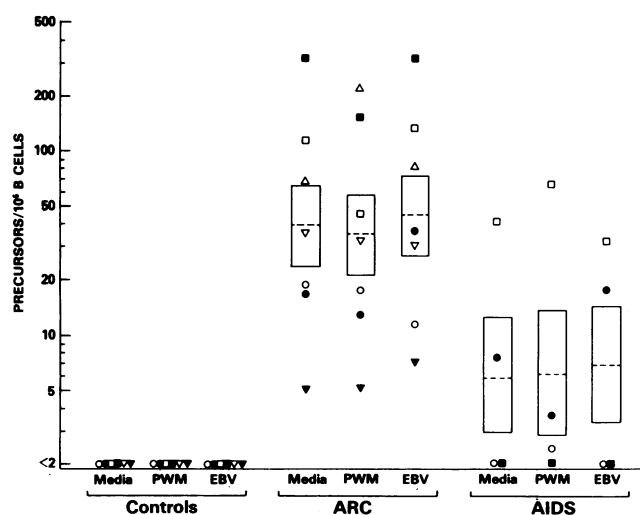


Figure 2. Precursor frequencies of B cells producing anti-HTLV-III antibody in normal controls, patients with ARC, and patients with AIDS. Precursor frequencies were determined as described from limiting dilution cultures established either with media alone, with PWM, or with EBV. Individual symbols are used to denote individual normals, patients with ARC, or patients with AIDS; these same symbols are used for each patient in each of the figures. Dotted lines denote the geometric means of the precursor frequencies obtained with each group, and the rectangles denote the limits of  $\times/\pm 1$  SEM.

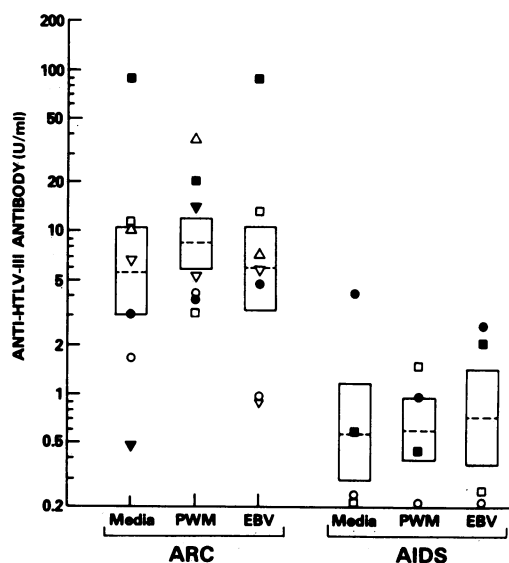


Figure 3. Total anti-HTLV-III antibody produced in cultures of B-enriched cells from patients with ARC or AIDS. Eight replicate cultures containing  $10^5$  B-enriched cells from patients with ARC or AIDS and  $10^5$  irradiated (2,500 rad) PBMC from a normal allogeneic control were established with media, PWM, or EBV. Supernatants harvested at 14 d were assayed for anti-HTLV-III antibody and the mean antibody calculated for each patient. As in Fig. 2, different symbols are used to denote different patients, and the geometric means  $\times/\pm 1$  SEM are shown for each group.

spontaneously producing precursors. As expected by their lack of exposure to HTLV-III, the B cells from the normal controls did not produce any anti-HTLV-III antibodies with any of the stimuli used (Fig. 2).

*Anti-A/Aichi influenza virus antibody production in cultures of B cells from patients with ARC or AIDS.* Circulating cells that spontaneously produced specific antibody have been detected in people recently immunized with tetanus toxoid (25) or infected with attenuated influenza virus (26), and it was possible that the circulating cells that spontaneously produced anti-HTLV-III antibody were the result of chronic antigenic stimulation by HTLV-III. To further investigate the specificity of the antibodies spontaneously produced by B cells from patients with HTLV-III infection, we assayed the same supernatants for the presence of anti-A/Aichi influenza virus antibody. A/Aichi influenza virus was prevalent in 1968, and antigenically cross-reacting H3N2 viruses have circulated since that time; both patients and controls were thus likely to have had previous exposure to this representative recall antigen. As shown in Fig. 4, all but three of the patients with HTLV-III infection had precursors that spontaneously produced anti-A/Aichi influenza virus antibody (mean  $7.28 \times / \pm 1.35$  precursors/ $10^6$  B cells in the ARC patients and  $3.69 \times / \pm 1.55$  precursors/ $10^6$  B cells in the AIDS patients). Thus, B cells that produce antibodies to recall antigens comprise a portion of the activated B cells in patients with AIDS or ARC. The addition of PWM to these cultures resulted in an increase in the number of precursors in two of the ARC patients; however, taken as a group, the addition of either PWM or EBV did not cause a significant increase in the number of precursors that produced anti-A/Aichi influenza virus antibody ( $P > 0.05$  in each case) (Fig. 4). In contrast, none of the normals had detectable precursors that spontaneously produced anti-A/Aichi in-

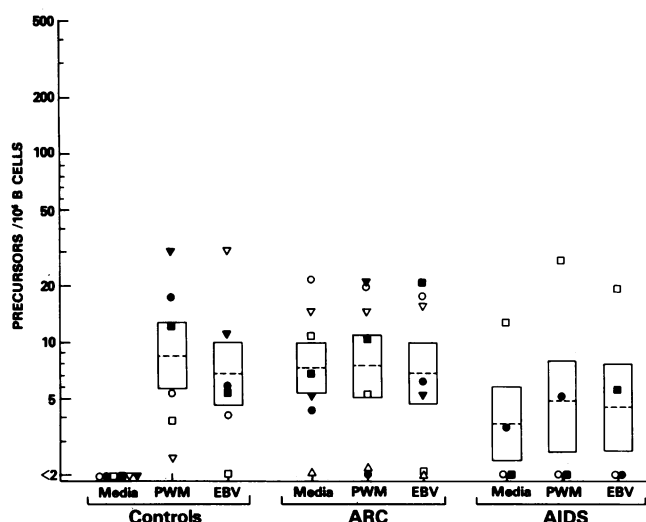


Figure 4. Precursor frequencies of B cells producing anti-A/Aichi influenza virus antibody in normal controls, patients with ARC, and patients with AIDS. Precursor frequencies of B cells either spontaneously producing anti-A/Aichi influenza virus antibody or producing antibody in the presence of PWM or EBV were determined. Symbols used are as in Fig. 2.

fluenza virus antibody (Fig. 4). However, in the presence of PWM, there were a mean of  $8.42 \times / \div 1.48$  precursors/ $10^6$  B cells that produced anti-influenza virus antibody, and in the presence of EBV there were  $6.78 \times / \div 1.47$  precursors/ $10^6$  B cells. Thus, unlike the patients with HTLV-III infection, normals had no B cells that spontaneously produced anti-A/Aichi influenza virus antibody, but did have "memory" B cells that could be activated by PWM or by EBV.

**Immortalization of B cells from patients with ARC or AIDS.** Patients with ARC or AIDS often have active infection with EBV (15), and we wondered if the B cells that spontaneously produced antibody might be EBV-infected cells, which would produce immortal cell lines in vitro. Such B cells that spontaneously produce immortalized cell lines are found in low numbers in EBV-seropositive normals, but not in EBV seronegative normals (27), and are known to be increased in EBV-induced infectious mononucleosis (28) and in patients with rheumatoid arthritis (29). To test this hypothesis, we assessed these limiting dilution cultures for immortalization/outgrowth after an additional 4 wk in culture (total 6 wk). As shown in Fig. 5, only one

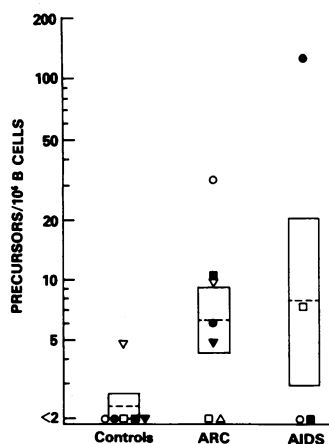


Figure 5. Spontaneously immortalizing cells in normal controls, patients with ARC, and patients with AIDS. The precursor frequencies of cells with outgrowth in culture at 6 wk (in the absence of exogenously added EBV) were determined from limiting dilution cultures of B-enriched cells from normal controls, patients with ARC, and patients with AIDS. Symbols used are as in Fig. 2.

of six normals tested had  $>2$  precursors for B cell outgrowth per  $10^6$  B cells. In contrast, the patients with ARC had a mean of  $6.2 \times / \div 1.46$  precursors for outgrowth per  $10^6$  B cells, and patients with AIDS had a mean of  $7.8 \times / \div 2.67$  precursors/ $10^6$  B cells ( $P < 0.05$  comparing patients with HTLV-III infection with normals). Each of the spontaneously growing lines in these patients produced Ig but none, however, produced antibody to either HTLV-III or A/Aichi influenza virus. Thus, patients with HTLV-III infection had an increased number of spontaneously immortalized B cells as compared with normals. These spontaneously immortalizing B cells, however, could not account for a significant portion of the anti-HTLV-III or anti-A/Aichi influenza virus antibody production, and were only a small fraction of the reported frequency (13) for B cells that spontaneously produced Ig in these patients ( $10^3$ – $10^4$ / $10^6$  lymphocytes). Thus, it is unlikely that EBV-infected outgrowing B cells account for all the spontaneous Ig production in patients with AIDS or ARC.

In contrast to the above, B cells from patients with HTLV-III infection had a markedly decreased ability to be immortalized by exogenous EBV added in culture. The six normals tested had a mean of  $3,082 \times / \div 2.06$  precursors forming immortalized cell lines per  $10^6$  B cells upon the addition of the B95-8 strain of EBV (Fig. 6). In contrast, the patients with ARC had  $<10\%$  as many B cells that were immortalized under these conditions ( $237 \times / \div 1.27$ / $10^6$  B cells), and patients with AIDS had  $<3\%$  as many ( $85 \times / \div 2.36$ / $10^6$  B cells). Cells from some of the patients were also cultured in the presence of anti-alpha IFN (sufficient to neutralize  $10^2$  U/ml); however, no increase in the frequency of B cells that formed immortalized cell lines was seen (Table II). Thus, patients with ARC or AIDS had an increased frequency of B cells that spontaneously formed cell lines, but a markedly decreased ability to be immortalized by exogenously added EBV.

**HTLV-III induces Ig production by normal PBMC.** The discrepancy between the number of cells that produce Ig in these patients and the number of spontaneously outgrowing cells suggested that EBV-infected B cells may not account for all the activated B cells in these patients. We asked whether HTLV-III might itself induce polyclonal Ig production. As shown in Table III, live HTLV-III (1,000 viral particles per cell) induced an average of 2,860 ng/ml of Ig compared with an average of 370 ng/ml produced in media alone. While this was somewhat less than the average of 7,820 ng/ml of Ig produced in response to

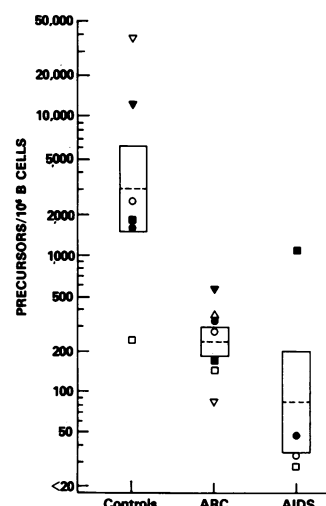


Figure 6. B cells from normal controls, patients with ARC, or patients with AIDS forming immortalized cell lines with exogenously added EBV. The precursor frequencies of cells with outgrowth at 6 wk were determined in limiting dilution cultures with exogenously added EBV. Symbols used are as in Fig. 2.

**Table II. Precursor Frequencies of Cells Immortalized by EBV Were not Affected by the Addition of Anti- $\alpha$  IFN Antibody**

Diagnosis of donor	Immortalized cells/ $10^6$ B cells*	
	Without antibody	With antibody
Normal control	1,602	1,743
ARC	348	222
ARC	149	124
ARC	340	283
AIDS	1,100	1,139

\* Precursor frequencies of B cells immortalized by the addition of the B95-8 strain of EBV were determined as described in Methods in the absence or presence of anti- $\alpha$  IFN sufficient to neutralize 100 U/ml.

PWM, it still represented substantial Ig production induced by HTLV-III.

We examined the cellular requirements for this HTLV-III-induced Ig production. As shown in Fig. 7, cultures of  $0.5 \times 10^6$  E-rosette-depleted cells (B cells and macrophages) failed to produce any detectable Ig in response to HTLV-III. In the presence of  $1.5 \times 10^6$  E-rosette-enriched cells (T cells), however, 1,480  $\times$   $\div$  1.58 ng/ml of Ig was produced. Thus, Ig production induced by HTLV-III requires the presence of both B cells and T cells. It is likely that this HTLV-III-induced Ig production contributes to the B cell activation in patients with AIDS or ARC.

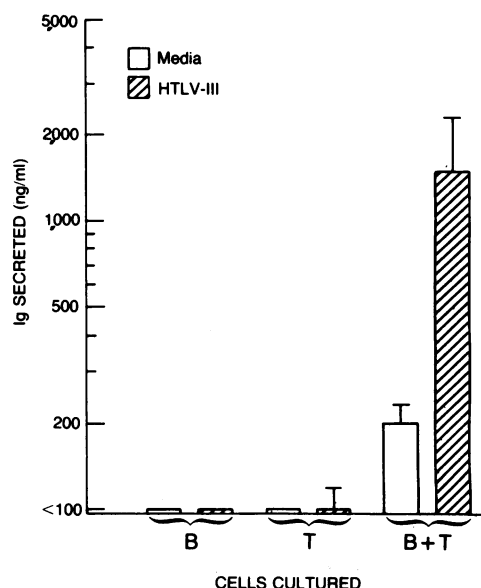
## Discussion

It has previously been shown that patients with AIDS have hypergammaglobulinemia and B cell hyperactivation with from  $10^3$  to  $10^4$  B cells that spontaneously produce Ig per  $10^6$  circulating lymphocytes (13). The results of the present study demonstrate that several mechanisms may contribute to this in vivo B cell hyperactivation, particularly chronic antigenic stimulation by HTLV-III, increased numbers of EBV-infected B cells, and HTLV-III-induced T cell-dependent B cell activation.

**Table III. HTLV-III Induces Ig Production by Normal PBMC\***

Stimuli	Cells cultured	Ig secreted	
		Exp. 1	Exp. 2
		ng/ml	ng/ml
Media	$2 \times 10^6$	376 (1.16)	363 (1.18)
PWM	$2 \times 10^6$	2,776 (1.61)	12,858 (1.01)
HTLV-III	$2 \times 10^6$	3,446 (1.24)	2,274 (1.25)
HTLV-III	None	<10	<10

\* Cultures of  $2 \times 10^6$  PBMC (from normals who were seronegative for HTLV-III) were established in triplicate with PWM (1:100; vol/vol), with live double-banded HTLV-III ( $10^9$  virions per culture), or in media alone. Supernatant collected at day 14 was assayed for total Ig by ELISA. Results shown are the geometric means (SEM) of triplicate cultures.



**Figure 7. T cells are required for HTLV-III-induced Ig production.** Triplicate cultures were established with  $0.5 \times 10^6$  E-rosette-depleted cells (B cells),  $1.5 \times 10^6$  E-rosette-enriched cells (T cells), or both in media alone ( $\square$ ) or with  $2 \times 10^9$  particles of HTLV-III per culture ( $\blacksquare$ ). Cumulative Ig production at the end of 14 d was measured by ELISA. Results shown are the geometric means of triplicate cultures; error bars show the range of 1 SEM.

We first undertook to examine the specificities of the antibodies produced by the spontaneously activated B cells, and showed that some of these cells were producing antibody to HTLV-III and that others were producing antibodies to antigens the patients had previously been exposed to. Circulating B cells that spontaneously produced specific antibody have been transiently observed after immunization with tetanus toxoid (25) or infection with attenuated influenza virus (26), and chronic antigenic exposure to HTLV-III probably contributed to the B cell activation in these patients (particularly for the B cells that produced anti-HTLV-III antibody). It is questionable, however, as to whether the antigenic stimulation by HTLV-III can totally account for activation of B cells that produce antibodies of other specificities. In patients infected with influenza virus, for example, the spontaneously secreted antibody is relatively specific for the stimulating antigen (26). Also, chronic antigenic stimulation may not in itself totally account for the B cells that spontaneously produce antibody to HTLV-III; patients with chronic active hepatitis, for example, do not have B cells that spontaneously produce anti-hepatitis virus antibody, but do have memory cells that can be activated by PWM (30). Thus, other factors besides antigenic stimulation by HTLV-III are likely to be involved in this process.

One mechanism that has been proposed to explain the spontaneously activated B cells in patients with AIDS is stimulation by viruses such as EBV or cytomegalovirus. EBV in particular is known to infect and activate human B cells even in the absence of T cell help (20), and patients with AIDS are known to have evidence of active infection with EBV as evidenced by the ability to isolate EBV from throat washings and the presence of antibodies to EBV early antigen (15). It is known that B cells latently infected with EBV in vivo will produce immortal cell lines when cultured in the absence of active suppressor or cytotoxic T cells,

and the frequency of such lines can be used as a measurement of the number of circulating EBV-infected B cells (27–29). Our results indicated that patients with HTLV-III infection had significantly more spontaneously outgrowing B cells than normals; a similar finding has recently been reported by Bix et al. (31). The frequency of such spontaneously outgrowing B cells was, however, only a small fraction of the frequency of cells reported to spontaneously produce Ig in AIDS patients (13) and approximately equal to the frequency of B cells that spontaneously produced antibody to A/Aichi influenza virus (one of many recall antigens). It is possible that even in the absence of suppressive T cell influences, not all of the EBV-infected B cells in these patients went on to produce cell lines in culture. A recent study, however, failed to detect Epstein-Barr viral nuclear antigen in B cells from homosexuals with generalized lymphadenopathy and circulating Ig-producing cells (32), providing further evidence that EBV-infected B cells did not account for all the activated B cells in these patients.

It is of course possible that antigenic stimulation by infection with agents such as cytomegalovirus or bacterial or fungal organisms contribute to the B cell activation in these patients. Such nonspecific B cell activation by antigenic stimulation, however, is generally mediated by helper T cells (even in the case of cytomegalovirus [33]), and in ARC or AIDS patients one thus has the seeming paradox of B cell stimulation in the face of a decreased number of OKT4<sup>+</sup> (helper/inducer) T cells. In part this may be the result of the preferential depletion of OKT4<sup>+</sup>-Leu 8<sup>+</sup> T cells (which are inducers of suppression) early in the disease process (reference 14, and also Redfield, R. R., and T. M. Chused, unpublished observations). As shown here, however, a more direct explanation is that HTLV-III itself (or some viral component) can induce Ig production.

As the experiments here show, PBMC from normals (seronegative for HTLV-III) produce Ig when exposed to HTLV-III in vitro, and this response requires the presence of both B and T cells. As this is observed in persons who have not been exposed to HTLV-III, it is not likely to be the result of secondary antigenic stimulation, but is instead an intrinsic property of the virus (or a viral component). It has previously been shown that T cell clones infected by another transactivating retrovirus, HTLV-I, induce polyclonal Ig production by B cells (34, 35), and it is possible that T cells infected by HTLV-III are similarly activated to provide help for Ig production. Even in patients with reduced numbers of OKT4<sup>+</sup> T cells, activation of the remaining OKT4<sup>+</sup> T cells by HTLV-III (perhaps in the time period between infection of these cells and their destruction by the cytopathic effect of HTLV-III) may contribute to the B cell hyperactivation.

In the present studies, the addition of PWM or EBV to the cultures of cells from the patients did not result in an increase in the number of B cells secreting antibody as compared with cultures in media alone. In addition, the patients had markedly fewer B cells that were induced to undergo immortalization by EBV than did normals, and the frequency of EBV-induced transforming cells was not increased by the addition of antibody to alpha IFN (which has been shown to inhibit the outgrowth of EBV-infected B cells [36] and is elevated in certain patients with ARC [37]). It is not clear why the cells from these patients are not activated by these stimuli; PWM and EBV are believed to act via different mechanisms and to activate different subpopulations of B cells. EBV activates small resting B cells but, although it can bind to them, does not induce outgrowth in

larger preactivated B cells (38). In contrast, PWM can activate both small and larger B cells (39). While the decreased number of B cells transformed by EBV in these patients may be due to the preactivation of their B cells, the failure of the cells to respond to PWM suggests further that these cells may be maximally activated in vivo.

The results observed here may have implications in the pathogenesis of the B cell lymphomas that are observed in patients with HTLV-III infection (10, 11, 40). Most normal adults have been infected with EBV and carry B cells that are latently infected by this virus (5). While these cells will spontaneously form cell lines in culture, they are believed to be prevented from doing so in vivo by regulatory T cells (20, 22, 41). The increased numbers of such cells in patients with ARC or AIDS suggests that these T cell regulatory mechanisms are defective, as one would expect, given their alterations in T cell functions. Such an expanded population of EBV-infected B cells may increase the chance of one of these cells undergoing additional malignant changes (e.g. chromosomal rearrangement) and developing into Burkitt's lymphoma (10, 42, 43). In addition, it has been observed that HTLV-III can superinfect EBV-transformed B cells in vitro (44), and integration of HTLV-III genetic material into the EBV-infected B cells may contribute to the malignant process. Finally, the B cell activation that is observed in these patients, and in particular the chronic antigenic stimulation, may lead to B cell lymphoma production by the mechanism of "receptor-mediated leukemogenesis" (45, 46). Indeed, Blattner et al. have recently found that the Ig (captured by hybridoma technology) of tumor B cells obtained from an HTLV-I-infected patient with B cell chronic lymphocytic leukemia had antibody activity against HTLV-I (47). Thus, there are several mechanisms that may all contribute to the increased incidence of B cell lymphomas in patients with HTLV-III infection.

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