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# HIV-1-SPECIFIC B CELL ACTIVATION

## A Major Constituent of Spontaneous B Cell Activation during HIV-1 Infection[1]

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**B cell activation is a well known consequence of HIV-1 infection, and seropositive subjects show high numbers of spontaneously activated Ig-secreting cells in circulation. To better define the importance of the HIV-1-specific response in this phenomenon, we first studied whether in vitro spontaneous anti-HIV-1 antibody production was accompanied by reactivation of memory B lymphocytes. Unstimulated PBL from HIV-1-infected individuals with prior history of hepatitis B and/or EBV infection did not consistently show spontaneous in vitro synthesis of antihepatitis B core Ag or anti-EBV antibodies; in addition, PWM-induced synthesis of anti-hepatitis B virus and anti-EBV antibodies was decreased compared to HIV-1-seronegative subjects. Moreover, in comparing the frequencies of activated HIV-1-specific B cell precursors and activated Ig-secreting precursors in limiting dilution experiments, a sizable fraction (20 to 40%) of circulating cells spontaneously secreting Ig produced antibody against HIV-1 determinants. The ratio between the two frequencies fitted in very well with the amount of Ig removed from unstimulated culture supernatants after HIV-1-specific antibody absorption with solid-phase HIV-1. These findings indicate that B cell activation during HIV-1 infection is mainly oriented toward a specific response to HIV-1 determinants; the possible relevance of this phenomenon to lymphomagenesis in AIDS patients is discussed.**

T cell dysfunction in AIDS is most likely mediated by a direct viral cytopathic effect and/or by an immune response against cells bearing HIV-1 Ag (1–5). However, complex B cell function disorders are a hallmark of most HIV-1-infected individuals (6–10). In this setting, the intense B cell activation observed in PBL from seropositive subjects (6, 8, 9) might have a pathogenetic significance, and could contribute to the immune damage that occurs during infection (11–13); moreover, it could be involved in the increased frequency of malignant B cell lymphomas observed in AIDS patients (14, 15).

Seropositive individuals have high numbers of B cells spontaneously secreting anti-HIV-1 Ab[3] in circulation (16, 17), but the importance of this response in the postulated polyclonal activation of the B cell system has not been fully explored. Although previous data suggest that reactivation of memory B cell clones may occur during HIV-1 infection, this phenomenon apparently is a minor event compared to HIV-1-specific B cell activation (17).

To better define this issue, we investigated whether in vivo activation of HIV-1-specific B lymphocytes was accompanied by reactivation of memory B cells in the peripheral blood of HIV-1-infected individuals. We also compared the frequencies of activated HIV-1-specific B cell precursors and activated Ig-secreting precursors. Our results indicate that activated HIV-1-specific B lymphocytes are a major constituent of the overall B cell stimulation seen in the peripheral blood of seropositive patients.

### MATERIALS AND METHODS

*Study population.* A total of 37 i.v. drug abusers (21 males and 16 females; mean age 25 yr), seropositive for HIV-1 as demonstrated by ELISA and Western blot assay, were staged according to the WR classification (18); 8 subjects had serologic evidence of previous HBV infection, because they were negative for HBs Ag and positive for serum Ab against HBs Ag and HBc Ag; 29 had serologic evidence of previous EBV infection. The control group consisted of HIV-1-seronegative age- and sex-matched i.v. drug abusers (WR 0), all with serologic evidence of a prior HBV and/or EBV infection (serum anti-EBV antibodies; serum anti-HBs and anti-HBc Ab titers comparable to those of the HIV-1-seropositive group). To control the reliability of the anti-HBc Ab detection assay, eight HIV-1-seronegative individuals not at risk for AIDS with acute or chronic HBV infection were also studied; in four cases, spot hybridization (19) detected viral DNA (HBV-DNA) in serum, whereas no evidence of active HBV replication was found in the others. Ten HIV-1-seronegative laboratory staff members served as normal controls in experiments to evaluate in vitro spontaneous Ig production.

*ELISA and Western blot assay.* Sera were tested by ELISA and Western blot assay as previously reported (20). The amount of HIV-1-specific Ab in serum samples was expressed as the absorbance value at 492 nm in ELISA at serum dilutions of 1/100.

*Cell cultures.* Cell cultures were performed as reported (16, 21). Briefly, following purification by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation, PBL were resuspended to $1 \times 10^6$/ml in RPMI 1640 medium supplemented with 10% FCS (Flow Laboratories, Irvine, U.K.), 1% L-glutamine, 1% nonessential aminoacids,

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[3] Abbreviations used in this paper: Ab, antibody; HBV, hepatitis B virus; HBc, HBV core Ag; HBs, HBV surface Ag; SN, supernatant; WR, Walter Reed staging classification; HIV-1 Seph, 0.1 ml Sepharose 4B-conjugated HIV-1; c. l., confidence limits.

50 μg/ml gentamycin, and $2 \times 10^{-5}$ M 2-ME (complete RPMI). Cultures were incubated at 37°C for 10 days in both the absence and presence of PWM (GIBCO, Grand Island, NY; 1/100 final dilution) in 48-well flat-bottom tissue culture plates (Costar Data Packaging, Cambridge, MA). Cell-free SN were then recovered by low-speed centrifugation and stored at −20°C until use.

*Limiting dilution assay of B cell precursors.* Activated B cell precursor frequency was determined according to minor modifications (16) of a previously described limiting dilution procedure (17, 22). Briefly, T and B cells were isolated by double rosetting with neuroaminidase-treated SRBC as reported elsewhere (23); graded numbers of the B enriched population (40 to 60% $sIg^+$ according to cytofluorographic analysis) were plated in 96-well U-bottom microtiter plates (Costar), and cultured without mitogenic stimulation in a total volume of 0.2 ml of complete RPMI in the presence of irradiated (3000 rad) autologous T cells ($2 \times 10^5$/well). In all cases, at least 30 replicate wells were set up for each cell concentration. After 14 days of incubation, SN aliquots were collected and assayed by RIA for anti-HIV-1 Ab, and total Ig content, as described below. Wells were scored as positive when radioactivity levels exceeded the mean value + 3 SD of control cultures (containing only irradiated feeder cells). The fraction of negative cultures was then plotted against the number of added B cells (calculated from the fraction of B lymphocytes in the B-enriched population), and the frequency of B cell precursors spontaneously secreting anti-HIV-1 Ab or total Ig was calculated by Poisson analysis; the $p$ of the fitness of the experimental values to a straight line equation was determined by the minimum chi square method (24), and tests where $p$ was >0.05 were discarded.

*Assays for anti-HIV-1 antibodies and total Ig in culture supernatants.* HIV-1-specific and total in vitro Ig production was determined in SN by solid-phase RIA. Briefly, the wells of 96-well flexible polyvinyl plates (Falcon, Grenoble, France) were coated with HIV-1 (HIV-1 lysate, Sclavo-Dupont, Siena, Italy) or rabbit anti-human Ig (Dakopatts, Copenhagen, Denmark) at a concentration of 100 μg/ml in carbonate buffer, pH 9.6 (50 μl/well). When the protein pattern of the commercial HIV-1 preparation used was examined on conventionally stained gels, we found no major contaminants compared to an HIV-1 preparation produced in our laboratory by double sucrose banding of virus harvested from HTLV-$III_B$-infected H9 cells (25); all *core*, *env* and *qaq* products were represented on Western blot assay. After overnight incubation at 4°C, the contents of the wells were recovered and saved for further use. The plates were saturated with 3% BSA in PBS, and after three washes with PBS, SN aliquots (50 μl) were added in triplicate to individual wells, which were left to stand for 3 h at room temperature. The plates were then washed again and incubated with $^{125}$I-labeled goat antihuman Ig $F(ab)_2$ (Amersham, Buckinghamshire, U.K.; sp. act. 19 to 74 TBq/mM, ~$2 \times 10^5$ cpm/well) for 4 h at room temperature. Finally, the plates were washed thoroughly with PBS, and each well counted in a γ-counter.

Due to the lack of a reference reagent for HIV-1-specific Ab, SN anti-HIV-1 Ab contents were expressed as mean cpm in triplicate wells; samples were scored as positive when bound radioactivity exceeded mean values + 3 SD of the background (obtained by testing SN on uncoated wells). In testing undiluted bulk culture SN from seronegative controls for spontaneous anti-HIV-1 Ab production, radioactivity levels did not exceed background values + 3 SD (~100 cpm in most experiments), whereas those in SN from HIV-1-infected patients ranged from 1000 to 6000 cpm. Supernatant Ig content was evaluated against a reference curve obtained by doubling dilutions of a standard human serum containing known Ig amounts. Standard curves in these RIA were linear between approximately 1 and 500 ng/ml; if Ig concentration exceeded this value, the SN was diluted so that Ig levels would fall into this range.

*Assay for anti-HBc antibodies in culture supernatants.* Antibody to HBc Ag was detected in culture SN by a competition RIA. The solid-phase was coated with purified HBc Ag (150 μg/ml in carbonate buffer, pH 9.6) prepared from serum Dane particles by ultracentrifugation as described (26), and incubated with SN in the presence of $^{125}$I-labeled anti-HBc Ab (CorAb reagent, Abbot Laboratories, North Chicago, IL). Samples were considered positive when they caused >50% inhibition of the binding of radiolabeled Ab to solid-phase HBc. Inasmuch as a reference anti-HBc Ab reagent is lacking, the sensitivity of this assay is undetermined; by using a previously characterized anti-HBc mAb (27), 50% inhibition was obtained up to a mAb dilution of 20 ng/ml. The specificity of this assay was demonstrated against a wide panel of anti-HBc $Ab^+$ and anti-HBc $Ab^-$ reference sera.

*Assay for anti-EBV Ab in culture supernatants.* EBV-specific Ab synthesis was detected by solid-phase RIA. The EBV Ag was isolated from the supernatant of 12-*O*-tetradecanoylphorbol-13-acetate-induced B95.8 cell line according to Nemerow and Cooper (28). The viral particles were purified on a discontinuous dextran T-10 (Pharmacia) gradient; a sample of the purified EBV batch was analyzed on an Uvicon 800 spectrophotometer (Kontron, Zurich, Switzerland) at 260 and 280 nm, to calculate the amount of virus particles in each preparation, as previously described (29). EBV was resuspended in coating buffer at a concentration of $2 \times 10^9$ viral particles/ml, and 50 μl of the suspension were seeded in RIA plates (Falcon). The assay was carried out as described above for the detection of HIV-1-specific antibodies. Results were scored as positive when bound radioactivity exceeded mean values + 3 SD of the background; $EBV^+$ and $EBV^-$ sera were run in parallel in each experiment.

*Absorption of HIV-1-specific antibodies on solid-phase HIV-1.* HIV-1 was coupled to CNBr-activated Sepharose 4B (Pharmacia) following the manufacturer's instructions; the actual coupling of HIV-1 proteins to Sepharose beads was demonstrated by indirect immunofluorescence using sera from HIV-1-infected patients. An unrelated Ag, an mAb (30) against ricin (75/3B12), was similarly linked to Sepharose and used as a control; about 370 μg HIV-1 and 1.0 mg 75/3B12 mAb were cross-linked per ml packed Sepharose 4B. Aliquots (0.5 ml) of unstimulated culture SN diluted 1/4 were incubated with HIV-1 Seph or control Sepharose for 2 h at room temperature under continuous shaking. Subsequently, SN were recovered by low-speed centrifugation and tested for HIV-1-specific Ab and total Ig content, as above. Results were expressed as percent of residual antibody (both HIV-1-specific and total) after absorption with HIV-1 Seph, in comparison to SN absorbed with control Sepharose.

*p24 Ag assay.* p24 Ag in serum was determined with an ELISA commercial kit (Dupont de Nemours, Wilmington, DE) according to minor modifications of the manufacturer's instructions. Samples were quantified by relating the absorbance value of the specimen to a standard curve prepared by testing serial dilutions of purified p24 Ag in the same assay. The average sensitivity of the assay was ~5 pg/ml, and the standard assay curve was linear to approximately 500 pg/ml.

*Immunofluorescence.* T cell subpopulations in the blood of seropositive patients were analyzed for phenotype by standard methods with mAb (OKT3, OKT4, OKT8, Ortho Diagnostics, Raritan, NJ) on an Epics C (Coulter Electronics, Hialeah, FL) cytofluorometer. The percentage of B lymphocytes in the B-enriched cell populations was evaluated by the same procedure with a panel of mAbs (B1, B2, B4, Coulter) directed against B cell surface Ag.

## RESULTS

*In vitro production of antibodies against HBc Ag.* We first studied whether spontaneous activation of HIV-1-specific B cells was accompanied by reactivation of B cells directed against recall Ag. As shown in Table I, PBL from HIV-1-seropositive individuals with a previous history of HBV infection spontaneously produced anti-HIV-1 Ab, but not anti-HBc Ab. No anti-HBc Ab synthesis was detected in unstimulated cultures from control subjects with previous history of HBV infection, nor in the four controls with acute or chronic hepatitis B but no serum HBV-DNA. As expected, spontaneous anti-HBc Ab synthesis was observed in all controls with ongoing HBV infection and HBV-DNA in serum. So, if reactivation of HBV-specific memory B cells occurs in HIV-1-infected subjects, its detection is beyond the sensitivity limits of our assay, which nonetheless invariably disclosed such a production in individuals where it was anticipated.

After PWM stimulation, anti-HIV-1 Ab production was again observed in all HIV-1-infected individuals (Table I), whereas anti-HBc Ab synthesis was found in only one subject. On the contrary, anti-HBc Ab synthesis occurred in most of the controls with ongoing hepatitis B (Table I), and in half of the controls with prior history of HBV infection; this is in line with the finding that PWM-induced anti-HBc Ag synthesis depends on the time of infection and the serum titer of anti-HBc Ab (31).

*In vitro production of antibodies against EBV.* To confirm these observations with another recall Ag, we studied spontaneous and mitogen-induced synthesis of antibodies against EBV by PBL from HIV-1-seropositive

TABLE I

*In vitro spontaneous and PWM-induced synthesis of anti-HIV-1 and anti-HBc Ag antibodies by PBL from HIV-1 seropositive and seronegative subjects*[a]

| Donors | HBV Infection Status[b] | In Vitro Antibody Production against | | | |
|---|---|---|---|---|---|
| | | HIV-1 | | HBc Ag | |
| | | Spontaneous | PWM-induced | Spontaneous | PWM-induced |
| HIV-1 seropositive | Prior HBV infection | 8/8[c] | 8/8 | 0/8 | 1/8 |
| HIV-1 seronegative | Prior HBV infection | 0/8 | 0/8 | 0/8 | 4/8 |
| | Ongoing HBV infection with serum HBV-DNA | ND | ND | 4/4 | 3/4 |
| | no serum HBV-DNA | ND | ND | 0/4 | 4/4 |

[a] Unfractionated PBL from individual patients were cultured at $1 \times 10^6$/ml in complete RPMI in the absence and in the presence of PWM. After 10 days of incubation, SN were recovered and assayed for anti-HIV-1 and anti-HBc Ag antibodies as described in *Materials and Methods*.

[b] The status of HIV-1 seropositive and seronegative donors with regard to the HBV infection was assessed by serologic marker studies as described in *Materials and Methods*.

[c] No. positive patients/no. tested.

and seronegative subjects with previous history of EBV infection. As shown in Table II, spontaneous synthesis of anti-EBV Ab was found in unstimulated PBL cultures from a minority (14%) of the HIV-1-infected subjects; this figure, however, was not different from that (22%) found in HIV-1-seronegative controls, and probably reflects reactivation of EBV infection in some individuals (7). After PWM stimulation, anti-EBV Ab synthesis was detected in a smaller fraction of HIV-1-infected subjects than controls, where such anti-EBV Ab production occurred in most individuals (Table II).

*Frequency of HIV-1-specific spontaneously activated B cell precursors.* The frequency of B cell precursors spontaneously producing HIV-1-specific Ab was evaluated by culturing B lymphocytes from HIV-1-infected individuals in a limiting dilution assay in the absence of mitogenic stimulation. After 14 days of incubation, the SN were tested for anti-HIV-1 Ab content, and precursor frequencies were calculated as described above. Results obtained in 14 consecutive patients disclosed a frequency range from 1/105 to 1/40,000 circulating B lymphocytes (Table III). Such a wide variation was also observed by Yarchoan et al. (17) in patients with AIDS and AIDS-related complex; in our case series, which also included patients with less advanced disease (stages WR 1 to 3), no differences were observed among the different groups (Fig. 1).

No apparent correlation was found between these values and serum Ig concentrations or HIV-1-specific Ab content (Table III). In fact, some patients (1 and 12 in Table III) had normal serum Ig levels and high precursor frequencies, whereas others (patients 9 and 14) showed high serum Ig and low frequencies. The same was true for anti-HIV-1 Ab, because patients (6 and 9 in Table III) with very high serum levels also had lower precursor frequencies than subjects showing low serum anti-HIV-1 Ab concentrations (patients 7, 11, and 12). Moreover, no correlation was observed between the frequency of HIV-1-specific B-cell precursors and serum p24 Ag levels (Table III).

*Comparison between the frequencies of B cell precursors spontaneously secreting anti-HIV-1 Ab and Ag.* We then compared the frequency of spontaneously activated HIV-1-specific B cell precursors with that of spontaneously activated Ig-secreting precursors. To this end, SN from limiting dilution experiments were simultaneously tested for the production of both virus-specific and total Ig as described above. Linear regression analysis of two representative experiments is shown in Figure 2, and the frequencies are compared in Table IV. Activated Ig-secreting precursor rates corresponded closely to figures obtained by Lane et al. (6) in seropositive patients, and were much higher than frequencies found in seronegative controls (not shown). In all the cases studied, HIV-1-specific and Ig-secreting activated B cell precursors showed relatively similar frequencies, regardless of the rate of spontaneously activated circulating B lymphocytes; on the average, 20 to 40% of the activated B cells produced anti-HIV-1 Ab. In patient 9 (Table IV and Fig. 2*a*), the calculated frequencies for virus-specific and total Ig-secreting B cell precursors overlapped, so in this particular case we can conclude that virtually all the circulating activated B lymphocytes generated Ab directed against HIV-1 determinants. However, patient 6 had similar numbers of activated Ig-secreting precursors (Table IV), but only about one-fourth of these cells synthesized HIV-1-specific Ab.

*Absorption studies.* To better substantiate these findings, we measured total Ig content in unstimulated culture SN before and after absorption with insolubilized HIV-1. As reported by others (4–6), significantly higher Ig amounts were present in unstimulated culture SN from

TABLE II

*In vitro spontaneous and PWM-induced synthesis of anti-HIV-1 and anti-EBV antibodies by PBL from HIV-1 seropositive and seronegative subjects*[a]

| Donors | In Vitro Antibody Production against | | | |
|---|---|---|---|---|
| | HIV-1 | | EBV | |
| | Spontaneous | PWM-induced | Spontaneous | PWM-induced |
| HIV-1 seropositive | 29/29[b] | 29/29 | 4/29 (13.8)[c] | 13/29 (44.8) |
| HIV-1 seronegative[d] | 0/9 | 0/9 | 2/9 (22.2) | 7/9 (77.8) |

[a] Unfractionated PBL from individual patients were cultured as reported in Table I, and SN were recovered and tested for anti-HIV-1 and anti-EBV antibodies by RIA as described in *Materials and Methods*.

[b] No. positive patients/no. tested.

[c] Percent of positive tests in parentheses.

[d] HIV-1-seronegative individuals at risk for AIDS (WR 0) with serologic evidence of previous EBV infection.

TABLE III

*Frequency of activated, HIV-1-specific B cell precursors in PBL from HIV-1-infected individuals*[a]

| Patient | Stage | Serum Ig (mg/100 ml) | | | Serum Anti-HIV-1 Ab[b] | Serum p24 (pg/ml) | HIV-1-Specific B Cell Precursor Frequency |
|---|---|---|---|---|---|---|---|
| | | IgG | IgM | IgA | | | |
| 1 | WR2 | 1,660 | 135 | 203 | 0.403 | 11.5 | 1/830 |
| 2 | WR2 | 1,200 | 129 | 199 | 0.119 | 21.5 | 1/9,900 |
| 3 | WR6 | 1,780 | 801 | 288 | 0.142 | 6 | 1/2,200 |
| 4 | WR2 | 1,560 | 360 | 140 | 0.111 | <5 | 1/40,000 |
| 5 | WR1 | ND | ND | ND | ND | 9.5 | 1/305 |
| 6 | WR3 | 2,390 | 143 | 264 | 0.608 | <5 | 1/2,170 |
| 7 | WR6 | 2,980 | 419 | 847 | 0.191 | ND | 1/500 |
| 8 | WR3 | 1,710 | 156 | 148 | ND | ND | 1/2,500 |
| 9 | WR3 | 3,680 | 283 | 141 | 0.650 | 7 | 1/2,400 |
| 10 | WR3 | 1,610 | 283 | 231 | 0.372 | 6 | 1/1,340 |
| 11 | WR2 | 2,470 | 208 | 145 | 0.314 | 9.5 | 1/105 |
| 12 | WR4 | 1,600 | 167 | 186 | 0.196 | 12.5 | 1/1,130 |
| 13 | WR2 | 958 | 104 | 152 | ND | ND | 1/8,700 |
| 14 | WR3 | 2,660 | 431 | 344 | 0.172 | 11.5 | 1/9,100 |

[a] Graded numbers of B lymphocytes were cultured without mitogenic stimulation in limiting dilution assay in the presence of autologous irradiated T cells. After 14 days of incubation, SN aliquots were recovered and tested for anti-HIV-1 production by RIA as described in *Materials and Methods*. Wells were scored as positive when radioactivity exceeded mean value + 3 SD of the control wells (containing only feeder cells), and the frequency of HIV-1-specific, spontaneously activated B cells was calculated by plotting the fraction of negative wells against the cell concentration.

[b] Expressed as absorbance at 492 nm of the serum (1/100 dilution) tested in ELISA for anti-HIV-1 Ab as reported elsewhere (20).

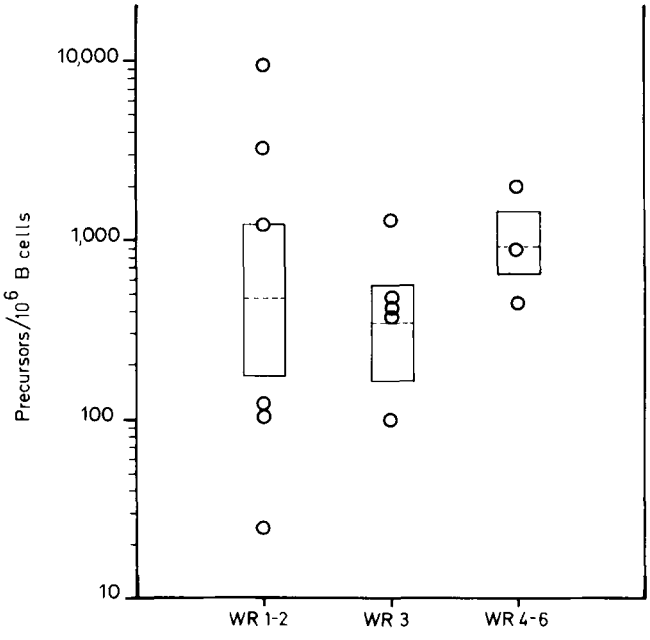


*Figure 1.* Precursor frequencies of B cells spontaneously producing anti-HIV-1 Ab in HIV-1-infected patients with different disease progression. Multiple replicate microcultures with $2 \times 10^5$ irradiated (3000 rad) autologous T cells and graded numbers of B cells were cultured in the absence of mitogenic stimulation, and after 14 days the culture SN were tested for anti-HIV-1 Ab by RIA. The fraction of cultures negative for HIV-1-specific Ab production were plotted against the number of added B cells, and the precursor frequencies were calculated as described in *Materials and Methods*. *Open symbols* denote individual patients; *dotted lines* denote the geometric means of the frequencies obtained within each group, and the *rectangles* denote the limits mean ± 1 SEM.

seropositive patients (1168 ng/ml ± 362 SEM, range 160 to 4000) compared to controls (72.0 ng/ml ± 16.0 SEM, range 25 to 170; $p = 0.002$). Incubation of unstimulated SN from three different HIV-1-infected individuals with HIV-1 Seph completely removed anti-HIV-1 activity (Fig. 3), whereas total Ig content was reduced from 400 to 305 ng/ml in experiment 1, from 480 to 320 in experiment 2, and from 180 to 125 in experiment 3; these figures imply that anti-HIV-1 Ab accounted for about 24, 33, and 31%, respectively, of the Ig spontaneously released in culture (Fig. 3). Similar studies could not be performed on patient 9 (Table IV), who had approximately equal numbers of spontaneously activated anti-HIV-1- and Ig-producing precursors, as he refused to participate further in this study.

## DISCUSSION

Polyclonal activation of any degree is invariably associated with Ag-specific responses; indeed, in the case of HIV-1 infection, the virus itself seems to trigger a polyclonal activation of B lymphocytes (32, 33). However, these results were obtained in vitro using far higher concentrations of stimulating HIV-1 Ag than those putatively involved in the in vivo phenomenon. Our findings indicate that HIV-1-specific Ab synthesis is a relatively major event in the overall B cell activation observed in PBL from HIV-1-infected subjects; several lines of evidence support this conclusion.

To discern whether spontaneous in vitro anti-HIV-1 Ab production was accompanied by synthesis of antibodies directed against recall Ag, we found that, as far as HBV and EBV are concerned, reactivation of memory B cells is not a consistent finding in PBL from the HIV-1-infected patients studied. In fact, even though spontaneous synthesis of EBV-specific Ab was observed in the group of HIV-1-infected patients, it occurred to almost the same degree in the group of seronegative controls; moreover, spontaneous production of anti-HBc Ab was not detected in any of the HIV-1-infected subjects tested. Although we cannot exclude the activation of a few B cell clones directed against recall Ag, undetectable in bulk culture SN testing, might have taken place in these individuals, our conclusions are in line with previously reported data; using influenza virus as a recall antigen, Yarchoan et al. (17) found that the frequency of circulating B cell precursors spontaneously producing anti-influenza Ab was much lower than that of B cell precursors spontaneously secreting anti-HIV-1 Ab. Therefore, if reactivation of memory B lymphocytes occurs, on the basis of these (17) and our own data it seems a negligible event compared to HIV-1-specific B cell activation.

Our data confirm previous reports that PBL from HIV-1-infected subjects show a reduced capacity to produce antibodies following mitogenic stimulation (6, 7, 16). In fact, we found PWM-induced Ab synthesis to both HBc Ag and EBV in a smaller fraction of such subjects compared to HIV-1-seronegative controls. Furthermore, in

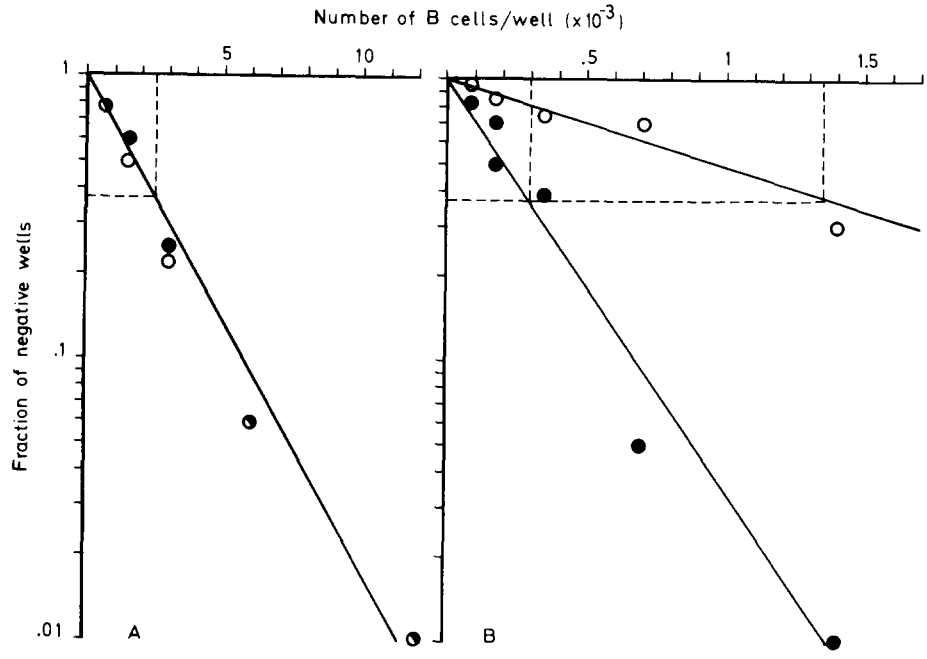


*Figure 2.* Representative frequency determinations of B cell precursors spontaneously producing anti-HIV-1 Ab (*open circles*) and total Ig (*closed circles*) in two HIV-1-infected individuals (*a* and *b*). Graded numbers of B cells were cultured for 14 days in the absence of mitogenic stimulation as described in Figure 1; thereafter, supernatants were assayed at one time for both anti-HIV-1 Ab and total Ig content by RIA. The fraction of wells negative for anti-HIV-1 Ab or Ig production was plotted against the number of added B cells, and the precursor frequency calculated as described. Patient a: HIV-1-specific B cell frequency 1/2400 (c.l. 1890 to 2843); Ig-secreting B cell frequency 1/2440 (c.l. 2011 to 2857). Patient b: HIV-1-specific B cell frequency 1/1340 (c.l. 980 to 1843); Ig-secreting B cell frequency 1/300 (c.l. 244 to 420).

TABLE IV

*Comparative evaluation of the frequencies of activated HIV-1-specific and Ig-secreting B-cell precursors in peripheral blood of HIV-1-infected patients*[a]

| Patient | HIV-1-Specific Precursors | Ig-Secreting Precursors | % of HIV-1-Specific Precursors[b] |
|---|---|---|---|
| 6 | 1/2,170 (460)[c] | 1/580 (1,730) | 26.6 |
| 9 | 1/2,400 (415) | 1/2,440 (410) | 101.0 |
| 10 | 1/1,340 (745) | 1/300 (3,370) | 22.1 |
| 11 | 1/105 (9,700) | 1/33 (30,300) | 32.0 |
| 14 | 1/9,100 (110) | 1/3,920 (255) | 43.1 |

[a] B cells were cultured without mitogenic stimulation in limiting dilution assay as described in Table II. Subsequently, culture SN were tested simultaneously for both HIV-1-specific Ab and total Ig production, and the relative frequencies were calculated as described in *Materials and Methods*.

[b] Calculated as the number of HIV-1-specific precursors/Ig-secreting precursors × 100.

[c] The absolute number of activated B cell precursors/$10^6$ circulating B lymphocytes is given in parentheses.

over 50% of the patients tested, PWM stimulation considerably reduced the amount of secreted anti-HIV-1 Ab compared to unstimulated cultures (data not shown); this observation is consistent with the suppressive effect of PWM on the spontaneous in vitro Ab production seen in recently immunized subjects (34). It is probable that a similar effect, mediated through the suppressive (cytotoxic?) action of large granular lymphocytes (34, 35), is responsible for our finding that specific anti-HBc Ab synthesis in unstimulated cultures was not invariably associated with mitogen-induced anti-HBc Ab production (Table I).

The other experimental approaches used provided more direct evidence for the importance of the HIV-1-specific response within the overall activation of the B cell system. In comparing the frequencies of spontaneously activated HIV-1-specific and Ig-secreting B cell precursors, very close rates were found; in every case, a considerable number of the circulating activated B lymphocytes synthesized anti-HIV-1 Ab. Although these results seem to be very clear-cut, the sensitivity of the two assays could constitute a major drawback to this approach. In fact, we

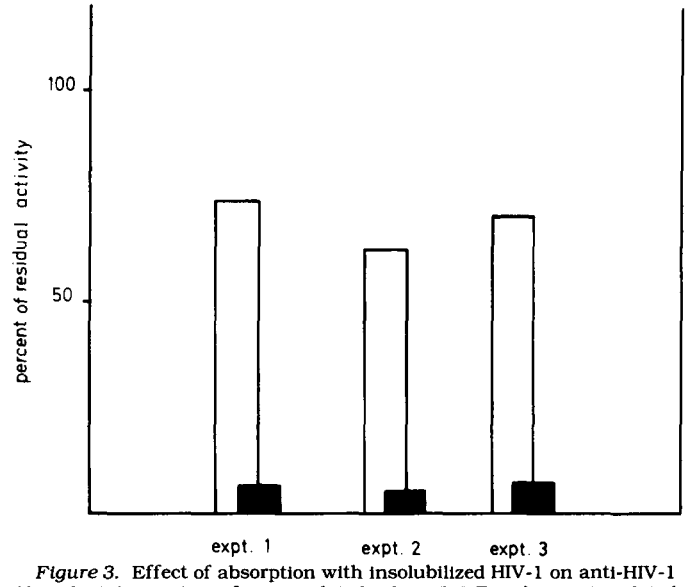


*Figure 3.* Effect of absorption with insolubilized HIV-1 on anti-HIV-1 Ab and total Ig content of unstimulated culture SN. Ten-day unstimulated SN from three different HIV-1-infected patients (not included in the precursor frequency studies) were incubated for 2 h at room temperature with CNBr-Sepharose coupled to HIV-1 and with control Sepharose as described in *Materials and Methods*. Subsequently, SN were recovered and assayed for both anti-HIV-1 Ab and total Ig content by RIA. Results are expressed as percent of residual Ig (*open columns*) and anti-HIV-1 Ab (*closed columns*) in comparison to control supernatants (100%).

have no information on just how much HIV-1-specific Ab we can detect in our assay, so it is conceivable that we overestimated the specific precursor frequency and/or underestimated the Ig-secreting precursors. However, these frequencies fitted the straight line typical of Poisson's distribution; moreover, the sensitivity of our RIA for Ig detection was similar to that reported by others (17, 36), and it seems doubtful that the sensitivity of the anti-HIV-1 Ab assay could be several orders of magnitude greater.

Nonetheless, we believed that these data needed additional support. To this end, we quantified spontaneous HIV-1-specific Ab production in bulk culture SN, and

found that the amount of Ig absorbed from unstimulated culture SN with solid-phase HIV-1 corresponded for the most part to the ratio between the two frequencies obtained in limiting dilution experiments. Hence, it seems reasonable to conclude that virus-specific B lymphocytes constitute a major component of the B cell activation observed in PBL of seropositive patients.

Why the HIV-1-specific B cells should undergo such an intense in vivo activation, and why this phenomenon varies so widely among seropositive individuals, is still a matter for conjecture. The triggering factor conceivably could be the antigenic stimulation by viral proteins; in this regard, it is noteworthy that spontaneous anti-HBc Ab synthesis was found only in the subjects with ongoing hepatitis B who showed serum HBV-DNA, which constitutes the most sensitive index of HBV replication (19). However, we did not find any correlation between HIV-1-specific B cell activation and p24 antigenemia. This discrepancy was not surprising, however, because the serum p24 level is not a completely reliable index of viral replication (37, 38); indeed, the availability of free p24 Ag in serum depends on the amount of circulating anti-p24 Ab, so that low serum p24 levels may simply reflect high titers of anti-p24 Ab and vice versa.

The lack of a correlation between activated B cell precursor frequency and serum anti-HIV-1 Ab or Ig levels is probably due to the relative stability of serum compared to more dynamic cellular events; moreover, our findings concern PBL, which constitute a limited district of the immune system, and contribute only minimally to serum Ab titers. Thus, as reported in many experimental models (39–41), we are probably dealing with a relatively compartmentalized immune response; it is worth mentioning that in the same patient dissimilar Western blot recognition patterns of the HIV-1 proteins were observed in SN of peripheral blood, bone marrow, and lymph node cell cultures (A. Amadori, and A. Del Mistro, unpublished observations). Therefore, it seems reasonable to advance that PBL findings, at least in some cases, might simply echo what is happening at a more central level of the lymphoid system.

Inasmuch as few reports address the analysis of B cell precursor frequency for human antiviral responses, comparisons with the figures reported here are not forthcoming; however, our rates of activated specific B cell precursors were similar to those observed for influenza proteins in recently immunized individuals (42), and much higher than those reported for the same Ag in nonintentionally immunized subjects (42, 43). Although recently immunized persons show transiently high numbers of Ag-specific activated B lymphocytes in circulation (42, 44), B cell system activation in HIV-1-seropositive individuals seems a long lasting event after initial virus exposure, as high frequencies of activated B cells were found in patients with limited disease progression. In addition to its possible down-regulating effect on T cell function (11–13), this phenomenon could be involved in events ultimately leading to the increased incidence of B cell lymphomas reported in AIDS patients (14, 15, 45). Oligo- or monoclonal Ig bands have been described in the sera of HIV-1-infected patients (46), but little information is available regarding their specificity (47). Indeed, in two cases of HTLV-I-associated B cell malignancies, Mann et al. (48) showed that the neoplastic B cells were specific for HTLV-I determinants. Lymphomagenesis is most likely a multistep process, where an expansion of activated B cell clones heightens the probability of chromosomal alterations which in turn lead to the acquisition of the neoplastic phenotype (49); the intensity of the activation of HIV-1-specific B lymphocytes certainly indicates that this population is the most likely target for malignant transformation.

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REFERENCES

1. **Popovic, M., M. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science 224:497.*
2. **Zagury, D., J. Bernard, R. Leonard, R. Chenier, M. Feldman, P. S. Sarin, and R. C. Gallo.** 1986. Long-term culture of HTLV-III-infected T cells: a model of cytopathology of T-cell depletion in AIDS. *Science 231:850.*
3. **Rook, A. H., H. C. Lane, T. Folks, S. McCoy, H. Atler, and A. S. Fauci.** 1987. Sera from HTLV-III/LAV antibody-positive individuals mediate antibody-dependent cellular cytotoxicity against HTLV-III/LAV infected T cells. *J. Immunol. 138:1064.*
4. **Lanzavecchia, A., E. Roosnek, T. Gregory, P. Berman, and S. Abrignani.** 1988. T cells can present antigens such as HIV gp120 targeted to their own surface molecules. *Nature 334:530.*
5. **Leonard, R., D. Zagury, I. Desportes, J. Bernard, J.-F. Zagury, and R. C. Gallo.** 1988. Cytopathic effect of human immunodeficiency virus in T4 cells is linked to the last stage of virus infection. *Proc. Natl. Acad. Sci. USA 85:3570.*
6. **Lane, H. C., H. Masur, L. C. Edgar, G. Whalen, A. H. Rook, and A. S. Fauci.** 1983. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med. 309:453.*
7. **Birx, D. L., R. R. Redfield, and G. Tosato.** 1986. Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. *N. Engl. J. Med. 314:874.*
8. **Mizuma, H., S. Litwin, and S. Zolla-Pazner.** 1988. B-cell activation of HIV infection: relationship of spontaneous immunoglobulin secretion to various immunological parameters. *Clin. Exp. Immunol. 71:410.*
9. **Mizuma, H., S. Zolla-Pazner, S. Litwin, W. El-Sadr, M. Marmor, B. Zehr, S. Weiss, and C. Saxinger.** 1987. Serum IgD elevation is an early marker of B cell activation during infection with the human immunodeficiency viruses. *Clin. Exp. Immunol. 68:5.*
10. **Zolla-Pazner, S.** 1984. B cells in the pathogenesis of AIDS. *Immunol. Today 5:289.*
11. **L'Age-Stehr, J., H. Teichmann, R. K. Gershon, and H. Cantor.** 1980. Stimulation of regulatory T cell circuits by immunoglobulin-dependent structures on activated B cells. *Eur. J. Immunol. 10:21.*
12. **Tosato, G., I. McGrath, I. Koski, N. Dooley, and M. Blaese.** 1979. Activation of suppressor T cells during Epstein-Barr virus-induced infectious mononucleosis. *N. Engl. J. Med. 301:1133.*
13. **Haynes, B. F., R. T. Schooley, C. R. Pawling-Wright, J. E. Grouse, R. Dolin, and A. S. Fauci.** 1978. Emergence of suppressor cells of immunoglobulin synthesis during acute Epstein-Barr virus-induced infectious mononucleosis. *J. Immunol. 123:2095.*
14. **Ziegler, J. L., J. A. Beckstead, P. A. Volberding, D. I. Abrams, A. M. Levine, R. J. Lukes, P. S. Gill, R. L. Burkes, P. R. Meyer, C. E. Metroka, J. Mouradian, A. Moore, S. A. Riggs, J. J. Butler, F. C. Cabanillas, E. Hersh, G. R. Newell, L. J. Laubenstein, D. Knowles, C. Odajnyk, B. Raphael, B. Koziner, C. Urmacher, and B. D. Clarkson.** 1984. Non-Hodgkin's lymphoma in 90 homosexual men. Relation to generalized lymphadenopathy and the acquired immunodeficiency syndrome. *N. Engl. J. Med. 311:565.*
15. Centers for Disease Control. Revision of the case definition of acquired immunodeficiency syndrome for national reporting-United States. 1985. *Mortality and Morbidity Weekly Report 34:373.*
16. **Amadori, A., A. De Rossi, G. P. Faulkner-Valle, and L. Chieco-Bianchi.** 1988. Spontaneous in vitro production of virus-specific antibody by lymphocytes from HIV-infected subjects. *Clin. Immunol. Immunopathol. 46:342.*
17. **Yarchoan, R., R. R. Redfield, and S. Broder.** 1986. 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. *J. Clin. Invest. 78:439.*

18. **Redfield, R. R., D. C. Wright, and E. C. Tramont.** 1986. The Walter Reed staging classification for HTLV-III/LAV infection. *N. Engl. J. Med. 314:131.*
19. **Alberti, A., P. Pontisso, G. Fattovich, E. Schiavon, L. Chemello, F. Bortolotti, F. Tremolada, and G. Realdi.** 1986. Changes in serum hepatitis B virus (HBV) DNA positivity in chronic HBV infection: results of a long-term follow-up study of 138 patients. *J. Infect. Dis. 154:562.*
20. **De Rossi, A., O. Dalla Gassa, A. Del Mistro, G. Faulkner-Valle, and L. Chieco-Bianchi.** 1986. HTLV-III and HTLV-I infection in populations at risk in the Veneto region of Italy. *Eur. J. Cancer. Clin. Oncol. 22:411.*
21. **Amadori, A., A. De Rossi, C. Giaquinto, G. Faulkner-Valle, F. Zacchello, and L. Chieco-Bianchi.** 1988. In vitro production of HIV-specific antibody in children at risk of AIDS. *Lancet 1:852.*
22. **Yarchoan, R., G. Tosato, R. M. Blaese, R. M. Simon, and D. L. Nelson.** 1983. Limiting dilution analysis of Epstein-Barr virus-induced immunoglobulin production by human B cells. *J. Exp. Med. 157:1.*
23. **Amadori, A., A. Del Mistro, A. De Rossi, D. Collavo, and L. Chieco-Bianchi.** 1986. Effect of HTLV-1 infection on the function of alloreactive T cell clones. *Immunol. Clin. Sper. 5:95.*
24. **Taswell, C.** 1981. Limiting dilution assay for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol. 126:1614.*
25. **Amadori, A., G. Faulkner-Valle, A. De Rossi, P. Zanovello, D. Collavo, and L. Chieco-Bianchi.** 1988. HIV-mediated immunodepression. In vitro inhibition of T-lymphocyte proliferative response by ultraviolet-inactivated virus. *Clin. Immunol. Immunopathol. 46:37.*
26. **Casini, A., A. Alberti, E. Banchetti, E. Schiavon, V. Patussi, P. Pontisso, C. Surrenti, and G. Realdi.** 1987. Cell-mediated immunity to HBc Ag in chronic HBV infection. *Am. J. Clin. Pathol. 88:494.*
27. **Mondelli, M., R. S. Tedder, B. Ferns, P. Pontisso, G. Realdi, and A. Alberti.** 1986. Differential distribution of hepatitis B core and E antigens in hepatocytes: analysis by monoclonal antibodies. *Hepatology 6:199.*
28. **Nemerow, G. R., and N. R. Cooper.** 1981. Isolation of Epstein-Barr virus and studies of its neutralization by human IgG and complement. *J. Immunol. 127:272.*
29. **Nemerow, G. R., F. C. Jensen, and N. R. Cooper.** 1982. Neutralization of Epstein-Barr virus by non-immune human serum. Role of cross-reacting antibody to herpes simplex virus and complement. *J. Clin. Invest. 70:1081.*
30. **Colombatti, M., V. G. Johnson, H. A. Skopicki, I. Fendley, M. S. Lewis, and R. J. Youle.** 1987. Identification and characterization of a monoclonal antibody recognizing a galactose-binding domain of the toxin ricin. *J. Immunol. 138:3339.*
31. **Hanson, R. G., G. M. Dusheiko, and J. H. Hoofnagle.** 1984. In vitro synthesis of antibody to hepatitis B virus antigens. In *Advances in Hepatitis Research.* F. V. Chisari, ed. Mason Publishing, Stoneham, MA, p. 123.
32. **Schnittman, S. M., H. C. Lane, S. E. Higgins, T. Folks, and A. S. Fauci.** 1986. Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency syndrome virus. *Science 233:1084.*
33. **Pahwa, S., R. Pahwa, R. A. Good, R. C. Gallo, and C. Saxinger.** 1986. Stimulatory and inhibitory influences of human immunodeficiency virus on normal B lymphocytes. *Proc. Natl. Acad. Sci. USA 83:9124.*
34. **Brieva, J. A., S. Targan, and R. H. Stevens.** 1984. NK and T cell subsets regulate antibody production by human *in vivo* antigen-induced lymphoblastoid B cells. *J. Immunol. 132:611.*
35. **Arai, S., H. Yamamoto, K. Itoh, and K. Kumagai.** 1983. Suppressive effect of human natural killer cells on pokeweed mitogen-induced B cell differentiation. *J. Immunol. 131:651.*
36. **Meuer, S. C., D. A. Cooper, J. C. Hodgson, R. E. Hussey, C. Morimoto, S. F. Schlossman, and E. L. Reinherz.** 1983. Immunoregulatory human T lymphocytes triggered as consequence of viral infection: clonal analysis of helper, suppressor inducer, and suppressor effector cell populations. *J. Immunol. 131:1167.*
37. **Lange, J. M. A., D. A. Paul, H. G. Huisman, F. DeWolf, H. Van der Berg, R. A. Coutinho, S. A. Danner, J. Van der Nordaa, and J. Goudsmit.** 1986. Persistent HIV antigenaemia and decline of HIV core antibodies associated with transition to AIDS. *Br. Med. J. 293:1459.*
38. **Tsiquaya, K. N., M. Youle, and A. C. Chanas.** 1988. Restriction of sensitivity of HIV-1-antigen ELISA by serum anti-core antibodies. *AIDS 2:41.*
39. **Lairmore, M. D., S. T. Butera, G. N. Callahan, and J. C. DeMartini.** 1988. Spontaneous interferon production by pulmonary leucocytes is associated with lentivirus-induced lymphoid interstitial pneumonia. *J. Immunol. 140:779.*
40. **Goud, S. N., N. Muthusamy, and B. Subbarao.** 1988. Differential responses of B cells from the spleen and lymph node to TNP-Ficoll. *J. Immunol. 140:2925.*
41. **Rothstein, T. L., and D. L. Kolber.** 1988. Peritoneal B cells respond to phorbol esters in the absence of co-mitogen. *J. Immunol. 140:2880.*
42. **Munoz, J. L., and R. A. Insel.** 1987. In vitro human antibody production to the *Haemophilus influenzae* type b capsular polysaccharide. *J. Immunol. 139:2026.*
43. **Yarchoan, R., and D. L. Nelson.** 1984. Specificity of in vitro anti-influenza virus antibody production by human lymphocytes: analysis of original antigenic sin by limiting dilution cultures. *J. Immunol. 132:928.*
44. **Kehrl, J. H., and A. S. Fauci.** 1983. Identification, purification and characterization of antigen-activated and antigen-specific human B lymphocytes. *J. Exp. Med. 157:1692.*
45. **Pelicci, P. G., D. M. Knowles, Z. A. Arlin, R. Wieczoreck, P. Luciw, D. Dina, C. Basilico, and R. Dalla Favera.** 1986. Multiple monoclonal B cell expansions and c-myc oncogene rearrangements in acquired immune deficiency syndrome-related lymphoproliferative disorders. *J. Exp. Med. 164:2049.*
46. **Grimaldi, L. M. E., R. P. Roos, S. G. Devare, W. G. Robey, J. M. Casey, M. E. Gurney, B. R. Apatoff, and A. Lazzerin.** 1988. Restricted heterogeneity of antibody to gp 120 and p24 in AIDS. *J. Immunol. 141:114.*
47. **Papadopoulos, N. M., H. C. Lane, R. Costello, H. M. Moutsopoulos, H. Masur, E. P. Gelmann, and A. S. Fauci.** 1985. Oligoclonal immunoglobulins in patients with the acquired immunodeficiency syndrome. *Clin. Immunol. Immunopathol. 35:43.*
48. **Mann, D. L., P. DeSantis, G. Mark, A. Pfeifer, M. Newman, N. Gibbs, M. Popovic, M. G. Sarngadharan, R. C. Gallo, J. Clark, and W. Blattner.** 1987. HTLV-1-associated B-cell CLL: indirect role for retrovirus in leukemogenesis. *Science 236:1103.*
49. **Lenoir, G. M., and G. W. Bornkamm.** 1987. Burkitt's lymphoma, a human cancer model for the study of the multistep development of cancer: proposal for a new scenario. *Adv. Viral Oncol. 7:173.*