

T Lymphocyte Colony Assay in Hemophiliacs

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Unexplained lymphadenopathy, with or without accompanying symptoms, known as the "lymphadenopathy syndrome," has been recognized in groups at risk for acquired immune deficiency syndrome (AIDS), namely, homosexuals and hemophiliacs. To date, however, no test has been defined that discriminates between asymptomatic individuals and those with adenopathy in these high-risk groups. The T colony assay, which measures T lymphocyte growth in soft agar and which allows selective T cell proliferation with minimal cell-cell contact, was evaluated in asymptomatic hemophiliacs. Significantly lower mean colony counts were found in eight hemophiliacs with adenopathy

(HA), 763 ± 348 (\pm SEM), than in 16 healthy hemophiliacs (HH) $3,044 \pm 661$ ($P < .005$), or than in 24 heterosexual control subjects, $3,964 \pm 395$ ($P < .005$). The in vitro addition of exogenous interleukin-2 (IL-2) restored normal colony growth in the HA population. These results indicate that the T colony assay can detect abnormal cell-mediated immunity among hemophiliacs and specifically discriminates between asymptomatic hemophiliacs (HH) and those with adenopathy (HA). In addition, IL-2 may be of potential benefit in improving T cell defects in AIDS or the "lymphadenopathy syndrome"; however, this remains to be proven.

THE HEMOPHILIAC POPULATION is considered to be one of the high-risk groups for developing the acquired immune deficiency syndrome (AIDS). However, hemophiliacs constitute only a small fraction (<1%) of the total number of those identified as having this syndrome. By contrast, AIDS has become a leading cause of death in hemophiliacs.¹

To date, the etiology is unknown, but mounting circumstantial evidence suggests that it is transmitted by a blood-borne infectious agent, probably a virus. The one common feature of all patients with this syndrome is defective cell-mediated (T cell) immunity. However, it has been difficult to define a specific laboratory test that will consistently identify those at risk for the syndrome. The most commonly used measure, the T lymphocyte helper/suppressor cell (H/S) ratio, is strikingly reduced in most patients with AIDS or the lymphadenopathy syndrome, which may be a prodrome of AIDS. In addition, it may also be abnormal in apparently healthy homosexuals or hemophiliacs. Thus, its discriminating value is limited.²⁻⁸

Previous studies from our laboratory suggest that the T colony assay is a highly sensitive test, capable of detecting abnormalities not apparent by other measures of cellular immunity.^{9,10} We have used this assay to evaluate T cell function in both asymptomatic healthy hemophiliacs (HH) and in hemophiliacs with chronic unexplained adenopathy (HA). Although the significance of the clinical abnormalities in this latter group are unknown, it may be the equivalent of the "prodromal" stage seen in the homosexual population. An unknown fraction of homosexual patients with this prodrome ultimately develop either opportunistic infections or Kaposi's sarcoma.

Data presented in this study indicate that the T cell colony assay is a more discriminating test for abnormal cell-mediated immunity among hemophiliacs than either proliferative responses to nonspecific mitogens

in suspension culture or the helper/suppressor ratio. Furthermore, in vitro abnormalities in colony growth in the HA population can be corrected by the addition of exogenous interleukin 2 (IL-2).

MATERIALS AND METHODS

Laboratory Data

Heparinized venous blood samples were obtained from 24 healthy heterosexual control subjects and from the 24 hemophiliac patients after written, informed consent. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and aspirated and washed twice in Hanks' balanced salt solution (HBSS). One aliquot of these cells was used in the T colony assay, and the other fraction was used in suspension cultures (see below).

Colony assay. This was performed by techniques previously described.^{9,10} Mononuclear cells were suspended in RPMI 1640 media containing HEPES buffer, glutamine (300 μ g/mL), penicillin (100 U/mL), streptomycin (100 μ g/mL), amphotericin B (0.25 μ g/mL), gentamicin (100 μ g/mL), and 10% pooled human AB serum. Cultures were prepared in 35-mm gridded plastic petri dishes: each plate contained a 1-mL base of 0.5% agar in RPMI 1640 medium, overlaid with 1 mL of 0.33% agar in culture media, containing 7.5×10^5 cells. For each plate, 5 μ L of stock phytohemagglutinin (PHA; Wellcome Research Laboratories, Research Triangle Park, NC) was added, and the plates were incubated for 7 days in a 5% CO₂-37°C humidified atmosphere. Previous studies have shown that this quantity consistently produces optimal colony growth.⁹ Colonies were enumerated with an inverted microscope, using multiple gridded squares for each plate, and correcting for the total area of the plate. Tight clusters containing at least 25 cells were scored as colonies. Interleukin-2 (IL-2) (Electronucleonics Laboratories, Silver Springs, Md) was prepared from PHA-stimulated lymphocytes, after which the PHA was removed by chromatogra-

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phy. This IL-2, of undetermined purity, was added to the T colony plates in a volume of 1.050 mL, making a final concentration of 5%/plate, then incubated for 7 days in a 5% CO₂-37°C humidified atmosphere, and colonies were again enumerated. Colony scores were taken as the average of 2 or 3 replicate plates.

Suspension cultures. Suspension cultures were performed with PHA, concanavalin A (Con-A), or pokeweed mitogen (PWM) in a microculture system, as previously described.^{9,13} In brief, mononuclear cells were incubated in RPMI 1640 medium, similar to that described above, except that it contained 5% fetal calf serum instead of human serum. This substitution, in our experience, does not significantly alter responses. Aliquots of 0.2 mL were added to round-bottom wells of tissue culture plates: in the PHA-stimulated cultures, 10 μ L of a 1:5, 1:10, or 1:25 dilution was added per plate; in the Con-A-stimulated culture, 100, 50, or 25 μ g/mL was added per plate (Sigma Chemical, St Louis); in the PWM-stimulated cultures, 10 μ L of a 1:4 stock solution (GIBCO, Grand Island, NY) was added per plate. For each assay, triplicate determinations were performed. Plates were incubated for 3 days in a 5% CO₂-37°C humidified atmosphere. Eighteen hours prior to harvest, 0.25 μ Ci of tritiated thymidine (³HTdR; New England Nuclear Corp, Boston), with a specific activity of 6.7 Ci/mmol/L, was added to each well. Cultures were harvested on filter paper with a semiautomatic cell harvester and allowed to dry overnight. To each vial was added 5 mL of Unogel scintillation solution (Becton Dickinson, Rutherford, NJ), and samples were counted in a Packard tri-carb scintillation counter. Responses were determined by subtracting the average activity in unstimulated cultures from those stimulated with mitogens.

Helper/suppressor ratios. Enumeration of T lymphocytes, including T helper and T suppressor cells, was performed, as previously described,^{7,14} by flow cytometry using the FACS IV instrument and monoclonal antibodies Leu-4 (total T), Leu-3a + b (T helper), and Leu-2a (T suppressor) (Becton Dickinson, Sunnyvale, Calif). Twenty healthy heterosexual individuals, different from those used in the above assays, served as control subjects.

Statistical analysis. Statistical analysis was performed by the two-tailed Student's *t* test, comparing mean colony counts of each hemophiliac group, HA and HH, with control subjects and with each other; similarly, mean H/S ratios of each group were compared with control subjects and with each other. Linear regression analysis was used to determine the correlation coefficient between helper/suppressor (H/S) ratios and T colony counts, and between H/S ratios and colony counts of PWM-suspension cultures.

Clinical Data

A total of 24 patients who are cared for by the Hemophilia Center of Western Pennsylvania were studied. Eight of the 24 hemophiliacs had adenopathy (HA), and all had hemophilia A treated with FVIII concentrate. The other 16 were healthy hemophiliacs with no evidence of adenopathy (HH). Included in this group were nine patients with hemophilia A: five of these were treated with FVIII concentrate, and four with inhibitors to FVIII were treated with FIX concentrate. The seven remaining HH patients had hemophilia B: four were treated with FIX concentrate and three were treated with fresh frozen plasma. The overall age range was 12-57 years, with an average age in the HA group of 23 years and in the HH group of 36 years.

All 24 hemophiliacs were clinically well, none having experienced fever, sweats, or weight loss. None of the patients had a history of intravenous drug use or bisexual or homosexual activity. Lymphadenopathy was defined as unexplained lymph node enlargement in at least two noncontiguous, extralingual lymph node chains for greater than 3 months. Five of the HA group also had unexplained splenomegaly: one additional patient with unexplained splenomegaly

but no detectable adenopathy was also included in the HA group. Lymph node biopsies performed on the first two documented HA patients, previously reported,¹¹ revealed benign nonspecific hyperplasia. Lymphoid cells from the lymph node of one of these patients showed vesicular rosettes by electron microscopy.¹⁵ Cultures of both lymph nodes for aerobic and anaerobic bacteria and cytomegalovirus (CMV) were negative, as were smears for *Mycobacterium*, both typical and atypical. Both patients were anergic to Candida, histoplasmosis, mumps, and purified protein derivative (PPD) skin test antigens. Three HA patients tested were "monospot" negative, and all eight HA patients were IgG-CMV seronegative. Because of the large dose of FVIII concentrate required, and because they were asymptomatic, lymph node biopsies were not performed on the remaining six HA patients.

Clotting studies revealed that FVIII levels were <0.01 U/mL (severe) in all 12 patients with hemophilia A. The hemophilia B patients included four with FIX levels <0.01 U/mL (severe), two with levels of 0.02 U/mL (moderate), and one with a level of 0.07 U/mL (mild). Anti-VIII was detected in four hemophilia A patients: the levels ranged from 0.5 to 4,100.0 Bethesda units. Chronic anti-HBs was found in 22/24 patients, two of whom had been immunized with Heptavax. The remaining two patients were persistently HBsAg positive and anti-HBs negative. Nineteen of the 24 had chronic anti-HBc. One hemophiliac in the HH group, who has been previously reported,⁷ also has thrombocytopenia with a platelet count of 60,000 cells/mL and a platelet-associated IgG of 21.9 ng/10⁶ platelets (normal <8.0 ng/10⁶ platelets), as determined by solid-phase radioimmunoassay.¹² Finally, one hemophiliac in the HH group has received chronic corticosteroid therapy in the form of prednisone, 10 mg qod, for chronic active liver disease, presumably secondary to transfusion hepatitis.

RESULTS

T lymphocyte colonies readily formed after plating 7.5×10^5 Ficoll-Hypaque-isolated mononuclear cells in agar. As can be seen in Table 1, the mean colony count in controls was $3,964 \pm 395$ (SEM). Of note, none of these had a value <1,000 colonies/plate, a value below which was arbitrarily defined as abnormal from previous work in this laboratory. Fourteen (88%) of the 16 hemophiliacs in the HH group had colony counts >1,000; one of the two other HH patients showed no growth. The mean value in the HH group was $3,044 \pm 661$ /plate, a value not significantly different from control subjects. By contrast, only five of the eight (62.5%) hemophiliacs in the HA group grew colonies. The mean value of all HA patients was 763 ± 348 /plate; this was significantly lower than either control subjects ($P < .005$) or the HH group ($P < .005$). Analysis of individual colony scores indicated that only two HA patients had counts >1,000 (1,800 and 2,900, respectively) (Fig 1).

Suspension cultures stimulated by either PHA or Con-A revealed no significant difference between the HA and HH groups. By contrast, there were reduced responses in the HA group to PWM when compared with control subjects ($P < .005$) or healthy hemophiliacs (HH) ($P < .005$) (Table 1). Analysis of individual PWM responses revealed that five HA patients had

Table 1. T Lymphocyte Colony Growth: T Colony Assays* and Suspension Cultures† With PHA, Con-A, and PWM

	Control Subjects (24)	HA (8)	HH (16)
T colony assay	3,964 ± 395	763 ± 348‡§	3,044 ± 661
T colony assay after interleukin-2	7,383 ± 624	6,571 ± 1,500¶	11,693 ± 1,406§
Suspension cultures			
With PHA	18,518 ± 3,590 (9)	23,427 ± 2,690 (7)	17,391 ± 2,359 (14)
With Con-A	12,977 ± 3,077 (9)	4,451 ± 2,080 (7)¶	7,650 ± 1,243 (14)¶
With PWM	12,139 ± 1,742 (9)	3,137 ± 835 (7)‡§	9,521 ± 1,311 (14)

*Results are expressed as the mean ± SE of the mean number of colonies per 7.5×10^5 cells plated. When fewer patients than the number indicated for each group were studied, the number is included in parentheses.

†Results are expressed as mean ± SE of the mean $^3\text{H}\text{TdR}$ uptake in cpm.

‡ $P < .005$, as compared with control, by Student's t test.

§ $P < .005$, as compared with HH group, by Student's t test.

¶ $P < .05$, as compared with control, by Student's t test.

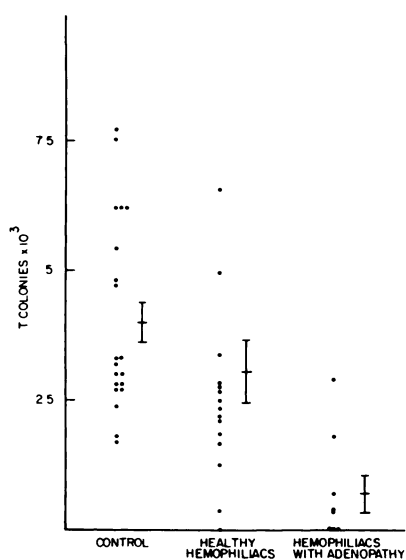


Fig 1. Individual T colony counts by the T colony assay in control subjects, healthy hemophiliacs (HHs), and hemophiliacs with adenopathy (HAs). The bars beside the data points represent the mean ± SEM for each group.

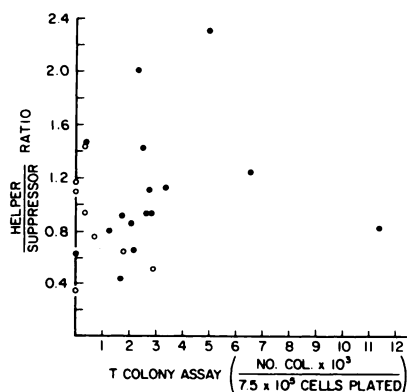


Fig 2. Scattergram showing the relationship between H/S ratio and colony count by the T colony assay. Open circles represent HA; solid circles are HH. The two measures of cellular immunity showed minimal correlation, with a correlation coefficient, $r = .159$.

>2,000 cpm, a value below which was arbitrarily defined as abnormal from previous work in this laboratory. Using the criteria for an abnormal T colony assay and an abnormal PWM culture, it appears that the T colony assay is more sensitive than PWM in detecting adenopathy. However, both tests are predictive.

All patients in both groups were evaluated for the ratio of T helper/suppressor cells. The mean helper/suppressor (H/S) ratio of the HA group, 0.87 ± 0.12 , was not statistically different from that of the HH group, 1.11 ± 0.12 , although both were significantly less than control subjects, 2.22 ± 0.16 ($P < .01$). The H/S ratio showed little correlation with the T colony scores, with a coefficient of correlation between these two variables, $r = .159$ (Fig 2). Similarly, there was poor correlation between the responses to PWM and the H/S ratio, $r = .165$ (Fig 3). The absolute number of lymphocytes did not differ significantly between the HA and HH groups, with a mean (\pm SEM) of $1,469 \pm 138/\mu\text{L}$ and $1,308 \pm 109/\mu\text{L}$, respectively; neither was the T helper subset nor the T suppressor subset able to discriminate between HA and HH, with mean T

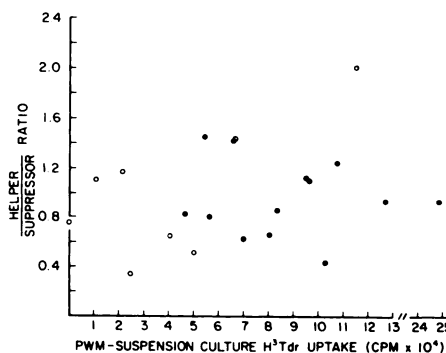


Fig 3. Scattergram showing the relationship between H/S ratio and PWM suspension culture $^3\text{H}\text{TdR}$ uptake in cpm. Open circles represent HA; solid circles are HH. The two immunologic parameters showed minimal correlation, $r = .165$.

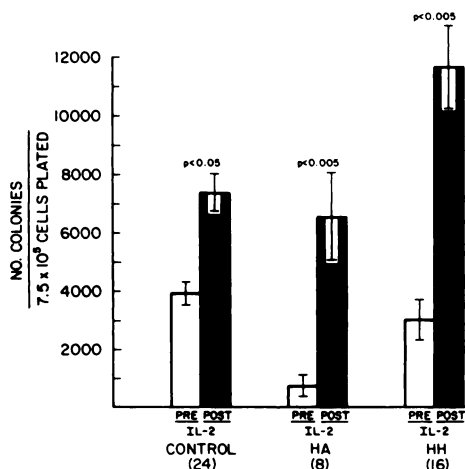


Fig 4. Response of T colony growth to the addition of 5% interleukin-2 (IL-2) in control subjects and two hemophiliac groups, HA and HH. Results are expressed as the mean \pm SEM number of colonies per 7.5×10^5 cells plated.

helper cells $415 \pm 44/\mu\text{L}$ v $377 \pm 33/\mu\text{L}$, and mean T suppressor cells, $556 \pm 107/\mu\text{L}$ v $416 \pm 70/\mu\text{L}$.

To determine the basis for the reduced growth seen in the HA group in the T colony assay, the effects of addition of exogenous IL-2 were examined (Fig 4). In the control group, colony counts rose 1.9-fold to $7,383 \pm 624$ ($P < .005$). Similarly, counts in the HH group increased 3.8-fold to $11,693 \pm 1,406$ ($P < .005$). Among the HA patients, there was a striking 8.6-fold increase from 763 ± 348 to $6,571 \pm 1,500$ ($P < .05$), a value not significantly different from that measured in the normal control subjects.

DISCUSSION

These results indicate that the T cell colony assay is a highly discriminating test for detecting cell-mediated defects in hemophiliac patients with chronic lymphadenopathy. Because patients with this bleeding disorder are considered to be one of the susceptible populations for AIDS, the chronic unexplained adenopathy in this group may be the equivalent of the prodromal phase described in homosexuals. To date, however, the ultimate risk of AIDS in the prodrome group has not been determined in either homosexuals or hemophiliacs.

Our previous studies have shown that the colony

assay may be able to detect cellular defects not apparent in either routine suspension culture assays or the T helper/suppressor cell ratio.^{9,10} This may be explained by several differences between the response in agar media and in suspension cultures. In the colony assay, it appears that growth is limited to a subpopulation of T cells, in particular, the T helper cell subset.^{16,17} By contrast, in suspension cultures, both T and B cells are capable of responding. Second, in the colony assay, the cells are immobilized in semisolid media; this serves to minimize cell-cell contact. Such contact is not restricted in suspension systems, and it is possible that direct interactions can obscure certain intrinsic cellular defects. Third, responses in suspension culture are measured by the incorporation of radiolabeled thymidine, a compound that is incorporated by any cell in division. By contrast, formation of a T cell colony requires that the stimulated cell undergo four to seven successive mitotic divisions.

The restoration of normal T cell colony growth in the T colony assay by the addition of interleukin-2 (IL-2) in hemophiliacs with adenopathy (HA group) is consistent with previous findings by other investigators.¹⁸⁻²¹ These data suggest that one of the basic abnormalities in AIDS or the lymphadenopathy syndrome is a deficiency in this interleukin, perhaps due to a lack of the appropriate helper-cell population. Whether the in vitro data has relevance to potential in vivo therapy for these patients remains to be determined.

Suspension cultures stimulated with PWM also discriminated between the HA and the HH groups. However, it appeared to be a less sensitive assay than the colony assay. PWM is considered to be a mitogen for both T and B cells; it is possible that the reduced reaction to this mitogen in the HA group may indicate dysfunction in B lymphocytes. Similar findings have been reported in homosexuals with AIDS.²² However, it is also possible that the abnormality results from a failure of T cell initiation of B cell response in this system.

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REFERENCES

- Centers for Disease Control: Personal communication
- Kornfeld H, Stouwe RAV, Lange M, Reddy MM, Grieco MH: T-lymphocyte subpopulations in homosexual men. *N Engl J Med* 307:729, 1982
- Stahl RE, Friedman-Kien A, Dubin R, Marmor M, Zolla-Pazner S: Immunologic abnormalities in homosexual men: Relationship to Kaposi's sarcoma. *Am J Med* 73:171, 1982
- Wallace JI, Coral FS, Rimm IJ, Lane H, Levine H, Reinherz EL, Schlossman SF, Sonnabend J: T-cell ratios in homosexuals. *Lancet* 1:908, 1982
- Centers for Disease Control: Persistent, generalized lymphadenopathy among homosexual males. *Morbidity Mortality Weekly Rep* 31:249, 1982
- Menitove JE, Aster RH, Casper JI, Lauer SJ, Gottschall JL,

- Williams JE, Gill JC, Wheeler DV: T-lymphocyte subpopulations in patients with classic hemophilia treated with cryoprecipitate and lyophilized concentrates. *N Engl J Med* 308:83, 1983
7. Ragni MV, Bontempo FA, Lewis JH, Spero JA, Rabin BS: An immunologic study of spouses and siblings of asymptomatic hemophiliacs. *Blood* 62:1297, 1983
 8. Ragni MV, Lewis JH, Spero JA, Bontempo FA, Rabin S: Decreased helper/suppressor cell ratios following treatment with blood products: factor VIII and IX concentrates and fresh frozen plasma. *Am J Med* 76:206, 1984
 9. Bernstein ML, Winkelstein A, Dobson SA: Depressed T cell colony growth in systemic lupus erythematosus. *Arthritis Rheum* 23:385, 1980
 10. Winkelstein A: Dependency of human T lymphocyte colony formation on soluble factors produced by accessory or tumor cells. *J Immunol* 130:2715, 1983
 11. Ragni MV, Lewis JH, Spero JA, Bontempo FA: Acquired-immune deficiency-like syndrome in two hemophiliacs. *Lancet* 1:213, 1983
 12. Hymes K, Shulman S, Karpatkin S: A solid-phase radioimmunoassay for bound anti-platelet antibody. *J Lab Clin Med* 94:639, 1979
 13. Winkelstein A, Brizzi JA: The effects of pharmacologic agents on mitogen-induced cellular cytotoxicity in guinea pigs. *J Immunopharmacol* 1:87, 1978-79
 14. Ledbetter JA, Rouse RV, Mecklem HS, Herzenberg LA: T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. *J Exp Med* 152:280, 1980
 15. Ewing EP, Spira TJ, Chandler FW, Callaway CS, Brynes RK, Chan WC, Basinger D: Ultrastructure of AIDS lymph nodes. *N Engl J Med* 309:1190, 1983 (letter)
 16. Claesson MH, Sponderstrup-Hansen G, Brix Poulsen P: Colony formation by subpopulations of human T lymphocytes. *Scand J Immunol* 13:395, 1981
 17. Pistoia V, Ghio R, Canonica GW, Colombatti M, Moretta L: Comparison of the colony-forming capacities of human T-lymphocyte subpopulations. *Clin Immunol Immunopathol* 21:289, 1981
 18. Rook AH, Masur H, Lane HC, Frederick W, Kashara T, Macher AM, Djeu JY, Manischewitz JF, Jackson L, Fauci AS, Quinnan GV: Interleukin 2 enhances the depressed natural killer and cytomegalovirus-specific cytotoxic activities of lymphocytes from patients with the acquired immune deficiency syndrome. *J Clin Invest* 72:348, 1983
 19. Ciobanu N, Kruger G, Welte K, Venuta S, Feldman S, Gold J, Moore MAS, Mertelsmann R: Defective T cell response to PHA and mitogenic monoclonal antibodies in male homosexuals with acquired immunodeficiency syndrome and its in vitro correction by interleukin 2. *Blood* 60:78a, 1982 (Abstr)
 20. Bussel J, Welte C, Fitzgerald P, Hilgartner M, Lopes C, Mertelsmann R: Defective cellular immunity in homosexual hemophiliacs: correlation in vitro with interleukin 2. *Blood* 60:209a, 1982 (Abstr)
 21. Tsuchiya S, Imaizumi M, Minegishi M, Konno T, Tada K: Lack of interleukin-2 production in a patient with OKT₄⁺ T cell deficiency. *N Engl J Med* 308:1294, 1983 (Letter)
 22. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS: Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N Engl J Med* 309:453, 1983