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Polyclonal B-cell activation reveals antibodies against human immunodeficiency virus type 1 (HIV-1) in HIV-1-seronegative individuals

(AIDS/immune response)

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ABSTRACT **Identification of human immunodeficiency virus type 1 (HIV-1)-infected individuals is of paramount importance for the control of the spread of AIDS worldwide. Currently, the vast majority of screening centers throughout the world rely on serological techniques. As such, clinically asymptomatic but HIV-infected, seronegative individuals are rarely identified. In this report we show that 18% (30/165) of seronegative individuals who were considered to be a unique cohort of patients at high risk for HIV infection had circulating B cells that, upon *in vitro* polyclonal activation with pokeweed mitogen, produced antibodies reactive with HIV. Furthermore, polymerase chain reaction analysis of DNA obtained from aliquots of the peripheral blood mononuclear cells from these seronegative but pokeweed mitogen assay-positive individuals tested revealed the presence of HIV-specific sequences in a significant number of samples. In addition, depletion of $CD8^+$ T cells from peripheral blood mononuclear cells of HIV-1-seronegative individuals prior to *in vitro* culture with pokeweed mitogen resulted in increased sensitivity for detecting HIV-reactive antibodies. This assay has obvious epidemiological implications, especially in the case of high-risk groups, and also provides a simple technique to enhance detection of HIV-infected individuals. Of further interest is the determination of the mechanisms related to the lack of HIV-specific antibodies in the serum of these infected individuals.**

Serological detection of antibodies against a variety of infectious disease agents is considered as evidence of exposure and/or active infection. Notably, serological screening techniques are being utilized worldwide for the detection of human immunodeficiency virus type 1 (HIV-1) infection. The current serological techniques, however, do not identify clinically asymptomatic individuals who are infected but lack detectable levels of HIV-1-reactive antibodies. The existence of such HIV-1-infected but seronegative individuals has recently been documented (1) by using the highly sensitive polymerase chain reaction (PCR) technique. A follow-up study indicated that there was considerable variation in the time interval between detection of HIV-1 infection as determined by PCR and seroconversion (2). A similar finding has recently been documented in studies of individuals infected with human T-lymphotropic virus type I (3).

We have been conducting studies on a nonhuman primate model of AIDS. Our studies show that about 75% of the colony of sooty mangabeys (*Cercocebus atys*) at the Yerkes Regional Primate Research Center are seropositive for a simian immunodeficiency virus (SIV). The virus isolated from these sooty mangabeys (termed SIV/SMM) has been shown to share 75% homology at the nucleotide level with HIV-2 and about 50% homology with HIV-1. Further studies on the *seronegative* sooty mangabeys provided two lines of evidence that suggest that, in fact, these *seronegative* mangabeys have either been previously exposed to SIV/SMM or are latently infected with SIV/SMM yet do not have detectable levels of antibodies reactive with SIV/SMM. First of all, $CD8^+$ cells from these *seronegative* mangabeys markedly inhibited the replication of endogenous and exogenously added SIV/SMM in autologous cultures of cells *in vitro* (J.D.P., unpublished results). This regulatory property was previously ascribed to $CD8^+$ cells from only HIV-1-infected or SIV-infected (but not HIV-1- or SIV-uninfected) humans and rhesus macaques. Second, when peripheral blood mononuclear cells (PBMC) from seronegative mangabeys were cultured *in vitro* with a polyclonal B-cell mitogen (pokeweed mitogen, PWM), the supernatant fluid contained significant levels of antibodies that specifically reacted with SIV both by ELISA and Western blot analysis (T.J.-C., unpublished results).

These findings prompted us to conduct similar studies on a cohort of human patients. Our study included 215 patients seen at the Grady Memorial Hospital in Atlanta, whose physicians requested testing for HIV-1 infection, following hospital protocol for counseling and consent. These patients, it must be emphasized, represent a very high-risk population. The results of these studies not only confirm previous data (1, 2, 4) on the occurrence of HIV-1-infected but seronegative individuals but, more importantly, describe a simple reproducible assay that can be used for the detection of occult HIV-1 infection. In addition, the results provide a unique opportunity to perform future longitudinal studies on these seronegative but HIV-1-infected individuals, in efforts to gain insight into the immunological basis of this interesting phenomenon.

MATERIALS AND METHODS

Patient Population. The pathology laboratory at the Grady Memorial Hospital routinely performs HIV-1 antibody testing on blood samples from individuals at the request of the physician, following hospital protocol and counseling. These individuals are considered at high risk for HIV-1 infection, and the seroprevalence rate is currently 24% for all samples tested during 1989, which is double the rate of the previous year. Blood samples from a total of 215 of these high-risk individuals comprise this study. It should be noted that only

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Abbreviations: HIV, human immunodeficiency virus; PCR, polymerase chain reaction; SIV, simian immunodeficiency virus; PBMC, peripheral blood mononuclear cells; PWM, pokeweed mitogen; EIA, enzyme immunoassay.

samples from patients for whom a complete blood count was requested were included in this study since the *in vitro* culture assay required PBMC. These samples represented about 60% of the total submitted for HIV-1 antibody screening; however, the seroprevalence rate was similar (about 30%) for samples for which a complete blood count was or was not requested. All samples were coded prior to receipt by our laboratory. For purposes of controls, for the ELISA test (cutoff values), the Western blot assay, and the PWM assay, blood samples from 15 individuals from the Grady Memorial Hospital who were not considered high-risk and from 20 normal adult healthy laboratory volunteers were used.

ELISA and Western Blot Assay. Each serum sample was screened for anti-HIV-1 antibody with an enzyme immunoassay (EIA) kit (Abbott). The sample was considered positive if the ratio (OD_{405} value/cutoff value for that assay) was ≥ 1.0 (per manufacturer's instructions). Samples considered positive were then subjected to Western blot analysis by using the Biotech/DuPont HIV-1 Western blot assay kit. The presence of three bands against the major HIV-1 proteins was considered evidence for seropositivity. For the purpose of this report, HIV-1-seronegative samples consisted of PBMC from patients whose sera were negative by ELISA and failed to demonstrate any band by Western blot analysis.

***In Vitro* Polyclonal Activation of PBMC with PWM.** Ficoll/Hypaque-purified PBMC from each sample were cultured *in vitro* in medium alone (control) or in medium containing a 1:500 final dilution of PWM (GIBCO) at a concentration of 2×10^6 viable cells per ml. Medium used throughout these studies consisted of RPMI 1640 supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 10% (vol/vol) heat-inactivated (56°C, 30 min) fetal calf serum (all purchased from GIBCO). Cultures were performed in triplicate in sterile test tubes and incubated for 4 days at 37°C in a 7% CO_2 humidified atmosphere. The cultures were then centrifuged, and the supernatant fluid was collected and assayed within 24 hr for HIV-1-reactive antibodies by the same EIA and Western blot kits described above. In contrast to serum samples, which were diluted 1:200 for the ELISA and 1:100 for the Western blot assay, the supernatant fluids from control and PWM-induced cultures were tested undiluted. Total protein and IgG contents were determined in the serum and supernatant fluids, in an effort to address questions regarding differences in the dilutions of each used for the assays. Serum samples contained 8.4 ± 0.3 mg of total IgG per ml (mean ± SD), and the supernatant fluids contained 1.9 ± 0.2 μg of total IgG per ml. The total protein concentration in the serum was 60.8 ± 8.6 mg/ml, and that in the supernatant fluid was 42.3 ± 1.7 μg/ml; thus, even undiluted, the supernatant fluids have lower IgG and protein contents than the diluted serum samples.

Depletion of $CD8^+$ Cells. Whenever enough PBMC were available, they were incubated with a previously determined optimal concentration of anti-OKT8-coated magnetic beads (Collaborative Research). The cell suspension was then exposed to a magnetic field, and the CD8-depleted population was collected, washed, and incubated at 2×10^6 viable cells per ml with medium or a 1:500 final dilution of PWM, as described above. Supernatant fluids from these cultures were similarly analyzed for reactivity against HIV-1 by ELISA and Western blot analysis.

PCR Assay. DNA was extracted from aliquots (when available) of the same PBMC sample that was used for the PWM assay and tested for HIV-1 sequences by using the PCR techniques as described by Ou *et al.* (5). The SK38/39 HIV-1 gag primer pair was used, and the samples were amplified by 35 cycles of denaturation, annealing, and extension. The amplified product was subjected to 1.8% agarose gel electrophoresis, blotted onto nitrocellulose, and hybridized with the ^{32}P-labeled SK19 probe. During the course of these studies, three basic patterns of reactivity emerged, which should be considered in the interpretation of the data. First, the samples were either totally negative or, second, clearly positive. Third, a significant number of samples were much lighter in density of reaction, from both HIV-1-seropositive and PWM assay-positive but seronegative individuals. The data presented in this communication consider samples PCR positive for HIV-1 only when they are clearly positive; the ones with lower degree of reactivity are described in the text. To ensure reproducibility, PCR analysis was performed on aliquots of each DNA sample at least three times.

RESULTS

As seen in Table 1, of the 215 samples studied, serum samples from 50 individuals were found to be HIV-1 positive by ELISA and confirmed by Western blot analysis. Supernatant fluid from PBMC cultured with PWM from 49/50 of these HIV-1-seropositive individuals also reacted with HIV-1 by ELISA, and 48 of these samples were confirmed to be positive by Western blot analysis and shown to react with more than three bands. Of the 165 samples that were HIV-1 seronegative, supernatant fluids from PWM-stimulated cultures of PBMC from 30 individuals showed significant HIV-1-reactive antibody titers. Approximately 50% (16/30) of these supernatant fluids showed distinct bands on the Western blot. Fig. 1 shows the typical staining pattern seen on HIV-1 Western blots of sera and supernatant fluids from cultures of PBMC stimulated with PWM. As illustrated, whereas sera from HIV-1-seropositive individuals show multiple bands (p17, p24, p31, gp41, p51, p55, p66, and gp120/160), sera from seronegative individuals do not demonstrate any reactive bands. Supernatant fluids from the PWM cultures of PBMC from seropositive individuals showed multiple bands, essentially similar to the pattern seen with sera from the same individuals. Supernatant fluids from PWM-stimulated cultures of HIV-1-seronegative individuals who were positive by ELISA, in contrast, showed predominant reactivity against p66 and gp120/160 on Western blots. Supernatant fluids from PBMC cultured with medium alone did not show any reactivity by ELISA or Western blot analysis. In addition, sera and supernatant fluids derived from PBMC cultured with PWM from the 15 individuals from the Grady Memorial Hospital who were not considered at high risk for HIV-1 and from the 20 normal adult laboratory volunteers failed to show any reactivity by either ELISA or Western blot analysis.

We hypothesized that there were at least three likely explanations for these findings. First, it was conceivable that these patients were not HIV-1 infected but may have been immunized against either HIV-1 or HIV-1 proteins (perhaps during the course of i.v. drug abuse), or perhaps there was a trivial explanation, such as cross-reactivity to other non-HIV-1 proteins. Second, it is quite possible that these pa-

Table 1. Detection of anti-HIV-1 antibodies in sera and in supernatant fluids from PBMC cultured with PWM *in vitro*

Assay	HIV^+	HIV^-
Conventional*	50	165
PWM supernatant†		
ELISA‡	49	30
Western blot	48	16

*All of the 215 sera were tested with the Abbott HIV-1 EIA kit and the Biotech/DuPont HIV-1 Western blot assay.

†Unlike the serum, which was diluted 1:200 for EIA and 1:100 for Western blots, the PWM-induced supernatant fluids were tested undiluted.

‡Positivity was calculated as a ratio of ≥ 1.0, per manufacturer's instructions (ratio = OD_{405} value/cut-off value for that assay).

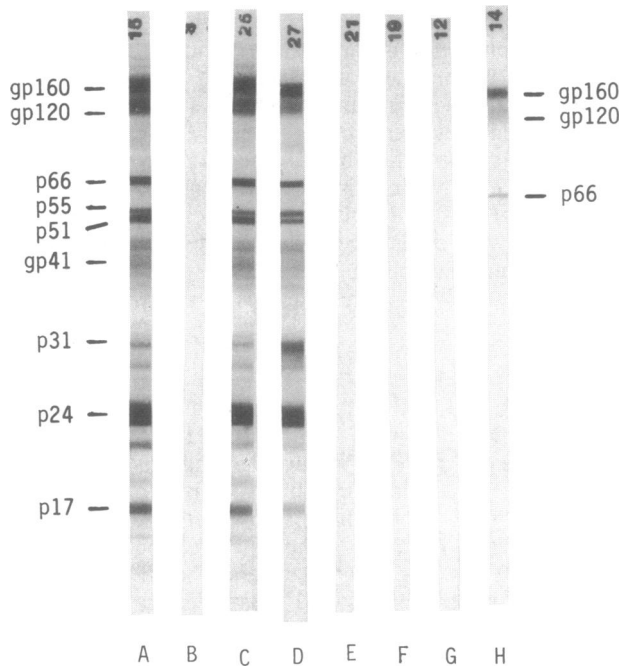

FIG. 1. Typical Western blot profile of sera and supernatant fluid of PWM-induced PBMC from HIV-seropositive and -seronegative individuals. Lane A, serum from the laboratory HIV-1-seropositive control; lane B, serum from the laboratory seronegative control; lanes C and D, serum and supernatant fluid, respectively, from an HIV-1-seropositive individual; lanes E and F, serum and supernatant fluid, respectively, from an HIV-1-seronegative, PWM-negative individual; lanes G and H, serum and supernatant fluid, respectively, from an HIV-1-seronegative but PWM-positive individual. Markers on either side reflect molecular size markers (in kDa) of the major HIV-1 proteins.

tients are similar to those described by Imagawa and coworkers (1, 2) in that they were infected but were in the process of seroconversion. Third, as in the case of conventionally seronegative mangabeys, it was reasoned that these patients were latently infected with HIV-1 but remained seronegative at the time of this study.

To address these issues, we used the highly sensitive PCR technique (5) on DNA samples from aliquots of the same PBMC as used for the PWM assay. As shown in Table 2, none of the 57 samples from patients who were HIV-1 seronegative and also negative by the PWM assay contained HIV-specific sequences as determined by PCR, and of the 26 samples from patients who were HIV-1 seropositive and also positive by the PWM assay, 25 were also positive by PCR. Of importance was our finding that 12 of the 26 samples tested from patients who were HIV-1 seronegative but positive by the PWM assay were also definitely positive by PCR. The qualifier definitely is used here because there were 9 additional samples from this group of 26 HIV-1-seronegative but PWM-positive samples that gave faint bands, which were hard to interpret and may represent either the quality of the DNA or limits of the sensitivity of detection. None of the DNA samples from the HIV-1-seronegative, PWM-negative individuals, however, gave such faint bands. Fig. 2 shows the hybridization reaction of the amplified product from representative samples. Lanes E, F, and G of Fig. 2 represent results of PCR analysis of DNA samples from three HIV-1-seropositive individuals who were also positive by the PWM assay. Lanes H, I and J of Fig. 2 represent results of PCR analysis of DNA samples from three HIV-1-seronegative individuals who were positive by the PWM assay. While it is clear that lanes E, F, G, H, and I of Fig. 2 represent positive signals, lane J shows a faint band, which unfortunately seems similar to the faint band

Table 2. Correlation of serum antibody results with the PWM assay and PCR analysis

HIV-1 serology	PWM assay	No. samples HIV-1$^+$ by PCR/total tested
+	+	25/26
−	−	0/57
−	+	12/26*

Serum was tested by the Abbott EIA kit and confirmed by Western blot assay as described in Table 1. Supernatant fluids of the PBMC cultured with PWM from the same individuals were similarly tested. In addition, DNA was extracted from aliquots of the PBMC (when enough cells were available) and tested for the presence of HIV-1 sequence by using the PCR assay. Enough cells were available from 26 individuals who were HIV-1 seropositive and positive by the PWM assay, 57 individuals who were negative for both assays, and 26 individuals whose sera were negative for HIV-1 by the EIA kit and Western blot analysis but positive by the PWM assay (ELISA). In brief, using the SK38/39 HIV-1 gag primer pair, the samples were amplified by 35 cycles of denaturation, annealing, and extension; separated on a 1.8% agarose gel; blotted onto nitrocellulose; and hybridized with ^{32}P-labeled SK19 probe as described (5).

*Twelve of the 26 samples tested were definitely positive by PCR, and 9 additional samples demonstrated faint bands, which were hard to interpret.

seen with the primer-pair-only control seen in lane B. However, this faint band type of reactivity is clearly visible and distinct from Fig. 2, lanes C and D, which represent results obtained with DNA samples from HIV-1-seronegative individuals who were also negative by the PWM assay. The results of Fig. 2, lane J, therefore, are difficult to interpret, as alluded to above, and are considered negative for the purposes of this paper. The double-blind nature of this study did not allow for such repeated testing. Future longitudinal studies on similar cohorts of individuals will help resolve these issues.

The demonstration that B cells can be induced to secrete HIV-1 antibodies *in vitro* in otherwise seronegative but infected individuals poses important questions as to the mechanisms involved. Within this context, it has been previously shown by Reinherz *et al.* (6), as well as others (7, 8), that CD8$^+$ suppressor T cells can effectively inhibit immunoglobulin secretion by B cells stimulated *in vitro* with PWM. To further examine the role of such CD8$^+$ T cells, aliquots of PBMC from 16 HIV-1-seronegative patients were cultured *in vitro* with PWM before and after depletion of CD8$^+$ T cells. As shown in Table 3, supernatant fluid from 6 of the 16 samples of unfractionated PBMC cultured with PWM showed positive reactivity against HIV-1. In addition, depletion of CD8$^+$ T cells prior to culture *in vitro* with PWM

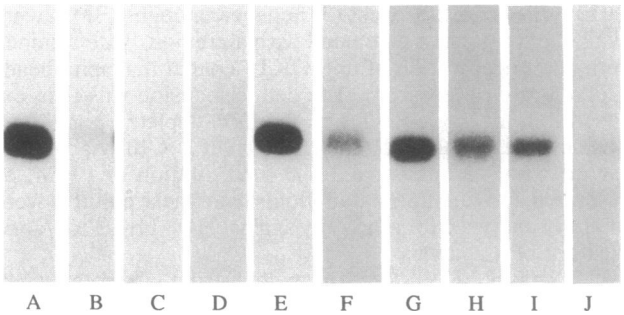

FIG. 2. DNA samples from PBMC of HIV-1-seropositive and -seronegative individuals were analyzed by PCR. Lane A, plasmid control; lane B, primer pairs only; lanes C and D, two HIV-1-seronegative individuals who were also negative by the PWM assay; lanes E–G, three HIV-1-seropositive individuals who were also positive by the PWM assay; lanes H–J, three HIV-1-seronegative individuals who were positive by the PWM assay.

Table 3. Effect of depletion of $CD8^+$ T cells on the induction of HIV-1-specific immunoglobulin biosynthesis by cells from HIV-1-seronegative individuals

PBMC sample number	Supernatant fluid from PWM cultures of: Unfractionated PBMC ELISA	Unfractionated PBMC Western blot	CD8-depleted PBMC ELISA	CD8-depleted PBMC Western blot	PCR results
1	−	−	−	−	−
2	+	+	+	+	+
3	−	−	−	−	−
4	−	−	+	+	+
5	−	−	+	+	+
6	−	−	−	−	−
7	−	−	−	−	−
8	−	−	−	−	−
9	−	−	−	−	−
10	−	−	−	−	−
11	−	−	+	+	+
12	+	+	+	+	+
13	+	+	+	+	+
14	+	+	+	+	+
15	+	+	+	+	+
16	+	+	+	+	−

Unfractionated PBMC or PBMC depleted of $CD8^+$ T cells (by using magnetic beads coupled to anti-CD8 antibody) from a total of 16 HIV-1-seronegative individuals were cultured at 2×10^6 per ml in a volume of 2 ml with a 1:500 final dilution of PWM. Cultures were incubated at 37°C in a 7% CO_2 humidified atmosphere for 4 days. Supernatant fluid was harvested and assayed for the presence of HIV-1 antibody as described in Table 1. Aliquots of PBMC from each sample were used to extract DNA that was analyzed for the presence of HIV-1 sequences by PCR as described in Table 2.

resulted in the identification of three more samples that were positive for HIV-1-reactive antibodies. DNA from 8 of the 9 samples that showed positive secretion of HIV-1-reactive antibodies were confirmed to be HIV-1 positive by PCR analysis. While it is clear that $CD8^+$ cells appear to regulate PWM-induced immunoglobulin biosynthesis specific for HIV-1, the specificity as well as the cellular and molecular basis for this *in vitro* suppression needs to be elucidated. This is especially relevant in these studies since spontaneous activation of B cells has been shown to be one of the manifestations inherent in patients with HIV-1 infection (9–12).

DISCUSSION

Data from these studies clearly define the existence of HIV-1-infected individuals who do not have detectable levels of serum antibodies that react with HIV-1, confirming the findings of Imagawa and coworkers (1, 2). The finding that PBMC from these individuals can be stimulated *in vitro* to secrete HIV-1-reactive antibodies provides for a relatively simple assay for detection of such individuals, one that does not require technically difficult procedures such as the PCR technique. The specificity of the *in vitro* assay is highlighted by the observation that all of the samples that were negative by the PWM assay were also negative by PCR, combined with the fact that PBMC from nearly all HIV-1-seropositive individuals were also positive by PWM ELISA (49/50) and Western blot (48/50) analysis. A significant number of PBMC cultures from seronegative individuals secreted HIV-1-reactive antibodies (30/165). At least 12 of the 26 samples tested from these 30 individuals were also determined by PCR analysis to be HIV-1 infected. The identification of individuals who are positive by the PWM assay but either failed to be detected by PCR or gave PCR results that were difficult to assess clearly dictates the need for further studies, which will require repeated testing and follow-up studies. The aim of this study was not to determine the prevalence of such individuals but merely to document our ability to detect such individuals by polyclonal activation of PBMC *in vitro*. A longitudinal study of similar cohorts of such individuals at multiple centers will be required before statistical evaluations of these data can be obtained and be meaningful.

It could be reasoned that our inability to detect serum antibody in the select group of these HIV-1-seronegative but HIV-1-infected individuals could be due to minimal virus load (dose of antigen), resulting in lack of a threshold of an immunogenic signal. This could be explained by a latently expressed HIV-1 genome. Alternatively, it might be argued that the PWM assay is merely identifying individuals who will shortly seroconvert and that ELISAs and Western blot assays of serum samples are not sensitive enough for the detection of low levels of HIV-1-reactive antibodies. Such an explanation is appealing in light of the high prevalence of infection in the select population comprising this study. Follow-up studies with such a population will certainly provide insights into such possibilities.

However, in light of the results obtained with depletion of $CD8^+$ cells (see Table 3), we would like to propose that, at least in some cases, the seronegativity observed may be due to peripheral tolerance, mediated perhaps by active suppression, which is overcome by polyclonal stimulation with the use of PWM. These thoughts are not without precedence since it has been previously shown that spleen cells from mice made experimentally tolerant to certain antigens can be shown to secrete antibody by polyclonal activation both *in vitro* and *in vivo* (13, 14). Thus, it is quite possible that these individuals, shortly after exposure, mount an immune response against HIV-1 proteins, which leads to the priming of B cells. However, the cross-reactivity of certain HIV-1 epitopes with self-proteins triggers the activation of regulatory $CD8^+$ T cells, leading to the nonspecific suppression of HIV-1-reactive B-cell clones. Such thoughts are supported by the observation of the homology between amino acid sequences of certain HIV-1 proteins and a number of immunologically relevant cell surface molecules (15, 16). Such antigenic mimicry may be responsible for these cases of unresponsiveness and account for the HIV-1-infected but seronegative state as described in this paper. The initial tolerant state can thus either maintain itself or, for a variety of reasons, can result in the breakdown of tolerance and subsequent seroconversion. If one accepts the former to be true, then there may be individuals who are, in fact, infected but remain seronegative and immune to HIV-1. In this regard, it is important to note that we have identified by PCR analysis several SIV-infected sooty mangabeys that have $CD8^+$ cells that markedly regulate SIV replication and remain seronegative, including one 12-year-old mangabey that is still seronegative. Such data suggest that certain forms of virus-specific immunity can persist in the absence of antibody production. The latter view would suggest that either repeated exposure to other HIV-1 variants or the replication of virus intracellularly to a sufficient level over a period of time results in the observed seroconversion. Further, it is possible that replication of HIV-1 to a significant level results in the release of peptides with sequences that are, in fact, polyclonal mitogens that lead to the breakdown of tolerance and subsequent seroconversion. Such sequences of the HIV-1 that induce polyclonal activation have indeed been described (12). In addition, such breakdown of tolerance may also lead to reactivation of the immune response to the HIV-1 sequences that cross-react with self-proteins (15, 16), resulting in autoimmunity, which has also been documented in patients with HIV-1 infection (17, 18).

The finding that PBMC from some individuals synthesize and secrete HIV-1-reactive antibodies only after depletion of $CD8^+$ cells prior to *in vitro* culture (see Table 3) suggests that

polyclonal activation by PWM does not totally overcome the tolerant state. It is possible that there are two levels of regulation in this assay. One involves B cells that have been tolerized, and the other concerns regulation of B-cell activation by $CD8^+$ T cells. Thus, PWM may induce the breakdown of B-cell tolerance, and depletion of $CD8^+$ cells may eliminate the influence of the regulatory property of these cells on the synthesis of immunoglobulin by B cells. The recent report by Nossal and Karvelas (19) clearly provides support for the presence of immunologically tolerant B cells.

The cell–cell interactions that are required for the activation of B cells to secrete HIV-1-reactive antibodies need to be examined in detail. Our preliminary data indicate that such induction of HIV-1-specific immunoglobulin biosynthesis *in vitro* requires both $CD4^+$ T cells and macrophages, which is similar to findings reported previously by several laboratories. Both these cell types, however, have been shown to be susceptible to HIV-1 replication (20–22) and thus reasoned to lead to quantitative or qualitative defects in the immune system. In addition, mitogenic responses to PWM have also recently been shown to be impaired in PBMC of not only HIV-1-infected AIDS patients but also HIV-1-infected asymptomatic individuals and those with AIDS-related complex (23, 24). Sera from these patients, however, have high levels of HIV-1-reactive antibodies. Cumulatively, these data suggest that immunoregulatory defects are clearly evident in PBMC from HIV-1-infected individuals. On the other hand, such defects may lead to increased immunoglobulin synthesis by augmentation of the necessary signals and/or cytokines necessary for the triggering of immunoglobulin-secreting B cells. The finding of similar amounts of total immunoglobulin synthesized by PWM stimulation of PBMC cultures from HIV-1-infected and non-HIV-1-infected B cells argues against such a hypothesis. It is more likely that there is a high precursor frequency of primed HIV-1-specific memory B cells that contribute to the release of HIV-1-specific antibodies. Within this context, it should be emphasized that the results reported in this paper reflect levels of IgG antibodies; thus, it is quite possible that if immunoregulatory defects play a role in the ability of $CD4^+$ T cells and macrophages from HIV-1-infected individuals to induce immunoglobulin biosynthesis upon stimulation by PWM, such defects may increase or decrease the synthesis and/or release of IgM antibodies but not IgG antibodies. The defects would thus characterize the triggering of primary but not secondary B-cell responses, suggesting distinct regulatory effects on primary antibody-forming cells and secondary B cells. Differences in such regulatory effects on primary and secondary B cells are not unusual since they have been shown recently to be generated from separate precursor B-cell subpopulations (25). The fact that IgG-specific responses are unimpaired indicates that memory B cells in HIV-1-infected individuals are, in fact, fully functional and not susceptible to changes in the function of $CD4^+$ T cells and macrophages. The finding of quantitatively high levels of HIV-1-reactive antibodies in supernatant fluids from PWM cultures of PBMC from HIV-1-seropositive individuals, as compared to HIV-1-infected but seronegative individuals, suggests that further studies are required to ascertain the precise cellular, subcellular, and molecular requirements that contribute to the synthesis and release of HIV-1-reactive antibodies in this assay; thus, different requirements may be operational in cells from patients at different stages of the disease. Such studies would include analysis of the effect of relevant cytokines [interleukin 1 (IL-1) (produced by macrophages), IL-2, IL-4, and IL-6, which are required for the chain of events that lead to immunoglobulin synthesis by B cells] and of the T-cell receptors that are involved in the response to PWM. Both CD2 and CD3 molecules have been shown to be involved as receptors on T cells for PWM-induced T-cell proliferation (26); CD3 is the receptor for low levels of PWM (24), and both CD2 and CD3 are receptors for high concentrations of PWM (26).

The ability to readily identify *seronegative* HIV-1-infected individuals may prove vital in the elucidation of the induction of the pathogenic mechanisms involved in AIDS. Finally, the practical application of our assay as a sensitive, cost-effective screening technique is clearly evident, and the epidemiological implications of these findings are readily apparent.

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