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CD20 Expression Is Increased on B Lymphocytes from HIV-Infected Individuals

*Frank J. T. Staal, *Mario Roederer, ‡Jeff Bubp, §Larry A. Mole, *Michael T. Anderson, *Paul A. Raju, †‡§Dennis M. Israelski, †Stanley C. Deresinski, *Wayne A. Moore, *Leonard A. Herzenberg, and *Leonore A. Herzenberg

**Department of Genetics, Stanford University School of Medicine, Stanford, California; †AIDS Community Research Consortium, Redwood City, California; ‡San Mateo County General Hospital and San Mateo County AIDS Program, San Mateo, California; and §Veterans Affairs Medical Center, Palo Alto, California, U.S.A.*

Summary: In studies presented here, we show that expression of the pan B cell marker CD20 is markedly increased on B lymphocytes from HIV-infected individuals and that this increase tends to be greater in individuals with more advanced disease. By using multiparameter FACS analyses to quantitate surface density of CD20 and intracellular glutathione (GSH) levels simultaneously, we further show that the distribution of intracellular glutathione (GSH) levels in B cells of HIV-infected individuals is more heterogeneous than in uninfected controls. Finally, we show that the intracellular GSH levels correlate with CD20 expression on a per-cell basis in all infected individuals. These findings suggest that CD20 expression, which can be precisely measured, may prove to be a useful surrogate marker for monitoring HIV infection.
Key Words: CD20—Glutathione—B cells.

Hypergammaglobulinemia, apparently the result of polyclonal B cell activation, is an early and common feature of HIV infection (1–3). This polyclonal activation is manifested as elevated levels of serum immunoglobins (Igs), increased numbers of spontaneous Ig-secreting cells in the peripheral blood, and an inability to mount an antigen-specific response following immunization. Such unresponsiveness has been ascribed to the fact that the B cells of HIV-infected individuals are already in a state of polyclonal activation (4).

In addition to surface Ig receptors for antigens, a number of B cell–associated surface molecules that may regulate B cell activation have been found. One such marker, the 35–37 kDa CD20 phosphoprotein (5), is expressed on all peripheral blood B cells, but not on T cells, NK cells, or monocytes. This pan B cell marker is commonly used to distinguish human B cells in FACS and fluorescence microscopy studies.

Gene-encoding CD20 has been cloned (5). The protein it encodes appears to be a calcium channel (6) that is phosphorylated differently in resting and activated cells (7). Several studies implicate this protein in B cell activation. For example, CD20 is present at a level up to fivefold higher on in vitro–activated B cells (T. Tedder, personal communication). In addition, certain antibodies against CD20 have been shown to trigger resting B cells to enter the cell cycle (8) and/or to induce IgM production (7), while other anti-CD20 antibodies have been shown to inhibit B cell activation (7).

In studies that led to the findings presented here, we established multiparameter FACS methods for

Address correspondence and reprint requests to Dr. Leonore A. Herzenberg at Beckman B007, Stanford University, Stanford, CA 94305, U.S.A.
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measuring intracellular glutathione (GSH) levels in individual peripheral blood mononuclear cell (PBMC) subsets. These studies showed that GSH levels in CD4 and CD8 T cells decrease significantly as a consequence of HIV infection (9,10) and that these decreases reflect the selective loss of cells that contain high levels of GSH, which are found in all normal individuals.

We further found that CD20 expression is increased on B cells from virtually all HIV-infected individuals and that this increase tends to be greater in individuals with low CD4 T cell counts and more severe infection. We also show that the HIV infection alters the regulation of intracellular GSH levels in B cells so that CD20 expression becomes correlated with intracellular (B cell) GSH in those infected with HIV. Thus we suggest that CD20 expression, perhaps in concert with GSH level measurements in B lymphocytes, may be a useful surrogate marker for following the progression of HIV infection.

METHODS

Subjects

We have previously presented data on a large cohort of HIV-infected and uninfected individuals (10). Partway through the study, we established the standard conditions for the measurement of surface CD20 levels presented here. The resulting subset comprises 60 HIV-infected people (in accordance with CDC classification, 15 subjects were asymptomatic, 17 were diagnosed with AIDS-related complex (ARC), and 28 were AIDS patients). Ten healthy, uninfected individuals were recruited in the San Francisco Bay Area. All subjects gave informed consent and were told about the study design. The presence of HIV infection was evaluated by a commercial clinical laboratory: the infection was documented by ELISA for antibodies to the p24 antigen; all positive results were confirmed by Western blot for antibodies to p24 and gp160 antigens. One AIDS patient with a B cell lymphoma was excluded from the study.

Staining and FACS Analysis

Multiparameter FACS analyses were performed using fluorochrome-coupled monoclonal antibodies detecting CD20 (Leu16), CD5 (Leu1), and CD25 (interleukin 2 receptor) (Becton Dickinson, San Jose, CA, U.S.A.). PBMCs from fresh blood samples (<4 h after drawing) were obtained by Ficoll-Paque (Pharmacia, Alameda, CA, U.S.A.) density centrifugation. Cells were washed and stained for intracellular GSH using a method employing monochlorobimane (MCB) (Molecular Probes, Portland, OR, U.S.A.) as described (9,11). Samples were washed, stained with monoclonal antibodies (saturation staining), and subsequently fixed and analyzed on a dual-laser FACStarPlus (Becton Dickinson). Data were collected on 30,000 cells, stored in list mode, and later analyzed with FACS-Desk software. One or two normal controls were included on each experiment day, as a reference for GSH levels. The clinical status of those infected with HIV was unknown at the time of the experiment and was only disclosed a week later, after all FACS measurements and calculations had been done. Together with the automated collection and standardization procedure, this practice prevents operator bias.

FACS Standardization

Because comparisons of antibody staining levels for different people are made over different days, precise standardization of FACS measurements are required. The automatic, computer-controlled data collection, standardization, and calibration procedures were developed at the Shared FACS Facility at Stanford and have been described (12). Briefly, cytometer conditions are standardized using fluorescent beads, i.e., photomultiplier voltages are adjusted such that the same fluorescence intensity for the beads is obtained at the beginning of each experiment and after any interruption of the experiment. This method results in highly reproducible fluorescences for a particular conjugated monoclonal antibody from day to day for the same individual. For example, the CD20 expression in one uninfected person measured nine times over a period of 5 months varied <3% (mean 64.6 ± 1.5 fluorescence units, range 61.9–66.3).

Calculations and Statistics

The absolute number of PBMCs per μl of blood was determined from the screening differential and white blood cell counts. This number was multiplied by the percentage of cells positive for a particular set of cell-surface markers (as found by FACS analysis) to obtain the absolute number of cells in that subset. Median GSH levels were calculated as the median of the MCB fluorescence value for a particular subset; CD20 expression was determined by the median fluorescence value from staining with an anti-CD20 monoclonal antibody. Statistical comparisons were computed with a single-factor analysis of variance (unbalanced design) and Scheffe F-test. Correlations were determined with a nonparametric Spearman rank test.

RESULTS

We examined B lymphocytes from 60 HIV-infected individuals (15 asymptomatic individuals and 17 ARC and 28 AIDS patients) for the expression of CD20, a pan-B cell marker that has been implicated in B cell activation. As Fig. 1 shows, there is very little variation in CD20 levels on B cells from uninfected individuals. Values for all uninfected individuals tested fall within a narrow range (63 ± 5). In contrast, median CD20 levels on B cells from those infected with HIV are greater than the upper bound of this range in all but one case.

The increased CD20 expression is greatest in the AIDS patients (on average almost twofold; see Table 1) but is also clearly detectable in those in the asymptomatic and ARC categories. Levels in the asymptomatic and ARC categories do not differ significantly from each other but are significantly higher than normal. Furthermore, levels in the AIDS category are significantly higher than in all other categories (Scheffe F test, 99% confidence

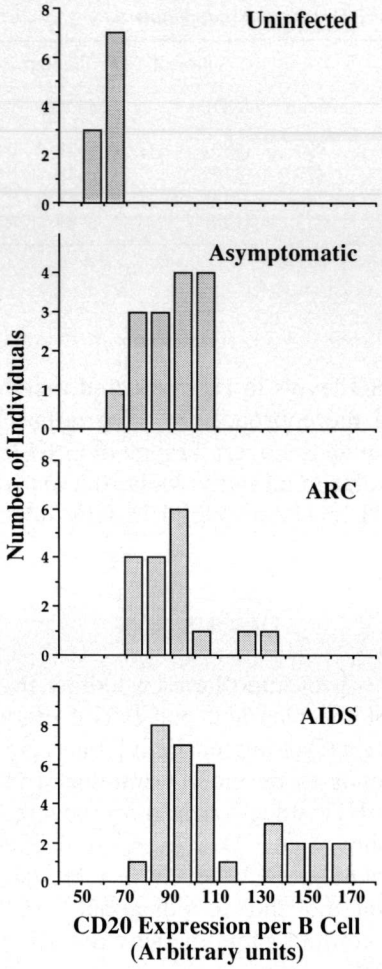

FIG. 1. CD20 expression on B cells is increased in HIV-infected individuals. Histograms of CD20 expression are shown (uninfected: n = 10; asymptomatic: n = 15; ARC: n = 17; AIDS: n = 28). CD20 expression was determined as the median fluorescein fluorescence from FITC-labeled anti-CD20 monoclonal antibodies. B cell CD20 levels in 27 uninfected individuals were also measured using PE-anti-CD20; the variation of CD20 expression was as highly conserved as that measured by the FITC-anti-CD20. The median CD20 expression increases with progression of the HIV infection to stages with clinical manifestations.

interval). Thus, on average, CD20 expression is higher in those with more advanced disease.

As these findings would predict, CD20 expression tends to be highest in HIV-infected individuals who have the fewest CD4 T cells (Table 1). Figure 2 shows the CD20 expression plotted as a function of the number of CD4 T cells in peripheral blood for all the subjects studied. There is a negative correlation between the number of CD4 T cells and the CD20 expression on the B cells in the HIV-infected population (nonparametric Spearman rank correlation test; $p < 0.0001$, $r = -0.52$ in a linear regression analysis). These values are not correlated in normal, healthy subjects ($p > 0.72$, $r = 0.10$).

The expression of other B cell markers (CD19, CD22, IgM) is not changed on B cells from these patients. Furthermore, there are no significant differences in size as measured by forward and side-scattered light (data not shown).

The small variation in CD20 expression in uninfected individuals was confirmed in a larger study using a differently conjugated monoclonal antibody. The anti-CD20 antibody used in this study is fluorescein (FITC)-conjugated Leu16. We have performed a similar analysis with phycoerythrin (PE)-conjugated Leu16 on 27 HIV-negative individuals. The coefficient of variation in CD20 expression, as measured by PE-anti-CD20 among the 27 individuals, was 4.5%, less than that found for the FITC-anti-CD20 measurements (7.5%). Furthermore, 11 of these people were measured a second time 3 weeks later; the average difference between the two measurements was 4.0%. Thus, the level of expression of CD20 on B cells is highly regulated and conserved among normal individuals.

We initially noted the increase in CD20 expression on B cells from HIV-infected individuals because it is accompanied by a striking change in the relationship between the level of surface CD20 and the level of intracellular GSH, as measured in multiparameter FACS analyses (Fig. 3). While these two parameters show no correlation in B cells from normal patients, they are correlated in B cells from HIV-infected individuals. This correlation, visible as a distinctive diagonal pattern in FACS contour plots of GSH versus CD20 levels, occurs in all HIV-infected individuals investigated (who have detectable numbers of B cells).

The change in CD20 and GSH expression appears to occur early after infection, in that it was already detectable in an HIV-infected individual known to have been infected only 2–3 weeks before analysis. In fact, one of the examples of the typical diagonal pattern for HIV-infected individuals shown in Fig. 3 is based on data from this person, whereas the other example presents data for an individual infected several years previously. The patterns for both are similar and are representative of all patients studied. This finding argues that the observed phenotype can occur in, but is not restricted to, acute HIV infection. At present, we do not know whether similar CD20 and GSH changes occur in other kinds of infections; however, the ubiquity of these

TABLE 1. *CD20 expression and number of CD4 T cells in HIV-infected individuals*

Group	CD20 expression per B cell[a]				No. of CD4 T cells[b]			
	Mean	(SD)	Median	(IQR)[c]	Mean	(SD)	Median	(IQR)[c]
Uninfected (n = 10)	63	(4.7)	64	(60–66)	998	(380)	921	(715–1,262)
Asymptomatic (n = 15)	92	(12)	95	(83–101)	479	(310)	448	(293–534)
ARC (n = 17)	92	(22)	97	(81–101)	244	(43)	188	(75–570)
AIDS (n = 28)	114	(27)	100	(91–140)	73	(120)	89	(20–201)

[a] Arbitrary fluorescence units.
[b] Per μl of whole blood.
[c] Interquartile range (25th–75th percentiles).

changes during HIV infection and their apparent early occurrence suggest their potential usefulness for monitoring infection. Measurement of CD20 expression is especially useful, because it can be done more precisely than absolute CD4 T cell counts. For example, one uninfected individual tested nine times over a period of 5 months had a 31% variation in CD4 T-cell count but only a 3% variation in median CD20 level per cell.

The dramatic change in the relationship between surface CD20 expression and intracellular GSH levels does not reflect an overall difference in GSH levels between HIV-infected and uninfected individuals, since the upper and lower bounds of the B cell GSH distribution are essentially the same in all individuals tested. Nevertheless, there is considerably more variability among median GSH levels in B cells across infected individuals. That is, while median GSH levels in uninfected individuals are quite closely clustered (interquartile range 95–103), median GSH levels in HIV-infected individuals are distributed more broadly in all groups (Table 2). Thus, although the overall range of GSH levels in B cells is similar in all individuals, the *distribution* of B cell GSH levels is altered in HIV-infected individuals.

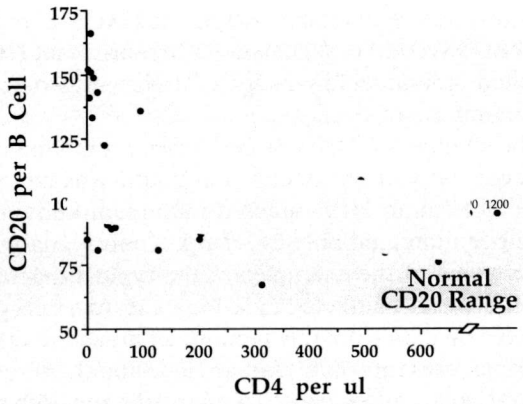

FIG. 2. Relationship between CD20 expression and the absolute number of CD4 T cells. Median expression of CD20 per B cell was determined by FACS measurements and is given as fluorescence units; the number of CD4 T cells was determined as described in Methods. Data are shown only for HIV-infected individuals. The normal range for CD20 expression is shown in gray. Two asymptomatic HIV-infected individuals had CD4 T-cell counts of 900 and 1,200 per μl.

DISCUSSION

In studies presented here, we show that the expression of the classical pan-B cell marker CD20 increases in HIV-infected individuals and that this increase tends to be more pronounced in those in whom the HIV infection is more severe. In addition, we show that CD20 expression is correlated with intracellular GSH levels in infected individuals, resulting in a shift to a diagonal FACS staining pattern in contour plots of the expression of GSH versus CD20.

The increase in CD20 expression may reflect the polyclonal B cell activation observed previously in HIV-infected individuals (1–4). For example, poly-

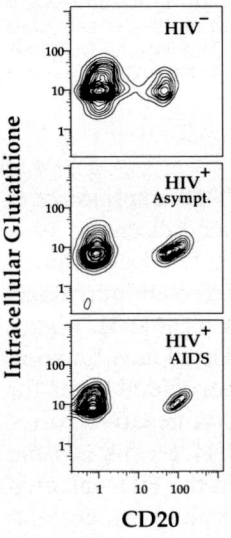

FIG. 3. CD20 expression changes with HIV infection and is correlated with the GSH levels in B cells. Dual-parameter plot of MCB fluorescence (measuring GSH) on ordinate versus CD20 fluorescence on abscissa. Note that the scale on both axes is logarithmic. **Top:** uninfected, healthy control. **Middle:** HIV-infected individual 2–3 weeks after infection. **Bottom:** HIV-infected individual at least several years after infection. In the HIV-infected individuals, as compared with the uninfected control, the level of CD20 expression is elevated and correlated with GSH level, and the range of CD20 expression is greater.

TABLE 2. *Intracellular GSH levels in B cells of HIV-infected individuals*

Group	B cell GSH level
Uninfected (n = 31)	100 (95–103)
Asymptomatic (n = 31)	88 (74–105)
ARC (n = 32)	82 (67–97)
AIDS (n = 71)	90 (77–110)

Data are given as median [interquartile range (IQR): 25th–75th percentile]. The IQR is substantially larger in the HIV-infected groups. The median values are not statistically significantly different from each other. These values were obtained from the larger cohort of individuals studied (see Materials and Methods), since GSH measurements were available for all individuals in the cohort.

clonal IgM production routinely occurs in HIV-infected individuals (4). Furthermore, Rieckmann and colleagues report that in vivo activated B cells from HIV-infected individuals produced cytokines that upregulate HIV expression (13). In addition, Cerny's group reported that B cells are required for induction of T-cell abnormalities in a murine AIDS model (14). As indicated earlier, the CD20 phosphoprotein has been shown to play an important role in B cell activation and to increase in expression upon activation.

The potential connection between B cell activation and the increased expression of CD20 that we found in HIV-infected individuals may also explain the tendency toward greater diversity in CD20 expression in these patients. CD20 expression is very homogeneous on B cells from normal, healthy people (range two- to fivefold within each individual). However, the range of (increased) CD20 expression on B cells from those infected with HIV varies from 10- to 25-fold. This could be because B cells tend to be activated to different extents in these individuals.

Alternatively, the increase and the greater diversity of CD20 expression on B cells could be due to the increased frequency of a (high CD20) B cell subset that is normally quite rare. That is, some individuals could have a relatively large number of B cells that express high levels of CD20 together with higher levels of intracellular GSH; others could have B cell populations in which most of the B cells express less CD20 and lower levels of intracellular GSH. The relative frequencies of these two types of B cells (more or less activated) would then determine the median GSH level for a given person. The FACS studies agree with this hypothesis, although the data are insufficient to resolve two types of B cells clearly.

This two subset hypothesis is useful because preliminary studies indicate that the B cells from HIV-infected individuals do not express the interleukin 2 receptor (CD25), an activation marker. CD25 becomes readily detectable when B cells from either HIV-infected or healthy (uninfected) individuals are activated in vitro with phorbol 12-myristate 13-acetate (data not shown). However, CD20 expression may merely be a better index of in vivo B cell activation than CD25 expression, at least the type of activation that occurs in HIV-infected individuals, since increased expression of other B cell activation markers has been reported on B cells from these individuals (15). Further study is required to resolve this issue.

From a practical standpoint, the increase in CD20 expression and the concomitant shift in the GSH/CD20 dual parameter contour patterns in B cells from HIV-infected individuals offer potentially useful tools for monitoring HIV infection and for evaluating experimental therapies. There is no reason to believe that these B cell changes are directly induced by the HIV virus or that they are found only in HIV infection; other viral infections or inflammatory conditions may induce similar increases in surface CD20 expression and/or changes in the distribution of B cell GSH levels. Nevertheless, the consistent occurrence of these B cell deviations in all HIV-infected subjects studied suggests that CD20 measurements may prove useful as surrogate markers for monitoring HIV infection.

The use of surrogate markers to replace mortality or development of severe opportunistic infections (OIs) as a measure of the progress of HIV infection or the effects of experimental therapies has been much debated (16); however, this approach has gained increasing support because HIV-infected individuals now tend to have fewer OIs and to survive for longer periods of time. The absolute number of CD4 T cells in peripheral blood is currently the only surrogate marker that is widely accepted. The relatively high standard error in this measurement detracts somewhat from its usefulness for charting HIV progression; however, no other marker has yet proved to have the value of the CD4 count.

CD20 measurements are particularly good for this purpose because they can be determined precisely and directly in FACS analysis by gating on a B cell population and determining the median level of CD20 expression. Determination of this median level (an intrinsic property of B cells) does not depend on the frequency or recovery of the B cells in

the sample (provided that enough B cells are present to make the measurement) and thus does not depend on the technique used to isolate the cells (e.g., Ficoll versus lysis). Therefore, measurements of CD20 levels are inherently simpler and certainly more precise than those of absolute CD4 T-cell counts, which require additional measurements (e.g., leukocyte counts and screening differential) to convert the FACS-determined CD4 T-cell frequency to the absolute CD4 T-cell count in the subject's blood.

The key question with respect to the use of CD20 as a surrogate marker, however, is whether CD20 levels (alone or in concert with GSH measurements) are better or worse than or equivalent to absolute CD4 T counts in terms of predicting clinical outcome. However, at a minimum, the data we have presented recommend further evaluation of CD20 as an adjunct to the measurement of absolute CD4 T-cell counts (and other potential surrogate markers) in evaluating experimental therapies for monitoring HIV infection.

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