CD5 expression by B lymphocytes and its regulation upon Epstein–Barr virus transformation

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Dim expression of CD5 on human B lymphocytes has been used to delineate B1 and B2 subsets. Nevertheless, others have suggested that the molecule is an activation marker and does not predicate a subset distinction. We have used enzymatic amplification staining, a technology that enhances the resolution of flow cytometric analysis of cell surface molecules by as much as 100-fold, to determine that essentially all human B cells express CD5. Furthermore, we show that this expression is regulated during Epstein–Barr virus transformation.

CD5, a 67-kDa surface glycoprotein of the scavenger receptor cysteine-rich family, appears on T lymphocytes early in their development and is abundantly expressed on all mature T cells. The expression of the molecule on B lymphocytes and its role in defining separate lineages of these cells is controversial (1, 2). It has been postulated that the low-level expression of CD5 by a subset of B lymphocytes defines a separate lineage that is associated with autoreactive antigenic specificity (2, 3). Alternatively, others have shown that CD5 expression on B cells can be enhanced by various activating agents, prompting the suggestion that CD5 is a B cell activation antigen and that B cells comprise a single lineage (4, 5).

We have developed a powerful technology, enzymatic amplification staining (EAS), that significantly enhances the resolution of flow cytometric analysis of cell surface molecules (6). By using EAS, we have achieved a 100-fold enhancement in the fluorescent signal. The enhanced signal allows for the detection of molecules that could not be observed previously. Thus, we have been able to define a subpopulation of peripheral blood cells stimulated *in vitro* that express Fas ligand (CD178), and we have demonstrated the validity of EAS in this case by correlating the staining with functional activity (6). EAS has also been used to provide a high-resolution immunophenotype of leukemic cells from patient samples. It was shown that enzymatic amplification gives a more powerful assessment of the clonality of the leukemic cells (7).

Some investigators have proposed that human B lymphocytes can be divided into B1 and B2 subsets based on the expression of CD5 (2, 3); however, others have shown that CD5 expression on B cells can be up-regulated by various activating agents, which suggests that CD5 is a B cell activation antigen (4, 5). To address these alternative models, we used EAS to assess the expression of CD5 on human B lymphocytes. We found that essentially all B cells express CD5 constitutively at low levels, and we also found that CD5 expression is regulated by Epstien–Barr virus (EBV) transformation.

Materials and Methods

Cells. Peripheral blood from healthy volunteers was obtained under a protocol approved by the Institutional Review Board, and mononuclear cells were obtained from the blood by discontinuous centrifugation over Ficoll/Hypaque. Cell lines were maintained in our laboratory in culture medium consisting of 10% FBS in RPMI medium 1640. Discarded samples of human spleen and tonsillar cells were obtained from the Diagnostic Immunology Laboratory of University Hospitals of Cleveland under approval by the Institutional Review Board. These samples did not contain malignant cells according to the analysis of the pathologists.

Monoclonal Antibodies. Biotinylated murine antihuman CD5 monoclonal antibodies were obtained from Becton Dickinson (clone L17F12), CalTag Laboratories (clone MHCD0515; Burlingame, CA), and BD Biosciences PharMingen (clone UCHT2; San Diego). Biotinylated murine IgG1 (isotype control) was obtained from BD Biosciences PharMingen. Murine antihuman CD19 monoclonal antibody conjugated with phycoerythrin was obtained from CalTag Laboratories. Murine anti-EBV latent membrane potential 1 (LMP-1) monoclonal antibodies were obtained from Dako.

Flow Cytometric Analysis. For standard amplification staining (indirect staining), we incubated cells with biotinylated primary antibodies followed by an incubation with streptavidin conjugated to fluorescein isothiocyanate. For EAS, we obtained kits from Flow-Amp Systems (Cleveland), and followed the manufacturer's instructions. The incubations were all performed for 10 min at room temperature for both procedures. The diluent used for washing and for the incubations was PBS, pH 7.5, with 1% BSA and 1% FBS. The standard amplification procedure and EAS used identical primary reagents and conjugated fluorochromes and differed only by the enzymatic amplification steps. The directly labeled anti-CD19 (conjugated to phycoerythrin) was added along with the streptavidin-fluorescein isothiocyanate for doubly stained cells. The stained cells were analyzed on a FACScan (Becton Dickinson) with CELLQUEST software. Compensation for overlapping FL1 and FL2 signals was set by using singly stained samples.

EBV Infection. Peripheral blood mononuclear cells from a healthy donor were isolated and the T cells and natural killer cells were eliminated by sheep red blood cell rosetting. The remaining cells were incubated at one million cells per ml in 50% RPMI medium 1640 with 10% FBS and 50% culture supernatant from the B95-8 cell line, which constitutively produces a transforming form of EBV (8).

Recombinant Retroviral Infection. JY(LCL), an EBV-transformed B cell line, was infected with a recombinant retrovirus, derived from pLXSN generously supplied by Dusty Miller (University of Washington, Seattle). An expression construct for human CD5 was produced by recombining the coding sequence (obtained from Jane Parnes, Stanford University, Palo Alto, CA) with pLXSN by using the *Eco*RI–*Bam*HI site. The packaging cell lines PE501 and PA317 were used consecutively to obtain recombinant, infectious virus coding for human CD5. JY(LCL)-CD5

Abbreviations: EAS, enzymatic amplification staining; EBV, Epstein–Barr Virus; LMP-1, latent membrane potential 1.

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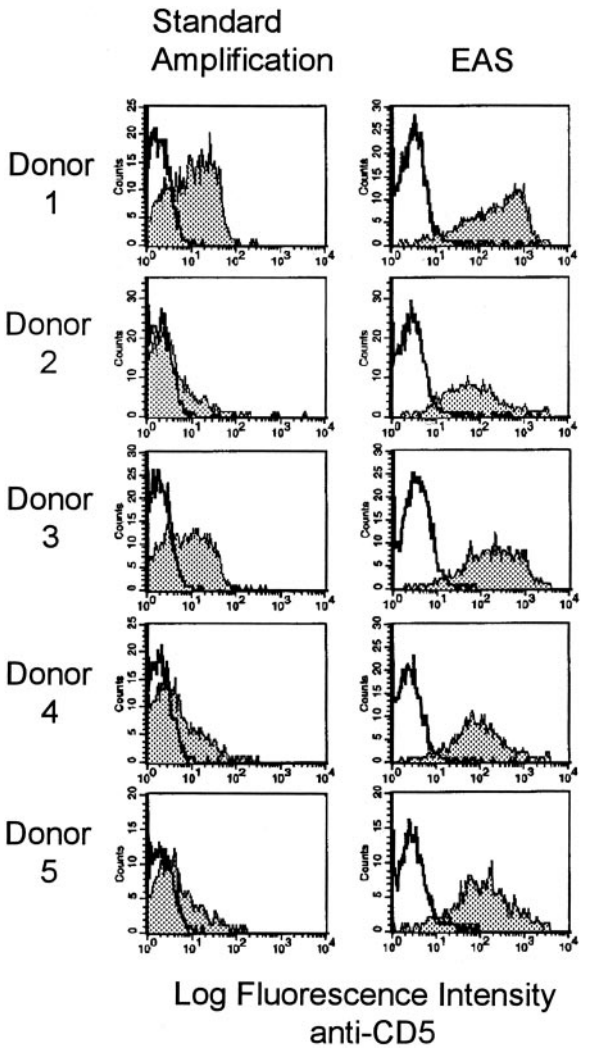

Fig. 1. Human B lymphocytic CD5 expression assessed by EAS and a standard amplification procedure. Peripheral blood cells from five healthy donors were simultaneously stained with anti-CD19 antibodies conjugated to phycoerythrin and with 100 ng of biotinylated anti-CD5 monoclonal antibody for conventional amplification (indirect staining) or with 10 ng of the same antibody for EAS. EAS was performed with a kit from Flow-Amp Systems. Equivalent amounts of isotype control biotinylated murine IgG1 (open histograms) were assessed in parallel with biotinylated anti-CD5 (shaded histograms). Fluorescein isothiocyanate was used as the detecting fluorochrome for both the standard and EAS procedures. CD5 expression on the B cells was assessed in the FL1 channel after gating on the CD19-expressing cells determined in the FL2 channel. Fluorescence in the FL1 channel due to phycoerythrin was eliminated by setting compensation using cells stained for CD19 alone. Similar results were obtained with cells from nine additional healthy donors.

cells were obtained by culturing the infected cells in neomycin and subcloning.

Results

Peripheral blood mononuclear cells were obtained from healthy volunteers and stained with anti-CD19 conjugated to phycoerythrin to define the B lymphocytes and with anti-CD5 either by a standard amplification format or EAS (Fig. 1). EAS was performed with a kit from Flow-Amp Systems. As has been reported (3, 9), conventional amplification procedures demonstrated that human peripheral blood B lymphocytes can be divided into two subpopulations defined by the expression of

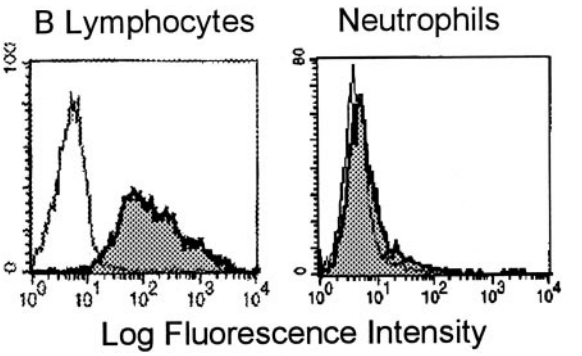

Fig. 2. Comparison between CD5 expression by B lymphocytes and neutrophils. Peripheral blood mononuclear cells and peripheral blood neutrophils were obtained by gradient centrifugation. The mononuclear cells were stained with anti-CD19PE to identify B lymphocytes, and the cells were stained simultaneously with either 10 ng of biotinylated control IgG1 (open histogram) or 10 ng of biotinylated anti-CD5 IgG1 (shaded histogram). The B lymphocytes are shown by gating on the lymphocytic population and the CD19-phycoerythrin-positive population. The neutrophils were stained with either 10 ng of biotinylated control IgG1 (open histogram) or 10 ng of biotinylated anti-CD5 IgG1 (shaded histogram). Binding of the biotinylated antibody was detected by EAS with a kit from Flow-Amp Systems.

CD5. However, B lymphocytic CD5 expression, as ascertained by the standard amplification procedure, was dim and there was no clear-cut demarcation between negative and positive cells. These results are similar to results obtained by others (3, 9). Conversely, detection of surface CD5 expression on B cells by EAS clearly indicated that essentially all of these cells expressed CD5. It should be noted that the level of CD5 expression by these cells varied considerably. B lymphocytes from 16 different donors were assessed and gave similar data, indicating that essentially all of the B cells expressed CD5. Similar results were obtained with two additional, distinct anti-CD5 monoclonal antibodies from different manufacturers (CalTag and BD Biosciences PharMingen). B cells from the peripheral lymphoid organs (spleen and tonsils) were also uniformly positive for CD5 (data not shown).

Although we have demonstrated that stain is not transferred from one cell to another with EAS, we considered the possibility that the high level of CD5 expression on T cells could affect the staining of the molecule on B cells. This inappropriate transfer of stain, bystander staining, was unlikely, because EAS demonstrated CD5 expression on essentially all B cells after T cells had been removed ($<3\%$ T lymphocytes) by sheep red blood cell rosetting (data not shown). Consequently, these results indicate that bystander staining could not account for the detection of the molecule on B cells. To assess the possibility that CD5 expression on B cells may have resulted from adsorbed soluble CD5 originating on T cells, we acid-treated the cells before staining and found that this treatment did not influence CD5 expression on the B cells. We ascertained that the acid treatment was effective because it removed previously bound antibodies from the cell surface (data not shown). Furthermore, neutrophils from the peripheral blood were assessed for CD5 by EAS and found to be negative, further indicating the specificity of EAS detection (Fig. 2). Thus, our results indicate that the B1 and B2 subset discrimination of human B lymphocytes based on a relatively insensitive analysis of CD5 expression cannot be corroborated by high-resolution immunophenotyping with EAS.

We also assessed the expression of CD5 on several different EBV-positive human B cell lines. EBV-transformed B cells from two different donors did not demonstrate surface expression of CD5 expression as determined by EAS (Fig. 3). These cell lines also did not contain CD5 mRNA as determined by reverse transcription PCR (data not shown). Analysis of 11 different

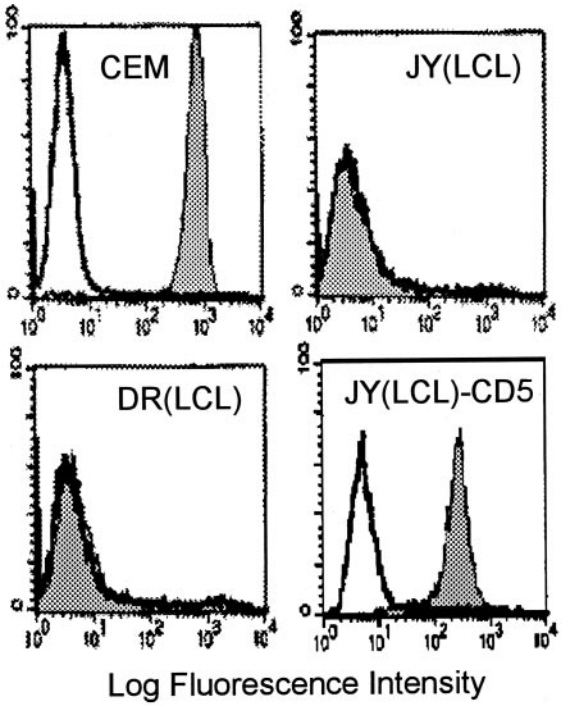

Fig. 3. Absent CD5 expression on EBV-transformed human B lymphocytes. CEM, a human T cell tumor line, two EBV-transformed human B cell lines, JY(LCL) and DR(LCL), and JY(LCL)-CD5, JY(LCL) cells transfected with a recombinant retrovirus encoding CD5 expression, were stained by EAS for CD5 expression by using 5 ng of biotinylated anti-CD5 monoclonal antibody for CEM, 100 ng for JY(LCL)-CD5, 500 ng for JY(LCL) and DR(LCL) (shaded histograms), or equivalent amounts of biotinylated control IgG1 (open histograms). For panels that appear to contain only one histogram, the shaded and open histograms precisely overlap. EAS was performed with a kit from Flow-Amp Systems. Results with equivalent amounts of biotinylated isotype control murine IgG1 are also shown.

EBV-positive B cell lines (DK, LL58, Akata-A.3, Namalwa, Daudi, MH, P3 h1, JR, JS, and CMC) by EAS revealed that none of them expressed CD5.

Because we had shown that peripheral blood B lymphocytes uniformly express CD5, we considered the possibility that EBV transformation resulted in the inhibition of CD5 expression. We exposed sheep erythrocyte rosette-negative peripheral blood cells to EBV-containing B95-8 culture supernatant and assessed the B cells for surface CD5 expression and the presence of latent EBV antigen over several weeks of culture. Our data demonstrate that EBV transformation, as assessed by the intracellular expression of the EBV-latent protein LMP-1 with EAS, was accompanied by the loss of CD5 surface expression (Fig. 4). Conversely, *in vitro* activation of B cells for 3 or 4 days in the presence of anti-CD40, IL-4, and/or anti-IgM did not reduce CD5 expression (data not shown). Thus, the loss of CD5 expression by EBV-transformed B cells in culture was not simply caused by *in vitro* activation.

It is possible that the EBV-transformed cells represent the outgrowth of the few peripheral blood B cells that did not express CD5. This possibility does not seem likely because we did not observe the gradual appearance of a distinct CD5-negative cell subpopulation and a concomitant diminution of the broad peak of CD5-positive cells; instead we saw a gradual decrease of CD5 expression over the entire population of B cells as seen most clearly in the day 16 analysis (Fig. 4).

It should be noted in Fig. 4 that the background surface staining of the cells undergoing transformation increased with time. We kept the voltage settings identical throughout the

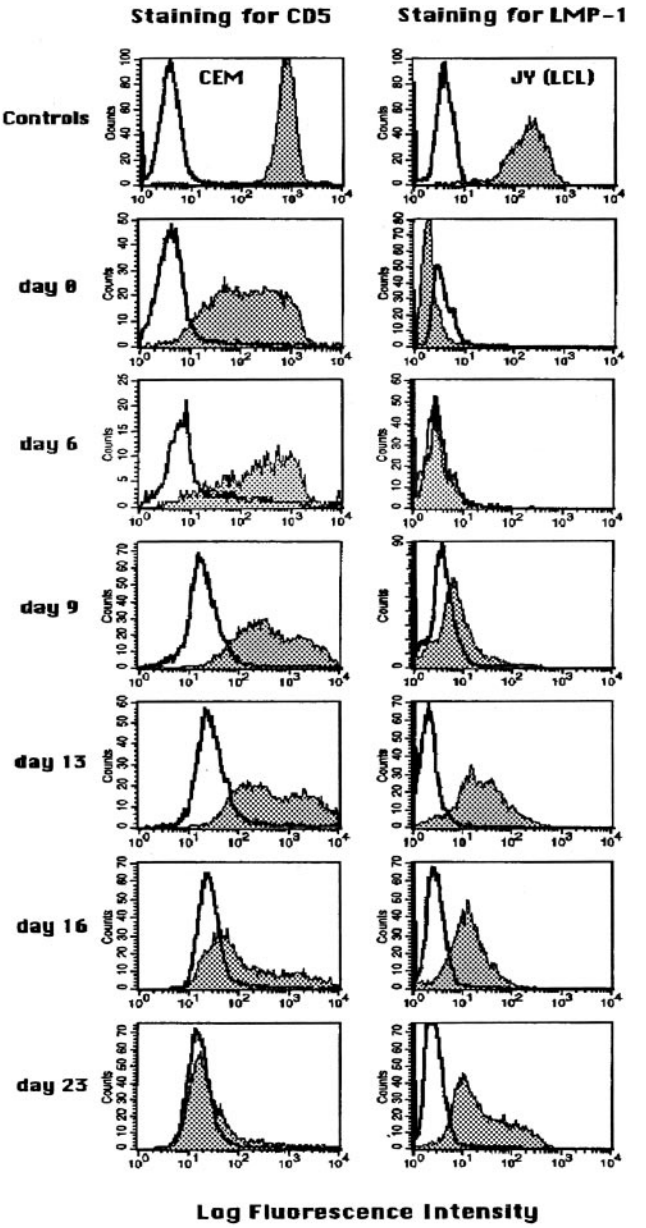

Fig. 4. Loss of CD5 expression by human B lymphocytes on EBV transformation. Peripheral blood mononuclear cells were obtained from a healthy volunteer, and T lymphocytes and natural killer cells were removed by sheep erythrocyte rosetting. The remaining cells, B lymphocytes and monocytes, were exposed to EBV-containing medium from the B95-8 cell line and assessed serially for both cell surface CD5 expression by EAS, gating on CD19 expressing cells, and intracellular EBV LMP-1 expression. The intracellular antigen expression was assessed by an EAS kit from Flow-Amp Systems. The anti-LMP-1 monoclonal antibody was obtained from Dako, and the validity of this technique was ascertained by staining EBV-positive and EBV-negative cell lines. The top panels show control staining for CD5 on CEM cells and for LMP-1 on JY(LCL) cells. Staining with isotype control antibodies is shown as open histograms, and staining with specific antibodies is shown as shaded histograms. These results are representative of three experiments.

experiment; consequently, the increase in the background surface staining is most probably a reflection of the increasing size of the cells concomitant with viral transformation. The increase in background first appeared on day 9, when the EBV antigen also first appeared. There was no increase in background staining for the intracellular antigen LMP-1. It is likely that the

failure to observe an increase in the background with these cells reflects the loss of intracellular fluorescent compounds because of the permeabilization of the cells by detergents.

To see whether an EBV-transformed cell could inhibit the expression of an exogenously introduced CD5 gene, we infected JY(LCL) with a recombinant retroviral vector that drives the expression of CD5 by means of a long terminal repeat promoter. CD5 was clearly expressed by these cells (Fig. 3). Because the wild-type JY(LCL) did not express CD5 mRNA, it seems likely that the regulation of CD5 expression by EBV occurs at the transcriptional level, which can be overcome by the retroviral construct.

Discussion

Our data conclusively demonstrate that nontransformed B lymphocytic subsets cannot be distinguished on the basis of CD5 expression. These results may account for the previous paradoxical finding that both CD5-expressing and CD5-nonexpressing B cells possess equivalent levels of CD5 mRNA (10). Moreover, our findings also help to explain and give credence to the prior demonstration of low levels of CD5 on all murine B lymphocytes (11, 12).

Importantly, the association of CD5-expressing B cells with the production of autoantibodies (1–3) cannot be a result of distinct subsets based on CD5 alone, but instead might be better explained as a quantitative phenomenon. Thus, the continuous presence of autoantigens or antigens derived from microbial flora may stimulate specific B cells so that they express more surface CD5 than B cells with specificities to antigens that are not continuously present in the body. B cells that express more CD5 are more likely to be detected as CD5 positive by the relatively insensitive conventional staining technology. In this sense, CD5 could represent an activation antigen. Consequently, the enhanced expression of CD5 on these cells may be related to their continual activation state. Nevertheless, it should be noted that we did not assess the possibility that CD5 acts as an activation antigen on B cells in the experiments reported in this article. Alternatively, the production of autoantibodies could be related to distinct B cell subsets based on criteria other than the surface expression of CD5.

CD5 has been shown to be important for the apoptosis of antigen-receptor induced B lymphocytes and for the maintenance of tolerance by anergic B cells (12, 13). The inhibition of CD5 expression by EBV transformation suggests that the virus down-regulates this molecule to prevent the apoptosis of the transformed cells, and thereby allows for the persistence of the latently infected cells.

The dim expression of CD5 on EBV-transformed cells has been reported (10); however, the analysis of the cells by these investigators occurred only 21 days after transformation. We showed loss of CD5 by day 23; however, with different initial viral inocula, it is likely that the time to transformation varies. Thus, the failure of these investigators to observe the complete loss of CD5 expression is probably a reflection of differential kinetics. Other scientists have suggested that EBV transformation may result in the inhibition of CD5 expression (14–16). In one study, none of the EBV-transformed cell lines that were derived expressed CD5, including 23 lines obtained from $CD5^+$ B lymphocyte cellular fractions. The results from another study indicated that only 1 of 50 EBV-transformed B cell clones expressed CD5. Similarly, EBV transformation of cord blood or fetal liver B cells, which express CD5, gave one clone that expressed CD5 compared with nine clones that did not. Our findings confirm the suggestions of these scientists that EBV transformation results in the inhibition of CD5 expression. By amplifying the fluorescent signal, we have been able to demonstrate the gradual loss of surface CD5 on B cells as they become transformed by EBV.

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