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Decreased expression of complement receptor type 2 (CR2) on neoplastic B cells of chronic lymphocytic leukaemia

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SUMMARY

Neoplastic cells from 49 patients with B cell chronic lymphocytic leukaemia (B-CLL) were studied and compared with normal peripheral and tonsillar B cells using CD21 monoclonal antibodies. Membrane expression of CR2 was quantified by calibrated flow cytometry and by binding analysis with radiolabelled antibody. Both assays indicate that B-CLL cells express only 30% of the CR2 found on normal B cells. These findings are further evidence of the aberrant phenotype of B-CLL cells.

Keywords CR2(CD21) B-CLL flow cytometry

INTRODUCTION

Human complement receptor type 2 (CR2, CD21) is a 145-kD glycosylated single polypeptide chain (Weis, Tedder & Fearon, 1984) found on the surface membranes of mature B lymphocytes in circulating human blood (Nadler *et al.*, 1981), follicular dendritic cells in lymphoid organs and some T lymphoblastoid cell lines. It is the receptor for complement proteolytic fragments C3d, C3d, g and iC3b (Ross *et al.*, 1983) and for the Epstein–Barr virus (EBV) which is involved in the aetiology of infectious mononucleosis, African Burkitt's lymphoma and nasopharyngeal carcinoma. Molecular cloning of the cDNA encoding CR2 using a Raji cell line library (Moore *et al.*, 1987) has shown a complement receptor locus on chromosome 1 at 1q32. The derived amino acid sequence places CR2 in a multigene family known as the regulators of complement activation (Hourcade, Holers & Atkinson, 1989). A common feature of this family is the presence of tandem repeats of 60–75 amino acids termed short consensus repeat (SCR) units showing homology with CR1, C4-binding protein and factor H (Weis *et al.*, 1986). The extracellular portion of CR2 comprises 15 SCRs (Weis *et al.*, 1988), the binding sites of C3d,g and EBV lying within the two SCRs at the amino terminus (Lowell *et al.*, 1989).

CR2 is implicated in transmembrane signalling. Occupancy of CR2 by sepharose cross-linked C3d, C3d,g in serum-free medium, T cell-independent EBV or monoclonal antibodies (MoAbs) to CR2 in the presence of T cell-derived B cell growth factors leads to B cell activation and proliferation. Following *in vitro* treatment of B cells with phorbol-12-myristate-13-acetate (PMA), anti-μ antibody or *Staphylococcus aureus* Cowan I strain, phosphorylation of CR2 occurs (Changelian & Fearon, 1986; Barel *et al.*, 1986). In addition, ligation of CR2 has been shown to induce the release of cytoplasmic free calcium and to augment B cell activation when stimulated by anti-μ (Carter *et al.*, 1988). This suggests that ligand-binding of CR2 is associated with *in vivo* immunoregulation.

We have studied the expression of CR2 on the B cells of patients with B cell chronic lymphocytic leukaemia (B-CLL). In this malignancy, there is a clonal proliferation of apparently immunologically incompetent B lymphocytes, which can be distinguished from normal B cells by light chain restricted expression and faint immunofluorescence of surface immunoglobulin and the presence of the T cell antigen CD5 (Boumsell *et al.*, 1978; Royston *et al.*, 1980). They are candidate malignant counterparts of autoantibody producing $CD5^+$ B cells found within the normal human B cell population (Casali *et al.*, 1987). This is supported by *in vitro* studies where B-CLL $CD5^+$ B cells are capable of producing autoantibody (Sthoeger *et al.*, 1989) and clinically by occurrences of autoimmune haemolytic anaemia and thrombocytopenia in these patients. Despite the morphologically mature appearance of the B-CLL B cell, the membrane phenotype includes immature B cell features such as the ability to form spontaneous rosettes with mouse erythrocytes as well as B cell activation markers signifying a degree of maturity. CR2 is also expressed on B-CLL cells, originally demonstrated by the binding of erythrocyte–antibody–complement complexes, EAC1-3d (Ross *et al.*, 1973). It is known that B cell function in B-CLL is defective, demonstrated by hypogammaglobulinaemia and lack of humoral response to foreign antigen (Hamblin, Jones & Peacock, 1975).

These features indicate disordered responses to immune stimuli, and since CR2 is implicated in transmembrane signal-

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ling, we have determined CR2 expression on the surface of B-CLL cells in an attempt to add to our understanding of this immune deficit.

MATERIALS AND METHODS

Samples

Heparinized blood (20 ml) was obtained from 49 patients with B-CLL defined by clinical, morphological and immunophenotypical characteristics as proposed by the FAB (French–American–British) Cooperative Group (Bennett *et al.*, 1989). All patients were Binet stage A or B (Binet *et al.*, 1981) and clinically stable at the time of testing. B cells from peripheral blood samples of healthy volunteers, buffy coats from donors at the South London National Blood Transfusion Centre, UK, and tonsils from routine tonsillectomies served as controls. All participants gave informed consent. The Burkitt's lymphoma-derived cell line, Raji, was used as a positive control for the expression of CR2.

Cell preparation

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll–Paque density centrifugation (Pharmacia, Uppsala, Sweden). B cell enrichment was achieved by depletion of adherent monocytes by incubation in plastic culture dishes for 1 h at 37°C. Some B cells adhered during this step. However, neither the proportion of B cells expressing CR2 (effectively 100%) nor the relative intensity of CR2 expression (see below) was altered in the post-adherence cell suspension as a result. The non-adherent cells were then T cell-depleted by AET-treated sheep erythrocytes rosette formation (Kaplan & Clark, 1974) and subsequent Ficoll–Paque separation. The cells at the interface were considered as enriched B cells.

Cell suspensions from human tonsils were centrifuged through Ficoll–Paque prior to T cell depletion of mononuclear cells at the interface as described for PBMC.

Viability, tested by trypan blue exclusion, was found to be >94%.

Monoclonal antibodies

Four MoAbs to the CD21 molecule (CR2) were employed in immunofluorescence labelling. CR2 (HB-5; Tedder, Clement & Cooper, 1984), an IgG2a, MoAb, was obtained from Becton Dickinson (Mountain View, CA) (henceforth called BD anti-CR2); OKB7 (Nemerow *et al.*, 1985), also an IgG2a MoAb, was obtained from Ortho Diagnostics (Raritan, NJ); B2 (Nadler *et al.*, 1981), an IgM MoAb, was obtained from Coulter Electronics (Hialeah, FL); and RFB6 (Campana *et al.*, 1985), an IgG1 MoAb obtained from the Royal Free Hospital Medical School, London, UK, was used in the form of supernatant for immunofluorescence staining and as ascites for radiolabelling. RFB6 ascites was affinity purified by the method of Ey, Prowse & Jenkins (1978). An IgG1 CD19 MoAb used as a *pan*-B cell marker was obtained from Becton Dickinson. MHM6 (CD23), a B cell activation marker (Rowe *et al.*, 1982) was a kind gift from Dr J. Gordon. FITC-conjugated rabbit anti-mouse IgG $F(ab')_2$ fragment was obtained from Dako (High Wycombe, UK).

Fluorescence staining

The indirect method of surface membrane antigen labelling was employed. Briefly, 50 μl of a 20×10^6/ml cell suspension were incubated with saturated binding concentrations of each CD21 MoAb at 4°C on ice for 30 min. After two washes in 3-ml volumes of washing solution containing 0·01 M phosphate-buffered saline (PBS), pH 7·3, 5% heat-inactivated fetal calf serum and 0·2% sodium azide, 100 μl of a 1/20 dilution of FITC-conjugated rabbit anti-mouse IgG $F(ab')_2$ were added to the cell pellet. After a further 30-min incubation at 4°C on ice, unbound FITC-conjugated rabbit anti-mouse IgG $F(ab')_2$ was removed by washing three times in washing solution. If cells were not analysed within 4 h, 400 μl of 2% paraformaldehyde solution were added to prevent loss of fluorescence (Lanier & Warner, 1981). Both direct and indirect fluorescence labelling were carried out using MoAbs B2 and B2 pre-conjugated to FITC (Coulter Electronics). The direct method involved incubation of test cells with the optimal dilution of B2FITC for 30 min at 4°C on ice then three washes in washing solution.

Negative controls were carried out by substituting the primary MoAb with either an irrelevant mouse immunoglobulin, tissue culture medium or by omitting the first layer.

Flow cytometric analysis

Standard fluorescent beads (Immuno-Brite, Coulter Electronics), standard I and II expressing 1000 and 31000 fluorescein equivalents, respectively, were employed for the daily calibration of an EPICS V (Coulter Electronics) flow cytometer equipped with an argon ion laser tuned to 488 nm and power of 300 mW. High voltage and gains adjustment were made in such a manner as to allow the greatest linear signal from the integral green fluorescence (IGFL) photomultiplier tube to fall into channel 255 when interrogating standard II (Fig. 1). Standard I, test and control cells were interrogated using the same settings. The mean number of fluorescein equivalents per cell was interpolated from the mean channel of brightness of beads and cells (see Results).

Radiolabelling and binding studies

Affinity purified RFB6 MoAb was radiolabelled with ^{125}I by the iodogen method (Fraker & Speck, 1978). The antibody was labelled to a specific activity of 0·35 μCi/μg of IgG.

The method of quantifying CR2 on B cells using ^{125}I-RFB6 was as described by Hogg *et al.* (1984). Binding-curve antibody dilutions were made in duplicate. Using a computer program designed to run on a Macintosh computer, non-specific bound ^{125}I-RFB6 was subtracted from total bound ^{125}I-RFB6 to determine specific cell-bound ^{125}I-RFB6. We achieved an enrichment of B cells for normal controls in the range of 41–65%. Therefore, a suitable correction was made for this in ascribing number of receptors per B cell when applying Scatchard analysis to determine receptor numbers and affinity, assuming that one molecule of RFB6 bound to one CR2 molecule.

Controls used to verify specific binding of RFB6 to CR2 on B cells were performed using the same techniques as for test cells, substituting cells from a patient with T cell chronic lymphocytic leukaemia (T-CLL) and blocking Fc receptors by prior incubation of patient B cells and tonsillar B cells in 0·01 M PBS, pH 7·3, containing 2% heat inactivated human **AB** serum for 15 min at 4°C on ice. Cells bound zero ^{125}I-RFB6.

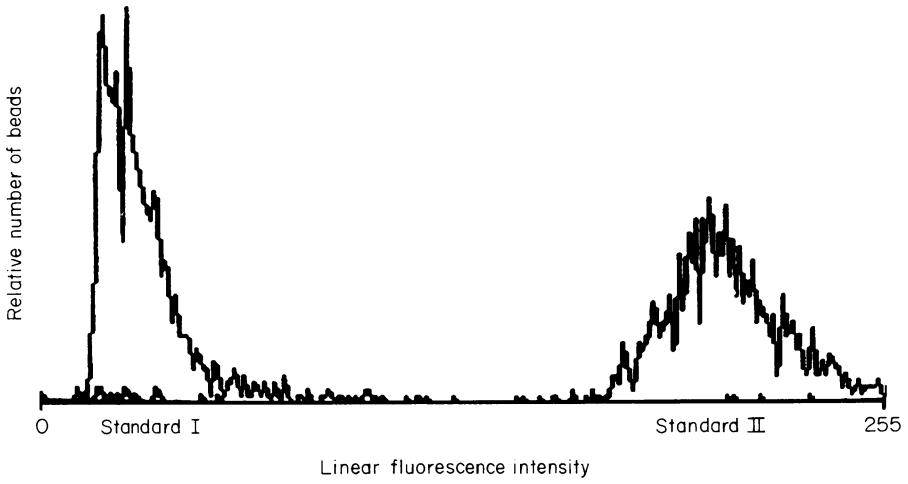

Fig. 1. Integral green fluorescence (IGFL) histograms of standards I and II representing 1000 and 31000 fluorescein equivalents, respectively. Mean channel of fluorescence for each standard is obtained from the EPICS V Stats package.

Statistical analysis

The Student's *t*-test was used to compare data from B-CLL patients, normal controls and tonsillar B cells.

RESULTS

Flow cytometric analysis

The histogram profile for calibration of the EPICS V flow cytometer employing the two sets of standard fluorescent beads (standards I and II) is shown in Fig. 1. The high voltage settings for this machine over a period of 12 months varied from 1510 to 1570 volts with a constant gain of 50. Analysing IGFL signals, the mean number of fluorescein equivalents of test and control cells was calculated by the formula:

$$\text{Mean number of fluorescein equivalents per cell} = \frac{(\text{Brightness of standard II} - \text{Brightness of standard I}) \times \text{Test IGFL mean channel}}{\text{IGFL mean channel of standard II} - \text{IGFL mean channel of standard I}}$$

where Test IGFL mean channel was obtained from the EPICS V Immuno statistical package and IGFL mean channel of standards I and II from the EPICS V Stats package. Typical values for mean channel fluorescence for standards I and II were 30 and 203, respectively.

Representative flow cytometry histograms from which the mean channel of fluorescence intensity of IGFL has been derived when using MoAbs to CR2 on peripheral blood B cells of B-CLL patients, normal control individuals and tonsillar B cells, and negative controls where the primary antibody was substituted with an irrelevant mouse immunoglobulin are shown in Fig. 2. Numerical values derived from these histograms are shown in Table 1 indicating that when using the RFB6 MoAb, leukaemic B cells express $28 \pm 19\%$ of CR2 found on normal control B cells. Similar results were obtained with three further MoAbs, namely BD-anti-CR2, OKB7 and B2, all directed against different epitopes on the CR2 molecule. An example of the close correlation between these CD21 MoAbs when related to B-CLL B cells is shown in Fig. 3a, where $r = 0{\cdot}964$ when comparing OKB7 and B2 with RFB6 and $r = 0{\cdot}928$ for BD-anti-CR2 with RFB6. A distribution histogram of CR2 fluorescein

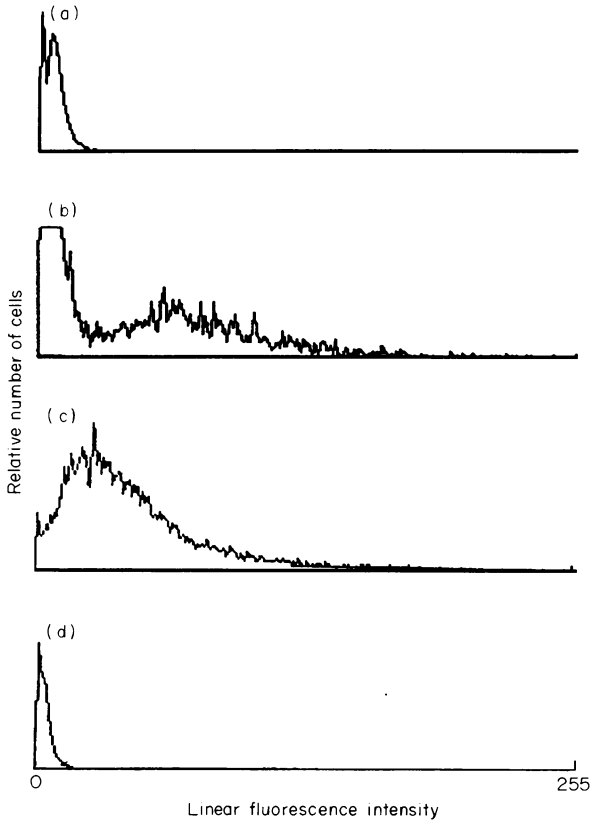

Fig. 2. Representative histograms of linear fluorescence intensity of B cells from (a) a B-CLL patient; (b) a normal control; (c) a tonsil, each labelled with the CD21 MoAb RFB6; and (d) B cells labelled with irrelevant mouse immunoglobulin. Mean channel of fluorescence for individual samples is obtained from the EPICS V Immuno statistical package.

Table 1. Fluorescein equivalents per B cell derived from mean channel fluorescence (mean ± 2 s.e.m.)

MoAb	B-CLL patient B cells ($n=49$)	Normal control B cells ($n=13$)	Tonsillar B cells ($n=13$)	Raji cells ($n=5$)
RFB6	3200 ± 500	11 400 ± 2600	7000 ± 1100	21 900 ± 6500
BD-anti-CR2	3500 ± 600	12 600 ± 3000	9100 ± 2000	25 000 ± 9600
OKB7	3500 ± 600	11 800 ± 3000	9200 ± 1900	23 300 ± 8800
B2	2100 ± 300	5900 ± 3500	5000 ± 700	16 900 ± 6600
B2FITC	3000 ± 400	7000 ± 2000	6000 ± 800	15 000 ± 6000
Pan-B cell CD19	8400 ± 900	16 300 ± 3200	8800 ± 1700	

Numerical results derived from integral green fluorescence (IGFL) histograms show that leukaemic B cells express approximately 30% of CR2 molecules found on normal B cells.

equivalents per B cell of B-CLL patients, normal individuals and tonsillar B cells with RFB6 is shown in Fig. 3b.

The B cells of 13 patients were tested with the activation marker MHM6 (CD23). This antigen was present in 12 out of 13 samples, being expressed on 66–99% of B cells.

Radiobinding studies

Figure 4 displays representative binding curves and Scatchard plots of the binding of ^{125}I-RFB6 to CR2 on the B cells of B-CLL patients and tonsillar B cells. Analysis of the Scatchard plots for binding of ^{125}I-RFB6 to CR2 on test cells gave a mean affinity of 0·4 nM^{-1}. Numbers of CR2 per B cell from normal controls, tonsils, patients with B-CLL and Raji cells are shown in Table 2.

DISCUSSION

We found that CR2 expression on the B cells of B-CLL patients was approximately 30% of that on normal control B cells. Close correlation between several CD21 MoAbs was achieved when using an amplification two-layer technique. These findings were further substantiated by radioligand binding assays. This results confirms and extends the observations of other investigators who found that a high proportion of the B cells of B-CLL patients commonly express CR2 (Freedman *et al.*, 1987) but to a lower intensity than normal peripheral B cells (Anderson *et al.*, 1984). CR2 numbers on normal B cells, tonsillar B cells and Raji cells are similar to those in other reports (Rasmussen *et al.*, 1988). The assignment of numerical values to fluorescence intensity allows prediction of the probability of an individual falling into the B-CLL or normal group. The result of lower numbers of CR2 when using the MoAb B2 may relate to the site of B2 epitope lying within SCRs 12–14 whereas the OKB7 and BD-anti-CR2 MoAbs bind within SCRs 1 and 2 (Lowell *et al.*, 1989); the binding site location for RFB6 used here both in immunofluorescence and radiobinding studies is unknown. Steric hindrance may be a further factor influencing disparate CR2 numbers as the B2 MoAb used was an IgM antibody, all others being IgG.

The significance of decreased numbers of CR2 on B-CLL B cells is unknown. The precise B cell lineage and developmental

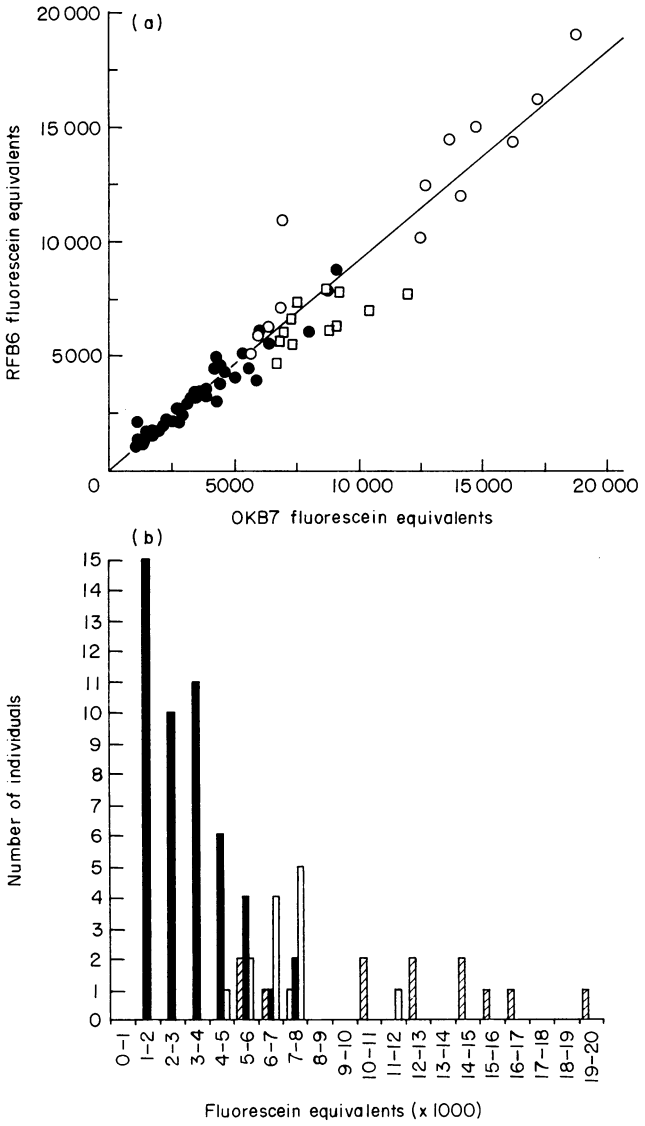

Fig. 3. (a) Comparison of CR2 fluorescein equivalents per B cell of B-CLL patients (●); tonsils (□); and normal controls (○), using MoAbs OKB7 and RFB6; (b) distribution histogram of CR2 fluorescein equivalents per B cell labelled with the CD21 MoAb RFB6 from 49 B-CLL patients (■); 13 tonsils (□); and 13 normal controls (■).

stage of these B cells indicated by the immunophenotype has been an enigma. The low expression of endogenous surface membrane immunoglobulin distinguishes B-CLL from other B lymphoproliferative disorders and is a useful diagnostic feature. The reproducible finding of low expression of CR2 on B-CLL B cells may be added to the diagnostic panel of tests to identify this disorder. Comparison of expression of CR2 in B-CLL with that in normal circulating human CD5$^+$ B cells would be of great interest. However, current cell separation methods do not allow sufficient purification of this minor population from adult human blood for quantitative binding analysis. Work on this problem is in progress, but meanwhile the clonally expanded B-CLL B cell can be regarded as displaying a homologous membrane phenotype to its physiological counterpart.

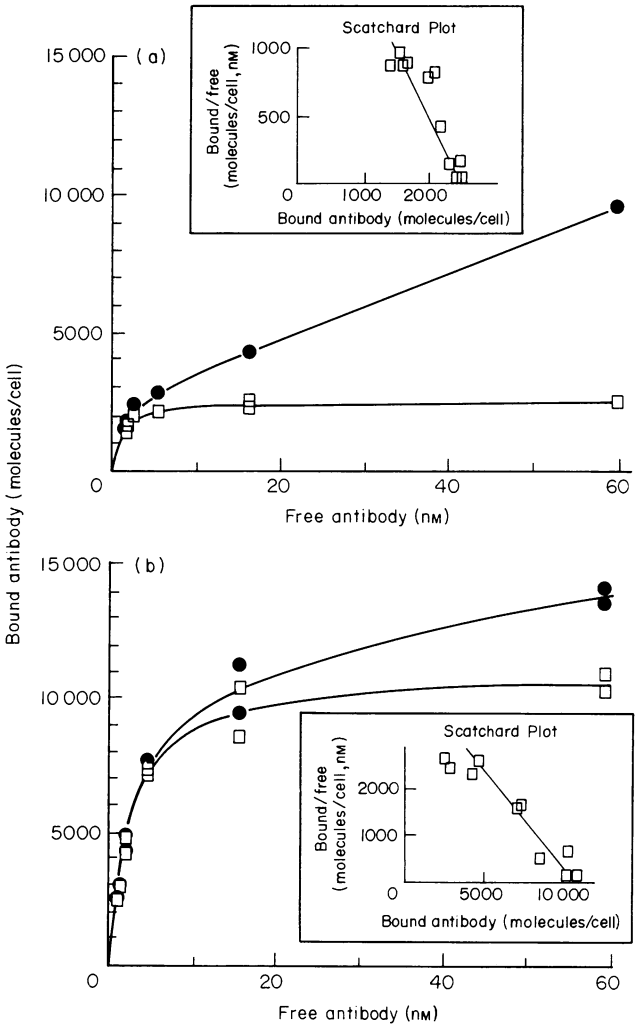

Fig. 4. Representative radiobinding curves and Scatchard plots of CR2 on B cells of (a) B-CLL patients and (b) tonsils. Binding curve antibody dilutions were made in duplicate. ●, Total; and □, specific antibody bound.

Table 2. CR2 per B cell derived from binding curves and Scatchard analyses (mean ± 2 s.e.m.)

	B-CLL patient B cells ($n=25$)	Normal control B cells ($n=2$)	Tonsillar B cells ($n=7$)	Raji cells ($n=4$)
Radiobinding of ^{125}I-RFB6	4200 ± 1500	17 600 ± 9400	12 000 ± 3300	28 900 ± 20 500

Numerical values of CR2 molecules per B-cell derived from binding curves and Scatchard plots.

Cell-surface CR2 molecules on normal peripheral blood B cells are essential for EBV-induced proliferation and differentiation (Tedder *et al.*, 1986). Despite the decreased expression of CR2 on B-CLL B cells, EBV is capable of stimulating these cell to proliferate and secrete monoclonal immunoglobulin in short-term culture (Deegan & Maeda, 1984). A contributory factor in this stimulation may be the requirement of only one infectious virus particle to cause immunoglobulin production in normal B cells (Yarchoan *et al.*, 1983). However, difficulty has been encountered in establishing immortalized cell lines from this source (Fu, Chiorazzi & Kunkel, 1979) suggesting CR2 density on these B cells may not permit this phenomenon. Nevertheless, it appears that determinants other than CR2 density affect EBV infection. For example, EBV DNA has been found by hybridization techniques in several cases of T cell lymphoma associated with chronic EBV infections (Jones *et al.*, 1988) and lethal midline granuloma (Harabuchi *et al.*, 1990); CR2 was expressed on the neoplastic T cells of only two out of eight cases.

Studies of normal B cell maturation show that CR2 is expressed in a stage-specific manner on early B cells (those with IgM alone) and on mature B cells (those with IgM + IgD) but not on pre-B cells or plasma cells (Bofill *et al.*, 1985; Campana *et al.*, 1985). The low expression of CR2 on neoplastic B-CLL B cells together with IgM expression (Grey, Rabellino & Pirofsky, 1971) or IgM + IgD (Fu, Winchester & Kunkel, 1974) is therefore anomalous. Opinions differ as to the origin of the $CD5^+$ B cell. Some think of it as a separate cell lineage (Hayakawa *et al.*, 1985), others as an activated B cell (Werner-Favre *et al.*, 1989). If the $CD5^+$ B-CLL B cell is viewed as the latter, and as CR2 is lost on activation of B cells (Stashenko *et al.*, 1981), this could be an explanation for the decreased expression of CR2 on B-CLL cells. In the present study, B cells of 92% of patients tested demonstrated positivity with the activation marker MHM6 (CD23). Alternatively, it has been suggested that in B-CLL, only the lower density CR2-expressing B cells are found in the circulating blood while higher density CR2-expressing B cells are trapped in the white pulp of the spleen where the microenvironment may influence CR2 expression, cell transformation and proliferation (Lampert, Hegde & Van Noorden, 1990).

Studies of quantitative expression of CR2 on B-CLL cells in response to immunoregulators and other stimuli are indicated in order to elucidate the CR2-related responses of these unusual B cells.

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