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Immune Complexes and Apoptosis in B-cell Chronic Lymphocytic Leukemia

ROMINA GAMBERALE*, JORGE R. GEFFNER and MIRTA GIORDANO

Laboratorio de Immunología, Instituto de Investigaciones Hematológicas, Academia de Medicina, Pacheco de Melo 3081-1425, Buenos Aires, Argentina

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The progressive accumulation of B-cell chronic lymphocytic leukemia (B-CLL) cells *in vivo* is attributed to resistance to apoptosis, although this can be modulated *in vitro* by a variety of cellular and humoral factors (cell–cell, cell–matrix interactions, cytokines). We have previously reported that IgG immune complexes (IC) delay B-CLL cell apoptosis through a paracrine mechanism, which depends on monocytes and NK cells. On the other hand, despite the fact that IC effectively bind to type II FcγRs expressed on B-CLL cells, they are unable to deliver transmembrane signals. We speculate that this lack of responsiveness of resting B-CLL cells to IC could be overcome by activation. The analysis of this possibility would be relevant since the presence of circulating IC is a common feature in B-CLL patients.

Keywords: B-CLL; Apoptosis; Immune complexes; FcγRs

INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) represents a neoplastic disorder caused by defective apoptosis, as opposed to increased cell proliferation [1–3]. It is characterized by the progressive accumulation of monoclonal CD5+ B cells, with the appearance of mature lymphocytes. Although no specific chromosomal alteration(s) responsible for this disease has been described until now, the gene setting of B-CLL appears optimally organized to avoid apoptosis. In fact, the great majority of B-CLL cases show an upregulated expression of the anti-apoptotic gene bcl-2 in the absence of the t(14;18) translocation [4,5]. Other members of the bcl-2 family with anti-apoptotic characteristics, like Mcl-1 and BAG-1, are also overexpressed in B-CLL cells [6,7].

Many studies have been devoted to describe the phenotypic and functional characteristics of B-CLL cells, however their origin and stage of differentiation remain questionable. They express most of mature B cell markers (CD19, CD20, HLA-DR), but their distinctive characteristic is the coexpression of CD5 and the very low levels of surface Ig, usually IgM [3,8,9]. Importantly, these monoclonal Igs often present polyreactive autoantibody activity and frequently behave as a rheumatoid factor, i.e. they bind to the Fc portion of IgG [10–12]. Another feature of B-CLL cells is their inability to respond adequately when stimulated through the B-cell receptor (BCR), which strictly correlates with an altered pattern of protein tyrosine phosphorylation and a defective Ca^{++} response [13–15]. Based on these characteristics, it has been proposed that the normal counterpart of B-CLL cells may be a mantle zone subpopulation of anergic self-reactive B lymphocytes with constitutive high levels of bcl-2 [16].

Despite their prolonged survival *in vivo*, B-CLL cells die by apoptosis when cultured *in vitro* in short-term incubations [4,17,18]. Therefore, it was proposed the existence *in vivo* of a survival-promoting microenvironment [3,19]. A variety of cellular and humoral factors have been reported to prevent apoptosis of B-CLL cells. Cytokines, like IL-4 [20,21], IL-8 [22] and interferons α [23,24] or γ [25], as well as cell–cell [26] and cell–matrix interactions via β1 and β2 integrins [27] or stromal cell-derived factor-1 (SDF-1) receptors [28] reduce spontaneous and drug-induced apoptosis of B-CLL cells.

*Corresponding author. Tel.: +54-11-4805-5759. Fax: +54-11-4803-9475.

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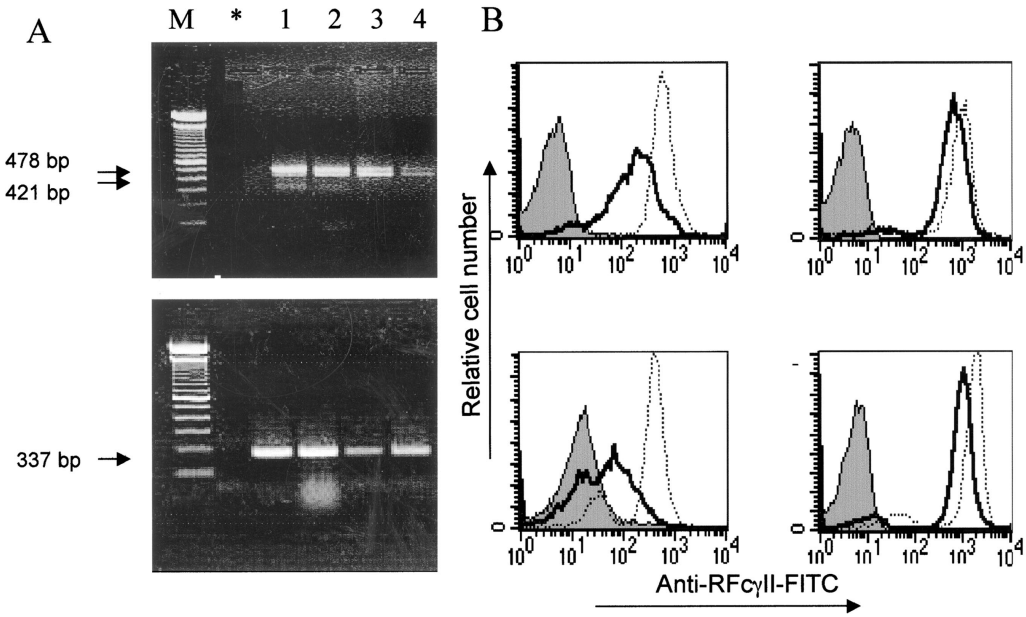

FIGURE 1 FcγRII are expressed in B-CLL cells. (A) RT-PCR analysis of FcγRII isoforms in purified B-CLL cells from four representative samples. IIb1/IIb2 (upper side) and IIa (lower side) PCR products were run on 2% agarose–ethidium bromide-stained gels. *M*: molecular weight marker. *Contamination-free reactions. (B) Membrane expression of FcγRII in B-CLL cells analyzed by flow cytometry, using two different mAbs: IV.3 (black line) or AT10 (dotted line). Shown are representative histograms from 4 of 18 patients studied. Control isotype in grey.

Receptors for the Fc Fragment of IgG (FcγR)

Most leukocytes are able to interact with IgG through membrane receptors which recognize its Fc portion. These receptors exist in three main forms: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) [29–31]. FcγRII, the most widely distributed class of FcγR, is a low affinity receptor for monomeric ligand which is able to interact with IgG only when forming an immune complex (IC) [32]. Molecular cloning has revealed two main FcγRII isoforms in humans that are highly homologous in their extracellular regions but exhibit a dramatic divergence in their cytoplasmic domains. Thus, FcγRIIa isoform bears a unique ~26 amino acid immunoreceptor tyrosine-based activation motif (ITAM) involved in activation functions while FcγRIIb isoform contains a unique 13 amino acid immunoreceptor tyrosine-based inhibitory motif (ITIM) [33–36]. Therefore, the effective interaction of IC with one or another isoform will have different, often opposite consequences in cellular behaviour. In mice, the unique FcγR expressed on B lymphocytes in FcγRIIb, which suppresses cellular activation triggered by cross-linking of the BCR when antigen is complexed to specific IgG antibodies [37]. Most of the data available on responses triggered through FcγRII in B cells come from studies performed in mice. However, since the IIa isoform is not present in mice, conclusions from these studies cannot be easily extrapolated to humans. In fact, the expression of the FcγRIIa isoform on human B cell membranes is still a matter of controversy [29,30]. Using a panel of B cell lines in different developmental stages, RNA transcripts for FcγRIIa were found in all the lines tested, being the only isoform present in pre-B cells [38]. Although it has been proposed to play a role during B cell development, there is no reported evidence on the functional relevance, if any, of FcγRIIa in B cells.

We have evaluated the expression of the different isoforms of FcγRII on B-CLL cells both, at protein and mRNA levels. By RT–PCR, we have found transcripts for the IIa, IIb1 and IIb2 isoforms in all the patients analyzed (Fig. 1a). To study surface expression of FcγRII, we employed two different monoclonal Abs: AT10, which recognizes both isoforms and IV.3, which has been claimed to recognize FcγRIIa, but not FcγRIIb [38–40]. Cytofluorometric analysis showed binding of both Abs in all the patients studied ($n = 18$) (Fig. 1b). In addition, FcγRI and FcγRIII were not found on leukemic cells.

Apoptosis and FcγR

Effector functions dependent on FcγRs, such as phagocytosis, antibody dependent cellular cytotoxicity (ADCC) and the release of inflammatory mediators, are well documented [29–31]. Likewise, there is increasing evidence for the inhibitory role of FcγRIIb not only in BCR-induced stimulation of B lymphocytes, but also on multiple immune responses that depend on the activation of ITAM-bearing FcRs, as confirmed in FcγRIIb-deficient mice which have enhanced sensitivity to IC-triggered inflammation [41–43]. More recently, it has been shown that FcγRs are also involved in the modulation of cell survival. In this regard, Ortaldo *et al.* [44] found that IL-2 activated NK cells undergo apoptosis as a consequence of FcγRIII cross-linking by aggregated IgG. In the case of granulocytes, FcγR ligands were able to not only induce but also to delay spontaneous apoptosis depending on the characteristics of the IC employed. Thus, we have shown

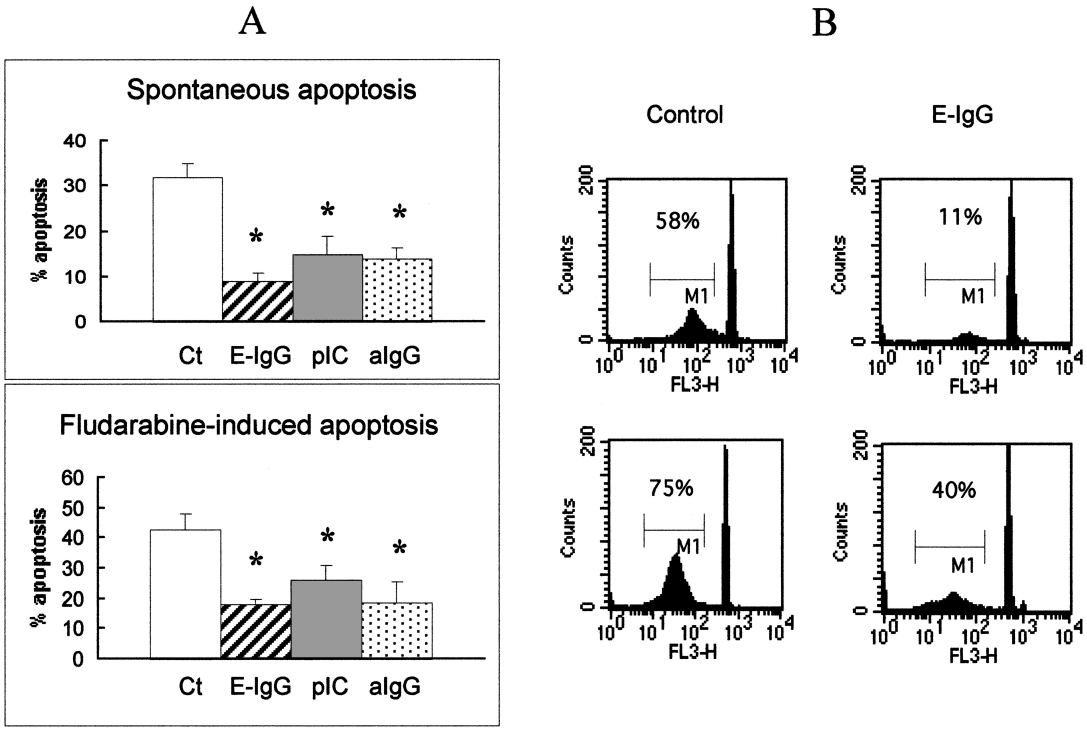

FIGURE 2 Immune complexes (IC) delays spontaneous and fludarabine-induced apoptosis of B-CLL cells. Peripheral blood mononuclear cells (2.5×10^6 ml^{-1}) were incubated for 24–72 h at 37°C with different types of IC: ovine erythrocytes coated with specific IgG (E–IgG) (hatched bars), precipitating ovalbumin-IgG anti-ovalbumin IC (pIC) (10 μg/ml) (grey bars) or human aggregated IgG (aIgG) (50 μg/ml) (dotted bars) or medium alone (open bars). Spontaneous and fludarabine (5 μg/ml)-induced apoptosis were determined by fluorescence microscopy (A) and flow cytometry (B). A: Results are expressed as the mean ±SEM of 10–25 patients. *Statistical significance ($P < 0.01$) compared to cells not treated with IC. B: Representative histograms showing the percentage ($M = 1$) of nuclei with hypodiploid DNA content.

that soluble IC enhance human neutrophil survival *in vitro*, while particulate IC accelerate apoptotic rates [45]. Similar results were observed by others in eosinophils [46]. In addition, engagement of FcγRIIb on murine B cells was found capable of generating an apoptotic signal which requires activation of Bruton's tyrosine kinase (Btk) and is blocked by SHIP [47].

Apoptosis and Immune Complexes in B-CLL

High levels of circulating IC have been previously reported in a considerable proportion of B-CLL patients [48–50] and our own observations). These observations, and the fact that leukemic cells express FcγRII on their membranes, prompted us to analyze whether IC were able to modulate B-CLL cell apoptosis. With this aim, we performed cultures of peripheral blood mononuclear cells (> 70% leukemic cells) in the presence of different types of IC and evaluated B-CLL survival. Our results showed that soluble and particulate IC significantly delay spontaneous apoptosis as well as that induced by chemotherapeutic drugs, such as fludarabine, chlorambucil and glucocorticoids, all of them widely used *in vivo* for B-CLL treatment [51–53] (Fig. 2). Interestingly, increased survival induced by treatment with IC resulted in higher expression of HLA–DR on B-CLL cells suggesting that inhibition of apoptosis was associated to cellular activation [54]. Given that B-CLL cells were not the only FcγR$^+$ population in peripheral blood of leukemic patients capable of interacting with IC, the possibility existed that prevention of apoptosis by IC was mediated through a paracrine mechanism rather than being the consequence of leukemic FcγRII crosslinking. Indeed, this was found to be the case. We showed that, in the absence of monocytes and NK cells, IC were unable to delay B-CLL cell apoptosis, although they effectively bound to leukemic cells. The protective effect exerted by accessory leukocytes partially involved the release of interferon γ and was evident even in those samples with very low percentages of monocytes and NK cells (< 5%).

While the paracrine effect of IC on B-CLL cells might play a role in disease progression, it was unclear why engagement of FcγRs expressed by leukemic cells was unable to induce apoptosis as described for murine B cells. As mentioned above, the interaction of IC with FcγRs can result not only in apoptosis, but also in cellular activation. For cells that express both activation and inhibitory receptors, as appears to be the case for B-CLL cells, the outcome of an interaction with an IC might be determined by multiple factors, such as the relative levels of each type of receptors [47], the isotype of the Ab in the IC [55,56] and the degree of clustering induced by the ligand [57], among others. Taking this into account, we assessed a number of cellular responses using different ways to crosslink FcγR on purified B-CLL cells. We evaluated proliferation, expression of activation markers such as

co-stimulatory molecules (CD80, CD86), HLA–DR, CD40, Fas (CD95), FasL (CD154), Bcl-2, as well as early events triggered by FcγR engagement, such as calcium mobilization and tyrosine phosphorylation. With regard to FcγR ligands, we employed soluble or particulate IC, immobilized IgG (IgG1, IgG2 and IgG3 isotypes) and Fab fragments of IV.3 which were extensively cross-linked with sheep anti-mouse IgG and goat anti-sheep IgG (both $F(ab')_2$ fragments). We were unable to modify any of these biological parameters in more than 20 B-CLL patients studied.

Defective signaling through FcγRs, as it was found for the BCR expressed by leukemic cells, may be a possible explanation for these negative findings. Further experiments are required to answer this question.

CONCLUSIONS

IgG IC are normally formed during the course of the antibody response. High levels of circulating IC have been previously reported in B-CLL patients despite the fact that most of them progressively develop a marked hypogammaglobulinemia [1–3,58,59]. Our results support the notion that IC may affect the course of B-CLL by virtue of their ability to increase leukemic cell survival through a paracrine mechanism. On the other hand, IC can effectively interact with FcγRII expressed by B-CLL cells, although they seem unable to deliver transmembrane signals. We are currently exploring the possibility that the activation status of these receptors could shift during course of B-CLL stimulation.

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