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Polyreactive Antibodies in CLL Correlate with the Level of Immunoglobulins Not the Number of B Lymphocytes

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Polyreactive antibodies are a major part of natural antibody repertoire and can react with different types of antigens both from the host (e.g., proteins, carbohydrates, lipids, DNA) as well as from the environment (e.g., bacteria, viruses). These antibodies have low affinities, are in germ-line or near germ-line configuration, and are predominantly IgM [1]. They are an important component of the innate immune system and a first line of defense before the adaptive immune system kicks-in[2]. Polyreactive antibodies also are important in phagocytizing the millions of cells that undergo apoptosis daily [3].

About 50% of B cells in the cord blood of newborns can make polyreactive antibodies as compared to about 20% of B cells in adult peripheral circulation [4]. It is estimated that as much as half of the natural IgM repertoire in sera is polyreactive and that normal sera contains millions of these antibodies which display different binding patterns and affinities. We recently developed an assay, using di-nitrophenol as a surrogate, to measure polyreactive antibodies and in mice we showed that the stimulation of toll-like receptors (e.g., TLR-4) profoundly enhanced the secretion of polyreactive antibodies into the circulation [5,6].

The present study was initiated to determine: (1) the level of polyreactive antibodies in the sera of Chronic Lymphocytic Leukemia (CLL) patients with un-mutated (U-CLL) and mutated (M-CLL) immunoglobulin heavy chain genes (*IGHV*) and (2) to evaluate the quantity of polyreactive antibodies secreted by cultured CLL cells stimulated with the TLR9 ligand CpG.

Sera from 40 CLL patients (median age 65 years, males 68%) enrolled in a natural history study (NCT00923507) at the National Institutes of Health (Supplemental Table 1) and 20

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Conflict of interest:

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age matched normal subjects (from NIH blood bank donors, collected with informed consent) were analyzed for the titer of polyreactive antibodies, serum IgM concentration, absolute lymphocyte count (ALC) and ratio of polyreactive IgM titer to IgM concentration by the methods described earlier [6,7]. Briefly, enzyme linked immunosorbent assay plates were coated with di-nitrophenol, two-fold-serially diluted serum samples were applied to each of the wells and the bound antibodies were detected using horse radish peroxidase coupled anti-human IgM secondary antibody. All the patients were treatment-naïve at the time of analysis and none had received intravenous immunoglobulin replacement.

We first compared the titer of polyreactive antibodies in CLL patients to age-matched normal controls. Fig. 1A shows that the titer of polyreactive antibody was significantly reduced in the sera from both un-mutated (U-CLL) and mutated (M-CLL) patients with CLL. IgM concentration was also significantly reduced in the sera from both un-mutated (U-CLL) and mutated (M-CLL) patients with CLL (Fig. 1B). However, the ratio of polyreactive antibody titers to IgM concentration was not statistically different in CLL patients compared to normal controls (Fig. 1C). Additional studies showed that the polyreactive antibody titers correlated with the concentration of IgM (Fig. 1D). No correlation between the polyreactive antibody titer and the ALC (Fig. 1E). In contrast, patients with advanced rai stage (3–4) had significantly higher polyreactive antibody titers than those with more indolent disease (rai stage 0–2) (Fig. 1F).

To determine whether B lymphocytes from CLL patients could be stimulated *in vitro*, lymphocytes from 44 patients (Supplemental table 2) were placed in culture and stimulated with the TLR ligand CpG. As seen in Fig. 2A, CpG increased significantly the polyreactive IgM in patients with U-CLL, but not in patients with M-CLL. However, the IgM concentration was increased in both U-CLL and M-CLL (Fig. 2B). Earlier reports suggested that the VH1–69 gene had a greater propensity to produce polyreactive antibodies than other members of the VH1 family [8]. Fig. 2C shows that CpG increased polyreactive IgM only in VH1–69 positive, but not in VH1–69 negative U-CLL cell cultures. However, the IgM concentration was increased significantly in the VH1–69 negative cell cultures and also in the VH1–69 positive U-CLL cell cultures, but did not reach statistical significance in the latter (Fig. 2D).

In conclusion, our studies show that despite the high number of lymphocytes in CLL, often in the hundreds of thousands, there was a marked decrease in both IgM concentration and polyreactive antibody titer compared to normal age-matched controls. In addition, while no correlation was found between the absolute number of CLL lymphocytes and the titer of polyreactive antibodies, patients with advanced rai stage had significantly higher polyreactive IgM than those with indolent disease, suggesting a link between polyreactive antibody production and disease state. Lastly, U-CLL in particular VH1–69 B cells have a greater capacity to secrete polyreactive antibodies than other members of the immunoglobulin gene family.

These findings are best explained by CLL B lymphocytes occupying the bone marrow and inhibiting the developments of normal B cells. Moreover, CLL lymphocytes have been shown to inhibit the secretion of antibodies from normal B cells [9]. Although the number of

subjects in our study was relatively small, certain immunoglobulin heavy chain genes (e.g., IgVH1–69), when stimulated with CpG, produced more polyreactive antibodies than other members of the *IGVH* gene family. This data adds to an earlier report that similarly showed that the IgVH1–69 gene is associated with secretion of higher levels of polyreactive antibodies [10].

Given the fundamental role of polyreactive antibodies in immune defense and homeostasis [2,3,11], therapeutic intervention that increases the titer of polyreactive antibodies in CLL patients might prove to be beneficial in the defense against foreign invaders and in the removal of apoptotic cells. This might explain the beneficial effect of ibrutinib, which increases polyreactive antibodies, in CLL patients [7].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Polyreactive antibody titers in CLL patients.

(A) Serum polyreactive IgM titer, (B) IgM concentration and (C) ratio of polyreactive IgM titer to IgM concentration in U-CLL (n=19) and M-CLL (n=21) patients compared to normal age matched subjects. (**=P<0.001, ****=P<0.0001, ns=not significant). (D) Correlation of polyreactive IgM titer with IgM concentration in CLL patients compared to normal age matched subjects. (Spearman correlation, $r=0.4969$, $P=0.0006$), (E) Lack of positive correlation between absolute lymphocyte count (ALC) and titer of polyreactive antibodies. (F) Comparison of polyreactive IgM antibody titer between patients with advanced Rai stage (3-4, n=8) and indolent disease (Rai stage 0-2, n=32).

Fig.2. CpG stimulates the secretion of polyreactive antibodies.

PBMC from U-CLL (n=24) and M-CLL (n=20) patients were cultured for 72 hours in presence of or absence of CpG: (A) polyreactive IgM level, (B) IgM concentration, (C) Comparison of the effect of CpG stimulation on polyreactive IgM level and (D) IgM concentration in cultures from VH1-69 (n=10) and non-VH1-69 (n=14) patients. Data is $\pm$ SEM of two independent experiments and (ANOVA, *= $P < 0.01$, **= $P < 0.001$, ns=not significant).