Functional Heterogeneity of B-CLL Lymphocytes: Dissociated Responsiveness to Growth Factors and Distinct Requirements for a First Activation Signal

By Saoussen Karray, Hélène Merle-Béral, Aimé Vazquez, Jean-Philippe Gerard, Patrice Debre, and Pierre Galanaud

We studied the effects of B cell directed growth factors on B lymphocytes from 11 patients with chronic lymphocytic leukemia (B-CLL). B-CLL lymphocytes were costimulated with anti-μ antibody (Ab) and with three growth factor preparations: recombinant IL2, B cell growth factor (BCGF) (20 kD BCGF) and a high molecular weight BCGF (50 kD BCGF). IL2 was the more active factor (in six of 11 patients). The effect of IL2 was dependent on a costimulation with anti-μ Ab or occurred independently of anti-μ Ab, according to the patients. This pattern of reactivity did not correlate with the presence or absence of the IL2 receptor (IL2-R) molecule on fresh B-CLL lymphocytes. Five patients responded to the 20 kD BCGF. Although four of them were also strong responders to IL2, one strongly responded to the 20 kD BCGF and did not respond to IL2. Only one patient responded to the 50 kD BCGF. When an anti-IL2-R Ab was introduced into the culture, only the responsiveness to IL2 was abolished: thus both 20 kD and 50 kD BCGFs activate B-CLL lymphocytes independently of the IL2-R. These results show that several B cell directed growth factors can act independently to support the proliferation of B-CLL lymphocytes.

Isolation of B-CLL lymphocytes. Mononuclear cells were isolated from B-CLL patients' blood samples by centrifugation on Ficoll-Hypaque. T cells were eliminated by two cycles of rosetting using aminooethyl isothiocyanate bromide treated sheep RBCs. T depleted cells were then depleted in adherent cells by 45 minutes incubation on plastic petri dishes in the presence of 10% fetal calf serum (FCS). These preparations were analyzed using a FACSTAR flow cytometer. They were homogeneous small cells and contained less than 1% cells labeled by the OKT3 antibody (Ortho Pharmaceuticals, Raritan, NJ) and <1% cells labeled by the OKM5 antibody (Ortho Pharmaceuticals). These cells will be referred to as B-CLL lymphocytes.

Growth factors. Recombinant IL2 was a gift of Biogen (Geneva). The preparation used had an estimated activity of 10^5 IU/mg protein and contained 97.3% IL2 by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) analysis. BCGF purified by chromatography according to Mehta et al was purchased from Cellular Products (Buffalo). This preparation did not contain detectable IL2 as assessed by the proliferation of mitogen activated human T cells and of the CTL-L murine T cell line. This growth factor will be referred to as 20 kD BCGF.

The 50 kD BCGF was prepared as already described. Conditioned medium was produced by activating normal peripheral blood lymphocytes with phytohemagglutinin. The supernatant was applied onto a concanavalin A sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) and the active material eluted with a-D-methyl mannoside (Sigma, St Louis) in 0.15 mol/L NaCl. This preparation contained no IL2 and supported B cell proliferation. The material used in the present work was further purified by two different chromatographic methods: gel filtration on an ACA 54 column (IBF, Villeneuve, France) and on a Sephacyr S-300 column (Pharmacia). The active fraction had an estimated activity of 10^5 IU/mg protein and contained 95% IL2 by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) analysis.

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Supported by grants from INSERM and Association pour la recherche contre le cancer-Villejuif. S. Karray was supported by a fellowship from Association pour la recherche contre le cancer.

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apparent MW of 50 kD) and affinity chromatography on a Biogel HPHT hydroxylapatite column (Bio-Rad, Richmond, CA) (the active fractions were eluted at phosphate concentrations between 160 and 180 mmol/L). This material, referred to as 50 kD BCGF, contained no IL2, IL1, or antiviral activities.

**Cell proliferation assay.** In costimulation assays insolubilized anti-μ Ab (Bio-Rad) was used at a final concentration of 10 μg/mL. This concentration was selected on the basis of preliminary experiments using normal B cells or B-CLL lymphocytes. B-CLL lymphocytes were cultured in 96 well flat-bottomed microtiter plates at a concentration of 10^5 cells per well in 0.2 mL culture medium. The latter was RPMI 1640 supplemented with 10% FCS and 5 x 10^-5 mol/L 2-mercaptoethanol. The cultures received graded concentrations of IL2, or serial dilutions of BCGFs in the presence or in the absence of anti-μ Ab. After three and six days of culture the proliferative response was assessed by the addition of 0.5 μCi (^3H)thymidine (CEA, France) during the last 16 hours of culture. Cultures were performed in duplicate and the standard deviation between replicates was <5%. When the day 3 and day 6 responses were considered, the day 6 response was identical to or higher than the day 3 response. Thus only the latter response was considered. A patient's lymphocytes were considered as responsive to a given factor when this factor induced a dose-related response, with a maximal thymidine incorporation at least five times that observed in the presence of anti-μ Ab and in the absence of the factor.

**Functional effect of anti-IL2-R antibodies.** In one case an anti-IL2-R Ab was used to inhibit the IL2 dependent proliferation of B-CLL lymphocytes. In this case we used the 33.B.3-1 Ab produced by Olive et al. This Ab is a rat IgG2 directed toward the IL2-R molecule and able to inhibit the IL2 dependent proliferation of normal T cells. The blocking experiments were performed as follows: B-CLL lymphocytes were cultured for three days at 2 x 10^5 cells per milliliter in the presence of anti-μ Ab (10 μg/mL). They were then washed exhaustively, cultured for three additional days in the presence of graded concentrations of IL2 or 20 kD BCGF or 50 kD BCGF and in the absence or in the presence of the 33.B-3-1 Ab. The 33.B-3-1 Ab obtained as ascitic fluid was used at a final dilution of 1/100 and added to the cultures two hours before the addition of IL2.

**Analysis of activation markers on patient's lymphocytes before and after three days of culture.** For determination of the presence of activation markers on B-CLL lymphocytes we used the following antibodies: OKT9, an Ab toward the transferrin receptor; anti-Tac Ab (a gift of T. Waldmann), directed toward a molecule associated with IL2-R; 4F2 (a gift of A. Fauci), an early activation marker for B lymphocytes; and the 1.35 anti-DR Ab, to quantify the expression of MHC class II products. Selected patients' lymphocytes were cultured for three days at 2 x 10^4 cells per milliliter with medium, with anti-μ Ab (10 μg/mL) or with IL2 (4 U/mL) according to the functional data obtained in previous experiments. Cells were washed and 0.7 x 10^6 viable cells were treated with antibodies at appropriate dilution in phosphate buffer saline (PBS) containing 2% bovine serum albumin (BSA) and 0.1% sodium azide (PBS/BSA/Azide). After 30 minutes incubation at 4°C, the cells were washed twice in cold PBS/BSA/Azide, resuspended in 50 μL of 1/10 dilution of FITC-conjugated goat F(ab')2 anti-mouse Ig (Tago, Burlingame, CA) and incubated for 30 minutes at 4°C. Thereafter, cells were washed twice in PBS/BSA/Azide. Fluorescence analyses were performed on a FACSTAR flow cytometer.

**RESULTS**

**Response of B-CLL lymphocytes to IL2.** B-CLL lymphocytes were cultured in the presence of (4 U/mL) of IL2 and in the presence or absence of anti-μ Ab (10 μg/mL). Both reagents were added on day 0 of the culture. In the absence of growth factor, anti-μ Ab had no effect (Table 2). In five cases IL2 did not induce thymidine incorporation in B-CLL lymphocytes, regardless of the presence of anti-μ Ab (POUB, BRU, ALB, and POU). The other six patients' lymphocytes were able to proliferate in the presence of IL2 and this response was dose-related. In five cases (BAT, LEG, LOI, RIA, and PER) a clearcut costimulatory effect of IL2 and anti-μ Ab was apparent. In one case (ROU) an optimal response to IL2 was obtained in the absence of anti-μ Ab, the addition of which had no additive or synergistic effect.

**Response of B-CLL lymphocytes to 20 kD BCGF.** The proliferative response to the 20 kD BCGF was tested in the same experiments. Comparable experimental conditions were used: concentrations of 20 vol/vol dilution of 20 kD BCGF were added to the cultures in the presence and absence of anti-μ Ab. Lymphocytes from five of 11 patients responded to the 20 kD BCGF. The pattern of reactivity of these five patients' lymphocytes to the 20 kD BCGF was heterogeneous. In one case this response was not consistently
The proliferative response was evaluated for three additional days with graded concentration of interleukins in the absence of anti-\(\alpha\) Ab or in the presence of anti-\(\alpha\) Ab (10 \(\mu\)g/ml) for three days. Cells were cultured at a concentration of 1 0 cells per milliliter with anti-M Ab (10 \(\mu\)g/ml) for three days. Cells were determined as described in Materials and Methods.

The lymphocytes from four patients responding to the 20 kD BCGF were cultured with IL2 (4 U/mL), with 20 kD BCGF (20% vol/vol), or with 50 kD BCGF (20% vol/vol), in the absence and presence of anti-\(\alpha\) Ab was tested in eight patients. RIA’s lymphocytes were able to respond to the 50 kD BCGF in the absence and presence of anti-\(\alpha\) Ab (BAT). In two cases the 20 kD BCGF exhibited a clearcut response to IL2 (BAT, LEG, RIA, and PER). Interestingly, the lymphocytes from one patient displayed a clearcut response to 20 kD BCGF, in contrast to no response to IL2 (POUB). The same discrepancy was observed when this patient’s lymphocyte response was measured on day 3 (data not shown).

Response of B-CLL lymphocytes to the 50 kD BCGF. The responsiveness of B-CLL lymphocytes to the 50 kD BCGF in the absence and presence of anti-\(\alpha\) Ab was tested in eight patients. RIA’s lymphocytes were able to respond to the 50 kD BCGF. This response was obtained regardless of the presence of anti-\(\mu\) Ab. Lymphocytes from this patient also responded to both 20 kD BCGF and IL2. However, the response to the former factor was anti-\(\alpha\) independent, whereas that to IL2 required costimulation with anti-\(\alpha\) Ab.

Involvement of IL2-R in the response of B-CLL lymphocytes. We wished to verify that the responsiveness to the BCGFs occurred independently of the IL2-R molecule. Lymphocytes from patient RIA, who responded to all three growth factors, were selected. They were cultured for three days in the presence of anti-\(\alpha\) Ab, washed and recultured for three additional days in the presence of any of the three growth factors (Fig 1). The addition of an antibody directed toward a functional epitope of the IL2-R molecule fully inhibited the response of IL2, without affecting the dose effect curves of BCGFs. Thus the 20 kD and 50 kD BCGFs interact with B-CLL lymphocytes independently of the IL2-R molecule.

Acquisition of activation markers by B-CLL patients’ lymphocytes. We examined the presence of activation markers on the lymphocytes from three patients selected as representative of a functional pattern of responsiveness. When their lymphocytes were examined before culture, they did not express Tac, 4F2 or T9 antigens (Table 3). The cells from these patients were cultured for three days in conditions previously shown to induce or not induce B cell proliferation. The phenotypic analysis was then performed (Table 4). Patient RIA’s lymphocytes were cultured with anti-\(\alpha\) Ab, a procedure that allows the response of these cells to IL2.

Table 2. Effect of IL2, 20 kD BCGF, and 50 kD BCGF on B-CLL Proliferation

<table>
<thead>
<tr>
<th>Patients</th>
<th>Without Anti-(\mu) Ab</th>
<th>With Anti-(\mu) Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL2</td>
<td>20 kD BCGF</td>
<td>50 kD BCGF</td>
</tr>
<tr>
<td>BAT</td>
<td>761</td>
<td>7,800</td>
</tr>
<tr>
<td>LEG</td>
<td>943</td>
<td>8,156</td>
</tr>
<tr>
<td>LOI</td>
<td>307</td>
<td>1,798</td>
</tr>
<tr>
<td>RIA</td>
<td>259</td>
<td>1,645</td>
</tr>
<tr>
<td>POU</td>
<td>1,714</td>
<td>3,051</td>
</tr>
<tr>
<td>ROU</td>
<td>1,245</td>
<td>53,627</td>
</tr>
<tr>
<td>PER</td>
<td>382</td>
<td>1,219</td>
</tr>
<tr>
<td>BRU</td>
<td>299</td>
<td>1,050</td>
</tr>
<tr>
<td>GUI</td>
<td>277</td>
<td>500</td>
</tr>
<tr>
<td>ALB</td>
<td>402</td>
<td>1,500</td>
</tr>
<tr>
<td>POU</td>
<td>370</td>
<td>1,336</td>
</tr>
</tbody>
</table>

Proliferation was measured by incorporation of \(^{3}H\) thymidine during 16 hours pulse. Data shown represent the mean of duplicate cultures.

Abbreviation: ND, not done.

*10° purified B-CLL lymphocytes were cultured with IL2 (4 U/mL), with 20 kD BCGF (20% vol/vol), or with 50 kD BCGF (20% vol/vol), in the absence or in the presence of anti-\(\mu\) Ab (10 \(\mu\)g/mL) for six days. Standard deviation was <5%.

Fig. 1. RIA’s lymphocytes were cultured at 2 x 10⁶ cells per milliliter with anti-\(\alpha\) Ab (10 \(\mu\)g/mL) for three days. Cells were washed extensively and cultured at a concentration of 10⁶ cells per well for three additional days with graded concentration of interleukins in the absence (---) or in the presence (-----) of 33B.3.1 (anti-IL2-R Ab). The proliferative response was evaluated by \(^{3}H\) thymidine incorporation.

Table 3. Lack of Activation Markers Expression Before Culture

<table>
<thead>
<tr>
<th>Patients</th>
<th>HLA-DR</th>
<th>Tac</th>
<th>4F2</th>
<th>OKT9</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>46.07</td>
<td>7.21</td>
<td>11.2</td>
<td>8.5</td>
</tr>
<tr>
<td>BRU</td>
<td>58.53</td>
<td>11.79</td>
<td>9.3</td>
<td>9.25</td>
</tr>
<tr>
<td>ROU</td>
<td>63.63</td>
<td>9.17</td>
<td>8.88</td>
<td>6.52</td>
</tr>
</tbody>
</table>
RIA's lymphocytes expressed the Tac, 4F2 antigens and an increased density of class II products upon culture with anti-\( \mu \) Ab, but not when cultured alone. Patient BRU's lymphocytes were unreactive to all three growth factors, even in the presence of anti-\( \mu \) Ab. In this case anti-\( \mu \) Ab did not modify the density of Tac antigen. Patient ROU's lymphocytes were directly reactive to IL2. On culture with this factor (but not without addition or with anti-\( \mu \) Ab) we observed the correlated increased expression of all activation markers tested: class II, Tac, 4F2, and T9 antigens (Fig 2).

**DISCUSSION**

The initial steps of B cell activation are usually thought to result from the combined effect of the antigenic signal and of T cell derived interleukins.\(^4\) Using anti-Ig antibodies to mimic the antigenic signal, several studies have characterized B cell directed growth factors, able to drive normal B cells into the S phase of the cell cycle.\(^5\) The cells from our patients were small sized (putatively resting) B lymphocytes. We verified in three selected cases that they did not express the activation markers Tac, 4F2, and T9 before culture. This was especially important in these three cases where B-CLL lymphocytes responded to growth factors in the absence of anti-\( \mu \) Ab. These results emphasize the functional heterogeneity of small B cells.\(^3\)

In another series of experiments we analyzed the conditions for the in vitro acquisition of the IL2-R molecule and its relationships with that of activation markers. When patient RIA's lymphocytes were cultured for three days with anti-\( \mu \) Ab (a procedure able to induce their responsiveness to IL2) they acquired the Tac antigen and increased their expression of 4F2, and major histocompatibility complex (MHC) class II products. In contrast, cells from the unresponsive patient BRU were not modified by the same incubation procedure.

The direct responsiveness to a given growth factor could be expected to correlate with the constitutive expression of the corresponding receptor. We examined this point for IL2 responsiveness using the IL2 responsive cells from patient ROU. These cells were Tac\(^+\) before culture. A three day culture in the absence of reagent or in the presence of anti-\( \mu \) Ab (which did not influence the responsiveness to IL2) did not induce Tac positivity. In contrast, these cells became Tac\(^+\) (and remained negative for the T3 marker) after a three-day culture in the presence of IL2. The presence of Tac\(^+\) cells on day 3 correlated with their responsiveness to IL2 on day 6. Our results confirm that IL2 can act positively on the expression of the IL2-R molecule\(^1\) and show that this expression may be functionally significant. Moreover, on culture with IL2, patient ROU's lymphocytes expressed the 4F2 and the T9 antigens and increased their expression of MHC class II molecules.

B-CLL lymphocytes are thought to be frozen at various maturation stages\(^2\) and these monoclonal populations may be representative of at least some aspects of the heterogeneity of normal B cells. It is therefore not surprising that the

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**Table 4. Surface Membrane Antigen on Activated B-CLL**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Inducers</th>
<th>HLA-DR</th>
<th>Tac</th>
<th>4F2</th>
<th>OKT9</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>Medium</td>
<td>49</td>
<td>8.70</td>
<td>15.28</td>
<td>12.13</td>
</tr>
<tr>
<td></td>
<td>Anti-( \mu ) Ab</td>
<td>80</td>
<td>24.42</td>
<td>22.46</td>
<td>13.82</td>
</tr>
<tr>
<td>BRU</td>
<td>Medium</td>
<td>76.77</td>
<td>13.43</td>
<td>9.02</td>
<td>5.54</td>
</tr>
<tr>
<td></td>
<td>Anti-( \mu ) Ab</td>
<td>110.35</td>
<td>13.21</td>
<td>7.37</td>
<td>5.68</td>
</tr>
<tr>
<td>ROU</td>
<td>Medium</td>
<td>146.78</td>
<td>15.63</td>
<td>11.45</td>
<td>8.82</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>IL2</td>
<td>348.25</td>
<td>35.51</td>
<td>21.50</td>
<td>16.88</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Medium</td>
<td>10.75</td>
<td>1.91</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-( \mu ) Ab</td>
<td>8.35</td>
<td>1.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2 \( \times 10^6/mL \) B-CLL lymphocytes from three selected patients were cultured with medium, with anti-\( \mu \) Ab (10 \( \mu g/mL \)), or with IL2 (4 U/mL) for three days.

**Fig 2.** ROU's lymphocytes were cultured with medium (-----) or with IL2 (-----) for three days then washed and stained as described in Materials and Methods.
RESPONSIVENESS OF B-CLL CELLS TO GROWTH FACTORS

reactivity to the three growth factors studied varied among our patients’ lymphocytes. From our results IL2 is the most frequently active growth factor. However, in five cases we observed a response to the 20 kD BCGF. This is in contrast to results of a recent report, showing the lack of reactivity of B-CLL lymphocytes to the same preparation of BCGF.1This discrepancy could be due to technical conditions or to patient selection. In our hands four of the five patients responding to 20 kD BCGF (among whom one responded to 50 kD BCGF) also responded to IL2. However, in one case (POUB), the response to IL2 was absent and contrasted with a clearcut response to the 20 kD BCGF. This latter result and the absence of IL2 in the BCGF preparations used clearly show that B-CLL lymphocytes may be responsive to BCGFs. On the other hand 20 kD BCGF has been shown to induce the proliferation of cells from another lymphoproliferative disease, namely hairy cell leukemia.3,4,5 Moreover, the additional study of one patient (RIA) shows that the reactivities to IL2 and to BCGFs are indeed representative of different B cell activation pathways. Patient RIA’s lymphocytes responded to BCGFs in the absence of anti-Ab, whereas they required anti-Ab to proliferate in the presence of IL2. More importantly a monoclonal Ab to the IL2-R molecule abolished the responsiveness to IL2 without affecting the dose-effect curves of BCGFs. The direct discrimination between the responsiveness to the 20 kD and to the 50 kD BCGFs could not be provided in this work. These growth factors differ with respect to their biochemical properties and several functional differences. Normal PBL large B cells (putatively preactivated in vivo) when cultured in the absence of anti-Ab do respond to the 20 kD BCGF, whereas they do not respond to the 50 kD BCGF.11 However, PBL B cells may be directly responsive to the 50 kD BCGF in some auto-immune situations8: such B cells may be a normal counterpart of patient RIA’s B-CLL cells. More importantly, the 50 kD BCGF does not support the 20 kD BCGF-dependent growth of a normal long-term B cell line27 (A. Vazquez, unpublished results, 1985). Our results do not exclude that a given B cell subpopulation may be selectively responsive to a given growth factor. However, they show that monoclonal B cells (and thus at least some individual normal B cells) can respond simultaneously to several growth factors.

As exemplified by recent results obtained with hairy cell leukemia lymphocytes,3,4,5 the analysis of the reactivity of lymphocytes in monoclonal lymphoproliferative disorders to growth factors may have important implications for the understanding and management of these diseases.

ACKNOWLEDGMENT

We thank Drs. F. Binet, G. Tchernia, and G. Tertian for providing blood samples from B-CLL patients; D. Olive for the gift of anti-IL2-receptor antibodies (33B.3.1); Biogen for the gift of recombinant IL2; and A. Michel for the technical assistance of FACS flow cytometer.

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