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CD20 and CD40 mediated mitogenic responses in B-lineage acute lymphoblastic leukaemia

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Summary. Activation of CD20, a cross-membrane ion channel, induces cell cycle progression from G_0 to G_1 in B lymphocytes. Subsequent activation of CD40, a membrane receptor of the nerve growth factor receptor superfamily, transits the B cells to the S phase. CD40 may also act synergistically in combination with IL-4 (B lymphocytes) or IL-3/IL-7 (B-cell precursors). We investigated the proliferative responses of B-lineage acute lymphoblastic leukaemia (ALL) cells to CD20/CD40 activation. In 18/56 ALL cases, CD20 activation resulted in significant increases in DNA synthesis. Similar, although more moderate, effects were seen of activation of CD40 in 10/44 cases. Responses to CD20 or CD40 activation were independent of co-stimulation with IL-3, IL-4 or IL-7, and various cocktails of the different growth stimuli did not act synergistically.

Keywords: CD20, CD40, acute lymphoblastic leukaemia, *in vitro* proliferation.

B-cell development is governed by an, as yet, incomplete characterized series of haemopoietic growth factors and other stimuli. Factors that exert growth stimulatory effects on normal B-cell precursors include interleukin (IL)-3 and IL-7 (Lee *et al*, 1989; Namen *et al*, 1988; Saeland *et al*, 1991; Skjonsberg *et al*, 1991).

CD20 is a 35/37 kD surface polypeptide expressed exclusively on B cells. Onset of expression of CD20 takes place before the appearance of cytoplasmic μ chains and is sustained during B-cell development until the plasma cell stage (Rosenthal *et al*, 1983; Stashenko *et al*, 1981). In normal B lymphocytes, activation of CD20 proteins with the antibody 1F5 induces cell cycle transition from G_0 to G_1 but is not sufficient to initiate DNA synthesis (Clark *et al*, 1985; Clark & Ledbetter, 1986; Clark & Shu, 1987; Golay *et al*, 1985; Smeland *et al*, 1985). Activation of CD20 results in its phosphorylation on serine and threonine residues (Oettgen *et al*, 1983; Tedder & Schlossman, 1988) and increases the influx of Ca^{2+} ions into the cells. The structural and functional properties of CD20 indicate that the protein acts as a cross-membrane ion channel (Bubien *et al*, 1993; Deans *et al*, 1993).

CD40, a receptor of the nerve growth factor receptor superfamily, is expressed on B cells, follicular dendritic cells, normal basal and thymic epithelium, and several carcinoma and melanoma cell lines (Clark *et al*, 1988; Paulie *et al*, 1989; Young *et al*, 1989). During B-cell development, the appearance of CD40 on the cell membrane precedes the expression of CD20 and immunoglobulin heavy chains (Uckun *et al*, 1990). Activation of CD40 by either the CD40 ligand or CD40 activating antibodies, in concert with IL-4, induces proliferation and maturation of B lymphocytes (Banchereau *et al*, 1991; Crawford & Catovsky, 1993; Defrance *et al*, 1992; Maliszewski *et al*, 1993; Spriggs *et al*, 1992). Also, co-activation of CD20 and CD40 results in a full mitogenic response of B lymphocytes (Gordon *et al*, 1987; Ledbetter *et al*, 1987). B-cell precursors (BCP) proliferate in response to CD40 activation only when co-stimulated with IL-3, IL-7 or IL-10 (Larson & Le Bien, 1994; Saeland *et al*, 1993).

Here, we show the proliferation inducing effects of CD20 and CD40 activation on B-lineage acute lymphoblastic leukaemia (ALL) cells, alone and in combination with growth factors (IL-3, IL-4 and IL-7). Activation of CD20 resulted in a proliferative response of ALL cells in 18/58 (31%) cases; activation of CD40 induced DNA synthesis in 10/44 (23%) cases. With the exception of one case, no synergistic effects of the various stimuli were seen. These data show that responses of ALL cells to CD20/CD40 activation are heterogenous and distinct from those of normal (pre-)B cells.

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MATERIALS AND METHODS

Isolation of cells. ALL cells were isolated from bone marrow or peripheral blood by Ficoll-Hypaque (Nygaard, Oslo, Norway), density gradient centrifugation as described previously (Touw *et al*, 1989). T cells were removed from the ALL cell samples by E-rosette formation using 2-aminoethylisothiouronium bromide (AET) treated sheep erythrocytes, followed by sedimentation through Ficoll-Hypaque (Madsen *et al*, 1980). Monocytes were removed by adherence to plastic petri dishes at 37°C for 1 h. In all cases informed consent was obtained. The ALL cell samples were subjected to routine diagnostic immunophenotyping with a panel of monoclonal antibodies (MoAb). Six cases were classified as null-ALL (patients 1–6), 36 as common-ALL (patients 7–41, 58), 11 as pre-B-ALL (patients 42–52) and five as B-ALL (patients 53–57) according to established criteria (van Dongen *et al*, 1988). Cells were used either fresh or after cryopreservation using a controlled-freezing apparatus (Planer Biomed, Sunbury-on-Thames) (Touw *et al*, 1989).

Enriched fractions of normal peripheral blood B-lymphocytes were obtained using the same protocol.

DNA synthesis assay. DNA synthesis was assessed by uptake of ^{3}H-thymidine (^{3}H-TdR, specific activity 2 Ci/mmol, Amersham International, Amersham) as described (Delwel *et al*, 1988). In brief, $0{\cdot}2 \times 10^5$ cells were cultured in triplicate in 96-well dishes (Greiner, Alphen a/d Rijn, The Netherlands) for 3 and 7 d in 100 μl serum-free medium (Touw *et al*, 1990). 18 h before harvesting on nitrocellulose filters using a Titertek cell harvester (Flow Laboratories, Irvine) $0{\cdot}1$ μCi ^{3}H-TdR was added to each well. ^{3}H-TdR incorporation was measured by liquid scintillation counting.

Growth factors and CD20 or CD40 activating antibodies. Human IL-3 (Gist Brocades, Delft, The Netherlands) was used at 100 U/ml; human IL-4 (Dr S. Clark, Genetics Institute Cambridge, Mass.) was added to the cultures at a 1:5000 dilution of COS cell supernatant; human IL-7 (Dr L. Park, Immunex Corp., Seattle, Wash.) was used at 100 U/ml. Anti-CD40 monoclonal antibody (MoAb) 14G7, provided by Dr R. A. W. van Lier (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam) was used at a 1:250 dilution of ascites. The CD20 activating MoAb 1F5 (Clark & Ledbetter, 1986; Clark *et al*, 1985) (Dr E. A. Clark, University of Washington, Seattle, Wash.) was used at a final concentration of 3 μg/ml.

Analysis of CD20 and CD40 expression. Expression of CD20 or CD40 was assessed by flow cytometry (FACScan) after labelling of cells with either anti CD20 MoAb 1F5 (Clark *et al*, 1985) or the anti-CD40 MoAb 14G7 and goat anti-mouse immunoglobulin coupled to fluorescein isothiocyanate (GAM/FITC, Nordic Tilburg, The Netherlands).

RESULTS AND DISCUSSION

CD20 activation of ALL cells

Dose titration experiments performed with ALL cells from four patients indicated that maximal stimulation of DNA synthesis is seen at 1F5 concentrations of 1–10 μg/ml. A representative experiment is shown in Fig 1A. Parallel incubations with control MoAb did not result in mitogenic responses (data not shown). Proliferative responses to 1F5 (3 μg/ml) were analysed in 58 cases of ALL. CD20 activation resulted in a significant increase of DNA synthesis in 18 cases (Table I). The (in)ability to induce DNA synthesis of 1F5 did not correlate with the immunologic subtype of ALL. Therefore differences of maturation phenotype of ALL did not explain the heterogeneity in responses to CD20 activation. A correlation was neither apparent between responses to CD20 activation nor responses to IL-3 or IL-7 (Fig 2). In fact, the random distribution of the plotted stimulation indices indicates that each of the individual responses is independent from any of the responses to the other growth stimuli. As expected, flow cytrometric analysis with anti-CD20 showed

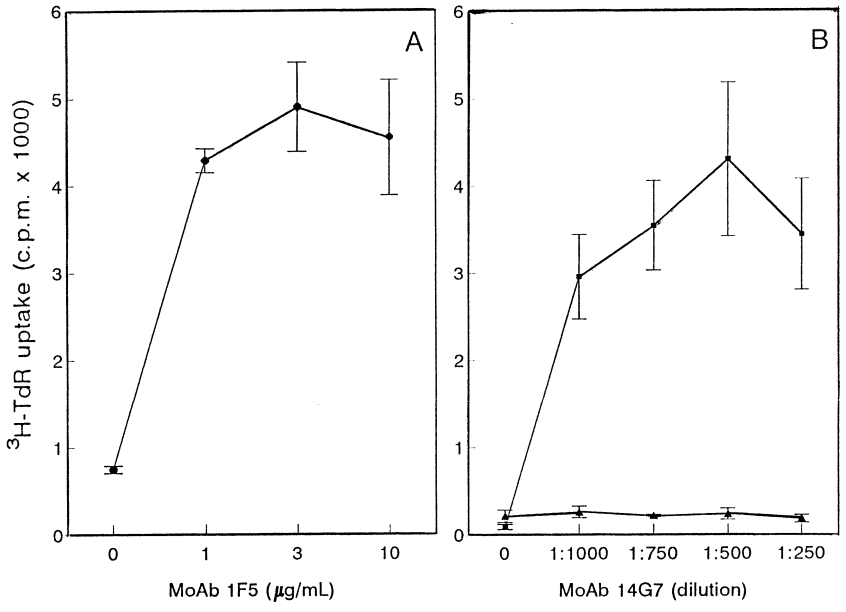

Fig 1. (A) Response of human BCP-ALL cells to titrated doses of the CD20 activating MoAb 1F5 in a ^{3}H-TdR uptake assay. (B) Response of peripheral B cells to serial dilutions of the activating CD40 MoAb 14G7 in a ^{3}H-TdR uptake assay in the presence (squares) or absence (triangles) of IL-4.

Table I. Proliferative responses of ALL cells to activation of CD20 in comparison to responses to IL-3 or IL-7.*

Pt	ALL subtype	No additive to culture	Anti-CD20	IL-3	IL-7	Irradiated cells‡
1	Null	1·3 ± 0·6†	10·9 ± 1·7	3·7 ± 1·8	2·4 ± 0·4	0·4 ± 0·1
6	Null	10·7 ± 0·2	25·2 ± 6·5	50·8 ± 7·6	18·0 ± 1·2	0·7 ± 0·1
10	Common	18·4 ± 0·6	29·8 ± 0·9	31·8 ± 1·0	22·4 ± 2·0	0·4 ± 0·1
13	Common	8·8 ± 0·2	59·1 ± 1·6	18·4 ± 1·3	17·1 ± 1·7	0·6 ± 0·1
15	Common	13·0 ± 0·8	55·5 ± 5·5	23·3 ± 3·0	20·3 ± 1·0	0·2 ± 0·0
16	Common	13·4 ± 0·9	20·6 ± 3·1	n.d.§	17·2 ± 1·0	0·2 ± 0·0
22	Common	3·8 ± 0·1	9·3 ± 2·1	18·4 ± 4·2	6·3 ± 0·5	0·4 ± 0·2
30	Common	5·9 ± 0·2	65·9 ± 11	11·4 ± 2·2	10·1 ± 1·8	0·7 ± 0·2
31	Common	5·9 ± 0·5	78·2 ± 7·2	20·0 ± 2·1	27·3 ± 3·5	0·5 ± 0·2
32	Common	3·1 ± 0·3	8·1 ± 2·2	14·8 ± 4·6	18·2 ± 0·4	0·3 ± 0·1
36	Common	2·6 ± 0·4	13·1 ± 2·3	6·6 ± 2·0	2·3 ± 0·6	0·7 ± 0·1
58	Common	7·9 ± 1·3	27·0 ± 2·7	9·8 ± 2·7	6·4 ± 1·1	0·3 ± 0·1
38	Common	7·1 ± 2·2	14·7 ± 3·3	21·0 ± 6·9	10·3 ± 1·9	1·4 ± 0·2
43	Pre-B	10·5 ± 1·8	23·3 ± 6·0	18·9 ± 5·1	19·1 ± 1·4	1·0 ± 0·0
44	Pre-B	29·6 ± 1·9	87·3 ± 9·7	58·1 ± 10	69·3 ± 3·0	0·3 ± 0·2
45	Pre-B	19·3 ± 1·0	39·0 ± 8·0	27·5 ± 1·4	31·0 ± 3·4	0·3 ± 0·2
50	Pre-B	2·2 ± 0·6	24·9 ± 1·0	6·6 ± 1·4	2·2 ± 0·4	0·4 ± 0·1
57	B	19·4 ± 1·8	32·0 ± 1·0	273 ± 14·7	19·6 ± 6·0	0·9 ± 0·2

*Only cases responsive to CD20 activation have been included. In these cases, stimulation values (1F5 supplemented versus non-supplemented controls) were >1·5.

†Data are from ^{3}H-thymidine uptake assays and are expressed as mean counts per minute × 100 ± standard deviation of triplicate cultures.

‡Cells were irradiated (25 Gy) before culture.

§Not determined.

that ALL cells of 1F5 responding cases expressed CD20. In 15/27 1F5 nonresponders analysed, the ALL cells expressed CD20, indicating that in these cases the absence of response to 1F5 was not caused by lack of CD20.

CD40 activation of ALL cells

In dose titration experiments, maximal stimulation of DNA synthesis by CD40 activating antibody 14G7 was reached at dilutions of 1 : 1000–1 : 250 (Fig 1B). In 10/44 ALL cases,

Table II. Proliferative response of ALL cells to activation of CD40 in comparison to responses to IL-3, IL-7 and CD20 activation.*

Pt	ALL subtype	No additive to culture	Anti-CD40	IL-3	IL7	Anti-CD20	Irradiated cells‡
1	Null	1·3 ± 0·6†	9·6 ± 2·4	3·7 ± 1·8	2·4 ± 0·5	10·9 ± 1·7	0·4 ± 0·1
11	Null	35·4 ± 1·5	70·8 ± 3·1	121 ± 4	33·5 ± 0·2	35·8 ± 1·4	0·4 ± 0·1
8	Common	349 ± 22	568 ± 45	341 ± 8	336 ± 12	389 ± 8	0·6 ± 0·1
14	Common	5·5 ± 0·9	15·6 ± 3·6	15·0 ± 2·0	5·9 ± 1·6	3·8 ± 1·2	0·4 ± 0·1
21	Common	5·6 ± 0·6	32·3 ± 1·3	16·8 ± 1·9	12·2 ± 1·5	8·9 ± 1·2	0·2 ± 0·1
26	Common	31·5 ± 1·0	45·5 ± 3·9	35·2 ± 1·6	27·0 ± 1·7	32·9 ± 0·4	1·4 ± 0·2
30	Common	5·9 ± 0·2	12·4 ± 1·9	11·4 ± 2·1	10·1 ± 1·8	65·9 ± 11	0·7 ± 0·2
48	Pre-B	17·6 ± 3·9	28·9 ± 0·8	25·7 ± 1·1	10·7 ± 4·0	19·8 ± 1·2	0·4 ± 0·1
50	Pre-B	2·2 ± 0·6	5·5 ± 0·2	6·5 ± 1·4	2·2 ± 0·4	24·9 ± 1·0	0·3 ± 0·1
52	Pre-B	3·2 ± 0·0	8·0 ± 0·6	16·2 ± 2·3	2·0 ± 0·4	3·6 ± 1·3	0·6 ± 0·1
55	B	1·8 ± 0·0	5·0 ± 0·3	1·8 ± 0·5	2·0 ± 0·2	1·9 ± 0·3	0·4 ± 0·1

*Only cases responsive to CD40 activation have been included. In these cases, stimulation values (14G7 supplemented versus non-supplemented controls) were >1·5.

†Data are from ^{3}H-thymidine uptake assays and are expressed as mean counts per minute × 100 ± standard deviation of triplicate cultures.

‡Cells were irradiated (25 Gy) before culture.

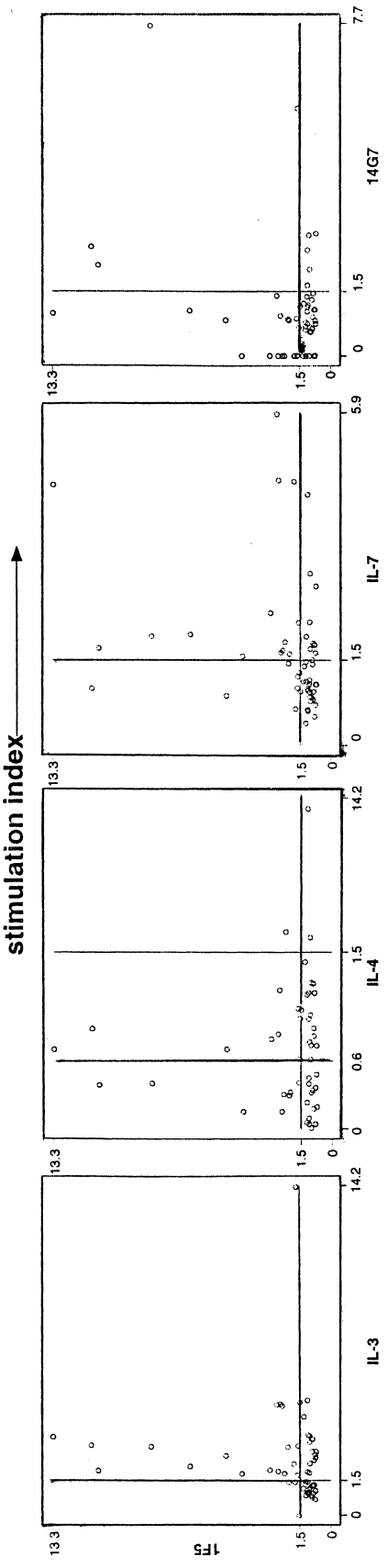

Fig 2. Proliferative responses of BCP-ALL cells in response to different growth stimuli plotted as stimulation indices (SI) in matrix mode. SI of 1F5 (top panel) or 14G7 (lower panel) on the *Y*-axis are plotted against SI of IL-3, IL-4, IL-7 and 14G7 on the *X*-axis. SI $>1{\cdot}5$ was defined as stimulation, whereas values $<0{\cdot}6$ were defined as inhibition.

DNA synthesis was moderately enhanced by 14G7 (1 : 250 dilution, Table II).

Previously, Law *et al* (1990) could not demonstrate mitogenic effects of CD40 activation in ALL cells. However, the latter study was based on the analysis of eight cases of ALL only (Law *et al*, 1990). The various immunological subtypes of B-cell ALL were represented among the 10 14G7 responsive cases. No correlation was apparent between responses to anti-CD40 and those to IL-3, IL-4, IL-7 or anti-CD20 (Fig 2). All 14G7 responders expressed CD40 at levels detectable by flow cytometry. In 6/8 14G7 nonresponders, ALL cells expressed CD40, suggesting that in the majority of cases non-responsiveness of ALL cells following CD40 stimulation is due to ineffective signalling.

Activation of CD20 and CD40 in combination with other stimuli

Certain stimuli act synergistically in inducing proliferative responses in nonleukaemic (pre-)B cells. Particularly, this has been demonstrated for the combined activation of CD20 and CD40 (Gordon *et al*, 1987; Ledbetter *et al*, 1987), and for activation of CD40 in combination with IL-4 (Banchereau *et al*, 1991; Defrance *et al*, 1992; Maliszewski *et al*, 1993; Spriggs *et al*, 1992), IL-3 or IL-7 (Saeland *et al*, 1993). Proliferative responses of ALL cells to different combinations of stimuli, in the context of activation of CD20 or CD40, are summarized in Table III. Although additive effects (1F5 + IL-3; 1F5 + IL-7; 1F5 + 14G7) were occasionally seen, none of the combinations tested were synergistic, except in a single case of ALL in which both the combinations 14G7 + IL-3 and 14G7 + IL-7 synergistically stimulated DNA synthesis.

The response of ALL cells to activation of CD20 or CD40, with or without the addition of cytokines, differ from those of nonleukaemic (pre-)B cells. Whether this would indicate that ALL cells generally display aberrant responses to CD20 and CD40 activation is not clear. In normal B lymphocytes activation of CD20 induces transition from G0 to G1 (Clark & Ledbetter, 1986; Clark *et al*, 1985; Golay *et al*, 1985; Smeland *et al*, 1985), whereas CD40 activation promotes the transition from G1 to S phase of the cell cycle. Activation of both of these molecules is required for transduction of a full mitogenic signal (Golay *et al*, 1991; Gordon *et al*, 1987; Ledbetter *et al*, 1987). The observation that in certain cases ALL cells proliferate in response to either CD20 or CD40 activation as a single growth stimulus could be suggestive of a partial loss of cell cycle control bypassing the necessity of cooperation of signals for a mitogenic response. Further elucidation of signalling properties of CD20 and CD40 in normal and leukaemic (pre-)B cells is needed to clarify these issues.

Table III. Summary of proliferative responses of ALL cells to different stimuli.

Stimulus	Frequency of response (%)	Type of response
IL-3	31/58 (53%)	Stimulation
IL-7	24/58 (41%)	Stimulation
IL-4	2/49 (4%)	Stimulation
	22/49 (45%)	Inhibition
MoAb 1F5 (CD20 activation)	18/58 (31%)	Stimulation
Moab 14G7 (CD40 activation)	10/44 (23%)	Stimulation
1F5 + IL-3	22/36 (61%)	Additive response in four cases; no synergy
1F5 + IL-7	25/50 (50%)	Additive response in nine cases; no synergy
1F5 + 14G7	22/36 (61%)	Additive response in five cases; no synergy
14G7 + IL-3	6/12 (50%)	Synergistic response in one case
14G7 + IL-7	5/12 (42%)	Additive response in two cases; synergistic response in one case
14G7 + IL-4	2/11 (18%)	Stimulation; no additive or synergistic responses
	5/11 (45%)	Inhibition

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