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Growth Inhibition of CD20-positive B Lymphoma Cell Lines by IDEC-C2B8 Anti-CD20 Monoclonal Antibody

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Treatment with IDEC-C2B8 (C2B8), the chimeric anti-CD20 antibody, was shown in a phase I-II study to be very effective for the treatment of low-grade B-cell lymphoma, in contrast to the results of most previous immunotherapies with monoclonal antibodies. In a study designed to elucidate the reason for this efficacy, two cell lines derived from lymphomas with BCL2 gene rearrangement (SU-DHL-4 and SU-DHL-6) showed remarkable growth inhibition and cell-death, and two other cell lines derived from a diffuse lymphoma (RC-K8) and a mantle cell lymphoma (SP-49) showed moderate growth inhibition, but neither a CD20 weakly positive cell line (NALL-1) nor a negative cell line (MOLT-4) showed any growth inhibition. An examination of the intensity of cell-surface CD20 expression showed no correlation between intensity and degree of growth inhibition among the four cell lines showing growth inhibition. Morphological examination revealed condensed and fragmented nuclei and budding of the plasma membrane, both characteristic of apoptosis, with some cells in these cell lines showing growth inhibition by C2B8. Such apoptosis was also confirmed by flow cytometric analysis, suggesting that, at least in part, apoptosis plays a role in this growth inhibition. This growth-inhibitory mechanism may thus account for the effectiveness of C2B8 antibody therapy.

Key words: B-cell lymphoma — Anti-CD20 antibody — Apoptosis

In the treatment of B-cell lymphoma, immunotherapy with monoclonal antibodies is an attractive alternative to chemotherapy or radiotherapy. In the 1980s, anti-idiotype monoclonal antibodies or other antibodies specific to cell-surface differentiation antigens were developed in order to treat lymphoma patients by means of an immunological cell killing mechanism.[1,2] Although treatments with anti-idiotype monoclonal antibodies[3–5] or anti-CDw52 antibodies (CAMPATH-1G and CAMPATH-1H)[6,7] have been applied clinically, the results have not necessarily been satisfactory. One of the major causes of poor responsiveness is the induction of an anti-globulin response in the patient.

However, human and mouse chimeric antibodies have been developed to minimize antiglobulin responses and to facilitate both the effector cell function and complement fixation. Anti-CD20 monoclonal antibody IDEC-C2B8 (C2B8) is one of these chimeric antibodies and consists of human immunoglobulin G (IgG) 1-κ constant regions and variable regions from murine anti-CD20 antibody IDEC-2B8 (2B8). Complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytolysis (ADCC) have been suggested as the mechanisms of the *in vivo* anti-tumor activity of C2B8.[8]

Clinical studies of C2B8 have demonstrated considerable anti-tumor activity and safety in patients with low-grade or follicular lymphoma.[9] In a single-agent Phase II clinical trial in relapsed patients with low-grade or follicular lymphoma, the overall response rate (complete remission and partial remission) was as high as 50% and the median time to progression was 10.3 months.[10] A subsequent combination chemoimmunotherapy study of C2B8 with CHOP demonstrated a 100% response rate in low-grade lymphoma.[11] Thus, these clinical data indicated that C2B8 has the potential to prolong lymphoma patients' survival.

These excellent clinical results with C2B8 prompted us to investigate in more detail its cell killing mechanism by focusing on the interaction of C2B8 with CD20 antigens on lymphoma cell lines, because CD20 has been characterized as a B-cell specific cell-surface molecule with four membrane-spanning domains and cytoplasmic N- and C-terminal domains, and it has been suggested that it may play an important functional role in B-cell activation, proliferation and differentiation.[12]

To examine whether a suppression mechanism exists, we studied the growth rates of six lymphoma cell lines in the presence of C2B8, and found that the growth of the four cell lines which strongly expressed CD20 was inhibited *in vitro*. Furthermore, apoptotic cell death accompa-

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nying the growth inhibition was observed by means of morphological and flow cytometric analysis.

MATERIALS AND METHODS

Monoclonal antibodies 2B8 is a murine IgG1 monoclonal antibody which is specific to CD20. This antibody was purified from a hybridoma cell line prepared by fusion of mouse myeloma cells and splenocytes from a mouse immunized with human lymphoblastoid cell line SB. C2B8 is a human-mouse chimeric IgG which consists of the variable regions from 2B8 and the constant regions from human IgG1-κ. 2B8 and C2B8 were supplied by IDEC Pharmaceutical Co. (San Diego, CA). The monoclonal antibodies anti-CALLA (CD10, Becton Dickinson, Franklin Lakes, NJ), HIB19 (CD19, PharMingen, San Diego, CA), Leu16 (CD20, Becton Dickinson) and CR3/43 (HLA-DR, DAKO-Japan, Kyoto) were used for the analysis of cell surface antigens with a FACScan flow cytometer (Becton Dickinson).

Cell lines Six cell lines were used for the present study and their surface markers and antibody binding capacity (ABC) to CD20 are summarized in Table I. SU-DHL-4[13] and SU-DHL-6[14] have been shown to contain a BCL2 rearrangement.[15] SP-49,[16] derived from mantle cell lymphoma, and RC-K8,[17] derived from diffuse large cell lymphoma, were kindly provided by Dr. Kubonishi of Kochi Medical University. NALL-1[18] was derived from pre-B type acute leukemia and MOLT-4[19] from T cell type leukemia. SU-DHL-4, SU-DHL-6, SP-49, NALL-1 and MOLT-4 were maintained in Iscove's modified Dulbecco's medium (IMDM) with 5% heat-inactivated fetal calf serum (FCS) and RC-K8 was maintained in IMDM with 10% FCS.

Quantitative analysis of CD20 antigen on the cell surface The ABC (sites/cell) of CD20 was measured by means of quantitative indirect immunofluorescence (QIFI) assay ("QIFIKIT," DAKO-Japan).[20, 21] Briefly, a mixture of five standard microbead populations with a known ABC for mouse IgG was reacted with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody and analyzed in a FACScan flow cytometer. A standard curve was then obtained from the resulting five fluorescence intensity values. Cell lines reacted with anti-CD20 antibody were incubated with the same FITC-conjugated anti-mouse IgG antibody as used for the standard, and the fluorescence intensity of each cell line was converted into ABC with "TallyCAL" (DAKO-Japan) software by using the standard curve described above.

Analysis of cell growth Cells were centrifuged at 1200 rpm for 3 min and the resulting pellet was suspended in a fresh culture medium. Five milliliters of a 2×10^5 cells/ml suspension was incubated in a 6-well clustered culture dish (Costar, Cambridge, MA) with concentrations of 0 (control), 1, 10, 100, or 1000 μg/ml of C2B8 or 2B8. The cells were cultured at 37°C with 5% CO_2 for 5–6 days and the viable cells and dead cells were counted daily by use of the trypan blue dye-exclusion test.

For morphological analysis, 2×10^5/ml cells were cultured in the presence or absence of 100 μg/ml of C2B8 for 3 days. Then 2×10^4 cells were cytocentrifuged on glass slides, fixed and stained with May-Grünwald-Giemsa.

Assay of apoptosis Cells were prepared and cultured under the same conditions as for the proliferation analysis in the presence or absence of C2B8 (100 μg/ml). At 0, 1, 4, 8, 24, 48 and 72 h from the start of the culture, cells in 300 μl of cultured suspension were washed and stained with annexinV-FITC (AN) and propidium iodide (PI)[22, 23] with the aid of an "Apoptosis Detection Kit" (R&D Systems, Minneapolis, MN). Four thousand cells were analyzed with a FACScan flow cytometer, and the AN–/PI–, AN+/PI–, and AN+/PI+ populations were counted. These populations were selected because cells expressing phosphatidylserine on the outer leaflet of cell membranes bind AN, and cells with a compromised cell membrane allow PI to bind to the cellular DNA. Thus, the three populations of AN–/PI–, AN+/PI–, and AN+/PI+ have been found to correspond to live cells, early apoptotic cells, and both late apoptotic cells and necrotic cells, respectively.[22, 23]

Table I. Cell Lines

Cell line	Cell origin	CD10 (%)	CD19 (%)	CD20 (%)	HLA-DR (%)	ABC[a] of CD20 ($\times10^3$ sites/cell)
SU-DHL-4	Diffuse histiocytic lymphoma	97	100	100	100	123.1
SU-DHL-6	Diffuse histiocytic lymphoma	96	86	99	100	86.4
SP-49	Mantle cell lymphoma	0	96	100	100	56.5
RC-K8	Diffuse large cell lymphoma	0	30	100	34	111.1
NALL-1	Pre-B ALL[b]	99	99	62	99	16.3
MOLT-4	T-ALL[b]	0	0	0	0	0.6

a) ABC: antibody binding capacity.
b) ALL: acute lymphoblastic leukemia.

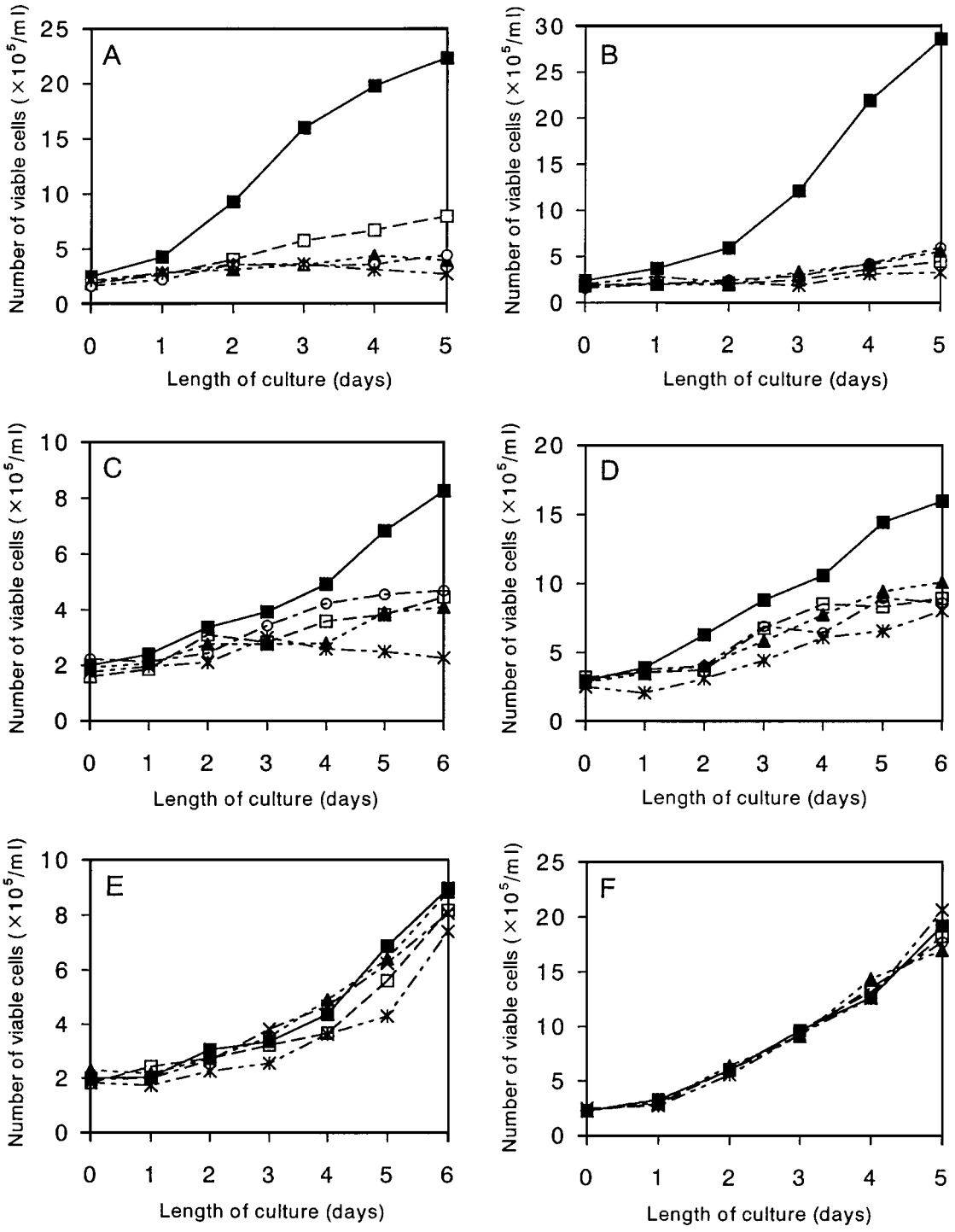

Fig. 1. Growth curves of the cell lines in the presence of various concentrations of C2B8. Each cell line (2×10^5/ml) was plated with various concentrations of C2B8 antibody. The number of viable cells was counted daily. C2B8 was used at concentrations of 1 μg/ml (□), 10 μg/ml (▲), 100 μg/ml (○), 1000 μg/ml (∗) or was absent (■). The curves represent one of the results of two repeated experiments. Both of the experiments gave similar results (data not shown). A, SU-DHL-4; B, SU-DHL-6; C, SP-49; D, RC-K8; E, NALL-1; F, MOLT-4.

Apoptosis was further analyzed by the TUNEL method using an "APO-BRDU" Kit (Phoenix Flow Systems, Inc., San Diego, CA). Briefly, at 24 h from the start of the culture, the cells were washed, fixed with 1% (W/V) paraformaldehyde in phosphate-buffered saline and then processed for labeling of DNA breaks with terminal deoxynucleotidyl transferase, bromodeoxyuridine triphosphate (Br-dUTP), and fluorescein-labeled anti-BrdU antibody and for counter-staining of the total DNA with PI/RNase A solution. Ten thousand cells were analyzed with a FACScan flow cytometer. By using the dual parameter display method, apoptotic cells and the cell cycle were determined.[24)]

RESULTS

Growth inhibition by C2B8 Growth curves of the six cell lines in the presence of C2B8 are shown in Fig. 1. SU-DHL-4 and SU-DHL-6 without the antibody had increased about ten times by day 5. In the presence of more than 1 μg/ml of C2B8, their growth was significantly retarded (Fig. 1, A and B). The dead cells increased day by day, and representative data are shown in Fig. 2. SP-49 also showed significant growth inhibition at an antibody concentration of 1000 μg/ml (Fig. 1C), but this inhibition became only moderate at lower antibody concentrations when compared to that obtained for SU-DHL-4 and SU-DHL-6 (Fig. 1, A, B and C). RC-K8 showed moderate growth inhibition at various antibody concentrations (Fig. 1D), and its growth rate remained close to half of that without the antibody. NALL-1 and MOLT-4, whose CD20 was weakly positive or negative, respectively (Table I), did not show any growth inhibition (Fig. 1, E and F). These data indicate that C2B8 had a growth-inhibitory effect on lymphoma cell lines expressing CD20.

The growth-inhibitory potential of C2B8 was compared with that of the original mouse monoclonal antibody 2B8 to examine if the modification of the Fc portion affected its activity. The growth-inhibitory curves obtained in the presence of either C2B8 or 2B8 at a concentration of 100 μg/ml were almost identical to each other in the case of SU-DHL-4, but no growth inhibition was seen with MOLT-4 (Fig. 3). This evidence suggested that binding to the same epitope in the CD20 molecule with the Fab portion of these two antibodies accounted for the growth inhibition of the lymphoma cell lines.

Anti-CD20 antibody-binding capacity in cell lines To examine the correlation between the expression of CD20 and the degree of growth inhibition in each of the cell lines, the number of binding sites for anti-CD20 antibody was measured by flow cytometry. The cell lines with significant growth inhibition, SU-DHL-4, SU-DHL-6, RC-K8, and SP-49, showed more than 56.5×10^3 sites/cell, while the cell lines with no growth inhibition, NALL-1 and MOLT-4, showed only 16.3 and 0.6, respectively (Table I). RC-K8 showed a similar number of antibody-binding sites to those of SU-DHL-4 and more binding sites than those of SU-DHL-6, but the growth inhibition was less than that observed with the other two cell lines (Table I and Fig. 1, A, B and D).

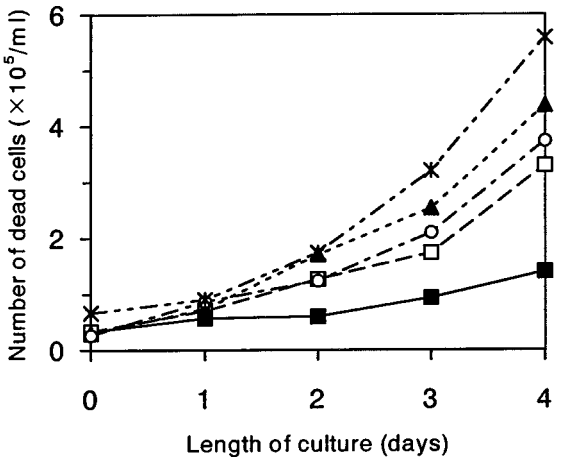

Fig. 2. Dead cell counts of the cell line SU-DHL-4 in the presence of various concentrations of C2B8. SU-DHL-4 (2×10^5/ml) was plated with various concentrations of C2B8 antibody. The number of dead cells was counted daily. C2B8 was used at concentrations of 1 μg/ml (□), 10 μg/ml (▲), 100 μg/ml (○), 1000 μg/ml (∗) or was absent (■). The curves represent one of the results of two repeated experiments. Both of the experiments gave similar results (data not shown).

Induction of apoptosis by C2B8 The morphological changes during growth inhibition and cell death induced by C2B8 were examined microscopically. Fig. 4 shows May-Giemsa staining of SU-DHL-4 incubated with 100 μg/ml of C2B8 for 72 h. A considerable number of cells had lost their normal nuclear and cytoplasmic structure, and representative cells are shown in Fig. 4A. The cell indicated by the arrow shows a reduced cell size with a condensed and fragmented nucleus, while budding of plasma membranes in other cells is also apparent. All these features are characteristic of apoptotic cells.[25)] No such findings were observed in the absence of the antibody (Fig. 4B).

To confirm the apoptotic changes, the proportion of necrotic or apoptotic cells was measured by flow cytometry. Fig. 5 shows a representative log fluorescence dot plot of AN (abscissa) and PI (vertical) after 72 h for SU-DHL-4 and MOLT-4. In the case of SU-DHL-4 with C2B8 antibody, the AN+/PI− (early apoptotic) population accounts for 32.0% and AN+/PI+ (late apoptotic and necrotic) for 25.1%, while the AN−/PI− (live) population is 41.4% (Fig. 5A). These changes were not observed in either SU-

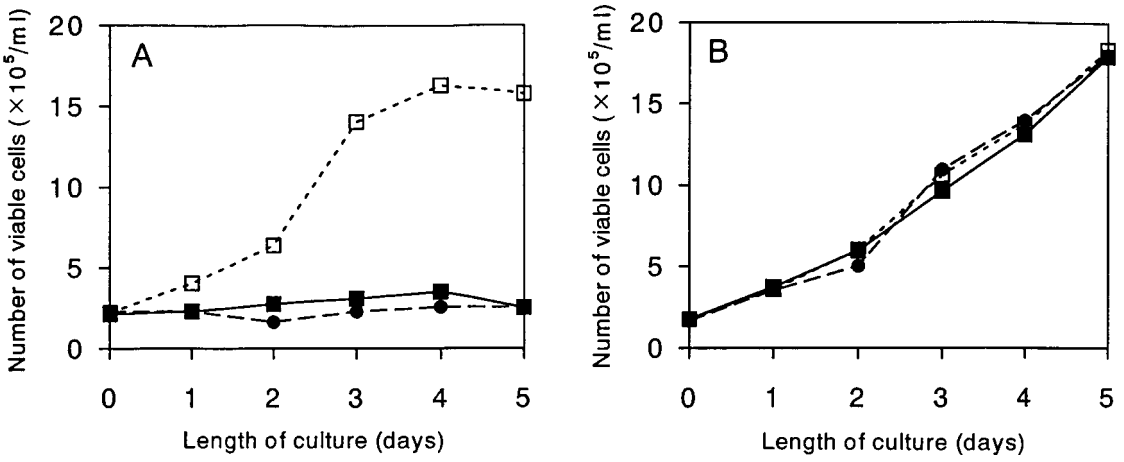

Fig. 3. Comparison of growth inhibition by C2B8 and 2B8. SU-DHL-4 and MOLT-4 were incubated with 100 μg/ml of C2B8 (●) or 2B8 (■), or in the absence of the antibody (□). Viable cells were counted daily by means of the trypan blue dye-exclusion test. A, SU-DHL-4; B, MOLT-4.

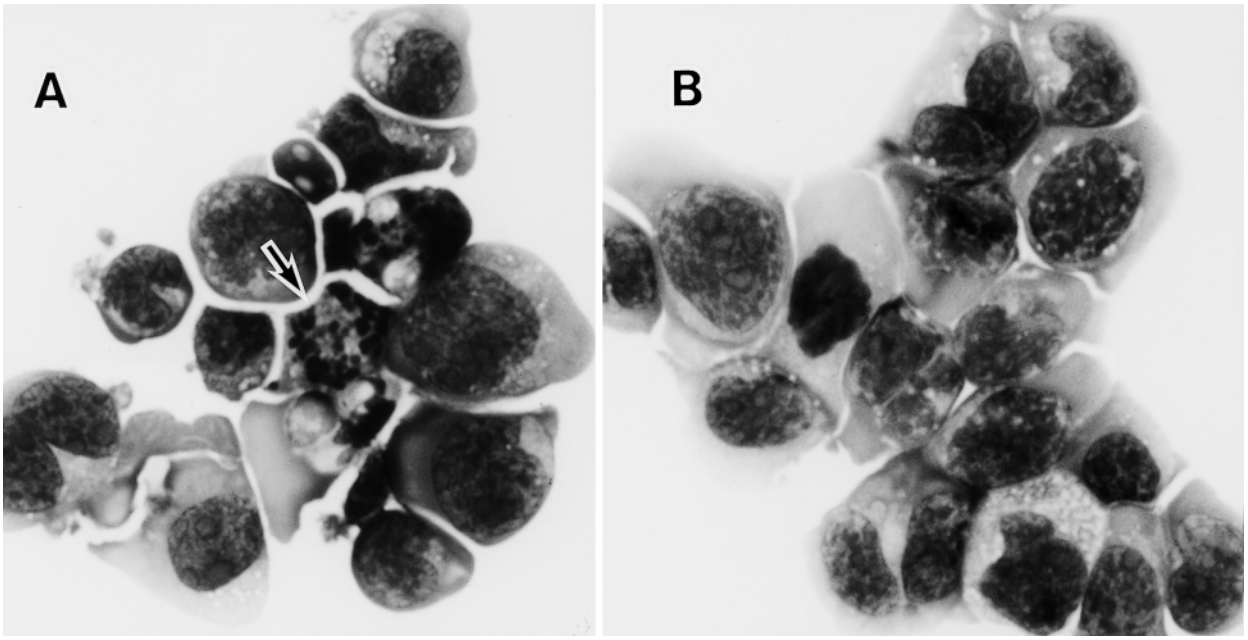

Fig. 4. Morphological changes induced by C2B8. SU-DHL-4 was incubated with 100 μg/ml of C2B8 (A) or in the absence of the antibody (B) for 3 days, and then fixed and stained with May-Grünwald-Giemsa (×400). The arrow indicates a typical apoptotic cell.

DHL-4 without antibody (Fig. 5B) or MOLT-4 with the antibody (Fig. 5C). Fig. 6 graphically summarizes the time course for SU-DHL-4. Before the addition of C2B8, more than 85% of the cells were located in the lower left (AN–/PI–) and stained cells accounted for less than 15%. During the early incubation period with C2B8, up to 48 h, the proportion of early apoptotic cells rapidly increased within the first 24 h, and then became steady. As for the proportion of cells in late apoptosis and necrosis, a rapid increase was also observed within 24 h from the start, followed by a plateau state. These changes were not observed for cells incubated without the antibody. These data clearly demonstrate that C2B8 induced apoptosis within 24 h.

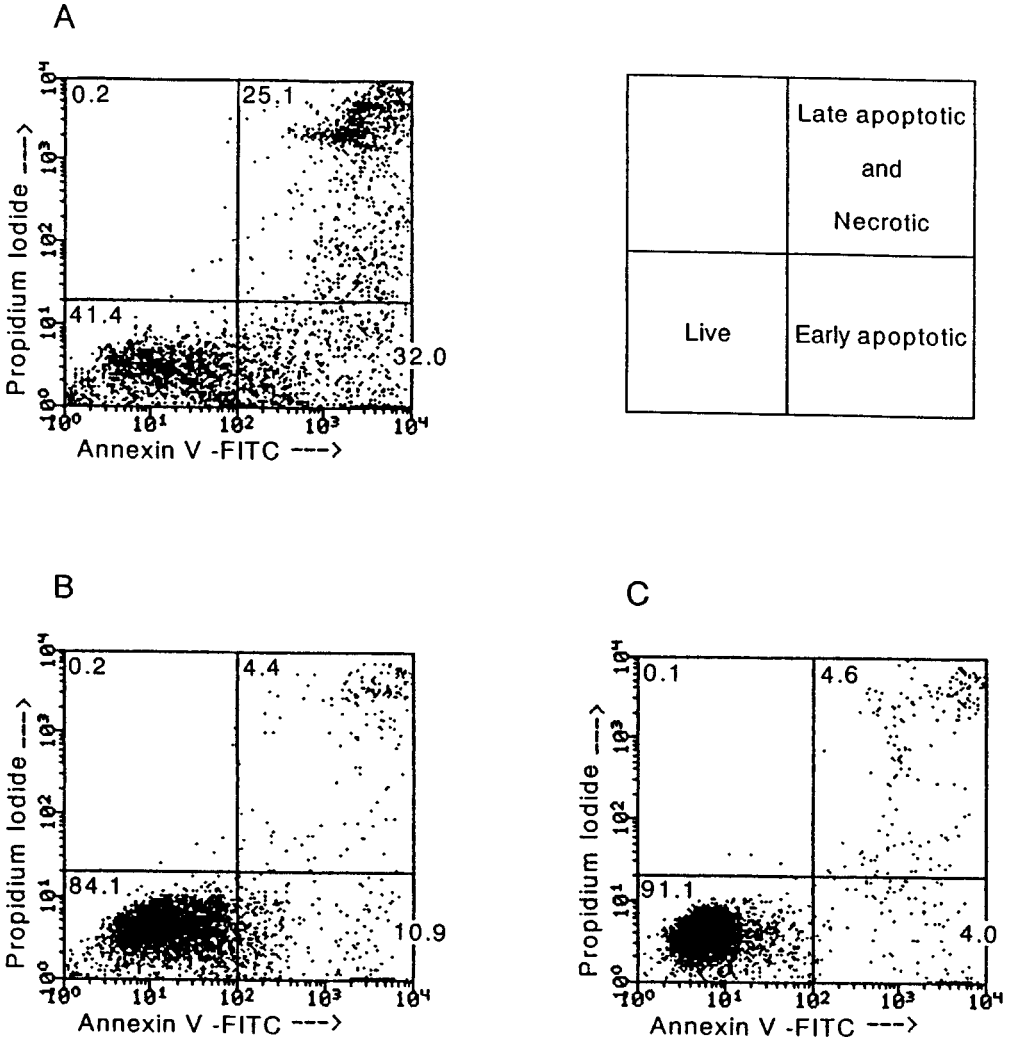

Fig. 5. Flow cytometric analysis of apoptosis. SU-DHL-4 cells were incubated with 100 μg/ml of C2B8 (A) or without C2B8 (B), and MOLT-4 with 100 μg/ml of C2B8 (C). At 0, 1, 4, 8, 24, 48 and 72 h from the start, apoptotic or necrotic cells were analyzed with a FACScan flow cytometer. The values shown are examples of the results at 72 h. The fluorescence intensities of AN (abscissa) and PI (vertical) have been plotted. The location of populations of live cells (AN−/PI−), early apoptotic cells (AN+/PI−) and late apoptotic cells and necrotic cells (AN+/PI+) are shown in the upper right panel. The numerical values in each of four regions of the panels indicate the proportion of each type of population.

In order to confirm further the apoptosis and cell cycle specificity, a method based on *in situ* DNA strand break labeling was used. Fig. 7 shows DNA strand breaks (vertical) and DNA content (abscissa) of SU-DHL-4 at 24 h following the addition of C2B8. With the antibody, the DNA strand breaks increased in G0/G1 phase cells, while such changes were not observed in the absence of the antibody. However, the percentage of apoptosis is small, suggesting that only a proportion of cells becomes apoptotic after the addition of the antibody.

DISCUSSION

C2B8 is reported to have a profound anti-tumor effect in relapsed patients with low-grade or follicular lymphoma.[9, 10] On the basis of a previous *in vitro* study using a CD20-positive human lymphoblastoid cell line SB, ADCC and CDC have been suspected to be the major mechanisms of cytotoxic activity of C2B8 antibody.[8] In this report, we examined CD20-positive cell lines of various origins, and demonstrated the growth-inhibitory effect

of C2B8. It is worth noting that the concentration of C2B8 inducing growth inhibition in the culture medium ranged from 1 μg/ml to 1000 μg/ml, the same range as that of the serum concentrations seen in patients after administration of C2B8.[8, 26] Therefore, it is conceivable that this growth inhibition mechanism could also function *in vivo*.

The fact that the addition of C2B8 had no effect on CD20-negative cell lines indicates that the association of the antibody with CD20 is necessary for growth inhibition. Furthermore, the fact that 2B8 and C2B8 showed similar inhibitory effects suggested that an antibody binding site for the CD20 molecule is essential for this growth inhibitory effect to occur, and the human IgG1-κ portions of the C2B8 antibody are not involved in this *in vitro* growth inhibition, although the *in vivo* effect could be different between the two antibodies if ADCC occurs.

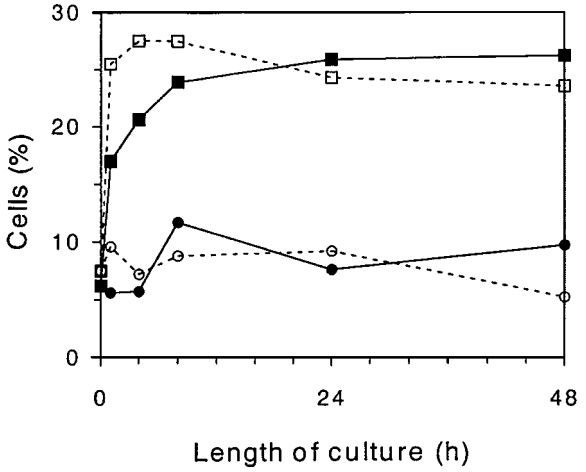

Fig. 6. Time course of apoptosis for SU-DHL-4 with or without 100 μg/ml of C2B8. At 0, 1, 4, 8, 24 and 48 h, the percentages of early apoptotic cells (AN+/PI–) with C2B8 (■) and in the absence of the antibody (●), or late apoptotic cells and necrotic cells (AN+/PI+) with C2B8 (□) and in the absence of the antibody (○) were determined by flow cytometry.

The level of growth inhibition was different for the four CD20 strongly positive cell lines. Remarkable inhibition was observed in both SU-DHL-4 and SU-DHL-6, which are known to contain a BCL2 gene rearrangement,[15] suggesting that these cell lines might be derived from cells originating in the follicular lymphoma. Growth inhibition in the mantle cell lymphoma, SP-49, and diffuse large cell lymphoma, RC-K8, was relatively mild, though at a concentration of 1000 μg/ml, strong inhibition was seen in SP-49. The fact that the maximum inhibitory effect was observed in the two follicular lymphoma-related cell lines suggested that cell type specificity may be critical. Furthermore, the inhibitory effect in SP-49 at 1000 μg/ml of the antibody indicates that a higher dosage of C2B8 might be effective for the treatment of mantle cell lymphoma.

RC-K8 did not show as profound a growth inhibition as did SU-DHL-4 and SU-DHL-6, in spite of the similar ABC of CD20, suggesting that the degree of CD20 expression does not necessarily correlate with growth inhibition. This evidence suggests that the circumstances of the intracellular progression to growth inhibition after the binding of C2B8 on CD20 may be different for the four CD20-positive cell lines examined. Although the factors which define the level of growth inhibition have not yet been clearly identified, it is nevertheless important

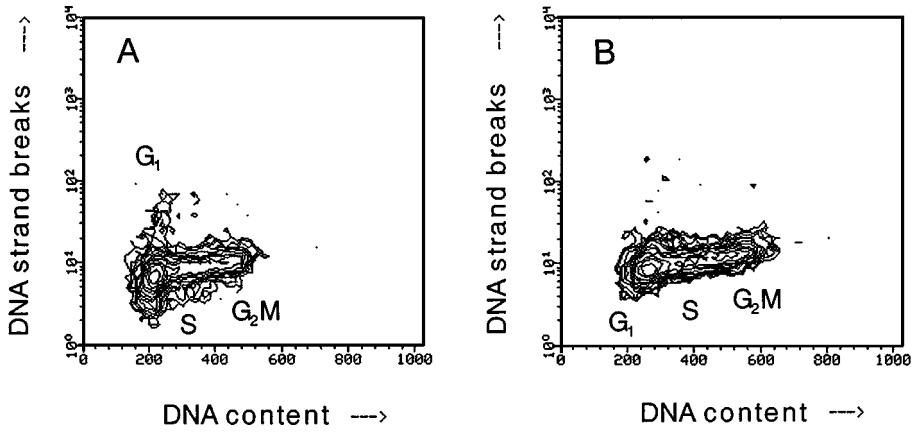

Fig. 7. Analysis of the cell cycle specificity of apoptosis by a method based on *in situ* DNA strand break labeling. SU-DHL-4 cells were incubated with (A) or without (B) 100 μg/ml of C2B8 and analyzed at 24 h from the start. DNA strand breaks (vertical) and DNA content (abscissa) were determined with a FACScan flow cytometer.

at this stage to study the sequence of events resulting from antibody binding to the CD20 molecule. The biological function of CD20 is not well understood, but it has been suggested that CD20 is associated with transmembrane Ca^{2+} conductance and plays an important functional role in B-cell activation, proliferation and differentiation.[12, 27–29] In normal B lymphocytes activated by mitogen, binding of an anti-CD20 antibody inhibited cell cycle progression from the G0 to the G1 phase.[29] In other experiments, some anti-CD20 antibodies inhibited B-cell differentiation or immunoglobulin secretion.[27, 28] It is thus conceivable, on the basis of this evidence, that anti-CD20 antibody has an inhibitory effect on proliferation in some B-cell lymphoma. Further studies of the cell cycle-modulating and cytodifferentiating effects of binding with C2B8 should cast more light on the mechanism of lymphoma cell growth inhibition.

It should be pointed out that C2B8 induced apoptosis. In the present study, the flow cytometric analysis of the time course showed that rapid induction of apoptosis was observed within 24 h of the addition of C2B8 (Fig. 6). In the growth curves of the four cell lines in the presence of C2B8, no reduction in the number of viable cells was observed in the presence of C2B8(Fig. 1), but dead cells increased day by day (Fig. 2). These findings indicate that C2B8 induces apoptosis in a part of the cell population. In fact, C2B8 induced apoptosis in a part of the G0/G1 phase cells in SU-DHL-4 (Fig. 7). This apoptosis seems somewhat different from that induced by the Fas-Fas ligand system, in which most of the cells were found to be dying.[30] An anti-CD20 antibody, 1F5, was reported to prevent anti-IgM-induced apoptosis in a B-cell line.[31, 32] It has also been reported that 1F5 recognizes an epitope distinct from that detected by other anti-CD20 antibodies.[31, 32] A recent report by Shan *et al*. also demonstrated that the B1 antibody (detecting a different CD20 epitope) needed to be cross-linked to induce apoptosis of Burkitt cell lines.[33] Although the sequence of events was different for different anti-CD20 monoclonal antibodies, the available evidence suggests that the CD20 molecule is involved in the programmed cell death pathway.

For the treatment of low-grade or follicular lymphoma, C2B8 clinical trials have shown promising results.[8–11] Although the precise *in vivo* mechanisms of the anti-tumor effect of C2B8 remain to be elucidated, it is very important from the therapeutic point of view that growth inhibition can be obtained with this antibody. This might be one reason why C2B8 has been more effective than the monoclonal antibodies previously employed for therapy. Further studies of the anti-tumor effect of C2B8 should provide new insights into more effective therapies for B-cell lymphoma.

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