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bcl-2 Regulation in Normal Resting Lymphocytes and Lymphoblasts

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Expression of the *bcl-2* protein and *bcl-2* mRNA at the individual cell level was semiquantitatively examined in normal quiescent peripheral blood lymphocytes and pokeweed mitogen- or concanavalin A and interleukin-2-induced lymphoblasts *in vitro* by microscopic fluorometry using immunofluorescence and fluorescein-labeled *in situ* hybridization. Approximately 90% of normal quiescent T and B lymphocytes expressed *bcl-2* protein at a level which was compatible with that of *bcl-2* mRNA. On the contrary, most mitogen-induced lymphoblasts showed a posttranscriptional suppression of *bcl-2* protein expression. However, *bcl-2* protein was not downregulated by the posttranscriptional suppression in all lymphocytes activated *in vitro*, but approximately 15% of the lymphoblasts still expressed *bcl-2* protein at a higher level than nontransformed quiescent small lymphocytes; thus *bcl-2* protein expression in lymphoblasts showed a distinct bimodal pattern. Furthermore, it was supposed that lymphoblasts with no detectable *bcl-2* protein might fall into apoptosis but the remainder, expressing high levels of *bcl-2* protein, could escape apoptosis. Thus, the *bcl-2* gene may play an important role as a regulator of apoptosis in the human immune system.

Key words: *bcl-2* — Apoptosis — Lymphocyte — Mitogen

The *bcl-2* gene has been implicated in B cell lymphomagenesis through its activation by the t(14;18) chromosomal translocation.[1-4] *bcl-2* protein prolongs the lifespan of certain growth factor-dependent cell lines and inhibits programmed cell death (apoptosis),[5-7] but the role of *bcl-2* gene in lymphomagenesis is still unclear. We and another group previously showed that germinal center cells in the lymph node paradoxically express abundant *bcl-2* transcripts despite the absence of *bcl-2* protein.[8,9] Previous studies of the regulation of *bcl-2* gene in normal human peripheral blood lymphocytes (PBL) demonstrated that, unlike quiescent PBLs containing little or no detectable *bcl-2* mRNA, lymphoblasts activated *in vitro* using phytohemagglutinin or anti-immunoglobulin μ chain antibody expressed markedly elevated levels of *bcl-2* mRNA.[10,11] However, in these studies the *bcl-2* protein and mRNA were evaluated in a whole lymphoblast population, and the *bcl-2* regulation in lymphoblasts at an individual cell level remains unclear. In the present study, we investigated the *bcl-2* gene regulation in both quiescent PBLs and lymphoblasts activated *in vitro* at an individual cell level. The role of *bcl-2* gene in clonal selection or deletion of lymphocytes in normal human immune systems through regulation of programmed cell death is discussed.

MATERIALS AND METHODS

Cells Mononuclear cells were isolated from healthy human peripheral blood by Ficoll-Hypaque density gradient centrifugation. They were used as PBLs after removal of monocytes by incubation in plastic culture vessels coated with human AB serum for 30 min at 37°C in a CO_2 incubator. PBLs were incubated in RPMI 1640 containing 10% fetal calf serum and 0.1% pokeweed mitogen (PWM) (Gibco, USA) or 10 mg of concanavalin-A (Con A) (Boehringer Mannheim, Germany) and 75 JRU/ml of interleukin-2 (IL-2) (Shionogi Co. Ltd., Osaka) for 72 h at 37°C in a CO_2 incubator and used as the source of lymphoblasts. PWM-induced lymphoblasts consisted of 95% T cells and 5% B cells as revealed by flow cytometric analysis and double enzyme immunohistochemistry using anti-CD3, CD19 and CD20 antibodies. Con A and IL-2-induced lymphoblasts included approximately 99% T cells. These preparations contained over 50% lymphoblasts. For Western blots, the lymphoblasts were further enriched to over 80% by means of non-sequential density gradient centrifugation with 50, 45, and 40% Percoll (Pharmacia LKB, Sweden).

Immunofluorescent staining *bcl-2* protein was detected by indirect immunofluorescent staining of acetone-fixed cytosmears using anti-*bcl-2* protein monoclonal antibody (MAb) (*Bcl-2* 100α) as described previously.[8] Controls

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were stained with MOPC-21 mouse myeloma $IgG_1\kappa$ protein (Sigma, USA).

Double enzyme immunohistochemistry After culture for 7 days in the presence of PWM, most lymphoblasts fell into apoptosis. Therefore, the cells which had formed apoptotic bodies were removed by Ficoll-Hypaque density gradient centrifugation, and only live cells were immunostained.

Microscopic fluorometry Using microscopic fluorometry, small lymphocytes that did not show a blastic change and lymphoblasts in the same specimen were semiquantitatively evaluated. The fluorescence signal on an individual cell of each sample was point-measured by use of a microscopic fluorometer (MPM, Zeiss, Germany). Total number of cells used for each fluorometric analysis was one hundred. Absorbance values were estimated as an integral value per cell after subtracting the mean value of the control.

Western blotting Cell lysates prepared from 10^7 cells were analyzed by Western blotting as described previously.[8]

***In situ* hybridization** Cytosmear specimens of quiescent PBLs and of lymphocytes stimulated with PWM in culture as described above were subjected to *in situ* hybridization to detect *bcl-2* mRNA using a digoxygenin(Dig)-labeled antisense RNA probe against *bcl-2* mRNA. The detection procedure was the same as described previously,[8] except that sheep anti-Dig antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-sheep IgG were used instead of alkaline phosphatase-conjugated anti-Dig antibody.

Northern blotting Total RNAs were extracted from 10^8 non-stimulated and PWM-stimulated PBLs, and 20 μg aliquots of these RNAs were analyzed by Northern blotting as described previously.[8] The intensity of expressed *bcl-2* mRNA of each cell sample on autoradiographed film was quantified using a densitometer after standardization by β-actin hybridization.

DNA fragmentation assay PBLs were cultured in the presence of PWM for 7 days and separated into live (mostly consisting of non-blastic small lymphocytes) and dead cells (mostly consisting of lymphoblasts) by Ficoll-Hypaque density gradient centrifugation. DNA fragmentation in these cells was assessed following the method described previously.[12] Briefly, low-molecular-weight DNA was extracted from the supernatants of lysed cells with phenol/chloroform and precipitated with ethanol/ammonium acetate. After digestion with RNase A, DNA fragments (3 μg) in each cell sample were loaded on a 2% agarose gel containing 0.01% ethidium bromide and electrophoresed. Bands were visualized by means of a UV transilluminator.

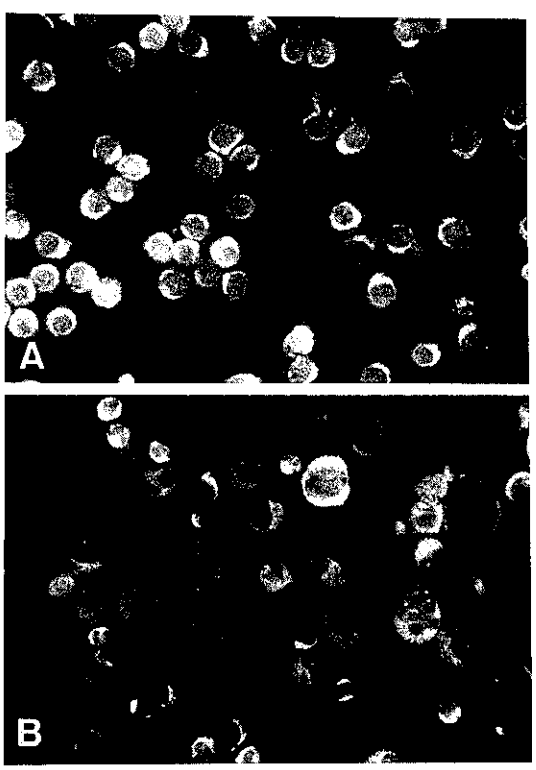

Fig. 1. Indirect immunofluorescence for *bcl-2* protein. A: Freshly isolated non-stimulated PBLs. B: PBLs stimulated with PWM in culture for 72 h. ×160.

RESULTS

Expression of *bcl-2* protein in normal resting lymphocytes freshly separated from PBL and lymphoblasts induced with PWM or with Con A and IL-2 for 72 h was examined by immunohistochemical means. Over 90% of normal quiescent PBLs expressed *bcl-2* protein with weak intensity (Fig. 1A), whereas most of the lymphoblasts did not express any detectable *bcl-2* protein except for a few strongly positive lymphoblasts consisting of both T and B cells (Fig. 1B). Small lymphocytes which did not show a blastic change after PWM or Con A and IL-2 exposure expressed *bcl-2* protein at a level comparable to that of non-treated PBLs. Double enzyme immunohistochemistry, using anti-*bcl-2* protein MAb and anti-CD3 or anti-immunoglobulin antibody, revealed that both quiescent T and B lymphocytes in the peripheral blood expressed *bcl-2* protein (data not shown).

Microscopic fluorometry revealed that approximately 85% of lymphoblasts had lost *bcl-2* protein, but that the remainder expressed fairly high levels of the protein, thus presenting a bimodal pattern (Fig. 2). The small lymphocytes, which did not show the blastic change upon PWM exposure, expressed *bcl-2* protein at a low level compara-

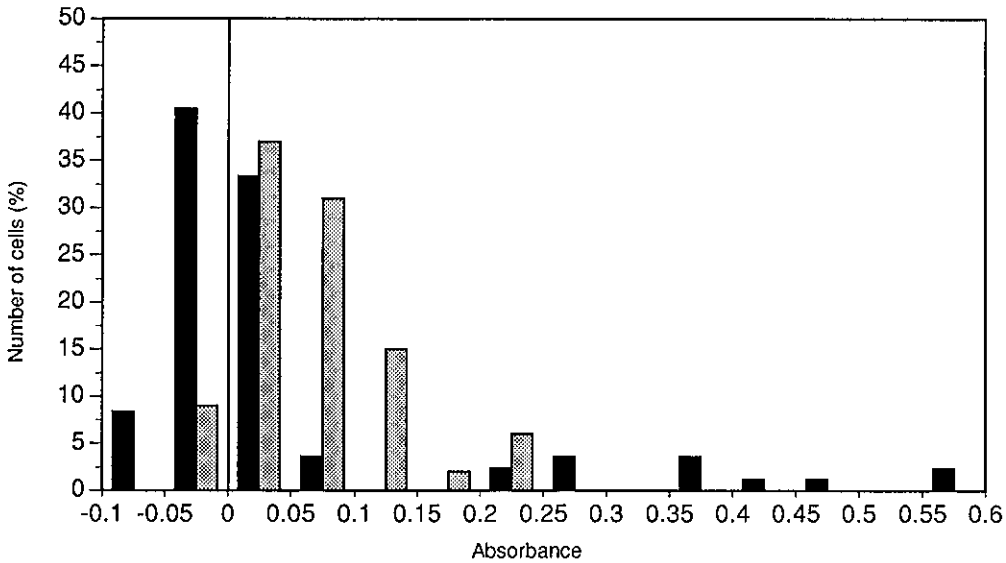

Fig. 2. Quantitative analysis of *bcl-2* protein expression in PWM-treated PBLs using microscopic fluorometry. ■: Lymphoblasts; ▨: Small lymphocytes.

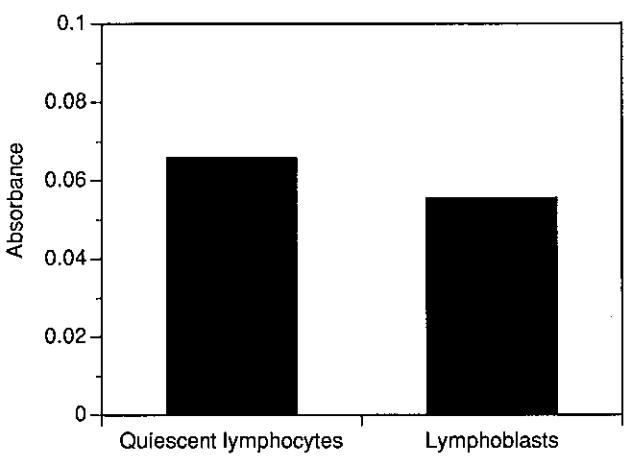

Fig. 3. *bcl-2* protein in both quiescent PBLs and PWM-induced lymphoblasts using microscopic fluorometry. Mean absorbance value of *bcl-2* signal of 100 cells in each sample is indicated as a bar.

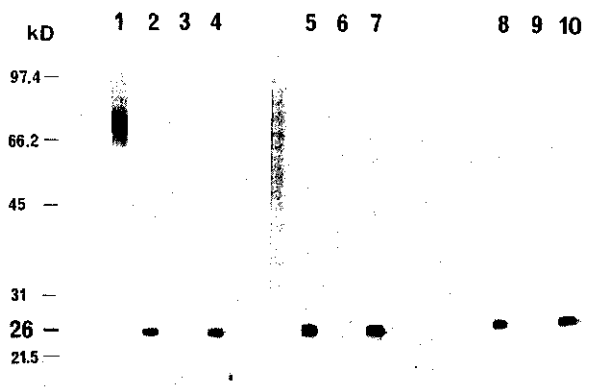

Fig. 4. Western blot analysis of *bcl-2* protein. Lanes 1–4 are the lysates from lymphoblasts induced by PWM. Lanes 5–7 are the lysates from small lymphocytes which did not transform into blasts in the presence of PWM. Lanes 8–10 are the lysates from resting PBLs which were cultured without PWM for 72 h. Lanes 2, 5 and 8 were incubated with anti-*bcl-2* protein MAb (*Bcl-2* 100α), lanes 4, 7 and 10 with anti-*bcl-2* protein MAb (*Bcl-2* 124α), lane 1 with anti-CD44 MAb, and lanes 3, 6 and 9 with MOPC-21 mouse myeloma protein ($IgG_1\kappa$) as a negative control antibody. In lanes 2, 4, 5, 7, 8, and 10, a single band corresponding to 26 kDa was recognized.

ble to that of PBLs which were cultured without PWM for the same period. However, the mean value of *bcl-2* protein evaluated by microscopic fluorometry was almost the same in both quiescent PBLs and PWM-induced lymphoblasts (Fig. 3). Western blotting revealed a 26 kDa band corresponding to *bcl-2* α protein in lysates extracted from both lymphoblasts and non-blastic lymphocytes in PWM-treated PBL cultures (Fig. 4). The band intensity was similar in both preparations although *bcl-2* protein was downregulated in more than 80% of the lymphoblasts. These results may be explained by the

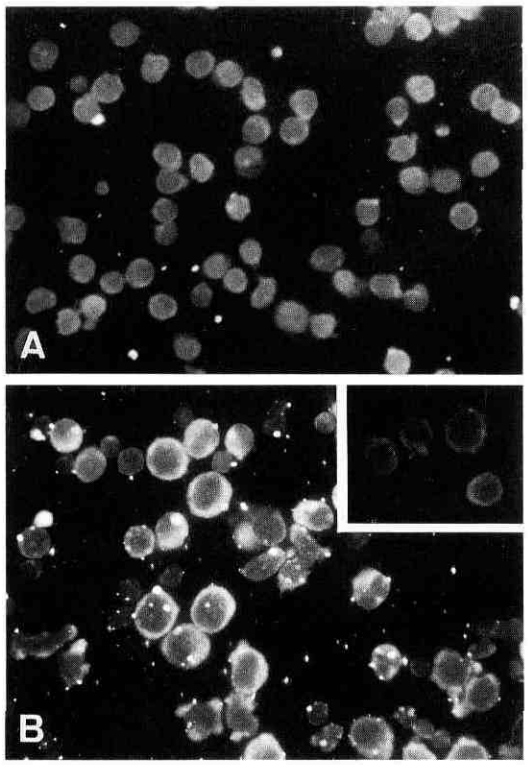

Fig. 5. Fluorescence *in situ* hybridization for *bcl-2* mRNA using a digoxygenin-labeled antisense and sense (inset) *bcl-2* probe. A: Freshly isolated non-stimulated PBLs. B: PBLs stimulated with PWM in culture for 72 h. ×160.

presence of a small number of lymphoblasts expressing *bcl-2* protein at higher levels, as shown in Fig. 1B.

bcl-2 mRNA was detected both in non-treated resting PBLs and PWM-treated lymphoblasts by fluorescence *in situ* hybridization. Over 90% of the quiescent lymphocytes expressed *bcl-2* mRNA with a weak or moderately intense FITC signal (Fig. 5A), whereas most of the

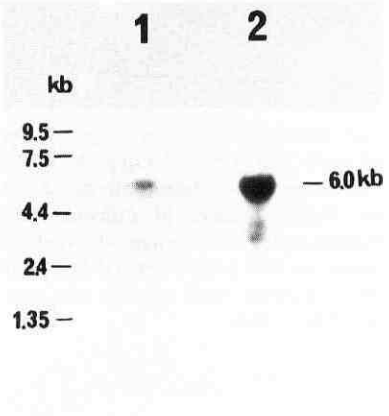

Fig. 6. Northern blot hybridization revealing *bcl-2* mRNA in resting (lane 1) and PWM-stimulated PBLs (lane 2). The transcripts in both lanes displayed a single band corresponding to the 6.0 kb normal transcript of *bcl-2* mRNA. The intensity of the transcript in PWM-stimulated PBLs was about 3.4-fold higher than that in resting PBLs.

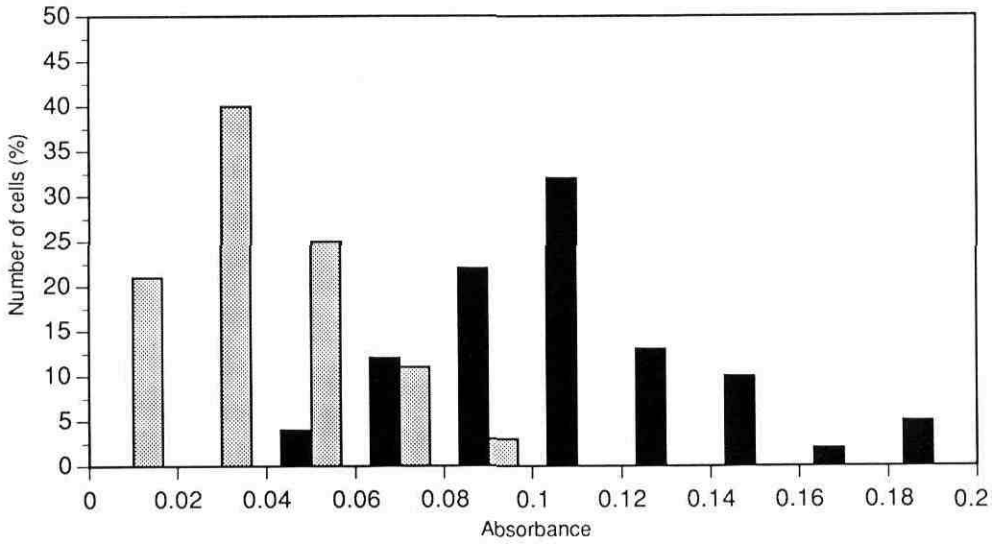

Fig. 7. Distribution of *bcl-2* mRNA expression in both PWM-induced lymphoblasts and non-transformed quiescent PBLs using microscopic fluorometry. A fluorescence signal on individual cells (total 100 cells) was point-measured with a microscopic fluorometer. ■: PWM blastic lymphocytes; ▨: PWM small lymphocytes

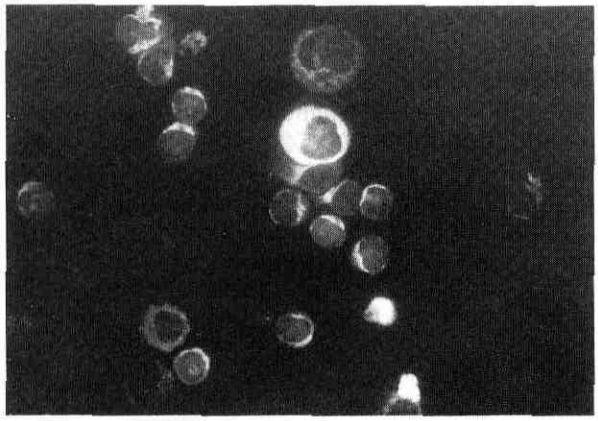

Fig. 8. Indirect immunofluorescence for *bcl-2* protein of the PBLs cultured with PWM for 7 days. Most PWM-induced lymphoblasts with low or no detectable *bcl-2* protein fell into apoptosis after about 7 days of culture, but small non-transformed lymphocytes still remained viable. Note the remarkable decrease in the proportion of lymphoblasts among the whole cells compared with that in the sample cultured with PWM for 72 h. ×160.

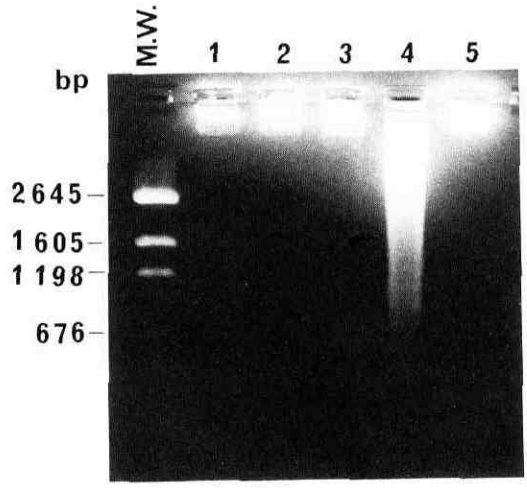

Fig. 9. DNA fragmentation assay. Lane M.W., pGEM digested with *Hin*fI, *Rsa*I, and *Sin*I as a molecular weight marker; lane 1, DNA extracted from PBLs cultured with PWM for 24 h, which showed no blastic change; lane 2, DNA extracted from PBLs cultured with PWM for 72 h, of which 53% showed blastic change; lane 3, DNA extracted from live small lymphocytes purified from PBLs cultured with PWM for 7 days; lane 4, DNA extracted from dead cells mainly consisting of clustered lymphoblasts separated from PBLs cultured with PWM for 7 days; lane 5, DNA extracted from PBLs cultured without PWM for 7 days.

lymphoblasts induced by PWM expressed high levels of *bcl-2* mRNA (Fig. 5B).

Northern blots revealed that both quiescent PBLs and PWM-treated lymphocytes including over 50% of lymphoblasts transcribed 6.0 kb of the normal length *bcl-2* mRNA. The intensity of *bcl-2* mRNA expression in the PWM-treated lymphocytes was amplified about 3.4-fold compared with that in the resting PBLs (Fig. 6), in reasonably good agreement with the results of microscopic fluorometry (Fig. 7). After 72 h, PWM-treated PBLs formed clusters consisting of transformed lymphoblasts, over 98% of which were viable as shown by the dye exclusion test. Almost all of these lymphoblasts were immunohistochemically positive for the Ki-67 antigen, which shows that they were in a cycling phase (data not shown). However, most of the lymphoblasts became apoptotic and died about 7 days after PWM exposure. Non-blastic lymphocytes cultured under the same conditions remained alive. Only 45% of PBLs cultured with PWM for 7 days were alive, among which approximately 10% of the cells were blastic (Fig. 8). DNA fragmentation assay of the lymphoblasts cultured with PWM for 7 days showed a ladder pattern which demonstrated that they had fallen into apoptosis (Fig. 9).

DISCUSSION

Previous studies showed that the level of *bcl-2* mRNA was very low in resting PBLs and increased markedly in PBLs stimulated by mitogens and lymphokines to proliferate in culture.[11, 12] The fact that there was little or no detectable *bcl-2* mRNA in quiescent PBLs *in vitro* is apparently in conflict with the immunohistochemical demonstration of *bcl-2* protein in resting PBLs and in mantle zone lymphocytes that represent quiescent recirculating memory B cells.[8, 9, 13]

bcl-2 mRNA is much more intensely expressed in lymphoblasts than in quiescent PBLs. However, most of the mitogen-induced lymphoblasts expressed no detectable *bcl-2* protein. This phenomenon observed *in vitro* is compatible with the inverse expressions of *bcl-2* protein and *bcl-2* mRNA observed in germinal center cells of the lymph nodes.[8] This finding differs from the previous report[14] that PBLs stimulated with Con-A and IL-2 for 72 h still expressed *bcl-2* protein. In that study, *bcl-2* protein and *bcl-2* mRNA levels were measured in unseparated stimulated PBL cultures by immunoblotting. We also detected a comparable amount of *bcl-2* protein in both quiescent PBLs and PWM-stimulated PBLs overall, by Western blotting analysis. However, semiquantitative microscopic fluorometry on individual cells revealed there are two different populations of lymphoblasts in PWM-stimulated PBLs: a small population intensively expressing *bcl-2* protein and the majority bearing little or no *bcl-2* protein. Interestingly, most of the lymphoblasts

clearly expressed a large amount of *bcl-2* mRNA. This inverse expression of *bcl-2* mRNA and *bcl-2* protein was also observed in germinal center cells of the lymph node.[8, 9] As shown in Figs. 8 and 9, most of the lymphoblasts fell into apoptosis, while small quiescent PBLs escaped apoptosis. A few lymphoblasts, which strongly expressed *bcl-2* protein, may be rescued from apoptosis. In this context, it is notable that there are a few centroblasts scattered within the germinal centers that continue to express *bcl-2* protein[8] and that stimuli such as cross-linking of the surface Ig of isolated germinal center cells cause them to express *bcl-2* protein.[15]

In conclusion, mitogen-stimulated lymphoblasts were divided into two populations, little or no *bcl-2* expressing cells (85%) and high *bcl-2* expressing cells (15%). The 7-day culture with mitogen resulted in the apoptotic cell death of the former population and in the survival of the latter population. These results suggest that *bcl-2* gene regulation plays an important role as an antidote to apoptosis in the human immune system.

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