Transfection of the c-myc Oncogene into Normal Epstein-Barr Virus–harboring B Cells Results in New Phenotypic and Functional Features Resembling Those of Burkitt Lymphoma Cells and Normal Centroblasts

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Summary

Activated c-myc gene was introduced into the cells of three normal Epstein-Barr virus (EBV)–positive lymphoblastoid B cell lines (LCL). The cells were monitored for the appearance of new phenotypic and functional features compared with the control LCL cells transfected with plasmid that did not contain the c-myc gene. The LCL-expressing c-myc constitutively did not arrest growth in low serum concentration. However, the cell number in the cultures failed to increase because of substantial cell death. Death was due to apoptosis as demonstrated by flow cytometric analysis of propidium iodide–stained cells, by typical DNA laddering in gel electrophoresis, and by the inspection of Giemsa-stained cell smears. Apoptosis was also induced by exposing the transfected cells to antibodies directed to the immunoglobulin μ chain (a-μ-ab) irrespective of the serum concentration in the culture. Exposure of the cells to CD40 ligand (CD40L) or CD40 monoclonal antibody prevented cell apoptosis. Upon transfection with c-myc, the LCL cells acquired a vacuolated morphology that was never observed in control cells. Moreover, the expression of CD10 and CD38 was upregulated, while that of CD39 and especially CD23 was downregulated. Unlike that observed in certain Burkitt lymphoma (BL) cell lines that share the same surface phenotype (CD10+CD38−CD23−CD39−), the c-myc–transfected cells expressed lymphocyte function–associated (LFA) 1, LFA-3, and intercellular adhesion molecule 1 and grew in large clumps rather than single-cell layers. Expression of CD10 and CD38 was particularly evident on the cells undergoing apoptosis, thus suggesting a correlation between the presence of these markers and the apoptotic process. Cells placed in conditions favoring in vitro apoptosis displayed downregulation of Bcl-2 protein. Bcl-2 expression was, however, upregulated when the cells were exposed to CD40L. These data indicate that the B cells expressing c-myc constitutively acquire some of the features of normal centroblasts and of BL cells, including the expression of CD10 and CD38, and the propensity to undergo apoptosis, which can be prevented by exposure to CD40L. Therefore, these cells can serve as a model system to study both BL lymphomagenesis as well as the process of B cell selection occurring in the germinal centers.

It is widely accepted that germinal centers (GCs) represent a major site for maturation of antibody affinity and the generation of B cell memory (1-4). Histologically, two different areas of the GC can be distinguished: the dark zone, which contains proliferating B lymphocytes, called centroblasts, and the light zone, which is populated mainly by nondividing B cells, called centrocytes (1). The centroblasts undergo random hypermutation of the genes encoding the H and L chain V regions of the Ig molecules and transform into resting centrocytes (1-4). Unless exposed to stimulatory signals, the centrocytes die by apoptosis (1-4), a form of programmed suicide caused by the activation of endonucleases (5, 6). This process of hypermutation and subsequent selection contributes to the diversification of the antibody repertoire.

Recently, it has become possible to obtain purified populations of human GC B cells in vitro (7, 8). These cells have a characteristic surface phenotype (CD38+CD10−CD23−CD39−) and die spontaneously in vitro by apoptosis. Studies on the agents capable of preventing this in vitro apoptosis
have elucidated in part the mechanism responsible for B cell selection that operates in the GC. Thus, antibodies to surface Ig and the CD40 ligand (CD40L) are able to prevent apoptosis (7, 9, 10). In addition, antibodies to CD38 or to CD21 inhibit apoptosis and prolong GC B cell survival (10, 11). Some of these stimuli, in combination with cytokines, may also induce proliferation of GC B cells and/or their differentiation into Ig-secreting cells (7, 10–13). Despite this information, the molecular mechanisms that induce apoptosis or cause hypermutation of the Ig V-region genes remain to be elucidated. Moreover, the function of the bcl-2 protooncogene (14, 15), which has a key role in preventing apoptosis of other cell types (16, 17), has been questioned in the case of GC B cells (9, 18–20). Part of the difficulties in elucidating these issues is generated by technical problems related to both the handling of the GC B cells in vitro and the relatively low yield of cell purification procedures. In this respect, the availability of continuous lines of cells sharing some or all of the features of the GC B cells would represent a substantial improvement.

A number of studies have shown that there are phenotypic and functional similarities between normal centroblasts and the malignant B cells of Burkitt lymphoma (BL) (8, 9, 21, 22). Two types of BL can be distinguished and are referred to as endemic BL (eBL) and sporadic BL (sBL) (23). While EBV infection is believed to play a major pathogenetic role in the eBL, the cells of sBL are generally EBV negative (23). Despite these differences, the cells of the two types of BL share some characteristic features: expression of a CD10−CD38+CD23−CD39− surface phenotype, absence or low expression of LFA-1, LFA-3, intercellular adhesion molecule (ICAM)-1 and HLA class I antigens, presence of typical intracytoplasmic vacuoles, and a propensity to undergo apoptosis (22–26). Some or all of these characteristics are retained by the cells of the continuous lines derived from the EBV-negative BL (23). In contrast, the lines from EBV-positive BL have the tendency to shift their phenotype in vitro from that of BL cells (generally referred to as the phenotype of group I BL cell lines) to that of normal lymphoblastoid EBV-positive cell lines, which express abundant CD23, CD39, LFA-1, LFA-3, ICAM-1, and class I antigens, and low levels of CD10 and CD38 (phenotype of group III BL cell lines) (22, 27).

Both eBL and sBL share similar cytogenetic and molecular abnormalities characterized primarily by the presence of a specific chromosomal translocation that results in the juxtaposition of the c-myc protooncogene with an Ig H or L chain gene (14, 23). Although there are differences in the topographic location of the chromosomal breakpoints of eBL and sBL (28), the translocation invariably causes a deregulation, which is frequently an upregulation, of c-myc expression (23, 29–33). This c-myc translocation is responsible for the acquisition of most of the malignant features displayed by BL cells (34, 35).

Considering these observations, it is possible that constitutive expression of c-myc may be responsible for some or all of the phenotypic and functional properties of BL cells, including the centroblast-like features and their propensity to undergo apoptosis. In an attempt to clarify this issue and with the aim of finding an in vitro system suitable for investigating the control of GC B cells, we have transfected and expressed the c-myc gene in normal EBV-positive lymphoblastoid B cell lines (LCL). We show that c-myc activation is sufficient to induce a change of phenotype from typical LCL to that characteristic of centroblasts. In addition, the transfected cells become prone to death by apoptosis, from which they can be rescued by exposure to CD40L. Thus, besides indicating a possible role for c-myc protooncogene in the physiology of GC cells, these data describe an in vitro model of potential value for investigating both the process of GC B cell selection and that of BL lymphomagenesis.

Materials and Methods

**Plasmid Construction.** Plasmid pHEBo-Eμ-myc contains a fragment derived from the Manca BL cell line in which the transcription of the c-myc gene is controlled by the Eμ enhancer (36). The plasmid also contains the hygromycin resistance gene. This plasmid, kindly provided by Dr. Carlo Croce (Jefferson Cancer Institute, Philadelphia, PA), was originally described by Lombardi et al. (34). The control plasmid, pHEBo-Eμ, was generated by removing the XbaI-SalI fragment containing the second and third c-myc exons from the original plasmid.

**Cell Lines and Cell Culture.** Two EBV-harboring LCL (Cor and Pte) were generated spontaneously from two healthy EBV-seropositive individuals. Per LCL was obtained from an HIV- and EBV-seropositive patient. The LCL were obtained by culturing the PBMC in the presence of 50 ng/ml of cyclosporin A (Sandos, Basel, Switzerland) as previously described (37). Small colonies of proliferating cells began to appear in 20–50 d. These were collected and subsequently expanded in RPMI-1640 medium (Seromed Biocrom KG, Berlin, Germany) containing 10% FCS (Seromed Biocrom KG). Daüdi was an EBV-positive malignant B cell line generated from a BL patient (23).

**Cell Transfection.** Transfection was performed by electroporation (38). 5×106 cells were resuspended in 0.2 ml of cold PBS, and 10 μg of plasmid DNA was subsequently added. The mixture was placed in an electroporation chamber and a high voltage (2.7–2.9 kV) pulse was applied. Thereafter, the cells were placed in RPMI with 10% FCS and cultured for 48 h before selection in the same medium containing 300 U/ml of hygromycin B (Calbiochem-Novabiochem Corp., San Diego, CA).

**Growth Curves.** To determine their growth curves, the cells were resuspended at a concentration of 3×105/ml in RPMI-1640 medium containing 10, 1, or 0.1% FCS, and placed in culture in 24-well plates. Growth was measured by cell count at different intervals. Cell viability was assessed by trypan blue exclusion.

**Cell Cycle Analysis.** Cell cycle analysis was carried out by flow cytometry according to the method described by Fried et al. (39). Briefly, 1.5×106 cells were centrifuged at 200 g and the cell pellets gently resuspended in 1.5 ml hypotonic fluorochrome solution (propidium iodide [PI] 50 μg/ml) [Sigma Aldrich, Milano, Italy], 0.1% sodium citrate, 0.1% Triton X-100) and placed in the dark overnight at 4°C before flow cytometry analysis (Cytoror Absolute; Ortho Pharmaceuticals, Raritan, NJ). The data were subsequently analyzed by use of a computer program (Cell Cycle Pro; Ortho; ABS version 1.6C).

**Apoptosis Assays.** Cells to be tested for their capacity to un-
dego apoptosis were washed twice in serum-free RPMI 1640 medium and plated in medium containing 10 or 0.1% FCS at concentration of 3 × 10^7/m. In selected experiments, apoptosis was induced by exposing the cells (in RPMI-10% FCS) to a monoclonal anti-μ antibody (μ-μ-ab) coupled to polyacrylamide beads (Bio-Rad Laboratories, Richmond, CA) as previously described (6). Apoptosis was measured by three different methods. First, the cells were harvested at intervals and subsequently stained with an rhodamine PI fluorochrome solution and placed in the dark overnight at 4°C. The amount of DNA fragmentation was measured by flow cytometry (40). Second, DNA laddering was measured according to the method of Paccinetti et al. (41). Briefly, 10^6 cells were resuspended in 5 ml of ice-cold lysing buffer (NaCl 150 mM, Tris 50 mM [pH 7.6], Triton X-100 1%, 10 mM EDTA, containing 10 μg DNase-free RNase [Boehringer Mannheim Biochemicals, Indianapolis, IN]); after 10 min on ice, the sample was centrifuged for 5 min at 2,000 rpm, and the supernatant containing DNA fragments was recovered. After phenol extraction and ethanol precipitation, the samples were resuspended in 50 μl TE (Tris-EDTA). After 5 min at 60°C, the fragments were loaded on a 1.5% agarose gel. The PCR products were detected by ethidium bromide staining, the gel was photographed with T55 film (Polaroid Corp., Cambridge, MA) and a densitometric analysis (LKB/Pharmacia Biotech, Brussels, Belgium) of the negative was performed.

**Western Blot Analysis.** Nuclear fractions were prepared by incubating the cells at a concentration of 10^7/ml in nuclear extract buffer (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM MgCl, 0.5 mM dithiothreitol, and 0.5 mM PMSE) for 10 min at 0°C. The cells were subsequently dounced in a homogenizer, and the resulting homogenates were layered over a cushion of 20% glycerol nuclear extraction buffer and then centrifuged at 250 g for 10 min. The pellets were solubilized by sonication in electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 4%, 2-ME, 10% glycerol, and 0.01% bromophenol blue) and boiled for 3 min (44). 10^6 nuclei of each sample were separated by discontinuous 8–16% SDS PAGE and electroblotted onto Hybond-C extra nitrocellulose membrane (Amersham International, Amersham, Bucks, UK). The membrane was subsequently probed by use of an anti-Myc hybridoma supernatant (9E10 clone, kindly provided by Dr. R. Sita, San Raffaele Institute, Milano, Italy [45]) or a rabbit antiserum to histone 4 (kindly provided by Dr. M. Romani, IST, Genoa, Italy). To detect the Bcl-2 protein, total cell lysates rather than purified nuclei were used (10). Briefly, 10^6 cells were resuspended in electrophoresis sample buffer, sonicated, and run on a 14% acrylamide gel. The membrane was probed with a Bcl-2-specific mAb (Dakopatts, Glostrup, Denmark). Second-step reactions were performed by a peroxidase-linked rabbit anti-mouse or a goat anti-rabbit antibody (Dakopatts). The antigens were visualized by chemoluminescence (ECL; Amersham International).

**Immunofluorescence.** The mAbs used were anti-CD19 (Leu 12), anti–human κ and anti–λ Ig L chains from Becton Dickinson & Co.; anti-CD10 (JS) and anti-CD23 (B6) from Coulter Corp. (Hialeah, FL); anti-CD38 (IB4), kindly provided by Dr. F. Malavasi (University of Torino, Torino, Italy) (46); anti-CD39, kindly donated by Dr. M. Rowe (University of Birmingham, Birmingham, UK) (22); anti-LFA-1 (CD11a) and –LFA-3 (CD58), kindly provided by Dr. A. Poggi (IST); and ICAM-1 (CD54) from Serotec (Oxford, UK). All of these mAbs were used in indirect immunofluorescence. The second FITC– or PE-conjugated antibodies to the appropriate murine Ig isotype were from Southern Biotechnology Associates (Birmingham, AL). Staining for cytoplasmic Bcl-2 was performed as previously described (10). Briefly, the cells were fixed in 1% paraformaldehyde/PBS solution, and the cell pellets were permeibilized for 3 min with 0.5 ml of 70% ethanol before indirect immunofluorescence was performed with an anti–Bcl-2 mAb (Dakopatts). All samples were analyzed by flow cytometry.

**Cell Separation.** Viable and apoptotic cells were physically separated by cell sorting (Epics-Elite flow cytometer; Coulter Corp.). The two populations were gated on the basis of forward and 90° light scatter parameters and, after separation, analyzed by flow cytometry and Western blotting for apoptosis and Bcl-2 expression.

**Results**

**Establishment of c-myc–transfected LCL.** Vectors containing c-myc (pHEBo Eμ-μyc) or control vectors (pHEBo Eμ) were transfected into three different normal LCL (Cor, Piz, and Per). The expression of the transfected c-myc gene was analyzed by RT-PCR by use of two synthetic primer pairs. The c-myc I/II primer pair amplified a 224-bp fragment that spanned the first and second exons of c-myc and was derived from the endogenous LCL gene. The c-myc II/III primer pair produced a 633-bp fragment, which spanned the second and third exons of c-myc and was derived from both the endogenous and transfected genes (Fig. 1 A). With these primers, it was possible to detect an increase of the II/III exon fragment over the I/II fragment in the c-myc–transfected LCL as compared to the LCL transfected with pHEBo Eμ (Fig. 1 A).
The expression of the Myc protein was analyzed by Western blot (Fig. 1 B). Cor LCL transfected with c-myc (Cor-myc) expressed much higher quantities of Myc protein than the corresponding control (Fig. 1 B). Less marked, but nevertheless substantial, differences were noticed when Per-myc and Piz-myc LCL were compared with their respective controls (Fig. 1 B).

To ensure that the transfection procedure did not select for subclone(s) with special properties, the ratio of κ:λ-producing cells and the karyotypes of the two pairs were analyzed. K and λ L chain–expressing cells were detected in the same ratio in the c-myc–transfected and control LCL. Karyotypic analysis failed to demonstrate chromosomal abnormalities in the c-myc–transfected and control LCL.

**Mode of Growth of c-myc-transfected LCL.** Next, the growth ability of each of the c-myc–transfected LCL was compared with that of the corresponding control LCL in cultures containing three different FCS concentrations. Fig. 2 shows representative results obtained with Cor LCL. The c-myc–transfected LCL had a growth ability superior to the control LCL in the cultures supplemented with 10% FCS, whereas the growth curves of the c-myc–transfected and control LCL were generally identical at 1% FCS concentrations (Fig. 2 A). The growth curves of the control LCL at 1 and 10% FCS were similar. At 0.1% FCS, the growth ability of the c-myc–transfected and control LCL slowed considerably and sometimes approached growth arrest. Occasionally, under these culture conditions, the growth of the c-myc–transfected cells was marginally superior to that of the control LCL, as is the case in the experiment reported in Fig. 2.

A comparative analysis of the cell cycle of the c-myc–transfected and control LCL was carried out by flow cytometry. In the control LCL kept in 0.1% FCS, the proportion of cells in the S phase of the cycle decreased by ~50% compared
with the LCL cultured in 10% FCS (Fig. 2 B). In contrast, in the c-myc-transfected LCL there was no significant difference between the proportion of cells in the S phase observed in 0.1 or 10% FCS (Fig. 2 B). Thus, unlike the control cells, which slowed their growth in low serum concentration, the c-myc-transfected cells continued to proliferate at 0.1% FCS. However, it is possible that the cultures failed to expand because of the death of a portion of cells and not simply because of lack of proliferation. Analyses of cell viability in the different cultures confirmed that this was the case. While the viability of the control cells assessed by trypan blue exclusion remained high in all culture conditions (90-98% of viable cells), the c-myc-transfected LCL, the cell viability at 72 h of culture decreased from 95% in 10% FCS to 60-70% in low-FCS concentrations.

Apoptosis of c-myc-transfected Cells. To explore the possibility that the death of the c-myc-transfected LCL cells in low serum concentration was caused by apoptosis, c-myc-transfected and control LCL were cultured in either 10 or 0.1% FCS, harvested at intervals, stained with PI, and analyzed by flow cytometry. Fig. 3 A reports the result of one representative experiment with Piz-myc LCL. The proportion of fragmented DNA in Piz-myc LCL was as high as 50% after 24 h in the cultures supplemented with 0.1% FCS. These values increased, albeit slowly, over the next 72 h. Under these culture conditions, the cell line was lost after ~1 wk. In contrast, apoptosis was very low when the c-myc-transfected cells were cultured in 10% FCS (Fig. 3 A). DNA laddering, a characteristic feature of apoptotic cells, was observed by agarose gel electrophoresis in the c-myc-transfected cells cultured in 0.1% FCS but not in the same cells cultured with 10% FCS (Fig. 3 B) or in the control LCL (not shown).

Apoptosis was also measured by morphological inspection of Giemsa-stained cytopsin. Cells with apoptotic nuclei were detected in the c-myc-transfected LCL kept at 0.1% FCS (Fig. 3 C), but were virtually absent from those kept in 10% FCS (not shown).

Exposure to a-μ-ab of c-myc-transfected cells kept in 10%
Figure 3. Apoptosis of c-myc-transfected cells. (A) Percentage of apoptosis determined on PI-stained cells by flow cytometry in Piz LCL transfected with pHEBo Eμ (Piz) or with pHEBo Eμ-myc (Piz-myc). The cells were cultured for different times with the indicated concentrations of FCS in the presence or absence of a-μ-ab. Apoptosis was measured by determining the percentage of fragmented DNA relative to the total DNA. The data are representative of those obtained with Cor and Per LCL. (B) Gel electrophoresis of total DNA from Cor and Per LCL transfected with pHEBo Eμ-myc to detect DNA laddering diagnostic of apoptosis. The cells were cultured for 48 h with the indicated concentrations of FCS. (C) Morphology of Per LCL transfected with pHEBo Eμ-myc. The cells were cultured in 0.1% FCS for 48 h before being stained with Giemsa. Apoptotic nuclei can be observed in the majority of the cells.

FCS also caused apoptosis. In this case, the percentage of fragmented DNA was as high as 80% after 72 h of culture (Fig. 3A). Serum deprivation and exposure to a-μ-ab had a synergistic effect on the inducement of apoptosis of the c-myc-transfected (but not control) cells. Thus, after 48 h, the percentage of apoptosis was >65% and reached values >95% by 72 h. After this time, the c-myc-transfected LCL were lost.

CD40L Prevents Apoptosis of c-myc-transfected Cells. The c-myc-transfected cells were cultured in 0.1% FCS in the presence or absence of CD40L, harvested at intervals, PI stained, and analyzed by flow-cytometry. Fig. 4 shows the results of one typical experiment carried out on Cor-myc LCL. The proportion of degraded DNA in the Cor-myc cells cultured in low serum concentration was as high as 55% after 18 h. The presence of CD40L substantially lowered the percentage of degraded DNA, although the values observed were still higher than those detected in the cells cultured in 10% FCS. CD40L prevented apoptosis in the first 18 h of culture. After this time, the percentage of apoptosis observed in the untreated and CD40L-treated cultures became similar. Readdition of CD40L after 18 h to the cultures of the cells that had been already exposed to CD40L did not further delay the apoptotic process (not shown). Exposure to CD40L also

Figure 4. Inhibition of apoptosis by CD40L. Cor LCL transfected with pHEBo Eμ-myc were cultured with 10 or 0.1% FCS in the presence or absence of the CD40L, as indicated. The cells were harvested at intervals and the percentage of apoptosis determined on PI-stained cells analyzed by flow cytometry. The results reported are representative of those obtained with Piz and Per LCL.
prevented apoptosis of c-myc-transfected cells treated with a-μ-ab in 10% FCS, with kinetics identical to those observed for the transfected cells deprived of serum (not shown). Exposure to CD40 mAb caused an inhibition of apoptosis of the c-myc-transfected cells similar to that observed with the CD40L (not shown).

Phenotypic Changes of c-myc-transfected Cells. The above experiments demonstrated that the constitutive expression of c-myc induced the ability to undergo apoptosis that was prevented by CD40L. This propensity to undergo spontaneous apoptosis is a characteristic functional feature of both normal centroblasts and BL cells (7, 9–11, 26). Therefore, the c-myc-transfected cells were examined for the appearance of the phenotypic features shared by centroblasts and BL cells. This hypothesis was first analyzed by immunofluorescent tests with different mAbs on cells cultured in 10% FCS. The results of one representative experiment on the Col LCL are shown in Fig. 5. The control Cor LCL cells expressed high levels of CD39 and CD23, low levels of CD38, and virtually no CD10 (Fig. 5 A). This is the phenotype of normal LCL (22, 26). In the Cor-myc LCL, there was downregulation of CD39 especially of CD23 expression, whereas the characteristic markers of BL cells and centroblasts, CD38 and CD10, were upregulated (Fig. 5 A). The constitutive expression of the c-myc oncogene also caused morphological changes. As is apparent from the Giemsa-stained preparations (Fig. 5 B), the c-myc-transfected LCL were comprised of numerous vacuolated cells similar to the malignant cells of BL (23). Vacuolated cells were consistently absent from the parental or control LCL (not shown).

The cells from the three c-myc-transfected LCL continued to grow in medium- to large-sized clumps as normal LCL do, rather than in single-cell carpets, as is typical of group I BL cell lines. Considering that the c-myc-transfected cells had undergone a significant change in phenotype toward that of BL cells (22, 23), this finding was somewhat unexpected. However, these data are consistent with the observation that the c-myc-transfected LCL expressed quantities of surface ICAM-1, LFA-1, or LFA-3 no different from those of the control or parental LCL (24) as assessed by immunofluorescence staining and flow cytometry analysis (data not shown). This is different from that reported for group I BL cells that downregulate the expression of these adhesion molecules (24).

Increased Expression of CD38 and CD10 and Downregulation of Bcl-2 in c-myc-transfected Cells Undergoing Apoptosis. Immunofluorescence tests were carried out on c-myc-transfected cells cultured at 0.1% FCS for 48 h to ascertain whether there was a difference in CD10 and/or CD38 expression by apoptotic cells compared with nonapoptotic cells. Two groups of cells could be distinguished by forward and side light scatter in Per-myc LCL cultured in 0.1% FCS (Fig. 6 A, Gates 1 and 2). Separation of each of the two groups by cell sorting followed by PI staining of the cells and reanalysis by flow cytometry disclosed that the apoptotic cells were concentrated within the cells in gate 1, whereas the cells in gate 2 were virtually devoid of apoptotic cells (Fig. 6 A). Moreover, the cells in gate 1 had low to nonexistent Bcl-2 protein levels, whereas Bcl-2 was readily demonstrable by Western blot in the cells in gate 2 (Fig. 6 C). The cells in gate 1 were characterized by the expression of higher levels of CD10 and CD38 and lower levels of CD39 and CD23 compared with the cells in gate 2 (Fig. 6 B). The figure also shows the results of staining for these markers of the control Per LCL. These cells were more brightly stained for CD23 and CD39 than the nonapoptotic cells in gate 2. Conversely, the expression of CD10 and CD38 was lower. Therefore, it appears that constitutive c-myc expression causes a shift in phenotype that becomes more apparent after the cells are induced into apoptosis. These apoptotic cells phenotypically resemble both the BL cells and the centroblasts.

Figure 5. Phenotypic changes induced by the constitutive expression of c-myc. (A) Surface phenotype of Cor LCL cells transfected with pHEBo Eμ-myc (black areas) or pHEBo Eμ (white areas). The cells were stained with the indicated mAb and analyzed by flow cytometry. (CTR) Control staining with an irrelevant mAb. (B) Morphology of Cor LCL transfected with pHEBo Eμ-myc. Numerous vacuoles are present in the cytoplasm of the transfected cells. The results reported are representative of those obtained with Piz and Per LCL.
Figure 6. Increased expression of CD10 and CD38 and downregulation of Bcl-2 after apoptosis. (A) Per LCL cells transfected with pHBo Ep-myc were cultured with 0.1% FCS for 48 h and analyzed by flow cytometry. Two populations of cells were detected by forward (vertical axis) and side (horizontal axis) light scatter and subsequently separated by appropriate gating. After separations, the cells in gates 1 or 2 were PI stained and analyzed by flow cytometry to detect degraded DNA, which is diagnostic of apoptosis. Apoptosis was almost exclusively observed only in the cells in gate 1. (B) Expression of surface markers by Per-myc LCL cells separated according to their capacity to undergo apoptosis (as depicted in A). The cells were stained with the indicated mAbs and analyzed by flow cytometry. Apoptotic cells (Gate 1, black areas) express more CD10 and C38 than nonapoptotic cells (Gate 2, white areas). These latter cells have more abundant CD23 and CD39. (Dotted lines) Flow cytometry profile observed with the same reagents in the parental Per LCL cells. (C) Western blot analysis of Bcl-2 expression by Per-myc LCL cells. The cells were cultured in 0.1% FCS for 48 h, separated as in A, and analyzed by Western blot. The results shown are representative of those obtained with Cor and Piz LCL.

Bcl-2 Expression by c-myc–transfected Cells. In these experiments, we investigated whether exposure to CD40L of the c-myc–transfected cells cultured in low serum concentration results in upregulation of Bcl-2 expression. Fig. 7 reports the results of one representative experiment carried out on Piz-myc LCL. In this experiment, the cells were cultured in 0.1% FCS in the presence or absence of CD40L, harvested at intervals, and studied for Bcl-2 expression by immunofluorescence. This technique was selected over the Western blot used above as it proved more reliable in measuring variations in the Bcl-2 expression as assessed in the course of preliminary experiments. With this fluorescent method, which requires cell permeabilization, it was not possible to distinguish the apoptotic from the nonapoptotic cells by forward and side light scatter as in the experiments shown in Fig. 6. After 1 h of culture in low FCS concentration, virtually no difference in the expression of Bcl-2 was noted between the cells exposed to CD40L and those that were not exposed (Fig. 7). Bcl-2 expression in the cells cultured in the absence of CD40L was decreased after 3 h, whereas this decrease of Bcl-2 expression was not detected in the cells exposed to CD40L. In fact, in these cells there was an increase, albeit a marginal one, of Bcl-2 expression compared with the values observed after 1 h of culture under the same conditions (Fig. 7). After 18 h of culture, the majority of the cells exposed to CD40L expressed Bcl-2, whereas in the cultures that did not contain CD40L, two group of cells could be distinguished. One was virtually negative for Bcl-2 expression, while the other had a Bcl-2 fluorescent intensity almost comparable to that of the cells exposed to CD40L. After 48 h, the vast majority of cells cultured in the absence of CD40L expressed very little or no Bcl-2 (Fig. 7). The cells exposed to CD40L displayed a higher level of Bcl-2 expression. However, the amount of Bcl-2 detected was clearly lower than that of the cells cul-

Figure 7. Induction of Bcl-2 expression by exposure to CD40L. Piz LCL cells transfected with pHBo Ep-myc were cultured for the indicated times in 0.1% FCS in the presence or absence of the CD40L. The cells were permeabilized, stained for Bcl-2, and analyzed by flow cytometry. (Black areas) Flow cytometry profile of the cells cultured with CD40L; (white areas) flow cytometry profile of the cells cultured without CD40L. (Dotted lines) Bcl-2 expression by Piz-myc cells cultured in 10% FCS. The results reported are representative of those obtained with Cor and Piz LCL.
tured for 18 h. Thus, prolonged culture, even in the presence of CD40L, resulted in a Bcl-2 downregulation consistent with the data on apoptosis shown in Fig. 4. It should be noted that the immununofluorescent method used generally results in a slight overestimation of the Bcl-2-positive cells in the suspension cultured for prolonged times, as the permeabilization method selectively eliminated dead cells. Because of this difficulty and poor cell recovery, determination of Bcl-2 at 72 h could not be performed. These findings are in line with those obtained with the Western blot analysis reported in Fig. 6 and corroborate the presence of cells that have low or absent Bcl-2 expression and are apoptosis prone, as well as cells that still express some Bcl-2 that are apoptosis resistant, at least over a short period of time.

Discussion

This study demonstrates that the constitutive expression of the c-myc oncogene induces a number of phenotypic and functional changes in the cells of normal EBV-positive LCL. These changes include (a) the expression of CD10 and CD38; (b) the downregulation of CD23 and CD39; (c) the acquisition of a vacuolated morphology; and (d) the capacity to undergo apoptosis. Exposure to CD40L or CD40 mAb prevents apoptosis from occurring. Because of these newly acquired features, the cells transfected with c-myc resemble the malignant cells of BL and normal centroblasts.

Apoptosis of the c-myc-transfected cells was observed in low serum concentration, a condition that caused an apparent in 10% FCS. The cells underwent apoptosis instead of cell division initiated by exposure to CD40L (or CD40 mAb), similar to that observed in the control LCL (Fig. 2). However, in the c-myc-transfected LCL, the number of cells in the S phase of the cell cycle did not decrease as it did in the control LCL, but remained as high as in the LCL kept in 10% FCS. The cells underwent apoptosis instead of progressing normally into the cell cycle. Thus, c-myc expression, combined with a block to proliferation, appears to be a potent inducer of apoptosis in B cells, similar to that described in cells of other tissue origin (47-49). This mechanism is likely to represent a general safety device for aborting a strophic event, like c-myc mutation, occurs. In the case of GC B cells, it may also have a special function in the selection of cells producing the highest affinity antibodies to the stimulating antigen(s).

Apoptosis of c-myc-transfected cells was prevented by exposure to CD40L (or CD40 mAb), similar to that observed with normal centroblasts (9-10). Moreover, anti-CD38 mAb that normally prevents apoptosis of centroblasts (10) similarly inhibits apoptosis of the c-myc-transfected LCL (data not shown). Thus, the c-myc-transfected LCL seem to retain the capacity to respond to some (or even all) of the signals that regulate GC B cell selection in vivo. The inhibition of apoptosis of c-myc-transfected LCL with CD40L (or CD40 mAb) was short-lived (Fig. 4). This is similar to what is observed with normal centroblasts, which can be, however, induced into the cell cycle by exposure to IL4 (10). This finding suggests that signaling via CD40 prolongs cell survival in preparation for other signals for proliferation. Unfortunately, despite the use of a battery of cytokines, we have so far been unable to reinduce cell proliferation of c-myc-transfected LCL after apoptosis has been inhibited by CD40L.

Exposure of the c-myc-transfected cells to CD40L in vitro was followed by upregulation of Bcl-2 expression (Fig. 7). In addition, Bcl-2 was normally present in the c-myc-transfected cells kept at high FCS concentration (Fig. 7), while it decreased substantially in those kept at low FCS concentration and was virtually absent from the cells purified after apoptosis had been induced (Fig. 6). Finally, the short-lived inhibition of apoptosis caused by exposure to CD40L correlated closely with the cell capacity to express Bcl-2 (see Fig. 7). Together, these data indicate a key role of Bcl-2 in preventing apoptosis of c-myc-transfected cells. Therefore, if the constitutive expression of c-myc predisposes the cell to either proliferation or apoptosis, then the bcl-2 protooncogene system appears to dictate the outcome of the options imposed on the cell (50, 51). This notion is consistent with the data obtained on cells of nonlymphoid origin (16, 17, 52, 53) and would provide a conceptual framework for the various processes of cell selection that occur within the immune system. The stimuli received by T or B lymphocytes through their surface antigen receptors are known to upregulate c-myc expression (32, 54-56). These stimuli would, therefore, predispose the cell toward either proliferation or apoptosis, the final decision between the two options eventually being dictated by signals that govern bcl-2 protooncogene expression. Evidence, albeit indirect, in favor of a mechanism of this type exists in the case of T cell selection in the thymus and the inhibition of excessive T cell expansion during viral infections (54, 55, 57, 58). In connection with this, it is of note that we have recently found that a large fraction (up to 60%) of freshly isolated GC B cells are in the S phase of the cell cycle and express high levels of Myc (Zupo, S., personal communication), thus indicating that c-myc upregulation may play a role in the selection of GC B cells.

Apoptosis of c-myc-transfected cells deprived of serum or exposed to a-μ-ab occurred over a relatively expanded period of time, and the LCL experienced increasing difficulty in growing before being definitively lost. These findings could indicate that the decision between apoptosis and progression into the cell cycle may be governed by certain stochastic factors. In addition, they may suggest the presence of certain as yet unidentified stimuli in the cultures capable of upregulating bcl-2 expression in a proportion of cells. Such a hypothesis is supported by the finding that certain cells expressed Bcl-2 even under culture conditions that favor apoptosis (Fig. 6).

Apoptosis of c-myc-transfected cells was induced by culturing the cells in low FCS concentration or by culturing in the presence of a-μ-ab irrespective of the FCS concentrations in the culture. The synergistic effect of the exposure to a-μ-ab and serum deprivation may suggest that the two signals follow somewhat different pathways in inducing cell apoptosis. Although the fine mechanisms involved are still largely unknown, some hypotheses amenable to further experimental testing can be presented. For example, in low FCS concentrations, the cells were likely to lack those stimula-
tory signals that are required to maintain adequate Bcl-2 expression in order to counterbalance the effect of Myc overexpression. In the case of a-μ-ab-induced apoptosis, ligation of surface IgM may have caused an increased expression of endogenous c-myc, thus altering the normal balance between Myc and Bcl-2. This hypothesis would offer a plausible, albeit incomplete, explanation as to why exposure to a-μ-ab causes apoptosis in certain B cells while preventing apoptosis and even inducing proliferation in others (7, 59). Studies along these lines are currently in progress.

The constitutive expression of c-myc caused changes in the cell phenotype consisting of downregulation of CD23 and CD39 and upregulation of CD10 and CD38. These findings demonstrate that of the various cytogenetic alterations of BL cells (23, 60), those involving the c-myc oncogene are responsible for the acquisition of a new cell phenotype. It is of interest in this connection that CD10 and CD38 expression was particularly evident in those cells that had been induced to undergo apoptosis in vitro, a finding that might suggest a role for the two markers in the apoptosis itself and/or its prevention. A number of observations would argue for this notion. First, both CD10 and CD38 are expressed by group I BL cell lines, which are apoptosis prone, and not by group III BL cell lines, which are resistant to apoptosis (26). Second, certain agonistic anti-CD38 mAbs prevent apoptosis of normal centroblasts (10) and c-myc-transfected LCL (data not shown). Third, malignant myeloid cells also express CD10 and CD38 when exposed to stimuli in vitro that render them prone to undergo apoptosis in vitro, a finding that might suggest a role for the two markers in the apoptosis itself and/or its prevention. A number of observations would argue for this notion. First, both CD10 and CD38 are expressed by group I BL cell lines, which are apoptosis prone, and not by group III BL cell lines, which are resistant to apoptosis (26). Second, certain agonistic anti-CD38 mAbs prevent apoptosis of normal centroblasts (10) and c-myc-transfected LCL (data not shown). Third, malignant myeloid cells also express CD10 and CD38 when exposed to stimuli in vitro that render them prone to apoptosis (61). Fourth, normal EBV-positive LCL cells, when infected by HIV in vitro, undergo apoptosis readily and concomitantly express surface CD10 (62). If c-myc controls some of the cells' phenotypic features as well as their propensity to undergo apoptosis, it is then possible that the differences in phenotype and apoptotic capacity of group I BL cells, group III BL cells, and c-myc-transfected cells may in part depend on the different levels of c-myc expressed. Studies along this line are currently in progress.

Previously, it was reported that transfection of c-myc into normal EBV-positive LCL induced the acquisition of malignant properties such as the capacity to form colonies in semi-solid agar or grow in nude mice (34), but did not usually cause phenotypic changes except for the possible downregulation of surface LFA-1 expression (63). Although we have been able to confirm that the c-myc-transfected cells acquired malignant properties (data not shown), we also have consistently observed the phenotypic changes described above, which do not include LFA-1 downregulation. These discrepancies may, in part, be due to the different plasmids used. Moreover, since both apoptosis and upregulation of CD10 and CD38 are best observed when a block to cell proliferation is imposed, the culture system used for cell selection and testing (particularly the FCS concentrations), may have influenced the outcome of the experimental observations.

The c-myc-transfected cells retained the capacity of normal LCL to express high-density surface adhesion molecules and grew in large cellular clumps. Moreover, the c-myc-transfected cells expressed the full set of EBV latent antigens. This is similar to the results observed for normal LCL and is different from those occurring in most BL cells that express only Epstein-Barr nuclear antigen 1 (EBNA-1) antigens (22). The EBV lytic antigens were, however, consistently absent, thus ruling out that the death of c-myc-transfected cells was caused by reactivation of a lytic infection (Pais, F., G. Cutrona, S. Roncella, and M. Ferrari, manuscript in preparation). Although the discussion of these issues goes beyond the scope of this study, a few points deserve special comment. First, these findings demonstrate that constitutive expression of c-myc does not result per se in the downregulation of the expression of either EBV latent antigens or of surface adhesion molecules. Therefore, it appears that in eBL cells, which express only EBNA-1 and have low surface adhesion molecules (23), these phenotypic features are acquired independently of the c-myc translocation during lymphomagenesis. The observations made on the malignant BL cells obtained from certain AIDS patients and on group III BL cell lines, all of which have a translocated c-myc and express the full set of EBV latent antigens and surface adhesion molecules, would argue for this notion (64–66). Second, it is widely accepted that in cells latently infected by EBV, EBNA-2 promotes the progression of the cells into the cell cycle and induces the expression of CD23 and CD39, whereas latent membrane protein-1 (LMP-1) upregulates bcl-2 protooncogene expression, thus preventing apoptosis (22, 26). These findings seem to indicate that these mechanisms, which promote cell proliferation and survival, may sometimes be overridden by the constitutive expression of c-myc. It is possible that the mode of expression of latent viral genes plays a crucial role in determining the cellular phenotype and the differences observed between group I BL cells, group III BL cells, and c-myc-transfected LCL. However, the mechanisms that regulate both viral gene expression and the mutual controls possibly existing between the c-myc oncogene and EBV gene expression remain to be elucidated. The c-myc-transfected LCL reported here may represent a useful investigative tool in this field.

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