Anti-IgM-mediated Regulation of c-myc and Its Possible Relationship to Apoptosis*

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Anti-IgM treatment of Burkitt’s lymphoma cells is followed by either growth arrest or induction of apoptosis. In this study we have explored the role of c-myc in these events. Our results in Ramos cells indicate the following. (a) The decline in c-myc mRNA occurs at about 4 h; inhibition of about 80% being observed. (b) The stability of c-myc message is involved since the half-life of c-myc mRNA is decreased from about 30 min in untreated cells to about 15 min following treatment with anti-IgM. In the presence of cycloheximide, a protein synthesis inhibitor, the half-life is increased to about 50 min and was unaltered by treatment with anti-IgM. (c) By contrast, nuclear run-on experiments indicated no change in transcription rates for c-myc message due to treatment with anti-IgM. (d) A decrease in c-myc causes apoptosis since specific repression of c-myc with antisense oligonucleotides decreases the levels of c-Myc, inhibits growth rate, decreases viability, and induces apoptosis. (e) Anti-CD40 inhibition of apoptosis occurs without alteration in anti-IgM-induced down-regulation of c-myc mRNA, suggesting that it acts distally to c-myc down-regulation. Other cell lines were also investigated. In Epstein-Barr virus (EBV)-positive cell lines (Daudi, Raji, and Namalwa), anti-IgM treatment for 24 h results in growth inhibition without induction of apoptosis. In EBV-negative cell lines (ST486 and CA46, as well as Ramos), a more heterogeneous pattern of responses to anti-IgM are observed. Ramos and ST486 cells both show growth inhibition and apoptosis upon anti-IgM treatment; CA46 cells shown only growth inhibition but not apoptosis. Anti-IgM causes a decline in c-myc mRNA levels in all of these lines, as well as in c-Myc protein level in the two lines investigated, Daudi and Ramos, regardless of apoptosis. Addition of antisense c-myc oligonucleotides to the cells reduced growth in both Daudi and Ramos cells lines, however it resulted in substantial apoptosis only in Ramos cells.

These results suggest that anti-IgM destabilizes c-myc mRNA by a process that involves mRNA turnover, rather than transcription rates. However anti-IgM exerts differential effects in EBV-positive and EBV-negative cell lines. EBV-positive cells are uniformly resistant to apoptosis, while EBV-negative cell lines show a tendency to apoptosis but with exceptions. Growth inhibition can be uncoupled from apoptosis in EBV-positive cell lines, but not in those EBV-negative cell lines prone to apoptosis. Furthermore, down-regulation of c-myc message correlates with growth inhibition in these cells, but is an insufficient link to apoptosis. By contrast inhibition of apoptosis by anti-CD40 occurs even though c-myc mRNA is decreased.

Anti-IgM cross-links the B cell antigen receptor and is an important regulator of B cell function (1–5). Depending on the type of cell system studied, anti-IgM may cause differentiation or growth arrest (6, 7), or induce apoptosis (1–5). We have examined the effects of anti-IgM on various B lymphocyte cell lines, particularly B lymphoblastoid cell lines (8–10). These results show that anti-IgM induces apoptosis in EBV-negative cell lines (including Ramos and ST486), but induces growth arrest without apoptosis in EBV-positive cell lines (including Daudi and Raji) (this report) (11–13). This in turn led us to examine in greater detail the molecular events which may precede anti-IgM-mediated induction of apoptosis in Ramos.

Results from many laboratories have strongly implicated the regulation of c-myc as an important event in a variety of meaningful cellular activities. Observations favoring the importance of c-myc include: (a) c-myc levels are modulated by growth factors which regulate entry into the cell cycle (14), (b) transient increases in c-myc have been observed in quiescent cells just prior to their entry into the cell cycle (15, 16) (reviewed in Ref. 17), (c) in other systems decreases in c-myc may be important causing cells to exit from the cell cycle (18–23), (d) in HL-60 cells, differentiation of the cell with associated decreases in c-myc (of up to 90%) is observed regardless of the signal used for differentiation (21–23). In agreement with these results, antisense oligonucleotides, which decrease c-Myc levels (24, 25), cause differentiation of HL-60 cells (18–21, 23). More recent experiments show antisense oligonucleotides to c-myc may also cause apoptosis (26). Thus anti-IgM-mediated regulation of c-myc as a possible mediator of growth arrest and apoptosis in B lymphoblastoid cells deserves to be further studied (27, 28). Furthermore, whether c-myc alone can play a role or whether it can play a role only in conjunction with other proteins (Ras (29), p53 (30, 31), c-Ab1 (32), or EBV proteins (10, 33)) needs to be determined. These proteins may also include known partners of c-myc itself, Max and Mad (14, 34, 35).

Regulation of mRNA stability is a major mechanism by which mRNA steady state levels are regulated (36–41) and this applies to c-myc mRNA in particular (17). Altered transcription rates can also play a role in c-myc regulation. Since EBV status of the cell is a major but not exclusive indicator of...
whether apoptosis occurs, we have used both EBV-positive and -negative cell lines in our study and have examined critically the relationship of growth to apoptosis. Our results suggest that c-myc down-regulation due to increased message turnover is a very likely contributor to subsequent cellular events, although the final outcome, growth or apoptosis, is in part dependent on the cell line. By contrast neither anti-CD40 nor the inhibition of anti-IgM-induced apoptosis by anti-CD40 are direct modulators of c-myc.

MATERIALS AND METHODS

DAudi (ATCC CCL 213), Ramos (ATCC CRL 1596), Raji (ATCC CCL 86), Namalwa (ATCC CRL 1432), ST486 (ATCC CRL 1647), CA46 (ATCC CRL 1648) cell lines and GAPDH plasmid (ATCC 57090) were obtained from American Type Culture Collection (Rockville, MD). Plasmid DNAs were prepared from the GAPDH plasmid, an HL-60 γ-actin clone (42), and a derivative of a c-myc clone (43). This c-myc derivative contains only the 0.4-kilobase pair PsiI fragment from within exon II. 1HThymidine was purchased from Dupont NEN. Goat anti-human IgM (μ chain specific) was obtained from Organon Technika (Dundee, UK). Mouse monoclonal antibody against human c-myc was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibody against human CD40 was from Genzyme Diagnostics (Cambridge, MA). DNA marker (HindIII digest of λ DNA) and DNA isolation kit were from U.S. Biochemical Corp. Calf fetal serum was from Gemini Bioproducts (Calabasas, CA), and cell culture medium from Irvine Scientific (Santa Ana, CA). All other reagents were obtained from Sigma.

Cell Culture and Treatment—The various Burkitt's lymphoma cell lines were cultured in RPMI 1640 medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), Fungizone (0.25 μg/ml), 10% heat-inactivated fetal calf serum, and Hepes (pH 7.4, 10 mM) at 37 °C in a humidified 5% CO₂ incubator (44). The cells were treated with anti-IgM (20 μg/ml) for various time periods as described in individual experiments. The number of cell culture passages appeared to have no effect on the results presented here.

Proliferation Assays—Proliferation of cells was assessed by the incorporation of radiolabeled thymidine or by determining the cellular uptake of the fluorochromes, triphenyltetrazolium chloride (MTT), and the 1,3-diphenylisooctylcarbinol (DPI). 1HThymidine uptake was determined by the addition of 1 μCi/ml thymidine to each well of a 96-well microtiter plate 24 h prior to harvest. The cells then were washed and harvested, and the DNA content was examined. The percentage of apoptotic cells is determined by nuclear DNA electrophoresis (47, 48). The DNAs were extracted from the cells using the U.S. Biochemical Corp. DNA isolation kit containing guanidine thioocynate, and the final DNA pellets were dissolved in 20 μl of sterile water and quantified by UV absorbance. Equivalent amounts of DNA (10 μg) were subjected to electrophoresis on 1.5% agarose gels containing bromphenol blue (5 μg/ml) at 5 V/cm in TBE buffer (45 mM Tris borate, 1 mM EDTA, pH 8.0) for 3 h. The gels were then visualized under UV light and photographed using Polaroid film 667. Gaps and/or markers consisting of a HindIII digest of λ DNA ranging in size from 2.3 to 0.5 kilobase pair were loaded simultaneously to indicate the size of the DNA bands.

Flow cytometry for determination of nuclear DNA content was carried out using a CycleTEST Kit (Becton Dickinson, Mountain View, CA). Suspensions of appropriately treated cells were stained with propidium iodide as follows: 200 μl of cells (2.5 × 10⁶/ml) were treated with trypsin buffer, mixed gently for 10 min at room temperature, after which period 1.5 ml of a solution containing trypsin inhibitor and RNase was added, and the incubation was continued for a further 10 min. The cells were stained with DNA iodine-staining solution which was then added (final concentration 125 μg/ml), and incubation was continued for 10 min more in the dark. The samples were then filtered through 50-μm nylon mesh in the dark, transferred to plastic tubes wrapped in foil for light protection, kept on ice, and analyzed within 3 h using a FACStar™ flow cytometer (Becton Dickinson). 20,000 events were acquired and the resulting histogram profiles were analyzed sequentially for ploidy analysis. The software generates a plot of a number of cells versus DNA content. The area under each peak, which corresponds to the number of events at each phase of the cell cycle, is determined. The apoptotic peak (A₉₀) is defined as that peak which precedes G0, and was analyzed separately as described by others (47, 49, 50). The samples were analyzed with or without gating, which excluded essentially similar results. Controls include analysis of nuclei derived from resting lymphocytes (single G₀ peak) and from Ramos under normal culture conditions which gives a characteristic G₀/G₁, S, and G₂/M distribution.

RNA Isolation—Total RNA was isolated as described previously (51) from exponentially growing treated cells by lysis using guanidium thiocyanate solution (22) and pelleting through a CsCl cushion. The RNA pellets were resuspended in 50–100 μl TES (10 mM Tris, 5 mM EDTA, 1% SDS, pH 7.4) and quantified by UV absorption. Alternatively, total RNA was isolated using a simpler RNA STAT-60 isolation procedure (TEL-TEST "B" Inc., Friendswood, TX).

Northern Blots—Equivalent amounts (25 μg) of total RNA from each sample were analyzed using gel retardation filters (53) as described previously (51). Samples were analyzed with probes for c-myc, actin, or GAPDH. Autoradiograms of the gels were then scanned with a densitometer (Bioimaging Instruments, Fullerton, CA) and the relative c-myc/actin or c-myc/GAPDH ratios of the RNA samples were compared to determine the changes in the c-myc mRNA level.

mRNA Degradation Rate—RNA synthesis was terminated in various treated cell cultures by addition of 5 μg/ml actinomycin D. At different times after actinomycin D addition, the cells were harvested, and total RNA was isolated. The amount of c-myc mRNA remaining was determined by Northern blotting and was used to calculate the half-life of c-myc mRNA. Data were fitted to an exponential decay model. Comparison of experiments were carried out by normalizing the starting c-myc mRNA levels to 100%.

Northern Run-up—The amount of nascent c-myc mRNA present in cells under different treatment conditions was determined by nuclear run-on. Following the appropriate treatments, cell nuclei were prepared and stored as described elsewhere (54). mRNA molecules in the process of transcription were isolated by incubating nuclei with T7TPP (55) to generate labeled RNA probes. Target cDNAs consisted of plasmid DNA for pUC19, the GAPDH clone (American Type Culture Collection ATCC 57090), and the c-myc derivative clone containing the 0.4-kilobase pair PsiI fragment from within exon II. These were prepared by taking 3 μg of each DNA template, linearizing by cleavage with appropriate restriction enzyme, and heating to 100 °C for 10 min in 10 × SSC. The denatured DNA was allowed to bind to Zeta Probe (Bio-Rad Laboratories, Hercules, CA) nylon membrane filters by vacuum filtration following which the DNA was cross-linked to the filter.

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with UV light (Stratalinker, 120,000 J/cm², Stratagene, Torrey Pines, CA). Blocking of the filters and hybridization of the DNA with the RNA probes was carried as described above for the Northern blots.

Western Blots and c-myc Protein Turnover—Cellular c-myc protein levels under various treatment conditions were determined by immunoblotting (56). Treated cells were washed with cold phosphate-buffered saline and lysed in TBS (20 mM Tris, pH 7.5, 0.15 M NaCl) containing 20 mM MgCl₂, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate. Cellular extracts were incubated on ice for 30 min and then centrifuged at 12,000 × g for 20 min. The supernatants were removed, and the protein concentration was determined using a Bio-Rad protein assay kit. Equivalent amounts of total cellular protein extract (20 mg) were fractionated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane using a Bio-Rad mini transblot apparatus. The membrane was blocked with TBS plus 5% skim milk powder, and incubated overnight with 2 μg/ml mouse monoclonal antibody to human c-myc (Santa Cruz Biotechnology, Inc.) in TBS plus 1% skim milk powder. Following three washes in TBS plus 0.1% Tween 20, the membranes were incubated with a second antibody for 2 h and then developed using an ECL kit (Amersham Corp.) according to the manufacturer's instructions. Protein bands were quantified by densitometric analysis.

For the c-Myc protein turnover experiments, cells were treated with cycloheximide (1 μg/ml) to stop further protein synthesis. At different times following this, the cells were harvested and protein was isolated. The amount of c-Myc remaining at different times after cycloheximide addition was determined and was used to calculate the half-life of c-Myc protein. Data were fitted to an exponential decay model.

Antisense Oligonucleotide Treatment of Cells—Antisense c-myc oligodeoxynucleotideAACCTGGAGGGGCAT (phosphorothioate) and sense c-myc oligodeoxynucleotideATGCCCTCTACGTT (phosphorothioate) were obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). Cells were cultured in 96-well microtiter plates, at a cell density of 0.5 to 2 × 10⁵ cells/ml, and incubated with various concentrations of oligonucleotide in OptiMEM (Life Technologies, Inc.) plus 2% fetal calf serum for 24 h. Parallel samples included untreated controls and anti-IgM-treated cultures. Treatment with antisense oligonucleotide plus anti-IgM was done by incubating with oligonucleotide for 6 h and then adding anti-IgM.

**RESULTS**

Ramos (EBV-negative) and Daudi (EBV-positive) cell lines were examined to determine the interrelationships of growth inhibition, cell cycle distribution and c-myc regulation. To further establish the generality of our conclusions, we selectively examined certain restricted aspects of other cell lines such as Namalwa and Raji (both EBV-positive), and CA46 and ST486 (both EBV-negative).

Anti-IgM Induces a Decline in c-myc mRNA Levels—Treatment of Ramos cells with anti-IgM (μ chain-specific antibody) results in a decrease in the expression of the c-myc oncogene. Anti-IgG (γ chain-specific) antibody had no effect on these cells (57). The time course of the change in the level of c-myc mRNA was examined. Following treatment with anti-IgM, cells were harvested at various times, and total cellular RNA was isolated. Equal amounts of RNA were analyzed by Northern blot to determine the amount of c-myc mRNA present at each time interval. These results shown in Fig. 1A demonstrate that c-myc mRNA levels are diminished between 2 and 5 h after treatment, reaching the lowest point at about 4 h. After this time, c-myc mRNA levels rise again. The time course of c-myc mRNA expression in Daudi cells is indistinguishable from that in Ramos cells. ¹

To ensure that these changes in c-myc mRNA levels are specific for this oncogene and not the result of changes in overall mRNA synthesis rates, the level of c-myc mRNA relative to that of control mRNAs, γ-actin and GAPDH was determined at 4 h after anti-IgM treatment. These results shown in Fig. 1B demonstrate that c-myc mRNA level is decreased relative to that of γ-actin or GAPDH, due to the anti-IgM treatment. Little change in the expression of either γ-actin or GAPDH could be discerned.²

Further demonstration that this effect on c-myc transcription is specific was obtained by examining the decreases in c-myc mRNA levels following different doses of anti-IgM treatment. c-myc mRNA was found to decrease to greater extents as the treatment dose increased.² The dose of anti-IgM needed to effect c-myc mRNA decreases is similar to that needed to effect Ca²⁺ mobilization or to effect c-fos mRNA induction (44, 51).

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**Fig. 1. Reduction of c-myc due to anti-IgM treatment.** A, time course. Ramos cells were treated with anti-IgM and the levels of c-myc mRNA were determined at various times. c-myc mRNA was observed to reach a minimum at 3 to 5 h, following which recovery was observed. B, the level of c-myc mRNA was determined relative to γ-actin or GAPDH mRNA levels at 4 h after treatment with anti-IgM. Both actin and GAPDH mRNA levels changed only slightly, whereas decreases in c-myc were observed. The ratio of the density of the c-myc mRNA band to that of the actin band decreased from 0.37 in the untreated sample to 0.05 in the anti-IgM-treated sample (cross-hatched bars). (Actin and c-myc mRNA levels were measured on parallel aliquots of the same sample.) In a second experiment, the ratio of the density of the c-myc mRNA band to that of the GAPDH band, measured on the same blot using a mixture of the two probes, decreased from 0.36 in the untreated sample to 0.01 in the anti-IgM-treated sample (diagonal hatched bars). C, protein levels. Ramos cells were treated with anti-IgM and c-Myc protein was determined at various times by Western blot. c-Myc protein was observed to decrease in parallel to the changes seen with c-myc mRNA. c-Myc protein was decreased by 3 h and remained low even up to 24 h.
Anti-IgM-induced Decline in C-Myc Protein Accompanies Decline in c-myc mRNA—In addition to the decline in c-myc mRNA level, a decline in the level of C-Myc protein could also be demonstrated. Extracts prepared from cells treated with anti-IgM were analyzed by Western blots for the presence of C-Myc protein. C-Myc protein was observed to decrease within 3 h after treatment with anti-IgM (Fig. 1C), paralleling but trailing the decline in c-myc mRNA. Recovery of C-Myc protein levels did not occur even by 24 h. At these later times apoptosis is well under way, thus suggesting that the decrease in C-Myc rather than an increase in C-Myc levels may be a contributing factor for the induction of apoptosis.

Comparison of c-myc Decline in Daudi and Ramos Cell Lines—c-myc is basally expressed at high levels in both Daudi and Ramos cells. A comparison of these two cell lines following anti-IgM treatment revealed that both mRNA and protein levels for c-myc declined. Northern blots for c-myc expression reveal that significant down-regulation of c-myc mRNA was observed at 4 h in both Daudi and Ramos cells treated with anti-IgM (Fig. 2, panel A). When comparing the c-myc mRNA levels to that of a reference mRNA, actin, in the same samples, a reduction of 60% in the c-myc/actin ratio was found in Daudi cells, while a reduction of 86% was observed in Ramos. Actin mRNA shows no change at 4 h. Similarly, when protein levels were examined by immunoblotting, a significant decrease in C-Myc protein level was found in both Daudi and Ramos cells 24 h after treatment with anti-IgM (Fig. 2, panel B). Thus a reduction of c-myc mRNA at 4 h is followed by a reduction in Myc protein levels extending to 24 h for Daudi as well as for Ramos.

Comparison of c-myc Declines in Other Cell Lines—Additional cell lines were also examined for c-myc expression following treatment with anti-IgM. The Northern blot analysis for these are shown in Fig. 2, panel C. In accord with the previous results, anti-IgM also causes down-regulation of c-myc in Namalwa, Raji, CA46, and ST486 cell lines to varying degrees. Thus, c-myc down-regulation is common to all the cell lines upon treatment with anti-IgM.

Basal c-myc mRNA levels can be decreased by several mechanisms. A specific decrease in c-myc mRNA synthesis rate could yield this result, but an increase in mRNA degradation would also result in a similar observation. The synthesis rate of c-myc mRNA was determined in Ramos cells by labeling newly synthesized mRNA in nuclear run-on experiments. The amount of labeled c-myc mRNA relative to that of a control mRNA was determined. Fig. 3 shows that the amount of labeled c-myc mRNA relative to that of the control mRNA was fairly constant. The c-myc sequence probed corresponds to a region which is within the coding sequence and is downstream of the reported elongation block (17, 21, 58, 59). This indicates that the rate of c-myc mRNA initiation appears to be unaffected by anti-IgM treatment for 4 h. Anti-IgM does not appear to act by controlling the synthesis rate of c-myc mRNA.

The degradation rate of c-myc mRNA was determined in both untreated and anti-IgM-treated cells. Cells were treated for 4 h and then mRNA synthesis was terminated by the addition of actinomycin D. The amount of c-myc mRNA remaining at different times following the actinomycin D addition was determined by Northern blotting and was used to calculate the half-life of c-myc mRNA (Fig. 4A). The $t_{1/2}$ of c-myc mRNA is 31.5 min in untreated cells, but this is shortened to 13.1 min when cells have been treated with anti-IgM. These differences were statistically significant (p < 0.05, n = 5 experiments for untreated cells and n = 3 for anti-IgM-treated cells). Thus c-myc mRNA stability is decreased following anti-IgM treatment.

Dependence of Change in mRNA Degradation Rate on New Protein Synthesis—To ascertain whether the increased c-myc mRNA turnover rate requires new protein synthesis, the dependence on protein synthesis of the changes in mRNA half-life was determined. Cells were treated with cycloheximide for 2 h to block protein synthesis and then treated with anti-IgM to see if the mRNA stability could be affected. Four hours after anti-IgM treatment, actinomycin D was added, and the amount of c-myc mRNA remaining at different times was followed by Northern blotting (Fig. 4B). Cells in which protein synthesis was blocked showed a 1.7-fold increase in c-myc mRNA levels.
Fig. 4. Basis for reduction in c-myc mRNA levels, mRNA degradation rate. A, Ramos cells were treated with anti-IgM for 4 h at which time actinomycin D was added to block further mRNA synthesis. At varying times following, RNA was isolated and the amount of c-myc mRNA remaining was determined by Northern blotting. The figure shows the means ± 1 S.E. The amount of c-myc mRNA present at different times was used to determine the mRNA half-life. Untreated samples (C) showed $t_{1/2} = 31.5$ min (data from five experiments), whereas the $t_{1/2}$ was 13.1 min in anti-IgM-treated cells (●) (data from three experiments). This difference in $t_{1/2}$ was significant. B, dependence of increased c-myc mRNA turnover on new protein synthesis. Ramos cells were treated with cycloheximide (5 μg/ml) for 2 h and then with anti-IgM for 4 h. At this time actinomycin D was added, and RNA was isolated at varying times. c-myc mRNA remaining was quantitated and $t_{1/2}$ for mRNA stability was determined as in panel A above. $t_{1/2}$ was increased from about 21 min for cells with no treatment (○) in this experiment to about 85 min due to cycloheximide treatment (with no anti-IgM treatment (●), $t_{1/2} = 73$ min, with anti-IgM treatment (■), $t_{1/2} = 98$ min). There was no statistical difference between the cycloheximide-treated and cycloheximide plus anti-IgM-treated sample; however, both of these were statistically different from the untreated sample. Anti-IgM treatment was no longer able to induce an increase in mRNA turnover in cycloheximide-treated cells.

Fig. 5. Degradation rates of c-Myc protein. Ramos cells were treated with anti-IgM for 4 h at which time, cycloheximide was added to stop further protein synthesis. Untreated cells were treated with cycloheximide in parallel with the anti-IgM-treated samples. c-Myc protein remaining at varying times was determined by Western blotting and used to determine half-lives for c-Myc protein. The slopes of these two plots are not significantly different. $t_{1/2}$ was 34 min in control cells (○) and 39 min in anti-IgM-treated (●) samples.

and an increase in c-myc mRNA stability ($t_{1/2}$ about 73 min). In cells that were treated with cycloheximide and then with anti-IgM, the $t_{1/2}$ of the c-myc mRNA was 98 min. This did not differ significantly from the cells treated with cycloheximide alone ($p > 0.05, n = 2$). However both the cycloheximide-treated and cycloheximide plus anti-IgM-treated cells were statistically different from the untreated cells. Anti-IgM treatment was no longer able to induce an increase in mRNA turnover in cycloheximide-treated cells. Thus c-myc mRNA degradation is modulated by a mechanism requiring protein synthesis.

Measurement of control mRNAs was carried out in parallel to the experiments with c-myc mRNA. Actin and GAPDH messages were stable after the addition of actinomycin D during the time period needed to measure the c-myc mRNA turnover rate. As before, anti-IgM treatment had no statistically significant effect on the levels of these messages.

C-Myc Protein Stability—Changes in c-myc mRNA levels are reflected by changes in c-Myc protein levels due to changes in synthesis (see Figs. 1 and 2). Whether changes in protein stability would be independently affected cannot be predetermined. c-Myc protein turnover was therefore determined in untreated and anti-IgM-treated Ramos cells (Fig. 5). Cells were treated with anti-IgM for 4 h. Cycloheximide was added, and cell extracts were prepared at different times and analyzed by Western blot to determine the amount of c-Myc remaining. c-Myc protein half-life was found to be 34 min in untreated cells and 39 min in anti-IgM-treated cells. These values were not statistically different. c-Myc protein stability is therefore unchanged upon treatment with anti-IgM. Thus the decline in c-Myc protein level seen in Figs. 1C and 2B is likely due only to alterations in synthesis due to declines in c-myc mRNA levels.

Anti-IgM-induced Apoptosis—Treatment of Ramos cells with anti-IgM results in the induction of apoptosis. This can be demonstrated by several methods. In Fig. 6A, cells were examined by fluorescence microscopy to morphologically determine the extent of apoptosis. In Fig. 6B, DNA from treated cells was examined by agarose-gel electrophoresis to determine the extent of characteristic nucleosomally sized DNA fragmentation which accompanies apoptosis. In Fig. 6C, determination of the distribution of cells in various stages of the cell cycle was performed by flow cytometry. This analysis can also reveal distributions of cells which contain DNA characteristic of apoptotic cells. Each of these analyses revealed that treatment of cells with anti-IgM was followed by induction of apoptosis.

Apoptosis Induction in Other Burkitt’s Lymphoma Cell Lines—Anti-IgM treatment led to induction of apoptosis in Ramos cells. Whether this phenomenon was general was determined by examining the induction of apoptosis in other Burkitt’s lymphoma cell lines following anti-IgM treatment.
When the basal levels of apoptosis were analyzed by flow cytometry, all cells show insignificant amounts of A0 peak (3–10%) with the exception of ST486 which shows ~20% A0 peak in the basal state. Upon anti-IgM addition, all of the EBV-positive cell lines (Daudi, Namalwa, and Raji) were relatively resistant to induction of apoptosis. EBV-negative ST486 cells showed an even greater induction of apoptosis than did Ramos cells. CA46 cells showed less susceptibility to apoptosis than did Ramos and probably resembles the EBV-positive cell lines. The increment in A0 peak due to anti-IgM treatment is shown in Fig. 7.

Growth Inhibition and Apoptosis—Besides having an effect on apoptosis, the reduction in c-myc expression due to anti-IgM may also have an effect on growth regulation. Growth was assessed for Daudi and Ramos cell lines by determining cell numbers (Fig. 8) and by thymidine incorporation with essentially similar results. Anti-IgM-mediated growth inhibition is observed for Daudi cells with doubling time increasing from about 1 day to about 4 days. In Ramos cells complete inhibition of growth is observed.

Growth rates of the other Burkitt’s lymphoma cell lines were measured simultaneously using an ELISA method and are also plotted in Fig. 8. Similarly to Daudi and Ramos cells, growth rates of Namalwa, Raji, CA46, and ST486 are all affected by anti-IgM. One noticeable difference between EBV-positive and EBV-negative cell lines was that EBV-negative cell lines all show declining cell numbers in the presence of anti-IgM, while EBV-positive cell lines show slowed but still positive growth.

Relationship of Anti-IgM-induced Declines in c-myc Expression and Anti-IgM-induced Apoptosis—The previous studies indicated that anti-IgM treatment of all of the Burkitt’s lymphoma cell lines induced a down-regulation of c-myc. Concomitant with this result, the EBV-positive cell lines showed a slowing in the growth rate, and very little induction of apoptosis, whereas the EBV-negative cell lines showed a decline in the cell numbers, and variable induction of apoptosis. To further examine the relationship between these responses, a number of additional experiments were performed.

Antisense Oligonucleotides to c-myc—Since c-myc seems to correlate with growth inhibition but not with apoptosis, the functional role of c-myc was further tested using an antisense c-myc oligonucleotide. Daudi and Ramos cells were treated with either 10 μg/ml anti-IgM or 5, 30, or 50 μM antisense c-myc oligonucleotide, or 50 μM sense c-myc oligonucleotide for 24 h and then analyzed. Treatment of Ramos cells for 24 h with antisense oligonucleotides causes a decrease in c-myc expression (Fig. 9A). This decrease is similarly observed in Daudi cells. Concurrently, as shown in Fig. 9, B–D, antisense oligonucleotide also causes a decrease in cell growth rate (cell number), and a decrease in viability (as determined by staining) in both cell lines, but an increase in apoptosis (morphologic examination) only in Ramos cells. Both anti-IgM treatment as well as antisense c-myc treatment are observed to induce apoptosis in Ramos cells but fail to induce apoptosis in Daudi cells. Addition of anti-IgM plus antisense c-myc oligonucleotide yielded no greater change in cell number, viability, or apoptosis than treatment with antisense oligonucleotide alone. Un-treated and sense oligonucleotide-treated cells showed none of these changes. Thus specific repression of c-myc is a sufficient condition to cause induction of apoptosis in Ramos cells. These results are in agreement with the previous findings that down-
regulation of c-myc by anti-IgM may be a major contributor leading to apoptosis in Ramos cells. However, down-regulation of c-myc is insufficient to cause apoptosis in Daudi cells.

Anti-CD40 Blocks Induction of Anti-IgM-induced Apoptosis in Ramos Cells but Does Not Block Anti-IgM-induced Declines in c-myc mRNA Level—Various agents are known which are capable of inhibiting the anti-IgM-mediated induction of apoptosis (60, 61). An attempt was therefore made to determine if repression of apoptosis induction was linked to abrogation of c-myc inhibition. Ramos cells were treated with anti-CD40 and anti-IgM. Parallel assays were performed for the determination of c-myc mRNA levels and for the induction of apoptosis (Fig. 10). As expected, anti-CD40 caused a decrease in the amount of apoptosis induced by the anti-IgM, and this decrease was related to the magnitude of the anti-CD40 dose. However, the results also indicate that anti-IgM treatment still resulted in a decrease in c-myc mRNA levels which could not be distinguished from the decrease seen in cells which had not been pretreated with anti-CD40. Thus a decrease in the induction of apoptosis could be effected without a concomitant change in the reduction of c-myc mRNA levels. Although a reduction in c-myc expression is required for induction of apoptosis, additional requirements, including the activity of CD40, may be needed to complete the induction process once the c-myc levels have been reduced.

FIG. 8. Cell growth in various Burkitt’s lymphoma cell lines treated with anti-IgM. Daudi and Ramos cells were seeded at a density of 1 × 10⁵ cells/ml in 200 μl in a 96-well microtiter plate and treated (10 μg/ml anti-IgM). Cell numbers were determined at indicated times using a Thomas hemacytometer. (○) untreated cells; (●) anti-IgM-treated. Raji, CA46, Namalwa, and ST486 cells were seeded at a density of 5 × 10⁴ cells/ml in 200 μl in 96-well microtiter plates and treated (10 μg/ml anti-IgM). Cell numbers at the indicated times were determined by an ELISA method. (□) untreated cells; (●) anti-IgM-treated.

FIG. 9. Antisense oligonucleotide to c-myc causes apoptosis, growth arrest, and loss of viability. A, total protein was extracted at 4 h from untreated, anti-Ig-treated, and from sense (S) and antisense (AS) myc oligonucleotide-treated Ramos cells. Total protein was fractionated by polyacrylamide gel electrophoresis and immunoblotted with anti-c-Myc antibody. The level of c-Myc protein observed in c-myc antisense oligonucleotide-treated cells was diminished in a dose-dependent manner relative to that seen in untreated cells. B, C, and D, cells were treated with sense or antisense oligonucleotide, or with anti-IgM. The apoptotic index (B), cell number (C), and viability (D) were determined simultaneously at 24 h after treatment. Anti-IgM and antisense oligonucleotide treatments both caused diminution of cell number and viability in both cell lines, and increase in apoptotic index only in Ramos. Sense oligonucleotide-treated cells resembled untreated cells. Combinations of 50 μM antisense oligonucleotide plus anti-IgM showed no greater change than that of 50 μM antisense oligonucleotide alone (J. S. Kaptein, C.-K. E. Lin, C. L. Wang, T. T. Nguyen, C. I. Kalunta, E. Park, F.-S. Chen, and P. M. Lad, unpublished data). Solid bars, Ramos cells; open bars, Daudi cells.

DISCUSSION

Our studies were directed at understanding the role of antigen stimulation in B cell function. As part of this goal the studies presented here have examined in some detail the regulation of c-myc by anti-IgM and an assessment of the role of c-myc regulation in determining the cellular effects of anti-IgM treatment.

The results suggest that c-myc mRNA levels decline sharply after anti-IgM treatment. Decreases of about 70% compared to control untreated cells are observed within a 3–6 h time period. The ratio of c-myc mRNA to actin mRNA, as well as c-myc mRNA to GAPDH mRNA, are both lowered to approximately the same extent. Since actin and GAPDH genes are stable to anti-IgM treatment the results imply that c-myc mRNA levels are specifically affected. The decline in mRNA is accompanied by a decline in protein levels as judged by Western blotting. We have also noted that the half-life of the c-Myc protein does not appear to be changed due to anti-IgM treatment (Fig. 5). c-Myc protein activity is thought to be regulated through phosphorylation by casein kinase (62, 63) and via interactions with associated proteins, Max and Mad (14, 34, 35, 64). Al-
though anti-IgM appears to have little effect on the turnover of c-Myc protein, its effects on c-Myc protein activity are unknown.

We have observed that c-myc mRNA levels recover after the initial 6 h but we believe that the relevant observation is the initial decline of the c-myc mRNA. The basis for this is that (i) a decline in c-myc mRNA levels rather than an elevation is the primary event; (ii) the mRNA decline is followed by a decline in the c-Myc protein level which does not recover, thus abolition of protein activity is the observed effect following anti-IgM stimulation; (iii) apoptotic changes can be observed prior to the recovery of the c-myc mRNA levels; and (iv) H7 and staurosporine treatments at low doses (10 μM and 10 nm, respectively) cause elevation of c-myc mRNA levels without inducing apoptosis, whereas higher doses (100 μM and 100 nm, respectively) cause both decreases in c-myc mRNA levels and induction of apoptosis. This study has therefore focused on the lowering of c-myc expression which occurs primarily in the 3–6 h following anti-IgM treatment.

The decline in c-myc mRNA level could occur by a number of various mechanisms involving mRNA synthesis or degradation. A decline in transcription rate due either to decreased initiation or to a block in transcription may play a role, however control of initiation and removal of the transcriptional block have predominantly been implicated as inductive mechanisms (58, 65–76). The presence of a block to elongation may also contribute to degradation rates of the message (17, 20, 77). Degradation of c-myc mRNA appears to play a major role in control of c-myc mRNA levels occurring predominantly in cases where c-myc needs to be down-regulated from a high basal state (15, 17, 36, 77–85). Factors that may contribute to c-myc mRNA stability include a stability-conferring region within the coding sequence (77, 86–90), polyadenylation (77, 91), and AUUUA motifs (77, 89). We have assessed both synthesis and degradation as potential mechanisms by which anti-IgM caused decreases in c-myc mRNA levels. Our nuclear run on experiments detected no alteration in the level of messages capable of hybridizing to a segment of the gene downstream of the reported elongation block. In view of the extent of the decline in message level, it is highly unlikely that changes in elongation or changes in an elongation block are responsible for the observed decline. Minor contributions however cannot be ruled out. By contrast however, dramatic changes in the stability of c-myc mRNA were observed following anti-IgM treatment. Therefore the decrease in c-myc message is due to enhanced degradation of c-myc mRNA and is unlikely to be due to alterations in transcription.

Our results are in accord with observations in other systems that indicate that c-myc message is unstable and that a change in the stability of this message is a critical regulator of its levels (15, 36, 77, 78, 80–82, 84). A comparison of half-lives of other mRNAs reveals that c-myc has a very short half-life (92, 93). The half-life of the protein is similarly short (94–96). Thus the observations made here are in qualitative agreement with observations reported by other investigators.

A requirement for protein synthesis is involved in the destabilization of c-myc mRNA. This may indicate a requirement for an ancillary protein or may indicate a need for the c-myc message itself to be translated in order to become unstable (77, 86–90, 93). In our experiments elimination of protein synthesis by pretreatment with cycloheximide alters the profile of c-myc mRNA turnover. Two changes are observed. The first is that cycloheximide itself significantly increases the half-life of the c-myc mRNA and this results in a higher c-myc mRNA level, thus destabilization and degradation are prevented or delayed. The second is that anti-IgM no longer can effect a change in the half-life of c-myc message.

A stabilizing element within the c-myc coding sequence has been reported (77, 86–90). This element appears to be dependent upon translation of c-myc mRNA. Translation appears to displace the stabilizing element leading to c-myc mRNA degradation, hence inhibition of translation using cycloheximide would lead to c-myc message stabilization. The nature of the protein involved and the interaction with the message which causes the stabilization are the subject of current investigations (17, 77, 89, 90, 97).

In view of the available results, there appear to be two mechanisms by which anti-IgM could lead to more rapid degradation of c-myc mRNA. (i) It could inactivate or cause removal of the stabilizing element or prevent its emplacement. This mechanism would be independent of translation of c-myc and hence would still be present even in the presence of cycloheximide. Our results do not support this as we clearly show that treatment with anti-IgM in the presence of cycloheximide results in the prolonged half-life of c-myc mRNA seen with cycloheximide alone. Anti-IgM does not cause an increased turnover of c-myc mRNA in the presence of cycloheximide. (ii) It could induce or activate a destabilizing element which would act after the translation-dependent removal of the stabilizing agent. The activity of this element would require the c-myc mRNA to be translated since cycloheximide alone prolongs the c-myc half-life. The effect of cycloheximide would be to override the anti-IgM effect in our experiments. This is compatible with our observations. The identity of the protein involved is conjecture at this time.

Therefore the cycloheximide experiments allow us to distinguish which of these two possibilities for anti-IgM action are relevant. Anti-IgM appears to act by inducing or activating a destabilizing agent which acts on the c-myc mRNA. This may be significant since anti-IgM triggers various signaling pathways. Which signaling pathway is pertinent to the activation of the destabilizing element is a highly relevant biological problem and experiments using anti-IgM are central to elucidating the data.

Relationship of c-myc to Apoptosis—Treatment of cells with anti-IgM causes significant apoptosis. The level of apoptosis in Ramos cells ranges from about 20 to 50% at 24 h with even higher levels being observed at 48 h. This represents a 10-fold increase in apoptosis over basal levels. An obvious question therefore is whether c-myc is involved in the regulation of apoptosis. Two lines of experimentation have been utilized. The first was to examine whether specific inhibition of c-myc was a sufficient condition to cause apoptosis. Our results using antisense oligonucleotides show clearly that specific inhibition of myc results in growth inhibition and the induction of apoptosis.
The role of c-myc in regulating cell growth, and the effect of EBV proteins in preventing apoptosis. Burkitt's lymphoma cells which are p53 mutant (103) and express high level of c-myc (10, 17, 105) can grow rapidly in vitro. Cross-linking of B cell antigen receptor by anti-IgM in these cells induces down-regulation of c-myc mRNA as well as Myc protein. Reduction of the c-myc level in these cells will propel the cells to exit the cell cycle. In the absence of EBV proteins (EBNAs, LMPs, and BHRF-1 (bd-2 homologue)), the cells will result in apoptosis (e.g. Ramos and ST486 cells). However, in the presence of EBV proteins, apoptosis is blocked resulting in growth arrest (e.g. Daudi, Raji, and Namalwa cells). Addition of antisense c-myc oligonucleotides to these cells demonstrated a similar result to that seen with anti-IgM.

in Ramos cells. The sense oligonucleotide control has neither effect. The combination of anti-IgM plus antisense oligonucleotide has no greater effect than that of antisense oligonucleotide alone. Thus, at least in this cell line, the repression of c-myc is a sufficient condition for induction of apoptosis. The second approach was to examine whether inhibition of apoptosis could be achieved without affecting the decline in c-myc mRNA levels. The results indicate that anti-CD40 which abolishes anti-IgM-induced apoptosis does not affect the anti-IgM-induced decline in the c-myc level. This suggests that cellular processes leading to inhibition of apoptosis are distal to the steps linking anti-IgM to c-myc. Experiments are ongoing to determine whether anti-CD40 inhibits antisense c-myc oligonucleotide-induced apoptosis as it does anti-lg-induced apoptosis. Our results so far indicate that antisense c-myc-induced apoptosis can be inhibited by anti-CD40 although the degree of inhibition has been equivocal. The reasons for this are being investigated.

The results in the EBV-positive cell lines are strikingly different from those of the EBV-negative ones. Anti-lgM treatment resulted in inhibition of c-myc mRNA levels for all of the cell lines tested. Apoptosis was observed in only two of the EBV-negative cell lines. Moreover, although antisense c-myc oligonucleotide caused induction of apoptosis in Ramos cells, it was incapable of inducing apoptosis in Daudi cells. Thus, although a decline in c-myc is a sufficient condition for subsequent induction of apoptosis in Ramos cells (27, 28) (above), additional factors such as bd-2 (98–100), or EBV homologues of bd-2 (BHRF-1) (10, 30, 102) are likely also to be involved in the regulation of apoptosis in other cell lines.

Relationship of c-myc to Growth Control—Other lines of investigation using various cell lines suggest that c-myc may be involved primarily in the control (along with many other factors) of cell growth rather than apoptosis (28).

Anti-IgM slows growth progression in all of the cell lines tested with clear apoptosis observed in only two of the EBV-negative cell lines. That c-myc is probably involved in growth control is indicated by the fact that the antisense c-myc oligonucleotide caused growth inhibition in both Ramos and Daudi cell lines. These results suggest that depression in c-myc alone may contribute significantly to growth inhibition although being insufficient to cause apoptosis.

Interactions of Growth Control and Apoptosis Induction—An obvious question is what other gene products may contribute to the patterns of growth and apoptosis observed here. A potential answer based on several recent studies would be that genes which initiate cell cycle progression such as p53 (30, 103) and those that inhibit apoptosis such as bd-2 (98, 99, 104) or the EBV homologue BHRF-1 (101, 102) may be important. A model integrating these observations is presented in Fig. 11. The main elements of the model are as follows. In “normal” cells p53 expression would cause a suppression of cell cycle progression and the cells would be in G0. In cells where a mutation occurs in p53, the block between G0 and G1 is removed. However progression in the cell cycle will be slow unless c-myc is expressed at high levels. Both conditions occur in the Burkitt's lymphoma cell lines studied here which are p53 mutants and express c-myc at high level. The next scenario to consider is the down-regulation of c-myc. Under these conditions c-myc is low (anti-IgM-treated cells or antisense c-myc treated cells) and the cells will progress slowly though the cell cycle. This growth inhibition will lead to apoptosis unless inhibitors of apoptosis are present. This is the case in EBV-positive cells where BHRF-1, though to be a homologue of bd-2, could serve as an inhibitor although other proteins may contribute to this process. The details of the interactions involved would require further studies of multiple genes. A model which proposes separate roles for c-myc in growth and apoptosis has been proposed (28). The studies presented here suggest that c-myc is a contributor to the elements of growth but is insufficient for apoptosis. Apoptosis may be independently modulated by cellular context and inhibition of apoptosis by anti-CD40 or other agents may bypass c-myc altogether acting on an as yet unidentified step.

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