Growth Factor and Bcl-2 Mediated Survival During Abortive Proliferation of Hybridoma Cell Line

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Abstract: Cultures of the CRL-1606 hybridoma (ATCC) have been reported to undergo continuous proliferation with simultaneous death during nutrient limited fed-batch fermentations. The *bcl*-2 proto-oncogene has been shown to prevent cell death under a variety of otherwise death inducing conditions. We were interested in elucidating the nature of the massive death observed in cultures of CRL-1606, specifically with respect to the possible environmental causes, and the ability of overexpressed human *bcl*-2 (h*bcl*-2) to mitigate cell death. Abortive proliferation, or continuous proliferation in the presence of continuous death, could be induced in serum free cultures of CRL-1606 through the withdrawal of insulin provided the culture was competent for cell proliferation. Culture competency for proliferation was found to be solely determined by the presence of cell culture nutrients. Abortive proliferation was defective in cultures transfected with h*bcl*-2 and the enhanced viability observed resulted from an increased viable cell population and at the expense of the nonviable cell population normally found in untransfected cultures. Abortive proliferation was also observed in serum containing cultures upon serum shiftdowns. Like the insulin-supplemented serum free culture system, h*bcl*-2 transfected cultures exhibited defects in the abortive proliferation process. These results suggest that the massive death observed during nutrient-limited fed-batch fermentation originate, in part, from growth or survival factor limitations. Hence, approaches to design cell culture media that account for the cell's proliferation requirements without accounting for the cell's survival requirements may represent a cell death sentence. Given the transformed nature of the hybridomas, we conclude that the abortive proliferation of CRL-1606 is a consequence of inappropriate cell cycle entry in a survival factor limited environment. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57:** 164–171, 1998.
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INTRODUCTION

Recent advances in our basic understanding of cell cycle progression controls and apoptosis (i.e., programmed cell death) have stimulated efforts to improve industrial cell culture performance using genetic engineering approaches (Renner et al., 1995). Such efforts in many ways complement much of the past research aimed at ''environmental design'' or media formulation. By designing both a cell and an environment that support extended cell proliferation and attenuated death, the ultimate goal is to obtain high cell densities while maintaining high cell viability. With respect to cell proliferation, efforts to effect improvements in cell culture generally fall into two categories: genetical engineering of cells to simplify proliferation requirements (Renner et al., 1995) and rational design of media to meet the material requirements of the cell and to sustain cell proliferation (Xie and Wang, 1994). With respect to cell death, the finding that the death phase of many industrial cell cultures is characterized by massive apoptosis has stimulated efforts to engineer cell lines defective in this process (Itoh et al., 1995; Murray et al., 1996). Attempts to effect this goal have centered around the overexpression of the *bcl-2* protooncogene (see Korsmeyer, 1992, for review), the best characterized member of the *bcl-2* family of apoptosis suppressor genes, in various cell lines. While the precise physiological role that Bcl-2 plays has not yet been identified, its overexpression has been shown to protect cells from a variety of otherwise death inducing stimuli (Deng and Podack, 1993; Korsmeyer, 1992; Miyashita and Reed, 1993; Nunez et al., 1990; Walton et al., 1993).

Yet, despite the apparent dichotomy between proliferation and apoptosis research, recent biological findings indicate that the processes of proliferation and cell death (Evans et al., 1992) are intimately related. The interrelated nature of proliferation and death can be deduced from recent research on the function of *c-myc* protooncogene. Under certain conditions the expression of the *c-myc* can induce apoptosis (Evans et al., 1992) (Fig. 1). Because *c-myc* encodes for a

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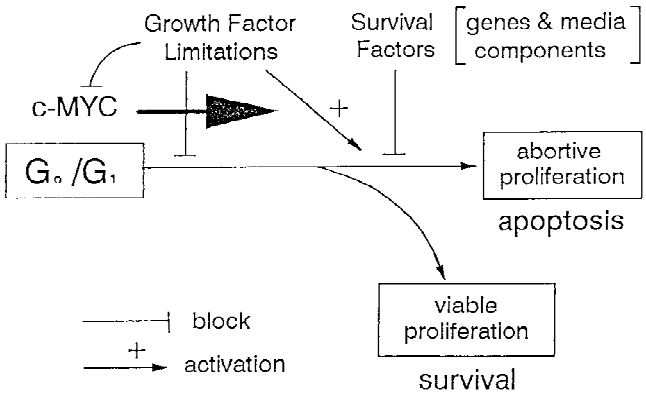

Figure 1. Model depicting the interrelated nature of proliferation and apoptosis as deduced from the work of Evans et al. (1992) on the ability of *c-myc* overexpression to induce apoptosis (see text for detailed explanation).

nuclear transcription factor whose expression is needed to drive quiescent cells into a cell cycle, in the past the expression of *c-myc* has been associated with cell proliferation (Evans et al., 1991). However, recent experiments where cell cycle entry was induced by the ectopic expression of *c-myc* indicate that once a cell enters a cycle, it can either die by apoptosis or survive. Whether or not a cycling cell successfully completes the cycle depends on a number of factors including the prevailing environmental conditions and the expression of survival genes. Hence, cell cycle entry neither precludes the activation of the death pathway nor guarantees that the required survival signals will be present. Under normal physiological circumstances, as approximated by the behavior of nontransformed cells in culture, the expression of *c-myc* is regulated by the presence of various growth factors in the environment. Because growth factors also appear to provide survival signals, the environmental conditions that induce *c-myc* expression in ''normal'' cells are often incompatible with those that activate the death pathway. Under such conditions, cells that enter a cycle can also be expected to survive. However, in cells that have entered a cycle under ''abnormal'' physiological circumstances, which might occur as a result of deregulated *c-myc* expression, it is unclear whether a cycling cell dies by apoptosis or survives. Insights into the factors that influence this decision are paramount to the rational design of cell lines and media for enhanced cell culture performance, and approaches to this problem are the subject of this article.

Given the transformed nature of most industrial cell lines, the need to sustain cell proliferation and enhance cell survival during industrial processes, and the observation that cultures of CRL-1606 exhibit massive death in the presence of continuous proliferation during nutrient feed fed-batch fermentations (Xie and Wang, 1994), we examined the behavior of the CRL-1606 hybridoma with the goal of addressing many of the issues motivated by the model depicted in Figure 1. A major complication associated with the previous studies with CRL-1606 has been the use of serum. Therefore, to examine the role of media components in effecting proliferation and survival, a defined serum free media formulation was adopted in our studies. We were also interested in examining how genetic modifications might effect culture behavior in well defined cultures. Specifically, we examined the ability of overexpressed Bcl-2 to enhance survival in serum free media. Through a systematic evaluation of the effects that environmental and genetic elements had on the proliferative and apoptotic responses, we hoped to identify minimal growth factor conditions that supported CRL-1606 proliferation, identify environmental conditions where the potential benefits of *bcl-2* overexpression might be realized, and provide explanations for the massive death observed in cultures of the CRL-1606 hybridoma.

In this article we report on the behavior of a mouse–mouse hybridoma (ATCC CRL-1606) cultured in serum free and serum containing media. Human Bcl-2 was found to extend the viability of cultures of CRL-1606 in both media. However, the efficacy with which Bcl-2 imparted protection was found to depend on whether the death inducing stimuli originated from nutritional or growth factor limitations. Cells in nutrient deprived cultures ceased to proliferate and rapidly lost viability. Human Bcl-2 provided only marginal, if any, protection in such cases. In contrast, cells in nutrient rich, serum free media deprived of insulin or in a low serum environment exhibited ''abortive proliferation'' whereby continuous proliferation occurred in the presence of continuous death. In such environments the effects of h*bcl-2* overexpression were most evident, because abortive proliferation was prevented in cultures overexpressing h*bcl-2*. Increased culture viability resulted from an increase in the number of viable cells over the level that is normally found in wild-type cultures. Similarities between the behavior of cultures in low serum media and in serum free media lacking insulin with respect to abortive proliferation and its prevention by *bcl-2* overexpression suggests that the massive death exhibited by cultures of CRL-1606 during nutrient limited fed-batch fermentations (Xie and Wang, 1994) results in part from survival factor limitations. Finally, attention is brought to the fact that the abortive proliferation, as induced by growth and survival factor withdrawal, displays striking similarities to the massive cell death observed in cultures inappropriately expressing the *c-myc* gene in low serum environments. Based on the propensity with which transformed cell lines exhibit aberrant *c-myc* expression (Waters et al., 1991) and the finding that the MOPC21 ancestral cell line contains a translocated *c-myc* gene (Shepard et al., 1978), we conclude that the abortive proliferation of CRL-1606 is a consequence of inappropriate cell cycle entry in a survival factor limited environment.

MATERIALS AND METHODS

Cultivation Conditions

CRL-1606 and the h*bcl-2* transfected cultures were grown in Iscove's modified Dulbecco's medium (IMDM, Sigma

Chemical Co.) supplemented with 4 m*M* L-glutamine (Sigma), 1 U/mL penicillin–1 μg/mL streptomycin (Sigma), and 5% fetal bovine serum (FBS, Sigma). The serum free medium formulation consisted of IMDM supplemented with 4 m*M* L-glutamine, 1 U/mL penicillin–1 μg/mL streptomycin (Sigma), 5 mg/L transferrin (Sigma), 10 μg/mL insulin (USB), 2.44 μL/L 2-aminoethanol, 3.5 μL/L 2-mercaptoethanol, and 12 mg/L protease free bovine serum albumin (BSA, Sigma). Prior to experiments, frozen aliquots were thawed and subsequently expanded in serum containing media. The cells were cultivated in T flasks in a 37°C humidified incubator (95% relative humidity) with a 10% CO_2 atmosphere. In all resuspension experiments the cells were washed and resuspended in media prewarmed to 37°C in a 10% CO_2 atmosphere. G418 selection pressure was released following the resuspension of the transfected cultures in the appropriate media. All experiments were performed on at least two different occasions.

Cell Lines

CRL-1606 was obtained from the American Type Culture Collection (ATCC). Transfections were performed via electroporation using a Biorad gene pulsar operating at 0.4 kV and 960 μF using 0.4-cm cuvettes. Exponentially growing cells were concentrated to 1E7 viable cells/mL in fresh IMDM supplemented with 4 m*M* L-glutamine and 0.5 mL was added to the electroporation cuvette. Plasmid DNA harboring the h*bcl-2* cDNA under the control of the cytomegalovirus (CMV) promoter was then added to the final concentration of 1 μg/mL. The cell mixtures were subsequently cooled on ice until electroporation. Following electroporation, the cells were diluted 10-fold with fresh IMDM (5% FBS) and allowed to recover for 2 days. G418 (Geneticin, Gibco-BRL, Gaithersburg, MD) was added to a concentration of 0.6 g/L and the cultures were maintained for 1 week. After G418 selection the cultures were expanded in IMDM containing 5% FBS, and frozen aliquots were prepared and maintained at −80°C or in liquid nitrogen.

The *bcl-2* expression vector consisted of the h*bcl-2* fragment subcloned into the NotI–ApaI sites of the polycloning region of the pRc/CMV expression vector (Invitrogen, Portland, OR) and was kindly provided by Scott Lowe (Cold Springs Harbor Laboratory, Cold Springs Harbor, NY). Following the expansion of the G418 resistant culture, the expression of h*bcl-2* was verified by Western blot analysis (Fig. 2). The Western blot detection system consisted of hamster anti-hBcl-2 primary antibody (10 μg/mL, Pharmingen, San Diego, CA), biotinylated mouse anti-hamster IgG cocktail (10 μg/mL, Pharmingen), and alkaline phosphatase conjugated streptavidin (1/1000 dilution, Pharmingen). The NBT-X-phosphate alkaline phosphatase substrate system was obtained from Boehringer–Mannheim and used according to the manufacturer's procedure. Using this detection scheme along with a primary antibody specific for mouse Bcl-2, we were unable to detect the expression of mouse Bcl-2 in the CRL-1606 cell line.

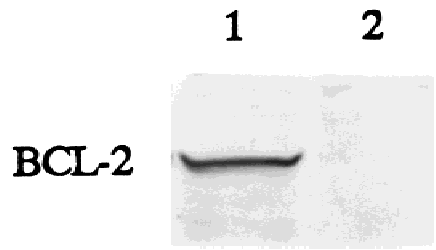

Figure 2. Western blot detection of human BCL-2 (26,000 kDa) in CRL-1606. Lane 1 represents the extracts from 4e5 cells of CRL-1606 electroporated with an h*bcl-2* gene under the CMV promoter. Lane 2 represents an extract from 4e5 wild-type CRL-1606 cells.

Cell Viability

Cell viability was determined microscopically by trypan blue exclusion. Cells were diluted using a solution of 0.4% (w/v) trypan blue in phosphate buffered saline (PBS). At least 200 cells were counted in each determination.

RESULTS

CRL-1606 Survival in Different Serum Free Media

To more precisely identify the role of medium components in mediating cell survival, resuspension experiments were performed whereby cell behavior was assessed in different combinations of our serum free medium base. CRL-1606 cells growing exponentially in IMDM containing 5% FBS were washed and resuspended in media formulations lacking either insulin, transferrin, or L-glutamine. The time course viability obtained from wild-type cultures (Fig. 3A) indicates that the absence of any of these components leads to a decrease in culture viability compared to control cultures. L-Glutamine deprivation induced the largest drop in culture viability when compared to cultures deprived of transferrin or insulin.

hBcl-2 provided partial protection against the death response induced upon resuspension in serum free media. Similar to the experiments summarized in Figure 3A, exponentially growing cultures of CRL-1606 transfected with h*bcl-2* were resuspended in a variety of serum free media formulations and the time course viability profiles were monitored (Fig. 3B). In all cases, h*bcl-2* transfected cultures exhibited extended culture viability relative to the wild-type controls. As with wild-type control cultures, the resulting culture viability varied in accordance with the particular component withdrawn; L-glutamine deprivation induced the most severe decline in culture viability. In contrast, the adverse effects that insulin and transferrin had on untransfected cells was almost completely absent in the h*bcl-2* transfected cultures over the time frame examined.

Growth Factor Requirements for CRL-1606 Proliferation

The results presented in Figure 3 indicate that hBcl-2 provides partial protection against the death response induced

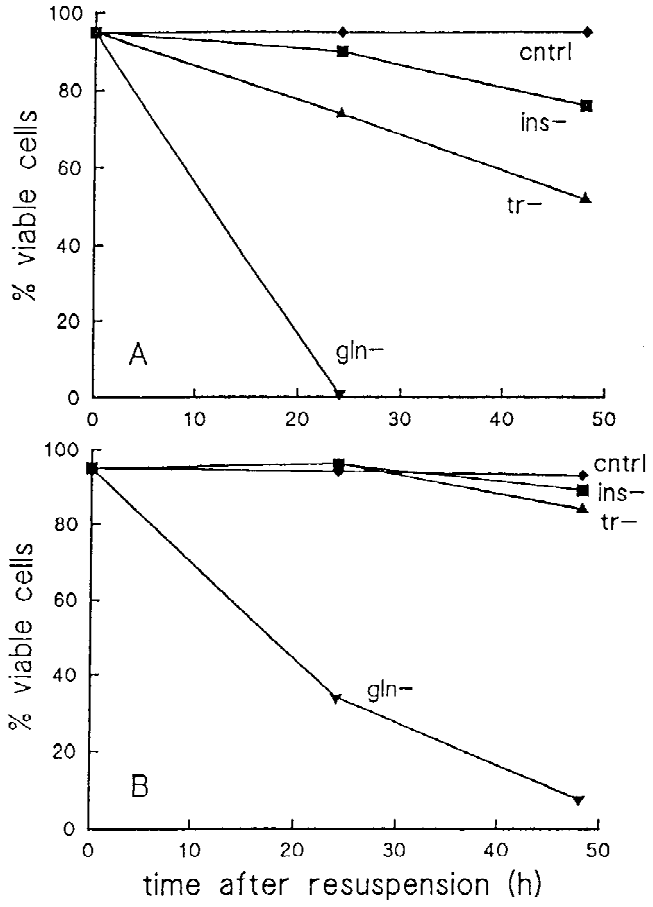

Figure 3. Time course percent viability profiles obtained from (A) CRL-1606 wild-type and (B) polyclonal cultures transfected with the human *bcl-2* gene following resuspension in serum free medium lacking either insulin (ins-), transferrin (tr-), or L-glutamine (gln-).

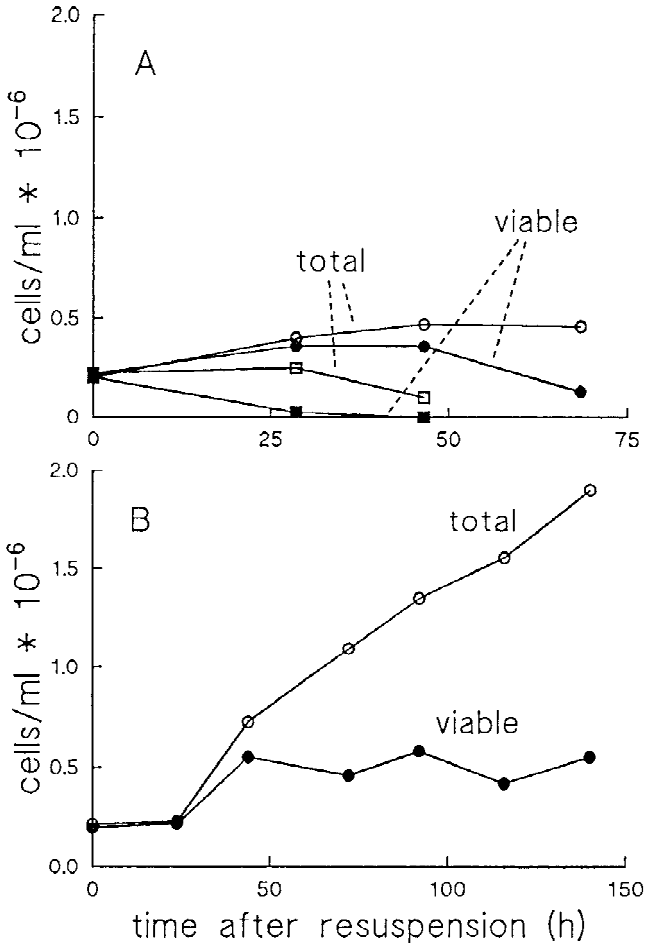

Figure 4. Time courses of cell numbers obtained from wild-type CRL-1606 grown in serum free medium lacking (A) glutamine (squares) or transferrin (circles) or (B) insulin.

upon resuspension in various serum free media formulations. During such experiments it was observed that, depending on the missing component, the loss in culture viability resulted from two qualitatively different cultures responses. In media lacking either L-glutamine or transferrin, the drops in the percent viability observed (Fig. 3) were not accompanied by an expansion in the total cell population as the cells ceased to proliferate (Fig. 4A). Hence, the loss in viability resulted from an increase in the number of dead cells at the expense of the original viable cell population. In contrast, in media lacking insulin the resulting decrease in viability was accompanied by an increase in the size of the total cell population despite a relatively constant viable cell population (Fig. 4B). This was reflected in an unabated increase of total cell numbers with time. Because the culture continued to proliferate in the absence of insulin, the loss in culture viability resulted from an increase in the number of nonviable cells. These results indicated that L-glutamine and transferrin are required for cell cycle entry by CRL-1606. In contrast, the absence of insulin does not preclude CRL-1606 cells from entering a cell cycle. Because the CRL-1606 transferrin requirement can be replaced by ferric citrate (data not shown), we conclude that nutrient deprivation poses a severe block to CRL-1606 cell cycle entry. Within the context of the model presented in Figure 1, the abortive and viable proliferation pathways are unavailable to the cells as proliferation ceases altogether.

Insulin and Bcl-2 Are CRL-1606 Survival Factors

Insulin enhances survival and accelerates cell cycle entry. By systematically supplementing our IMDM base nutrient media in the previous experiments we found that CRL-1606 proliferation could take place essentially in the absence of growth factors. However, induction into a cell cycle far from guaranteed cell survival as noted by the behavior of insulin deprived cultures. Because insulin is needed for proliferation by many cell lines, we then examined effects that insulin had on CRL-1606 survival. Wild-type cultures of CRL-1606 grown in serum containing media were washed then resuspended in serum free media in the presence and absence of insulin. The resulting time profiles of viable and total cell densities reveal two functions of insulin (Fig. 5A,B). Comparisons between the total and viable time course density profiles between the two cultures reveal that

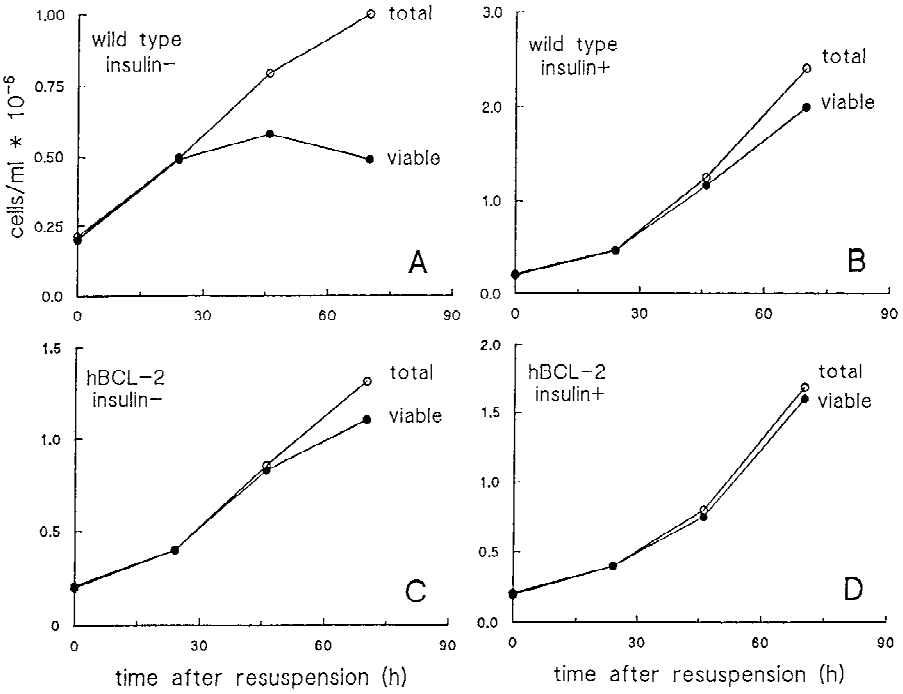

Figure 5. Time course cell density profiles obtained from (A, B) wild-type CRL-1606 and (C, D) polyclonal cultures transfected with the h*bcl-2* gene following resuspension in serum free medium (B, D) with and (A, C) without insulin.

insulin rescues cells from the abortive proliferation pathway and thus acts as a survival factor. In the culture supplemented with insulin, the total and viable time course density profiles are approximately parallel (Fig. 5B). In addition to imparting survival function, proliferation was also enhanced: the insulin supplemented culture exhibited an approximate twofold increase in total cells relative to unsupplemented cultures.

Similar to insulin, hBCL-2 extends the viability of insulin deprived cultures by decreasing the fraction of cells that initiate abortive proliferation (Fig. 5C). CRL-1606 transfected with the h*bcl-2* gene growing exponentially in IMDM supplemented with 5% FBS were washed and resuspended in serum free media deprived of insulin, and the time courses of viable and total cell densities were monitored. h*bcl-2* transfected cultures were defective in the abortive proliferation as noted by the approximately parallel increase in the time course of total and viable cell densities (Fig. 5C). Under similar conditions, the viable cell concentration of wild-type cultures levels off (Fig. 5A), while cells in the h*bcl-2* transfected cultures continue to viably proliferate. Hence, the enhanced viability observed in h*bcl-2* transfected cultures grown in media deficient in insulin occurs as a result of the survival of cells that would have died in the absence of hBcl-2 or insulin. In the context of the model presented in Figure 1, we conclude that insulin and hBcl-2 both function as survival factors in CRL-1606 cultures. Both serve to redirect cycling cells from the abortive proliferation pathway. As expected, h*bcl-2* transfected cultures grown in the presence of insulin also exhibit enhanced viability (Fig. 5D). However, the results presented in Figure 5D also identify environmental conditions where no obvious survival benefits are observed by *bcl-2* overexpression. Interpreted in the light of the model in Figure 1, insulin and Bcl-2 are both functionally redundant with respect to CRL-1606 survival.

Serum Contains CRL-1606 Survival Factors

The previous experiments indicate that cultures deprived of insulin continue to abortively proliferate in serum free media as long as ample nutrients are available. These observations suggests that the massive death observed in the presence of continuous cell proliferation during nutrient feed based fermentations with CRL-1606 in serum containing media (Xie and Wang, 1994) might reflect growth or survival factor depletion. To further explore this hypothesis and to identify the functional role(s) of serum in the context of the model presented in Figure 1, serum titration experiments with CRL-1606 were performed. An inoculation regime similar to the previous experiments was utilized. CRL-1606 cells growing exponentially in IMDM containing 5% FBS were washed then resuspended in IMDM containing different serum concentrations.

Comparisons of the time courses of viable and total cells obtained from wild-type cultures grown in 5 and 0.5% FBS indicate that serum shiftdowns induce behavior qualitatively similar to insulin deprivation in serum free media cultures (Figs. 6A vs. 5A). Serum shiftdowns resulted in continuous cell proliferation with simultaneous death. As such, the viable cell density ceased to increase despite a continuous increase in the size of the total cell population (Fig. 6A).

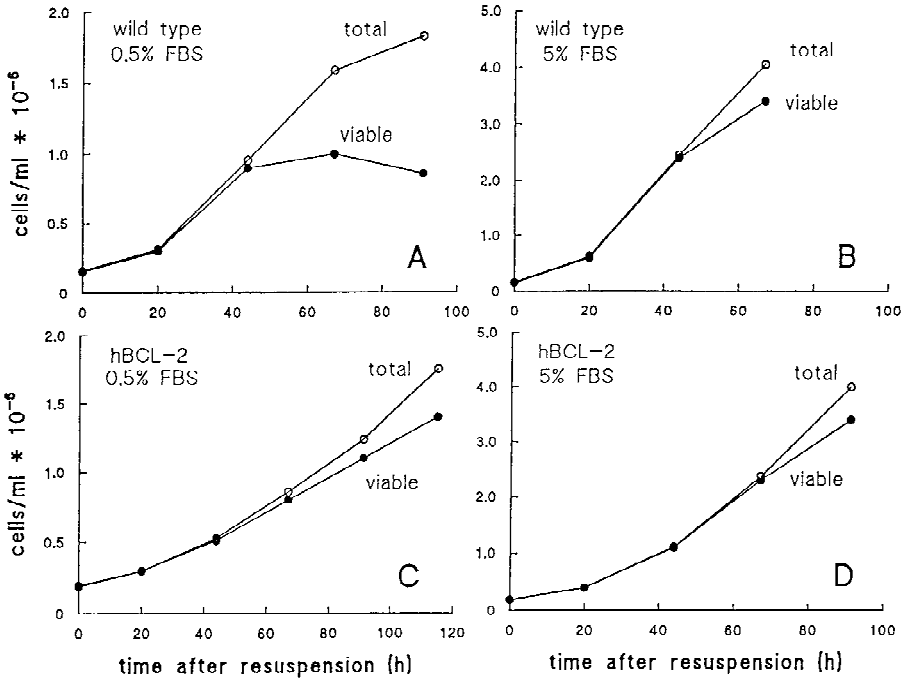

Figure 6. Time course cell density profiles obtained from (A, B) wild-type CRL-1606 and (C, D) polyclonal cultures transfected with the h*bcl-2* gene following resuspension in medium with (B, D) 5% FBS and (A, C) 0.5% FBS.

This phenomenon was not observed in control cultures growing in 5% serum (Fig. 6B), as noted by the parallel profiles of viable and total cell densities. The similarities exhibited between low serum cultures and insulin deprived serum free cultures implicate growth factor limitations as the cause of abortive proliferation observed in low serum environments. The fact that the 0.5% FBS culture continued to proliferate over time argues against the possibility that nutrient limitations induced the phenomenon of abortive proliferation.

The results presented thus far (Figs. 4, 5) indicate that continuous, yet abortive proliferation can be induced upon growth factor withdrawal or serum reduction. The similar wild-type behavior induced by these regimes suggested a similar cause: growth factor limitations. Given the ill defined nature of serum, we were interested in gathering further evidence that might ascertain whether growth factor limitations or nutrient limitations might be the cause of the behavior exhibited by wild-type CRL-1606 in reduced serum environments. Should the hypothesis concerning growth factor limitations be true, then one would expect hBcl-2 to enhance the viability observed in low serum environments by increasing the number of viable cells. h*bcl-2* transfected cultures did exhibit behavior consistent with this hypothesis. This is illustrated by the parallel time courses of the total and viable cell densities obtained from *bcl-2* transfected cultures growing in 5% FBS; abortive proliferation was also prevented (Fig. 6D). Taken together these results strongly support the notion that the abortive proliferation often observed in cultures of CRL-1606 result principally from growth factor limitations.

DISCUSSION

Using a serum free medium, *bcl-2*, and a conceptual model relating proliferation and apoptosis, we clarified the role(s) that nutrients, insulin, serum, and Bcl-2 play in promoting proliferation and survival of the CRL-1606 hybridoma in culture. The realization of hBcl-2 imparted benefits under our experimental conditions depended strongly on two factors: the ability of the culture environment to support cell cycle entry and the environmental role in predisposing cycling cells to apoptosis. Proliferation of cultures of CRL-1606 was induced by the availability of nutrients, irrespective of the growth factor composition of the media. Culture deprived of nutrients ceased to proliferate and eventually died. Bcl-2 provided a marginal effect on viability following nutrient withdrawal. Cells predisposed to apoptosis resulted when cells were driven into a cell cycle in the absence of survival factors such as insulin or certain serum components. Cultures subject to this regime exhibit a phenomenon that we termed abortive proliferation, which is characterized by continuous cell proliferation in the presence of continuous cell death. Under such conditions, h*bcl-2* overexpression was found to increase culture viability by preventing the death of cells that would have ordinarily died at some point in the next division cycle.

In this study our interpretation of the results focused primarily on the behavior of cells during the rounds of replication that immediately follow factor withdrawal. This allowed us to examine how the cell's molecular machinery governs the chance of successful division (i.e., viable proliferation) without complications associated with nutrient

depletion, waste product accumulation, or the polyclonal nature of the culture. Such complications were obvious during insulin supplementation experiments where major differences in nutrient uptake and waste product buildup were observed (data not shown). Evidence of the role that population heterogeneity plays in influencing the observed culture response was also apparent in the viability time courses of *bcl-2* transfected cultures. These were found to first exhibit a biphasic behavior characterized by high viability followed by a decline in viability, and an eventual leveling of viability between 30 and 50%, sustainable even after 7 days (data not shown). While *bcl-2* transfected cultures were found to exhibit enhanced viability at longer times in all of the experiments summarized in Figures 5 and 6 (data not shown), the unknown influences of the aforementioned factors makes it difficult to interpret such results in the context of the model presented in Figure 1. Additionally, we have no evidence suggesting that the spontaneous death observed in a nondividing cell is equivalent to the abortive death associated with an actively dividing culture.

Our interpretation of the results presented in this study is inherently static. We have interpreted the effects of insulin and Bcl-2 solely in terms of the cells' outcome, upon factor withdrawal, after each division cycle (Fig. 1). However, an important question that remains unresolved is whether the rate of cell proliferation affects the rate of death. Insulin supplement and *bcl-2* transfected cultures exhibited differences in the rates of proliferation. Given the polyclonal nature of the *bcl-2* transfected cultures, these results cannot provide insights into this question. Because Bcl-2 was recently found to play a role in proliferation, it would be interesting to examine how variations in gene dosage might affect the proliferation and death responses under conditions that support continuous cell proliferation. Considerable evidence suggests that gene dosage affects the magnitude of the death response observed in culture.

The ability of CRL-1606 to proliferate despite the absence of growth factors highlights an important feature of plasmacytomas and transformed cell lines in general, namely, the subverted nature of their cell cycle controls. Among other aberrations, transformed cell lines are generally characterized by altered regulation of *c-myc*, a protooncogene whose expression is needed to drive quiescent cells into cell cycles (Kelly et al., 1983). In plasmacytomas, altered *c-myc* regulation is generally associated with chromosomal translocations (Shepard et al., 1978; Vaux et al., 1988). In nontransformed cells in culture, the expression of *c-myc* is mediated by growth factors present in the media. Therefore, cell proliferation as a result of *c-myc* expression in the absence of growth factors is physiologically abnormal and has been found to result in massive cell death. Evans and coworkers (Evans et al., 1992) found that when *c-myc* is overexpressed in fibroblasts cultured under low serum environments, massive apoptosis results. The behavior exhibited by cultures of CRL-1606 in serum free media lacking insulin and in low serum environments is thus remarkably similar to the behavior exhibited by cultures that inappropriately express *c-myc*. In both cases cell death results from regimes that normally induce cell cycle arrest and this death could be prevented by the overexpression of h*bcl-2*. Based on these similarities and the propensity with which plasmacytoma derived cell lines exhibit aberrant *c-myc* expression, it could be suggested that the abortive proliferation observed in cultures of CRL-1606 reflects the aberrant expression of *c-myc* in a survival factor deprived environment. However, regardless of the c-myc status of CRL-1606, our results do indicate that cell cycle entry occurs in the absence of the necessary survival signals. The ability to eliminate such conflicting signals is critical in improving cell culture performance. The model introduced in Figures 1 and 7 served as a useful framework to study proliferation and death in Chinese hamster ovary and other hybridoma cultures because abortive proliferation was also found to be induced in these cultures (unpublished results).

The likelihood that the model of Figures 1 and 7 describes the aberrant growth control exhibited by CRL-1606 and other industrially relevant cell lines has important implications for the design of cell lines and environments to improve cell culture performance. With respect to the environment, cell culture media are often optimized to promote sustained cellular proliferation. Because most, if not all, cells used as recombinant hosts are transformed, the environmental requirements for sustained proliferation are less demanding due to aberrant cell cycle controls. This was found to be the case with cultures of CRL-1606 that proliferated in the absence of growth factors. Given that the inappropriate expression of the *c-myc* gene product can induce apoptosis, environments that sustain the proliferation of transformed cell types do not necessarily satisfy the requirements for cell survival. Therefore, the quality of a medium cannot be judged solely on the ability to promote proliferation but must also be assessed by the presence of potential apoptotic ''priming'' signals. Hence, a well designed medium must not only provide cells with the ingredients needed for continuous proliferation, but also the components needed for cell survival.

Regarding the genetic engineering of robust cell lines,

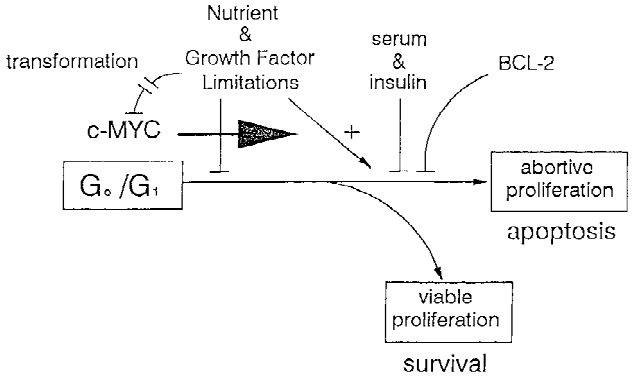

Figure 7. Alterations of the model presented in Figure 1 to explain the specifics of CRL-1606 behavior as deduced from the experimental results obtained in this study.

mounting evidence indicates that environmental factors play a key role in regulating the expression of positive and negative mediators of apoptosis. *bcl-2* is one such gene whose expression has been found to be naturally upregulated in many cell lines in response to certain growth factors and cytokines (Otani et al., 1993). In certain malignant plasma cells its expression even appears to be constitutive (Pettersson et al., 1992). Therefore, the ability to extend culture viability via *bcl-2* overexpression under certain circumstances can be expected to depend on how environmental conditions affect endogenous cell survival defenses. In our study this was displayed by the lack of any obvious effect of *bcl-2* overexpression on cell survival in the presence of insulin. Such a possibility could also provide an explanation for the mixed results obtained by other researchers who sought to extend the viability of cultures by overexpressing *bcl-2* (Itoh et al., 1995; Murray et al., 1996). Additionally, growth factors can induce other mediators of the apoptosis process. These include among other genes, the *p53*, *c-myc*, and *bax* gene products (Hall et al., 1993; Kelly et al., 1983; Miyashita et al., 1994). p53 and c-myc play important roles in regulating normal cell cycle progression; however, under certain conditions they can also promote apoptosis (Evans et al., 1992; Lowe et al., 1993). The means by which Bax induces apoptosis appears to be more direct, because heterodimerization with Bcl-2 leads to a loss of Bcl-2 function (Oltavi et al., 1993). These observations suggest that the cell's decision to induce apoptosis reflects the net effect of positive and negative mediators of the process. Therefore, an understanding of how this balance is influenced by culture conditions is likely to play a major role in the design of genetically engineered cell lines capable of exhibiting extended viability.

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