Apoptosis-resistant NS/0 E1B-19K myelomas exhibit increased viability and chimeric antibody productivity under cell cycle modulating conditions

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Abstract

Lymphoid cells expressing sufficient levels of Bcl-2 or E1B-19K are known to resist to induction of apoptosis in glutamine-free or nutrient-limited batch cultures. However, despite the increased viability and prolonged stationary phase achieved in batch culture, product yields are not necessarily improved. Here we have found that expression of E1B-19K in NS/0 myeloma cells cultivated in the presence of certain cell cycle modulators could result in a significant increase in MAb productivity as compared to untransfected control cells. The use of E1B-19K significantly enhanced cell survival in the presence of osmolytes (sorbitol, NaCl), DNA synthesis inhibitors (hydroxyurea, excess thymidine), and the cell culture additive OptiMab^{TM}. E1B-19K myelomas cultivated in the presence of NaCl or OptiMab^{TM} accumulated in the G₁ phase, while those arrested with excess thymidine were blocked in all phases. Interestingly, control NS/0 cells treated with these agents were found to die in a cell-cycle specific manner. Thus, while all G₁ and most S phase cells quickly underwent apoptosis, G₂/M cells remained alive and maintained MAb secretion for more than 10 days if supplied with adequate nutrients. For both control and E1B-19K cells, incubation with sorbitol or hydroxyurea was detrimental for MAb secretion, while addition of NaCl, excess thymidine and OptiMab^{TM} resulted in an increased specific MAb productivity as compared to the batch culture. However, this increase resulted in an improvement of final MAb yields only in the case of OptiMab^{TM}. The extension of viability conferred by E1B-19K allowed to further improve the final MAb yield obtained using OptiMab^{TM} with a 3.3-fold increase for E1B-19K cells as compared to 1.8-fold for control NS/0 cells.

Abbreviations: HT – Hypertonic; HU – Hydroxyurea; IT – Isotonic; MAb – Monoclonal antibodies; PI – Propidium Iodide; q_{MAb} – Specific MAb production rate; TTP – Thymidine triphosphate; XST – Excess thymidine; X_t – Total cell density; X_v – Viable cell density; μ – Specific growth rate.

Introduction

In the last fifteen years, increasing demand for proteins derived from eukaryotic cells has led to many

developments in the field of animal cell technology. The appreciation of the apoptotic nature of many cell types used in the production of biopharmaceuticals has stimulated efforts to generate genetically engineered apoptosis-resistant cell lines in an attempt to

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increase viability and productivity in culture processes (reviewed in Mosser and Massie, 1994; Mastrangelo and Betenbaugh, 1998). Genes such as Bcl-2 (Fassnacht et al., 1998; Itoh et al., 1995; Murray et al., 1996; Singh et al., 1996), Bcl-2 in combination with Bag-1 (Terada et al., 1997) and adenoviral E1B-19K (Mercille et al., submitted) have been tested into production cell lines. While some researchers have observed that Bcl-2 overexpression could result in improved MAb productivity in batch culture (Simpson et al., 1997; Suzuki et al., 1997), others have found no effect (Fassnacht et al., 1998). In a previous study, we found that the dose-dependent enhancement of viability conferred by E1B-19K in batch cultures of NS/0 cells did not lead to significant antibody productivity throughout the prolonged stationary phase (Mercille et al., submitted). We postulated that this could be explained by the early exhaustion of glutamine or other limiting nutrients essential for protein synthesis, and that cultivation of apoptosis-resistant cell lines in other modes of culture could result in both improved viability and increased MAb yields. Indeed, we have recently demonstrated that E1B-19K-expressing myeloma cells grown in perfusion culture exhibited a significant increase in MAb production (Mercille and Massie, submitted). Cultivation of apoptosis-resistant production cell lines in culture medium supplemented with cell cycle modulators such as osmolytes or inhibitors of DNA synthesis may also lead to significant increases in antibody concentration as compared to apoptosis-susceptible cell lines grown under similar conditions.

A number of studies have shown that hypertonic stress can increase the specific MAb productivity of hybridoma cells (Oh et al., 1993; 1995; Øyaas et al., 1994a; 1994b; Ozturk and Palsson, 1991; Reddy et al., 1992; Reddy and Miller, 1994; Yang et al., 1996). This has been attributed in part to increased amino acid uptake via the Na⁺-dependent symport (Oh et al., 1995). The use of osmolytes such as NaCl, KCl, sucrose, choline chloride, and sorbitol usually results in apparent growth arrest and accumulation of cells in the G₁ phase of the cell cycle (Fuhrman Conti et al., 1990; Pellicciari et al., 1995). However, hypertonic conditions are a potent inducer of apoptosis in a variety of cell lines (Pandey et al., 1996; Qin et al., 1997; Matthews and Feldman, 1996; Mathews et al., 1997; Anderson, 1997; Ortiz et al., 1997) such as S49 lymphocytes, primary thymocytes (Bortner and Cidlowski, 1996), SK-N-SH and HeLa cells (Galvan and Roizman, 1998). In neurons, the

levels of Bcl-2 have been shown to modulate the susceptibility to induction of apoptosis in response to hypertonic conditions. Singleton et al. (1996) reported that osmotic stress decreases total Bcl-2 by 4-fold and correlates with the processing of neuronal ced-3/ICE-like proteases. In these cells, type I insulin-like growth factor receptor activation maintains normal Bcl-2 levels, enhances the negative death regulator Bcl-X_L and prevents apoptosis.

Another approach aiming at increasing the productivity of cultured cells has been through the inhibition of DNA replication with the use of hydroxyurea or excess thymidine (Terada et al., 1997; Suzuki and Ollis, 1990; Takahashi et al., 1994; Al-Rubeai et al., 1990). Inhibition of ribonucleotide reduction either by increasing dATP and dTTP pools by addition of deoxyadenosine and thymidine to culture medium, or by hydroxyurea, leads to a rapid cessation of DNA synthesis (Stubbe, 1990; Fontecave et al., 1992). However, stalled replication forks are unstable and DNA repair mechanisms become rapidly incapable of coping with long periods of DNA synthesis arrest. To avoid extensive DNA damage accumulation, apoptosis is rapidly induced following prolongation of deoxynucleotide depletion (Paulovich et al., 1997; Barbiero et al., 1995). Hydroxyurea has been extensively studied as a suitable chemotherapeutic agent capable of inhibiting tumor cell growth by induction of apoptosis (Schrell et al., 1997; Anand et al., 1995). However, since Bcl-2 and Bcl-X_L are expressed in a variety of tumors, their expression can modulate the sensitivity of tumor cells to chemotherapeutic agents such as hydroxyurea (Simonian et al., 1997). In an effort to increase MAb yields and prevent induction of apoptosis following addition of excess thymidine to culture medium, genes such as Bcl-2 (Simpson et al., 1997) and Bcl-2 in combination with Bag-1 (Terada et al., 1997) have been transfected into hybridoma cells. Although expression of those genes was successful in improving host cell survival (Terada et al., 1997) and increasing specific MAb productivity (Simpson et al., 1997), it still has not been demonstrated that this resistance can result in increased final MAb yields in growth-arrested conditions as compared to normal batch culture conditions.

The culture media additive known as OptiMab^{TM} (Gibco, Grand Island, NY) is a MAb production enhancer containing a cell cycle modulator and a nutrient supplement of proprietary composition. Enhancements in both specific MAb productivity and yields have been previously reported with the use of

this agent (Fike, 1990; Fike et al., 1991a; 1991b; Jayme, 1991). In a previous study, we have shown that addition of OptiMAb^{TM} to batch cultures of D5 hybridoma cells resulted in an increase in the G₁ phase population, in a 50% increase in specific MAb production rate and in an overall enhancement of MAb concentration (Mercille et al., 1994). However, this increase in productivity was only transient since cell viability declined after 2 days in culture. In the present paper, we have examined whether expression of E1B-19K in myeloma cells could prevent induction of apoptosis and increase final MAb yields in growth-modulating culture conditions achieved using agents such as sorbitol, NaCl, hydroxyurea, excess thymidine or OptiMAb^{TM}.

Materials and methods

Cell lines, media and additives

The NS/0 myeloma cell line used in this study was derived from a NS1/1.AG4.1 clone originally obtained from Caesar Milstein (Cambridge University, U.K.). The construction of the E1B-19K and chB43 chimeric monoclonal antibody expressor plasmids, the transfection protocol, and the establishment of stably transfected antibody-producing control NS/0 and E1B-19K cell lines have been described elsewhere (Mercille et al., submitted). Selection of the final sub-clone constitutively expressing both E1B-19K and chB43 was not only done on the basis of high levels of secretion, but also on the basis of high stability of chB43 expression and best apoptosis resistance. Although we initially tried to obtain control NS/0 and E1B-19K clones with similar MAb secretion levels in batch, we considered that stability of MAb and E1B-19K expression was paramount for long-term culture. Unfortunately, following the various rounds of cloning and selection of stable clones, the best E1B-19K clone selected for this study turned out to have a lower secretion in batch as compared to the control NS/0 clone. Both parental and E1B-19K myelomas used in the present study tested negative for mycoplasma contamination using a Hoechst staining and co-culturing detection assay (ATCC quality control methods for cell lines, 1st edition, 1985, pp. 12–15). Cells were cultivated in high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah), and maintained at 37 °C in a 5% CO₂

atmosphere. Spinner flask cultures were supplemented with 0.1% Pluronic F68 (Gibco) for protection against shear stress. Every culture was started from the same batch of inoculum vials kept in liquid nitrogen.

Cell cycle modulators

Hypertonic medium (HT) medium was prepared with sorbitol or NaCl as osmolytes. All osmolarity measurements were made using a micro-osmometer (Model 3MO+, Advanced Instruments Inc, Needham Heights, MA). The osmolarity of isotonic (IT) DMEM was 335 mOsm. Using sorbitol, 1400 mOsm medium was prepared by dissolving one mole of sorbitol into DMEM and filtering with a 0.22 µm filter (Millipore). Three different HT media with osmolarities of 385, 425, and 475 mOsm were also prepared by 1:150, 1:100 and 1:75 addition of a 5 M NaCl stock solution (prepared in water) into isotonic DMEM. Given that 110 mM NaCl is already present in DMEM, the final NaCl concentration of these three HT media was 143, 160 and 176 mM, respectively. In order to achieve inhibition of DNA synthesis, cells were grown in medium supplemented with hydroxyurea (HU) or excess thymidine (XST). Medium containing 10 mM HU (Bianchi et al., 1986) was prepared by 1:100 addition of a 1 M HU stock solution (prepared in water) into isotonic DMEM. Three different XST media were prepared by 1:50, 1:30 and 1:15 addition of a 150 mM thymidine stock solution (prepared in water) into DMEM. The final thymidine concentration of these three media was 3, 5 and 10 mM, respectively. In order to maintain the thymidine concentration, medium replacements were performed every 2 days by centrifuging the cells and resuspending them in fresh thymidine-supplemented medium. Both component A and B of the OptiMAb^{TM} antibody production enhancer were added in concentrations prescribed by the manufacturer (Gibco). Batch controls and cultures supplemented with sorbitol, NaCl, HU and XST were performed in 6-well plates. OptiMAb^{TM} experiments and their controls were performed in 100 mL spinner flasks.

Analytical methods

Viable and total cell concentrations were evaluated using erythrosin B (Sigma) dye exclusion tests and haemocytometer counts. The concentration of human immunoglobulin G₃ in culture supernatants was measured using a standard ELISA protocol described elsewhere (Gervais et al., 1998; Mercille et al., submitted).

Cell cycle distribution was assessed by measuring the DNA content of propidium iodide stained cells. Conditions of cell fixation, coloration, flow cytometric analyses, data collection and analysis and quantification of cell cycle phases are detailed elsewhere (Mercille and Massie, submitted; Mosser et al., 1997). All the samples selected for determination of cell cycle profiles have been analyzed during the same session. Appropriate controls such as DNA beads have been used before and after the analysis to ensure that no drift had occurred. Throughout the analysis run, G₁ and G₂/M peaks in all samples did not move, exhibiting constant PI staining intensity (DNA content). Two methods were used for quantification of apoptosis. In the first method, cell suspensions were mixed with fluorescent DNA-binding dyes and examined by fluorescence microscopy to visualise and count cells with apoptotic nuclear morphology (Duke and Cohen, 1992; Mercille and Massie, 1994). In the second method, apoptotic cells were quantified by determination of the sub-G₁ population following cytometric cell cycle analysis. Cell fixation in ethanol followed by rinsing in aqueous buffers (Hotz et al., 1994; Telford et al., 1992) leads to extraction of the mono- and oligonucleosomes produced consequent to the action of endonucleases (Sokolova et al., 1992). Subsequent staining with DNA fluorochromes such as propidium iodide (PI) results in a discrete peak with reduced fluorescence as compared to G₁ non-apoptotic cells of the same ploidy. Since it can be difficult to discriminate between apoptotic cells and debris in the sub-G₁ population, we have used a logarithmic instead of a linear scale on the x-axis (DNA content/intensity of fluorescence from PI) to achieve a better discrimination between debris and apoptotic cells. This slight modification to the established protocol is documented in Mercille and Massie (submitted). Similar results were obtained with these two methods.

Computational methods

Specific growth rate (μ) was defined as $(dX_t/dt)(1/X_v)$ where X_t ($\times 10^8$ cells L⁻¹) is the total cell density (including both viable and dead cells) and X_v ($\times 10^8$ cells L⁻¹) is the viable cell density. Considering the fact that the control and test clones did not secrete equivalent levels of MAbs in batch culture, we did not make direct comparisons between control NS/0 and E1B-19K cells under arrested conditions. The levels of secreted MAbs and specific MAb productivity (q_{MAb}) of control NS/0 and E1B-19K cells were com-

pared on the basis of ratios obtained by dividing the MAb concentrations or q_{MAb} value of a given clone under cell cycle modulating conditions by the MAb or q_{MAb} value obtained with the same clone in batch culture in isotonic medium. Specific MAb production rate (q_{MAb}) was defined as $(dMAb/dt)(1/X_v)$ where MAb is the antibody concentration (mg L⁻¹). For both μ and q_{MAb} , rates were evaluated at each data point by taking 3-point slopes as a function of time (days). For example, in the case of μ : $dX_t/dt = \Delta X_t/\Delta t$. This slope was then divided by the corresponding average value of X_v (middle point) to obtain the specific rates.

Results

Effect of E1B-19K on viability and MAb productivity in hypertonic medium

In order to assess whether E1B-19K could protect NS/0 myeloma cells from induction of apoptosis in hypertonic (HT) medium and could allow for increased MAb concentration, both control NS/0 and E1B-19K-expressing cells were cultivated in medium supplemented with sorbitol or NaCl osmolytes. Sorbitol was used to temporarily increase the osmolarity of the culture medium to 1400 mOsm. Cells seeded at 8×10^5 cells mL⁻¹ were incubated for 2 hr in medium containing 1 M sorbitol and then reincubated in isotonic (IT) medium for 18 hr. Following the 18-hour incubation period, control NS/0 cells exhibited a viability of 15% while E1B-19K cells had a viability of 68%. Various clones containing different levels of E1B-19K were also tested similarly. The resistance to induction of apoptosis following a 2-hour sorbitol osmotic shock and 18-hour recovery was found to be proportional to the amount of E1B-19K expressed in a given clone (data not shown). For both cell lines, dead cells were found to exhibit apoptotic morphology. E1B-19K therefore partially protected the cells from apoptotic death following sorbitol osmotic shock. In order to achieve an HT medium more suitable for long term cell cultivation and antibody production, the osmolyte NaCl was used instead of sorbitol. Concentrations of 33, 50 and 66 mM were tested on the cells but only the latter was sufficient to stop the increase in viable cell density (data not shown). Supplementation with 66 mM NaCl resulted in an increase in the osmolarity from 335 to 475 mOsm. For both control NS/0 and E1B-19K cells, a batch culture performed in IT medium was included in the experiment. This batch

Figure 1. Effect of E1B-19K on cell survival, MAb productivity and cell cycle distribution of NS/0 myelomas grown in hypertonic medium (supplemented with 66 mM NaCl). This figure presents the evolution of viable (X_v) and total (X_t) cell density, viability (%), MAb concentration and specific MAb productivity (q_{MAb}) of control NS/0 (left panels) and E1B-19K-expressing cells (right panels) cultivated throughout a 12-day period in isotonic (IT) and hypertonic (HT) medium (supplemented with 66 mM NaCl). Bottom panels present cell cycle profiles of control NS/0 (left panel) and E1B-19K cells (right panel) after 5 days in HT medium. Each lower panel presents both log and linear (inset) scales of the same sample. Log scale profiles allow to distinguish the sub- G_1 apoptotic peak while linear scale insets present the cell cycle distribution into G_1 , S and G_2/M phases of non-apoptotic cells, excluding the sub- G_1 cells which make up for a large fraction of the sample, especially in the case of control NS/0 cells.

control was important since large variations are often observed in the specific growth rates, maximal viable cell densities, and specific MAb productivities of different clones. The viable density of control NS/0 cells exposed to NaCl leveled off initially around 10×10^5 cells mL^{-1} for 3 days and then decreased steadily until day 8 (Figure 1, left panels). Immediately after exposing the cells to the HT medium, a rapid and linear decrease in viability was observed concomitant with the accumulation of sub-G₁ apoptotic cells (Figure 1, bottom left panel). Although viable cell density quickly leveled off and declined, the growth of control NS/0 myeloma cells was not arrested. Indeed, the accumulation of total cells up to 28×10^5 cells mL^{-1} throughout the culture indicated that cells were still dividing. A constant specific growth rate (μ) of 0.28 day^{-1} was measured for control NS/0 cells during the first 5 days of culture in HT medium, as compared to a μ of 0.51 day^{-1} for exponentially growing control NS/0 cells in IT medium. Therefore, a leveling-off or decrease in the viable cell density in HT medium does not necessarily imply that cells are undergoing growth arrest, in contrast to what is sometimes reported in the literature (Oh et al., 1993; 1995). At day 5, control NS/0 cells in HT medium were collected for cell cycle analysis. The sub-G₁ cells made up for 65% of the population (Figure 1, bottom left panel) and the resulting viability estimate of 35% obtained with this cytometric technique was close to the 27% measurement made using the standard dye exclusion test. The remaining viable control NS/0 cell population at day 5 (excluding sub-G₁ cells) was composed of 1% G₁, 9% S and 90% G₂/M phase cells. At the beginning of the experiment (day 0), exponentially growing control NS/0 cells used as inoculum were composed of 32% G₁, 52% S and 16% G₂/M phase cells. Thus at day 5, all G₁ and most S phase cells had disappeared, concomitant with a large increase in the sub-G₁ cells, whereas the G₂/M phase cells had increased from 16 to 32% of the total population. Indeed, on day 5, only 35% of the total population was viable and 90% of these viable cells were composed of G₂/M, making up for a resulting percentage of 32%. For control NS/0 cells, the evolution of the MAb concentration followed the same trend in both HT and IT media. As is typically observed in batch culture of lymphoid cells, MAbs were produced in IT medium throughout the exponential growth phase and further accumulation could be observed in the 2 to 3 days that followed the onset of the decline phase. In HT conditions, MAbs were produced continuously until day 5

when the viable cell number started to decrease drastically. A 2-fold increase in specific MAb productivity (q_{MAb}), from $0.8 \text{ mg}/10^8 \text{ cells day}^{-1}$ in IT conditions as compared to $1.5 \text{ mg}/10^8 \text{ cells day}^{-1}$ in HT conditions, was concomitant with the 2-fold decrease in specific growth rate described above. However, due to the continuous production of a very large number of dead cells in the HT conditions, a significant contribution from the passive release of antibodies from dying cells may have led to an overestimation of q_{MAb} . In both IT and HT media, the MAb concentration reached a maximum of 80 to $85 \text{ } \mu\text{g mL}^{-1}$ at day 5 and no real gain was observed.

E1B-19K-expressing NS/0 cells exhibited very different growth kinetics in HT medium. Indeed, upon incubation with the osmolyte, the viable density of E1B-19K cells leveled off before decreasing gradually (Figure 1, right panels), but at a much lower rate than the control NS/0 culture. Thus, after 7 days in HT medium, 52% of the E1B-19K cells were still viable, as compared to only 7% in the control NS/0 culture. Furthermore, in contrast to control cells, a greater reduction in μ from IT to HT conditions was observed for E1B-19K-expressing cells. Indeed, a constant μ of 0.07 day^{-1} was measured for E1B-19K cells during the first 8 days of culture in HT medium, as compared to a μ of 0.40 day^{-1} (measured at day 2) for the same cells in IT medium. The cell cycle profile of E1B-19K cells in HT medium at day 5 was markedly different from the control NS/0 cells; only 31% of the population appeared as apoptotic sub-G₁ cells while the viable cell population (excluding sub-G₁ cells) was composed of 64% G₁ cells, 21% S phase cells and 15% G₂/M cells. Cells transfected with the E1B-19K gene product and exposed to HT medium were therefore able to accumulate in the G₁ phase without extensive apoptosis. Fluorescent microscopic evaluation of the culture revealed that non-viable E1B-19K cells nonetheless exhibited apoptotic morphology (data not shown), as confirmed by the presence of the sub-G₁ peak. The evolution of the MAb concentration of E1B-19K cells followed similar trends in both IT and HT conditions with a similar maximal MAb concentration of $28 \text{ } \mu\text{g mL}^{-1}$ at day 5. As for control NS/0 cells, an increase in q_{MAb} was observed for E1B-19K cells in HT conditions. However, the greater reduction in specific growth rate achieved under HT conditions with the E1B-19K cell (6-fold reduction for E1B-19K cells versus 2-fold reduction for control NS/0 cells) did not result in a greater increase in q_{MAb} , and was not translated into a greater antibody concentration. In fact,

although the E1B-19K cells retained a much higher viability in HT conditions throughout the culture, they stopped producing antibodies at day 5, at which point they were still 60% viable.

Effect of E1B-19K on viability and MAb productivity under DNA synthesis inhibition

The effect of E1B-19K on apoptosis protection and MAb productivity under conditions of DNA synthesis inhibition was tested using 10 mM HU or 10 mM XST. Immediately after exposing control NS/0 cells to HU, the viable cell density decreased rapidly and most cells died from apoptosis within 2 days (Figure 2, left panels). The negligible increase in the total cell number throughout the culture indicated that HU inhibited DNA replication and prevented cell division. As for control NS/0 cells, incubation of E1B-19K cells in HU resulted in total growth arrest (Figure 2, right panels). In this case, the decrease in viability was significantly slower than in the control NS/0 culture. Thus, after 4 days in HU, 65% of the E1B-19K cells were still viable, whereas the control NS/0 cells were all dead. E1B-19K cells therefore exhibited resistance to apoptosis induced by inhibition of DNA replication. No cell cycle profiles are presented in Figure 2 for HU conditions as all control NS/0 cells were dead when the analysis was performed on day 5. MAb concentrations measured at day 9 were as follows: 81.6 and 25.1 $\mu\text{g mL}^{-1}$ for control NS/0 cells in batch and HU conditions respectively; 21.1 and 5.6 $\mu\text{g mL}^{-1}$ for E1B-19K cells in batch and HU conditions respectively. Since MAb productivity in the presence of HU was considerably reduced for both cell lines, the use of this cell cycle modulator presented little advantage.

In an effort to find more physiological conditions under which long-term DNA synthesis inhibition would not result in inhibition of MAb synthesis, another agent, XST, was subsequently tested at three different concentrations: 3, 5 and 10 mM. Concentrations of 3 and 5 mM were not sufficient to inhibit DNA synthesis or prevent accumulation of viable cells (data not shown). Experiments were therefore performed in medium supplemented with 10 mM thymidine with medium replacements every 2 days to insure that thymidine remained in excess. As usual, a batch control culture was included in the experiment for both control NS/0 and E1B-19K cells. The viable density of control NS/0 cells exposed to XST conditions increased during the first 2 days, decreased from day 2 to day 5 where it reached 5×10^5 cells mL^{-1} and

only marginally increased afterwards (Figure 3, left panels). Immediately after exposing the cells to XST medium, a rapid decrease in viability could be observed associated with the accumulation of apoptotic sub- G_1 cells (Figure 3, bottom left panel). This decrease in viability lasted for 5 days where it reached 36% and leveled-off afterwards until the experiment was terminated. The small increase observed between day 8 and day 10 was probably artefactual and can be explained by the loss of a fraction of the dead cells upon medium changes every two days. The negligible increase in the total cell number throughout the culture indicated that XST inhibited DNA replication and prevented cell division. Indeed, in contrast to HT conditions (NaCl), the specific growth rate of control NS/0 cells in XST conditions was considerably reduced. A constant μ of 0.13 day^{-1} was measured for control NS/0 cells during the first 5 days of culture in XST medium, as compared to of 0.61 day^{-1} for exponentially growing control NS/0 cells. At day 5, the sub- G_1 cells made up 68% of the control NS/0 cell population in XST and the remaining viable cell population (excluding sub- G_1 cells) was composed of 2% G_1 , 42% S and 56% G_2/M phase cells (Figure 3, left bottom panel). The G_1 cell population that made 32% of total cells at day 0 had disappeared almost totally after 5 days of exposure to XST, concomitant with the increase in the sub- G_1 cells. The S phase cells also decreased from 55% at day 0 to 13% of the total population at day 5. Indeed, on day 5, only 32% of the total population was viable and 42% of these viable cells were composed of S phase cells, making up for a resulting percentage of 13%. The percentage of G_2/M cells present at day 0 (16%) remained stable at 18% on day 5. In control batch conditions, MAbs reached a maximum of 98 $\mu\text{g mL}^{-1}$ on day 5. In XST conditions, the cumulative evolution of the MAb concentration was computed by adding the MAb concentration found in the successive medium replacements performed at 48-hour intervals. The evolution of the cumulative MAb concentration followed the same trend as in standard medium until day 5. However, the S and G_2/M phase cells that remained viable after day 5 kept on secreting antibodies until day 10, accumulating up to 155 $\mu\text{g mL}^{-1}$. A 3-fold increase in q_{MAb} from 0.75 $\text{mg}/10^8$ cells day^{-1} in standard batch conditions to 2.25 $\text{mg}/10^8$ cells day^{-1} in XST conditions was concomitant with the 4-fold decrease in growth rate described above.

Myeloma cells expressing E1B-19K exhibited very different growth kinetics in XST medium. While incu-

Figure 2. Effect of E1B-19K on cell survival of NS/0 myelomas grown in the presence of the DNA synthesis inhibitor hydroxyurea. This figure presents the evolution of viable (X_v) and total (X_t) cell density and viability of control NS/0 (left panels) and E1B-19K cells (right panels) cultivated throughout a 9-day period in standard (batch) and 10 mM hydroxyurea (HU) medium.

bation of E1B-19K cells in XST medium also resulted in an arrest in growth rate (Figure 3, right panels), the viability remained between 80–90% throughout the culture. Thus, after 5 days in XST medium, the viability of the E1B-19K cells measured by dye exclusion test was 89%, as compared to 36% for the control NS/0 cells at day 5. Similarly as for the HT (NaCl) condition, the cell cycle profile of E1B-19K cells at day 5 was totally different from the control NS/0 cells (Figure 3, right bottom panel). Indeed, only 16% of the population appeared as sub- G_1 apoptotic cells and the cell cycle profile of viable cells (excluding sub- G_1 cells) was similar to what was found for exponentially growing cells with 32% G_1 , 51% S and 17% G_2/M phase cells. The cell cycle profile of E1B-19K cells seeded at day 0 in XST medium therefore remained unchanged for 5 days while G_1 cells and S phase cells were protected from apoptosis. Such an arrest in all phases of the cell cycle is consistent with what others have found following a single prolonged treatment with excess thymidine (Becker and Bartholomew, 1979; Cos et al., 1996).

The evolution of the cumulative MAb concentration of E1B-19K cells in the XST medium followed the same trend as for control NS/0 cells, with continuous production until day 10. A final concentration of

$35 \mu\text{g mL}^{-1}$ was found for XST medium as compared to $20 \mu\text{g mL}^{-1}$ for standard batch condition. Similarly as for control NS/0 cells, an increase in the q_{MAb} of E1B-19K cells was observed in XST conditions as compared to the control batch culture.

Effect of E1B-19kD on viability and MAb productivity in medium supplemented with the monoclonal antibody production enhancer OptiMAb™

As can be observed on Figure 4 (left panels), the viable density of control NS/0 cells exposed to OptiMAb™ slowly increased on the first 2 days in culture and then decreased steadily until day 8. Two days after exposing these cells to OptiMAb™, a rapid decrease in viability could be observed concomitant with the accumulation of sub- G_1 apoptotic cells (Figure 4, bottom left panel). Although viable cell density quickly declined, the growth of control NS/0 myeloma cells was not arrested. Indeed, as for cells exposed to HT (NaCl) conditions, the significant accumulation of total cells throughout the culture up to 20×10^5 cells mL^{-1} indicated that cells were still dividing. A μ of 0.24 day^{-1} was measured for control NS/0 cells exposed to OptiMAb™ at day 3 (at which point the viable cell number was steadily declining), as compared

Figure 3. Effect of E1B-19K on cell survival, MAb productivity and cell cycle distribution of NS/0 myelomas grown in the presence of excess thymidine (DNA synthesis inhibitor). This figure presents the evolution of viable (X_v) and total (X_t) cell density, viability (%), MAb concentration and specific MAb productivity (q_{MAb}) of control NS/0 (left panels) and E1B-19K-expressing cells (right panels) cultivated throughout a 10-day period in standard (batch) and excess thymidine (XST) medium. Bottom panels present cell cycle profiles of control NS/0 (left panel) and E1B-19K cells (right panel) after 5 days in XST medium. Each lower panel presents both log and linear (inset) scales of the same sample. Log scale profiles allow to distinguish the sub-G₁ apoptotic peak while linear scale insets present the cell cycle distribution into G₁, S and G₂/M phases of non-apoptotic cells only. The evolution of the MAb concentration in XST conditions is cumulative and has been computed by addition of the MAb concentration found in the successive medium replacements performed at 2-day intervals.

Figure 4. Effect of E1B-19K on cell survival, MAb productivity and cell cycle distribution of NS/0 myelomas grown in medium supplemented with the monoclonal antibody production enhancer OptiMAb^{TM}. This figure presents the evolution of viable (X_v) and total (X_t) cell density, viability (%), MAb concentration and specific MAb productivity (q_{MAb}) of control NS/0 (left panels) and E1B-19K-expressing cells (right panels) cultivated throughout a 11-day period in standard (batch) and OptiMAb^{TM}-supplemented medium. Bottom panels present cell cycle profiles of control NS/0 (left panel) and E1B-19K cells (right panel) after 5 days in OptiMAb^{TM}-supplemented medium. Each lower panel presents both log and linear (inset) scales of the same sample. Log scale profiles allow to distinguish the sub-G₁ apoptotic peak while linear scale insets present the cell cycle distribution into G₁, S and G₂/M phases of non-apoptotic cells only.

to a μ of 0.72 day^{-1} for exponentially growing control NS/0 cells in standard medium. At day 5, the sub- G_1 cells made up 80% of the control NS/0 population and the remaining viable cell population (excluding sub- G_1 cells) was almost entirely composed of G_2/M cells (87%) with some remaining S phase cells (13%). The G_1 phase cells present at inoculation totally disappeared concomitantly with the increase in the sub- G_1 cells. The S phase cells decreased from 52% at day 0 to 3% of the total population at day 5. Similarly as for XST conditions, the percentage of G_2/M cells present at day 0 (16%) remained stable at 17% on day 5. In OptiMab^{TM} conditions, MABs were produced continuously until day 5 when the viable cell number started to decrease drastically, as compared to normal batch culture where MAB accumulation was observed for 3 to 4 days. Antibody concentration was higher in the OptiMab^{TM} condition with an average final MAB concentration of $156 \mu\text{g mL}^{-1}$ as compared to a maximum of $87 \mu\text{g mL}^{-1}$ for the batch condition, a 1.8-fold increase in antibody concentration. Interestingly, a 3-fold increase in q_{MAB} from $1.22 \text{ mg}/10^8 \text{ cells day}^{-1}$ in batch to $3.52 \text{ mg}/10^8 \text{ cells day}^{-1}$ in OptiMab^{TM} was concomitant with the 3-fold decrease in μ described above.

Like for control NS/0 cells, incubation of E1B-19K cells in OptiMab^{TM} medium resulted in a much slower accumulation of viable cells (Figure 4, right panels). However, viability decreased at a lower rate than in the control NS/0 culture. Indeed, after 7 days in OptiMab^{TM} medium, 78% of the E1B-19K cells were still viable, as compared to only 2% in the control culture. As observed in HT and XST conditions, the cell cycle profile of E1B-19K cells in OptiMab^{TM} at day 5 was totally different from the control NS/0 cells. Thus, only 21% of the population appeared as sub- G_1 apoptotic cells whereas the viable cell population (excluding sub- G_1 cells) was composed of 76% G_1 , 12% S and 12% G_2/M phase cells. E1B-19K cells exposed to OptiMab^{TM} could therefore accumulate into G_1 presumably because they underwent apoptosis at a much reduced rate. As in all other conditions, the non-viable E1B-19K cells (sub- G_1) exhibited morphology typical of apoptosis (data not shown). A 4-fold increase in q_{MAB} was obtained through the use of OptiMab^{TM} for E1B-19K cells. MABs were produced continuously until day 8 when the viable cell number and viability started to decrease. At day 5, the gain in antibody concentration of E1B-19K cells (1.8-fold increase from to $33 \mu\text{g mL}^{-1}$ in batch to $60 \mu\text{g mL}^{-1}$ in OptiMab^{TM}) was similar to that of control

NS/0 cells (1.8-fold increase from to $87 \mu\text{g mL}^{-1}$ in batch to $156 \mu\text{g mL}^{-1}$ in OptiMab^{TM}). However, the extension of viability beyond day 5 resulting from expression of E1B-19K allowed to further extend the gain of productivity obtained. Thus, the final antibody concentration of E1B-19K cells at day 11 was even higher at $107 \mu\text{g mL}^{-1}$ as compared to $33 \mu\text{g mL}^{-1}$ for the batch condition, accounting for the final 3.3-fold increase in antibody concentration compared to only 1.8-fold for control NS/0 cells.

Discussion

The results presented above indicate clearly that antibody-producing E1B-19K myeloma cells are more resistant than control NS/0 cells against apoptosis induced by cell cycle modulating conditions such as incubation in HT medium (achieved with sorbitol or NaCl osmolytes), inhibition of DNA synthesis (achieved by supplementation with HU or XST), or use of the production enhancer OptiMab^{TM}. The dose-dependent apoptosis-protective effect of E1B-19K observed in the presence of some of these cell cycle modulators is consistent with the dose-effect observed previously in glutamine-deprived cultures (Mercille et al., submitted). While supplementation with sorbitol and HU were detrimental in terms of their effect on MAB secretion, the use of NaCl, XST and OptiMab^{TM} resulted in an increase of q_{MAB} as compared to the batch culture in both control NS/0 and E1B-19K-expressing cells. However, this increase in q_{MAB} was translated in significantly higher MAB yields only in the case of OptiMab^{TM}, and that, for both cell lines. Moreover, the inhibition of apoptosis conferred by the protective action of E1B-19K allowed to further extend the production period, and that resulted in an additional enhancement in productivity.

Cell cycle kinetics of control NS/0 cells cultivated in the presence of cell cycle modulators showed that non-resistant G_1 and S phase cells were much more susceptible than G_2/M cells to induction of apoptosis. After 5 days in the presence of NaCl and OptiMab^{TM}, all G_1 and most S phase cells had disappeared, while the G_2/M phase cells had either increased in number (in the case of NaCl) or remained stable (in the case of OptiMab^{TM}). These results, along with the progressive increase in the total cell density, indicate that under these conditions: (i) S phase cells retained the ability to progress into the G_2/M phase, showing that these agents were not inhibitory for DNA synthesis,

(ii) G₂/M cells could still enter mitosis and produce G₁ cells, and (iii) G₁ cells were unable to initiate another cycle and died by apoptosis. This confirms previous observations which showed that HT conditions arrest the cells and induce apoptosis in G₁ (Pellicciari et al., 1995; Pandey et al., 1996; Qin et al., 1997; Matthews and Feldman, 1996; Matthews et al., 1997; Anderson, 1997; Ortiz et al., 1997) and is in agreement with the 'grow or die' cell cycle model of De la Broise et al. (1991). In sharp contrast with the cell cycle profile of control NS/0 cells at day 5, a high percentage of apoptosis-resistant E1B-19K cells was found in the G₁ phase. Again, this is consistent with the notion that the apoptotic death of control NS/0 cells occurs preferentially in the G₁ phase of the cell cycle.

In the case of control NS/0 cells supplemented with XST, most of the G₁ cells disappeared, S phase cells decreased from 55% to 13% of the total population and G₂/M cells remained stable. Considering that the evolution of total cells was negligible in this case, these results demonstrate that upon addition of XST to non-protected cells: (i) most S phase cells died by apoptosis, (ii) G₂/M and a small fraction of S phase cells remained viable without undergoing apoptosis for more than 10 days if adequately fed; (iii) G₁ cells were unable to initiate another cycle and died by apoptosis. We can therefore conclude that, for all three conditions where cell cycle distribution was analyzed (66 mM NaCl, OptiMAb^{TM}, XST), the apoptosis susceptibility of non-protected lymphoid cells exposed to cell cycle modulators was cell cycle dependent; while G₁ and most S phase cells readily died by apoptosis, G₂/M phase cells could remain viable for more than 10 days. In contrast to cells arrested using cell cycle modulators, there was no selective survival of G₂/M cells in the control batch cultures. Thus, the cell cycle profile of the remaining viable control NS/0 cells at day 5 in a standard batch culture consisted of 55% G₁, 40% S and 5% G₂/M phase cells while for E1B-19K cells, the distribution was 51% G₁, 42% S and 7% G₂/M phase cells (data not shown).

Curiously, we observed that the increase in the total cell concentration of NaCl- and OptiMAb^{TM}-treated E1B-19K cells was slower than in the control NS/0 cells, in contrast to exponential batch cultures where both cell lines exhibited similar growth rates. This indicates that, in addition to protection against apoptosis, expression of E1B-19K altered the growth kinetics of NS/0 cells under cell cycle modulating conditions. This behavior of E1B-19K cells is consistent with what has been recently reported by O'Reilly et al.

(1996) who showed that E1B-19K, and its functional homologues Bcl-2 and Bcl-x_L, could exert a growth-inhibitory effect predominantly during transition from the quiescent G₀/G₁ state into the initial S phase, while having little or no effect once cells are continuously cycling.

As reported in other studies (Oh et al., 1993; Ozturk and Palsson, 1991; Reddy et al., 1992; Ryu and Lee, 1997), the increase in q_{MAb} observed in HT conditions did not result in an increase in antibody concentration. Indeed, despite the fact that E1B-19K cells retained a much higher viability in HT conditions throughout the culture, they stopped producing antibodies on day 5 in the culture, at which point they were still 60% viable. It has been reported that cells grown under stressful conditions can exhibit increased nutrient consumption rate (Oh et al., 1995). In such a case, nutrient exhaustion could have impaired the protein synthesis machinery by limiting the availability of some essential amino acids. While supplementation with nutrients throughout the culture could prevent nutrient limitation, increased accumulation of lactate and ammonia may in turn inhibit secretion. Increased antibody concentration may also be achieved through gradual adaptation of the cells to HT conditions (Oh et al. 1993, 1995) or by supplementation with osmoprotective agents such as glycine betaine (Øyaas et al., 1994a, b).

As mentioned above, E1B-19K cells exposed to OptiMAb^{TM} accumulated into the G₁ phase and exhibited a 4-fold increase in q_{MAb} . In a previous study we have shown that treatment of D5 hybridoma cells with OptiMAb^{TM} had also resulted in increased q_{MAb} and final MAb concentration, associated with prolongation of the G₁ phase (Mercille et al., 1994). Although we did not analyze the cell cycle distribution of control NS/0 cells in OptiMAb^{TM} conditions during the peak production period (day 2 to day 4), it is also likely that the increased q_{MAb} of control NS/0 cells (3-fold) was associated with enrichment in G₁ phase cells. This is in agreement with a number of studies reporting that synthesis and secretion of immunoglobulins are maximal in late G₁/early S phase (Byars and Kidson, 1970; Garatun-tjeldsto et al., 1976; Suzuki and Ollis, 1989; Al-Rubeai et al., 1990; Ramirez and Mutharasan, 1990; Richieri et al., 1991; Cazzador and Mariani, 1993). With the use of OptiMAb^{TM}, it was possible to establish that through an appropriate combination of cell cycle modulator and nutrient supplements, one can benefit from the use of an apoptosis-resistant cell line via an extension of

the productive period, thus leading to significant gains in antibody concentration. Is it also possible that the prolongation of the production period from day 5 to day 8 (Figure 4, right panels) achieved here through the use of OptiMAb™ could have been extended further with appropriate feeding strategies. Indeed, we have recently shown that, under slow growth conditions in perfusion culture, E1B-19K cells exposed to a constant supply of nutrients exhibited an increased productivity that could be maintained for an extended period of time (Mercille and Massie, submitted).

In the case of DNA synthesis inhibition with XST, although cumulative productivity was greater than in batch for both control NS/0 (155 vs 98 $\mu\text{g mL}^{-1}$) and E1B-19K cells (35 vs 20 $\mu\text{g mL}^{-1}$), it is difficult to compare XST and batch control conditions as medium replacements were only performed in the XST condition. However, a comparison of the q_{MAb} and cell cycle profiles of control NS/0 and E1B-19K cell lines in XST medium leads to interesting speculations. While G_1 and a fraction of S phase control NS/0 cells died from apoptosis within the first 5 days (Figure 3, left panels), nutrient-fed G_2/M and a fraction of S phase cells remained viable for up to 10 days, secreting MAbs with a q_{MAb} 3-fold higher than in standard batch culture. These observations are consistent with studies reporting that a peak in synthetic activity can not only be associated with the late G_1 /early S phase but also with the G_2 phase (Watanabe et al., 1973; Cherlet et al., 1995; Kronemaker and Srienc, 1991). These studies also point to the fact that both the rate of synthesis and secretion are at the lowest level in the S phase. This is also substantiated by our results since, for E1B-19K cells, the cell cycle profile of viable cells after 5 days in XST was similar to exponentially growing cells with more than 50% cells in S phase (Figure 3, right bottom panel), and the q_{MAb} from day 4 to day 8 was similar to what was found during the exponential phase of the batch culture. Thus, whereas G_2/M -enriched control NS/0 cells kept secreting MAbs in XST at a q_{MAb} 3-fold higher than in standard batch culture, S phase-enriched E1B-19K cells showed no increase in q_{MAb} . Since non-protected control NS/0 myeloma cells in G_2/M can remain viable under apoptosis-inducing conditions and exhibit such an elevated q_{MAb} , strategies could be developed to specifically block the cells in the G_2/M phase using cytostatic agents such as mitomycin C (Herrera et al., 1991) or gene products such as vpr from HIV (He et al., 1995). In order to avoid possible nutrient limitations, G_2/M cell cycle blocking agents and/or genes

could be used in conjunction with feeding strategies such as perfusion cultures (Fong et al., 1997; Mercille and Massie, submitted).

Conclusions

Although E1B-19K protected against apoptosis induced by all cell cycle modulators tested in this study, only in one condition did we see a significant improvement in antibody productivity. This could be explained by pleiotropic effects of these modulators which could not only induce apoptosis but also interfere with MAb synthesis through unknown mechanisms. Therefore, in order to exploit the extended viability conferred by anti-apoptotic genes, a careful optimization of all culture conditions (concentration of cell cycle modulators, medium formulation, etc.) has to be achieved. Alternatively, cell engineering targeting modulation of the cell cycle (Fussenegger et al., 1997) could alleviate the putative pleiotropic effects of chemical modulators. Work is in progress to explore this avenue.

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