Effects of hydroxyurea on monoclonal antibody production induced by anti-mIgG and LPS stimulation on murine B cell hybridomas

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Abstract Chemical treatment with hydroxyurea (HU) has been selected as a simple and low cost strategy to generate a cell population enriched for the G1 phase. After the chemical treatment with HU, cells were stimulated with anti-mIgG to test if the positive effects of anti-mIgG on CD40 expression and specific IgG2a production rate were improved upon a cell population with a higher percentage of cells in G1 phase at the beginning of the cell culture. In addition, other treatments assayed in this work were the cell stimulation with Lipopolysaccharide (LPS) both before and after the HU treatment. It has been observed that the use of HU under conditions able to maintain the cells in viable state (0.1 mM for 20 h), has a negative effect on CD40 expression and specific IgG2a production rate induced by anti-mIgG. The positive effect of LPS on cell stimulation induced by anti-mIgG is reduced on cells treated with HU.

Keywords Murine B cell hybridoma · CD40 · Hydroxyurea · Lipopolysaccharide · Anti-mIgG

Introduction

Numerous strategies have been proposed to increase the production of protein by animal cells through the cell growth regulation and its relationship with cell cycle control, since if it can be shown that a particular subpopulation of cells within a process is more productive than another, control strategies can be developed to favour the more productive fraction. These strategies have been divided by some authors (Carvalho et al. 2003) into four categories: (1) genetic, (2) environmental, (3) chemical, and (4) physical strategies. Although all these strategies may improve the protein production rate, addition of stimulatory agents is considered as a simple and low cost method, which could quickly impact on protein productivity. Chemical drugs as aphidicolin, mimose (Lalande 1990; Sukhorukov et al. 1994), rapamycin (Balcarcel and Stephanopoulos 2001), doxorubicin (Sukhorukov et al. 1994), staurosporine (Abe et al. 1991), hydroxyurea (Fallon and Cox 1979), dimethyl sulfoxide (Fiore et al. 2002; Ling et al. 2003), and butyrate (Kruh 1982; Ganne et al. 1991; Chen et al. 1993; Cherlet and Marc 2000; Mimura et al. 2001) are commonly used as substances able to generate a cell population that is enriched for a particular phase of the cell cycle, which can eventually increase the protein production.

Hydroxyurea (HU) is well known as an inhibitor of DNA synthesis but allowing continued RNA and protein synthesis (Yarbro et al. 1965; Adams et al.

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1966; Adams and Lindsay 1967; Cohen and Studzinski 1967; Young et al. 1967a, b). It is believed that its biological effects are based mainly on its inhibition of ribonucleoside diphosphate reductase (enzyme M2), the essential enzyme for the de novo synthesis of all four DNA purines and pyrimidines by converting ribonucleotide diphosphates to deoxyribonucleotide diphosphates (Lewis and Wright 1974; Yarbrow 1992). Accordingly, HU is a S-phase-specific cytotoxic and antineoplastic drug which disrupts the cell cycle at the G1 and S phases (Yarbrow 1992). It has been used in the treatment of an extensive variety of neoplastic and haematological malignancies (Donehower 1992; Kennedy 1992) and nowadays, it is utilized to reduce the painful attacks in sickle cell anaemia patients by inducing the synthesis of foetal haemoglobin (Cokic et al. 2003; Platt 2008) and decreasing sickle haemoglobin erythrocyte aggregation (Johnson and Telen 2008). HU has been used quite early as an effective in vitro synchronizing agent, producing rapid blockade and release of DNA synthesis. It is assumed, in the most of synchronization studies, that the cells are blocked at the G1/S boundary progressing more or less synchronously through the cell cycle following removal from HU block (Adams and Lindsay 1967; Gottifredi et al. 2001). However, other authors found that a portion of the cell population of mammalian cells entered in permanent S phase stasis after release from prolonged arrest with inhibitors of DNA such as HU or aphidicolin (Borel et al. 2002).

Cell cycle arrest, particularly in G1 phase, has been often used as a strong strategy to enhance productivity of biomolecules in commercially significant cell lines such as hybridomas and CHO by extending the main production phase. In spite of the mechanism by which the control of cell cycle regulates the protein secretion is still unclear, some authors think that there is a relationship between the protein production and the phase of the cell cycle, since those genes involved in ribosome synthesis and protein translation are expressed mainly in the G1 phase (Al-Rubeai and Emery 1990; Al-Rubeai et al. 1992). Other workers consider the G1 as the ideal phase of the cell cycle to increase the productivity of monoclonal antibodies (mAb) and recombinant proteins with rising biopharmaceutical relevance (Kromenaker and Srien 1991; Trummer et al. 2006).

CD40 is a key signalling molecule belonging to the tumour necrosis factor receptor superfamily

(TNF), expressed mainly by antigen-presenting cells of the immune system (Banchereau et al. 1994). The up-regulation of CD40 was incorporated into this assay since signalling through this molecule also up-regulates the expression of certain molecules such as immunoglobulins. Addition of anti-mouse surface immunoglobulin G antibody (anti-mIgG) to the culture medium has been shown to increase CD40 expression as well as specific mAb productivity on 55-6 hybridoma cells (Martín-López et al. 2007a, b, c). This effect was improved by increasing the proportion of cells in the G1 phase with thymidine synchronization (Martín-López et al. 2007a). In addition, there was a good correlation between the increase in specific mAb productivity and the increase in CD40 expression.

The purpose of this study was to test if the positive effects of anti-mIgG were improved upon a 55-6 hybridoma cell population with a higher percentage of cells in G1 phase at the beginning of the cell culture induced by the chemical treatment with HU. We have observed that the use of HU under conditions able to provide the highest percentage of cells in G1 phase without affecting viable cell density (0.1 mM for 20 h) does not enhance neither CD40 expression neither the specific IgG2a production rate induced by the anti-mIgG stimulation. The positive effect of LPS on cell stimulation induced by anti-mIgG was also reduced on cells treated with HU.

Materials and methods

Cell line and cell maintenance

The cell line used, a mouse-mouse B cell hybridoma, designated 55-6 (ATCC: CRL-2156), produces IgG2a monoclonal antibodies to human immunodeficiency virus (HIV) glycoprotein 120 (gp120). The cells were grown in static T-culture flasks in the medium previously described (Martín-López et al. 2007b).

Antibodies and reagents

Goat anti-mouse IgG (whole molecule) (Sigma–Aldrich, Inc.); anti-mCD40: FITC (Serotec). LPS from *Escherichia coli* 0111:B4 (LPS); hydroxyurea (HU); ribonuclease A (RNase A); propidium iodide (PI); phosphate-buffered saline (PBS); PBS/albumin

bovine serum (PBS/BSA); ethanol and trypan blue; all from Sigma–Aldrich, Inc.

Hydroxyurea treatment

To determine the most appropriate concentration of HU and incubation time, it was established a set of cultures treated with a wide range of concentrations of HU (0, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 5, 10 mM). For each culture cell cycle and viability were analyzed twice a day. The conditions selected for this paper were 0.1 mM of HU for 20 h. Cells from the exponential phase were adjusted to a concentration of 1.5×10^5 cells mL⁻¹ and transferred into medium containing 0.1 mM of HU. After 20 h HU was removed and the cells transferred into fresh medium before beginning activation assays.

Cell activation

Cells (5×10^4 cells mL⁻¹ untreated and treated previously with 0.1 mM of HU for 20 h) were incubated with anti-mIgG (230 µg mL⁻¹), HU (0.05 mM) and LPS (1.4 µg mL⁻¹) in different combinations summarizes in Table 1. Cell concentration and viability were measured using the trypan blue dye exclusion test. The experiments were carried out in triplicate.

Flow cytometry analysis

CD40 surface antigen was determined by direct immunofluorescence staining for flow cytometry. To assess the changes in cell-cycle distribution cellular DNA content were analyzed by PI staining and flow cytometry. The following protocols were previously described (Martín-López et al. 2007b). Data analysis employed software from Coulter Corporation (System IITM) and histograms were analysed with the algorithm developed by Watson et al. (1987).

Determination of antibody concentration

IgG2a concentration was measured by the sandwich-type ELISA using goat anti-mouse IgG-coated plates and goat anti-mouse IgG peroxidase conjugate as the second antibody.

To check the capture of secreted IgG2a by free anti-mIgG antibody added to the culture medium, a control solution of IgG2a was incubated with increasing concentrations of anti-mIgG antibody for 1 and 24 h. After incubation, IgG2a concentration was measured by the sandwich-type ELISA. The capture of secreted IgG2a by free anti-mIgG antibody added to the culture medium was determined as described previously (Martín-López et al. 2007a).

Table 1 Summary of the different treatments which cells were subjected to

Treatment number	Pre LPS ^a	Hydroxyurea block ^b	Anti-mIgG ^c	Hydroxyurea ^d	LPS ^e
T1	–	–	–	–	–
T2	–	–	+	–	–
T3	+	–	–	–	–
T4	+	–	+	–	–
T5	–	+	–	–	–
T6	–	+	+	–	–
T7	–	+	+	+	–
T8	–	+	+	+	+
T9	+	+	–	–	–
T10	+	+	+	–	–

^a Cell preincubation with 5 µg mL⁻¹ LPS for 48 h prior to HU block

^b Cells treated with 0.2 mM of HU for 20 h (HU block)

^c Cell stimulation with 230 µg mL⁻¹ of anti-mIgG after release from HU block

^d Cell stimulation with 0.05 mM of HU after release from HU block

^e Cell stimulation with 1.4 µg mL⁻¹ of LPS after release from HU block

Statistical analyses

Comparison of treatments using the nonparametric paired sign test (95% confidence level, $\alpha = 0.05$), multifactor anova analysis, and multiple linear regression analysis were performed in STATGRAPHICS Plus v4.1 (StatPoint, Herndon, VA, USA). Tests were conducted for measured mAb specific productivities and CD40 expressions.

Results

Cell growth

Since HU is an antiproliferative agent, which can be cytotoxic and induces apoptosis, depending on its concentration in the culture medium, it was essential to determine the effect of the concentration of HU and incubation time on cell growth and survival. In a preliminary study, 55-6 cells were incubated for various days with increasing concentrations of HU, from 0 to 10 mM in order to find out the lowest concentration of HU that provides the highest percentage of cells in G1 phase without affecting cell growth and viable cell density. This criterion was achieved in cells incubated with 0.1 mM of HU for 20 h (Table 2). These results are in agreement with those reported in a previous work by Cress and Gerner (1977), where cell survival in cells treated with 0.1 mM of HU for greater than 13 h was not

reduced. This concentration (0.1 mM of HU) was therefore selected to generate a cell population that was enriched for the G1 phase of the cell cycle. After 20 h, cells were washed and released from HU treatment, resuspended into fresh medium and treated with various agents as shown in Table 1.

Maximum specific growth rates ($\mu_{\max}$) versus different treatments are displayed in Fig. 1. $\mu_{\max}$ did not increase after stimulation with anti-mIgG or LPS compared to control culture. Nonetheless, LPS in combination with anti-mIgG enhanced the $\mu_{\max}$ over both control culture (untreated cells) and cultures with anti-mIgG or LPS alone. All the cultures treated with HU presented a similar growth rate. Therefore, these data demonstrated that cell treatment with 0.1 mM of HU during 20 h did not have an inhibitory effect on cell growth.

Cell cycle

Figure 2 shows the effect of HU and LPS preincubation on cell cycle phase distribution. As can be seen, cells treated with 0.1 mM of HU for 20 h (T5–T10) showed higher percentage of cells in G1 phase than cells without HU treatment (T1–T4). LPS preincubation before HU treatment (T9, T10) slightly decreased the percentage of G1 cells with respect to cells arrested with HU without LPS preincubation (T5–T8). A lower percentage of cells in G1 phase led to a higher percentage in both S and G2/M phase.

Table 2 Summary of the different treatments with HU which cells were subjected to determine the optimal HU concentration (results are for 20 h of incubation time)

HU concentration (mM)	Cell viability (%)	Cells in G1 phase (%)
0	92	30
0.05	93	33
0.1	92	66
0.25	68	62
0.5	58	64
1.0	55	66
2.0	52	68
3.0	41	71
5.0	34	70
10.0	25	69

Fig. 1 Exponential growth rate for all the treatments shown in Table 1. Values shown are the average $\pm$ SD of three independent cultures

Fig. 2 Effect of HU treatment and LPS preincubation on cell cycle phase distribution. Data for T1–T4 correspond to the inoculum culture used for experiments T1–T4 before any treatment, data for T5–T8 to the same inoculum culture after incubation with HU, and data for T9, T10 to the same inoculum culture after LPS preincubation and HU treatment. After 20 h of treatment, cells were washed and release from the HU block. The DNA contents were determined by flow cytometry after staining the cells with PI

CD40 expression

In Fig. 3a the expression of CD40 per cell, calculated as Mean Fluorescence Intensity (MFI), is presented. The expression of CD40 was insignificant in cells without anti-mIgG stimulation (T1, T3, T5, T9) in comparison with cells stimulated with anti-mIgG (T2, T4, T6, T7, T8, T10). These data clearly suggest that anti-mIgG was the main determinant of the increase in CD40 expression.

The significant decrease of CD40 expression after 21 h is consistent with the progressive decrease in free anti-mIgG initially added to the culture medium, as previously described in “Material and methods”. It was found that concentration of free anti-mIgG initially added to the culture medium decreased progressively over time due to the capture of anti-mIgG by secreted IgG and at about 70 h was exhausted (see Fig. 3a). This decrease in free anti-mIgG concentration could be therefore involved in the down-regulation of CD40 expression.

No significant influence of LPS alone (T3) is observed on CD40 expression. However, cells preincubated with LPS (T4) were more responsive to anti-mIgG stimulation. The effect of preincubation with LPS declined over 48 h of incubation with anti-mIgG.

Fig. 3 CD40 expression. **a** Expression of CD40 per cell (measured as Mean Fluorescence Intensity, MFI) for different treatments shown in Table 1. Dead cells were excluded by PI staining. **b** Maximum expression of CD40 relative to the control. *Black bars*: experiments without HU. *Grey bars*: experiments with HU. Data are representative of three independent experiments

Treatment of cells with HU decreases the level of CD40 expression induced by anti-mIgG stimulation (Fig. 3b). On the other hand, the effect that LPS exerts on anti-mIgG-induced stimulation seems to keep on cells treated previously with HU, since cells preincubated with LPS before the anti-mIgG stimulation (T10) showed higher CD40 expression than non-preincubated cells (T6). By contrast, the presence of LPS in the culture medium after the HU block (T8) decreased the CD40 expression with respect to cells without LPS (T6). However, the addition of a smaller dose of HU (0.05 mM) to the culture medium after HU block (T7) improved the CD40 expression in comparison with both treatments (T8, T6).

mAb productivity

Figure 4a shows the specific IgG2a production rate for the experiments summarized in Table 1. This figure shows that specific production rate on cells stimulated with LPS plus anti-mIgG (T4) was higher than in cells stimulated with LPS alone (T3) and anti-mIgG alone (T2). It can be seen that the treatment of cells with HU (T5) decreased by itself the IgG2a specific productivity almost 10% with respect to untreated cells (T1). However, cell stimulation with anti-mIgG (T6) and preincubation with LPS before the anti-mIgG stimulation (T10) clearly improved mAb specific productivity, suggesting that the

Fig. 4 IgG2a production. **a** Specific IgG2a production rate. **b** Maximum specific IgG2a productivity relative to the control. *Black bars*: experiments without HU. *Grey bars*: experiments with HU. Values are representative of three independent cultures

observed positive effect of anti-mIgG and LPS on IgG2a specific productivity and CD40 expression is also preserved on cells arrested with HU.

As seen above for CD40 expression, HU has a negative effect on IgG2a productivity. Cells treated with HU (T5) showed lower IgG2a productivity than control culture (T1) and cells treated with HU before the anti-mIgG stimulation (T6) showed lower IgG2a productivity than untreated cells (T2). With a second dose of HU (0.05 mM) after HU block (T7, T8) a further decrease on IgG2a productivity was observed. This effect was also observed in cells preincubated with LPS (T10) since the increase in IgG2a specific productivity with respect to control culture in cells without HU treatment (T4) was almost 70% and only 41% in cells with HU treatment (T10). The negative effect of HU is also evident in experiment T9, since when cells were preincubated with LPS without HU treatment (T3), the increase in IgG2a specific productivity with respect to the control (T1) was over 30%, and when HU is used after LPS preincubation (T9) a decrease of 22% relative to the control (T1) is observed.

Nevertheless, the observed increase in specific mAb productivity was consistent with that obtained in the expression of CD40 (Fig. 5a), in a similar way to that observed in a previous work for thymidine synchronization (Martín-López et al. 2007a). Both the expression of CD40 and specific productivity declined abruptly after achieving the maximum value, probably provoked both by the loss of synchronization and by the decrease in the concentration of anti-mIgG in the culture medium since, as previously shown, the free anti-mIgG initially added to the culture medium was bound to IgG2a as this was secreted by cells.

Since different treatments have been used in both this and the previous study, that affect CD40 expression and mAb production, a multifactor anova analysis was done. This analysis showed that there are not statistically significant differences between the previous results for thymidine synchronization and the results obtained in this study for HU synchronization. In order to model the relationship between CD40 expression and mAb productivity, a multiple linear regression analysis was done using seven categorical factors (preLPS treatment, thymidine synchronization, HU synchronization, anti-mIgG treatment, a second dose of thymidine, a

Fig. 5 CD40 expression and IgG2a productivity. **a** Maximum specific IgG2a productivity vs maximum expression of CD40 per cell. **b** Observed maximum specific IgG2a productivity against proposed model predictions. *Solid symbols* correspond to cultures with LPS, anti-mIgG and/or HU obtained in the present work. *Open symbols* correspond to data from Martín-López et al. (2007a). These data were obtained for cultures treated with LPS, anti-mIgG and thymidine in different combinations

second dose of HU, LPS treatment) and one quantitative factor (CD40). The results showed that some of the factors (HU synchronization, anti-mIgG treatment, a second dose of thymidine, a second dose of HU and LPS treatment) affected stronger CD40 expression (p value < 0.05), and the increase or decrease in CD40 expression led to increased or decreased mAB productivity; but others factors (preLPS treatment and thymidine synchronization) affected stronger mAB productivity (p value < 0.05), probably involving metabolic routes different from those of CD40. This shows a complex relationship between mAB productivity and CD40 expression that can not be represented by a simple linear regression as proposed previously (Martín-López et al. 2007a).

According with these results, the following multiple linear model may be proposed:

$$\text{Specific IgG2a productivity} = 509 - 102 \cdot x_1 + 114 \cdot x_2 + 10 \cdot \text{CD40}$$

where $x_1 = -1$ if preLPS treatment was done and $+1$ otherwise and $x_2 = -1$ if thymidine synchronization was done and $+1$ otherwise the term $10 \cdot \text{CD40}$ represents the effect of the rest of the treatments in specific IgG2a productivity through CD40 expression.

In Fig. 5b, the predictions of the model are shown. The model explains 82.09% ($r^2 = 0.8209$) of the variability in mAb productivity, being 73% of this variation due to CD40 expression (calculated from F -ratio values). Therefore, the variation in mAb productivity is mainly due to CD40 expression.

Discussion

The effects of LPS and anti-mIgG on B cells are well known, but very little is known about their effects on B cell hybridomas. Previous works in our laboratory have demonstrated that addition of LPS and anti-mIgG to B cell hybridoma cultures markedly increased both mAb production and relevant surface antigen expression such as CD40 (Martín-López et al. 2007a, c). These data showed that LPS and anti-mIgG, which are widely considered as activating-agent of B cells (Kearney and Lawton 1975; Klaus et al. 1984; Lenschow et al. 1991; Oliver et al. 1999), may trigger the same metabolic routes in hybridoma as in B cells to increase their proliferation and mAbs production. Moreover, this stimulatory effect of LPS and anti-mIgG was higher during G1 phase of the cell cycle (Martín-López et al. 2007b).

In this paper, HU has been used to increase the percentage of cells in phase G1 before the stimulation with these activating-substances (LPS and anti-mIgG). The selected HU concentration and time of incubation were those able to get the higher percentage of cells in G1 phase without affecting cell survival.

Reduction of hybridoma growth by controlled strategies has been positively associated by some researchers with the enhancement of mAbs production (Hayter et al. 1992). However, negatively-, or

non-growth associated mAbs production rates have been observed by others authors (Franěk et al. 1998). Overall, the common definition of mAbs production as growth-associated or non-growth associated seems to be correlated with production dependence upon S phase or G1 phase, respectively. Nevertheless, some authors have shown that an S-phase or G1-phase accumulation was not always accompanied by an improvement of product production; factors such as cell metabolism may be crucially important (Carvalho et al. 2003). In other cases, the time of the agent addition has been optimised to preserve the stimulation effect of this component on antibody production, minimizing its negative effect on cell growth (Franěk et al. 1998; Cherlet and Marc 2000). In this paper, we have preferred to maintain the cells in viable state after HU treatment since a positive correlation between the specific growth rate, CD40 expression and specific mAbs production rate has been shown previously for hybridoma cells stimulated with LPS and anti-mIgG (Martín-López et al. 2007a, b, c).

The high percentage of cells in G1 phase obtained with HU treatment for 20 h (Fig. 2) decreased rapidly after release from HU block, reaching the lowest value at about 20 h. Cells therefore resume its normal distribution of DNA content over time after the removal of HU. This decrease in % G1 phase was accompanied with an increase in % S phase just after release from HU block (data not shown). This short time for cell recovery after the block with HU seems to be a normal behaviour according to some authors, since although the treatment of cells with HU generally blocks cells at the G1/S boundary (Meyn et al. 1973), 1–4% of the total DNA can be replicated during the block (Meyn et al. 1973; Walters et al. 1976). Walters et al. (1976) have further suggested that cells traversing G1 in the presence of HU are able to enter S phase since DNA $<1 \times 10^7$ d made during the block can be chased into bulk DNA within 3 h after the removal of HU.

The effect of HU on antigen- and mitogen-stimulated lymphocyte responses has been studied in vitro by some investigators. Benito et al. (2007) have demonstrated that the effect of HU on the expression of different activation markers is not direct but seems to be mediated through its effect on cell proliferation. Nariuchi and Adler (1979) observed that when spleen cells were stimulated with LPS in the presence of HU, dissociation between

product production and the proliferative response could be shown. HU reduced the product production, but proliferation was, if anything, usually augmented. Similarly, in this paper HU does not exert negative effects on the survival of both non-stimulated and stimulated cells (Fig. 1); but CD40 expression and specific IgG2a production rate were reduced.

It is clear from Fig. 3 that only anti-mIgG is able to induce the expression of CD40. However, this expression is significantly lower in experiments with HU treatment (Martín-López et al. 2007a, b, c). Although the effects of HU on cell surface molecules is not clear, some authors have reported significant influence of HU on their expression. Benito et al. (2007) reported a reduction in CD25 and CD38 in T cells treated with HU. Styles et al. (1997) found that HU decreased the expression of both VLA-4 and CD36 in reticulocytes. This finding has been corroborated by other authors (Covas et al. 2004; Gambero et al. 2007). Most recently, Odièvre et al. (2008) have found an increase in surface molecules as CD47 and CD147 and a decrease in VLA-4 and CD36 in erythroid progenitors following HU treatment. These authors propose that another mechanism other than modulation of membrane molecules expression may mediate the effects of HU, and suggest that HU may affect signalling pathways leading to surface molecules activation rather than by reducing molecule expression.

As other membrane molecules, CD40 gene expression occurs through a range of processes that operate at three different levels of gene expression: transcriptional, post-transcriptional, and post-translational regulation. CD40 maximum transactivation can be decreased by the combination of several negative feedback pathways such as the inactivation or down-regulation of transcription factors or other transcription mediated molecules. In addition, post-transcriptional regulation is involved in the production of different CD40 isoforms by alternative splicing (Tone et al. 2001). By a mechanism of post-translational regulation, the protein products from these CD40 isoforms block signalling through the functional CD40 isoform (Type I). In this paper, the down-regulation of CD40 expression could be due to the HU negative effect at any of the three levels of CD40 gene expression. Nonetheless, the mechanism by which HU affects surface molecules expression is still not clear and further study is warranted.

With regard to the specific IgG2a production rate, HU exerts a similar effect. In this case, the observed positive effect of anti-mIgG and LPS upon IgG2a productivity was inhibited by HU, probably because of its negative effect on surface receptor for LPS and anti-mIgG. Although this effect has been observed by other workers for B cells (Shinohara and Kern 1976; Shinohara et al. 1976; Nariuchi and Adler 1979), the mechanism of the HU effect on the specific IgG2a production rate is still unclear.

It is important to note that, in spite of the negative effect that HU exerts on both CD40 expression and mAb production, the increase in specific mAb productivity was consistent with the increase in CD40 expression (Fig. 5a), similarly to that observed in a previous work concerning thymidine synchronization (Martín-López et al. 2007a).

CD40 is a cell surface receptor with a critical role in T cell-dependent humoral immune responses. CD40 activation of B cell in vitro has been demonstrated to have major effects on many steps of the B cell activation. Out of its biological effects on B lymphocytes, the activation of cell proliferation, cell survival and Ig production makes it an ideal target for monoclonal antibodies technology by using B lymphocyte hybridoma cells. CD40-mediated signals induce soluble factor synthesis such as cytokines which activate transcription factors that enhance the transcription of Ig genes and therefore Ig synthesis. Cytokines may also affect RNA processing to increase the amount of transcripts encoding the secretory form of Ig. CD40-mediated biological effects on B lymphocytes are regulated by signalling pathways that activate the transcription of an important number of genes. The main signalling pathways involved are mediated by NF- κ B, STAT3 transcription factors and the Mitogen-Activated Protein (MAP) kinase complex. Although the CD40 gene is constitutively expressed in most non-stimulated cell types in low levels, the expression of this protein can be up-regulated by mitogen factors such as LPS, anti surface immunoglobulins antibodies, phorbol esters and calcium ionophores; all of them are able to enhance NF- κ B transcription factor and MAP kinase complex which are involved on the regulation of CD40 expression. Therefore, activation of these transcription factors either by using mitogen factors or by triggering CD40 molecules may be a key factor to consider when dealing with mAb production process development.

While this study was focused on 5S-6 cell line, the CD40 up-regulation by chemical stimulation has been tested in our lab on hybridoma cells from transgenic mice secreting human mAb (unpublished). The preliminary results obtained with these cells suggest that these protocols might find applicability with other hybridoma cells not constitutively expressing CD40.

Conclusions

Hydroxyurea has been selected to generate a cell population that is enriched for G1 phase, under conditions able to maintain the cells in viable state. Under these conditions cells were subjected to different treatments with LPS and anti-mIgG that have previously been shown to increase the expression of membrane receptor CD40 and specific IgG2a productivity in this cell line. Viable cell concentration and growth rate were not significantly affected by HU. However, HU affected negatively the expression of membrane receptor CD40 and specific IgG2a productivity, which were reduced compared to similar cultures without HU treatment. In spite of this reduction, a multiple linear regression analysis showed that the variation in specific mAb productivity was mainly due to CD40 expression. Thus, membrane receptor CD40 may be an important factor to consider in mAb production process development.

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