Cytotechnology (2005) 48:15–26
DOI 10.1007/s10616-005-2926-9

Techniques for dual staining of DNA and intracellular immunoglobulins in murine hybridoma cells: applications to cell-cycle analysis of hyperosmotic cultures

Kathleen M. McNeeley[1,2], Zhe Sun[1,3] and Susan T. Sharfstein[1,4,*]
*[1]Department of Bioengineering, University of Toledo, Toledo, OH 43606, USA; [2]Present address: Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0535, USA; [3]Present address: Department of Medical Physiology, Texas A&M University System Health Science Center, College Station, TX 77843, USA; [4]Present address: Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Ricketts Building, 110 8th Street, Troy, NY 12180-3590, USA; *Author for correspondence (e-mail: sharfs@rpi.edu; phone: +1-518-276-2166; fax: +1-518-276-4285)*

Received 4 October 2004; accepted in revised form 20 August 2005

Key words: Cell cycle, Flow cytometry, Hybridoma, Hyperosmotic stress, Immunoglobulin, Monoclonal antibody

Abstract

Flow cytometry was used to evaluate the effects of hyperosmotic stress on cell-cycle distribution and cell-associated immunoglobulins for murine hybridoma cells grown in batch culture. Paraformaldehyde/methanol fixation substantially increased the fluorescence signal for intracellular immunoglobulins compared to ethanol fixation. For surface immunoglobulins, similar fluorescence signals were observed regardless of fixation method. Dual staining of immunoglobulins and cellular DNA was employed to determine immunoglobulin pool size as a function of cell-cycle phase. The intracellular immunoglobulin pool sizes increased as the cells progressed through the cell cycle for both control and hyperosmotic cultures. For control cultures, the immunoglobulin pool size increased during the exponential phase of culture, followed by a decrease as the cultures entered stationary phase. In contrast, hyperosmotic cultures showed an initial decrease in immunoglobulin pool size upon the application of osmotic shock, followed by an increase to a level above that of control cultures. This behavior was observed in all phases of the cell cycle. In addition, hyperosmotic cultures exhibited an increase in cell size when compared to control cultures. When normalized for cell size, the intracellular immunoglobulin concentration in hyperosmotic cultures was initially lower than in control cultures and subsequently increased to slightly above the level of control cells. Cells in all phases of the cell cycle behaved in a similar manner. There was no apparent relationship between the intracellular antibody concentration and the rate of antibody secretion.

Introduction

With the increasing demand for monoclonal antibodies for a variety of diagnostic and therapeutic applications, research has focused on strategies to improve monoclonal antibody production from murine hybridoma cells. Strategies to improve monoclonal antibody production can be grouped into two broad categories: (1) improving overall productivity by increasing culture density and

longevity, and (2) improving specific antibody productivity (i.e. antibody production per cell). Efforts to increase cell density and longevity have included various culture configurations such as fed-batch, hollow fiber, perfusion, and continuous modes. Enrichment of nutrient media has also been employed to improve cell density and longevity. More recently, several investigators have attempted to extend culture longevity by inhibiting apoptosis through genetic manipulation of the cells (Simpson et al. 1998; Simpson et al. 1999) or supplementation of culture medium with specific factors (e.g. interleukin-6) (Chung et al. 1997). Approaches to improve specific antibody production have included adding a variety of factors such as mouse peritoneal factors (Mikama et al. 1991), growth inhibitors (Suzuki and Ollis 1990), autocrine factors (Pendse and Bailey 1990), and cyclic mononucleotides (Dalili and Ollis 1988) into the culture medium. Application of environmental stresses such as alterations in temperature (Sureshkumar and Mutharasan 1991) and dissolved oxygen tension (Reuveney et al. 1986) have also been employed to improve specific productivity.

Several investigators have shown that the induction of hyperosmotic stress in culture increases specific antibody production. Ozturk and Palsson observed about a two-fold increase in specific antibody production after increasing the medium osmolarity from approximately 300 mOsm to 450 mOsm through the addition of sodium chloride (Ozturk and Palsson 1991). However, this increase in specific productivity was not accompanied by an increase in overall yield, since the viable cell number was decreased substantially. Osmotic stress has also been seen to slow the growth of a hybridoma culture (Ozturk and Palsson 1991) and to increase the relative cell size (Ozturk and Palsson 1991; Coco-Martin et al. 1992; Oh et al. 1996). These effects could be due to alterations in the cell-cycle distribution of the osmotically stressed hybridoma cells.

The mechanisms by which osmotic stress increases specific antibody production are still unclear. Oh and coworkers have demonstrated an increase in sodium-dependent amino acid uptake using non-metabolizable amino acid analogs (Oh et al. 1995). Both Oh and coworkers and Ozturk and Palsson observed an increase in overall metabolic rates. However, Ozturk and Palsson noted that, contrary to what would be expected, the osmotically stressed cells were substantially larger than the control cells. Consequently, some of the increase in metabolism may be explained by the increase in cell volume. Hence, the net increase in metabolic rate, on a per protein (or dry cell weight) basis is somewhat lower than observed on a per cell basis. Moreover, deZengotita et al. (2002) observed no increase in glucose metabolism at elevated osmolarity, indicating that metabolic changes are not necessarily related to the increase in antibody production.

Many investigators have explored the roles of growth (Dalili and Ollis 1990; Heath et al. 1990; McKinney et al. 1991) and, more specifically, cell-cycle kinetics (Al-Rubeai and Emery 1990; Hayter et al. 1992; Kromenaker and Srienc 1994; Park and Ryu 1994) on antibody production in an effort to improve antibody productivity. The results of these studies were quite variable. In some cases, antibody production was constant throughout a batch culture. In other cases, it increased during stationary and death phases, suggesting that lower cell growth rates improve antibody production. Cell-cycle studies by Hayter et al. (1992) found maximum production in G1 and G2 phases while Al-Rubeai and Emery (1990) found maximum production in G1 and S phases. These studies suggest that manipulation of the cell cycle may enhance the specific antibody production and that the cell-cycle alteration may play a role in the increased antibody productivity due to hyperosmotic stress.

We previously reported a temporary alteration in the cell-cycle distribution between unstressed and osmotically stressed cultures (Sun et al. 2004). This shift in cell-cycle distribution lasted from about 18 h to between 32 and 48 h after osmotic shock. We observed an increase in the fraction of cells in S phase in the osmotically stressed culture at 18, 24, and 32 h after application of osmotic stress with a corresponding decrease in the G0/G1 population. There was no significant change in the percentage of cells in G2/M phase. At 48 h after osmotic stress the difference disappeared, and the cell-cycle distributions between the control and stressed cultures were similar once again. As anticipated, the fraction of cells in G0/G1 increased for both samples as the culture progressed and the cells entered stationary phase. However, the increase in specific antibody synthesis was initiated before this time frame and continued after the cell-cycle distribution in the hyperosmotic

culture returned to that of the control population. This calls into question whether or not the change in cell-cycle distribution plays a role in the change in specific antibody production.

Several investigators have examined the relationships between either surface or intracellular immunoglobulins and specific productivity for both individual cells and the population as a whole. Sen et al. (1990) found a linear correlation between the mean surface fluorescence of the population as a whole and the specific productivity of the culture. Another study found a correlation between mean surface fluorescence and total cell-associated IgG as determined by ELISA; however, they were unable to find a relationship between surface fluorescence and specific productivity (Meilhoc et al. 1989). In contrast, when cells with a higher surface fluorescence were sorted from the population as a whole, they had a higher specific productivity than the entire culture or cells with a lower surface fluorescence (McKinney et al. 1991). However, there was no correlation between surface fluorescence and intracellular antibody as measured by fluorescent staining. A variety of different techniques were used for staining, permeabilization, and fixation which we hypothesize may have led to some of the discrepancies between the previous studies. Pollice et al. (1992) found that for dual staining of proteins and DNA, sequential paraformaldehyde and methanol fixation and permeabilization gave significantly better intracellular fluorescence for an anti-tubulin antibody than either paraformaldehyde or methanol alone or methanol followed by paraformaldehyde, presumably by preventing the leaching of cytoplasmic proteins during the staining steps. Based upon this, we report an optimization of dual staining protocols for surface and intracellular immunoglobulins and DNA and the application of these protocols to investigations of cell-cycle variation and its effects on antibody productivity in response to hyperosmotic stress.

Materials and methods

Cell line and cell culture

Hybridoma cell line 167.4G5.3 (a mouse: mouse hybridoma with plasmacytoma parent P3X63-Ag8.653, a generous gift from J.L. Clafin, University of Michigan) which produces an IgG_1 (κ light chain) directed against phosphorylcholine (Briles et al. 1984) was cultured in Iscove's Modified Dulbecco's Medium (IMDM, Sigma) supplemented with 5% fetal bovine serum (FBS, Hyclone). Cell cultures were incubated at 37 °C in a 7% CO_2 incubator in T-flasks (Marsh Biomedical).

Chemicals

Trypan blue solution (0.4% in normal saline), bovine serum albumin (BSA), and ribonuclease A (RNase A) were purchased from Sigma. FITC-conjugated rabbit anti-mouse IgG_1 (0.75 mg ml^{-1}, Zymed Laboratories) and propidium iodide (PI) (Calbiochem) were used to stain for IgG_1 and DNA, respectively. Paraformaldehyde was purchased from Fisher Chemicals.

Cell counting

A small amount of culture sample was removed and mixed with an equal volume of trypan blue solution. The stained cells were loaded on a hemacytometer (Bright-line) and observed under a phase contrast microscope (Olympus). Viable and dead cells (~200–500 cells/sample) were counted to determine cell density and viability.

Induction of osmotic shock

All cells were inoculated into control medium (~290 mOsm) at a density of 1×10^5 cells ml^{-1} into a T-150 flask. The cells were allowed to grow to midexponential phase (30–39 h after inoculation) before the culture was split into two T-75 flasks. Osmotic shock was induced in one of the flasks by adding sterile 5 M sodium chloride to 110 mOsm above control.

Flow cytometry and measurement of fluorescence

A Becton Dickinson Fluorescence Activated Cell Sorter (FACSCalibur) was used to analyze the samples. The FACSCalibur was equipped with an air-cooled argon-ion laser tuned to 488 nm and operated at 15 mW output. PI and FITC fluorescence were detected using a 585/42 bandpass and

a 530/30 bandpass filter, respectively. Data storage and analysis were performed with CELL Quest (Becton Dickinson) and ModFit LT (Verity Software House) software on a Macintosh G3 computer.

Dual staining of IgG and DNA

Several sets of experiments were performed in an attempt to determine the optimal dual-color staining procedure. Different wash and fixation techniques were compared in an effort to ensure preservation of intracellular IgG. Finally, experiments were conducted to determine the proper dilution of the FITC-conjugated rabbit anti-mouse IgG_1. In all cases, $1.0–1.5 \times 10^6$ cells were removed from culture, washed, fixed, and stained. In the case of surface staining, cells were stained before fixation (as described below). Chinese hamster ovary (CHO) cells that do not produce monoclonal antibodies were used as a negative control.

Ethanol fixation
Samples were centrifuged and resuspended in 500 μl IMDM medium; subsequently 500 μl FBS was added to the cell suspension. The cells were fixed by slow, dropwise addition of 3 ml ice-cold absolute ethanol while vortexing. Cell solutions were incubated for 30 min at 4 °C and then washed with PBS (8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 2.72 g l^{-1} $Na_2HPO_4 \cdot 7H_2O$, 0.24 g l^{-1} KH_2PO_4, pH 7.4).

Paraformaldehyde–methanol fixation
Cell suspensions were centrifuged, resuspended in 1.4 ml PBS, and fixed by the addition of 200 μl of 2% paraformaldehyde prepared in PBS to yield a final paraformaldehyde concentration of 0.25%. After incubating in the dark at 4 °C for 20 min, the cells were centrifuged, resuspended in 300 μl PBS, and permeabilized with 700 μl 70% methanol. Solutions were then incubated for 1 h at 4 °C and subsequently washed with PBS.

Staining procedure
Intracellular staining. Samples were washed with cold PBS or cold 1% BSA in PBS and then fixed either with ethanol or paraformaldehyde–methanol as described above. Washed samples were resuspended in 100 μl PBS and mixed with 20 μl FITC-conjugated rabbit anti-mouse IgG_1 solution. After incubating at 4 °C for 30 min in the dark, the cells were washed with PBS and then resuspended in 50 μl RNase A solution (5 mg ml^{-1}). DNA was stained by mixing the cells with 450 μl PI solution (50 μg ml^{-1}) and incubating at 4 °C in the dark for a minimum of 30 min but not more than 3 h until ready to be analyzed by flow cytometry.

Surface staining. Cells were washed with cold PBS or cold 1% BSA in PBS and resuspended to 100 μl with cold PBS. Extracellular IgG_1 was stained by mixing the samples with 20 μl FITC-conjugated rabbit anti-mouse IgG_1 solution and incubating at 4 °C for 30 min in the dark. After staining, the samples were washed with PBS and then fixed with ethanol or paraformaldehyde/methanol as outlined above. Washed cell pellets were resuspended in 50 μl RNase A solution (5 mg ml^{-1}), stained for DNA content with PI (as above), and analyzed using flow cytometry.

Determination of optimal FITC dilution
Experiments were conducted using varying dilutions of FITC-conjugated rabbit anti-mouse IgG_1 to ensure complete saturation of the intracellular IgG. Three samples of 1×10^6 cells were taken from the hybridoma culture approximately 40 h after inoculation. The cells were fixed according to the paraformaldehyde–methanol fixation procedure. Washed samples were resuspended in 100 μl PBS. The FITC-conjugated rabbit anti-mouse IgG_1 was mixed with PBS at the following dilutions: 1:1, 1:2, 1:5, before being added to the samples. Each sample received 20 μl of a respective dilution and was incubated at 4 °C for 30 min. The samples were then washed with PBS and mixed with 50 μl RNase A solution followed by 450 μl PI solution (50 μg ml^{-1}). The cells were incubated at 4 °C for 30 min before being analyzed by flow cytometry. A solution diluted 1–1.5 with PBS was determined to be most effective in ensuring saturation while conserving antibody.

Dual staining of hyperosmotic cultures

Osmotic shock was induced as described above. At selected time points (immediately before osmotic shock and 2, 4, 8, 24, 48 h after osmotic shock), $\sim 1 \times 10^6$ cells were removed from both osmotically stressed and control cultures. The cells were fixed

according to the paraformaldehyde–methanol fixation procedure. The fixed cells were stored at 4 °C in the dark in a solution of PBS and methanol until analysis (the staining and flow cytometry analysis was conducted within 72 h after fixation).

Prior to flow cytometric analysis, the fixed cells were centrifuged and washed once with PBS. Cells were then stained for intracellular IgG and cellular DNA as described above using a solution of FITC-conjugated rabbit anti-mouse IgG_1 diluted 1:1.5 with PBS followed by PI staining. Immediately after incubation, the cellular DNA contents and intracellular IgG were analyzed by flow cytometry.

Results and discussion

We had previously reported a change in the cell-cycle distribution in response to hyperosmotic stress in murine hybridoma cells (Sun et al. 2004). To determine whether this change in cell-cycle distribution played any role in the increase in specific antibody production, we evaluated the cellular antibody concentrations as a function of cell-cycle distribution.

Optimization of intracellular and surface IgG staining

Initial experiments focused on optimizing the intracellular and surface IgG staining to obtain an accurate reflection of the cellular immunoglobulin concentration.

1% BSA in PBS wash vs. cold PBS wash

Different wash techniques were compared because there was concern that some of the IgG may have been lost during the washing of the cells. It has been suggested that BSA prevents the loss of surface proteins by trapping them in the cellular membrane (Coco-Martin et al. 1992). By incorporating BSA into the PBS wash, the loss of protein may be prevented. A wash technique involving cold PBS with 1% BSA was developed and compared to the standard cold PBS wash to determine whether loss of protein was occurring and if it could be prevented.

In these experiments, both surface IgG and intracellular IgG were examined separately. The CHO cells were used as a negative control to ensure that proper separation of the background signal (due to nonspecific binding) and the actual data could be obtained through manipulation of the flow cytometer configuration. When we compared the two wash techniques (performed separately) for the intracellular and surface IgG stained cells, very little difference was observed between the two washes (compare Figure 1a, b). Therefore, it can be concluded that either no loss of IgG occurred or that the BSA wash was not effective in preventing the loss. From these results, it was determined that adding 1% BSA to the PBS wash was unnecessary.

Figure 1 does reveal a difference between the intracellular IgG concentration and the surface IgG concentration. McKinney and Belfort (1996) observed that the surface immunoglobulin is about 3% of the total cell-associated immunoglobulin in hybridoma cells. Hence, a larger fluorescent signal would be expected from the intracellular IgG. As the intracellular staining protocol should stain surface immunoglobulins as well as the intracellular immunoglobulins, at a minimum, we would expect to see the same signal intensity for the intracellular staining protocol as for the surface protocol. However, as seen in Figure 1, this is not the case. These plots indicate that either the surface IgG concentration is greater than the intracellular IgG concentration or the signal intensity is somehow being altered. A possible explanation is that intracellular IgG is being lost. Another possibility is that the intracellular signal intensity is being reduced due to quenching inside the cell. The fluorescence signal could also be altered by interference or amplification from the fixatives. In addition, the avidity of the antibody for the fixed immunoglobulins may be reduced in comparison with the avidity for the unfixed target. Additional experiments were conducted to determine the cause of this unexpected result.

Ethanol vs. paraformaldehyde–methanol fixation experiments

An alternate fixation technique was investigated in an effort to decrease the amount of intracellular IgG assumed to be lost during fixation. Pollice et al. (1992) compared paraformaldehyde and methanol to ethanol for fixing intracellular antibodies. Their results showed an increase in the immunofluorescence signal exhibited by cells fixed with paraformaldehyde followed by permeabilization with methanol. A technique similar to that used by

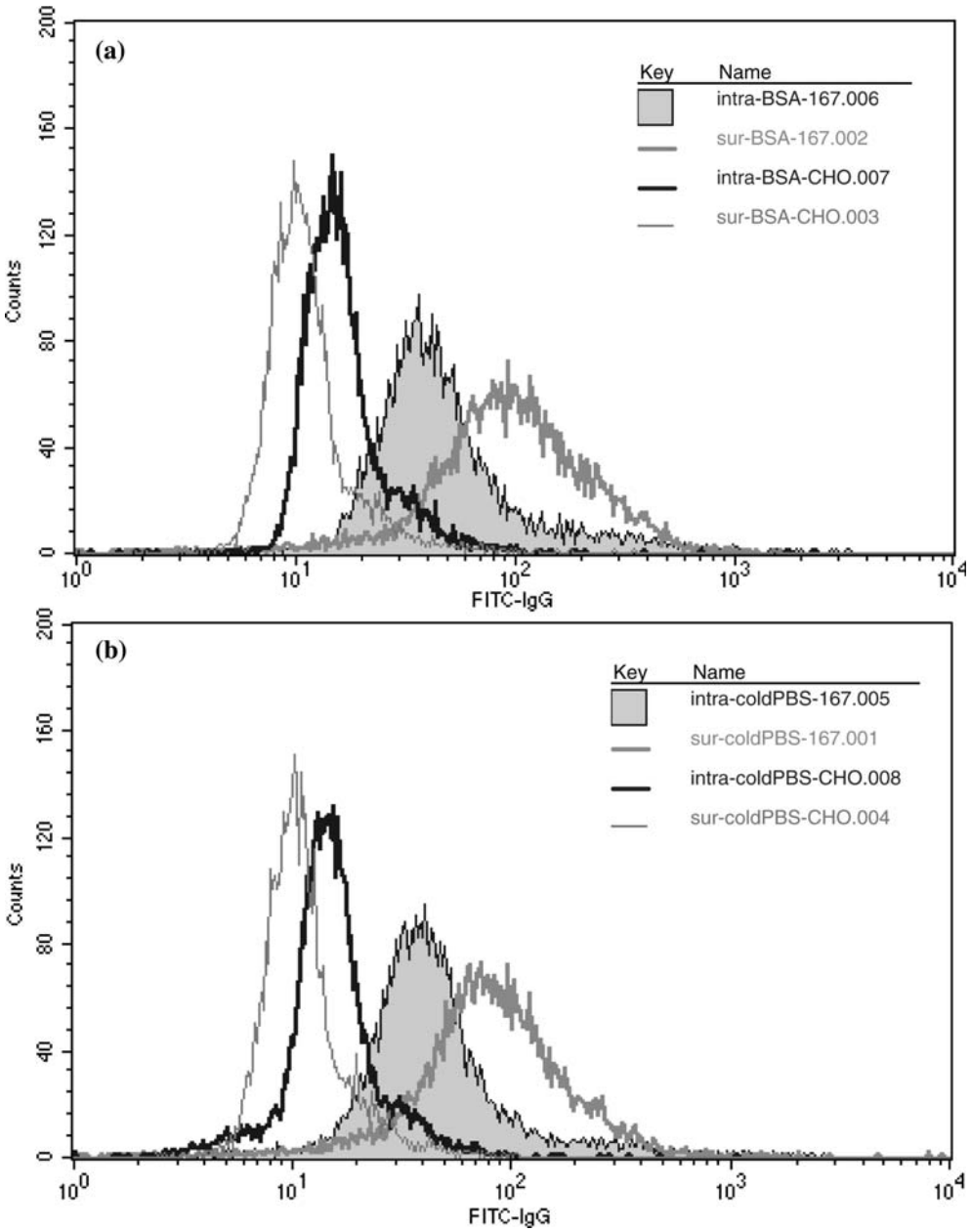

Figure 1. Comparison of surface and intracellular IgG for ethanol-fixed hybridoma (167) and control (CHO) cells stained with FITC-conjugated rabbit anti-mouse IgG washed with PBS + 1% BSA (a) or standard cold PBS (b).

Pollice et al. was developed and compared to the ethanol fixation procedure.

The results from these experiments are shown in Figures 2 and 3. The ethanol fixation samples show a higher IgG signal in the surface-stained samples than in the intracellularly-stained samples (Figure 2a). However, in the paraformaldehyde–methanol fixation trials, the intracellularly-stained samples exhibit a larger immunofluorescence signal (Figure 2b) as would be expected. A higher signal was expected from the intracellular IgG because the inner volume of the cell (where the intracellular IgG resides) is greater than the surface area occupied by the surface IgG. Figure 3a shows an increase in the intracellular immunofluorescence signal for the cells fixed with paraformaldehyde

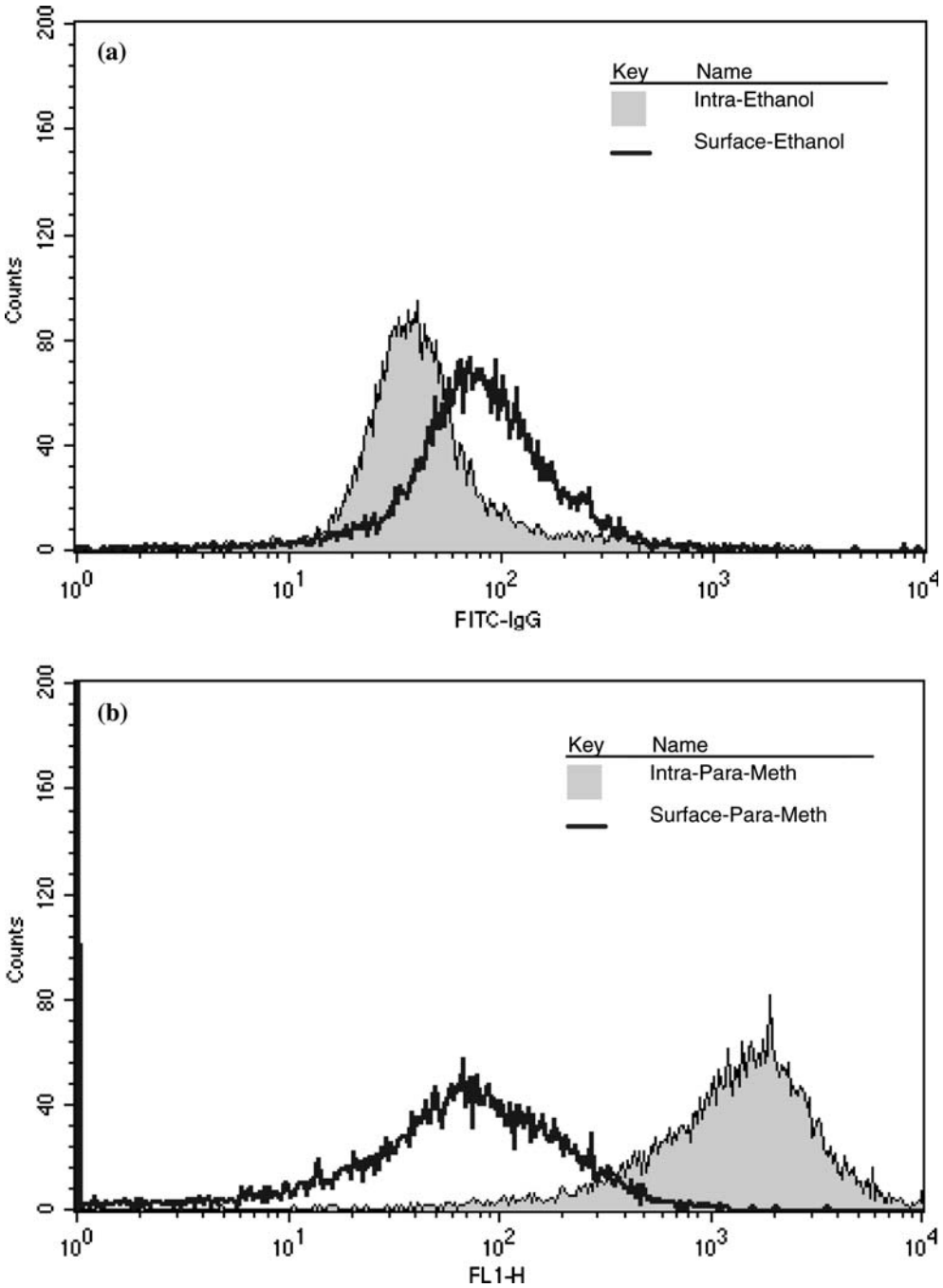

Figure 2. Comparison of intracellular and surface IgG for hybridoma cells fixed with ethanol (a) or methanol/paraformaldehyde (b).

and methanol when compared with ethanol fixation. The surface IgG signal, however, is similar whether the cells are fixed with ethanol or with paraformaldehyde–methanol (Figure 3b). The increase in the intracellular immunofluorescence exhibited by the cells fixed with paraformaldehyde–methanol suggests that there was a significant loss of intracellular IgG when the cells were fixed with ethanol alone. These comparative studies indicate that the paraformaldehyde–methanol fixation

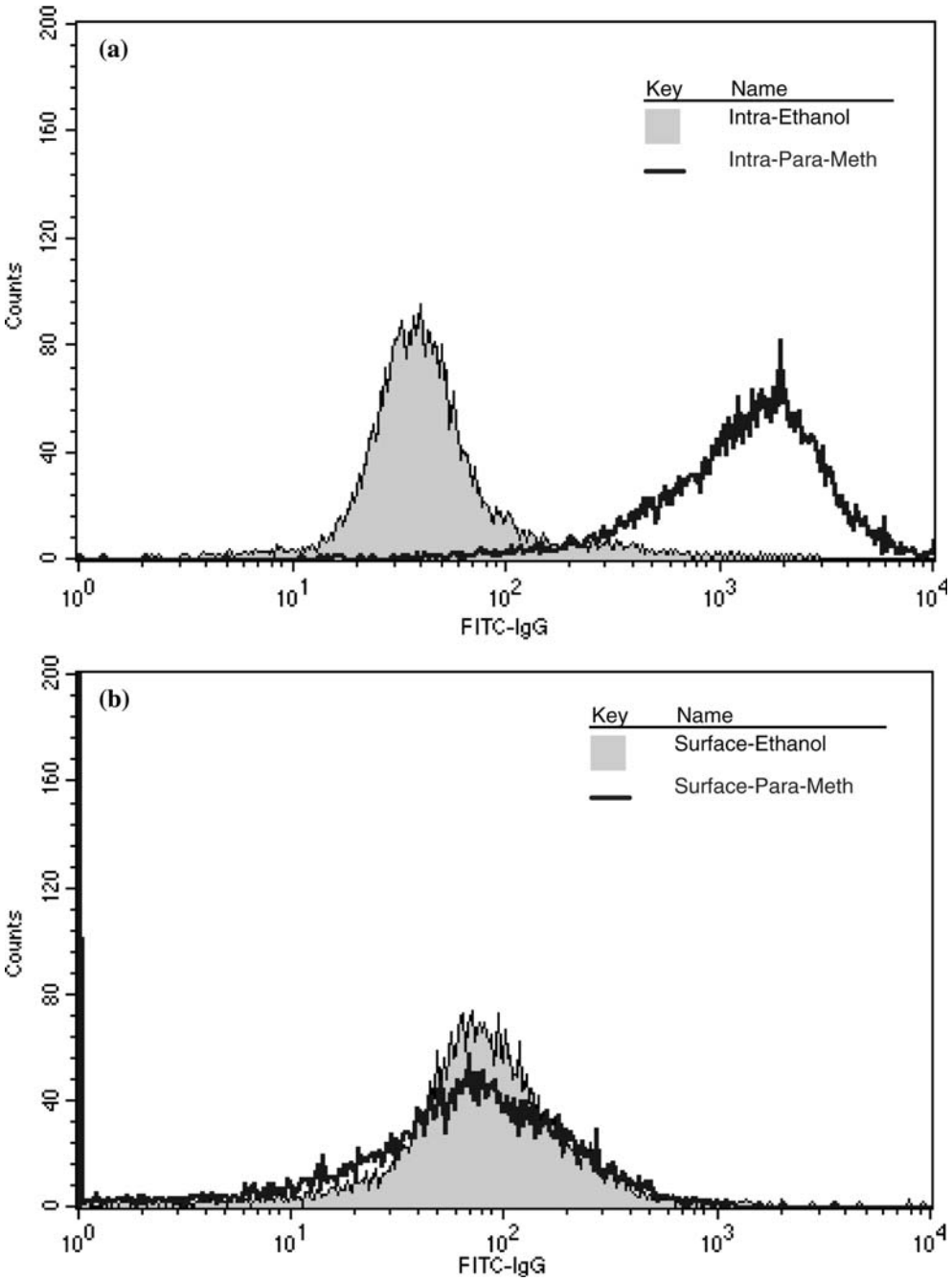

Figure 3. Comparison of ethanol and methanol/paraformaldehyde fixation for intracellular staining (a) or surface staining (b).

procedure provides better cell fixation conditions than the use of ethanol alone by preventing the loss of intracellular IgG. When we attempted to dual stain for cellular DNA and surface IgG, we found that regardless of the fixation procedure, the FITC signal was too small to be separated from the bleed-through of the PI signal on the FITC channel. Consequently, all dual-staining analysis was performed with intracellular immunoglobulins.

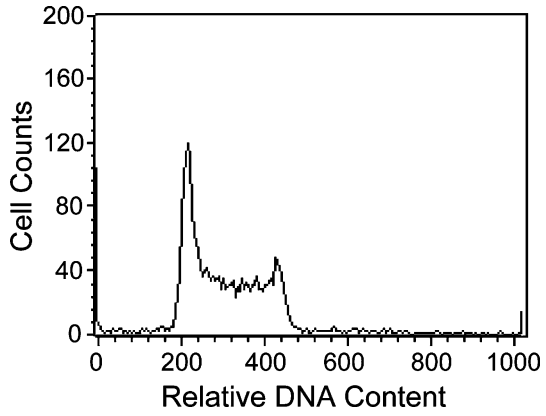

Figure 4. Typical histogram of DNA staining for cell cycle analysis. Histogram is for control cultures at 8 h after the application of osmotic stress. Cells were fixed with paraformaldehyde–methanol and dual stained for antibody and cellular DNA as described in Methods and Materials. Cell-cycle phases were identified using ModFit LT.

Changes in immunoglobulin concentration with cell-cycle phases

After development of an acceptable fixation and intracellular staining technique, quadruplicate experiments were conducted to examine the dependence of the intracellular IgG pool on the cell cycle and how changes in the cell-cycle distribution (due to osmotic shock) affect the overall IgG pool. Studies were conducted over a 48-hour period (after induction of osmotic shock) to observe the long-term effects resulting from exposure to hyperosmotic conditions.

Figure 4 shows a typical histogram of DNA content for a dual-stained population. Cell-cycle phases were assigned using ModFit as shown in Figure 5a, b. The intracellular IgG pool increases as the cells progress through the cell-cycle phases from G0/G1 to S to G2/M for both the control and hyperosmotic cultures. In the control cultures, the intracellular antibody increases during exponential phase and then drops as the cells enter stationary phase (Figure 5a). In contrast, the intracellular antibody decreases in the stressed cultures immediately after application of osmotic shock, possibly as a result of the increase in osmotic pressure (Figure 5b). The intracellular antibody then increases to ultimately reach a higher level than in the control cultures as seen in Figure 5c. The temporal behavior of the intracellular antibody is similar for all phases of the cell cycle.

We previously reported an increase in total protein in hyperosmotic cultures relative to control (Sun et al. 2004). This is substantiated by the larger cell size observed by measuring the mean forward scatter (FSC-Figure 6a). While the increase in cell size with hyperosmotic stress seems counterintuitive, it has been observed repeatedly by others (Ozturk and Palsson 1991; Oh et al. 1996). In particular, Ozturk and Palsson (1991), using the same cell line as our experiments, observed that cells placed in hyperosmotic media initially shrink as would be expected, but within 24 h in growth medium, attain a larger size, which is maintained for the entire culture. In addition, the cell size increases as the cell cycle progresses in both the hyperosmotic and control cultures (Figure 6b). To determine an approximate intracellular antibody concentration, the mean intracellular pool for each phase was normalized by the mean FSC for each phase. As seen in Figure 7a, the relative intracellular IgG initially drops after osmotic shock for approximately 10 h, then increases, becoming slightly larger in the hyperosmotic cultures sometime after 24 h. There is no substantial difference between the different cell-cycle phases, indicating that the change in expression of IgG is not specific for any cell-cycle phase. While the alteration in cell-cycle distribution cannot be conclusively ruled out as contributing to the increase in antibody productivity, there is no evidence to suggest that it does play a role.

A key question is whether the intracellular immunoglobulin profiles can be correlated with antibody secretion. As seen in Figures 2 and 3, ethanol fixation leads to substantial loss of intracellular immunoglobulin. This loss was dramatically reduced by the use of paraformaldehyde and methanol; hence by applying the paraformaldehyde/methanol fixation, we are more likely to observe a correlation between intracellular immunoglobulin and antibody secretion. However, that does not appear to be the case. Figure 7b shows a typical ratio for antibody secretion between hyperosmotic and control cultures. (These data were previously published in Sun et al. (2004); details of the experimental methods can be found in that publication.) The relative antibody secretion rate increases rapidly after osmotic shock and remains elevated well into stationary phase. While the relative secretion rate is largest on a per cell basis, when the rate is normalized for cell size or total protein,

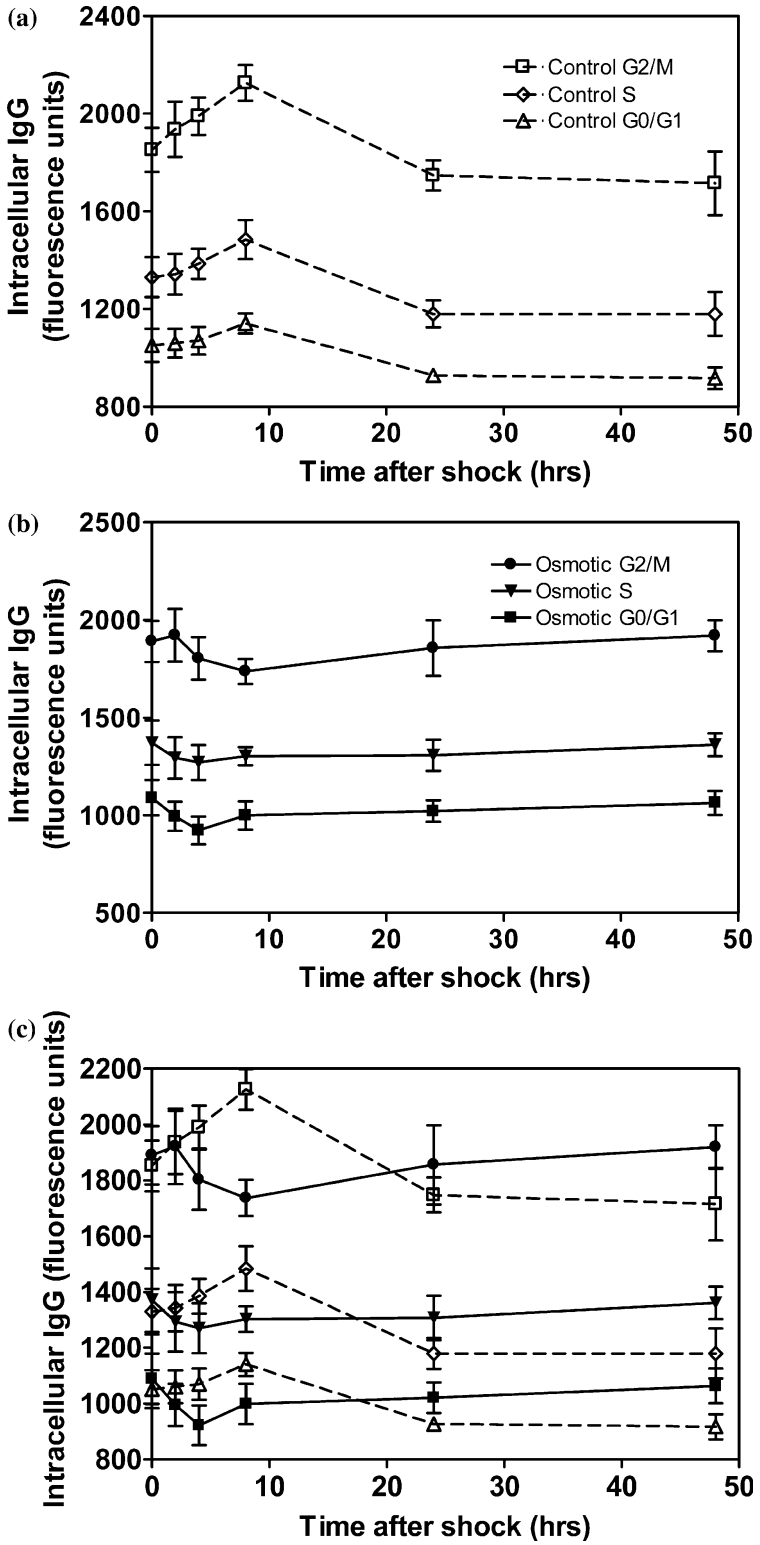

Figure 5. Mean intracellular IgG as a function of cell-cycle phase for control cultures (a), hyperosmotic cultures (b) or both (c). Error bars represent the standard error of the mean for 2–4 replicates per sample.

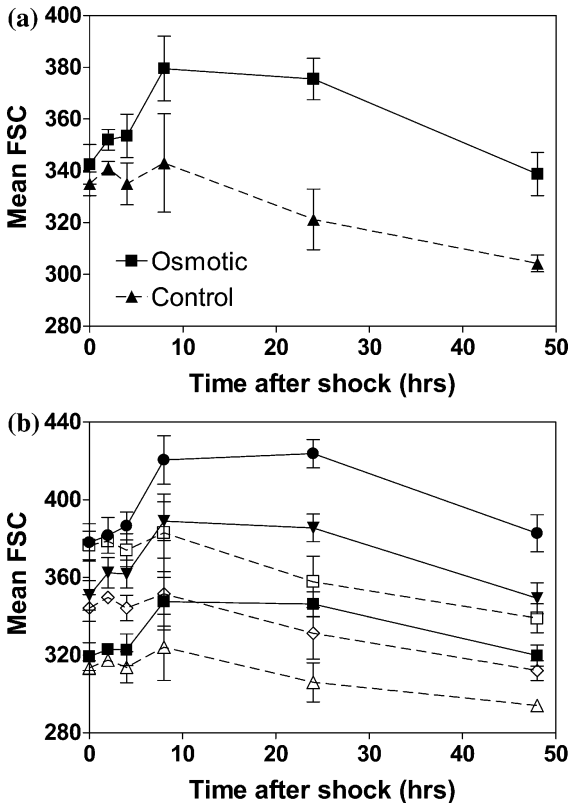

Figure 6. Mean forward scatter (FSC) for osmotic and control cultures. (a) Total population; (b) Different cell-cycle phases (symbols are given in Figure 5). Error bars represent the standard error of the mean for 2–4 replicates per sample.

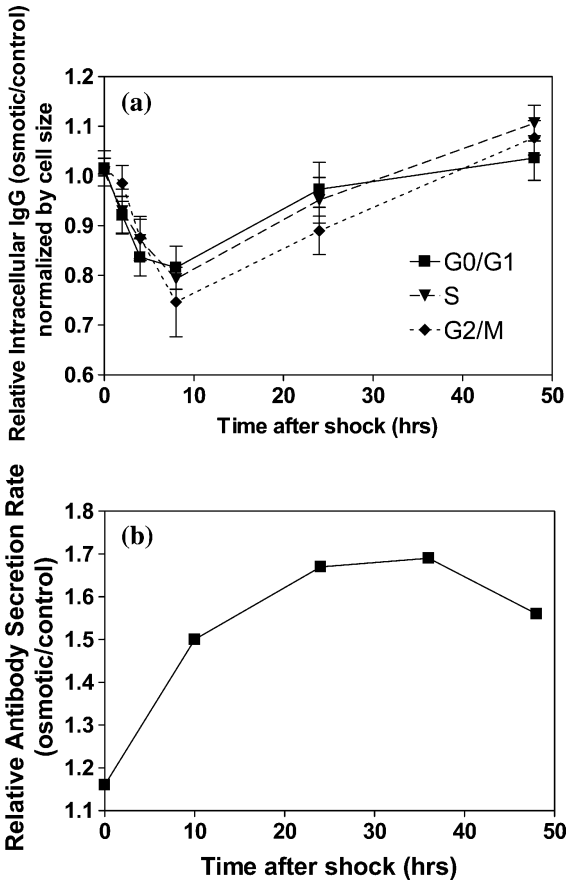

Figure 7. (a) Relative intracellular IgG (osmotic/control) as a function of cell-cycle phase. Error bars represent the standard error of the mean for 2–4 replicates per sample. (b) Relative antibody secretion rate for similar cultures.

the cells in hyperosmotic cultures still show an elevated secretion rate and the same time profile (data not shown). In contrast, the ratio of intracellular antibody concentrations drops and then increases, reaching a final value slightly larger than one (compare Figure 7a, b). This result should not detract from previous studies which found that sorting hybridoma cells for highly fluorescent populations selected for high producers; however, it suggests that use of intracellular antibody concentrations as a tool to assess productivity in response to extracellular stimuli may not be appropriate.

Conclusions

Through this study we have determined that paraformaldehyde/methanol fixation provides significantly better retention of intracellular immunoglobulins than ethanol fixation. Paraformaldehyde/methanol fixation was used for dual staining of intracellular immunoglobulins and DNA. Intracellular antibody increased as cells progressed through the cell cycle for both control and hyperosmotic cultures. Variations of the cell cycle with hyperosmotic stress do not appear to have any correlation with the increase in specific productivity in response to hyperosmotic stress. Most significantly, intracellular immunoglobulin concentrations do not appear to correlate with specific productivity in response to environmental stimuli.

Acknowledgements

This work was supported in part by a deArce Memorial Endowment Fund Grant from the University of Toledo and by the National Science

Foundation (BES-9984918, BES-9731073). KMM was supported by a University of Toledo graduate fellowship. We would like to thank Dr J.L. Clafin for supplying the hybridoma cell line used in this work and Dr Patty Relue for assisting with figure preparation.

References

Al-Rubeai M. and Emery A.N. 1990. Mechanisms and kinetics of monoclonal antibody synthesis and secretion in synchronous and asynchronous hybridoma cell cultures. J. Biotechnol. 16: 67–86.

Briles D.E., Forman C., Hudak S. and Claflin J.L. 1984. The effects of idiotype on the ability of IgG1 anti-phosphorylcholine antibodies to protect mice from fatal infection with *Streptococcus pneumoniae*. Eur. J. Immunol. 14(11): 1027–1030.

Chung J., Zabel C., Sinskey A. and Stephanopoulos G. 1997. Extension of Sp2/0 hybridoma cell viability through interleukin-6 supplementation. Biotechnol. Bioeng. 55(2): 439–446.

Coco-Martin J.M., Oberink J.W., van der Velden-de Groot T.A.M. and Beuvery E.C. 1992. The potential of flow cytometric analysis for the characterization of hybridoma cells in suspension cultures Cytotechnology 8: 65–74.

Dalili M. and Ollis D.F. 1988. The influence of cyclic nucleotides on hybridoma growth and monoclonal antibody production. Biotechnol. Lett. 10: 781–786.

Dalili M. and Ollis D.F. 1990. A flow-cytometric analysis of hybridoma growth and monoclonal antibody production. Biotechnol. Bioeng. 36: 64–73.

deZengotita V.M., Schmelzer A.E. and Miller W.M. 2002. Characterization of hybridoma cell responses to elevated pCO(2) and osmolality: intracellular pH, cell size, apoptosis, and metabolism. Biotechnol. Bioeng. 77(4): 369–380.

Hayter P.M., Kirkby N.F. and Spier R.E. 1992. Relationship between hybridoma growth and monoclonal antibody production. Enzyme Microb. Technol. 14: 454–461.

Heath C.A., Dilwith R. and Belfort G. 1990. Methods for increasing monoclonal antibody production in suspension and entrapped cell cultures: biochemical and flow cytometric analysis as a function of medium serum content. J. Biotechnol. 15: 71–90.

Kromenaker S.J. and Srienc F. 1994. Cell cycle kinetics of the accumulation of heavy and light chain immunoglobulin proteins in a mouse hybridoma cell line. Cytotechnology 14: 205–218.

McKinney K.L. and Belfort G. 1996. Use of flow cytometry for monitoring antibody productivity and isolating high-secreting hybridoma cells. In: Al-Rubeai M. and Emery A.N. (eds), Flow Cytometry Applications in Cell Culture. Marcel Deckker Inc., New York, pp. 17–37.

McKinney K.L., Dilwith R. and Belfort G. 1991. Manipulation of heterogeneous hybridoma cultures for overproduction of monoclonal antibodies. Biotechnol. Prog. 7: 445–454.

Meilhoc E., Wittrup K.D. and Bailey J.E. 1989. Application of flow cytometric measurement of surface IgG in kinetic analysis of monoclonal antibody synthesis and secretion by murine hybridoma cells. J. Immunol. Methods 121(2): 167–174.

Mikama T., Makishima F. and Suzuki E. 1991. Enhancing *in vitro* antibody production rate per cell by applying mouse peritoneal factors. Biotechnol. Lett. 13: 255–260.

Oh S.K.W., Chua F. and Al-Rubeai M. 1996. Flow cytometric studies of osmotically stressed and sodium butyrate treated hybridoma cells. In: Al-Rubeai M. and Emery A.N. (eds), Flow Cytometry Applications in Cell Culture. Marcel Dekker Inc., New York pp.101–119.

Oh S.K.W., Chua F.K.F. and Choo A.B.H. 1995. Intracellular responses of productive hybridomas subjected to high osmotic pressure. Biotechnol. Bioeng. 46: 525–535.

Ozturk S.S. and Palsson B.O. 1991. Effect of medium osmolarity on hybridoma growth, metabolism, and antibody production. Biotechnol. Bioeng. 37: 989–993.

Park S.H. and Ryu D.D. 1994. Cell cycle kinetics and monoclonal antibody productivity of hybridoma cells during perfusion culture. Biotechnol. Bioeng. 44: 361–367.

Pendse G.J. and Bailey J.E. 1990. Effects of growth factors on cell proliferation and monoclonal antibody production of batch hybridoma cultures. Biotechnol. Lett. 7: 487–492.

Pollice A.A., McCoy J.P.Jr., Shackney S.E., Smith C.A., Agarwal J., Burholt D.R., Janocko L.E.., Hornicek F.J., Singh S.G. and Hartsock R.J. 1992. Sequential paraformaldehyde and methanol fixation for simultaneous flow cytometric analysis of DNA, cell surface proteins, and intracellular proteins. Cytometry 13(4): 432–444.

Reuveney S., Velez D., Miller L. and Macmillan J.D. 1986. Factors affecting cell growth and monoclonal antibody production in stirred reactors. J. Immunol. Methods 86: 53–59.

Sen S., Hu W.S. and Srienc F. 1990. Flow cytometric study of hybridoma cell-culture – correlation between cell-surface fluorescence and IgG production-rate. Enzyme Microb. Technol. 12(8): 571–576.

Simpson N.H., Singh R.P., Emery A.N. and Al-Rubeai M. 1999. Bcl-2 over-expression reduces growth rate and prolongs G1 phase in continuous chemostat cultures of hybridoma cells. Biotechnol. Bioeng. 64(2): 174–186.

Simpson N.H., Singh R.P., Perani A., Goldenzon C. and Al-Rubeai M. 1998. In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the bcl-2 gene. Biotechnol. Bioeng 59(1): 90–98.

Sun Z., Zhou R., Liang S., McNeeley K.M. and Sharfstein S.T. 2004. Hyperosmotic stress in murine hybridoma cells: effects on antibody transcription, translation, posttranslational processing, and the cell cycle. Biotechnol. Prog. 20(2): 576–589.

Sureshkumar G.K. and Mutharasan R. 1991. The influence of temperature on a mouse–mouse hybridoma growth and monoclonal antibody production. Biotechnol. Bioeng. 37: 292–295.

Suzuki E. and Ollis D.F. 1990. Enhanced antibody production at slowed growth rates: experimental demonstration and simple structured model. Biotechnol. Prog. 6: 231–236.