BIOTECHNOLOGY TECHNIQUES
Volume 7 No.4 (April 1993) pp.271-276
Received 19th January

EFFECT OF FIXATION TEMPERATURE ON FLOW CYTOMETRIC MEASUREMENT OF INTRACELLULAR ANTIBODY CONTENT OF HYBRIDOMAS DURING BATCH CULTURE

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ABSTRACT

In order to investigate the effect of fixation temperature on flow cytometric measurement of intracellular antibody content of hybridoma cells, cells in different growth stages during a batch culture were fixed and stored at 4 and -20 °C, respectively. Flow cytometric analysis indicates that both fixation temperatures can be used in monitoring the changes in intracellular antibody content of the cells during a batch culture. However, it is better to fix and store the cells at -20 °C than 4 °C with regard to preservation of intracellular antibody and storage stability.

INTRODUCTION

The major advantage of the flow cytometric technique is that specific biochemical and immunological changes in each cell within a population are obtained, rather than an average value of these changes in an entire population as obtained by other techniques.

Flow cytometric techniques have been specifically applied for studying antibody production kinetics in hybridomas (Meilhoc *et al.*, 1989; Dalili and Ollis, 1990; Sen *et al.*, 1990; Al-Rubeai *et al.*, 1991; McKinney *et al.*, 1991) and population dynamics between antibody producer and non-producer populations (Lee and Palsson 1990; Ozturk and Palsson, 1990; Chuck and Palsson, 1992; Chuck *et al.*, 1992) or high and low producer populations (Altshuler *et al.*, 1986; Heath *et al.*, 1990). The changes in surface and intracellular antibody levels of hybridomas during the culture are quantified for this purpose. Quantification of antibodies is made by measuring the fluorescence intensity induced by immunofluorescent labelling of antibodies. Immunofluorescent labelling of surface antibodies does not necessarily require fixing the cells. However, in order to label the intracellular antibodies of hybridomas, the external membrane of the cells should be first permeablized (Shapiro, 1988). A fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin can then diffuse into the cells and bind to the intracellular antibody.

Among many different fixation protocols, ethanol and formaldehyde are widely used in flow cytometry (Shapiro, 1988; Watson, 1991). The fixation process can cause major problems in immunofluorescence staining with reduction or loss of antibody binding due to epitope modulation induced by the fixation (Levitt and King, 1987). Various fixative solutions were found to have different denaturing effects on cell surface antigen of mouse T-lymphocytes (Van Ewijk *et al.*, 1984). Fixation temperature may also influence the degree of denaturation of antibody. The cells are often fixed at either 4 °C (Clevenger et al., 1985; Levitt and King, 1987; Dalili and Ollis, 1990; Heath *et al.*, 1990) or -20 °C (Jacobberger *et al.*, 1986; Chuck and Palsson, 1992; Reddy *et al.*, 1992).

In this study, S3H5/γ2bA2 hybridomas were fixed at both 4 and -20 °C using 70%(v/v) ethanol, and the effect of fixation temperature on flow cytometric measurements of intracellular antibody content of the cells during a batch culture was monitored. In addition, we investigated the storage stability of intracellular antibody at these two fixation temperatures.

MATERIALS AND METHODS

Cell line, medium, and culture maintenance Two murine hybridoma cell lines were used in this study. One cell line was S3H5/γ2bA2 provided by Dr. Mark Kaminski from the University of the Michigan Medical Center. The antibody produced by this cell line is IgG_{2b} anti-idiotype antibody, directed against a carcinogen-induced B cell tumor (38C13) (Bergman and Haimovich, 1977). The fusion partner used was P3/x63/Ag8.653. The other cell line used as a negative control was S3H5/NP. This cell line was originally derived from S3H5/γ2bA2, and lost its antibody productivity (Lee and Palsson, 1990). The medium for culture maintenance was Iscove's modified Dulbecco's medium (IMDM, Sigma, St.Louis, MO) supplemented with 10%(v/v) fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Sigma). The cells were maintained in 25 cm^2 T-flasks (Bellco Glass, Inc., Vineland, NJ) in a 5% CO_2 /air mixture, humidified at 37 °C. The cells were diluted 1:5 with fresh medium every other day.

Cell cultures Exponentially growing cells were inoculated into three 75 cm^2 T-flasks containing 30 ml of IMDM supplemented with 10% (v/v) FBS. The initial cell concentration was ca. 10^5 cells/ml. Since the cell concentration in the early culture was not high enough, 10 ml of cell culture suspension was taken from the flasks to prepare flow cytometric samples. Experiments were repeated three times. All the experiments were performed in a humidified CO_2 incubator adjusted to 37 °C.

Quantification of intracellular antibody The intracellular antibody content of the cells was quantified using flow cytometry as follows. $1x10^6$ cells taken from the T-flasks were washed twice by centrifugation (5 min, 4 °C, 200*g*) with phosphate buffered saline (PBS) and centrifuged again. After discarding the supernatant, the cell pellet was resuspended in cold ethanol and stored at 4 °C and -20 °C, respectively. Cell samples accumulated during a batch culture were stained and analyzed at the same time and conditions in order to avoid day-to day variation in flow cytometer measurements. We did not see any significant decrease in antibody content during storage over a month regardless of fixation temperatures used (refer to Fig. 4(A)). Ethanol-fixed cells were washed twice by centrifugation with PBS. Cells were resuspended in 0.4 ml of a 1:30 dilution of fluorescein-isothiocyanate (FITC)-labeled goat antimouse IgG2b (heavy chain specific, Southern Biotechnology Associates, Birmingham, AL), and then incubated at 4 °C for 45 min. After centrifugation, the cells were washed and resuspended in PBS. Prior to flow cytometric analysis, cell suspensions were filtered through a 40-μm-nylon-mesh filter (Tetko Inc., Elmsford, NY). Green fluorescence was monitored in a Coulter EPICS 751 flow cytometer (Coulter Corporation, Hialeah, FL). The flow cytometer was calibrated with microspheres of four varying fluorescence intensities (EPICS intensity standard kit for calibration of fluorescence intensity, Coulter Corporation) prior to running samples each day. Gates were set on forward angle light scatter (FALS) and 90° light scatter plots to eliminate any fluorescence from cell debris. A logarithmic amplifier was used to accumulate green fluorescence data with a large dynamic range.

Analytical methods Cell growth was monitored by counting viable cells with a hemocytometer. Viable cells were distinguished from dead cells by trypan dye exclusion method. The cell culture suspension was

centrifuged. The supernatant was aliquoted and kept frozen at -80°C. The total secreted antibody, IgG_{2b}, was quantified using an enzyme linked immunosorbent assay (ELISA) as described previously (Lee *et al.*, 1989). The specific antibody production rate were evaluated using an integral method as described by Renard *et al.*, 1988.

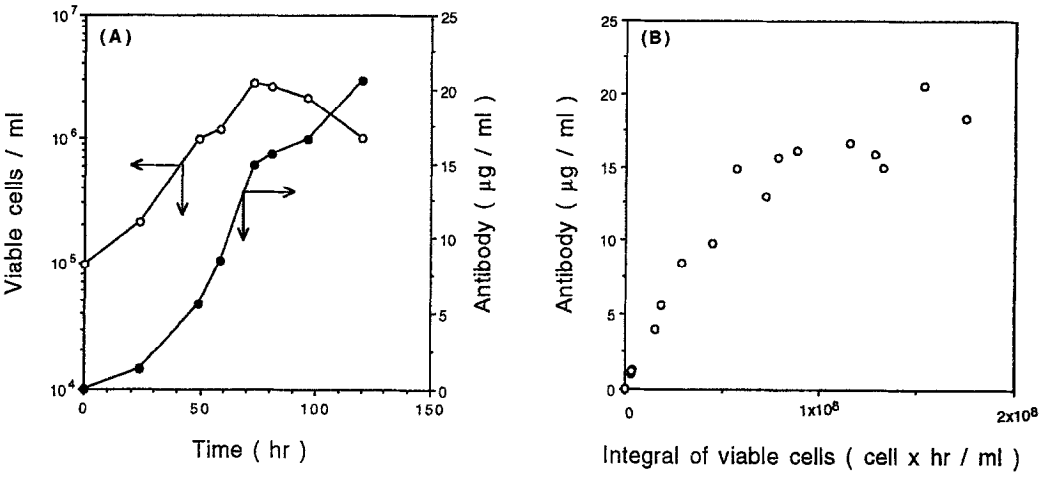

Figure 1. Batch cultivation of S3H5/γ2bA2 hybridoma (A) Growth and antibody production. (B) Antibody versus integral of viable cells with respect to cultivation time.

RESULTS AND DISCUSSION

In order to study the effect of fixation temperature on the flow cytometric measurement of intracellular antibody during batch culture, we first cultivated S3H5/γ2bA2 hybridoma in T-flasks. The cell growth and secreted antibody in the medium are shown in Fig.1(A). The cell viability remained at over 90% during exponential growth but significantly dropped during decline phase of growth. The cells in the decline phase continued to secrete antibody. In order to calculate specific antibody productivity, antibody concentration versus integral of viable cells with respect to culture time is plotted in Fig. 1(B) as described by Renard *et al.* (1988). Constant specific antibody productivity was not observed throughout the batch culture. The specific antibody productivity, which is calculated from the slope of curve in Fig. 1(B), was approximately 0.19 pg/cell/hr until the cell concentration reached a maximum and thereafter, significantly dropped to 0.04 pg/cell/hr. Although there are some exceptions (Ozturk and Palsson, 1990), many hybridoma cell lines during batch culture have been reported to produce MAb at slower rates after the time integral of viable cells reached $1x10^8$ cell/ml (Savinell *et al.*, 1989).

When the intracellular IgG_{2b} content is quantified using flow cytometry, FITC-conjugated goat antimouse IgG_{2b} is employed to give fluorescence by specifically labelling the heavy chain of IgG_{2b}. Thus, the fluorescence intensity indicates the content of intracellular antibody. However, FITC-conjugated goat antimouse IgG_{2b} also binds nonspecifically. This nonspecific binding of FITC-conjugated goat antimouse IgG_{2b}, which also causes fluorescence, necessitates the proper negative control to evaluate intracellular antibody accurately. Membrane composition and intracellular property of the cell may change during a batch culture resulting in variations of nonspecific binding of FITC-conjugated goat antimouse IgG_{2b}. In addition, fixation temperature may also influence the nonspecific binding. Thus, we cultivated S3H5/NP hybridoma to monitor the changes of nonspecific binding during batch culture. The growth of

S3H5/NP hybridoma, which was similar to that of S3H5/γ2bA2 hybridoma is shown in Fig. 2(A). No antibody was detected in a cell culture solution and S3H5/NP hybridoma did not have mRNA for heavy chain of IgG2b (Chuck *et al.*, 1992). The changes in intracellular antibody content of S3H5/NP hybridoma during a batch culture which were measured by flow cytometry are shown in Fig. 2(B). The cells sampled during a batch culture were fixed at both 4 and -20 °C. Fluorescence intensity of S3H5/NP hybridoma was almost constant during a batch culture regardless of fixation temperature used, indicating that both possible changes of the cells and fixation temperature do not influence the degree of nonspecific binding of FITC-conjugated goat antimouse IgG_{2b}. Accordingly, the changes in fluorescence of S3H5/γ2bA2 hybridoma directly reflect the changes in the intracellular antibody of the cells.

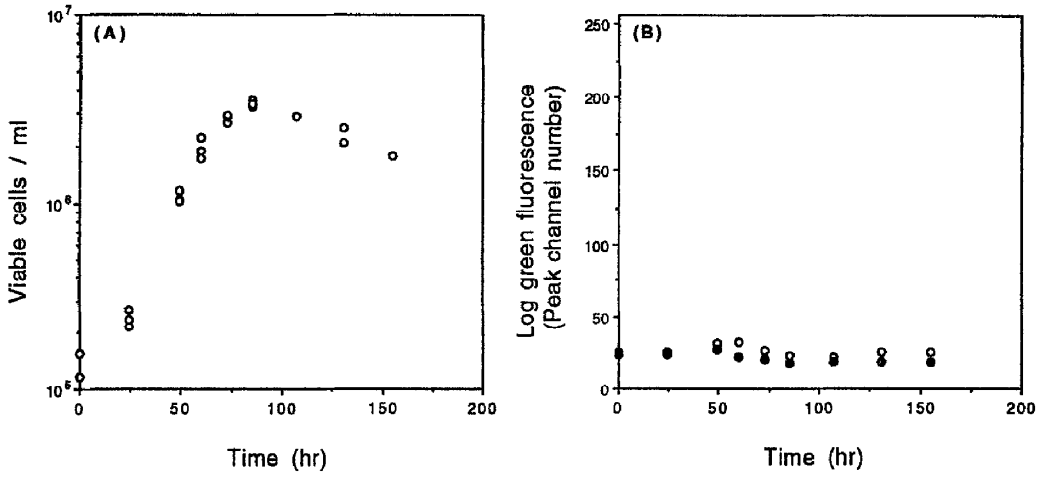

Figure 2. Batch cultivation of S3H5/NP hybridoma (A) Cell growth. (B) Fluorescence intensity of the cells fixed at 4°C (-o-) and -20°C (-●-), respectively.

The changes in intracellular antibody contents of S3H5/γ2bA2 hybridoma during the batch culture are shown in Fig. 3. The intracellular antibody contents of S3H5/γ2bA2 hybridoma was almost constant until the cell concentration reached the maximum, and thereafter, significantly dropped. Similar observations on the changes in intracellular antibody content during a batch culture were made by other investigators using different cell lines (Meilhoc *et al.*, 1989; Al-Rubeai *et al.*, 1991). Since the changes in specific antibody production rate of S3H5/γ2bA2 hybridoma during a batch culture are similar to those in the intracellular antibody content (refer to Fig. 1(B)), it appears that there is a good correlation between specific antibody production rate and intracellular antibody content in this case.

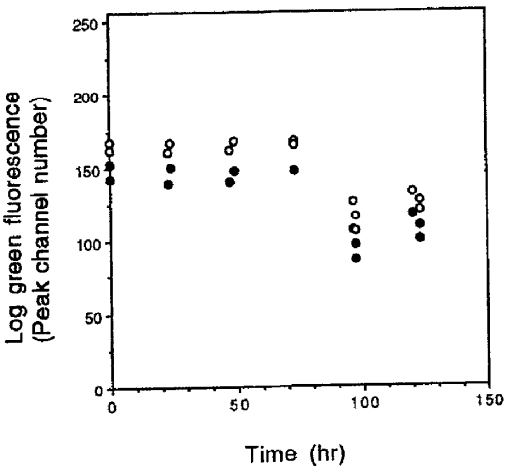

Figure 3. Changes in intracellular antibody contents of S3H5/γ2bA2 hybridoma during a batch culture shown in Fig. 1(A): -●- cells fixed at 4°C, -o- cells fixed at -20°C.

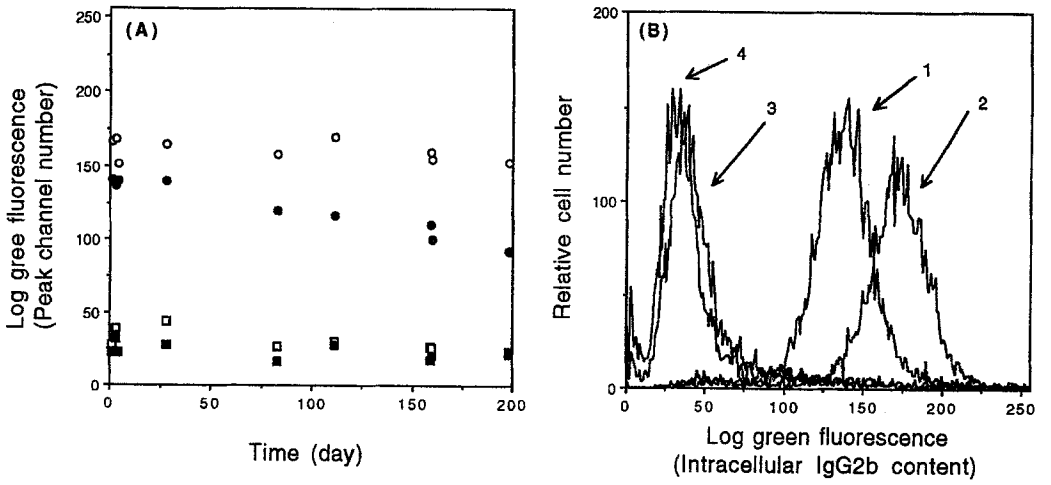

Figure 4. (A) Storage stability of intracellular antibody contents of the cells in the late exponential phase of growth at 4 and -20°C: -●- S3H5/γ2bA2 hybridoma fixed and stored at 4°C, -o- S3H5/γ2bA2 hybridoma fixed and stored at -20°C; -□- S3H5/NP hybridoma fixed and stored at 4°C, -■- S3H5/NP hybridoma fixed and stored at -20°C. (B) Histogram of the fixed cells after 3 day of storage: Histogram 1 and 2 represent S3H5/γ2bA2 hybridoma fixed at 4°C and -20°C, respectively. Histogram 3 and 4 represent S3H5/NP hybridoma fixed at 4°C and - 20°C, respectively.

However, since the intracellular antibody content also depends on net synthesis of antibody and dilution of intracellular antibody by cell growth (Meilhoc *et al.*, 1989), it does not always correlate the specific antibody productivity (Lee *et al.*, 1991). The fixation temperature did not influence the pattern of changes in intracellular antibody content of S3H5/γ2bA2 hybridoma, indicated by the fluorescence intensity, during a batch culture. However, it did influence the fluorescence intensity of S3H5/γ2bA2 hybridoma. The cells fixed at -20 °C showed higher fluorescence intensity than those fixed at 4 °C. Accordingly, in order to preserve intracellular antibody, it is desirable to fix the cells at -20 °C rather than 4 °C. During the fixation process, the intracellular antibody may denature faster and/or leak more out of the cells at 4 °C than at -20 °C.

In order to examine the storage stability of the intracellular antibody of the fixed cells, the cells fixed at 4 °C and -20 °C were stored at 4 °C and -20 °C, respectively. Fluorescence intensity , i.e. intracellular antibody content, vs storage time is plotted in Fig. 4(A). As shown in Fig. 4(A), fluorescence intensity of S3H5/NP hybridomas caused by nonspecific binding was constant during a storage period of over 6 months regardless of storage temperature. This result indicates that the changes in fluorescence of S3H5/γ2bA2 hybridoma directly reflect the changes in the intracellular antibody of the cells. The intracellular antibody content of S3H5/γ2bA2 hybridoma stored at 4 °C decreased slowly during a storage period while that of the cells stored at -20 °C did not decrease noticeably during a storage period of over 6 months. Accordingly, storage stability of intracellular antibody at -20 °C was better than that at 4 °C. Figure 4(B) shows typical histograms of S3H5/γ2bA2 and S3H5/NP hybridomas which were fixed at both 4 °C and -20 °C.

In conclusion, by monitoring the changes in intracellular antibody content of S3H5/γ2bA2 hybridoma during a batch culture using flow cytometry, it is more desirable to fix and store the cells at -20 °C than 4 °C with regard to preservation of intracellular antibody and storage stability.

Acknowledgments The authors thank Dr. Mark Kaminski for supplying the cell line. The authors also thank Mehran Shahabi for his help in flow cytometry. This work was supported by National Science Foundation (BSC-9009389) and KAIST.

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