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EFFECT OF MECHANICAL AGITATION ON HYBRIDOMA CELL GROWTH

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Abstract

Hybridoma cells (S3H5/γ2bA2) were grown in spinner flasks at different agitation speeds. It was found that cells in stationary and decline phases of growth were sensitive to shear force caused by agitation but cells in growth phase seemed less sensitive to the shear forces introduced. The death rate was found to be 0.007 hr^{-1} in T flasks but 0.018 hr^{-1} and 0.028 hr^{-1} at 100 and 200 rpm, respectively, while the growth rate was about 0.05 hr^{-1} for all cases.

INTRODUCTION

In vitro cultivation of hybridoma cells is a common means for the production of monoclonal antibody (MAb). It has been frequently noted that hybridoma cells secrete MAb throughout batch growth and a significant portion of MAb is produced during stationary and decline phases (Birch *et al.*, 1983; Velez *et al.*, 1986; Brennan *et al.*, 1988). This production does not seem to be due to the release of antibody from dying cells but due to active secretion from viable cells throughout the growth cycle (Renard *et al.*, 1988; Walker *et al.*, 1988). Usually hybridoma cells die rapidly after reaching maximum cell density and it is, therefore, important to maintain cell viability during the stationary and decline phases. Numerous studies have been carried out to achieve this goal, especially by adding nutrients and removing waste products (Luan *et al.*, 1987; Dodge *et al.*, 1987; Lindberg *et al.*, 1988). Though many studies deal with the effect of shear forces on animal cells, the importance of shear effect on cells in stationary and decline phases of growth has gone unnoticed (*e.g.*, de St. Groth, 1983; Dodge *et al.*, 1986). Here, we have investigated the effect of shear caused by agitation in spinner flasks on the maintenance of cell viability during stationary and decline phases. Cell growth in T flask was used as a reference. Furthermore, the MAb production, nutrient uptake and waste product formation were measured at the different agitation speeds.

MATERIALS AND METHODS

Cell line, medium and culture maintenance The murine hybridoma used was S3H5/γ2bA2. The antibody produced by this cell line is of the γ2b isotype, and the specificity of the antibody is against the anti-idiotype on the surface of the 38C13 lymphoma (Kaminski *et al.*, 1987).

The cell culture medium was RPMI 1640 (Sigma, St.Louis, MO) supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and 100 units/ml of each penicillin and of streptomycin (Sigma). The cells were maintained in T 25 cm^2 plastic cell cuture flasks (Bellco Glass, Inc., Vineland, NJ) at 37°C in a humidified CO_2 incubator. The cells were diluted 1:4 with fresh medium every other day.

Cell cultures Exponentially growing cells were inoculated into 25 cm^2 T flasks and spinner flasks (Bellco) at the initial density of 10^5 cells/ml. The total working volumes of T flasks and spinner flasks were 10 ml and 50 ml, respectively. Ten T flasks were inoculated and one flask was taken each time for assays. Two sets of experiments were performed using spinner flasks. For the first set, cell cultures were carried out at different agitation speeds (100 and 200 rpm). The second set of experiments were performed as follows: at first, cells were grown in 8 spinner flasks at 150 rpm. After 60 hr cultivation, the cell culture suspension was collected and mixed well. Then, 10 ml of cell suspension were added to each T flask and 50 ml of cell suspension were added to each spinner flask which was placed on the magnetic stirrer (Bellco) with different agitation speeds. All the experiments were performed in a humidified CO_2 incubator.

Analytical methods Cell growth was monitored by counting viable cells with a hemocytometer. The viable cell population was distinguished from dead cells by the trypan blue dye exclusion. The cell culture suspension was centrifuged and the supernatant was aliquoted and kept frozen at -80^oC. Glucose and lactate were measured enzymatically using Sigma diagnostic kits (No.315 and No.826-UV); ammonia was measured using ammonia electrode (Orion Research Inc., Model 95-12).

The antibody, γ2b, was quantified using an enzyme linked immunosorbent assay (ELISA). Anti-mouse IgG (Sigma) was used to coat wells of plates at a concentration of 10 μg/ml of PBS overnight at 4^oC and the wells were blocked with PBS-1%BSA for 1 hr. After these plates were washed 4 times, various dilutions of triplicate samples with proper concentration of standard (purified MAb, S3H5/γ2bA2) were added to plates. After 45 min incubation at room temperature, plates were washed and alkaline phosphotase-labelled affinity purified goat antibody against mouse IgG2b (Southern Biotechnology Associates,Inc., Birmingham, AL) was added to plates. After 45 min, the plates were washed and alkaline phosphotase substrate was added. The optical density at 410 nm of each well was recorded by ELISA reader (Molecular Devices, Palo Alto, CA).

RESULTS AND DISCUSSION

In many large scale suspension culture apparatus, mechanical agitation is necessary to ensure good heat and mass transfer. However, agitation should not be so vigorous that the fragile cell membranes are disrupted. Since it is important to maintain the cell viability for the production of MAb, the effect of mechanical agitation on cell growth and viability was investigated. Cell cultures in T flasks were used as a reference for shear-free conditions.

As shown in Fig.1(a) & (b), no significant differences in cell growth rate and cell viability in exponential growth phase were observed in all three cases tested. The viability in exponential growth was above 90% in all cases. However, the cell death rate and cell viability in decline phase of cell growth were very sensitve to agitation speed. Viable cell number and viabilty in case of 200 rpm were reduced much faster than in T flasks which means cells in decline phase are more shear sensitive than cells in exponential phase. The rapid decrease of cell viability at 200 rpm resulted in lower MAb concentration (140μg/ml) than in T flasks (250μg/ml). The decrease of viable cell number after reaching maximum density seems to follow first order kinetics with respect to viable cells (Fig.1(a)). The specific death rates were determined from the slope of the growth curve during decline phase, *e.g.* as in Fig.1(a). Specific growth rates in case of 100 rpm and 200 rpm , 0.051 and 0.049 hr^{-1}, respectively, were comparable with that in T flasks, 0.052 hr^{-1}. However, specific death rate in 200 rpm (0.028 hr^{-1}) was much higher than that (0.007 hr^{-1}) in T flasks and at 100 rpm (0.018 hr^{-1}). The specific death rates and growth rates for the various conditions are summarized in Table 1.

rpm	*specific growth rate, hr^{-1}*	*specific death rate, hr^{-1}*
0 (T flask)	0.052	0.007
100	0.051	0.018
200	0.049	0.028

Table 1: Specific growth rate and specific death rate at different agitation speeds.

As shown in Fig.1(a) & (c), hybridoma cells (S3H5/γ2bA2) produce a significant amount of MAb during the decline phase. To confirm that MAb accumulation during the decline phase was due to active secretion of MAb from viable cells , MAb concentration versus the integral of viable cell count with respect to time was plotted (Luan *et al.*, 1987; Renard *et al.*, 1988). As can be seen in Fig.1(d), the relation was almost linear except in the region of over $1{\cdot}10^8$ cells·hr/ml in T flasks where essential precursors for synthesizing MAb might be depleted. This supports the hypothesis that the viable cells in the decline phase of growth produce MAb at a constant rate as long as essential precursors for synthesizing MAb are present in the medium. The specific MAb production rate was about 1.6 pg/cell·hr, as evaluated from the slope in Fig.1(d).

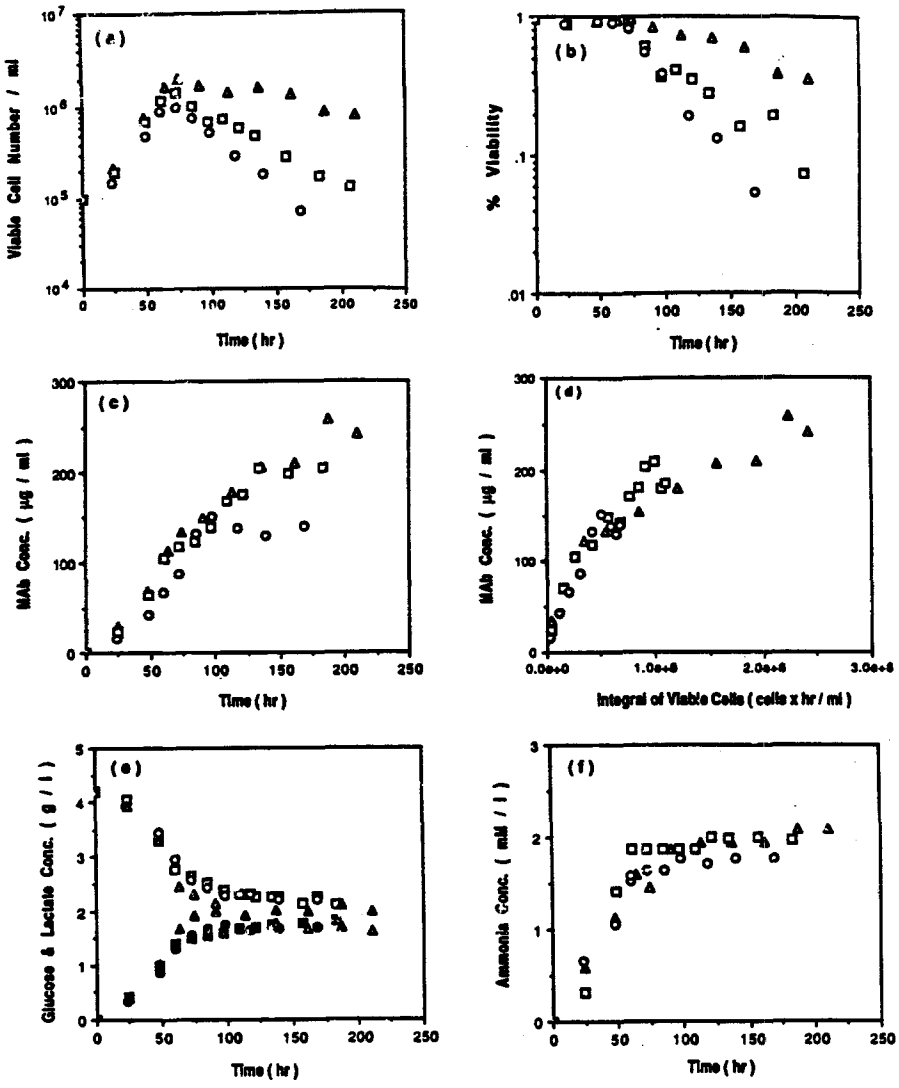

Figure 1: Cell growth in T flasks and spinner flasks at different agitation speeds : (a) cell growth curves; -▲- flask, -□- 100 rpm, -○- 200 rpm, (b) cell viability, (c) antibody concentration, (d) correlation between the integral of viable cells and MAb production, (e) open symbol, glucose concentration, closed symbol, lactate concentration and (f) ammonia concentration.

Since lactate and ammonia are believed to influence cell viability, we measured both lactate and ammonia concentrations (Lindberg *et al.*, 1988). As shown in Fig.1(e) & (f), lactate and ammonia concentrations were similar for the three cases and even slightly higher in T flasks. This observation indicates that the rapid decrease of viable cell number during agitation at 200 rpm was not due to metabolic differences or the buildup of waste, but rather due to the differences in shear force. The final lactate and ammonia concentrations in T flasks were 2.0 g/l and 2 mM/l, respectively, and the ratios of lactate produced to glucose consumed were in the range of 0.77 and 0.84 g/g in three cases.

We excluded the hypothesis that the viability of cells during the decline phase could be influenced by the agitation speed during exponential growth. After cultivating cells at 150 rpm for about 60 hrs, cells were cultivated at 100 rpm and in T flasks. As shown in Fig.2(a), the cell death rate could be lowered by reducing the agitation speed. When cells were transferred to T flasks and cultivated, the cell death rate was 0.007 hr^{-1} which was the same cell death rate as when cells were cultivated in T flasks from the beginning (Table 1). MAb concentration was also increased by prolonging cell viabilty. When agitation rates were lowered from 150 rpm to static in T flasks and 100 rpm, MAb concentrations were increased from 160 to 190 and 220 μg/ml, respectively. There were no significant differences in lactate and ammonia concentrations in three cases tested.

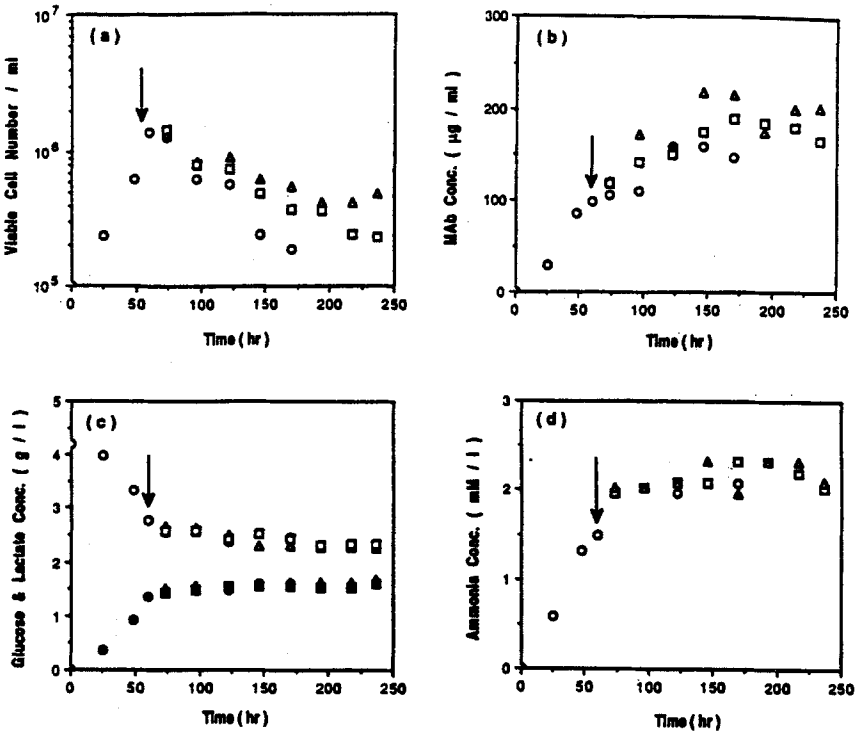

Figure 2: Shift of agitation from 150 rpm to various agitation speeds : (a) cell growth curves; -▲- T flasks, -□- 100 rpm, -○- 150 rpm, (b) MAb concentration, (c) glucose and lactate concentrations, (d) ammonia concentration

In conclusion, hybridoma cells (S3H5/γ2bA2) produce a significant amount of MAb in the decline phase of growth by active secretion from viable cells. Therefore, it is important to maintain cell viability to increase the MAb production. Since cells in decline phase of growth appear to be very shear sensitive, severe agitation should be avoided in this phase of growth to achieve this goal.

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