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Examination of serum and bovine serum albumin as shear protective agents in agitated cultures of hybridoma cells

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Summary

A murine hybridoma cell line (167.4G5.3) was cultured in batch mode using IMDM containing different serum concentrations and bovine serum albumin (BSA). Cell growth and death, metabolism and antibody production were studied in these cultures. The cells were more susceptible to shear in the stationary and in the decline phase of growth as evidenced by higher death rates. Cell growth was best at high serum concentrations with high specific growth and low specific death rates. When BSA was used instead of serum in IMDM, no protective effect was observed. Cell metabolism and monoclonal antibody production rates were not influenced by the level of serum or by BSA. The use of serum in commercial serum-free media (OPTI-MEM) also resulted in no change in both growth and death rates.

Hybridoma; Cell culture; Serum; Albumin; Shear; Agitation

Introduction

Monoclonal antibodies are produced in large quantities by growing hybridoma cells in agitated bioreactors. Mechanical or bubble-induced agitation is applied in stirred tank reactors or in air-lift reactors to keep the cells in suspension and to provide sufficient oxygen transfer to the cells. These reactors are simple to operate

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and to control because of the uniform environment provided by the agitation. Hybridoma cells can be grown successfully in these reactors. However, the cells can be damaged by the shear forces resulting from agitation. The shear sensitivity of the cells limits the extent of mixing that can be used and therefore the mass transfer of nutrients, most importantly, oxygen. The number of cells, and thus the amount of product made in the bioreactor may, therefore, be limited by the degree of agitation that can be used without causing damage to the cells.

The response of cells to shear stress and the cell damage under agitation-induced shear have been studied extensively in the past for anchorage-dependent cells under defined stress fields (McIntire et al., 1987; Levesque et al., 1989) and for microcarrier cultures under agitation-induced stress environments (Croughan et al., 1987; Cherry and Papoutsakis, 1989). The effects of shear on cells growing in suspension, including hybridoma cells, are relatively less well understood. Blood cells have been exposed to shear in capillaries to study their response to hydrodynamic forces (see, for instance, Hellums and Hardwick, 1981). Hybridoma cells have also been exposed to shear in capillaries (McQueen et al., 1987) and in viscosimeters (Smith et al., 1987; Petersen et al., 1988) and cells were lysed at high turbulent intensities. In agitated bioreactors, cells experience shear due to mechanical agitation and gas sparging. Mechanical agitation has been shown to damage the cells (Dodge and Hu, 1986). Sparging of gas to increase the mass transfer of oxygen also damages the cells by various mechanisms (Kilburn and Webb, 1968; Hunscher and Onken, 1988; Handa-Corrigan et al., 1985, 1989).

A number of medium additives have been used to protect cells from adverse shear forces. The addition of phospholipids increased the growth of myeloma cells in spinner flasks (Murakami et al., 1983). Addition of bovine serum albumin (BSA) and pluronic F-68 decreased the detrimental effect of sparging (Mizrahi, 1984; Hunscher and Onken, 1988; Handa-Corrigan et al., 1989; Murhammer and Goochee, 1988). In addition to its growth stimulating function, serum has also been reported to protect insect cells from shear stresses (Tramper et al., 1986). The shear sensitivity is more pronounced in serum-free media (Griffiths, 1985). Kunas and Papoutsakis (1989a,b) studied the shear protective effect of serum in agitated hybridoma cell cultures. In studying the shear-induced death in capillary flow systems McQueen et al. (1989) reached the same conclusion.

In this work we studied the effect of serum and bovine serum albumin (BSA) on hybridoma cell growth and death in agitated cultures. Cell metabolism and antibody production rates were studied. Further, protective effects of serum in serum-free media were investigated in order to help us understand the mechanism of protection.

Materials and Methods

Cell line, media and culture maintenance

The murine hybridoma cell line (167.4G5.3) used in this study was provided by Dr. Latham Claflin from the Medical Center at the University of Michigan. The

antibody produced by these cells is IgG_1 subclass against phosphorylcholine (PC). Hybridoma cells were made by fusion of BALB/c spleen cells with the nonsecreting plasmacytoma fusion line P3X63-Ag8.653. Antibody was generated from mice immunized with PC-keyhole limpet hemocyanin (KLH) (Briles et al., 1984). The cells were obtained at an unknown passage number. For the experiments one vial was thawed and the cells were recovered. The cells were maintained in humidified incubators (5% CO_2) at 37°C. The medium used was IMDM with 5% fetal bovine serum (FBS) supplemented with penicillin and streptomycin. The cells were kept in exponential phase of growth by passing them every two days with a dilution factor of 1 : 4.

Effect of serum on cell growth and death rates

One week before commencing the experiments, cells were passed every day with a dilution rate of 1 : 2. This procedure was found to be useful in preventing changes in media composition during inoculation. By the daily passages, cells were exposed to 'almost-fresh' media and they started growing immediately after inoculation (Ozturk and Palsson, 1990). The cells were spun down at 200 *g* for 10 min and the spent media were discarded. Cells were then washed with IMDM without serum and inoculated into 100 ml spinner flasks (Bellco) containing IMDM with different serum levels. Six serum levels were used: 0.625%, 1.25%, 2.5%, 5%, 10% and 20%. The volume in spinners was 50 ml and the agitation speed was 100 rpm. The cells were grown at 37°C and under 5% CO_2 environment in a humidified incubator. Samples were taken twice daily. After the cell counts, the samples were centrifuged and the supernatants were stored at −80°C for analysis of metabolite and antibody concentrations.

Effect of BSA on cell growth and death rates

The protocol for the effect of BSA on cell growth and death was the same as outlined above. Two types of BSA were used: standard BSA fraction V from Gibco and fatty acid-free BSA (ICN Biochemical, Cleveland, OH). In both cases BSA was added to IMDM containing 1% FBS at concentrations of 1% and 5%. Cells were grown in spinner flasks in these media.

Use of serum in serum-free media

We also studied the effect of serum in a commercial serum-free media. OPTI-MEM (Gibco) was used with 0%, 2.5% and 10% FBS. Penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) were added. Cells were inoculated into 50 ml of media in spinner flasks and were grown in batch mode using the same protocol as described above for the experiments with IMDM.

Analytical methods

Cell concentrations were determined using a hemacytometer. Trypan blue dye exclusion method was used to assess the viability. Glucose and lactate concentra-

tions were measured using YSI glucose/lactate analyzer (Yellow Springs) model 2000. Glutamine and ammonia were determined using gas sensing electrodes by the method described by Ozturk et al. (1989). Antibody, IgG_1 was quantified with an ELISA.

Mathematical analysis

The growth and death rates were determined from the concentration time profiles of viable cells, total cells, and dead cells:

$$\frac{\mathrm{d}X_v}{\mathrm{d}t} = \mu_{app} X_v = (\mu - k_d) X_v \tag{1}$$

$$\frac{\mathrm{d}X_d}{\mathrm{d}t} = k_d X_v \tag{2}$$

where X_v and X_d are the viable and death cell concentrations, t time, and μ and k_d are the specific growth and death rates, respectively. The difference between the apparent growth rate μ_{app} and actual growth rate μ is important at high death rates. In the above equations we neglect cell disrupture due to cell lysis. This assumption was tested later and under the agitation speed of 100 rpm no cell disruption was detected (see Fig. 3A).

The growth rates were evaluated in the exponential phase only by the semi-log plot of viable cells versus time. Note that this gives the apparent rate and the death rate has to be evaluated to assess the actual growth rates. The death rates were evaluated from Eq. (2) using an integral method. If k_d is constant over the time period of concern, then we have:

$$X_d = k_d \int_0^t X_v \, \mathrm{d}t \tag{3}$$

Hence a plot of dead cell concentration versus the time integral of viable cell concentration should yield a straight line whose slope is the death rate.

The antibody production rates were also evaluated using an integral method. Using

$$\frac{\mathrm{dMAb}}{\mathrm{d}t} = q_{Ab} X_v \tag{4}$$

we get

$$\mathrm{MAb} = q_{Ab} \int_0^t X_v \, \mathrm{d}t \tag{5}$$

if the specific antibody production rate, q_{Ab}, is constant. Hence, a plot of the antibody concentration, MAb, as a function of the time integral of viable cells should yield a straight line with the specific antibody production rate, q_{Ab}, as the slope. This has been shown to be the case by several investigators (Renard et al., 1988; Ozturk and Palsson, 1990).

Results

Cell physiology in different serum concentrations

The results of spinner flask experiments in different serum concentrations are summarized in Fig. 1. Cell growth proceeded for about 100–150 h, depending on the serum concentration used, and a maximum in cell concentration was reached (Fig. 1A). The growth rate and the maximum cell number were dependent on serum concentration. Higher serum concentrations led to higher growth rates and higher maximum cell concentrations. After a maximum in the viable cell number was reached, cells entered the decline phase. The cell viabilities in these experiments are summarized in Fig. 1B as functions of time. The cell viability decreased rapidly during the decline phase, and the rate of decrease in the viability was more pronounced for the low serum-containing cultures. This indicates that serum has an

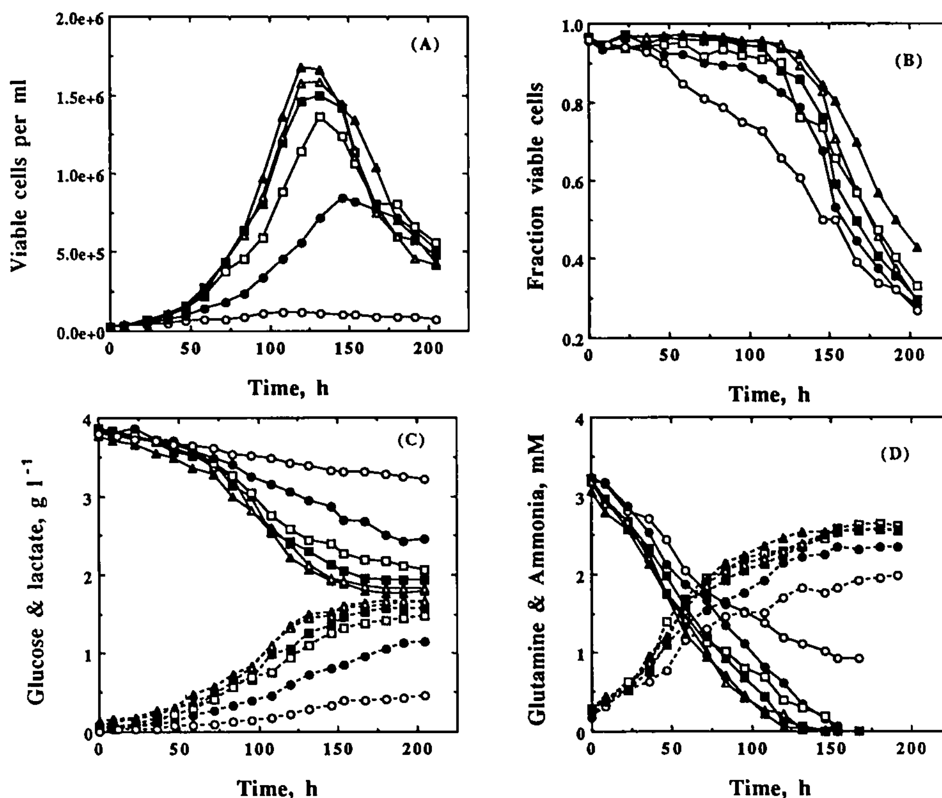

Fig. 1. The time profiles of (A) viable cell concentration, (B) cell viability, (C) glucose and lactate concentrations and (D) glutamine and ammonia concentrations for batch cultures carried out at different serum levels. Legend: 0.625% FBS (open circles), 1.25% FBS (closed circles), 2.5% FBS (open squares), 5% FBS (closed squares), 10% FBS (open triangles) and 20% FBS (closed triangles).

effect on cell death. Higher serum results in lower death rates and therefore higher cell viabilities.

The metabolite concentration profiles are presented in Fig. 1C for glucose and lactate, and in Fig. 1D for glutamine and ammonia. Both glucose and glutamine were consumed. Cell growth was not limited by the supply of glucose since about 50% of the glucose remained at the end of the culture. The high serum-containing cultures consumed more glucose and more lactate was produced in these cultures. This higher glucose uptake and higher lactate production were due to the higher cell concentrations.

Glutamine was depleted in the cultures at the same time as maximum cell concentration was reached (Fig. 1D). Thus, the cultures were nutritionally limited by glutamine. The 0.625% serum culture was, however, not glutamine limited. Growth ceased in this culture possibly due to the low serum level and shear forces.

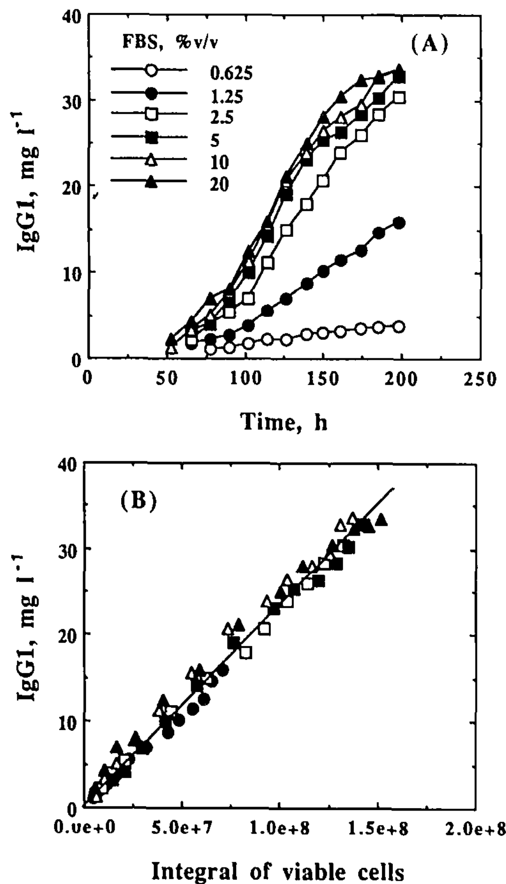

Fig. 2. (A) Antibody concentrations obtained in batches with different serum concentrations and (B) the plot for obtaining specific antibody productivities. The same symbols were used as in Fig. 1. Plot (B) shows that the antibody productivity, q_{Ab}, is a constant and has a value of 0.2 pg per cell per h.

Ammonia was produced in parallel with the glutamine consumed in all the cultures. The kinetics of glutamine consumption and ammonia production were similar to glucose and lactate. However, the consumption of glutamine and production of ammonia were less dependent on the serum concentration and the resulting cell numbers. Note that chemical decomposition of glutamine also contributes to the disappearance of glutamine and accumulation of ammonia.

Antibody production is presented in Fig. 2. Antibody production continued into the stationary and the decline phases. The level of antibody concentration was again serum dependent and higher antibody titers were obtained at higher serum concentrations. This was attributed to the higher cell concentrations present in higher serum batches since the specific antibody production rate is a constant as seen from Fig. 2B (recall Eq. 5).

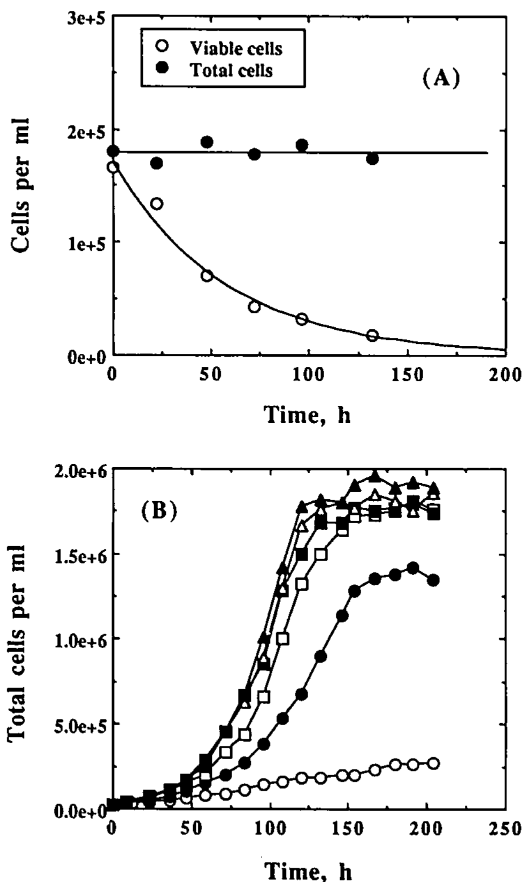

Fig. 3. (A) Cell death in IMD media without serum and (B) total cell concentrations in the experiment presented in Fig. 1.

Kinetics of cell death

When cells lose their viability they might lyse due to the shear forces that they experience. In an additional experiment, cell death kinetics and the degree of cell lysis were examined. Cells were cultured in IMDM without serum and glutamine at a level of 10^5 cells per ml. Cell counts were performed in a hemacytometer using trypan blue to differentiate dead and viable cells. The cells did not grow in this medium and viable cell concentration decreased constantly (Fig. 3A). The total cell concentration (viable plus dead cells counted) was, however, constant. This result shows two important features of cell death under these conditions. First, loss of viability is a first order process as indicated by straight line on the semi-log plot of viable cell concentration over time. This result is in agreement with the data of Dodge et al. (1987). Second, the dead cells do not disrupt under the agitation speed used (100 rpm) since the total cell concentration is constant.

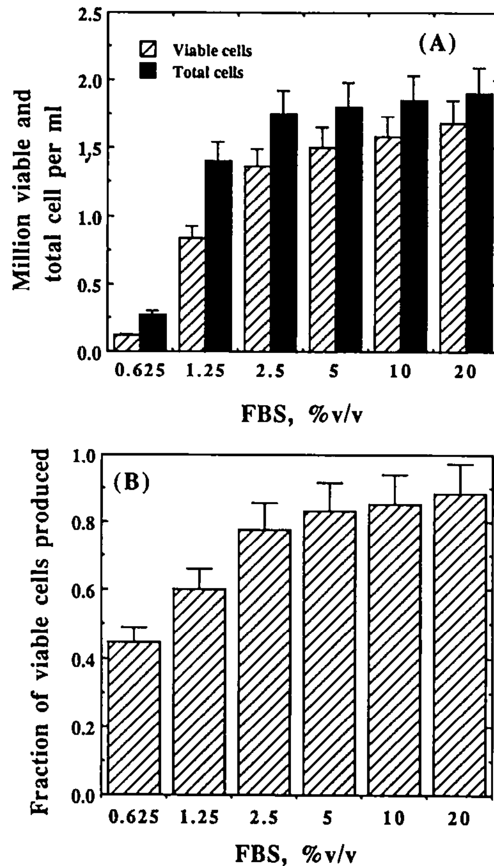

Fig. 4. (A) The maximum total and viable cells obtained and (B) fraction of viable cells at these maximum values, in the experiment presented in Fig. 1.

Further evidence for negligible cell disruption comes from the total cell counts for the experiment summarized in Fig. 1. The total cell concentrations in these batches are presented in Fig. 3B. Total cell concentration increases in the growth phase (between 0 and 150 h) but remains relatively constant during the decline phase. This data further illustrates that there is negligible cell lysis under these conditions and all non-viable or dead cells can be counted by the trypan blue technique. Then Eq. 2 can be used to describe cell death under these conditions.

Effect of serum on cell death

An immediate effect of serum on cell death can be seen by comparing the number of the cells attainable in the batch mode and their viability. The maximum cell concentrations are presented in Fig. 4A for different serum concentrations. Both viable and total cell concentrations are higher at higher serum concentrations.

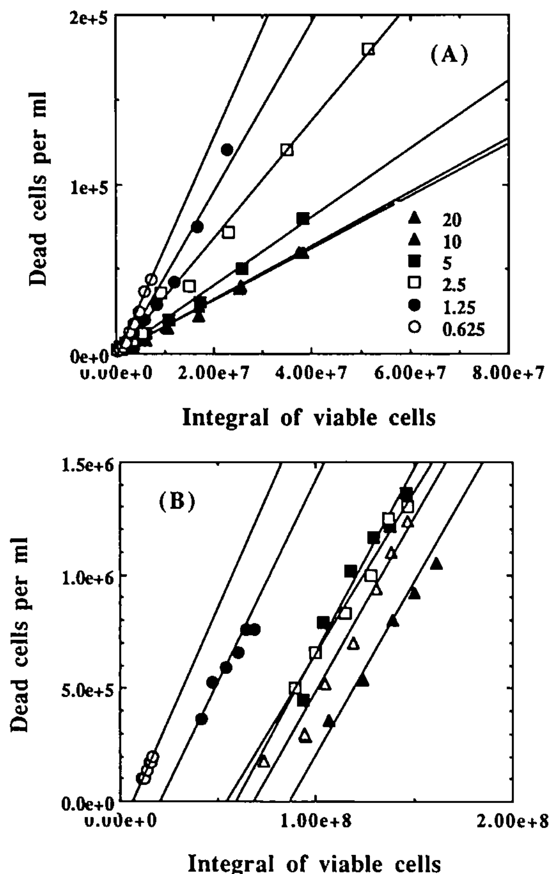

Fig. 5. The evaluation of death rates by the integral method. (A) Data during the exponential phase, (B) data during the decline phase.

Using the data in Fig. 4A we calculated the ratio of maximum viable cells to the total maximum cells. This ratio represents the overall health of the culture and is related to the relative death and growth rates. Fig. 4B shows that the fraction is higher at higher serum levels. The culture at 0.625% serum resulted in low cell concentrations (Fig. 4A) and only 44% of these cells were viable. Growth in 20% serum yielded more cells and 88% of the total cells were viable. As all cultures were started at the same inoculum size, the higher total cell concentrations indicate higher growth rates, and higher fraction of viable cells produced indicates lower death cells. For the hybridoma cell line used in this work, the result presented in Fig. 4 reflects not only the growth-stimulating effect of serum but also the shear protecting effect of serum. These two effects can be separated by the evaluation of growth and death rates by kinetic analysis of cell growth data.

The apparent growth rates were evaluated from the semi-log plot of cell concentrations against time. This plot was linear only over the first 75 h of growth. The actual growth rates were found by adding the death rates to the apparent growth rates (Eq. 1). The discrimination of actual and apparent growth rates and the necessity of this correction is especially important in low serum-containing media.

The death rates were evaluated by the integral method as described in Materials and Methods. The dead cell concentration, when plotted against the time integral of viable cells, formed a curve that has two linear segments, one representing the exponential phase and the other the death phase. For each part we obtained straight lines with regression coefficients higher than 95% (Fig. 5). Thus, we could evaluate the death rates in the exponential and death phases separately. Table 1 shows the death rates as a function of serum concentration. The death rate was one order of magnitude higher during the death phase than during the exponential phase (Table 1).

The exponential phase growth rates were then corrected using the death rates evaluated during the exponential phase and actual specific growth rates were obtained (Table 1). The growth rates increased with increased serum concentration, while the death rates in the exponential phase decreased. Fig. 6 summarizes the growth and death rates in the exponential phase as a function of serum concentration used. We can conclude from this figure that serum promotes cell growth both

TABLE 1

Growth and death rates for the hybridoma cell used in different serum concentrations; the ratio, k_d/μ, in the last column is evaluated during the exponential growth phase

Serum FBS % v/v	Apparent growth rate h^{-1}	Exponential phase death rate h^{-1}	Decline phase death rate h^{-1}	Actual exponential growth rate h^{-1}	k_d/μ
0.625	0.011	0.0065	0.0196	0.018	0.36
1.25	0.024	0.0050	0.0144	0.029	0.17
2.5	0.032	0.0035	0.0143	0.036	0.10
5	0.039	0.0021	0.0145	0.041	0.05
10	0.043	0.0015	0.0152	0.045	0.03
20	0.044	0.0015	0.0132	0.046	0.03

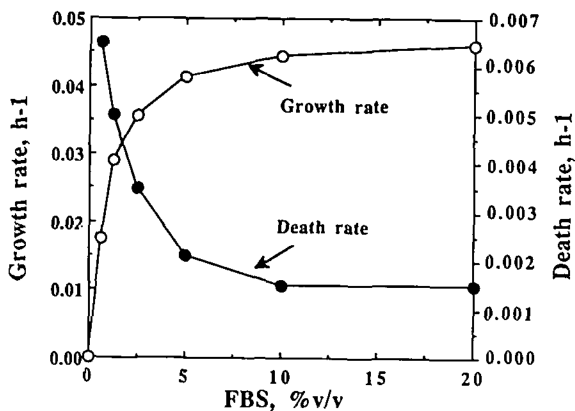

Fig. 6. The influence of growth and death rates by the serum level. The values are evaluated in the exponential phase.

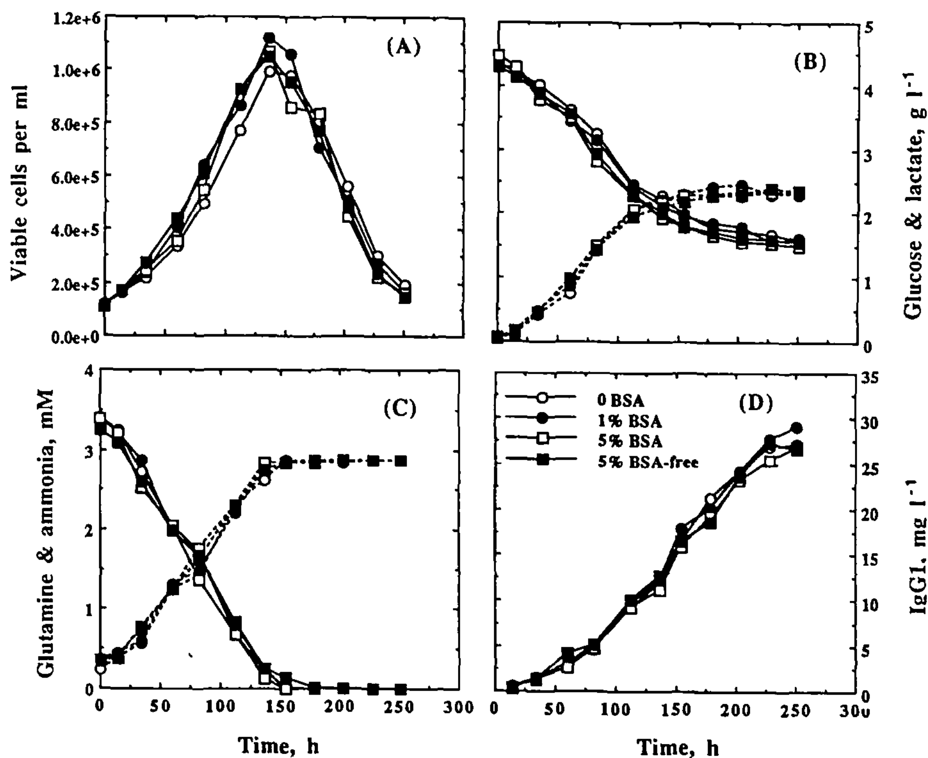

Fig. 7. Effect of BSA on cellular kinetics: (A) viable cells, (B) glucose and lactate, (C) glutamine and ammonia and (D) monoclonal antibody concentrations for the batches of different BSA concentrations. Legend: no BSA (control, open circles), 1% BSA (closed circles), 5% BSA (open squares) and 5% fatty acid-free BSA (closed squares).

by increasing the specific growth rate and by decreasing the specific death rate. Both effects are more pronounced at low serum concentration. Above 5% FBS, there was little change in both growth and death rates.

The specific uptake rates of glucose and glutamine, and the specific production rates of lactate and ammonia calculated from the time profiles presented in Fig. 1 were found to be independent of serum concentration (data not shown). The antibody production rates were also independent of serum concentration. As presented in Fig. 2B, the slope of the curves of antibody as a function of integral of viable cells were very close to each other. As explained in Materials and Methods, these slopes are the specific antibody production rates, q_{Ab}.

Effect of BSA on cell growth and metabolism

The growth characteristics in BSA-containing cultures are presented in Fig. 7 for the case of fatty acid carrying BSA. Similar data was obtained with fatty acid-free

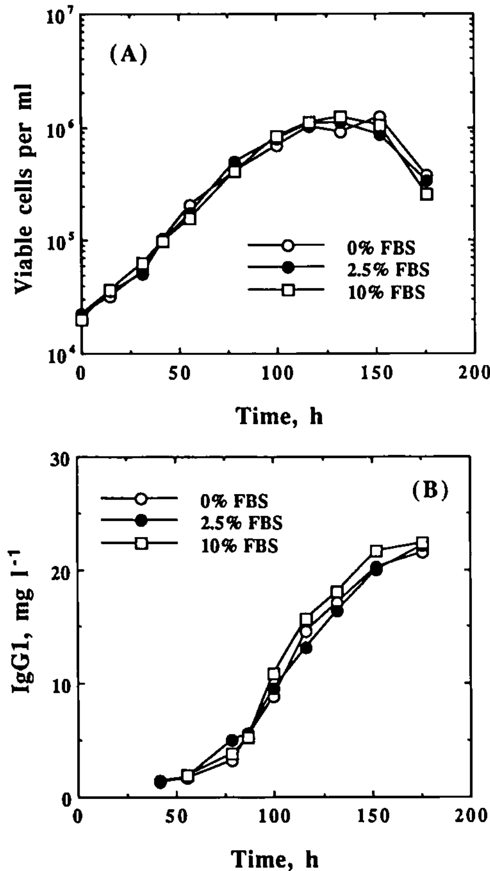

Fig. 8. Effect of serum in OPTI-MEM. (A) viable cells and (B) monoclonal antibody concentrations for the batches of different serum concentrations.

BSA. The addition of BSA did not have any effect on cell growth, death, cell metabolism and antibody production at even relatively high concentrations (5%). The specific growth and death rates evaluated in the exponential phase remained unaltered at $\mu = 0.019$ h^{-1} and $k_d = 0.007$ h^{-1}, respectively. These results suggest that the shear protective effects of serum are due to some specific action of the proteins present in serum.

Effect of serum in serum-free media

Cells were grown in OPTI-MEM media at different serum concentrations. The results are presented in Fig. 8 for viable cell and antibody concentrations. The addition of serum to OPTI-MEM did not affect the cell growth and cell death. The specific growth and death rates in OPTI-MEM were $\mu = 0.042$ h^{-1} and $k_d = 0.0016$ h^{-1}, respectively. These values did not change by the addition of 2.5% and 10% serum. Antibody production was also not influenced by the addition of serum to OPTI-MEM media as presented in Fig. 8B. The calculated specific antibody production rate in OPTI-MEM and in IMDM was similar (data not shown).

Discussion

Shear sensitivity of hybridoma cells is an important problem in producing antibodies in suspension bioreactors. We have studied cell growth and death and examined serum and BSA as possible shear protective agents in agitated cultures.

Serum was observed to promote the cell growth and to suppress the cell death. The influence of serum on specific growth and death rates was more pronounced at low serum concentrations. A similar response of growth rate to serum was reported in the literature (Glacken et al., 1988; Dalili and Ollis, 1989; Ozturk and Palsson 1990). A similar decrease in death rate by serum was also observed by Kunas and Papoutsakis (1989a,b) and by McQueen et al. (1989) and our data is consistent with their findings.

We have evaluated the specific death rates in both exponential and death phases. The death rate during the decline phase was higher than that observed during the exponential growth phase (Table 1). This shows that cells are more sensitive to shear in the decline phase. It is important to note that the death rates during the decline phase are relatively independent of serum concentration (Table 1). These data suggest that the protective effect of serum is only effective during the growth phase.

Serum albumin is a major component of serum. BSA was used as a shear protective agent by previous investigators (Hunscher and Onken, 1988; Handa-Corrigan et al., 1989) in sparged bioreactors. The shear protective effect of serum may be related to the presence of BSA. It has been suggested that BSA may form a protective layer around the cell membrane. Also important to note is that one of the functions of serum albumin is to carry lipids. The supply of lipids may also be responsible for the shear protective effect of serum. Cells may form more stable cell

membrane when fatty acids are provided externally. The addition of BSA may also affect the cellular kinetics.

We tested these hypotheses by employing both fatty acid-carrying and fatty acid-free BSA. There was no change in either growth or death rates by the addition of BSA. Hunscher and Onken (1988) reported no effect of BSA in surface aerated reactors. There was, however, an influence of serum in sparged reactors. The influence of BSA in their experiment then should be attributed to the action of BSA at the gas-liquid interface as suggested by Handa-Corrigan et al. (1989).

The hypothesis of formation of a more stable cell membrane by the presence of BSA or BSA carried lipids also failed as the data for lipid-carrying and lipid-free BSA was almost identical. Kunas and Papoutsakis (1989b) have observed that the protective effect of serum is very fast and their results also contradict this hypothesis.

The shear protective effect of serum was not due to the protein and the lipid content of serum and possibly not to a physical phenomenon such as the coating of cells with a protein layer. The protective effect seems to be related to other components of serum; specific components, such as growth factors. Our hypothesis is that these growth stimulatory factors may change the structure of the cells, i.e., the cytoskeleton, making the cells more sturdy or more flexible. The action of these factors is known to be fast. Some of the growth factors are included in commercial serum-free media, such as the OPTI-MEM formulation, to support growth. These same factors seemed to protect the cells from shear. The growth and death rates in OPTI-MEM without serum was comparable to the values in IMDM with 5% FBS. As we have seen earlier, these parameters do not change significantly by the addition of more serum. It then means that the growth stimulatory and shear protective agents both have saturation kinetics. The concentrations of these agents in OPTI-MEM are most probably at saturation levels as no additional increase was observed by the addition of serum. Kunas and Papoutsakis (1989) have also reported that the addition of serum did not change the growth and death rates of hybridoma cells growing in a serum substituted culture.

In addition to studying growth and death rates in the presence of serum and BSA, we have analyzed the cell metabolism and antibody production. We have seen no significant effect of serum and BSA on these parameters.

Acknowledgements

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