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FLOW EFFECTS ON THE VIABILITY AND LYSIS OF SUSPENDED MAMMALIAN CELLS

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ABSTRACT

A mouse myeloma cell line growing in suspension was subjected intermittently to flow through a sudden contraction and turbulent flow in a capillary tube. The probability of lysis per pass through the capillary tube increased with average wall shear stress level and with residence time per pass in the tube. Lysis was first observed at a threshold average wall shear stress level of 1800 dyn/cm^2. Although the flow caused lysis, it had no effect on cell viability.

INTRODUCTION

One of the major challenges in scaling up mammalian cell culture is avoiding cell damage caused by mixing. Agitation is required in bioreactors to provide adequate oxygen and nutrient supply and to keep cells suspended. Since circulation is essential to many bioreactor designs, it is important to understand the effects of flow on mammalian cells.

Effects of laminar and turbulent flows on anchorage-dependent cells have been well documented. Most previous work on cells attached to a substrate was concerned with monolayers of endothelial cells (e.g. Stathopoulos *et al.*, 1985, and Davies *et al.*, 1986). In addition, there have been many studies of cells on microcarriers in stirred tanks, such as those of Croughan *et al.* (1986) and Cherry *et al.* (1987). Much less information is available on flow effects on cells in suspension. Studies in well-defined flow are difficult to do with suspended cells, because the cells must circulate with the medium and therefore often encounter different hydrodynamic environments during the experiment. Settling of the cells is another practical problem in such experiments.

Studies of cells in suspension include investigations of insect cells (Tramper *et al.*, 1986) and mouse hybridoma cells (Smith *et al.*, 1987) in laminar simple shear flow. Previous work on suspended cells in turbulent flow include experiments by Tramper *et al.* (1986) in a stirred tank and one study by Augenstein et al. (1971) in which cells are subjected to multiple passes through a capillary tube. The latter study reported viable cell counts and compared the behavior of two cell lines: HeLa S3 and mouse L929. Cell viability decreased as the number of passes through the capillary tube increased, and the two cell lines exhibited very different sensitivities to flow effects. However, Augenstein *et al.* (1971) did not determine a threshold value of average wall shear stress at which significant loss of viability began to occur since they did not investigate sufficiently small values of average wall shear stress. One objective of the experiments in the present work was to determine this threshold value of average wall shear stress for a mouse myeloma cell line in suspension, using a similar experimental apparatus. As well as viable cell counts, a complementary assay, based on the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the medium, was used to monitor cell lysis. This assay was also used by Smith *et al.* (1987).

MATERIALS AND METHODS

Cell culture

A mouse myeloma cell line (ATCC TIB 18) was chosen because myeloma cells are used to make hybridoma cells which produce monoclonal antibodies. The cells were grown in Dulbecco's modified Eagle's basal medium, high glucose option (4.5 g/L glucose) (Irvine Scientific, Irvine, CA) with 20% defined horse serum (HyClone, Logan, Utah) and antibiotics (430 units/mL of penicillin and 22μg/mL of streptomycin) (Irvine Scientific, Irvine, CA), in an incubator kept at 37°C and 10% CO_2. The cells were maintained in exponential growth for 3 days prior to the start of the experiment. Once the cell concentration had reached 5 to 8 $\times$ 10^5 cells/mL, 170 mL of the cell suspension was transferred into the flow loop. The cell suspension was then circulated around the flow loop for two and one half hours, the duration of the experiment.

Flow Loop

The flow loop consisted of (i) a syringe-type Harvard infusion-withdrawal pump, which was used to drive the cells around the flow loop; (ii) the flow device, which provided the flow trauma to which the cells were exposed intermittently; and (iii) a holding flask, which enabled sampling and gas exchange to take place. The holding flask had two bottom ports through which the cell suspension entered and exited. A humidified, filtered 10% CO_2 in air gas mixture was gently sparged over the surface of the cell suspension. A sample tube, inserted into the rubber stopper used to seal the flask, was used to collect a sample aseptically with a needle and syringe. The three components of the flow loop were connected with silicone tubing having an inside diameter of 0.32 cm. The flask and a considerable amount of the tubing were placed in a 37°C water bath. Given the flowrate of 114 ml/min and the total flow loop volume of 170 mL, 150 minutes in the flow loop corresponded to 100 passes of the cell suspension through the flow device. Samples were taken after 5, 50 and 100 passes through the flow loop. The residence time per pass in the flow device was only a very small fraction of the loop circulation time, as will be discussed next.

Flow devices

Two flow devices were used. Most of the results presented were obtained with a capillary tube flow device. This flow device consisted of a sudden contraction into a short length L of stainless steel capillary tubing of inside diameter D (Fig. 1A). Several different capillary tube geometries were studied. The ratio of the tube inside diameter to the cell diameter of 10 microns varied between 50 and 80. The ratio of the silicone tubing inside diameter of 0.32 cm to the capillary tubing inside diameter varied between 4 and 6. Table 1 lists the various diameters used and the corresponding velocities and Reynolds numbers. The tube lengths are also listed in Table 1, together with the corresponding residence time per pass in the flow device, t_{res}. The same length was used for all tube diameters except 0.060 cm. This meant that the residence time per pass varied inversely with the velocity. For the 0.060 cm tube diameter, another residence time per pass was tried at constant average wall shear stress by using an additional tube of this diameter but of different length. Since the Reynolds number is above the critical value of 2200 for flow in a tube (Davies, 1972), the flow is potentially turbulent. Therefore, the turbulent average wall shear stress, $\tau_{w,ave}$, is a useful parameter for characterizing the experiments. The values of $\tau_{w,ave}$ for each of the diameters used are listed in Table 1. From the ratios of length to diameter used, it is clear that the flow is not fully developed turbulent at the outlet of the tubes, since this requires an entrance length of at least 25 to 40 tube diameters (Hinze, 1959). Consequently, $\tau_{w,ave}$ in Table 1 is only an approximate value of the actual average wall shear stress level. It is possible that the average wall shear stress level is higher in the developing turbulent boundary layer.

The second flow device, which was used with the same flow loop, had an opposing jets geometry (Fig. 1B). The diameter of the exit tubes of the opposing jets flow device was varied like that of the capillary tubes, leading to a similar range of flow parameters (Reynolds number and $\tau_{w,ave}$) for the flow in these tubes.

Assays of Flow Effects on Cells

A control flask was left in the incubator for the duration of the experiment. Cells which had been circulated around the flow loop and cells from the control flask were assayed in parallel. The total cell count was measured by hemacytometer.

Percent Viability Measurement

The percent viability was determined using viability dye (eosin yellow) exclusion. Whereas live cells exclude the dye, dead cells, whose membranes are permeable, become stained.

Percent Cell Lysis Measurement

The percent cell lysis was obtained by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the surrounding medium. The enzyme activity in the medium was determined as follows. 25 μL of medium was diluted in 1.4 mL of MOPS buffer. NADH and pyruvate were added to final concentrations of 1.5×10^{-4} mM and 4.2×10^{-3} mM, respectively. The decrease in the absorbance of NADH (at 340 nm) was measured at 37°C. The relative enzyme activity (REA) values, which were equal to the slopes of the records of absorbance as a function of time, were substituted into the following equation for the percent cell lysis.

$$\text{Percent Cell Lysis} = \frac{(\text{N} - \text{NC})}{(\text{S} - \text{NC})} \times 100$$

where NC was the REA value of the unsonicated control sample (not subjected to flow), N was the REA value of the unsonicated experimental sample (subjected to flow), and S was the REA value of the sonicated experimental sample (subjected to flow) (Rhee, 1987). This equation will give the percent cell lysis provided that all cells are lysed in the sonicated sample.

Growth rate assay on the surviving cells

After the end of the experiment, a sample of the surviving cells from the flow loop was allowed to grow in a flask placed in the incubator. The number of viable cells as well as the DNA synthesis rate were determined 24, 36 and 48 hours later.

DNA synthesis rate assay

The DNA synthesis rate was determined by [methyl ^{3}H] thymidine incorporation. The incorporation into triplicate aliquots (4×10^5 cells) was determined as previously described (Meilhoc *et al.*, 1987). The final pellet was solubilized in a mixture of 50 μL of water and 300μL Scintigest solubilizer (Fisher Scientific Company) for 5 hours at 55°C. The radioactivity incorporated into the acid-insoluble material was determined by liquid scintillation spectrometry.

Diameter D, cm	**Velocity U, cm/s**	**Reynolds Number**	$\tau_{w,ave}$ **dyn/cm^2**	**Length L, cm**	**Residence Time per pass, t_{res}, seconds**
0.050	920	4780	4290	0.22	0.00024
0.056	775	4340	2880	0.22	0.00028
0.060	672	4030	2250	0.22	0.00033
0.060	672	4030	2250	0.66	0.00099
0.064	590	3780	1780	0.22	0.00037
0.080	378	3020	784	0.22	0.00058

Table 1: Flow parameters

RESULTS

The flow loop design meant that the cells experienced damaging flow effects intermittently rather than continuously. Control experiments indicated that the flow loop, without the flow device, had no effect on any of the cell parameters studied. Therefore, flow effects are considered to be confined to the flow device.

Cell Damage in the Capillary Tube Flow Device

After subjecting cells to capillary tube flow devices having average wall shear stress levels between 800 and 4300 dyn/cm^2, it was found that the flow had no effect on the percent viability of the cells as measured by dye exclusion. However, multiple passes through the flow device did cause a decline in the total cell number, as determined by cell counts in the hemacytometer, and an increase in the percent cell lysis, as measured by LDH release into the medium. This means that, when subjected to the flow, the cells either broke or remained viable. The results obtained from cell counts and from lysis measurements were in good agreement, and both were reproducible from experiment to experiment. Only cell lysis measurements will be presented in the following figures.

Cell lysis measurements indicated that the viable cell number decreased exponentially rather than linearly as a function of the number of passes (Fig. 2). Therefore, the data fits the form:

$$log\ \frac{x}{x_o} = -kN$$

where N is the number of passes and x/x_o is the fraction of viable cells remaining (not lysed). k is the specific lysis rate (units of (number of passes)$^{-1}$). Alternatively, k may be interpreted as the probability of lysis per pass through the flow device. As shown in Fig. 2, the specific lysis rate depends on the average wall shear stress (noted by each data set).

No cell lysis occured at the lowest average wall shear stress level studied, 800 dyn/cm^2. The threshold level of average wall shear stress, defined as the level at which significant lysis began to occur, was 1800 dyn/cm^2. Above this threshold level of average wall shear stess, the specific lysis rate increased as the average wall shear stress level increased.

In order to assess the relative contributions of the flow through the sudden contraction and the turbulent flow in the tube to cell damage, a preliminary investigation was conducted on the effect of increasing the residence time per pass (i.e. the tube length) in the capillary tube flow device. The damage due to the flow through the sudden contraction is unchanged in these experiments. In Fig. 3, the percent cell lysis is shown as a function of the number of passes for different residence times per pass. It is evident that increases in tube length, at a constant level of average wall shear stress, result in significant increases in cell lysis. This indicates that there is a contribution of the turbulent flow in the tube to cell lysis, as well as (probably) a contribution of the flow through the sudden contraction.

Cell Damage in the Opposing Jets Flow Device

In the opposing jets flow device, at the high flowrates required to obtain lysis, the flow was turbulent and therefore not well defined in this geometry. Similar to the results obtained with the capillary tube flow device, the opposing jets flow device had no effect on cell viability but caused substantial lysis which increased as the diameter of the jets decreased. The opposing jets flow device is included because of a number of important experimental results obtained using it. Two additional assays were performed on the samples subjected to this alternative flow device. The growth rate and DNA synthesis rate of the surviving cells (which were not lysed) were measured for a further 48 hours after the end of the flow loop experiment. It was found that, for a size of the opposing jets at which considerable lysis occurred, there was no effect on either the growth rate or the DNA synthesis rate of the remaining cells measured 24, 36 or 48 hours after the end of the experiment. This means that, in the flow loop with the alternative flow device, cells either lysed or were unaffected by the flow. It remains possible that a transient effect occured during the first 24 hours and this is still under investigation. Although these assays have not been performed so far in experiments with the capillary tube flow device, it is hypothesized that the same results would be obtained under analogous experimental conditions.

DISCUSSION

The flow geometry examined here consisted of flow through a sudden contraction and through a short length of capillary tubing. The Reynolds number for the flow in the tube was greater than the critical Reynolds number, indicating that the flow was potentially turbulent. Since the length of the tube was less than the required entrance length, the turbulent flow was not fully-developed even at the outlet of the tube. However, the results obtained in this flow device will be compared to measurements made in both laminar flow through a sudden contraction and fully developed turbulent flow in a tube, even though this flow device only approximates those flow types. In addition, the present work will be compared with work on flow effects on cells for other flow types, with other cell lines.

Flow Through a Sudden Contraction

The flow through a sudden contraction (an abrupt entrance into a smaller diameter tube) is an extensional flow. This implies that the magnitude of the rate of strain tensor is greater than the magnitude of the vorticity tensor, rather than equal to it, as it is in simple shear flow. In an extensional flow, by definition, fluid elements and particles suspended in the fluid are extended. In the case of flow through a sudden contraction, the cell will be stretched in the axial (flow) direction and compressed in the radial (transverse) direction. This is because the velocity of the fluid increases and the streamlines of the fluid are squeezed in order to fit through the smaller cross-sectional area of the capillary tube. Han *et al* (1978) studied drops subjected to laminar flow through a sudden contraction. They observed the drops through the transparent walls of the flow device and found that the drops elongated in the contraction, recoiled just beyond it and then assumed an equilibrium configuration in the fully developed region of the flow. If the forces on the drop were sufficient, the drop did not recover from the extensional flow but burst. Drop burst occured at a critical value of the wall shear stess level.

Bentley and Leal (1986) studied drop deformation and burst in a 4-roll mill, which could produce both 2-D pure extensional flow and 2-D flows having nonzero vorticity (approaching simple shear flow). They found that drop burst occurred at a lower critical shear stress level in extensional flow than in flows with vorticity. Both of these studies suggest that extensional flow should be more effective in breaking cells than simple shear flow. Hence, the contribution of the extensional flow in the entry region of the capillary tube flow device may be sufficient to cause cells to lyse in spite of the short residence time in the capillary tube.

Turbulent Flow in a Tube

Augenstein *et al* (1971) used a flow apparatus very similar to that used in this work. The threshold average wall shear stress level found in the present work was below the least damaging conditions used by Augenstein *et al* (1971), all of which caused substantial cell lysis.

Correlation of Cell Lysis with the Kolmogorov Length Scale

The Kolmogorov length scale, which characterizes the size of the smallest, energy-dissipating eddies, was calculated for the tube diameters and velocities used, employing relations determined empirically for fully developed turbulent flow in a tube (Davies, 1972). Since the flow is not fully developed in this case, these values of the Kolmogorov length scale are only approximations to the true values in these tube sizes. In Fig. 4, the specific lysis rate is plotted as a function of the Kolmogorov length scale. It can be seen that the threshold value of the Kolmogorov length scale, at which the specific lysis rate became detectably greater than zero, was 3.5 microns. The cell diameter has been determined by Coulter counter measurements to be 10 microns on average, with some cells as small as 5 microns. Therefore, cell lysis began to occur when the smallest turbulent eddy size fell below the cell diameter. This conclusion is remarkably similar to that of Croughan *et al* (1986), who examined flow effects on cells on microcarriers in a stirred tank. They found that significant loss of viability began to occur when the Kolmogorov length scale of the turbulence in the stirred tank reached a value somewhat smaller than the microcarrier diameter.

Residence Time Required to Obtain Lysis

In the present work, 50 passes through the flow device (total residence time 0.02 seconds) were required to obtain significant lysis at the threshold average wall shear stress level of 1800

dyn/cm^2. In contrast, approximately one hour of continuous shear is required to obtain lysis in laminar simple shear flow experiments on other suspended cell lines. The threshold shear stress values obtained by Tramper *et al.* (1986) for insect cells and by Smith *et al.* (1987) for mouse hybridoma cells were 15 and 8.7 dyn/cm^2 respectively. This difference in residence time required to obtain lysis is probably due to different mechanisms of cell damage in the two cases. Rather than a gradual weakening of the membrane, the cell breaks abruptly in the flow device studied here, due to the severity of the flow conditions.

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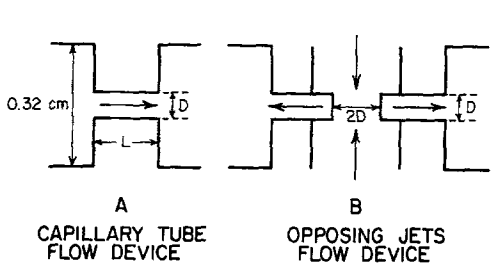

Figure 1: Schematic diagrams of flow devices

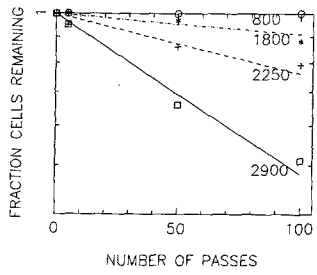

Figure 2: Effect of number of passes through the capillary tube flow device on the percent lysis of mouse myeloma cells. Different symbols denote measurements at different values of average wall shear stress (dyn/cm^2, indicated below each data set).

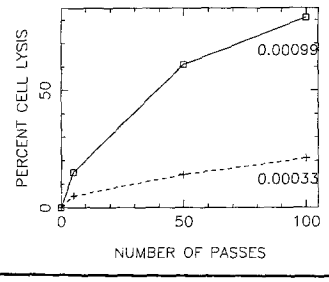

Figure 3: Effect of residence time per pass (seconds, indicated on the figure) in the capillary tube flow device on percent cell lysis as a function of the number of passes.

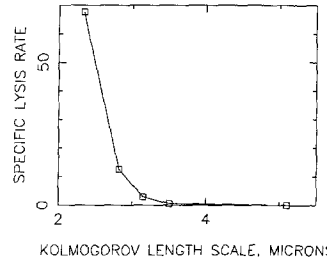

Figure 4: Relationship between the specific lysis rate (units (number of passes)$^{-1}$) of mouse myeloma cells and the approximate Kolmogorov length scale (size of the smallest turbulent eddies) in the capillary tube flow device.