

## Inhibition of mouse x mouse hybridoma growth by milk and colostrum

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**Summary** — A mouse x mouse hybridoma was grown in media containing colostrum (buffalo, cow), milk (buffalo, cow) and a reduced level of fetal calf serum (FCS). When these supplements (2% level) were added in combination with 1% FCS, growth of hybridoma and antibody production were drastically reduced as compared to cells grown in 1% FCS alone. These studies indicated the presence of some inhibitory factor(s) in colostrum or milk. The inhibitory factor appeared to be heat stable large molecule(s) as neither dialysis nor heat inactivation helped in overcoming inhibition by colostrum. The inhibitory effect of buffalo colostrum persisted even when hybridoma cells were cultivated in a collagen-treated 24-well plate. In all cases, binding of hybridoma cells to surface was affected resulting in flocculation and death of hybridoma cells.

**hybridoma culture / hybridoma inhibition / milk / colostrum / inhibitory factor**

**Résumé** — **Inhibition de la croissance d'hybridomes de souris par le lait et le colostrum.** Une culture d'hybridomes de souris a été utilisée pour étudier la croissance cellulaire sur milieux de culture contenant soit du colostrum de bufflesse ou de vache, soit du lait de ces mêmes espèces, en remplacement partiel du sérum de veau fœtal (SVF). L'ajout de ces suppléments au taux de 2% au milieu contenant 1% de SVF réduisait de façon drastique la croissance des hybridomes et la production d'anticorps par rapport aux cellules cultivées dans le milieu contenant seulement 1% de SVF. Les résultats obtenus démontrent la présence d'un facteur d'inhibition dans le colostrum et le lait. Ce facteur d'inhibition apparaît être une macromolécule thermostable puisque ni la dialyse ni l'inactivation par la chaleur ne permettait de lever l'inhibition par le colostrum. L'effet inhibiteur du colostrum de bufflesse persistait même quand les cellules d'hybridomes étaient cultivées dans des boîtes de 24 puits traités au collagène. Dans tous les cas, l'adhésion des cellules d'hybridomes à la surface était affectée, ce qui conduisait à la flocculation et à la mort des cellules.

**culture d'hybridome / inhibition / lait / colostrum / facteur d'inhibition**

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## INTRODUCTION

Monoclonal antibodies (mAbs) are among the most important products derived from large-scale mammalian cell culture (Lee and Palsson, 1990). Since mAbs have enormous potential for application in biological sciences, the demand for mAbs has increased tremendously. This has generated interest in more economical production of mAbs. The economics of large-scale *in vitro* culturing of hybridomas is mainly dependent on the media formulations. Serum is a complex mixture of hormones, growth factors, vitamins, binding proteins, amino acids, transport proteins, carbohydrates and inhibitors (Glassy *et al*, 1988; Lee and Palsson, 1990). Traditionally, hybridomas have been grown in various media or combination of media containing 10–20% fetal calf serum (FCS) (McHugh *et al*, 1983; Brown, 1987). But, in hybridoma culture in big reactors and with a high cell concentration ( $> 10^5$  cells/ml), the addition of 2–3% FCS is often sufficient to enhance growth (Linardos *et al*, 1992; Martens *et al*, 1992; van der Pol *et al*, 1992). FCS is fairly expensive and can account for upto 84% of the cost of media formulations (Griffiths, 1986). Limited supply and batch-to-batch variation of FCS have also demanded efforts to reduce or completely eliminate its supplementation. As a result, several serum-free (completely defined) media for hybridoma cell culture are being tested and some of them are even commercially available (Barman and Rajput, 1993). Downstream product purification from completely defined serum-free media is convenient and less expensive (Glassy *et al*, 1988). But so far, unlike serum, completely defined serum-free media are not available which can support growth of all hybridoma cell lines. This necessitates testing of each hybridoma cell line for its growth in completely defined serum-free media before such media could be employed for large scale *in vitro* culture.

This encouraged some workers (Ramirez *et al*, 1990; Derouiche *et al*, 1990; Pakkanen *et al*, 1992) to test other less expensive biological fluids such as milk, colostrum, whey fraction and colostrum ultrafiltrate which were added exclusively or in combination with reduced level of FCS. Preliminary results from these studies indicated that such fluids could prove to be an attractive alternative to serum. The present study was also an attempt in this direction but it was observed that growth of a mouse x mouse hybridoma was inhibited by milk or colostrum.

## MATERIALS AND METHODS

### Cell culture

A mouse x mouse hybridoma VID1D3 producing antibody against milk alkaline phosphatase was used. Fusion partner was PAIOP3. Like PAIOP3, hybridoma VID1D3 adhered to surface in static culture. Basal medium used was RPMI-1640 (Sigma Chemical Co, USA) containing  $\text{NaHCO}_3$  (28.6 mmol/l), HEPES (10 mmol/l), Na-pyruvate (1 mmol/l), L-glutamine (2 mmol/l), penicillin (100 IU/ml) and streptomycin (100  $\mu\text{g/ml}$ ). Cells were routinely cultured in basal medium containing 10% fetal bovine serum (Sigma Chemical Co, USA) in a  $\text{CO}_2$ -incubator (37°C, 5%  $\text{CO}_2$ ).

### Preparation of colostrum and milk

Colostrum (within 8 h of birth) from buffalo (Murrhah) and cow (Karan Swiss) was collected from the Institute's farm. Colostrum was immediately brought to the lab and centrifuged at 11 000 rpm (13 000 g) for 30 min at 5°C. The top fat layer and sediment were discarded. Middle layer was collected and kept frozen at -70°C till further use. Frozen sample of colostrum was thawed and then ultracentrifuged (L8-55 Ultracentrifuge, Beckman, USA) at 45 000 rpm (165 000 g) for 90 min at 5°C. The top fat layer (if any), sediment and a turbid layer just above the sediment were discarded. Transparent middle layer was collected

and frozen at  $-70^{\circ}\text{C}$  till further use. In some experiments, buffalo colostrum (ultracentrifuged) was modified in two different ways: i) dialyzed against 200 volumes of phosphate buffer saline (PBS) containing penicillin (100 IU/ml) and streptomycin (100  $\mu\text{g}/\text{ml}$ ) at  $5^{\circ}\text{C}$  for 24 h; and ii) heat inactivation of colostrum was achieved by treating it at  $56^{\circ}\text{C}$  for 30 min.

### **Preparation of media with colostrum and milk**

Colostrum and milk samples were sterilized by filtration after dilution with RPMI-1640 medium. Filtration was achieved by using both coarse filter as pre-filter and fine filter (0.2  $\mu\text{m}$ ) under positive pressure. Then appropriate volume of serum was added to give its 1% concentration. These media were used immediately after preparation.

### **Hybridoma cell culture**

Hybridoma cells growing in log phase in RPMI-1640 containing 10% serum were used in all experiments. Since hybridoma VID1D3 adheres to the surface, spent medium was removed by decantation. Cells were harvested by repeatedly flushing RPMI-1640 (without serum) basal medium on the surface of 25  $\text{cm}^2$ -tissue culture flask (Costar, USA). Cells were counted after staining with trypan blue dye (1:1). Cell suspension was then appropriately diluted with RPMI-1640 to obtain a concentration of  $2 \times 10^5$ -cells/ml. 100  $\mu\text{l}$  of cell suspension were plated into each well of 24-well tissue culture clusters (Costar, USA). Each well was then fed with 1 ml media prepared in different ways and placed in  $\text{CO}_2$ -incubator. Cells were harvested from duplicate wells on every or alternate days by repeatedly flushing each well with spent medium from the same well and centrifuged. Supernatant was collected and used for antibody assay. Cell pellet was tapped and 0.5 ml of RPMI-1640 medium was added and live cells were counted.

Culturing of hybridoma cells was also attempted in collagen (rat-tail) treated 24-well plate (Konigsberg, 1979). Stock solution of rat-tail collagen (UV irradiated for 24 h) was prepared by suspending 4 mg collagen in 10 ml 1.5 mol/l acetic acid for 2 days. A portion of col-

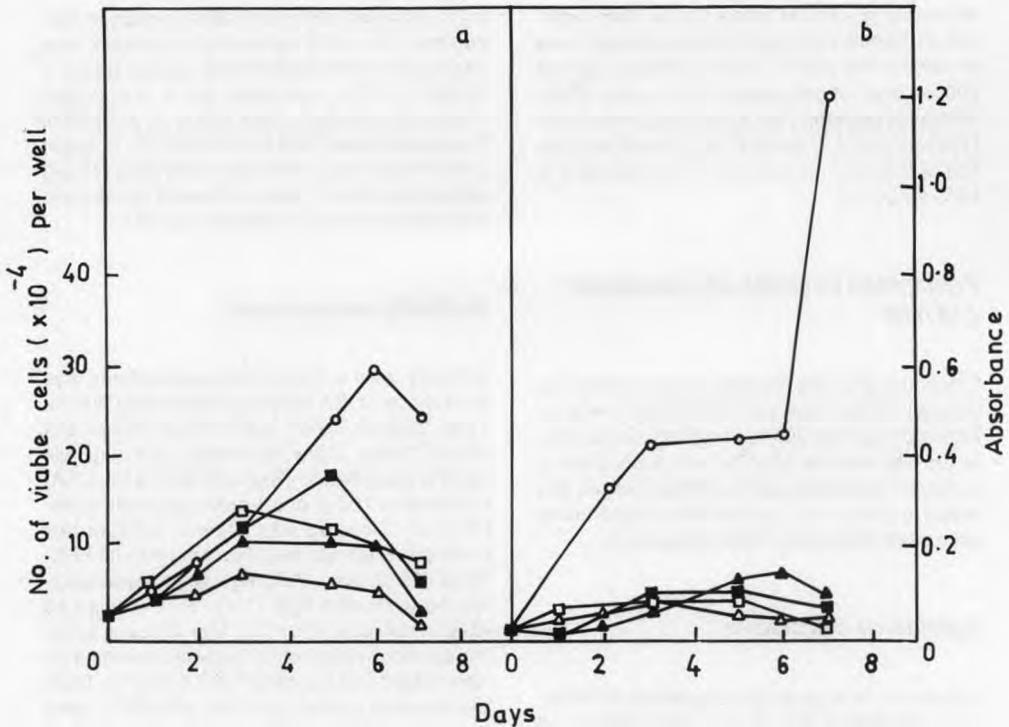
lagen dissolved while some fibres remained suspended. Ten ml of acid soluble collagen was diluted with 40 ml distilled water. Subsequently, 2 ml NaCl (3.76%) was added and 1 ml of diluted collagen was added to each well of 24-well plates. These plates were kept for 24 h at  $37^{\circ}\text{C}$ . Collagen solution from each well was aspirated off and plates were dried. Collagen-treated plates were then sterilized by UV-irradiation for 24 h.

### **Antibody measurement**

Antibody level in hybridoma supernatants was assayed by ELISA technique (Smith and Wilson, 1986; Barman, 1992). Each well of 96-well EIA plates (Costar, USA) was coated with 1  $\mu\text{g}$  milk alkaline phosphatase (Sigma Chemical Co, USA) dissolved in 100  $\mu\text{l}$  of 0.1 mol/l carbonate buffer (pH 9.6). Coating was done in refrigerator overnight. Each well was then washed with PBS-Tween 20 (0.05%). Blocking was achieved by filling the wells with BSA (1%) -PBS-Tween 20 (0.05%) solution ( $37^{\circ}\text{C}$ , 1 h). After removing blocking solution, 100  $\mu\text{l}$  of hybridoma supernatants were added and incubated for 2 h at  $37^{\circ}\text{C}$ . Each well was then washed four times with PBS-Tween 20 (0.05%). After washing, 100  $\mu\text{l}$  of antimouse immunoglobulin (raised in goat)-peroxidase conjugate (Sigma Chemical Co, USA) diluted 1:350 with BSA-PBS-Tween 20 were added and plates were kept for 2 h at  $37^{\circ}\text{C}$ . Each well was again washed 5 times with PBS-Tween 20. After washing, 100  $\mu\text{l}$  substrate (4 mg o-phenylene diamine dissolved in 10 ml of 50 mmol/l sodium citrate, pH 5.0 containing 0.01%  $\text{H}_2\text{O}_2$ ) was added and plates were incubated for 30 min at  $37^{\circ}\text{C}$ . Reaction was stopped by 100  $\mu\text{l}$  4 N  $\text{H}_2\text{SO}_4$  and absorbance was recorded in an ELISA plate reader (Toyo, Japan) using mode 2.

## **RESULTS AND DISCUSSION**

Figure 1a illustrates the influence of colostrum (2%) and milk (2%) from both buffalo and cow on the growth of hybridoma in RPMI-1640 medium containing 1% FCS. It is clear that supplementation of either colostrum or milk to medium resulted in inhibition of hybridoma. Also, antibody level is significantly lower when colostrum or milk



**Fig 1.** Growth response (a) and antibody production (b) of VID1D3 in various media containing 1% FCS (O), 1% FCS and 2% buffalo colostrum ( $\Delta$ ); 1% FCS and 2% buffalo milk ( $\blacktriangle$ ); 1% FCS and 2% cow colostrum ( $\square$ ), and 1% FCS and 2% cow milk ( $\blacksquare$ ).

*Croissance (a) et production d'anticorps (b) des cellules d'hybridomes VID1D3 dans différents milieux contenant 1% de SVF (O), 1% de SVF et 2% de colostrum de bufflesse ( $\Delta$ ), 1% de SVF et 2% de lait de bufflesse ( $\blacktriangle$ ), 1% de SVF et 2% de colostrum de vache ( $\square$ ), et 1% de SVF et 2% de lait de vache ( $\blacksquare$ ).*

were supplemented to medium (fig 1b). Further, microscopic examination from day 5 onwards revealed that in presence of colostrum or milk, many of the hybridoma cells were seen floating leading to flocculation and death (fig 2b,c). Under identical conditions, these hybridomas remained healthy in RPMI-1640 containing 1% FCS (fig 2a). Pakkanen *et al* (1992) observed that, whereas 1% defatted colostrum supported growth of mouse hybridoma, a higher level of added colostrum (5%, 10%, 15%, 20%) was inhibitory. Also, in other but similar studies, Ramirez *et al* (1990) noticed

that colostrum at 2.2% level was optimum for growth and higher level (> 2.2%) resulted in inhibition. In the present study, colostrum at even 2% level was observed to be inhibitory. Differences in the level of colostrum inhibitory to cell growth might be related to differences in levels of inhibitory factor(s) present in colostrum preparation. Inhibitory factor(s) appear to be non-dialysable and heat stable as both dialyzed and heat inactivated colostrum inhibited VID1D3 growth. Pakkanen *et al* (1992) had shown that colostrum ultrafiltrate containing significantly lower levels of immunoglob-

ulins, total proteins and endotoxins was effective for hybridoma cell growth at a relatively broad concentration range of 5–15% while cell growth was significantly reduced at 20% ultrafiltrate. On the other hand Derouiche *et al* (1990) used whey frac-

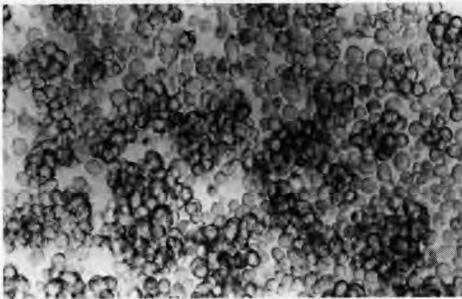
tions over a wide concentration range of 5–20% for routine culturing of hybridomas indicating absence of inhibitory factor(s) in whey fractions. It appears that milk or colostrum requires its modification for removing inhibitory factor(s) before these could be used as an alternate to FCS.

Propagation of non-hybridoma cell lines in colostrum or milk supplemented media has also been attempted by other workers (Klagsbrun, 1980; Steimer and Klagsbrun, 1981). Inhibition by colostrum was reported to be cell line dependent. Colostrum did not support human and rat fibroblasts in long-term culture, whereas canine kidney epithelial cell (MDCK) grew exponentially (Klagsbrun, 1980). Steimer and Klagsbrun (1981) suggested that milk was deficient in attachment factor such as fibronectin. This may not be the only reason for inhibition of VID1D3 hybridoma by colostrum since coating of 24-well plate with collagen did not improve binding and multiplication of hybridoma.

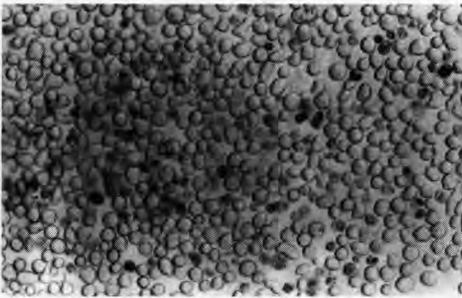
In the present case, VID1D3 hybridoma is producing antibodies against milk alkaline phosphatase and would carry incomplete immunoglobulins on their surface with the same specificity which may act as receptors for alkaline phosphatase present in colostrum preparation. This may evoke a reaction between milk alkaline phosphatase and hybridoma and perhaps may be an inhibitory reaction.



**a**



**b**



**c**

**Fig 2.** Growing VID1D3 at day 5 in RPMI-1640 with (a) 1% FCS; (b) 1% FCS and 2% cow colostrum, and (c) 1% FCS and 2% cow milk. *Culture à 5 jours de cellules d'hybridomes VID1D3 dans le milieu RPMI-1640 additionné de : (a) 1% de SVF, (b) 1% de SVF et 2% de colostrum de vache, et (c) 1% de SVF et 2% de lait de vache.*

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