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Viable™ AC-2, a New Adult Bovine Serum- and Colostrum-Based Supplement for the Culture of Mammalian Cells

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ABSTRACT

In this study we have shown that Viable™ AC-2, a medium based on an ultrafiltrate fraction of bovine colostrum and adult bovine serum, can be used successfully as a fetal bovine serum (FBS) substitute in the culture of several anchorage-dependent and independent cell lines. Of the 15 cell lines cultured in 8% Viable AC-2 in microplates, 10 reached the maximum cell density of 65%–123% of that in 10% FBS, 4 cell lines reached maximum cell density of 35%–65% of that in 10% FBS and only one cell line, a human osteosarcoma G-292, grew slowly in Viable AC-2. In a small-scale suspension culture, 8%–15% Viable AC-2 supports the growth of Chinese hamster ovary cells (CHO-K1) on microcarriers in spinner flasks significantly better than 10% FBS. Shionogi mouse mammary tumor cell line (S115) transfected with human α_2-adrenergic receptor subtype C2 was used as a model to study recombinant protein production in Viable AC-2-supplemented medium. The results showed that in cell culture flasks and in an ACUSYST-R™ bioreactor, the α_2-C2 receptor expression level per mg of total protein was similar in both Viable AC-2 and FBS.

INTRODUCTION

Traditionally, bovine serum has been used in mammalian cell cultures to support cell growth and production of different biochemical components. Both in small- and large-scale cultures, the most frequently used serum type is fetal bovine serum (FBS). However, there has been great interest in trying to replace FBS with other cell culture supplements because of fluctuating quality and availability of FBS (3,11–13,19,20, 26,28,29).

One of the most interesting candidates for the culture of mammalian cells is bovine colostrum, the milk secreted during the first few days after calving. Colostrum resembles FBS in that it contains growth factors, hormones and other nutrients that are essential for mammalian cells (9,10,16,24,27). It has been shown that bovine colostrum supports the growth of hybridomas (22,23,25) and some anchorage-dependent cells (14,15,21,30,31) in vitro, but normal bovine milk is significantly less effective than colostrum as an FBS substitute (15,25,30). On the other hand, raw colostrum contains extremely high amounts of proteins (45–130 g/L) and immunoglobulins (40–60 g/L) (9,24,27) but has a negligible amount of attachment factors (31). In addition, raw colostrum is usually contaminated with endotoxins derived from gram-negative bacteria (5). This makes the use of raw colostrum inappropriate, as such, as an FBS substitute in cell culture.

We have recently shown that an ultrafiltrate fraction of bovine colostrum (UF), with low total protein (1.16 g/L), IgG (0.24 g/L) and endotoxin [<0.24 endotoxin unit (EU)/mL] contents, supplemented with human transferrin, supports the growth and antibody production of anchorage-independent hybridoma cells (22,23). On the other hand, anchorage-dependent cells usually cannot be cultured in colostrum without adding attachment factors like fibronectin (30,31). However, UF supplemented with adult bovine serum (BS) and human transferrin supports the growth of Vero and Chinese hamster ovary cells (CHO-K1) as well as FBS (21).

In this paper we show that several cell types can be cultured successfully in Viable™ AC-2, which is a medium based on UF and BS (21).

MATERIALS AND METHODS

Cells

African green monkey kidney cells (Vero; ATCC No. CCL-81), Chinese hamster ovary cells (CHO-K1; ATCC No. CCL-61), human epidermoid carcinoma cells (HEp-2, ATCC No. CCL-23), BALB/c mouse embryo fibroblasts (3T3 Clone A 31; ATCC No. CCL-163), human cervical carcinoma cells (HeLa; ATCC No. CCL-2) and mouse myeloma cells (X63-Ag8-653) were all purchased from Flow Laboratories, Rickmansworth, England, UK. A mouse-mouse hybridoma cell line, LPC1, has been described elsewhere (22). Human osteosarcoma cells (MG-63, ATCC No. CRL-1427; KHOS/NP, ATCC No. CRL 1544; KHOS-240S, ATCC No. CRL-1545; KHOS-312H, ATCC No. CRL-1546), human osteogenic sarcoma cells (HOS, ATCC No. CRL-1543; MNNG/HOS, ATCC No. CRL-1547) and osteosarcoma cells (G-292, ATCC No. CRL-1423) were purchased from ATCC (Rockville, MD, USA). Human keratinocytes (HaCaT) were obtained as a gift from Dr. Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany). Mouse mammary tumor cell line S115 was from Drs. P.D. Darbre and R.B.J. King (The Imperial Cancer Research Fund, London, England, UK) (7). An S115α_2-C2 recombinant cell line, using the inducible mammalian expression vector pMAMneo (CLONTECH Laboratories, Palo Alto, CA, USA), produces human α_2-adrenergic receptor subtype C2 (17,18). The cells were tested for the absence of mycoplasma using the HybriComb™ Mycoplasma Kit (Biological Industries, Bet Haemek, Israel) or the Myco-Tect® Kit (Life Technologies, Gaithersburg, MD, USA).

Cell Cultures in Microplates

The basal medium used for CHO-K1 was Dulbecco's modified Eagle essential medium F12 (DMEM/F12), and for the other cells we used DMEM (both from Flow Laboratories) that was supplemented with glutamine (4 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL). Stock cultures were maintained in 75-cm^2 plastic flasks (Corning Costar, Cambridge, MA, USA) and supplemented with 10% FBS (HyClone Laboratories, Logan, UT, USA). Adult bovine serum was from HyClone, and Viable AC-2 was from Viable Bioproducts, Turku, Finland.

For subculture, cells growing at the mid- or late-exponential phase were washed once with the basal medium, detached with 0.1% trypsin, 1.06 mM EDTA-4Na (TE) (Life Technologies), and resuspended in the basal medium. Aliquots of the cell suspension were plated in the test media into 24-well microplates (Corning Costar) at a concentration of 13000 cells per mL (1.5 mL per well) and incubated for different time periods without a medium change. Only the media of HaCaT cell cultures were changed every counting day.

At the time points indicated, the medium was removed from each well, and the cells were detached by adding 150 µL TE. After incubation for 10 min at 37°C, the medium was added back into the corresponding wells, and the cells were suspended by pipetting. Cell counts were done in a hemocytometer using the trypan blue exclusion test to determine viability. All culture experiments were performed in duplicate, and each well was counted only once.

Cell Cultures in Spinner Flasks

CHO-K1 cells were cultured on gelatin microcarriers (CultiSpher™-G; HyClone) in spinner flasks (Bellco Glass, Vineland, NJ, USA) in 50 mL DMEM/F12 supplemented with 8%–15% Viable AC-2 or 10% FBS. Initial cell number in the spinner flasks was 0.5×10^5 cells per mg dry beads. Half of the medium was changed every day. At the time points indicated, samples (0.5 mL) were taken from the spinner flasks. After sedimentation of the beads, 0.3 mL of the supernatant was withdrawn. The beads were then dissolved by adding 0.8 mL protease solution (Dispase grade II; Boehringer Mannheim, Mannheim, Germany). The mixture was incubated at 37°C for 30 min to dissolve the beads, and samples were taken from the single-cell suspension and counted in a hemocytometer using the trypan blue exclusion test to determine viability. Duplicate samples were taken from each spinner flask.

Human α_2-Adrenergic Receptor Production in S115 Cells

In the first experiment, S115α_2-C2 cells were cultured in 25-cm^2 flasks in DMEM supplemented with penicillin (50 U/mL), streptomycin (50 µg/mL), 20 mM HEPES (Sigma Chemical, St. Louis, MO, USA), 20 mM $NaHCO_3$, 10 nM testosterone (Sigma Chemical), a neomycin analog, geneticin (G418) (150 µg/mL) (Sigma Chemical) and 5% heat-inactivated FBS (Harlan Bioproducts for Science, Madison, WI, USA) or 2.5%–15% Viable AC-2. For cell counting and α_2-adrenergic receptor measurements, the cells were detached with TE. The cells were counted in a hemocytometer using the trypan blue exclusion test to determine viability. Each flask was counted only once.

In the second experiment, S115α_2-C2 cells were cultured in an ACUSYST-R™ (surface area 0.8 m^2) bioreactor (Endotronics, Minneapolis, MN, USA) in DMEM supplemented in the intracapillary space with penicillin (50 U/mL), streptomycin (50 µg/mL), 20 mM HEPES, 20 mM $NaHCO_3$ and 10 nM testosterone. In the extracapillary space, the same medium was supplemented with 5% heat-inactivated FBS (Harlan) and 150 µg/mL geneticin (G418). Before inoculation of the cells, the fibers of the bioreactor were treated with FBS to remove toxins and to coat them with attachment factors. The cells (6×10^8) were inoculated into the extracapillary space and were allowed to attach without the medium flowing through the extracapillary space for 20 hours. When 5% Viable AC-2 was allowed to run through the extracapillary space, after a few days of cultivation, the cells started to detach from the fibers into the medium flow. Therefore, we replaced Viable AC-2 with 5% FBS after four days of cultivation. After another culture period of four days, FBS was replaced with Viable AC-2, after which the medium was switched every fourth day during cultivation. The total duration of the cultivation was 30 days. Alternatively, the cells were cultured continuously in 5% FBS. CO_2 was shut off after the cell density reached a level where the pH stayed stable. During cell growth, the pH was followed daily, and the glucose level was measured with BM-Test BG test strips (Boehringer Mannheim) on the Hypocount® MX glucose detector (Design Centre, London, England, UK).

α_2-Adrenoceptor Ligand Binding and Protein Determination

S115α_2-C2 cells were detached from the culture flasks and growth casettes of the bioreactor with TE to measure the concentration of adrenergic receptors as described previously (2).

Protein concentration was determined according to Bradford (4) using bovine serum albumin as a standard.

RESULTS

Cell Culture in Microplates

To determine the growth-promoting activity of FBS and Viable AC-2, 15 cell lines were cultured in the presence of the supplements for 10–14 days. Of the cell lines, LPC1 (mouse-mouse hybridoma) and X63-Ag8-653 (mouse myeloma) were anchorage-independent cells, whereas the other cell lines were anchorage-dependent. The cells were transferred directly from 10% FBS-supplemented stock cultures into Viable AC-2-supplemented media without any adaptation period.

The maximum cell numbers obtained in both supplements are shown in Table 1. The maximum density of Hep-2, HeLa, HaCaT, Vero, CHO-K1, X63-Ag8-653, LPC1, HOS, KHOS-240S and KHOS-312H cell lines in 8% Viable AC-2 was 65%–123% of that in 10% FBS, and the maximum density of KHOS-NP, MNNG/HOS, MG-63 and 3T3 cell lines in 8% Viable AC-2 was 35%–65% of that in 10% FBS. Figure 1 shows the growth curves of HaCaT and KHOS-312H cells that grew in 8% Viable AC-2 as well as in 10% FBS. MNNG/HOS required a lag period of five days after seeding from 10% FBS into 8% Viable AC-2 before its growth started (Figure 2). Only the growth of G-292 remained low in Viable AC-2 (Figure 2). On the other hand, the G-292 cells were still alive on the last counting day, which indicates that Viable AC-2 did not support cell proliferation.

Cell Culture in Spinner Flasks

To study the use of Viable AC-2 in a suspension cell culture with continuous stirring, CHO-K1 cells were cultured on microcarriers in spinner flasks for 10 days. The results show that 8% and 15% Viable AC-2 supported the growth of CHO-K1 cells significantly better than 10% FBS (Figure 3). The maximum cell density in 8% and in 15% Viable AC-2 was 61% and 71%, respectively, higher than that in 10% FBS.

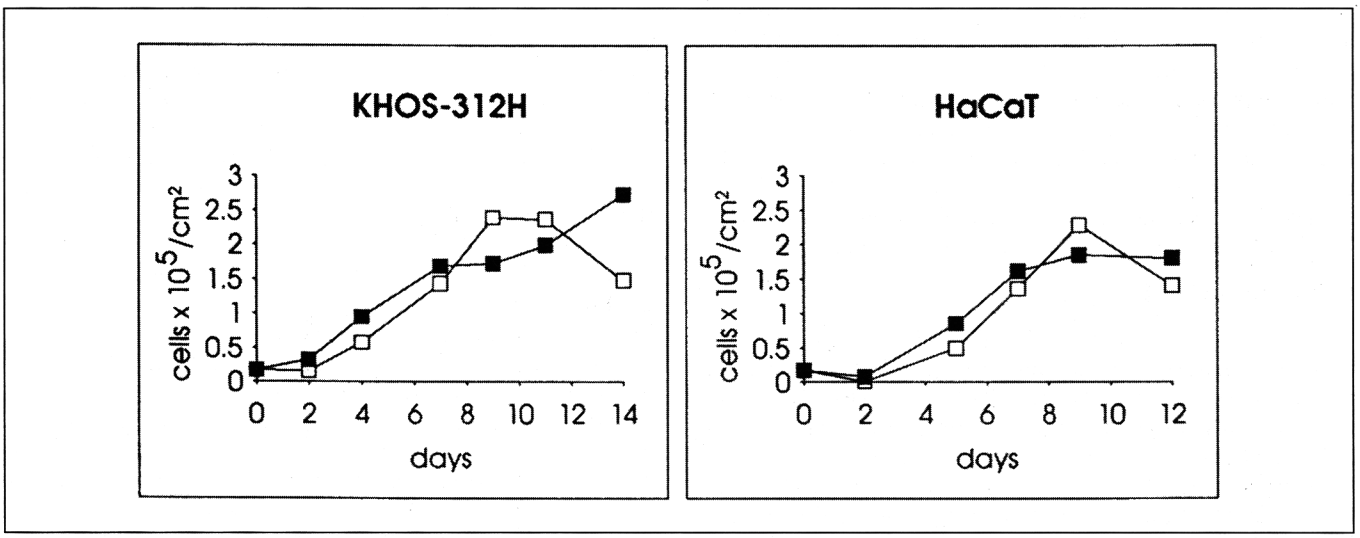

Figure 1. KHOS-312H and HaCaT cells, grown in 10% FBS, were detached with TE and plated in the test media, 8% Viable AC-2 (□) or 10% FBS (■), in 24-well microplates at a concentration of 17 200 viable cells/cm² and incubated for 12–14 days.

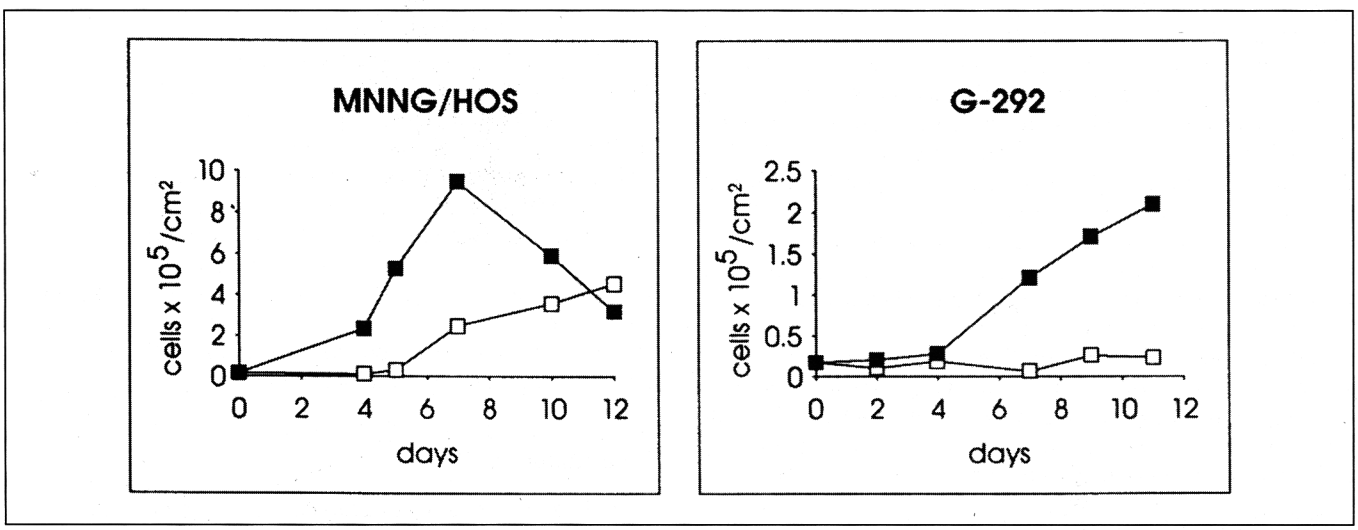

Figure 2. MNNG/HOS and G-292 cells, grown in 10% FBS, were detached with TE and plated in the test media, 8% Viable AC-2 (□) or 10% FBS (■), into 24-well microplates at a concentration of 17 200 viable cells/cm² and incubated for 11–12 days.

Table 1. Maximum Cell Numbers Obtained in 10% FBS and 8% Viable AC-2 Supplemented Media

	FBS	Viable AC-2	
Cell Line	Max. cell number (cells/cm^2)	Max. cell number (cells/cm^2)	%[a]
KHOS-312H	273 000	239 000	88
KHOS-240S	158 000	132 000	84
HOS	383 000	265 000	69
KHOS/NP	450 000	249 000	55
MNNG/HOS	944 000	452 000	48
MG-63	268 000	101 000	38
G-292	210 000	26 000	12
LPC1	1 170 000[b]	890 000[b]	76
X63-Ag8-653	950 000[b]	765 000[b]	81
CHO-K1	1 100 000	1 140 000	104
3T3	923 000	483 000	52
HeLa	497 000	368 000	74
Vero	1 250 000	1 160 000	93
HaCaT	185 000	228 000	123
Hep-2	865 000	825 000	95

[a](cell number in Viable AC-2/cell number in FBS) × 100%
[b]Maximum cell number/mL

Growth and Expression of Adrenergic Receptors of S115α_2-C2 Cells in Cell Culture Flasks

The growth of S115α_2-C2 cells in 2.5%–15% Viable AC-2 and 5% FBS is shown in Figure 4. The optimum concentration of Viable AC-2 was 5%, whereas in 10%–15% Viable AC-2, the cells started to lose their fibroblastic form and resembled epithelial cells. The expression of adrenergic receptors in S155α_2-C2 cells in 2.5%–15% Viable AC-2 was at the same level as that in 5% FBS (Table 2).

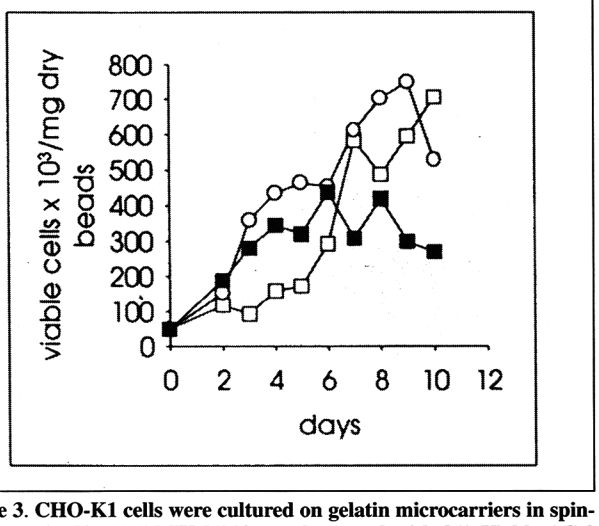

Figure 3. CHO-K1 cells were cultured on gelatin microcarriers in spinner flasks in 50 mL DMEM-F12 supplemented with 8% Viable AC-2 (□), 15% Viable AC-2 (○) or 10% FBS (■). The initial cell number was 0.5×10^5 viable cells per mg dry beads. Half of the medium was changed daily.

Growth and Expression of Adrenergic Receptors of S115α_2-C2 Cells in the ACUSYST-R Bioreactor

In the bioreactor, the cells cultured in 5% Viable AC-2 started to detach from the fibers after four days of cultivation. When Viable AC-2 was replaced with 5% FBS, the cells attached again. This indicated that the amount of attachment factors in 5% Viable AC-2 was probably not high enough to keep the cells attached on the fibers in the medium stream of the bioreactor. In cell culture flasks without medium flow, the cells grew well even in 2.5% Viable AC-2 alone. It should be noted that the amount of BS in 5% Viable AC-2 is only 0.6%. Long-term cultivation of the cells in the bioreactor was, however, possible when Viable AC-2 was replaced periodically with 5% FBS.

After 30 days of cultivation, the total amount of protein produced by S115α_2-C2 cells in 5% Viable AC-2, 5% FBS and in 5% FBS alone was 2.1 g and 2.0 g, respectively, whereas the expression level of the α_2-C2 receptor in 5% Viable AC-2, 5% FBS and in 5% FBS alone was 5.9 pmol/mg protein and 5.2 pmol/mg protein, respectively. This indicates that switching the media did not have any effect on receptor production.

DISCUSSION

The traditional medium for culturing mammalian cells is FBS, but there is an increasing demand for alternative supplements. Several FBS substitutes are available and most have been modified for cultivation of hybridoma cells (19,20). However, most of the other cell types are anchorage-dependent and therefore require additional attachment factors like fibronectin and collagen. Purified attachment factors, excluding collagen, are expensive, which usually makes their use in large quantities in cell culture media economically inappropriate. We have also made attempts to eliminate serum in Viable AC-2 through the addition of purified fibronectin and collagen, but in these experiments the test cells (CHO-K1) did not attach well (data not shown). Different cell types probably require a different set of attachment factors and/or protease inhibitors, which together are essential for cell growth. The aim of this study was to develop an FBS substitute that could be used for a variety of different cells; therefore, we used adult bovine serum as a source for the attachment factors.

In this paper we have shown that a combination of a growth factor-rich fraction of bovine colostrum and adult bovine serum supports the growth of several different types of anchorage-dependent cells. In addition, this mixture supports the production of a model recombinant protein, human α_2-adrenergic receptor subtype C2, as well as FBS. The cell lines were transferred directly from FBS-supplemented medium into Viable AC-2-supplemented medium without any weaning period. Of the cell lines tested, MNNG/HOS, MG-63, KHOS/NP, HaCat and S115α_2-C2 cells displayed a lag period of 2–5 days before their growth started. This indicated that adaptation of these cell lines to Viable AC-2 might have led to even better growth.

The UF used in Viable AC-2 was prepared as described previously (22,23). Briefly, casein was removed from defatted colostrum by acid precipitation at 56°C following the addition of sufficient 2 M HCl to adjust the pH to 4.6. The pH of the cleared whey was adjusted to 7.0 with 4 M NaOH before filtration through ultrafiltration plates with 100 kDa of nominal molecular weight cutoff (Millipore, Bedford, MA, USA). Undiluted Viable AC-2 contains an optimal composition of both UF (87%) and BS (13%) for the culture of Vero and CHO-K1 cells (21). In the ACUSYST-R bioreactor, S115α_2-C2 cells obviously require higher amounts of additional attachment factors than there are in 5% Viable AC-2. When the cells, cultured in 5% Viable AC-2, started to detach from the fibers of the bioreactor, the medium was periodically replaced with 5% FBS, which made it possible to perform long-term cultivation. On the other hand, the cells grew well even in 2.5% Viable AC-2 cell culture flasks. By increasing the amount of BS in 5% Viable AC-2, the cells should grow well in the bioreactor also. However, it should be noted that switching of the medium during the bioreactor cultivation did not have any significant effect on the recombinant protein production compared with that in 5% FBS.

It is generally known that the best natural cell culture supplement is FBS. Adult bovine serum and calf serum are usually less effective. There are also many other "synthetic" FBS substitutes available, but many of them are designed for cultivation of specialized cell types like hybridomas (19,20). Development of completely defined media has been difficult, since animal cells require numerous different components to be able to grow in vitro. It might be possible to enhance the growth-promoting activity of adult bovine serum by adding purified growth factors, but, in practice, this is very

Table 2. Expression of Adrenergic Receptors in FBS and Viable AC-2 Supplemented Media

Sample	α_2-C2 Receptor Expression (pmol/mg protein)
5% FBS	4.0
2.5% Viable AC-2	3.7
5% Viable AC-2	4.5
8% Viable AC-2	4.7
10% Viable AC-2	4.1
15% Viable AC-2	4.4

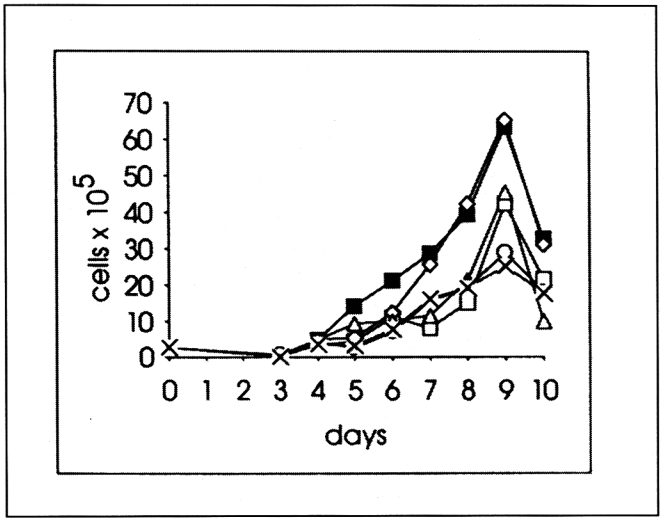

Figure 4. S115α_2-C2 cells, grown in 5% FBS, were detached with TE and seeded in the test media, 2.5% (□), 5% (◊), 8% (Δ), 10% (○), 15% Viable AC-2 (×) or 5% FBS (■), into 25-cm^2 cell-culture flasks at a concentration of 2.5 × 10^5 cells per flask and incubated for 10 days.

expensive. A natural source of bovine growth factor is colostrum. It can be collected and processed in large quantities at a moderately low price, just like adult bovine serum, since one cow produces several liters of colostrum and serum. In contrast, less than one liter of FBS can be collected from one bovine fetus, which makes collection and quality control of large FBS batches very complicated. The good supply of colostrum and adult bovine serum also reduces fluctuations in quality. For example, it should be relatively easy to collect the raw materials from farms that are free of viral diseases such as bovine viral diarrhea (BVD) or infectious bovine rhinotracheitis (IBR) viruses. The above-mentioned facts make the use of serum and colostrum also economically feasible.

Some attempts have been made to culture cells in media supplemented with normal bovine milk or its fractions. Although fractions of bovine milk have been shown to stimulate cell growth (1,6), normal milk is significantly less effective than colostrum, probably because of its lower growth factor content (15,25,30). This is also supported by the finding that a small amount of additional FBS (0.5%) was required in long-term cell cultures in a medium supplemented with fractions of normal bovine milk (6). One report describes a protein-rich fraction prepared from milk that was successfully used in long-term storage of hybridoma cells at -80°C (8). Further studies are required to test the use of Viable AC-2 as a storage medium.

In summary, the results in this paper show that a mixture of a growth factor-rich fraction of bovine colostrum and adult bovine serum (Viable AC-2) provides an attractive alternative to FBS as a growth-promoting supplement for mammalian cells.

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