IN VITRO Vol. 19, No. 11, November 1983


# A SERUM-FREE MEDIUM THAT SUPPORTS THE GROWTH OF PISCINE CELL CULTURES

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(Received March 21, 1983; accepted June 27, 1983)

## SUMMARY

An undefined, serum-free medium was developed for use with fish cell cultures. Lactalbumin hydrolyzate, trypticase-soy broth, Bacto-peptone, dextrose, yeastolate, and polyvinylpyrrolidone were initially combined in 100 ml of distilled $H_2O$, autoclaved, and added to 5% of the final volume of Medium 199. In addition, filter sterilized bovine pancreatic insulin, glutamine, and nonessential amino acids were added to the medium. The addition of insulin was observed to be unnecessary. Five fish cell lines [goldfish-derived CAR cells, fathead minnow (FHM) cells, epithelioma papillosum cyprini (EPC) cells, chinook salmon embryo (CHSE-214) cells, and a new cell line from goldfish air bladders (ABIII)] were all capable of growth in the serum-free medium at rates equivalent to cells grown in fetal bovine serum (FBS). The morphology of all cell lines, except CHSE-214 cells, was identical to cells grown in FBS. All cell lines were capable of long-term growth in the serum-free medium. The CAR, ABIII, EPC, and CHSE-214 cells in the serum-free medium supported the replication of goldfish virus-2 at levels equivalent to cells grown in FBS.

*Key words:* serum-free medium; fish cell culture; goldfish virus-2.

## INTRODUCTION

Numerous formulations for serum-free media have been developed in recent years; the majority have been used for mammalian cell cultures (1-8), with some developed for use in invertebrate cell culture (9,10). The removal or reduction of serum in cell culture media may eliminate a number of potential problems associated with serum use. Differences between lots of serum exist, which may alter the reproducibility of experiments performed in cell culture (11,12). It has been proposed by Sato (13) that one of the major roles of serum is to provide groups of hormones for cell growth; if differences exist in batches of serum the supplementation of hormones would also be variable, leading to alteration of cellular function. Serum may also be a possible source of contamination with viruses (14,15) or mycoplasmas (16).

The media and sera utilized for growth of fish cell cultures are basically the same as those for many mammalian cells (17). These similarities between mammalian and piscine cell culture systems have encouraged us to adapt a serum substitute, previously developed for mammalian cells (18), for use in fish cell cultures. To our knowledge no such serum substitute for piscine cell culture has been reported previously.

In this report we describe a serum substitute adapted for use with a variety of fish cell lines. This substitute is comprised of autoclavable digests, with or without the addition of bovine pancreatic insulin. The serum substitute described allows for long-term cultivation of five fish cell lines, with growth rates comparable to cells grown in fetal bovine serum (FBS). Cells grown in this serum substitute are also capable of supporting viral replication at levels comparable to serum-grown cells.

## MATERIALS AND METHODS

*Cells.* Goldfish-derived CAR cells (ATCC CCL 71) and fathead minnow (FHM) cells (ATCC

CCL 42) were obtained from the American Type Culture Collection, Rockville, MD. Epithelioma papillosum cyprini (EPC) and chinook salmon embryo (CHSE-214) cell lines were a generous gift of Dr. F. Hetrick (University of Maryland, Baltimore, MD). Air Bladder III (ABIII) cells, an uncharacterized cell line derived from the air bladder of an adult goldfish, were initiated by T. Yano in this department. Cells CAR, ABIII, and EPC were incubated at 24° C, whereas CHSE-214 cultures were incubated at 15° C and FHM cells at 30° C.

*Culture medium containing serum.* All cell lines were grown routinely in Medium 199 supplemented with 10% FBS, 2 m*M* glutamine, and 0.1 m*M* nonessential amino acids. Medium also contained penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml).

The CAR cells were tested for the ability to grow in Medium 199 supplemented with 2, 5, or 10% horse, newborn bovine, calf, or human serum. Other media components remained the same as described for FBS-containing media.

*Medium containing serum substitute (SSTS-199-4).* For serum-free growth of cells, Medium 199 was supplemented with 5% serum substitute and 0.02 mg/ml of bovine pancreatic insulin, plus the other supplements for FBS-media described above, and this media was termed SSTS-199-4. The serum substitute consisted of: 1 g lactalbumin hydrolyzate (GIBCO, Grand Island, NY); 3 g trypticase-soy broth (Difco Laboratories, Detroit, MI); 5 g Bacto-peptone (Difco); 1 g dextrose (Difco); 1 g yeastolate (Difco); 3.5 g polyvinylpyrrolidone (average mol wt of 360,000) (Sigma Chemical Co., St. Louis, MO); and 100 ml of deionized water. This solution was autoclaved for 15 min at 15 pounds per square inch and stored at 4° C. A stock solution of bovine pancreatic insulin (2 mg/ml) was prepared in 0.1 *N* HCl and stored at −10° C. Insulin was added to media by filtration through a 0.22 μm filter.

*Culture methods.* Transfer of cultures from FBS to SSTS was performed as follows: Cells were grown to confluency in FBS media, rinsed three times with Hanks' balanced salt solution (HBSS), and SSTS-199-4 was added. After 5 d incubation in SSTS-199-4, cells were removed from flask by trypsinization. Trypsin was removed by centrifugation of cells at 1000 rpm for 10 min. Cells were then dispersed in SSTS-199-4 at a ratio of 2:3. Cells were subsequently passaged when confluent (usually at intervals of 1 to 2 wk) at split ratios of either 1:2 or 2:3. Medium was not changed between passages.

*Determination of growth rates.* After a minimum of 1 month of cultivation in the absence of serum, $1 \times 10^6$ cells were planted in 5 ml of SSTS-199-4 in 25 $cm^2$ flasks. Control flasks, consisting of cells continuously cultivated in FBS, were planted with the same cell numbers. At intervals (2, 4, 7, and 10 d), duplicate cultures were rinsed with HBSS, trypsinized, and counted in a hemocytometer with trypan blue dye. The only exception to the above procedure was CHSE-214 cells, which grew in suspension after the 2nd passage. The CHSE-214 cells were counted by removing media with cells and directly counting in a hemocytometer.

*Virus susceptibility studies.* The CAR, ABIII, EPC, FHM, and CHSE-214 cells grown in SSTS-199-4 (for 9, 6, 3, 3 months and 1 passage, respectively), or cells grown continuously in FBS, were planted in 24 well trays at a density of $1 \times 10^5$ cells/well. Twenty-four hours later media were removed. Cultures were rinsed with HBSS, and 0.5 ml of goldfish virus-2 (GFV-2) was added to each culture at a multiplicity of infection of 10. GFV-2 is a recently described iridovirus originally isolated from healthy goldfish (19). Virus was allowed to adsorb for 2 h. Before adding the growth media, the inocula were removed and cultures were rinsed with HBSS and incubated at the respective temperatures described above. At 4 h and 1, 2, 3, 4, 5, and 6 d postinfection duplicate cultures were harvested for titration of infectious virus as follows: Media were removed, cultures were rinsed with HBSS, and cells were trypsinized. The medium, HBSS, and cells from each flask were recombined and stored at −70° C for viral assay. Assay of infectious virus was performed by an endpoint dilution microtiter system (19) and titers were determined by the method of Reed and Muench (20).

## RESULTS

*CAR cell growth in serum-supplemented medium 199.* The CAR cells were given media with fetal bovine, horse, newborn bovine, calf, or human serum after the cells had reached confluency; the results are shown in Table 1. Media with 2 to 10% FBS was supportive of maintenance and replication with constant fibroblastic morphology. Cells grown in 2% FBS were observed to replicate at a slightly slower rate but major differences were not noted.

Media with horse, newborn bovine, calf, or human serum did not support the replication of CAR cells (Table 1). At 10% levels, CAR cells did not survive with any of the above sera. At 2 and 5%, newborn bovine, calf, and human sera did not support survival of CAR cells. Cells with these supplements were observed to accumulate granules and eventually detach from the surface. Two percent horse serum was capable of CAR cell maintenance and rare cellular division, and cell morphology was epithelioid rather than fibroblastic. Two percent horse serum plus 8% lactalbumin hydrolyzate added to the media restored the fibroblastic nature of CAR cells and supported short-term maintenance. The addition of 1% newborn bovine serum to media containing 10% of the serum substitute developed by Lasfargues et al. (18) supported CAR cell maintenance and replication. No other combinations of sera were found to be supportive of CAR cells.

*Growth rates of CAR, EPC, FHM, ABIII, and CHSE-214 cell lines in SSTS-199-4.* The growth rates of all five fish cell lines in medium with 5% SSTS-199-4 were comparable to those obtained in medium with 10% FBS (Fig. 1). The CAR cell line with FBS had tripled in cell numbers by Day 5, after which little increase was noted. In SSTS-199-4, the CAR cells had doubled by Day 5 and continued rapid growth through Day 7. By Day 10 the cell numbers had more than tripled and reached a population equivalent to FBS-grown cells.

The FHM cell numbers were observed to double by Day 2 in medium with FBS, and the cell population continued to increase through Day 10. By comparison, FHM cells grown in SSTS-199-4 did not double by Day 2, but by Day 7 obtained equivalent cell numbers as those grown in FBS. The FHM cells continued growth in SSTS-199-4 through Day 10.

The EPC cell line, in FBS, was observed to grow rapidly through the entire 10 d period, reaching a population nearly 10-fold greater than initially planted. In contrast, the EPC cells grown in SSTS-199-4 did not increase in number until after Day 2, followed by rapid growth through Day 10, reaching a population comparable to FBS-grown cells.

The uncharacterized goldfish air bladder cell line, ABIII, grew in both FBS and SSTS 199-4 from Day 2 and reached nearly equivalent cell numbers by Day 10. The CHSE-214 cell line, grown in FBS or SSTS-199-4, grew rapidly through Day 7, reaching equivalent numbers by Day 10.

*Cellular morphology in SSTS-199-4.* The CAR, FHM, EPC, and ABIII cells all grew to monolayers in SSTS-199-4 by Day 10. The morphology

TABLE 1

EFFECTS OF VARYING THE SUPPLEMENTATION IN MEDIUM 199 ON THE GROWTH OF CAR CELLS

| Supplementation | Maintenance of CAR Cells[a] | Replication of CAR Cells[b] | Morphology of CAR Cells[c] |
|---|---|---|---|
| FBS | | | |
| 2, 5, or 10% | ++ | ++ | fibroblastic |
| Horse serum | | | |
| 2% | + | +/— | epithelioid |
| 5 or 10% | — | — | DNS[d] |
| Human, calf, or newborn bovine serum | | | |
| 2, 5, or 10% | — | — | DNS |
| 2% Horse serum plus 8% lactalbumin hydrolyzate | + | + | fibroblastic |
| 10% SSK[e] | — | — | DNS |
| 1% Newborn bovine serum plus 10% SSK | + | + | fibroblastic |
| SSTS-199-4 | | | |
| 5% | ++ | ++ | fibroblastic |
| 10% | +/— | +/— | epithelioid, granular, DNS |
| Insulin, 0.02 mg/ml | + | — | granular, DNS |
| Medium 199 alone | + | — | granular, DNS |

[a] ++, Ability to maintain cells without any degeneration as in FBS controls; +, maintenance with some degeneration, no longer than 2 wk; —, no ability to survive.

[b] ++, Ability to increase in cell numbers as in FBS controls; +, some replication without reaching a confluent monolayer; —, no replication.

[c] CAR cells are normally fibroblastic in FBS.

[d] DNS, Did not survive, cells started as a monolayer at the time of supplementation.

[e] SSK, Serum substitute developed by Lasfargues et al. (18).

of these cell lines was identical to FBS cultivated cells. The CHSE-214 cells in FBS remained attached to the growth surface throughout the studies, whereas CHSE-214 cells in SSTS-199-4 did not attach to the surface of the growth vessel after the 2nd passage in SSTS-199-4. The CHSE-214 cells continued to grow in suspension for the remainder of the study. On numerous occasions CHSE-214 cells were transferred to SSTS-199-4 with the same result.

*Effects of altering SSTS-199-4.* Medium prepared with 10% SSTS did not support the growth of any of the cell lines in this study. Cells in the presence of 10% SSTS accumulated cytoplasmic granules, and attempts to passage these cells were unsuccessful.

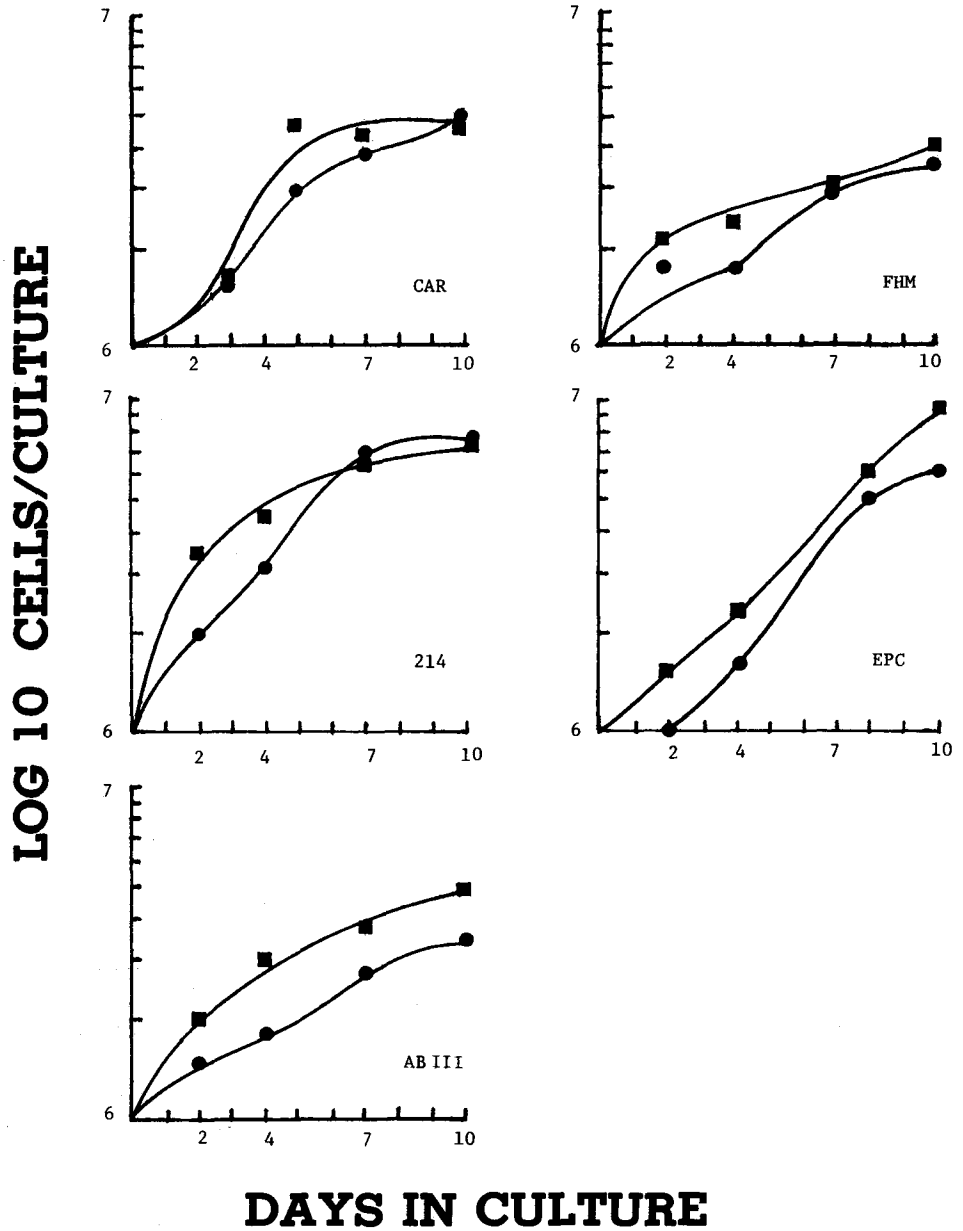


FIG. 1. Comparative growth rates of five piscine cell lines in Medium 199 supplemented with 10% FBS (■) or 5% SSTS (●). For procedures *see* Materials and Methods.

TABLE 2

CONTINUOUS GROWTH OF FIVE FISH CELL LINES IN SSTS-199-4

| Cell Lines[a] | Months in SSTS-199-4[b] | Number of Passages |
|---|---|---|
| CAR | 9 | 20 |
| ABIII | 6 | 15 |
| EPC | 3 | 5 |
| FHM | 3 | 5 |
| CHSE-214[c] | 3 | 5 |

[a] CAR, ABIII, EPC, and FHM cell lines have retained morphology identical to that observed in FBS grown cells.

[b] All cell lines are continuing growth beyond data shown.

[c] CHSE-214 cells have grown only in suspension after the second passage in SSTS-199-4.

Neither the deletion of insulin from SSTS-199-4 nor its addition to media containing FBS altered the growth rates of any of the cell lines studied. All cell lines were capable of at least three passages in SSTS-199-4 without insulin although no further passages were attempted.

*Long-term cultivation of cell lines in SSTS-199-4.* All cell lines in this study were cultivated continuously in SSTS-199-4 for extended periods of time (Table 2). Thus far, CAR cells have been grown in SSTS-199-4 for 9 months (20 subcultures); ABIII cells in SSTS-199-4 have grown continuously for 6 months (15 subcultures); and EPC, FHM, and CHSE-214 cells have each grown for 3 months (5 subcultures). The morphology of each type of cell, with the exception of CHSE-214 cell line, has remained identical to cells grown in FBS. The CHSE-214 cells have continued to grow in suspension in SSTS-199-4 after the second passage. There has been no indication of senescence in any of the cell lines.

*Susceptibility of cells grown in SSTS-199-4 to GFV 2.* The CAR, ABIII, EPC, and CHSE-214 cells grown and infected in SSTS-199-4 with GFV-2 supported the replication of GFV-2 at levels comparable to FBS-grown cells (Fig. 2). The GFV-2 initially replicated in CAR and EPC cells at a slightly more rapid rate in SSTS-199-4 than in Medium 199 with FBS, but the final virus yield was higher in Medium 199 with FBS in CAR and EPC cells (Fig. 2). The CHSE-214 cells replicated GFV-2 at a slightly more rapid rate throughout the study and the final virus yield was greater in Medium 199 with FBS (Fig. 2). The FHM cells were found to be completely refractory to GFV-2 infection. The cytopathology of the GFV-2 infection was observed to be identical in both media.

## DISCUSSION

The CAR cells were not capable of growth in media supplemented with 10% horse, newborn bovine, calf, or human serum. This observation may be due to the presence of a factor(s) in the serum that is toxic to CAR cells, or these sera may be lacking some nutritional requirement(s) for CAR cell survival. The ability of 2% horse serum to support short-term maintenance of CAR cells might indicate the former possibility. The ability of 2% horse serum plus 8% lactalbumin hydrolyzate to maintain and replicate CAR cells

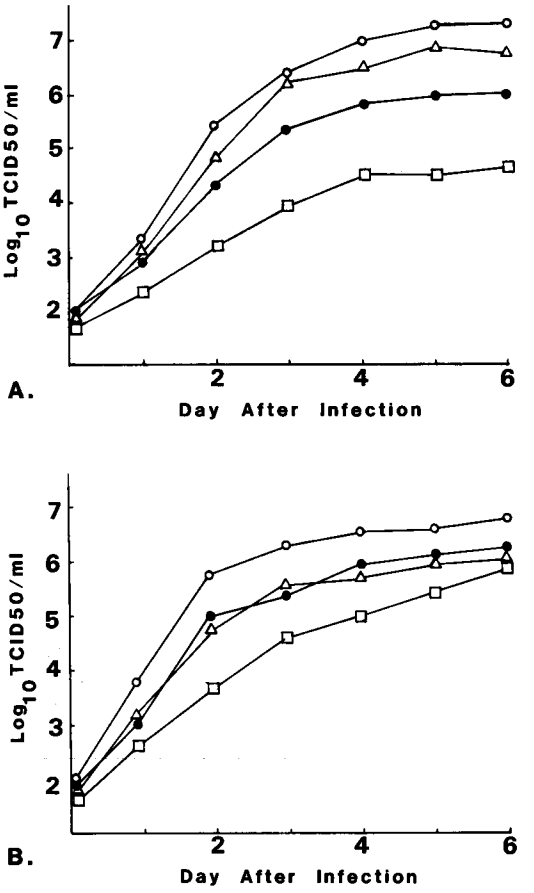


FIG. 2. *A*, Compatative replication of GFV-2 in CAR (○), ABIII (△), EPC (●), and CHSE-214 (□) cells in Medium 199 supplemented with 10% FBS. For procedures *see* Materials and Methods. *B*, Comparative replication of GFV-2 in CAR (○), ABIII (△), EPC (●), and CHSE-214 (□) cells in SSTS-199-4. For procedures *see* Materials and Methods.

indicates the latter possibility. Both possibilities are supported by the observation that 1% newborn bovine serum plus 10% serum substitute-SSK (18) supported growth of CAR cells when neither component alone would support growth or maintenance.

Cell growth rates for fish cell lines in medium with a 5% serum substitute were comparable to those obtained for cells grown in 10% FBS. The cell lines utilized represent cells derived from normal tissue (CAR, FHM, CHSE-214, and ABIII), and abnormal tissue (EPC). It seems that SSTS-199-4 may be useful for various types of piscine cells. In addition, SSTS-199-4 supported long-term cultivation of all the cell lines in this study. The CHSE-214 cells were the only cell line to exhibit morphological changes under short- or long-term cultivation. Although CHSE-214 cells would not attach to the surface of the culture vessel after the 2nd passage in SSTS-199-4, the growth rate of these cells was not deficient compared to FBS-grown cells. The addition of attachment factors may alter this pattern, but we have not yet explored this possibility.

This serum substitute is based upon that developed by Lasfargues et al. (18). This formulation developed by them for mammary tumor cells proved unsuitable for growth of piscine cells in our initial experiments. However, their formulation combined with 1% newborn bovine serum proved suitable for CAR cell growth. The addition of trypticase-soy broth, and the alteration of the concentrations of other components of the Lasfargues et al. (18) formulation, were essential to support growth of our piscine cultures. Other reports have described the use of soybean extracts as ingredients in serum-free medium (3,4). Deletion of polyvinylpyrrolidone resulted in the loss of cultures, which is consistent with previous reports (18,21,22). This suggests that polyvinylpyrrolidone, although inert, imparts a viscosity to the medium, thereby acting as a protective colloid, and enhances hormone activity (18).

Insulin, at the concentrations utilized in this study, was not essential for growth of fish cells for at least the first three passages. We have not attempted any longer-term cultivation of any of these cells in the absence of insulin. Insulin has been an important and necessary component of nearly all the serum-free media formulations up to this time [for review *see* (23)]. In this study, the use of enzymatic digests, yeastolate, and Bactopeptone may provide insulin, although it is unlikely that insulin would retain any activity after autoclaving (24,25). The ability of these piscine cell lines to grow without insulin may represent a difference in requirements for piscine cells compared to mammalian cells, or it may simply represent a short-term ability of these cells to grow without insulin. Medium supplemented with insulin alone maintained these cells for as long as 2 wk, but the cells were lost upon passage. This result was also obtained with Medium 199 alone.

Transferrin has also been present in most serum-free formulations for mammalian cells (25). We did not add transferrin, but it may be present in one or more of the enzymatic digests utilized in our study, although it is unlikely that transferrin activity would remain after autoclaving. Again, this could also reflect a difference in requirements of fish cell cultures.

We have found cells grown in SSTS-199-4 capable of replicating GFV-2 at levels equivalent to the replication observed in FBS-grown cells. Weiss et al. (26) reported increased production of enteric viruses with the use of a defined media and Nagle and Fine (27) reported maximum expression of mouse mammary tumor virus in cells propagated in serum-free medium. The removal of serum may provide a better system for the replication of viruses in cell cultures due to the possible contamination with inhibitory agents in the serum (14–16). The serum-free medium described here may help to reduce the variability introduced by the use of serum in fish cell culture systems. This medium would also provide substantial cost savings through the elimination of FBS and use of less expensive components.

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The authors thank Drs. F. Hetrick and J. Gabliks for providing cell lines used in this study; Dr. G. S. Jones for review of the manuscript; and S. Edmunds for preparation of the manuscript.