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The Media Used in Primary Cell Cultures of Prawn Tissues: A Review and a Comparative Study

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Abstract

Published literature were reviewed to determine the optimal medium used for the culture of penaeid prawn cells. Of the 19 publications, 11 indicated that Leibovitz's L-15 medium was the most suitable in the preparation of primary prawn cell cultures. From the literature *Penaeus monodon* has an osmotic pressure of 687 (range of 687 to 750) mOsmol·kg^{-1}. The isosmotic point for *Penaeus merguiensis,* that coexists in the same environments as *P. monodon* is 27 ppt (range 20 to 30 ppt). In culture flasks, the optimum pH for hepatopancreatocytes of *Penaeus chinensis* was 6.5 (range 6.0 to 7.2). Two media almost met these conditions, L-15 with Itami's salts and L-15 with Hsu's salts. Double strength L-15 did exceedingly well across a number of combinations excluding the heavy addition of extra salts. Experimentally, L-15 with Itami's salts performed as was expected from the theoretical literature analysis. Furthermore, the analysis of 2X L-15 suggested why so many authors have had partial success with this media. Established immortal cells that could be used as fusion partners with prawn cell lines were also exposed to different media to determine their tolerance levels to altered salinity and osmolarity conditions. In decreasing order of adaptability, insect, fish and mammalian cells survived in foreign media.

Introduction

Leibovitz's L-15 medium has been a popular choice for prawn cell culture in published literature. In a selection of 17 publications and two

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conference abstracts on prawn cell culture, the authors of 11 papers suggested that L-15 was the most appropriate choice for use with a variety of prawn tissues in cell culture. Similarly, Walton and Smith (1999) used L-15 for the production of primary crab cell cultures.

These authors, together with the authors of five of the 11 prawn cell culture papers, selected L-15 in preference to other media trialed at the same time (Nadala et al. 1993; Hsu et al. 1995; Tong and Miao 1996; Fraser and Hall 1999; Owens and Smith 1999). Authors of the other six papers elected to use L-15 without comparison to other media (Chen et al. 1986; Chen et al. 1988; Chen et al. 1989; Ellender et al. 1992; Najafabadi et al. 1992; Kasornchandra et al. 1998).

Of the other eight publications, the authors of five selected M199 or a variant of this medium to support prawn cell growth (Machii et al. 1988; Ke et al. 1990; Rosenthal and Diamant 1990; Sato et al. 1998; Itami et al. 1999). However, Toullec et al. (1996) stated that M199 was best for the culture of ovarian tissues while Grace's Insect Medium was best for the culture of limb bud regeneration tissue.

Of the two remaining papers, Luedeman and Lightner (1992) elected Grace's Insect Medium as the most suitable in comparison with other media, while Sano (1998) used a specially prepared medium. Of the twelve publications wherein the authors stated that L-15 produced the best results with the primary cell cultures, only two used single-strength medium (Hsu et al. 1995; Walton and Smith 1999). The other ten used double-strength L-15. FBS was a common supplement, as was prawn haemolymph or homogenized prawn muscle.

The choice of growth medium is important as each medium has different characteristics with regard to pH, salinity and osmolarity. The latter is a crucial factor for prawn cell culture as the prawns, unlike many established cell lines from mammals, live in an environment with high osmolarity. This is reflected in the measurements of the osmotic pressure for different species of prawns. The osmotic pressure for adult *P. monodon* has been variously determined to be approximately 687 mOsmol·kg^{-1} (Owens and Smith 1999) and 750 mOsmol·kg^{-1} (Cheng and Liao 1986; Ferraris et al. 1986). The osmotic pressure was determined to be 800 mOsmol·kg^{-1} for *P. penicillatus* (Cheng and Liao 1986), 707 mOsmol·kg^{-1} for *P. chinensis* (Chen and Lin 1994) and 780 mOsmol·kg^{-1} for *P. indicus* (Parado-Estepa et al. 1987).

Working with juvenile prawns, Castille and Lawrence (1981) determined that the haemolymph was isosmotic to seawater at 745 mOsmol·kg^{-1} in *P. aztecus*, 768 mOsmol·kg^{-1} in *P. duorarum*, 680 mOsmol·kg^{-1} in *P. setiferus*, 699 mOsmol·kg^{-1} in *P. stylirostris* and 718 mOsmol·kg^{-1} in *P. vannamei*. Some of these figures are in conflict with those quoted by McFarland and Lee (1963) who reported that the osmotic values for *P. aztecus* and *P. setiferus* were between 830 and 850 mOsmol·kg^{-1}, which exceed those quoted by Castille and Lawrence. However, McFarland and Lee conducted their work with subadult prawns whereas Castille and Lawrence worked with juvenile prawns.

It is notable then, that common to all of the cell culture publications, including those that selected M199 or Grace's Insect Medium, with the exception of Sano (1998), is some form of reference to the osmolarity of the culture medium. However, although not specifically mentioned in the text, the recipe provided by Sano indicated that the salt content was higher than the usual for standard cell culture. For the authors using L-15, osmolarity ranged from 472 mOsmol·kg^{-1} (Hsu et al. 1995), through 675 mOsmol·kg^{-1} (Owens and Smith 1999) and 720 mOsmol·kg^{-1} (Chen et al. 1986, 1988, 1989; Kasonchandra et al. 1998; Fraser and Hall 1999) up to 760 mOsmol·kg^{-1} (Nadala et al. 1993). Walton and Smith (1999) used a significantly higher osmolarity (925 mOsmol·kg^{-1}) for their work with crab cell cultures.

Those researchers who favored M199 and its derivatives also worked with osmolarities ranging from 682 mOsmol·kg^{-1} (Itami et al. 1999), through 750 mOsmol·kg^{-1} (Luedeman and Lightner 1992; Toullec et al. 1996), to 923 mOsmol·kg^{-1} (Rosenthal and Diamant 1990). It should be noted that the latter commented on the osmolarity of their culture medium in reference to the prawns having been raised in brackishwater with very high salinity. The osmolarity of the culture medium was adjusted using a variety of sterile salt solutions ranging from the simple addition of NaCl (Hsu et al. 1995), to the more complex recipes of Chen et al. (1988, 1989), Itami et al. (1999) and Ellender et al. (1992).

Also noted in many papers was the pH of the medium. However, this was simply a record of the pH measured in the media, rather than a deliberate attempt to optimize the pH for the cell cultures. The pH was generally within the range of 7.0 to 7.4 (Machii et al. 1988; Rosenthal and Diamant 1990; Luedeman and Lightner 1992; Tong and Miao 1996; Toullec et al. 1996; Fraser and Hall 1999). The only outstanding pH was that used by Hsu et al. (1995), with the pH ranging from 7.63 to 8.10. Again, this probably reflects the unusually salty conditions for rearing prawns.

Since the major species of prawn to be used to attempt to produce cell lines in this current study was *Penaeus monodon*, data pertaining specifically to the species was targeted. For example, Owens and Smith (1999) reported the osmotic pressure of *P. monodon* to be 687 mOsmol·kg^{-1} while Cheng and Liao (1986) reported it to be 750 mOsmol·kg^{-1}. Therefore, the optimum osmotic pressure should be 719 mOsmol·kg^{-1} with a range of 687 to 750 mOsmol·kg^{-1}. No data exists for the isosmotic point of *P. monodon*.

However, the isosmotic point for *Penaeus merguiensis*, that coexists precisely in the same environments as *P. monodon*, has been calculated to be 27 ppt (Dall 1981) with an osmotic capability range from 20 to 30 ppt. No direct measurements of the pH of the haemolymph of *P. monodon* or any penaeid could be found. However, Huang et al. (1999) have calculated, in culture flasks, the optimum pH for hepatopancreatocytes of *Penaeus chinesis* as 6.5 with a suggested range of 6.0 to 7.2.

This study was conducted to determine the pH, salinity and osmolarity of various media that have been used to support prawn cell culture. Furthermore, it also aimed to investigate if there were any, observable effects on prawn cells in culture during exposure to media that was modified with

regard to salinity and osmolarity. The ultimate aim of this project was to develop an immortal prawn cell line using cell hybridization technology. Therefore, there was the need to identify a medium that would be appropriate for both the primary prawn cells and for the hybrid cells. As the hybrid cells were going to be formed using existing immortal cells such as fish, moth and mouse myeloma cell lines, the medium shoud be able to compromise between the requirements of the two vastly different parental cells used in the fusions. Hence, further experiments were conducted using the established immortal cell lines in variously modified growth media.

Materials and Methods

Preparation of the salt solutions

The salt solutions were prepared as published. For Chen's salt solution, to 500 mL of distilled water, the following salts were added: 51.2 g NaCl, 0.9 g KCl, 5.4 g $MgSO_4 \cdot 7H_2O$, 5.9 g $MgCl_2 \cdot 6H_2O$ and 2.55 g $CaCl_2 \cdot 2H_2O$ (Chen et al. 1988, 1989). The working solution was prepared by adding 5 mL of salt solution per 100 mL total medium volume (Najafabadi et al. 1992).

For the AKN salt solution (Ellender et al. 1992), a double strength solution containing 51.2 g $C_6H_5Na_3O_7 \cdot 2H_2O$, 14.7 g $MgCl_2$ and 13.5 g $CaCl_2$ in 500 mL was prepared. This solution was used as an additive of 10 ml per 100 mL total medium volume. The published recipe used 20% of a 1X solution added to the growth medium.

With Itami's salt solution, the published recipe was designed in such a way that the salts are added as the medium is being made (Itami et al. 1989; Itami et al. 1999). As a compromise, a 10X solution was prepared and used as an additive with 10 mL being added per 100 mL total volume of medium. To prepare the 10X solution, to 500 mL of distilled water the following salts were added: 55 g NaCl, 2 g KCl, 15 g $MgSO_4 \cdot 7H_2O$, 15 g $MgCl_2 \cdot 6H_2O$, 4.5 g $CaCl_2 \cdot 2H_2O$ and 0.25 g $NaH_2PO_4 \cdot 2H_2O$.

Hsu et al. (1995) adjusted their media to 735 $mOsmol \cdot kg^{-1}$ with the addition of 15 g NaCl per liter of medium. The Hsu's salt solution used in the present study consisted of a 10X solution prepared using 75 g NaCl per 500 mL. The working strength required the addition of 10 mL of salt solution per 100 mL total volume.

Synthetic sea salt powder (Ocean Nature®, Aquasonic, Australia) was mixed with distilled water to make 1X, 2X and 5X seawater solutions. These were added at 10 mL per 100 mL final volume of media.

Growth media

Eight different growth media were tested including both standard media and specialized insect cell culture media. The standard media included Commonwealth Serum Laboratories version of Dulbecco's Modification of Eagles Medium (CSL) as described in their publication Cell Focus (Vol.1 (1) July

1986), Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco BRL/Life Technologies), Leibovitz's L-15 (L-15) (Gibco BRL/Life Technologies), Rosewall Park Memorial Institute (RPMI) (Multicel/Trace) and Bio-Rich 1 (BR1) (ICN Biomedicals). The insect media included ExCell™ 401 (ExCell™) (JRH Biosciences), Sf-900 II SFM (Sf900) (Gibco BRL/Life Technologies) and TC-100 (Gibco BRL/Life Technologies). All media with the exception of the specialized serum-free insect media, Sf900 and ExCell™, were supplemented with 10% foetal bovine serum (FBS) (Trace Biosciences) where noted.

The media were prepared according to the manufacturer's instructions. The solutions were filter-sterilized under positive pressure using a peristaltic pump (Masterflex® easyload® model 7518-00, Cole Parmer Instrument Co.) and MediaKap®-2 0.2mm hollow fibre media filters (Microgon). Antibiotics were added to a final concentration of penicillin 200 units·mL^{-1}, streptomycin 200 µg·mL^{-1}, kanamycin 80 µg·mL^{-1} and polymyxin B 5 µg or 30 units·mL^{-1}. Fungizone® (amphotericin B) was also used at a final concentration of 2.6 µg·mL^{-1}.

Measurements

Measurements of pH were made using a pH 20 Meter (ATI Orion). Osmolarity was measured using a 5500 Vapor Pressure Osmometer (Wescor). Salinity was measured using a Salinity Refractometer (Atago Co., Ltd.). Measurements were made of the media as well as of four tanks in which the prawns were held prior to dissection.

CELL LINES

The established immortal cell lines used in tests included the fish cell lines Bluegill Fry (BF2) and Fathead minnow (FHM), the moth cell lines Sf9 (derived from the army fall worm Spodoptera frugiperda) and High-Five™ (derived from the cabbage looper moth Trichnoplusia ni), and the mouse cell lines Sp2/0 and NS1. The cells were cultured in their normal media, this being DMEM or L-15 supplemented with 5 to 10% FBS for the BF2 and FHM cells, either TC-100 supplemented with 10% FBS or in Sf900 or ExCell™ without FBS supplementation for the moth cells, and CSL supplemented with 10% FBS for the mouse cells. Only the BF2 and FHM cells are attached, anchorage-dependent cells. These cells required treatment with trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA, w/v with antibiotics and Fungizone® as per cell culture medium) to remove them from the plastic surface of the flasks.

PRIMARY PRAWN CELL CULTURE

Primary cell cultures of prawn cells were set up using mature prawns obtained from grow-out farms in northern Queensland. Generally, the *Penaeus monodon* prawns weighed around 20 g, while "spawner prawns", when available, weighed around 50 g. The prawns were transported from

the farms in a 500 L tank that was aerated slowly with bubbled oxygen. At the laboratory, the prawns were held in 1000 L Reln tanks, filled to 400 L, that were fitted with trickle-tower filtration and aerators. Water changes were conducted as required using seawater collected from the local seashore. The prawns were fed on a diet of Monodon Grower Pellets (Ridley Agriproducts, Australia).

Prior to dissection, the prawns were chilled in iced seawater for 20 min and then surface sterilized by immersion in 70% ethanol for 15 min. The prawns were pinned on a foam board and the upper carapace were peeled off using sterile forceps and scissors. The organs of the cephalothorax area removed included the heart, the dorsal aorta, the haematopoietic tissue that forms membranes over the heart area, the lymphoid or Oka organs, and the hepatopancreas. The gonads were also retrieved at this time if the prawn was at an appropriate developmental stage. The exoskeleton plates covering the tail area were removed next, allowing an incision to be made along the midline of the tail muscle. The hindgut caecum could be located by following the intestinal tract towards the last tail segment. Nerve tissue was removed via a ventral incision along the underside of the tail. Samples of tail muscle were easily snipped off.

Haemocytes were collected from the heart sinus prior to dissection using a 25 gauge needle fitted to a 1 mL syringe containing 200 µL of citrate-EDTA (EDTA 0.01M; trisodium citrate 0.03 M; citric acid 0.026 M; NaCl 0.45 M; glucose 0.1M; adjusted to pH 4.6 and sterilized by filtration using a 0.2 µm Sartorius Minisart filter) (Owens and O'Neill 1997).

Thus removed, the tissues were homogenized using a Dounce ground glass homogenizer with twelve strokes of the A pestle that has a clearance of 100 µm. The dispersed cell suspensions were seeded into 24-well plates, 6-well plates and 25-cm^3 flasks containing one of the media supplemented, if required, with 10% FBS. The cultures were incubated at 28°C in a normal air incubator. Half medium changes were conducted on alternate days.

Cultures were set up on at least six occasions for the lymphoid organ and haemoctyes, five for the heart and the haematopoietic tissue, three for the hepatopancreas, aorta and tail muscle, twice for the hindgut caecum and nerve, and once each for the ovary and testis. The primary cultures were set up in a variety of different media including CSL, 2X CSL, DMEM, L-15, Bio-Rich 1, ExCell™, Sf900, TC-100 and 2X TC-100. The media were supplemented where appropriate with 10% FBS and with the different salt solutions. The cultures were observed for seven days for differences in cell appearance. Photographs were taken at various intervals, generally three hours, 24 hours, 48 hours, 72 hours, four days and six days.

Experiments with media

SWITCHED MEDIA

The normal growth media for the immortal cells were switched with the media used on other immortal cell lines. That is, mouse NS1 and Sp2/0 cells

and fish FHM cells were transferred to flasks containing the specialized insect growth medium Sf900, and the moth cells were transferred to the standard growth medium CSL that was supplemented with 10% FBS. FHM cells were also passaged into "blended" medium consisting of 50% of L-15 supplemented with 10% FBS and 50% Sf900 medium. Controls were set up with the cells in DMEM and L-15, both supplemented with 10% FBS. In addition, the mouse cells were also passaged into double-strength CSL medium supplemented with 10% FBS. The cells were incubated as per normal and were observed daily for signs of stress.

EFFECT OF SALT ON IMMORTAL CELLS

Experiments were conducted to determine the salt tolerance of the immortal cells by adding extra salt to their normal growth medium. The BF2, FHM and Sf9 cells were passaged in equal numbers into each of four flasks. One flask was retained as a control containing only the normal growth medium, while the other three contained media supplemented with either Chen's, Itami's or Hsu's salt solution. The cells were incubated for seven days afterwards, total cell counts and viability were determined.

EFFECT OF BIO-RICH 1 ON IMMORTAL CELLS

Bio-Rich 1 and L-15 media were compared using the established fish cells BF2 and FHM. The two types of cells were passaged into Bio-Rich 1 medium and observed after three and six days, with counts being performed on day six. The cells were counted again after 13 days. Controls were set up in L-15 medium. Both media were supplemented with 10% FBS.

Results

Salt solutions

Najafabadi's salt mixture (AKN) was to be investigated. However, the 2X mixture crystallized shortly after preparation and therefore was not used. When a 1X solution was prepared, it too precipitated out and could not be used. The synthetic sea salt solutions were also problematic with both the 2X and 5X solutions producing high levels of precipitates within days of production. Therefore, cell culture tests were conducted only on Itami's salts, Chen's salts and Hsu's salt solutions.

Parameters of different media

Examination of the osmolarity of the various media and salt solutions highlighted some combinations close to the range considered optimum for *P. monodon* (Table 1). Of note is that four media with Hsu's salts were optimum (DMEM, L-15, RPMI, Sf900) and two with Itami's salts

(ExCell, TC100). Eight other media combinations were just outside the optimum range (Table 1). Notably, 2X L-15 was acceptable with three other combinations (fresh, 10% FBS, Chen's salts).

Salinities of the media were considered optimum if they were within 2 ppt of the isosmotic point of 27 ppt. Five combinations were found to be desirable and included 2X L-15, TC-100+10% FBS, L-15 +Itami's salts, L-15 and RPMI with Hsu's salts (Table 2). Many media combinations were within the probable range (20 to 30 ppt) of capability of the intact prawn.

Only two media combinations (TC-100 +Chen's salts, TC-100 + Hsu's salts) were within 0.1 pH of the optimum as suggested by Huang et al (1999) but many were within the range of 6.0 to 7.2 pH (Table 3). Surprisingly, many media were too alkaline to be within the desired range.

Table 1. Osmolarity readings (in mOsmol·kg^{-1}) of media used in primary prawn cell cultures.

Osmolarity	Fresh	+10% FBS	+Chen's	+Itami's	+Hsu's
CSL	331	349	466	617	648
2 X CSL	612	584	—	—	—
DMEM	355	313	449	620	702*
L-15	328	359	468	676-	722*
2X L-15	684-	661-	779-	825	1110
RPMI	312	327	436	492	709*
Bio-Rich 1	269	282	370	421	556
ExCell™	395	n/a	545	726*	795
Sf900	367	n/a	522	606	739*
TC-100	378	382	509	688*	763-
2X TC-100	673-	622	769-	782-	1089

Asterisks (*) indicate media within the desired range (687 to 750) while (-) indicate media deemed to be acceptable but just outside the preferred range.
— not tested.
n/a (not applicable) – ExCell™ and Sf900 are serum-free media and not designed for supplementation with FBS.

Table 2. Salinity (in parts per thousand, ppt) of media used in primary prawn cell cultures.

Salinity	Fresh	+10% FBS	+Chen's	+Itami's	+Hsu's
CSL	20	32	37	44	44
DMEM	16	18	22	30	30
L-15	14	17	20	29*	27*
2X L-15	28*	30	32	35	40
RPMI	11	15	20	22	28*
Bio-Rich 1	11	15	18	21	24
ExCell™	33	n/a	37	45	45
Sf900	36	n/a	41	45	45
TC-100	24	28*	32	38	38
2X TC-100	42	43	46	47	53

Asterisks (*) indicate media close to the presumed isosmotic point (27 ppt) of *Penaeus monodon*.
n/a (not applicable) – ExCell™ and Sf900 are serum-free media and not designed for supplementation with FBS.

Contamination with chytrids only occurred on one occasion. Bacterial contamination of cultures was also uncommon. This could be attributed to careful dissection following the thorough surface-sterilization achieved by immersion in 70% alcohol.

The best primary cell cultures were obtained from the Oka (lymphoid tissue), the haematopoietic tissue and the haemocytes. These tissues were observed to routinely produce cell cultures that demonstrated cell attachment and survival for seven days or more. The ovary and testis also produced very good cultures, with the ovary in particular forming cultures that appeared viable for several weeks. However, these cultures could not be established on a regular basis due to the difficulty in obtaining prawns containing developed gonads.

Of the other tissues, the hepatopancreas and the heart also produced interesting cultures that contained mixtures of loose and attached cells. The heart cells were generally fibre-like and featured striations. The cells were observed to migrate out from unhomogenized chunks of tissue. However, neither of these tissues produced as dense nor as persistent cultures as the lymphoid organ and haematopoietic tissue. The hepatopancreas was also unreliable due to the presumptive presence of enzymes that discolored the medium and appeared to adversely affect the cell cultures. Cultures obtained from the dorsal aorta, the hindgut caecum, the nerve, and the tail muscle were generally very poor and contained large amounts of cell debri with few viable-looking cells.

The best results with the cultures of lymphoid cells, haematopoietic cells and haemocytes, were observed using single-strength L-15 medium, supplemented with 10% FBS and 10% Itami's salt solution (ISS). DMEM was also a very good culture medium. Neither CSL nor 2X CSL produced as good cultures as the other standard media. This may be attributed to the CSL requiring greater quantities of sodium bicarbonate to buffer appropriately.

Table 3. pH of media used in primary prawn cell cultures.

pH	Fresh	+10% FBS	+Chen's	+Itami's	+Hsu's
CSL	7.30	7.68	7.53	7.44	7.52
DMEM	8.30	7.96	7.62	7.54	7.77
L-15	7.29	7.40	7.32	7.07	7.37
2X L-15	7.10	7.25	7.22	7.15	7.20
RPMI	6.85	6.86	6.86	6.80	6.86
Bio-Rich 1	7.18	7.29	7.21	7.16	7.20
ExCell™	6.15	n/a	6.15	5.96	6.21
Sf900	6.27	n/a	6.12	6.00	6.09
TC-100	6.30	6.30	6.51*	6.34	6.43*
2X TC-100	5.70	5.78	5.76	5.75	5.75

Asterisks (*) indicate media close to the presumed optimal pH point for *Penaeus monodon*. Many media are within the range (6.0 to 7.2) suggested by Huang et al. (1999) for *P. chinensis*.

n/a (not applicable) – ExCell™ and Sf900 are serum-free media and not designed for supplementation with FBS.

The Bio-Rich 1 media did not produce any spectacular results with the prawn cell cultures despite enhancing the growth of immortal cells in other experiments. The specialized insect media did not produce as healthy-looking cultures as the standard media. This was particularly the case for the double-strength insect media and the insect media supplemented with the salt solutions. This could be attributed to the very high salinity (Table 2) in these instances, that adversely affect the cells.

HAEMOCYTE CELL CULTURE

The haemocytes were initially observed to be spherical to elliptical in shape and of a very small size. However, within three hours, about half the number of cells displayed a fibroblastic morphology. The two types of cells persisted in culture up to seven days. The cells appeared to be healthier in the L-15 medium supplemented with 10% FBS and 10% ISS when compared with the other media combinations. Interestingly, the cells in the insect media TC-100 and Sf900 did not display any fibroblastic morphology at all.

HAEMATOPOIETIC TISSUE CELL CULTURE

The haematopoietic tissue from over the heart region produced a culture that consisted initially of numerous very small spherical and elliptical cells. Within three hours, about 10% of the cells were observed to display fibroblastic morphology. As with the haemocytes, this was observed particularly in the L-15, DMEM and CSL medium, with very few cells in the specialized insect media displaying fibroblastic morphology. The cultures persisted for six days before the cells would indicate early signs of pyknosis. On several occasions, the cells were retained for five weeks, with no further discernable changes occurring over that time. The cells did not appear to replicate, nor did the pyknosis advance to give the appearance of a dead culture. Trypan blue staining indicated that the cells were viable however there was no evidence of proliferation. As before, the best medium appeared to be the L-15 supplemented with 10% FBS and 10% ISS.

LYMPHOID ORGAN CELL CULTURE

The cells of the lymphoid organ were initially spherical and uniform in size, however within three hours, the cells attached and started to adopt fibroblastic morphology. Within 24 hours, most of the cells in the culture were fibroblastic in shape. Cells were observed to be actively migrating out from the edges of any chunks of tissue present in the flasks to form a cell monolayer. Maximum confluency of about 70% was attained between 24 to 48 hours. As observed with the cultures of haemoctyes and haematopoietic tissue, the standard media L-15, DMEM and CSL produced cultures far richer in fibroblastic cells than the specialized insect media, and the tissues in the insect media did not appear to have as many cells migrating out from them. The cell cultures maintained a healthy appearance for around seven days, at

which time the cells started to show pyknosis with the fibroblastic cells beginning to look thin and stringy. Over the next three days, the monolayers were observed to deteriorate, after which the cultures were discarded. Of the media, L15 and DMEM supplemented with 10% FBS and 10% ISS produced the best monolayers of fibroblastic cells.

Experiments with media

SWITCHED MEDIA

When the NS1 and Sp2/0 mouse cells were switched from their normal growth medium of CSL supplemented with 10% FBS to the insect growth medium Sf900 the cells were initially observed to replicate rapidly. However, over seven days, the cells loosened from the plastic surface, crenated and died. The FHM cells crenated almost immediately upon being placed in insect cell culture media and did not recover. In the reverse situation, moth Sf9 cells fared much better after being transferred to CSL medium supplemented with 10% FBS. Although the cells dislodged more readily from the plastic surface during passage, the cells looked normal and healthy and continued to thrive over seven days before being passaged into their normal medium.

The FHM cells were observed to tolerate the blended media very well. There were no observable differences between the cells in the normal growth media of L-15 and DMEM and the cells in the blended media containing 50% specialized insect medium mixed with 50% normal growth medium.

The cells in double-strength CSL did not tolerate the change well. Both the NS1 and Sp2/0 cells were observed to crenate overnight, and after 48 hours, the majority of the cells in all flasks were crenated and detached from the plastic. The cells were transferred back to single-strength medium at this point. The Sp2/0 cells did not recover, but some of the NS1 cells did. Within 48 hours, colonies of cells were observed in the NS1 flask.

EFFECT OF SALT ON IMMORTAL CELLS

The addition of Itami's salt solution and Hsu's salt solution led to massive cell die off for all three cell types (Table 4). The number of live cells in the

Table 4. Total cell count per ml and percentage of live cells (BF2, FHM and Sf9) after culture in L-15 medium supplemented with salt solutions.

Cell type	Initial	Control	+Chen's salt	+Itami's salt	+Hsu's salt
BF2	3.08×10^6	7.54×10^6	2.00×10^6	4.92×10^6	4.16×10^6
	89.60%	98.28%	88.00%	6.10%	14.28%
FHM	6.27×10^6	9.76×10^6	6.40×10^6	3.52×10^6	4.16×10^6
	99.52%	100.00%	100.00%	6.82%	0.00%
Sf9	1.45×10^6	1.36×10^6	5.32×10^6	2.59×10^6	3.08×10^6
	61.38%	69.59%	64.47%	0.00%	0.00%

flasks decreased markedly. Chen's salt solution was not as harmful, since the percentage of viable cells was still high. For both the BF2 and FHM cells, the total number of cells in all treatments was low when compared to the controls. This may be attributed to the cells having a low division rate. This was not observed in the Sf9 cells, that experienced an increase in cell number in the flask containing Chen's salts as opposed to the control flask.

EFFECT OF BIO-RICH 1 MEDIUM ON IMMORTAL CELLS

After three days in Bio-Rich 1 medium, the FHM cells were observed to be growing better than the cells in L-15, with more progress towards a confluent layer of cells being evident. The cell count conducted on day six indicated a 329% increase in the number of the cells in Bio-Rich 1 medium. After 13 days growth in the medium, cell counts of replicate flasks indicated that a mean increase in cell numbers of 344% had occurred for the cells growing in Bio-Rich 1 medium.

The BF2 cells in Bio-Rich 1 medium came from two groups of cells. One group was being treated with 6-thioguanine (6TG) and the cells did not grow well. The other group was composed of the control cells and they grew very well. After transfer to Bio-Rich 1 medium, it was observed the 6TG-treated cells improved in appearance and growth rate. By contrast, there was no noticeable effect on the normal, healthy BF2 cells.

Discussion

Based on a compilation of literature, a medium that had an osmotic pressure of 719 mOsmol·kg^{-1} (range of 687-750 mOsmol·kg^{-1}), an isosmotic point of 27 ppt (range from 20 to 30 ppt) and a pH of 6.5 (range of 6.0 to 7.2) should have been close to optimum. Two media almost met these conditions, L-15 with Itami's salts and L-15 with Hsu's salts. Double strength L-15 did exceedingly well across a number of combinations excluding the heavy addition of extra salts. It was gratifying to see that experimentally, L-15 with Itami's salts performed as expected from the theoretical analysis. Furthermore, the analysis of the composition of 2X L-15 explained why many authors have had partial success with this medium.

Bio-Rich 1 is a relatively new medium in the market. It is touted to have the ability to improve cell growth. The FHM and BF2 cells were selected for the experiment with the Bio-Rich 1 medium since both were exhibiting poor health at the time. The FHM cells had been exhibiting slow growth in both DMEM and L-15 media. The BF2 cells were being treated with 6-thioguanine (6TG), that had a deleterious effect on their health. Normal, healthy BF2 cells did not exhibit any enhanced growth performance, whereas both the poorly growing FHM and the 6TG-affected BF2 cells exhibited vastly improved growth when transferred to Bio-Rich 1 medium. Thus, it would appear that Bio-Rich 1 may be useful for cells that are not growing well at the time. These results indicate that Bio-Rich 1

medium could be very useful for use with primary prawn cell cultures, as these are generally poorly growing cells. However, in combination with the salts tried herein, Bio-Rich 1 did not perform well with primary prawn cells.

With regard to the switching media experiment, it would appear that the insect cells in the mammalian medium fared much better than the mammalian cells in the insect medium. However, it is possible, that the cells could become adjusted to the medium if given a gradual change from one to the other.

Salt levels could be a key to primary prawn cell culture. Certainly, insect media have proved very popular in the literature, and these are very high in salinity and osmolarity. Also, double strength media have significantly increased salt levels. The increase in cell number observed by the Sf9 in Chen's salt could be attributed to the cells having a requirement for high salinity media. As seen in table 2, insect media is significantly saltier than standard media. On the other hand, the low tolerance of the mammalian cells to high osmolarity medium was illustrated by the rapid death of the Sp2/0 cells in the 2X CSL medium.

As pointed out by Nadala et al. (1993), the maintenance of proper pH is essential for the successful growth of cells, particularly primary cells. This further supports the findings that L-15 is the most suitable medium for prawn cells as it has strong buffering capacity, especially around the optimum pH of 6.5. Insect media are designed to operate at lower pH, that is more suited to insect cells in culture (Grace, 1982). In fact, TC-100 medium precipitates out at a pH greater than 6.2. Moreover, conversations with Dr. Dwight Lynn (Insect Biocontrol Laboratory, Beltsville) suggested that phosphate buffered media were often preferable to CO_2 buffered media for the establishment of insect cell lines. The actual buffering mechanisms of many of the media used here were protected by copyright and information was not available. However, L-15 is known to be a phosphate buffered medium. The effect of different buffers needs more serious scientific attention.

It is intriguing to note that fewer fibroblastic prawn cells grew in the two insect media compared with the other media. This observation was consistent across cultures derived from haemocytes, haematopoietic tissues and lymphoid organ cultures. Differences in salts and pH may be influencing the physical morphology and adhesion of the prawn cells or may be encouraging the proliferation of a specific cell type.

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