In Vitro Cell. Dev. Biol.—Animal 41:325–329, November–December 2005

1071-2690/05 $18.00+0.00

SELECTION OF A STANDARD CULTURE MEDIUM FOR PRIMARY CULTURE OF *LIMULUS POLYPHEMUS* AMEBOCYTES

LENKA V. HURTON, JIM M. BERKSON,[1] AND STEPHEN A. SMITH

Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 (L. V. H.), National Marine Fisheries Service RTR Unit at Virginia Tech, Blacksburg, Virginia 24061 (J. M. B.), Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 (S. A. S.), and Horseshoe Crab Research Center, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 (L. V. H., J. M. B., S. A. S.)

(Received 17 July 2005; accepted 2 September 2005)

SUMMARY

This study provides information relevant to future research aimed at producing *Limulus* Amebocyte Lysate (LAL) in vitro, which would potentially reduce the need to harvest and bleed horseshoe crabs as in the current methods of LAL production. To address the need for primary culture of horseshoe crab amebocytes, this study tested the effects of a variety of standard insect cell culture media on amebocyte morphology and viability after 7 d of maintenance. Amebocyte morphology was least altered from in vivo form in Grace's Modified Insect Medium, with no observed degranulation of cells, as compared to the other media tested. There were significant differences in amebocyte viability among the six insect cell culture media tested. Grace's Modified Insect Medium sustained viability of 77.2 ± 5.1% (mean ± standard deviation) of amebocytes, followed distantly by Grace's Insect Medium with 35.1 ± 8.7% amebocyte viability. Results indicate that Grace's Modified Insect Medium with horseshoe crab serum supplementation was the best candidate of the six media tested for future medium optimization for *Limulus* amebocyte requirements.

Key words: amebocyte culture; horseshoe crab; amoebocyte; morphology; viability; in vitro.

Free-living horseshoe crabs (*Limulus polyphemus*) are collected for the biomedical industry for the production of *Limulus* Amebocyte Lysate (LAL). LAL is used for detecting the presence of endotoxins pathogenic to humans in pharmaceutical, medical, and dental products (Mikkelsen, 1988; Novitsky, 1991). Although alternate tests exist for the detection of endotoxin, the LAL assay is the most sensitive, capable of detecting as little as 10^{-15} of a gram of endotoxin (Mikkelsen, 1988; Novitsky, 1991). The LAL test is used worldwide to protect public health, and horseshoe crabs are the only natural source of this substance.

LAL is extracted from amebocytes, the predominant blood cell type circulating in horseshoe crabs (Suhr-Jessen et al., 1989). These amebocytes contain intracellular granules filled with clotting factors (Levin and Bang, 1964) that are triggered to degranulate by the presence of Gram-negative bacterial endotoxin. This provides the basis for the LAL test, a clinically and commercially significant assay (Ding and Ho, 2001).

The manufacture and application of LAL have become widespread, but there are a number of problems inherent in producing LAL from its natural source. The biomedical bleeding process is laborious, involving collection of horseshoe crabs, transport, holding, bleeding, and return of the crabs to the ocean or, sometimes, to bait fishermen. Mortality rates of up to 20% have reportedly resulted from this process (Rudloe, 1983; Thompson, 1998; Kurz and James-Pirri, 2002; Walls and Berkson, 2003). Furthermore, biomedical bleeding facilities must comply with various regulations regarding collection and use of horseshoe crabs (HCTC, 1998; Schrading et al., 1998; ASMFC, 2001). These regulations have arisen in response to concerns of overharvest of horseshoe crabs by the commercial fishery and a potential population decline. LAL production is also restricted by the seasonal capture of horseshoe crabs (Pearson, 1980). Another important issue with naturally derived LAL is the fluctuation of its quality. There is significant variation in the sensitivity and specificity of the lysate between manufacturers, batches, and even seasons (Pearson, 1980; Frieberg et al., 1991; Ding and Ho, 2001). Development of alternative LAL sources could potentially reduce or circumvent problems associated with producing LAL from captured horseshoe crabs.

An alternative to naturally derived LAL is an in vitro culture system. Between 1979 and 1992, there were several attempts to culture amebocytes. Past studies (Pearson and Woodland, 1979; Ding et al., 1988; Chen et al., 1989; Frieberg et al., 1991; Gibson and Hilly, 1992) were either nonreproducible or yielded results conflicting with other studies. Pearson and Woodland (1979) reported to have optimized medium, pH, and temperature of culture conditions with resulting amebocyte growth. Pearson and Woodland (1979) worked with untransformed cells, and likely did not achieve any significant amebocyte replication because a mitotic index of less than 1 per 2000 amebocytes was reported by Sherman (1981), with other studies (Armstrong, 1985; Copeland and Levin, 1985) finding no mitosis present in circulating amebocytes of intermoult adults. Pearson (1980) estimated total DNA to as-

[1] To whom correspondence should be addressed at National Marine Fisheries Service RTR Unit at Virginia Tech, 100 Cheatham Hall, Blacksburg, Virginia 24061-0321. E-mail: Jim.Berkson@noaa.gov

certain growth. However, this method is not a direct measure of cell division, because DNA synthesis is not always indicative of mitosis (Mather and Roberts, 1998). Pearson and Woodland (1979) also reported to have maintained amebocytes up to 30 d, but vacuolation and degranulation became pronounced after 10 d. Ding et al. (1988) attempted to reproduce Pearson and Woodland's methods, but in the study by Ding et al. (1988), the culture did not remain viable for more than 2 wk. Other amebocyte culture studies (Ding et al., 1988; Chen et al., 1989; Frieberg et al., 1991; Gibson and Hilly, 1992) reported results from the use of various culture techniques using different media under different temperatures, pH levels, and osmolalities.

Over a decade later, Joshi et al. (2002) explored the potential of amebocyte culture using an organ culture technique. Based on information that suggested the gill flaps of the horseshoe crab are the source of amebocytes (Gibson and Hilly, 1992), Joshi et al. (2002) cultured gill lamellae in a standard insect cell culture medium, and reported amebocyte harvests for a period of 6–8 wk (Joshi et al. 2002). Renewed interest in this topic is fueled by difficulties associated with naturally produced LAL that continue to challenge the biomedical industry. Additional information on improvements in amebocyte and gill-flap culture media and techniques is still needed before commercial in vitro amebocyte production can occur.

A variety of culture media and conditions have been used individually for previous amebocyte culture studies; however, an optimal medium for amebocyte culture has not been developed. This study's objectives were to test a variety of standard insect cell culture media for their effects on amebocyte morphology and viability to identify the most appropriate medium as a base for further medium optimization.

All culture media used in this study were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). The following commercially available serum-free cell culture media were tested for their ability to maintain viable *L. polyphemus* amebocytes in vitro: Leibovitz L-15 Medium (catalog number 12–700) (L-15); L-15 double-strength (catalog number 12–669) (L-15 [2×]); Grace's Insect Medium (catalog number 04–457) (GIM); Grace's Modified Insect Medium (catalog number 04–649) (GMIM), also known as TNM-FH; IPL-41 Insect Medium (catalog number 04–759) (IPL-41); and Insect-Xpress–protein-free culture medium (catalog number 12–730) (IX). Of the six media tested, three had been used in previous amebocyte culture studies: L-15 (Pearson and Woodland), L-15 (2×) (Joshi et al., 2002), and GIM (Pearson and Woodland, 1979; Ding et al., 1988; Gibson and Hilly, 1992).

Hemolymph samples from one intermoult adult male horseshoe crab were used for the culture inoculations. The specimen was bled via cardiac sinus puncture. The exposed arthrodial membrane was swabbed with a 70% ethanol-soaked cotton swab to disinfect the surface. An 18-gauge needle was inserted through the membrane into the cardiac sinus to extract the needed amount of hemolymph into a syringe. The syringe then was sprayed with 70% ethanol to disinfect the surface and moved into a laminar flow cabinet. The used needle was replaced with a new sterile needle, which was then ready for dispensing whole hemolymph into the culture media. Amebocytes were seeded into the selected culture media within 5 min after harvest.

To test the effects of the different media on amebocyte maintenance in vitro, the six previously described culture media were tested in triplicate using pyrogen-free 12-well culture plates (B-D Falcon, Fisher Scientific, Hampton, NH). Wells were filled with 950 μl of the designated medium and inoculated with 50 μl of whole *Limulus* hemolymph. In this way, all of the media were supplemented with 5% v/v horseshoe crab hemolymph. Preliminary work suggested that horseshoe crab serum–supplemented cultures supported higher cell viability than did serum-free cultures (Hurton, 2003). The plates were then incubated at 21° C in air and in the dark. Cultures were assessed using morphological observations and cell viability counts on d 7 after inoculation.

Amebocytes were observed in vitro using an inverted microscope at ×400 magnification. Cells were photographed in culture to record cell morphology using an Olympus C-3000 digital camera fitted to the inverted microscope's camera port. Cultures were treated with trypsin to dissociate the cells, because most cultures formed monolayers. Stock trypsin–ethylenediaminetetraacetic acid (10×) (Cambrex) was diluted to 1× using phosphate-buffered saline without calcium or magnesium (Cambrex) (Mitsuhashi, 2002). Culture medium was aspirated and replaced with the diluted 1× trypsin–ethylenediaminetetraacetic acid solution. After 15 min of incubation, cells were suspended by pipetting. Viability of cells was assessed using the trypan blue dye–exclusion method (Mitsuhashi, 2002). The cell suspension and 0.4% trypan blue solution (Cambrex) were mixed in a volume ratio of 1:1. This mixture was loaded into a Neubauer-type hemocytometer (Hausser Scientific, Horsham, PA) where stained and unstained amebocytes were counted.

Data were processed with the aid of SAS (Statistical Analysis System, Version 8, Cary, NC) using a significance level of $\alpha = 0.05$. To test for differences in amebocyte viability between the six selected culture media, normality of the data was checked using the Shapiro–Wilk test. Subsequently, a square-root transformation was applied to the data, which then were analyzed by performing an analysis of variance for a completely randomized design. Tukey's Studentized Range test was used to make pair-wise comparisons among the six population means.

In the artificial environment of in vitro culture systems, most cells will be rounded when grown in suspension and flattened when grown on a surface like tissue culture plastic (Mather and Roberts, 1998). In mammalian cell culture, the detachment and rounding-up of cells is indicative of morphological deterioration (Freshney, 1987). However, some invertebrate cells may show increased function when they are allowed to maintain a rounded shape (Mather and Roberts, 1998). For example, cultured hemocytes from the hard clam *Meretrix lusoria* and the colonial protochordate *Botryllus schlosseri* did not adhere to the substrate and even multiplied in suspension (Mitsuhashi 2002).

In this study, three main forms of amebocytes were observed: (1) contracted—relatively spherical cells with little attachment to the surface, (2) granular flattened—partially flattened cells attached to the surface with refractive granules present, and (3) degranulated flattened—completely flattened cells attached to the surface without granules present. It was also noted that cultures with greater proportions of observed contracted cells also had greater viability than did cultures filled with primarily degranulated flattened cells, suggesting that the contracted amebocyte morphology is the most desired form in vitro. This inference regarding cell morphology is supported by Chen et al. (1986), who documented a progression of amebocyte morphology in vitro. Chen et al. (1986) observed amebocytes initially in the contracted form, which after a period of

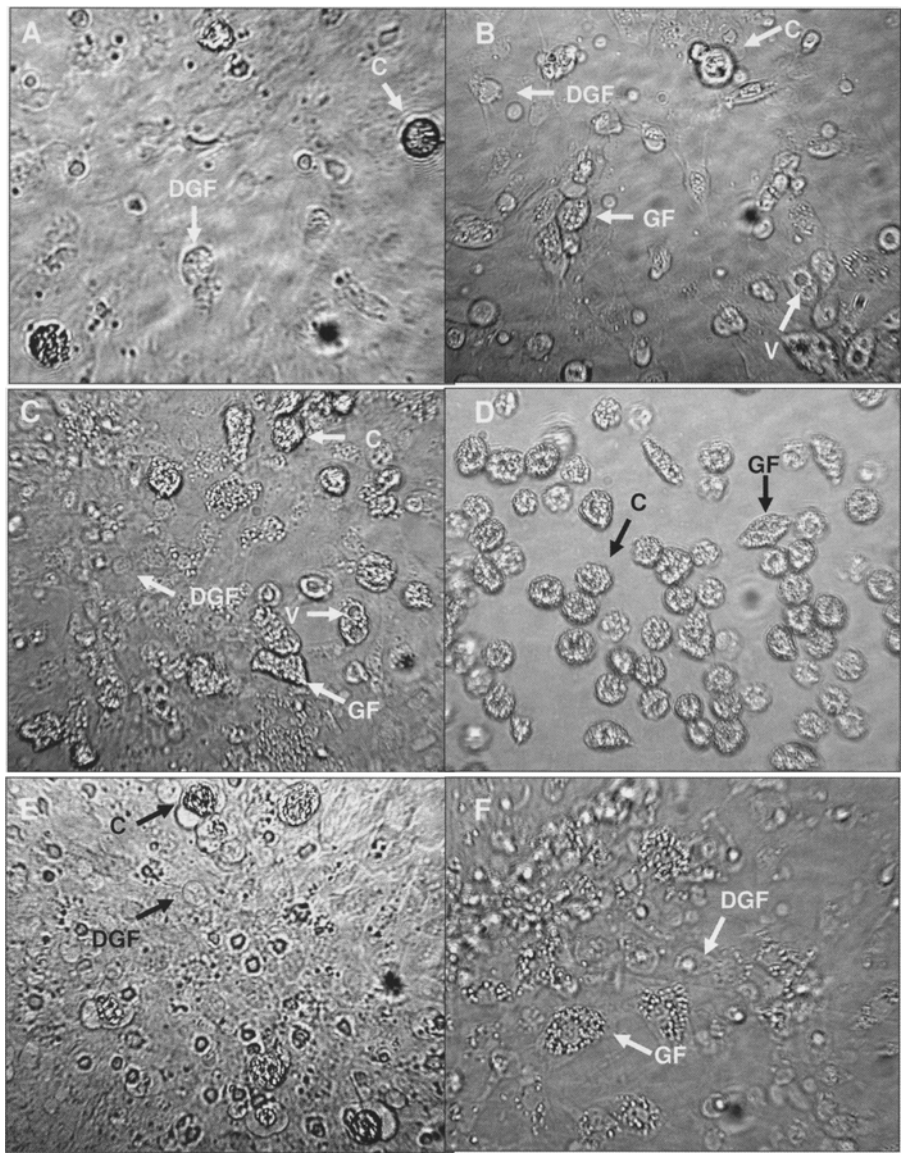

FIG. 1. Comparison of *Limulus* amebocytes maintained for 7 d in various culture media supplemented with 5% v/v horseshoe crab serum. (*A*) L-15. (*B*) L-15 (2×). (*C*) GIM. (*D*) GMIM. (*E*) IPL-41. (*F*) IX. C: contracted form, GF: granular flattened form, DGF: degranulated flattened form, V: vacuole. (×400)

hours or days in culture flattened on the substratum. The flattening of the cells was reversible, but the cells flattened irreversibly once degranulation occurred. In addition, there were many vacuoles, instead of granules, in cells after spontaneous degranulation (Chen et al., 1986). In other cell types, vacuolation is often a sign of morphological deterioration (Freshney, 1987). These observations provided the criteria used in assessing the success of amebocyte cultures on a morphological basis. However, morphological evaluation of amebocytes was coupled with cell viability results for an overall assessment of culture success.

In testing the effects of the six different media on amebocyte maintenance in vitro, different frequencies of amebocyte morphology were observed among the media by d 7. The L-15 medium contained very few contracted cells, with the majority of cells in the degranulated flattened form (Fig. 1*A*). More contracted and granular flattened cells were observed in L-15 (2×). However, there were cells that were vacuolated, and the majority of cells in the field of view were in the degranulated, flattened form (Fig. 1*B*). GIM had a number of cells in either the contracted or granular flattened states, but vacuolated and degranulated flattened cells were also observed (Fig. 1*C*). GMIM had a large number of cells in the contracted form, with no observed degranulation by d 7 (Fig. 1*D*). IPL-41 contained a few contracted and granular flattened cells, with the rest of the amebocytes in degranulated flattened form (Fig. 1*E*). No contracted cells were observed in the IX medium; few granular flattened cells were seen, with the majority of amebocytes in the degranulated, flattened form (Fig. 1*F*).

In the six culture media, amebocyte viability was 77.2 ± 5.1% (mean ± standard deviation) in GMIM, 35.1 ± 8.7% in GIM, 15.2 ± 5.7% in L-15 (2×), 13.3 ± 4.9% in IPL-41, 10.3 ± 2.4% in L-15, and 1.1 ± 1.9% in IX (Fig. 2). Cell viability data from the six insect cell culture media deviated significantly from a normal distribution ($P = 0.001$). The square root–transformed data met the normality criteria. The analysis of variance results indicated that at least one of the treatment means was different from the others ($N = 36$; $P < 0.0001$). Tukey's Studentized Range test showed signif-

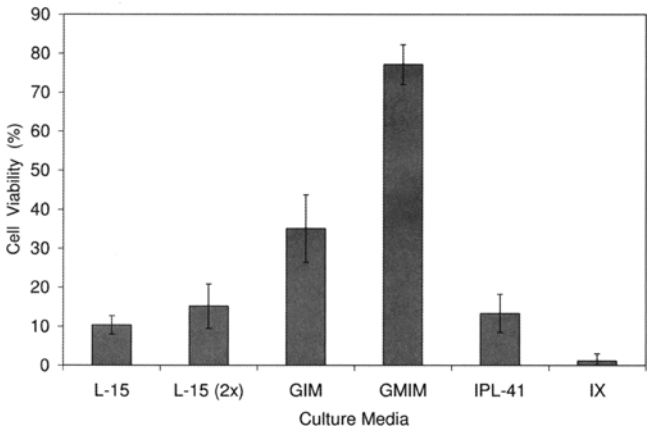

FIG. 2. Effects of various cell culture media on amebocyte viability. Cell viability was determined on d 7 of culture. Values are means ± standard deviation.

icant differences in cell viability between GMIM, GIM, and IX. The L-15, L-15 (2×), and IPL-41 media did not exhibit significant differences in cell viability, but were grouped together as differing significantly from the other three media.

Among several standard insect cell culture media, one medium in particular provided results distinctive from those of all other media tested. GMIM maintained amebocytes that retained their contracted form and did not appear to spontaneously degranulate for the duration of the culture (Fig. 1*D*). Compared to all other cultures tested, GMIM contained amebocytes that exhibited very light attachment to the substrate, with cell viability considerably higher than in the other culture media (Fig. 2), lending support to our morphological observations. With GMIM outperforming all other media tested, it is recommend that this medium, supplemented with horseshoe crab serum as in our study, be used for future amebocyte culture and further media optimization.

Cells in both GMIM and GIM surpassed those in other media in morphological quality and viability, thereby warranting further work with media composition. These two media have higher levels of calcium, potassium, and magnesium salts relative to the other media tested (Table 1), bringing the medium closer to being isotonic to horseshoe crab hemolymph, which has 12.5 mEq/L of potassium, 39.0 mg/dl of calcium, and 96 mg/dl of magnesium (Smith et al., 2002). The dramatic improvement in amebocyte morphology and viability in GMIM relative to GIM suggests a critical component is present in GMIM. The difference could be due to the presence of lactalbumin hydrolysate, yeastolate, and/or an increased level of magnesium salts (Table 1). This is contradictory to the results of Pearson and Woodland's (1979) study, where there were no improvements with lactalbumin hydrolysate and inhibitory effects from yeastolate. These observations provide a starting point for medium modification. Even without much modification, GMIM supplemented with horseshoe crab serum could possibly improve amebocyte culture and harvest duration or numbers with the culture technique described by Joshi et al. (2002), because in our study GMIM provided better results than did L-15 (2×). Optimization of the culture medium could possibly further enhance the results they obtained.

This study recommends GMIM as a culture medium suitable for optimization to *Limulus* amebocyte requirements, contributing to the progress of amebocyte culture. The appropriate medium and culture methods have potential for scale-up and commercial production of LAL. In this way, in vitro production of LAL could provide a more stable product regarding sensitivity and specificity, as well as drastically reduce the need to harvest horseshoe crabs.

ACKNOWLEDGMENTS

This work is a result of research sponsored in part by the NOAA Office of Sea Grant, U.S. Department of Commerce, under Grant NA96RG0025 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. Cambrex Bio Science Walkersville, Inc. generously supplied the horseshoe crabs and cell culture media used in this study. We wish to thank Dr. Eric Hallerman of the Department of Fisheries and Wildlife Sciences of Virginia Tech for his helpful comments throughout this project. In addition, we thank Xin Zhong and Li Wang of the Department of Statistics at Virginia Polytechnic Institute and State University for their statistical consulting.

TABLE 1

COMPARISON OF MEDIA COMPONENTS AND CHEMISTRIES OF THE SIX MEDIA USED FOR PRIMARY CULTURE OF *LIMULUS POLYPHEMUS* AMEBOCYTES. NOTE THE DIFFERENCES IN COMPONENTS PRESENT IN SUBSTANTIALLY HIGHER OR LOWER QUANTITIES IN GMIM THAN IN THE OTHER TESTED CULTURE MEDIA. ALL MEDIA FORMULATIONS WERE OBTAINED FROM CAMBREX (WALKERSVILLE, MD).

Media component/chemistry	L-15	L-15 (2×)	GIM	GMIM	IPL-41	IX
Inorganic salts (mg/L)						
Calcium chloride	0	0	0	0	500	n/a
Calcium chloride hydrate	140	280	993	993	0	n/a
Potassium chloride	400	800	4100	4100	1200	n/a
Magnesium-based salts	191	383	3638	5060	918	n/a
Sodium chloride	8000	16,000	0	0	1160	n/a
Sodium phosphate hydrate	0	0	1008	1008	0	n/a
Other compoenents (mg/L)						
Lactalbumin hydrolysate	0	0	0	3330	0	n/a
Sucrose	0	0	26,680	26,680	1650	n/a
Yeastolate	0	0	0	3,330	0	n/a
Chemistries						
pH	7.67	7.52	6.00	5.17	6.15	6.20
Osmolality (mOsm)	324	644	353	407	325	364

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