Title: IN VITRO CULTURE OF AMOEBOCYTES FROM TACHYPLEUS GIGAS IN LEIBOVITZ CULTURE MEDIUM

Abstract: The present invention provides a process for large scale in vitro production of amoeboocytes of Indian Horseshoe Crab (Tachypleus gigas) from dissected gill flaps of T. gigas, in Leibovitz culture medium concentration (2X L-15), having enhanced generation of amoeboocytes, said process comprising the steps of: dissecting gill flaps of T. gigas; washing the gill flaps with an antibiotic solution followed by alcohol; culturing the gill flaps in tissue culture plates of sterile saline on a Rocker platform; culturing further the gill flaps in Leibovitz culture medium (2X L-15); purging the gill flaps with Tween 80 solution; and purging again the gill flaps with horse crab serum, keeping the culture viable for 90 days by feeding with fresh medium at an interval of 10-15 days to enable the enhanced release of amoeboocytes both within and outside the gill flaps.

[Continued on next page]
IN VITRO CULTURE OF AMOEBOCYTES FROM TACHYPLEUS GIGAS IN LEIBOVITZ CULTURE MEDIUM

FIELD OF INVENTION

The present invention relates process for the in vitro culture for the production of Indian horseshoe crab (Tachypleus gigas) amebocytes.

BACKGROUND AND PRIOR ART REFERENCES

The blood or haemolymph of the horseshoe crab is an important source for the preparation of a diagnostic reagent - the amebocyte lysate useful in the detection gram-negative bacteria.

The preparation of amebocyte lysate where the haemolymph is directly collected from the wild Indian horseshoe crab, though the technology is viable, as the technology requires regular supply of the blood from the live horseshoe crabs which may lead to indiscriminate killing.

The process of production of amebocyte in vitro from the American horseshoe crab (Limulus polyphemus) was also patented (US Patent 5,082,782).

Reference may be made to a publication wherein amebocytes were cultured in liquid media from open surface of the gill lamellae of Limulus polyphemus. The drawback of the process was short termed culture (seven days only of the amebocytes (Hilly J. B; Gibson III D. G.,1989. Culture of amebocytes on opened gill lamellae of the horseshoe crab, Limulus polyphemus. American Zoologist, 29 (4) 112 A.

Reference may be made to another publication wherein culture of amebocyte was carried out in a nutrient mist bioreactor with little growth amebocyte restricted to only seven day. The drawback of this process was fungal contamination (Friberg J. A; Weathers P. J; Gibson III D.G.,1992. Culture of amebocytes in a nutrient mist bioreator. In vitro 28 A3 pp 215-217.

Reference may be made to a publication wherein gill flaps removed from American horseshoe crabs were cultured using Grace's Insect medium at pH 7.6, maintaining the
culture on a shaker table for 5-12 days at 23-37º C and obtaining the amoebocytes by pulsing with a solution of 10-20% Limulus serum (Daniel G. Gibson, III, Teaticket, Joan B., Hilly, Uxbridge, 1992, US Patent No 5,082,782. The drawback of the present invention is that the Grace's Insect medium retains the culture viable only for 5-12 days.

Reference may be made to our earlier patents where the amoebocytes were separated from the haemolymph directly collected from the wild horseshoe crab. The drawbacks of the invention were primarily fluctuating trend in the availability of the horseshoe crab for the collection of haemolymph, seasonal variations in the sensitivity of lysate and threat for depletion of this valuable animals due to over exploitation (Chatterji, A. 1997, An improved process for the preparation of Tachypleus Amoebocyte Lysate (TAL) useful for detection of pyrogens in vitro, NF-235/97).

Accordingly, the present invention provides a new medium for the production of Indian horseshoe crab (Tachypleus gigas) amoebocytes in vitro to one reported earlier wherein Grace's Insect Medium was used to cultivate the gill flaps. In the new medium, the Grace's Insect medium was replaced by sterilized normal saline with 720 mOsm, followed by culturing in (2X) L-15 medium Leibovitz medium (L-15) 2X concentration.

**OBJECTS OF THE INVENTION**

The main object of the present investigation is to provide a process for the production of Indian horseshoe crab (Tachypleus gigas) amoebocytes in vitro.

Another object of the present invention is to provide a new medium to keep amoebocytes viable for 90 days without any morphological change.

Yet another object of the present invention is to obtain large-scale production of amoebocyte in vitro from horseshoe crab.

Still another object of the present invention is to prepare high quality amoebocyte lysate without seasonal variability.

Yet another object of the present invention is to avoid batch to batch variability in the sensitivity of amoebocyte lysate.
Still another object of the present invention is to continuously produce amoebocyte in vitro reducing the cost of production of amoebocyte lysate.

Summary of the invention

The present invention comprises dissecting gill flaps from the Indian horseshoe crab (T. gigas), washing the organ at first with an antibiotic (5% betadine) solution for 10 minutes, further washing the organ with 70% alcohol for 10 minutes, culturing the organ in 6 well tissue culture plates containing sterilized normal saline 720 mOsm on a Rocker platform at 23-28°C for 48 hours, followed by culturing in 2X L-15 for another 48 hours. Gill flaps were purged with 1% Tween 80 solution followed by further purging with 10% horseshoe crab serum (haemolymph without amoebocyte), leading to release of amoebocyte within and outside the gill flaps. The cultures were fed at an interval of 5-10 days with fresh medium. In this present invention, the culture of amoebocyte was maintained for 90 days and there was no change observed in their morphology and viability.

Detailed description of the invention

Accordingly, the present invention provides a process for large scale in vitro production of amoebocytes of Indian Horseshoe Crab (Tachypleus gigas) from dissected gill flaps of T. gigas, in Leibovitz culture medium concentration (2X L-15), having enhanced generation of amoebocytes, said process comprising the steps of:

(a) dissecting gill flaps of T. gigas;
(b) washing the gill flaps initially with an antibiotic solution for 10 minutes followed by alcohol;
(c) culturing the gill flaps in tissue culture plates of sterile saline on a Rocker platform at a temperature ranging between 23-28°C;
(d) culturing further the gill flaps of step (c) in Leibovitz culture medium (2X L-15);
(e) purging the gill flaps with Tween 80 solution; and
(f) purging again the gill flaps with horse crab serum, keeping the culture viable for 90 days by feeding with fresh medium at an interval of 10 – 15 days to enable the enhanced release of amoebocytes both within and outside the gill flaps.

In an embodiment of the invention provides a process, wherein in step (a) the gill flaps from the Indian horseshoe crab (T. gigas) are removed and dissected under highly aseptic condition.
Still another embodiment of the invention, the preferable antibiotic for washing of gill flaps in step (b) is selected from betadine 5-10% solution.

Still another embodiment of the invention, alcohol used for further washing in step (b) is having a concentration ranging between (70-90%) for about 10-15 minutes.

Still another embodiment, the gill flaps in step (c), are cultured in 6-well tissue culture plates containing sterilized normal saline 720 mOsm on a Rocker platform for a period of 48-72 hours.

Yet another embodiment of the invention, further culturing of gill flaps in step (d) are performed in 2X L-15 for a period of 48 hours.

Yet another embodiment, purging of gill flaps in step (e) is done with 1-5% Tween 80 solution.

Yet another embodiment of the invention, further purging in step (f) is performed with 10-15% horseshoe crab serum, leading to enhanced release of amoebocytes within and outside the gill flaps.

Yet another embodiment, the horse crab serum in step (f) is a haemolymph without amoebocytes.

Yet another embodiment of the invention, the culture is in step (f) maintained for 90 days with intermittent feeding of fresh medium.

Yet another embodiment, the said process is performed in pyrogen free atmosphere.

In an embodiment, the gill flaps from the Indian horseshoe crab (*T. gigas*) is removed under highly aseptic condition.

In an embodiment, the gill flaps are washed with an antibiotic (5-8% betadine) solution for 10-15 minutes.

In an embodiment, the gill flaps are further washed with 70-90% alcohol for 10-15 minutes.

In an embodiment, the gill flaps are cultured in 6 well tissue culture plates containing sterilized normal saline 720 mOsm on a Rocker platform at 23-28 °C for 48-72 hours, followed by culturing in 2X L-15 medium.

In an embodiment, the gill flaps are purged with 1-5% Tween 80 solution followed by further purging with 10-15% horseshoe crab serum (haemolymph without amoebocyte), leading to release of amoebocyte within and outside the gill flaps.

In an embodiment, the culture of amoebocyte is maintained for 90 days and the cultures are fed at an interval of 5-10 days with fresh medium.
1) Removal of gill flaps: The gill flaps from the Indian horseshoe crab (*T. gigas*) are removed under highly aseptic condition.

2) Sterilization of gill flaps: The gill flaps are washed with an antibiotic (5% betadine) solution for 10 minutes followed by further washing with 70% alcohol for 10 minutes.

3) Culturing of gill flaps: The gill flaps are cultured in 6 well tissue culture plates containing sterilized normal saline 720 mOsm on a Rocker platform at 23°C for 48 hours, followed by culturing in (2X) L-15 for another 48 hours. The culture of amoebocyte is maintained for 90 days and the cultures are fed at an interval of 5-10 days with fresh medium.

4) Collection of amoebocytes: The gill flaps are purged with 1% Tween 80 solution followed by further purging with 10-15% horseshoe crab serum (haemolymph without amoebocyte) which lead to release of amoebocyte within and outside the gill flaps. The aforesaid process requires absolute precaution for pyrogen contamination at all processing steps. All apparatus and reagents must therefore, be pyrogen free.

Main advantage of the present invention:

The novelty and advantage of the present invention is the production of horseshoe crab amoebocyte *in vitro* from the gill flaps making use of simple salt solution initially followed by 2X L-15 nutrient media thus eliminating the chances of contamination and economizing the process.

Another advantage of the present invention is that the new medium maintains the culture viable for 90 days without any change in the morphology of the amoebocytes.

**Example-1**

Gills of Indian horseshoe crab were maintained in L-15 (2X). After 48 hours of incubation, the gills were purged with Tween 80 and haemolymph. Amoebocytes were seen releasing from the gills and termed as amoebocyte harvest. Count was taken after subsequent harvest. Gills were maintained *in vitro* for a period of 30 days with six intermittent harvests. After each harvest, gill lamellae were maintained in plain 2X L15 for 48 hours and then again purged with Tween 80 and haemolymph. The culture was maintained for 90 days and fed at an interval of 5-10 days with fresh medium.
CLAIMS

1. A process for large scale in vitro production of amoeocytes of Indian Horseshoe Crab (Tachypleus gigas) from dissected gill flaps of T. gigas, in Leibovitz culture medium concentration (2X L-15), having enhanced generation of amoeocytes, said process comprising the steps of:
   (a) dissecting gill flaps of T. gigas;
   (b) washing the gill flaps initially with an antibiotic solution for 10 minutes followed by alcohol;
   (c) culturing the gill flaps in tissue culture plates of sterile saline on a Rocker platform at a temperature ranging between 23-28 °C;
   (d) culturing further the gill flaps of step (c) in Leibovitz culture medium (2X L-15);
   (e) purging the gill flaps with Tween 80 solution; and
   (f) purging again the gill flaps with horse crab serum, keeping the culture viable for 90 days by feeding with fresh medium at an interval of 10 - 15 days to enable the enhanced release of amoeocytes both within and outside the gill flaps.

2. A process according to claim 1 wherein in step (a), the gill flaps from the Indian horseshoe crab (T. gigas) are removed and dissected under highly aseptic condition.

3. A process according to claim 1 wherein in step (b), the preferable antibiotic for washing of gill flaps is selected from betadine 5-10% solution.

4. A process according to claim 1 wherein in step (b), alcohol used for further washing has a concentration ranging between (70-90%) for about 10-15 minutes.

5. A process according to claim 1 wherein in step (c), the gill flaps are cultured in 6-well tissue culture plates containing sterilized normal saline 720 mOsm on a Rocker platform for a period of 48-72 hours.

6. A process according to claim 1 wherein in step (d), further culturing of gill flaps are performed in 2X L-15 for a period of 48 hours.

7. A process according to claim 1 wherein in step (e), purging of gill flaps is done with 1-5% Tween 80 solution.

8. A process according to claim 1 wherein in step (f), further purging is performed with 10-15% horseshoe crab serum, leading to enhanced release of amoeocytes within and outside the gill flaps.

9. A process according to claim 1 wherein in step (f), the horse crab serum is a haemolymph without amoeocytes.

10. A process according to claim 1 wherein in step (f), the culture is maintained for 90 days with intermittent feeding of fresh medium.
11. A process according to claim 1, wherein said process is performed in pyrogen free atmosphere.
AMENDED CLAIMS
[received by the International Bureau on 21 July 2003 (21.07.03);
original claims 1-11 replaced by new claims 1-8 (1 page)]

1. A process for large scale in vitro production of amoebocytes of Indian Horseshoe Crab (Tachypleus gigas) from dissected gill flaps of T. gigas, in Leibovitz culture medium concentration (2X L-15), having enhanced generation of amoebocytes, said process comprising the steps of:
   (a) dissecting gill flaps of T. gigas;
   (b) washing the gill flaps initially with an antibiotic betadine solution of concentration ranging between 5-10% for 10 minutes followed by alcohol;
   (c) culturing the gill flaps in tissue culture plates of sterile saline 720 mOsm on a Rocker platform at a temperature ranging between 23-28 °C for time duration ranging between 48-72 hrs.;
   (d) culturing further the gill flaps of step (c) in Leibovitz culture medium (2X L – 15);
   (e) purging the gill flaps with Tween 80 solution; and
   (f) purging again the gill flaps with horse crab serum, keeping the culture viable for 90 days by feeding with fresh medium at an interval of 10 – 15 days to enable the enhanced release of amoebocytes both within and outside the gill flaps.

2. A process according to claim 1 wherein in step (a), the gill flaps from the Indian horseshoe crab (T. gigas) are removed and dissected under highly aseptic condition.

3. A process according to claim 1 wherein in step (b), alcohol used for further washing has a concentration ranging between (70-90%) for about 10-15 minutes.

4. A process according to claim 1 wherein in step (d), further culturing of gill flaps are performed in 2X L-15 for a period of 48 hours.

5. A process according to claim 1 wherein in step (e), purging of gill flaps is done with 1-5% Tween 80 solution.

6. A process according to claim 1 wherein in step (f), further purging is performed with 10-15% horseshoe crab serum, leading to enhanced release of amoebocytes within and outside the gill flaps.

7. A process according to claim 1 wherein in step (f), the horse crab serum is a haemolymph without amoebocytes.

8. A process according to claim 1, wherein said process is performed in pyrogen free atmosphere.
STATEMENT UNDER ARTICLE 19(1)

The Applicant respectfully submits that he has modified the claims. These modifications would clearly bring about the novelty aspect of the instant application. Further, the applicant submits that the invention is totally distinct from the cited art US Patent No.5,082,782. The culture medium of the instant application is Leibovitz medium (2X L-15), whereas, that in the cited art is modified Grace’s insect medium. Further, the antibiotic used in the instant application is betadine, whereas, that in the cited art its Aleide expor.

The cited art shows a laborious and time consuming process of inserting glass needle into the blood channel of the gill to open the gill lamellae. The applicants have simplified this procedure and maintained intact gill lamellae as organ culture without opening them and thus, retaining the tissue architecture. This is been made possible by the simulation of the in vivo condition using rocker platform.

In addition, the L-15 (2X) medium permitted applicants to maintain the osmolarity of the seawater (720 mOsm) which resulted in the successfully release of amoebocytes in vitro for a long period without decrement in the cell numbers.

Further, the use of betadine as an antibiotic has considerably reduced the incidence of contamination. Also, use of Leibovitz medium (2X L-15) improvises the nutritional status of the culture and thus, brings about desired osmolarity.

Thus, we respectfully request the Examiner to withdraw the rejections set in the International Search Report.
# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 7 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>US 4 229 541 A (PEARSON FREDERICK C) 21 October 1980 (1980-10-21)</td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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- **A** document defining the general state of the art which is not considered to be of particular relevance
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- **O** document referring to an oral disclosure, use, exhibition or other means
- **P** document published prior to the international filing date but later than the priority date claimed

**T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search: 17 December 2002

Date of mailing of the international search report: 22/01/2003

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