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Growth OF Trout Hematopoietic Cells in Fibrin Clots

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INTRODUCTION

This hematopoietic cell assay uses fibrinogen and thrombin to form a fibrin three-dimensional network of semisolid medium. Trout kidney cells have been grown in this medium. Growth of trout hematopoietic cells to give colonies was accomplished in only one step. The number of mitogen-stimulated colony forming cells detected in the trout kidney and their morphological type depended on the mitogen, the time of incubation and the individual fish. Phytohemagglutinin (PHA) was the best inducer of trout kidney leukocyte colony formation followed by Concanavalin A (Con A). They both gave rise to four different homogeneous types of colonies formed by large-nucleated cells (blasts), cells with eccentric nuclei, multinucleated (lobulated nuclei) cells and lymphocytes. In addition, large adherent cells (macrophages) proliferated and/or differentiated in response to the addition of viral antigens.

Liquid culture has generally been used to examine the response of fish peripheral blood leukocytes to mammalian lymphocyte mitogens. The few reports that exist on fish (carp and/or trout) leukocyte proliferation in semisolid medium employed the 2-step soft-agar technique (liquid culture at a high cell concentration followed by semisolid culture at a lower cell concentration).

The *in vitro* fibrin-clot technique allows stimulation not only of proliferation (colony formation) but also differentiation (heme accumulation). It has been applied to the cloning of mouse myelomas and hybridomas (Rueda & Coll, 1988) and to study growth and differentiation of trout hematopoietic cells under various conditions including mitogen-induced kidney leukocyte colonies (Estepa & Coll, 1992a), kidney susceptibility to viruses (Estepa *et al.*, 1991b), macrophages (Estepa *et al.*, 1992), stroma (Diago *et al.*, 1993), immunostimulants for anti-viral responses (Estepa and Coll, 1992b), properties of kidney blast colonies (Estepa & Coll, 1993a), behaviour of melanomacrophages (Estepa & Coll, 1993b), and anamnesic responses to purified or recombinant rhabdoviral proteins (Estepa *et al.*, 1991a; Estepa *et al.*, 1994). Anamnestic responses can be used in diagnosing previous exposure to rhabdovirus (Sanz & Coll, 1992).

MATERIALS

Table 1
Preparation of Salt Free Fibrinogen

Step	Instructions	Amount	Temp	Time
1	Dissolve the content of one bottle* in distilled water	100 mL		
2	Dialyze against distilled water with agitation	1 L	4 °C	2 hr.
3	Repeat dialysis	4 - 5 L	4 °C	2 hr
4	Centrifuge to eliminate precipitated material	10,000 xg	4 °C	30 min
5	Filter to sterilize	first 0.45 µm second 0.22 µm		
6	Distribute in aliquots	1 mL		
7	Lyophilize			overnight
8	Reconstitute with water several aliquots to estimate protein content			

*Human fibrinogen (Kabi grade L) comes as a lyophilized powder, one bottle contains 1 g fibrinogen, 0.4 g sodium chloride and 1 g sodium citrate with a coagulability of 90% of the total protein content. After lyophilization, the final concentration of soluble reconstituted material should be about 8 mg/mL. Dilute and redissolve the fibrinogen in RPMI-1640 medium for a few minutes with agitation. The solution is stable for only a few hours at 4°C. After step 6, aliquots can also be frozenat -20°C and kept frozen until use.

Table 2.
Preparation of the Cell Culture Medium

Component (Source)	100 mL volume	Final Concentration
RPMI-1640 Dutch (Flow Lab, Ayrshire, Scotland)	78	78%
Pretested Fetal calf serum (Flow Lab.)	10	10%
Pretested Pooled trout serum/ plasma	0.5	0.5%
Hepes 1 M (Sigma Chem. Co. St. Louis, MO)	2	20 mM
Pyruvate x100 (Flow Lab.)	1	0.5 mM
Glutamine x100 (Flow Lab.)	1	4 mM
B-mercaptoethanol 1 M (Sigma Chem.)	0.005	0.05 mM
Gentamicin 50 mg/mL (Schering, Kenilworth, NJ)	0.1	50 g/mL
Fungizone 50 g/mL (Flow Lab.)	2	1 µg/mL
*Fibrinogen 8 mg/mL (Kabi, Stockholm, Sweden)	5	0.4 mg/mL
**Thrombin 200 NIH U/mL (Miles Lab, Elkhart, IN)	2 µL to 100 µL	4 NIH U/mL

*Other sources of fibrinogen and thrombin could also be used.
Hepes is added to the medium to avoid excessive changes of pH during the cell manipulations
The medium is prepared by adding and mixing all the components (except the fibrinogen) and kept at 4 °C.
On the day of use, lyophilized fibrinogen is reconstituted with RPMI-1640 and added to the medium.
Fibrinogen-containing medium should be well agitated to distribute all the fibrinogen and it is only stable for a few hours at 4 °C.
Other components, such as insulin, oxaloacetate, etc. can be added to the medium according to each laboratory recipe.
**Thrombin is not added to the medium but to the cell culture flask or well, before adding the fibrinogen-containing medium.

MATERIALS cont.

1. Lyophilized human fibrinogen is obtained from A.B. Kabi (Stockolm, Sweden).
   Reconstitute and dialyzed extensively against distilled water and lyophilize and/or store at -20 °C in aliquots of 8 mg fibrinogen/mL RPMI 1640 (20X) (see Table 1).
2. Thrombin (Miles, Elkhart, Indiana)
   Reconstitute with sterile distilled water and keep frozen at 200 National Institutes of Health Units (NIH U) per ml (50X).
3. Gentamicin (Shering, Kenilworth, NJ).
4. Mitogens
   Phytohemaglutinin (PHA)
   Concanavalin A (Con A)
   E.coli 026: B6 lipopolysaccharide (LPS) (Difco, Detroit, Mi. USA)
   Reconstitute to 1 mg/mL with RPMI-1640 and store at -20°C.
5. **Cell culture medium**
   RPMI-1640 Dutch modification (290 mOsm/kg, 20mM Hepes) (Flow Labs Ayrshire, Scotland) ***supplemented with:***
   4 mM L-glutamine
   0.5 mM sodium pyruvate
   1 µg/mL Fungizone (amphotericin)
   50 µg/ml gentamicin,
   50 mM ß-mercaptoethanol
   10% pretested fetal calf serum
   0.5% pretested pooled rainbow trout serum (see Table 2).
6. Fetal calf serum batches (Flow Lab.)
7. Pooled trout serum or plasma (pretested)
   Selected by testing the growth of PHA-induced colonies by the technique described (**important!**).
8. Toluidine blue (0.25% in 0.01 M sodium borate, pH 8)

Equipment

1. Two µL Pipettors (100 µL total volume, Hamilton).
2. Sterile 96-well tissue culture plates
3. Flow cabinets or hood
4. Inverted microscope
5. Low-temperature incubator
6. Plastic bags
7. Cylinders of 5% CO_2 in air.

METHODS

Preparation of fibrinogen and thrombin

1. Lyophilized human **fibrinogen** should be prepared as described in Table 1.

2. Lyophilized **thrombin** should be reconstituted with sterile distilled water and kept at -20 °C in 50X aliquots. Cell suspensions are prepared in the cell culture medium as described in Table 2.

3. Just before use, RPMI-1640 is reconstituted. Fibrinogen (completely dissolved) is added to the cell culture medium containing the cells and mixed well.

   Fibrinogen takes a few minutes to dilute.

   Clot formation occurs due to the reaction of fibrinogen with thrombin at low volumes.

   The concentration of these components is not critical (0.2-0.4 mg fibrinogen/mL or 2-4 NIH U of thrombin/mL), but fibrinogen has to be kept below 1 mg/mL and thrombin must be kept below 8 NIH U/mL to avoid toxic effects).

4. Fibrinogen-containing cell culture medium is poured into the thrombin-containing plastic container and the mixture agitated gently for only few seconds.

   Do not agitate while the clot is forming.

5. After clotting (approx 1 min), an equal amount of fibrinogen-free cell culture medium can be gently poured over the clot to prevent evaporation. This is particularly important when using large containers, (24-well plates, Petri dishes or ≥ 25 cm^2 flasks) and /or for long incubation periods.

   Not necessary for 96-well plates.

   Mitogen concentrations should be taken into account when adding medium and adjusted accordingly.

Cells from trout kidney

1. Trout (5-20 g body weight and free of infectious pancreatic necrosis virus) are held in 30 L aquaria containing dechlorinated water at 12-18°C. Cultures of trout kidney cells are always prepared from **individual fish** to avoid any possible mixed leukocyte reactions.

2. Fish are placed on ice and bled from the caudal vessel to reduce the blood content of the kidney.

3. The anterior kidney (pronephros) is removed under sterile conditions and macerated with scissors in a Petri dish containing culture medium(RPMI) at about 20°C.

4. Clumps of cells are dissociated by passing the suspension through a 20 gauge needle

5. The cell suspension is decanted from the undissociated tissue, centrifuged at 1000xg for 10 min and washed twice in culture medium(RPMI).

6. The cell concentration is determined with a hemocytometer and adjusted to the desired cell concentration by adding cell culture medium.

   The optimal number of cells is 1-3x10^5/ml. Mature erythrocytes, should not be included in the counts. By using the above procedure, contamination with mature erythrocytes is ≤10%. The relative abundance of erythrocytes depended mostly on the completeness of the bleeding of the trout prior to the kidney. Round cell (leukocyte) viability as determined by trypan blue exclusion was 80%. The cells could be kept from 1 day (89.2% of viability) to 1 week (75.3% of viability) (n = 6) in the cell culture medium at 4 °C.

Cell culture in fibrin-clots

Figure 1 illustrates the steps involved in the culture of fibrin clots.

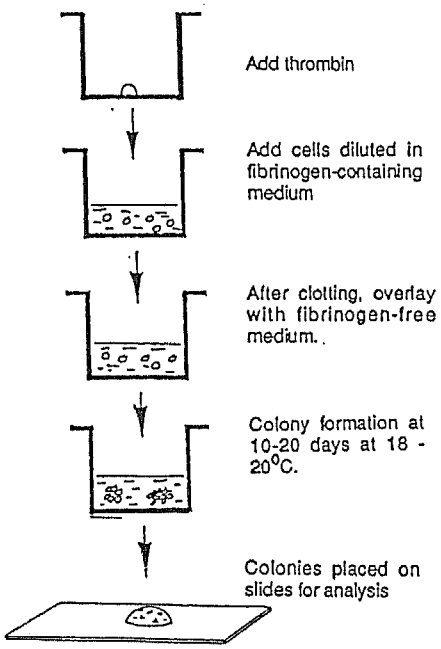

Figure 1. Procedure for fibrin-clot cultures.

1. Once the cell suspension is prepared, 2 µL of thrombin is added to each well of a 96-well plate followed by 100 µL of the fibrinogen-containing cell suspension.

   There is no need to add liquid medium to prevent evaporation in 100µL wells for ≤ 20 days in 96-well plates.

2. After clotting occurred (in about 60 sec), the mitogens, antigens and/or other additives to be tested are diluted in RPMI-1640 or sterile water and pipetted over the clot in each well, to a maximum volume of 10 µL.

3. The plates are then placed in a 20x12 cm plastic bag, heat-sealed and gassed with 5% CO_2 in air (with the aid of a needle). The puncture is resealed with adhesive tape.

4. The plates are incubated at 18-20°C for 1-2 weeks.

Fixing and staining of cells

1. After incubation, the fibrin-clots are removed from the wells of the microtiter plate onto a labeled frosted-end glass slide using the small end of a spatula.

2. One - two clots/plate are then partially dehydrated by overlaying with a rectangular piece (2 x 0.5 cm) of Whatman number 1 filter paper.

3. A second piece of filter paper (the size of the slide) is placed on top of the first (a maximum of 12 clots can be fixed on a regular microscope slide) and allowed to remain long enough for the second paper to become moist (2-3 min).

4. 10-20 drops of 1.7% glutaraldehyde in 0.01 M sodium phosphate and 0.15 M sodium chloride, pH 7.4 are then added to the filter papers and allowed to stand for 10 min.

5. Excess glutaraldehyde is then blotted with a thin piece of filter paper and discarded.

6. The filter papers are gently removed and the clots washed with water and air dried.

7. Stain clots for 10 min with 0.025% toluidine blue in 0.01 M sodium borate (pH 8) and wash with running tap water for 10 min.

8. Air dry and overlay with a coverslip using Permount®.

Calculation of cloning efficiency

1. Cell colonies containing more than 8 cells can be scored at a magnification of 100X.

$$Cloning\ Efficiency = \frac{Number\ of\ Colonies}{Number\ of\ Cells^*} \times 100$$

**Number of cells counted in fixed clots at time 0.*

2. The initial suspension of cells plated is counted using a hemocytometer and dilutions made and dispensed into each well.

3. Colonies can be scored with an ocular eyepiece (100X).

3. Cells are counted and identified microscopically at 400X.

Morphology and properties of mitogen-induced cultures

At time 0, about 10-20% of the round cells were of the erythrocyte lineage as determined by their cytoplasmic staining and/or the patched nuclei.

The average composition of the non-erythrocytic round cells, were lymphocytes: 30.9% (range, 11-57%), large-nucleated cells: 16.6% (range, 10-19%), multilobulated nuclei cells: 29.5% (range, 11-55%), eccentric-nuclei cells:16.6% (range, 7-20%), and adherent cells: 6.4% (range, 2-11%)

In the presence of mitogens, all the cells appearing in the clot after culture (Figure 2) could be classified into the following types: small lymphocytes, large nucleated cells, multinucleated (multilobulated nuclei) cells, eccentric-nuclei cells and large adherent cells (20-30µm). Large-nucleated cells formed colonies and individual cells in PHA and ConA containing cultures (Figure 3). Cells with eccentric nuclei formed colonies in larger numbers in the presence of PHA . About 100 µg/mL of ConA in the final cell culture medium (10 µg of ConA/well) was needed to obtain a comparable colony formation. Lymphocytes, present at the beginning of the cultures disappeared after 1 week in culture (Figure 3).

The number of colonies were dependent on the number of cells plated and the amount of mitogen used. For instance, in ConA cultures (50 µg/mL), the total number of colonies increased from 0 to 68 and 109 when $1x10^4$, $1.7x10^4$ and $2.5x10^4$ cells/well where plated, respectively. The numbers of PHA or Con A-induced colonies, increased (1 to 2 weeks in culture) when 2 µg/mL or 100 µg/mL, respectively were used. However, the PHA-induced colonies reached their maximum counts earlier (after 1 week) if 10 µg/mL instead of 2 µg/mL were used.

The kidney cells if incubated in liquid culture for 1 week, could be divided into adherent and

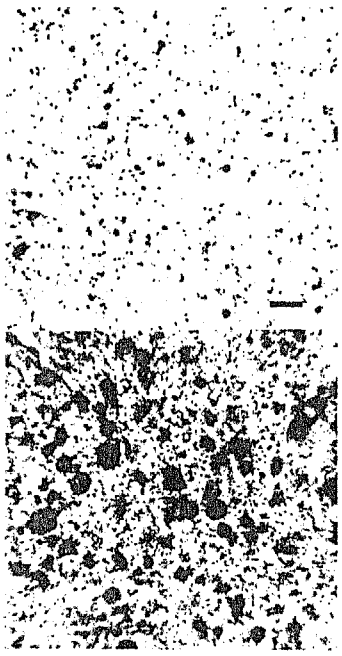

Figure 2. The effect of lymphocyte mitogens on the number of cells or colonies (2.8×10^4 trout kidney cells/100 µL were seeded). (a).Cells immediately after seeding (b).Colonies after 1 week in culture in the presence 2 µg/mL of PHA (Estepa and Coll, 1992a). Scale bar = 100µm

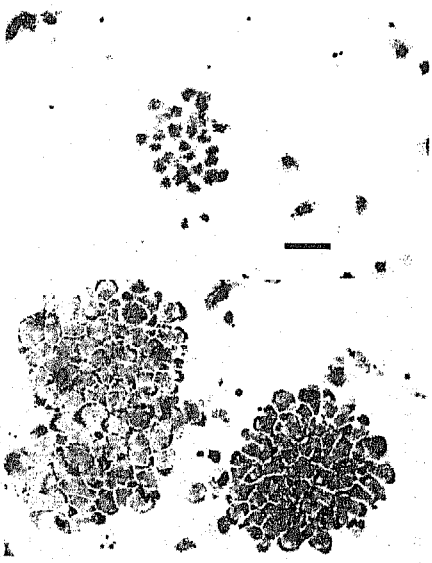

Figure 3. (a) Morphology of PHA-induced lymphocyte colonies. (b) large nucleated colonies Scale bar = 20 µm (Estepa *et al.*, 1992).

non-adherent cells. The adherent cells washed with medium, removed by agitation, centrifuged and placed onto a fibrin clot for fixing and staining, have a considerable amount of cytoplasm and a round nucleus. Some of these cells were filled with either brown material (melano-macrophages) or other cells and/or nuclei in their cytoplasm. Their cell surface showed finger-like projections. Their morphology was identical to the cells found in the kidney described as large cells and to some of the cells found in cultures stimulated by LPS. The adherent cells appeared individually dispersed in most of the mitogen containing cultures and only a few groups of 3-4 cells appeared in some of the cultures. Even though examination of the clots immediately after seeding showed no cell groups or clusters (Figure 2), to confirm that the colonies were not clusters of cells, their size was estimated at two different times during the culture. The average size of the colonies increased from 10 cells at day 7, to 19 cells on day 14. On the other hand, the 1 week old colonies had an average size of 6 cells per colony at 14°C, compared to 10 cells per colony at 20°C. Metaphase figures only appeared in some colonies (2-5% of the cells in the colony). Most of the colonies appearing in the clots were homogeneous, composed of only one cell type per colony (Figure 3).

In the absence of mitogens only dead cells, debris, and 0-5% of the initial round cells remained in the clots. Most often the large adherent cells were the only cells present, after two weeks in culture.

DISCUSSION

Trout cell culture in fibrin-clots (obtained by the reaction of fibrinogen and thrombin) has all the advantages of growing in a semi-solid medium (Rueda & Coll, 1988).This adds a reproducible and highly efficient alternative to soft-agar techniques (Estepa *et al*, 1991a).

The fibrin-clot method allows the study not only of proliferation, cell survival and/or differentiation without any cell division (Estepa *et al.*, 1991a), mitogens and other growth factors such

as the stimulation of cell survival that seems to be the first step to any proliferation induced by the same and/or other factors in mammalian hemopoietic cells. Table 3 is a summary of problems and possible solutions of the fibrin cloning technique.

The method of using kidney cells is simpler than the 2-step agar techniques described to culture blood lymphocytes (Finegan & Mulcahy, 1987). The 2-step agar techniques first use liquid culture at high cell concentrations to generate blast cells followed by cloning in soft-agar at lower cell concentrations to generate colonies. No colonies were formed if the first step was not entirely successful. Furthermore, even with agar gels setting at low temperatures, the trout cells could be sensitive to molten agar above 20 °C and batches of agar are highly variable (some may even be toxic). The method described here does not have these problems and, yet has all the advantages of culture in a semi-solid medium. Furthermore, the fibrin-clot technique allows a permanent record of the results, making possible the direct comparison between experiments.

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Table 3. Trouble Shooting

Problem	Possible Cause	Comments
Soft /no-clot	Incomplete dialysis of fibrinogen salts	Prepare fibrinogen again
	Low fibrinogen concentration	Increase fibrinogen Check reconstitution
	Low thrombin concentration	Add more thrombin
	Incomplete mixture of fibrinogen and thrombin	Agitate plates gently and horizontally
	Low calcium concentration*	Add $CaCl_2$ to 2 mM
Clot-lysis after culture	Too high cell concentration	Reduce cell number
	Low thrombin or fibrinogen concentration	Increase thrombin concentration
Clots dry out during culture	No overlay medium	Add overlay
	Low humidity in incubator	Check incubator
Low plating efficiency	Component is missing	Prepare medium again
	Feeders required	Make feeders
	Wrong serum batch (calf and/or trout)	Test other serum

* The calcium concentration in RPMI-1640 is 0.42 mM. Some medium components can chelate calcium thus preventing or slowing down normal clotting

References.

Coll, J. & Ingram, V.M. (1978). Stimulation of heme accumulation and erythroid colony in cultures of chick bone marrow cells by chicken plasma. J. Cell. Biol. 76: 184-190.

Diago, M.E., Estepa, A., López-Fierro, P., Villena, A. & Coll, J.M. (1993). The *in vitro* infection of hematopoietic stroma of trout kidney by haemorrhagic septicemia rhabdovirus. Viral Immunol. 6:185-191.

Estepa, A., Basurco, B., Sanz, F. & Coll, J.M. (1991a). Stimulation of adherent cells by the addition of purified proteins of viral haemorrhagic septicaemia virus to trout kidney cell cultures. Viral Immunol. 4: 43-52.

Estepa, A., Frías, D. & Coll, J.M. (1991b). *In vitro* infection of trout kidney cells with infectious pancreatic necrosis and viral haemorrhagic septicaemia viruses. Bull. Eur. Ass. Fish. Pathol. 11: 101-104.

Estepa, A. & Coll, J.M. (1992a). Mitogen induced proliferation of trout kidney leukocytes by one-step culture in fibrin clots. Vet. Immunol. Immunopathol. 32: 165-177.

Estepa, A.& Coll, J.M. (1992b). *In vitro* immunostimulants for optimal responses of kidney cells from healthy trout and from trout surviving viral haemorrhagic septicaemia virus disease. Fish Shellfish Immunol. 2: 53-68.

Estepa, A. & Coll, J.M. (1993). Properties of blast colonies obtained from trout head-kidney in fibrin clot cultures. Fish Shellfish Immunol. 3:71-75.

Estepa, A., Frías, D. & Coll, J.M. (1993). Susceptibility of trout kidney macrophages to viral haemorrhagic septicaemia virus. Viral Immunol. 5: 283-292.

Estepa, A., Thiry, M. & Coll, J.M. (1994). Recombinant protein fragments from haemorrhagic septicaemia rhabdovirus stimulate trout amnestic responses *in vitro*. J. Gen. Virol. 75:648-658.

Estepa, A. & Coll, J.M. (1993b). Reduction of melanomacrophage numbers in stimulated trout kidney cell cultures. Fish Shellfish Immunol.3:371-381.

Finegan, C.B. & Mulcahy, M.F. (1987). Successful culture of rainbow trout lymphocytes in soft agar. Dev. Comp. Immunol. 11:419-425.

Rueda, A. and Coll, J.M. (1988). Cloning of myelomas and hybridomas in fibrin clots. J. Immunol. Meth. 114: 213-217.

Sanz, F. & Coll, J.M. (1992). Techniques for diagnosing viral diseases of salmonid fish. Dis. Aquatic. Org. 13: 211-223.

Sanz, F., Basurco, B., Babin. M., Dominguez, J. & Coll, J.M. (1993). Viral haemorrhagic septicaemia virus isolates studied with monoclonal antibodies against its structural proteins. J. Fish Dis. 16:53-63.