Fish & Shellfish Immunology (1993) **3,** 71–75

SHORT COMMUNICATION

Properties of blast colonies obtained from trout head-kidney in fibrin clot cultures

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(*Received 11 June 1991, accepted in revised form 30 December 1991*)

Key words: blast colonies, *Oncorhynchus mykiss*, fibrin clots.

Previously the development of an *in vitro* fibrin-clot system to study mitogen induced proliferation (as measured by colony formation) of different morphological types of trout leucocytes was reported (Coll, 1990). Phytohaemagglutinin (PHA) was the best inducer of colony formation giving rise to, at least, four different homogeneous types of colonies composed of large-nucleated cells, eccentric-nucleated cells, multinucleated cells and small-lymphocytes. In addition, PHA (Coll, 1990) and some proteins from the viral haemorrhagic septicaemia (VHS) virus (Estepa *et al.*, 1991*a*) also prolonged survival (stimulated) of adherent cells identified as macrophages and melanomacrophages

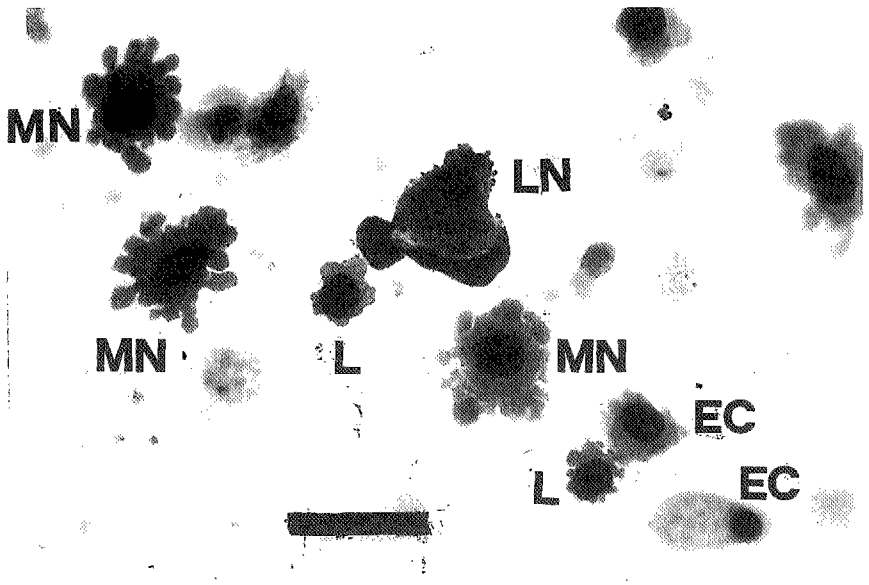

Fig. 1. Large-nucleated scattered cells obtained by culturing trout head-kidney cells in the presence of ConA. The horizontal black bar indicates 10 μm. LN, large-nucleated cell (blast); L, lymphocytes; EC, eccentric nucleated cells; MN, multinucleated cells. Cells classified as in Coll (1990).

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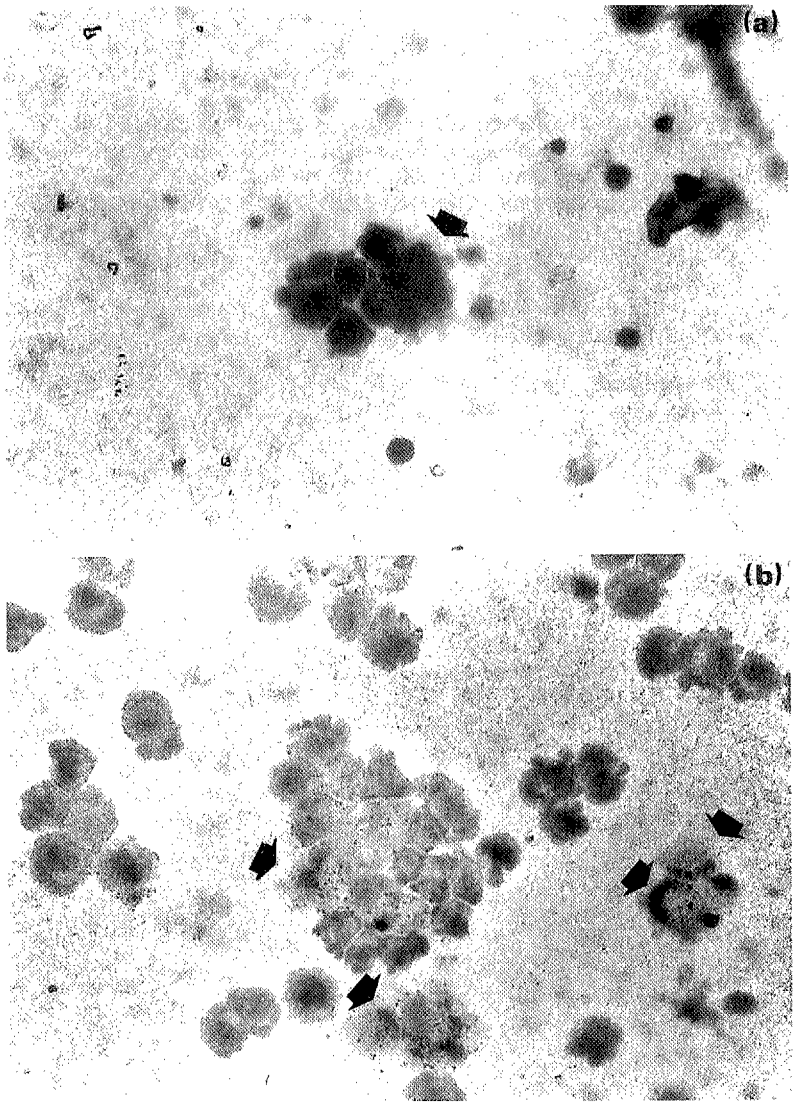

Fig. 2a–b.

(Estepa & Coll, 1991*a*). All these types and their mitogen-stimulated colonies were targets of VHS virus (Estepa *et al.*, 1991*b*). Eccentric-nucleated cells and multinucleated cells seem to belong to immunoglobulin (Ig) containing leucocytes (Estepa & Coll, 1991*b*), but there has not yet been any complete characterisation of the large-nucleated cells (blasts) detected in these cultures. We report here some peculiar characteristics of this cell type and its *in vitro* behaviour in the fibrin-clot culture.

Trout (*Oncorhynchus mykiss*) were purchased from commercial farms that are known to be free of IPNV. The trout were held during several months, fed with commercial pellets, in 100-l aquaria with dechlorinated free-flowing water at 12–18° C until use. The trout were chilled to 4° C and bled by the tail vein to reduce the blood content of the kidney. The anterior kidney (pronephros) was removed under sterile conditions and the cell suspension was prepared for culture as previously described (Coll, 1990). Cells were counted with a haemocytometer and adjusted to 2×10^5 round cells ml^{-1}. The culture

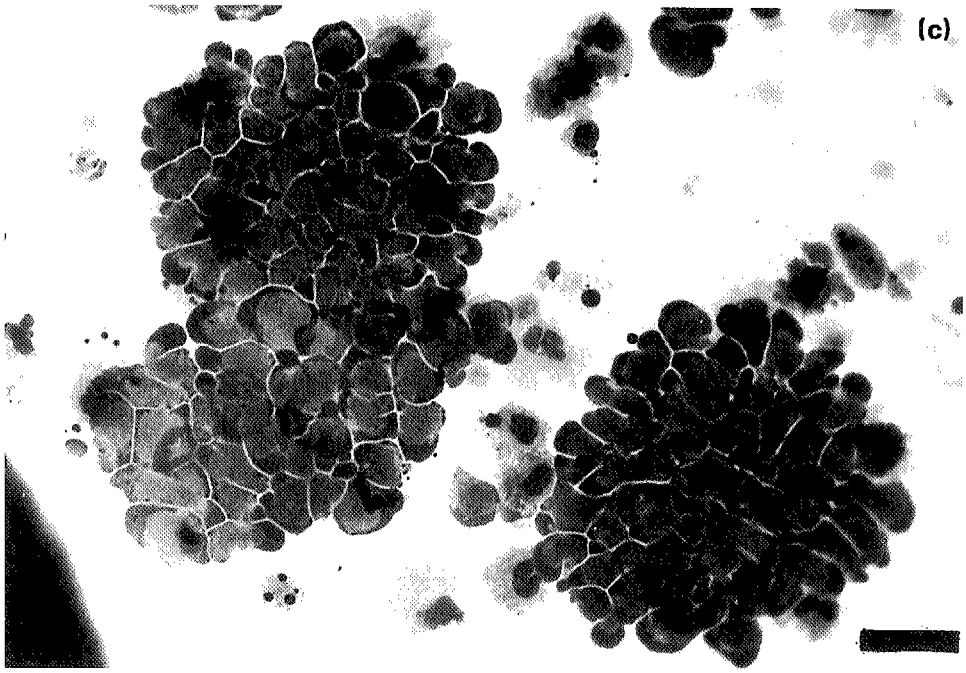

Fig. 2c.

Fig. 2. Trout head-kidney cultured in the presence of ConA. (a), 3-day liquid culture at 2×10^6 cells ml^{-1}; (b), cells from the 3-day liquid culture, plated at 2×10^5 cells ml^{-1} after 10 days in fibrin culture (two-step culture); (c), large-nucleated colonies appearing when cells were cultured at 2×10^5 cells ml^{-1} in the presence of ConA after 10 days in fibrin culture (one-step culture). The arrows indicate the presence of melanomacrophages. The horizontal black bar in (c) indicates 10 μm.

medium was RPMI-1640 (Dutch modification, Flow) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1·2 $\mu g\ ml^{-1}$ amphotericin, 50 $\mu g\ ml^{-1}$ gentamicin, 20 mM Hepes, 50 μM mercaptoethanol, 10% pretested foetal calf serum and 0·5% pooled rainbow trout serum. Fibrinogen (A. B. Kabi, Stockholm, Sweden) was added to the medium just before use to a final concentration of 0·2 mg ml^{-1}. Thrombin (Miles Lab, Elkhart, Indiana) was added to the wells of a 96-well plate (Costar, The Netherlands) to a final concentration of 2–4 NIH U ml^{-1} and 100 μl of the cell suspension were pipetted into each well. After clotting occurred (in about 30 s), the mitogens diluted in sterile water, were pipetted in a maximum volume of 10 μl onto the top of the clot. Phytohaemagglutinin (PHA) and Concanavalin A (ConA) were from Flow Labs (Ayrshire, Scotland). Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 was from Difco (Detroit, MI, U.S.A.). The conditions for mitogen and monoclonal antibody (MAbs) culture were as previously described (Estepa & Coll, 1991*a*). The plates were then sealed in a 20 × 12 cm plastic bag (Vaessen, Schoemaker Indtal, S.A., Sant Boi de Llobregat, Barcelona, Spain), gassed with 5% CO_2 in air, and incubated at 20° C for 2 weeks in the presence or absence of PHA, ConA, LPS or MAbs (see below). After the incubation, the fibrin clots were removed from the wells onto a 75 × 25 mm frosted-end glass slide, fixed and stained as previously described (Coll, 1990). After air drying, the preparations were permanently mounted with Permount (Fisher Sci. Co, NY, U.S.A.). Other details were as previously described (Rueda & Coll, 1988).

Acridine orange and anti-Igs MAb fluorescence staining methods have been described previously (Estepa & Coll, 1991*a*). Non-specific esterase and lipid were detected by the naphthyl-acetate and the Sudan-Black-B/ethylene-glycol methods, respectively, as described by Plytycz *et al.* (1989). For peroxidase staining, diaminobenzidine tablets of

Table 1. Characteristics of the large-nucleated cells

Characteristics	Results	Reference
Adherence to plastic surface	–	Coll (1990)
Granules in the cytoplasm	–	Coll (1990)
Trout red blood cell phagocytosis	–	Estepa & Coll (1991*b*)
Acridine orange staining	–	Estepa & Coll (1991*b*)
Fluorescence with anti-Ig	–	Estepa & Coll (1991*b*)
Prolonged survival by MAbs anti-Igs†	–	Estepa & Coll (1991*b*)
Prolonged survival by VHSV nucleoprotein N†	–	Estepa *et al.* (1991*a*)
Prolonged survival by VHSV glycoprotein G†	–	Estepa *et al.* (1991*a*)
Cell susceptibility to VHSV *in vitro*	+	Estepa *et al.* (1991*b*)
Colony susceptibility to VHSV *in vitro*	+	Estepa & Coll (1991*a*)
Peroxidase staining	–	Estepa & Coll (1991*b*)
Non-specific esterase staining	–	Estepa & Coll (1991*b*)
Sudan black B staining	–	Estepa & Coll (1991*b*)
Nitroblue tetrazolium staining	–	Estepa & Coll (1991*b*)
Proliferation by PHA or ConA*	+	Coll (1990). This work
Large-groups by two-step culture with ConA	+	This work
Prolonged survival by PHA or ConA†	+	Coll (1990). This work
Prolonged survival by LPS†	–	This work

*Proliferation, as measured by the presence of colonies.
†Prolonged survival, when the counts of scattered large-nucleated cells after culture were both equal to their counts before culture and higher than the cultured controls.

10 mg (Sigma Chemical, St. Louis, MO) were dissolved in 1 ml ethanol/water 1:1. Then a 10-fold dilution was made in PBS, pH 7·4, containing 0·01% merthiolate and 3 mM H_2O_2. Slides were held in the stain for 10 min, rinsed in water and mounted in glycerol PBS (1:1). Phagocytosis was estimated by uptake of red blood cells in at least 30 cells per culture.

Large-nucleated cells were abundant in fresh anterior kidney, being up to 16·6 ± 2·9% ($N=4$) of total leucocyte counts. In the absence of mitogens they disappeared after 2 weeks in culture. In the presence of PHA (2–10 $\mu g\ ml^{-1}$) or ConA (50–100 $\mu g\ ml^{-1}$) following 2 weeks in culture, they either appeared as scattered cells at up to 10 ± 9 or 16·1 ± 12% ($N=4$) of total cell counts, respectively (Fig. 1) or they formed colonies at up to 21·7 ± 15 or 24 ± 20% ($N=4$) of total colony counts, respectively [Fig. 2(c)]. In a few cases (two of 20 trout), the large-nucleated cell colonies were the only type of colonies found in PHA stimulated cultures. Lipopolysaccharide (LPS) for *E. coli* at concentrations of 200 $\mu g\ ml^{-1}$ seemed to have no clear effect on the large-nucleated cells nor to induce colonies. LPS obtained from aquatic bacteria (*Vibrio anguillarum, Aeromonas salmonicida, Yersinia ruckeri, Aeromonas hydrophila, Aeromonas fluorescens* and *Aeromonas sobria*) prolonged survival of some adherent cells but not of the large nucleated-cells.

The addition of each one of a panel of 20 anti-trout serum Ig MAbs (Estepa & Coll, 1991*b*), obtained as described by Sanchez *et al.* (1991) to the cultures of trout-kidney cell, did not prolong survival of the large-nucleated cells after 2 weeks. Only eccentric-nucleated, multinucleated and adherent cells increased as a result of the addition of the MAbs relative to the control cultures (without any addition) (Estepa & Coll, 1991*a*). Large-nucleated cells were not recognised by anti-trout Ig MAb 1G10 (MAb recognising more than 85% of the total trout serum Ig, Sanchez *et al.*, 1991), were not stained by Sudan Black, nitroblue tetrazolium or peroxidase and were never observed to phagocytose trout red blood cells (Table 1).

To date, liquid culture has been generally used to examine the response of fish leucocytes to mammalian lymphocyte mitogens. There are only two reports of fish leucocyte proliferation induced by PHA or LPS in semisolid medium in carp (Caspi *et al.*, 1982) and in trout (Finegan & Mulcahy, 1987). Both studies employed peripheral blood leucocytes and a two-step agar cloning technique that consisted of a first step in liquid culture at high cell concentration followed by a second step in agar culture at lower cell concentration.

In such two-step cultures, only when blasts appeared in the first step, were colonies seen in agar in the second step. Though in the present study, leucocyte colonies could be found without the two-step requirement, a series of experiments explored the usefulness of the two-step method. First, the cells were cultured in liquid medium in the presence of the mitogens PHA ($2\,\mu g\ ml^{-1}$), ConA ($20\,\mu g\ ml^{-1}$) or LPS ($200\,\mu g\ ml^{-1}$) at high cell concentrations (2×10^6 cells ml^{-1}) for 3 days, then they were plated at 2×10^5 cells ml^{-1} in fibrin clots in the presence of the mitogens. Figure 2(a), shows one of the many small groups of macrophages (melanomacrophages) and large-nucleated cells obtained after 3 days in liquid culture in the presence of ConA. Figure 2(b), shows one of the many large groups seen after culturing the above-mentioned small groups for 10 days in the fibrin culture in the presence of ConA. Both macrophages (indicated by arrows in the figure) and large-nucleated cells were seen in these large groups. Figure 2(c), shows the appearance of two colonies made of large-nucleated cells obtained by the 1-step fibrin culture method in the presence of ConA (similar colonies formed by the one-step method in the presence of PHA). Since PHA caused clumping of the cells in liquid culture, this precluded any further analysis of the effects of this mitogen in the two-step method. Also LPS did not stimulate any cells or colonies in these conditions. The large group- and colony-forming large-nucleated cells in our cultures were morphologically similar to the blast cells appearing in the PHA-stimulated peripheral blood leucocytes from carp (Caspi *et al.*, 1982) and trout (Finegan & Mulcahy, 1987) and whose appearance in liquid culture was required for colonies to appear in the agar culture. These cells also resembled those identified as blast cells by Plytycz *et al.* (1989).

Our continuing research is aimed at determining whether the large-nucleated cells (blasts) are the precursor of other cells and/or colony types found in long-term cultures in the presence of mitogens and the use of the fibrin-clot technique should facilitate this.

We appreciate the technical assistance of D. Frías, the isolation of LPS from aquatic bacteria by J. Barrera, and the help of J. Coll Perez in typing and drawing. This work was supported by Research Grants 8568 and 8171 from the Instituto Nacional de Investigaciones Agrarias del Ministerio de Agricultura (Spain).

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