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Mitogen-induced proliferation of trout kidney leucocytes by one-step culture in fibrin clots

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ABSTRACT

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This work describes a trout kidney leucocyte mitogen-stimulation assay using fibrinogen/thrombin. Cloning was obtained by only one step instead of by two steps, as required by the agar method described to date. Cultures were stimulated not only with phytohemagglutinin (PHA) and *Escherichia coli* lipopolysaccharides (LPS) but also with Concanavalin A (Con A) and six LPS from aquatic pathogenic bacteria. The number of colony-forming cells detected, and their morphological type depended on the mitogen, the time of incubation and the trout. PHA was the best inducer of trout kidney leucocyte colony formation followed by Con A, giving rise to four different homogeneous types of colonies formed by large-nucleated cells, cells with eccentric nuclei, multinucleated cells and lymphocytes.

ABBREVIATIONS

Con A, Concanavalin A; CV, coefficients of variation; LPS, lipopolysaccharides; NIH, National Institute of Health; PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

INTRODUCTION

Fish are the most primitive animals to have an adaptative immune system characterized by the existence of lymphocytes and immunoglobulins (Sanchez and Coll, 1989). It is, therefore, of interest to study the immune system in this primitive model and see whether or not it requires the cell types and interactions typical of mammalian immunity. To date, liquid culture has generally been used (Faulmann et al., 1983) to examine the response of fish leucocytes to mammalian lymphocyte mitogens (Blaxhall and Sheard, 1985; Kaattari et al., 1986). There are only two reports on fish leucocyte prolifera-

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tion induced by phytohemagglutinin (PHA) and lipopolysaccharides (LPS) in semisolid medium either in carp (Caspi et al., 1982) or in trout (Finegan and Mulcahy, 1987). Both reported methods employed a two-step agar cloning technique (liquid culture at high cell concentration to generate blasts followed by cloning) developed for mammalian hematopoietic cells and both were applied only to fish peripheral blood leucocytes.

We report here a new application of the fibrin-clot technique to the growth of leucocytes from trout in only one step. We applied this system to clone leucocytes from the trout kidney (its major hematopoietic forming tissue) for the first time and report the colonies induced not only with PHA and *Escherichia coli* LPS but also with Concanavalin A (Con A) and LPS obtained from six pathogenic aquatic bacteria. The use of fibrin clots (Rueda and Coll, 1988) for these studies allows both the preservation of the morphology and the possibility of studying both cell proliferation and differentiation. This technique forms the basis of present studies of immune responses to viral infections affecting trout (Estepa and Coll, 1991).

MATERIALS AND METHODS

Chemical and reagents

Medium RPMI-1640 (Dutch modification, 290 mOsm kg^{-1} and 20 mM Hepes) and fetal calf serum were obtained from Flow Laboratories (Ayrshire, UK). Fetal calf serum batches (Flow) were selected by testing the growth of PHA-induced colonies by the technique described below. Lyophilized human fibrinogen was obtained from A.B. Kabi (Stockholm, Sweden); it was reconstituted and dialyzed extensively against distilled water, then lyophilized and kept at $-20°C$ in aliquots. Other chemicals used were: gentamicin (Shering, Kenilworth, NJ), thrombin (Miles, Elkhart, IN), PHA, Con A, amphotericin (Flow) and *E. coli* 026: B6 LPS (Difco, Detroit, MI).

*Cells from trout (*Oncorhynchus mykiss*) kidney*

Trout were purchased from commercial farms (5–20 g body weight) after several annual tests indicated that they were free of infectious pancreatic necrosis virus (Jimenez et al., 1988). The different trout were obtained from August to June. The trout were held in 30 l aquaria with dechlorinated free-flowing water at 12–18 °C until used; they 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 cut into piecies with scissors in a Petri dish. Clumps of cells were dissociated by passing the suspension through a 20 gauge needle. The cell suspension was decanted briefly from the undissociated tissue, centrifuged at $1000 \times g$ for 10 min and washed in

cold cell culture medium twice. The cell concentration was determined with a hemocytometer and adjusted to 2×10^5 round cells ml^{-1}. Mature erythrocytes were not included in the counts. Contamination with mature erythrocytes was lower than 10%. Cell viability, as determined by trypan blue exclusion, was higher than 80%.

Cell culture in fibrin clots

The cell culture medium consisted of RPMI-1640 with 2 mM L-glutamine, 1 mM sodium pyruvate, 1.2 μg ml^{-1} amphotericin, 50 μg ml^{-1} gentamicin, 50 μM mercaptoethanol, 10% pretested fetal calf serum and 0.5% pooled rainbow trout serum. Fibrinogen was added to the medium just before use to a final concentration of 0.2 mg ml^{-1}. Thrombin was added to the wells to a final concentration of 2–4 National Institute of Health (NIH) U ml^{-1}.

Cultures of leucocytes were always prepared from individual fish to avoid any possible mixed leucocyte reactions (Kaastrup et al., 1988). The cell suspension was prepared, thrombin was added at 0.2–0.4 NIH U per well in 2 μl volume, and 100 μl of the cell suspension were pipetted into each well of a 96-well plate (Costar, Netherlands). After clotting occurred (in about 30 s) the mitogens, diluted in sterile water, were pipetted into each well in a maximum volume of 10 μl on top of the clot. The plates were then sealed in a 20 cm $\times$ 12 cm plastic bag (Vaessen, Schoemaker Indtal, S.A., Sant Boi de Llobregat, Barcelona, Spain), gassed with 5% CO_2 in air, sealed and incubated at 20°C for 1–2 weeks.

After the incubation, the fibrin clots were removed from the wells onto a 75 mm $\times$ 25 mm frosted-end glass slide with a spatula. The clots were partially dehydrated by placing a rectangular piece of Whatman No. 1 filter paper on their surfaces. Then, five drops of 1.7% glutaraldehyde in 0.01 M sodium phosphate, 0.15 M sodium chloride (pH 7.4) were added and allowed to stand for 10 min. The excess of glutaraldehyde was absorbed with filter paper. The papers were removed with gentle pressure and the fixed clots were washed in water and air dried. The clots were stained with 0.025% toluidine blue in 0.01 M sodium borate (pH 8) for 10 min and washed in running tap water for 10 min. After air drying, the preparations were permanently mounted with Permount (Fisher Sci. Co., NY). Other details were as described by Rueda and Coll (1988).

Colonies were scored with the aid of an ocular eye piece at 100$\times$. Cells were counted and identified at 400$\times$. Four clots were used for each point and one field was counted for each clot. Averages and standard deviations were used to calculate coefficients of variation (CV). Intra-assay CV of the number of cells vary between 10% and 74.4% and intra-assay CV of the number of colonies vary between 12.5% and 23.3%.

Cytological stains

Staining for non-specific esterase was made by the α-maphthyl acetate method as described by Plytycz et al. (1989). The following medium was prepared just before use: 10 mg of α-maphthyl-acetate were dissolved in 250 μl of acetone, diluted to 20 ml with phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M sodium chloride; PBS) (pH 7.4) and 20 mg Fast Blue BB added. The mixture was added to the slides, the slides incubated for 15 min at room temperature, rinsed three times with distilled water, and mounted in glycerol/PBS (1:1).

For Sudan Black B staining, 0.7 g of stain were dissolved in 100 ml of ethylene glycol, heated at 100°C and filtered. The slides were incubated with the stain for 30 min at room temperature, then rinsed briefly in 50% ethanol and distilled water and mounted in glycerol/PBS (1:1).

For peroxidase staining, 10-mg diaminobenzidine tablets (Sigma Chemical Co., 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 (Coll, 1989). Slides were kept in the stain solution for 10 min, rinsed in water and mounted in glycerol PBS (1:1).

Acridine orange (100 μg ml^{-1}) in culture medium was used to stain live cells for 1 min, followed by a brief rinse in medium; it is specific for lysosomes of the monocyte/macrophage cell lines (Bayne, 1986). Lysosomes appeared orange and nucleus green under the fluorescence microscope.

RESULTS

Composition of kidney cells as studied in fixed clots

The composition of trout kidney cells varied from trout to trout as determined by examination at time 0 in the fixed and stained clots.

About 5×10^4 round cells (leucocytes) could be obtained per gram of rainbow trout kidney (5–20 g trout, $n=9$). The round cells varied from 70 to 90% of the total number of cells, the rest being erythrocytes. The abundance of erythrocytes depended mostly on the completeness of the bleeding of the trout prior to the kidney extraction. 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 compositions of the non-erythrocytic round cells ($n=9$) were: 30.9% (range 11–57%) for lymphocytes; 16.6% (range 10–19%) for large-nucleated cells; 29.5% (range 11–55%) for multinucleated cells; 16.4% (range 7–20%) for eccentric-nuclei cells; 6.4% (range, 2–11%) for big cells (Table 1). 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.

TABLE 1

Individual trout variability of the mitogen response of kidney leucocytes in fibrin clots.
Trout kidney cells were incubated at 2.2×10^4 cells per well for 1 week at 20°C in the presence of 5 μg ml^{-1} of PHA, 50 μg ml^{-1} of Con A or 200 μg ml^{-1} of LPS. All the numbers of cells and colonies were expressed per 100 μl of culture (per well). Four trout were used and results pooled as indicated. Averages ± standard deviations are given in the table

	Cell types	Time 0	Time, 1 week			
			Control	PHA	Con A	LPS
Cells	L	6925 ± 3555	–	–	–	–
	LN	3725 ± 675	200 ± 141	3233 ± 3120	2800 ± 2194	166 ± 94
	MN	6625 ± 1525	2425 ± 1460	4833 ± 2875	6250 ± 3373	491 ± 471
	EN	3675 ± 3096	1250 ± 934	18533 ± 6298	6955 ± 1792	3433 ± 873
	Ad	1450 ± 763	1600 ± 353	4633 ± 2663	1325 ± 1063	2533 ± 703
Colonies	L	–	–	5 ± 3	–	–
	LN	–	–	206 ± 145	21 ± 18	9 ± 7
	MN	–	–	52 ± 29	41 ± 31	–
	EN	–	–	613 ± 420	18 ± 16	10 ± 8
	Ad	–	–	71 ± 66	7 ± 6	5 ± 4

L, lymphocytes; LN, large-nucleated cells; MN, multinucleated cells; EN, eccentric nuclei cells; Ad, adherent cells.

Morphology and staining properties of the mitogen-induced cells and colonies

Because of their morphology, all the cells appearing in the clot after culture in the presence of mammalian mitogens, could be classified into the following categories: small lymphocytes or simple lymphocytes, large-nucleated cells, multinucleated cells, eccentric-nuclei cells and big cells (20–30 μm). If no mitogens were added, after 2 weeks in culture at 20°C only dead cells, cellular debris, and 0–5% of the initial round cells remained in the clots. In the cases where some cells appeared morphologically intact, they were multinucleated, eccentric-nuclei and big cells; but most often the big cells were the only cells present (Fig. 1).

The kidney cells, when incubated in plastic flasks in liquid culture for 1 week, could be divided into adherent and non-adherent cells. The adherent cells were washed with medium, removed by agitation, centrifuged and placed onto a fibrin clot for fixing and staining. These cells possessed considerable cytoplasm (about 30 μm in diameter) and a round nucleus. Some of these cells were filled with either brown corpuscles or other cells and/or nuclei in their cytoplasm. They showed staining with acridine orange, peroxidase and non-specific esterase but not with Sudan-Black B or nitroblue tetrazolium. Their cell surface showed finger-like projections. Their morphology was identical to the cells found in the kidney described as big cells and to some of the cells found in cultures stimulated by LPS (Fig. 1). The adherent cells ap-

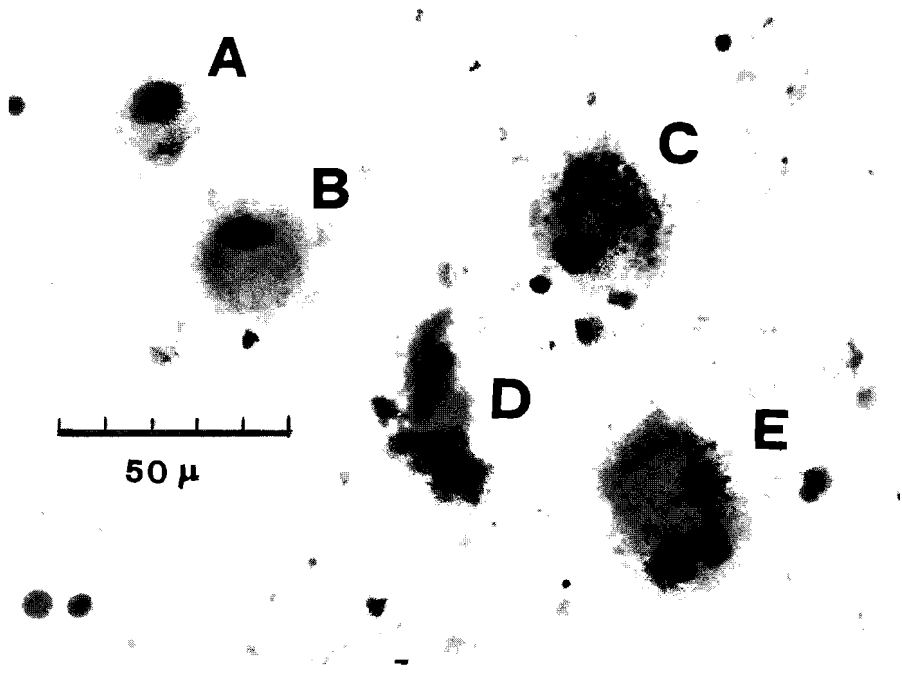

Fig. 1. Morphology of adherent trout head kidney cells. Trout cells were incubated in the presence of 200 μg ml^{-1} of LPS. Cells could be classified as eccentric-nuclei and adherent cells. Adherent cells were very polymorphic (A, B, D, E) and some contained brown granules in their cytoplasm (C).

peared individually dispersed in most of the mitogen-containing cultures and only a few groups of three to four cells appeared in some of the cultures.

Both isolated cells and colonies were present in the cultures made in the presence of mammalian mitogens. Large-nucleated cells formed colonies and dispersed individual cells in PHA- and Con A-containing cultures (Fig. 2A). Cells with eccentric nuclei formed colonies in large numbers in cultures made in the presence of PHA (Fig. 2B). Multinucleated cells formed colonies both in PHA- and Con A-stimulated cultures (Fig. 2C). None of these cells were stained by any of the staining procedures used (see Materials and Methods). Lymphocytes, present at the beginning of the cultures disappeared after 1 week in culture. Only a few rare lymphocyte colonies were present in some cultures (Fig. 2D). Some lymphocytes appeared positive to peroxidase and non-specific esterase staining.

Optimal mitogen concentrations

About 70% of the PHA-induced colonies were made of eccentric cells and the rest were made of either multinucleated or large-nucleated cells.

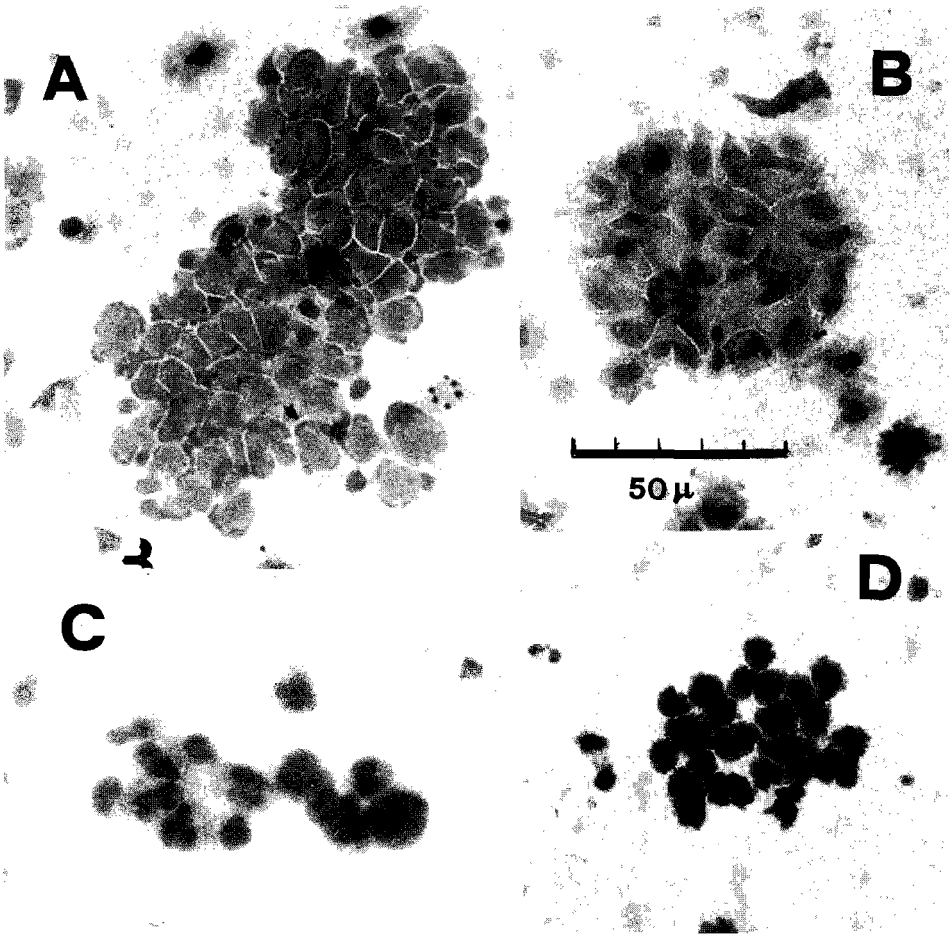

Fig. 2. Morphology of PHA-stimulated trout head kidney colonies. Trout kidney cells were incubated in the presence of 2 μg ml^{-1} of PHA. A, colonies formed by large-nucleated cells; B, colonies formed by eccentric-nuclei cells; C, colonies formed by multinucleated cells; D, colonies formed by lymphocytes.

Con A-induced cells belonged also to the eccentric and multinucleated type. Most of the Con A-induced colonies were made of eccentric-nuclei cells. PHA induced the highest number of colonies (more than 800 per well) when compared with Con A or LPS. About 100 μg ml^{-1} of Con A were needed to obtain a comparable effect to the one obtained with 2 μg ml^{-1} of PHA.

LPS did not seem to have any major effect upon the cells or to induce as many colonies as the other mitogens up to 200 μg ml^{-1}. Adherent cells seem to be relatively more abundant in these than in the other mitogen-containing cultures. LPS obtained from other aquatic bacteria (*Vibrio anguillarum,*

Aeromonas salmonicida, Yersinia ruckeri, Aeromonas hydrofila, Aeromonas fluorescens and *Aeromonas sobria*) induced some cell survival but no colony formation (not shown).

The number of colonies was also dependent on the number of cells plated. For instance, in Con A-containing cultures (50 μg ml^{-1}), the total number of colonies increased from 0 to 68 to 109 when 1×10^4 cells per well, 1.7×10^4 cells per well and 2.5×10^4 cells per well where plated, respectively.

Time course

The number of cells and colonies was studied at 1 or 2 weeks in culture in the presence of optimal concentrations of mitogens. The total number of lymphocytes present at time 0 in the culture decreased drastically during the first days in culture (Fig. 3). The numbers of PHA- or Con A-induced colonies,

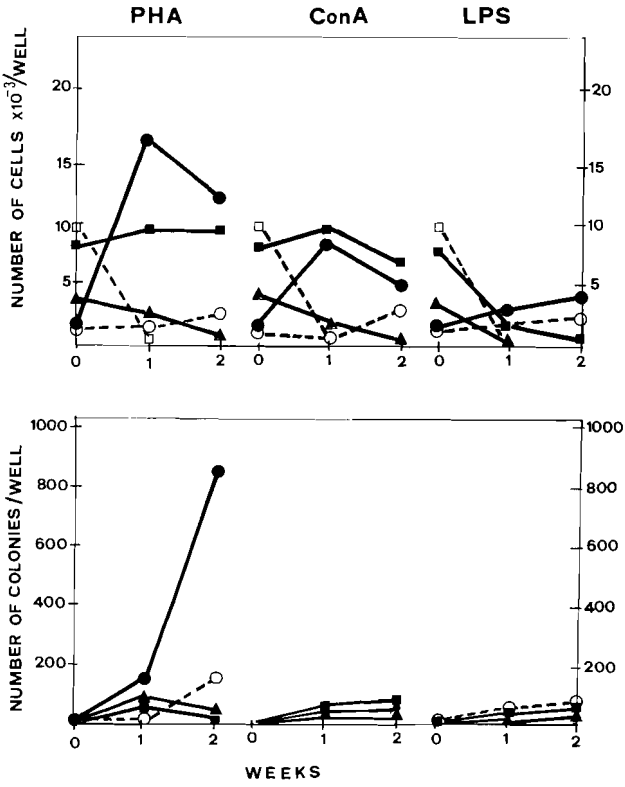

Fig. 3. Time-course of the appearance of cells and colonies in the presence of lymphocyte mitogens. Trout kidney cells were used at 28 000 cells per well. Concentrations of mitogens used were: 2 μg of PHA ml^{-1}, 20 μg of Con A ml^{-1}, and 200 μg of LPS ml^{-1}. ●, eccentric-nuclei cells; ■, multinucleated cells; ▲, large-nucleated cells; □, lymphocytes; and ○, adherent cells.

increased from 1 to 2 weeks in culture when 2 μg ml^{-1} (Fig. 3) or 100 μg ml^{-1} (not shown), respectively, were used. However, the PHA-induced colonies reached their maximum counts earlier (after 1 week) if 10 μg ml^{-1} instead of 2 μg ml^{-1} were used (Fig. 4).

Even though examination of the clots immediately after seeding showed no cell groups or clusters, their size was estimated at two different times during the culture to confirm that the colonies were not clusters of cells. The average size of the colonies increased from 10 cells at Day 7 to 19 cells at Day 14 ($n=50$). On the other hand, the 1-week-old colonies had an average size of six cells per colony ($n=50$) at 14°C, compared with 10 cells per colony ($n=50$) at 20°C. Metaphase figures only appeared in some colonies, being between 2 and 5% of the cells in the colony. Most of the colonies appearing in the clots were composed of only one cell type. However in cultures made in the presence of Con A, groups of both large-nucleated and adherent cells were

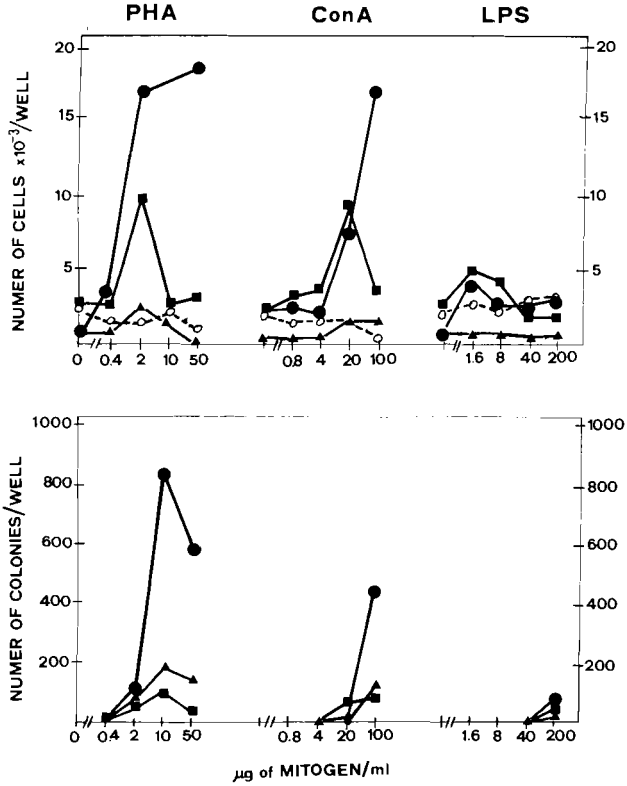

Fig. 4. The effect of the concentration of lymphocyte mitogens on the number of cells or colonies. Trout kidney cells were used at 28 000 cells ml^{-1}. The culture was at 1 week. ●, eccentric nuclei cells; ■, multinucleated cells; ▲, large-nucleated cells; and ○, adherent cells.

observed after 3 days in culture. These groups develop to colony bursts after 10 days in culture (not shown).

Individual trout variability

To study the influence of individual trout in the number and type of mitogen-induced cells and colonies, four trout were used in four individual experiments and results were pooled. The number of the cells varied between the different trout used but increased when compared with controls with no added mitogens. The number of colonies was dependent on the presence of PHA or Con A in the cultures. Most of the mitogen-stimulated colony-forming cells were of the eccentric-nuclei cell type although large-nucleated and multinucleated cells were also found forming colonies. The number of PHA-induced colonies formed by eccentric-nuclei cells was 613 ± 420 per 22 000 kidney trout leucocytes ($n=4$).

The average CV of the mitogen-stimulated cells varied from 51% to 61.5% and the average CV of stimulated colonies varied from 71.2% to 83% (Table 1).

DISCUSSION

This is the first report on the cloning of trout kidney leucocytes. The concentrations of mitogens necessary to obtain the maximum number of kidney colonies were the same as those needed to obtain the maximum incorporation of tritiated thymidine in parallel liquid cultures (M. Torroba, personal communication, 1990).

Thymidine incorporation induced by PHA has been used to study T-lymphocyte heterogeneity in teleosts, including the trout (De Luca et al., 1983; Faulmann et al., 1983; Blaxhall and Sheard, 1985). However, the heterogeneity of the PHA-induced kidney colonies reported here, the PHA-induced peripheral blood Ig-containing cells forming colonies (Caspi et al., 1982) and the PHA-induced stimulation of antibody synthesis (Kaattari et al., 1986), demonstrated that the fish cellular response to T-specific mitogens is not so simple. At least four different types of colonies could be distinguished only on morphological grounds (Fig. 2).

That some of the cells were proliferating was strongly suggested by the appearance of colonies made by only one cell type (Fig. 2), their increase in size with time, the 2–5% metaphases found in the colonies (as compared with the 0.6–3.9% reported by Caspi et al. (1982) in carp peripheral cultures) and the more than 10-fold stimulation indexes found when using PHA or Con A in parallel studies by the tritiated thymidine method (M. Torroba, personal communication, 1990). With LPS obtained from *E. coli*, and/or other aquatic bacteria, however, no such strong evidence of cell proliferation has been ob-

tained. As the fibrin-clot method allows the study of not only proliferation, as with tritiated thymidine (De Luca et al., 1983; Warr and Simon, 1983), but also of cell survival or differentiation without any cell division (as it appears in LPS-stimulated cultures), it may allow studies of other growth factors. For instance, the stimulation of cell survival rather than of proliferation seems to be a first step to any additional effects exerted by the same and/or other factors, as has been shown for hematopoietic cells (Williams et al., 1990).

The method employed in this work to culture kidney cells is simpler than the two-step agar techniques described to culture blood lymphocytes from fish (Caspi et al., 1982; Finegan and Mulcahy, 1987). The two-step agar techniques had a first step in liquid culture at high cell concentrations to generate blasts 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 temperature, the trout cells could be vulnerable to molten agar above 20°C and batches of agar are highly variable (some even may be toxic). The method described here has, therefore, all the advantages of growing in semi-solid medium and the use of more physiological substances (fibrinogen and thrombin). In addition, the fibrin-clot technique allows a permanent record of the results, making direct comparison between experiments possible.

According to the morphology of the subpopulations of trout kidney cells defined by Plytycz et al. (1989), the staining characteristics, and other ultrastructural studies (Suzuki, 1984; Dogget and Harris, 1989), the LPS-stimulated adherent cells are similar to the macrophages and the melanomacrophages. The granulocytes seem to be absent from the mitogen-induced cultures (Sudan-Black B and nitroblue tetrazolium negatives). The large-nucleated cell-forming colonies in our cultures are very similar to some of the blasts appearing in the liquid cultures of peripheral blood leucocytes from carp prior to cloning (Caspi et al., 1982). These cells appeared as Con A-induced colonies bursting from small groups of large-nucleated cells and macrophages. The eccentric-nuclei cells were similar to the surface and the agar colonies (Caspi et al., 1982; Finegan and Mulcahy, 1987) but no colonies of multinucleated cells have been reported before. Most probably this last type of cell does not form colonies from peripheral blood but, as happens with the macrophages, they are only obtained when the kidney is used as a source of leucocytes. The complete understanding of the function of all these cells must await further research. For instance, other experiments showed on the one hand, specific stimulation and immunostaining of multinucleated and eccentric-nuclei cells with a panel of monoclonal antibodies against trout serum immunoglobulins, and on the other hand, adherent cell stimulatory responses to the addition of isolated viral hemorrhagic septicemia viral proteins (Estepa et al., 1991).

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REFERENCES

Bayne, C.J., 1986. Pronephric leucocytes of *Cyprimus carpio*: isolation, separation and characterization. Vet. Immunol. Immunopathol., 12: 141–151.

Blaxhall, P.C. and Sheard, P.R., 1985. Preliminary investigation of the characteristics of fish lymphocytes separated on a Percoll discontinuous gradient. J. Fish Biol., 26: 209–216.

Caspi, R.R., Rozenszajn, L.A., Gothelf, Y., Pergamenikov-Litvak, T. and Avtalion, R.R., 1982. The cells involved in the immune response of fish II PHA-induced clonal proliferation of carp lymphocytes in soft agar culture. Dev. Comp. Immunol., 6: 683–692.

Coll, J.M., 1989. Addition of reducing agents to the peroxide-o-phenylenediamime buffer reduces background of enzyme immunoassays. Rev. Esp. Fisiol., 45: 41–46.

De Luca, D., Wilson, M. and Warr, G.W., 1983. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol., 13: 546–551.

Estepa, A., Basvrco, B., Samz, F. and Coll, J.M., 1991. 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. and Coll, J.M., 1991. Infection of mitogen-stimulated trout leucocytes with salmonid viruses. J. Fish Dis., in press.

Doggett, T.A. and Harris, J.E., 1989. Ultrastructure of the peripheral blood leucocytes of *Oreochromis mossambicus*. J. Fish Biol., 33: 747–756.

Faulmann, E., Cuchens, M.A., Lobb, C.J., Miller, N.W. and Clem, L.W., 1983. An effective culture system for studying in vitro mitogenic responses of channel catfish lymphocytes. Trans. Am. Fish Soc., 112: 673–679.

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

Jimenez, J., Marcotegui, M.A., San Juan, M.L. and Basurco, B., 1988. Diagnosis of viral diseases in Salmonid farms in Spain. Bull Eur. Assoc. Fish Pathol., 271: 3–4.

Kaastrup, P., Nielsen, B., Horlyck, V. and Simonsen, M., 1988. Mixed lymphocyte reactions (MLR) in rainbow trout (*Salmo gairdneri*) sibling. Dev. Comp. Immunol., 12: 801–808.

Kaattari, S.L., Irwing, M.J., Yui, M.A., Tripp, R.A. and Parkins, J.S., 1986. Primary in vitro stimulation of antibody production by rainbow trout lymphocytes. Vet. Immunol. Immunopathol., 12: 29–38.

Plytycz, B., Flory, C.M., Galvan, I. and Bayne, C.J., 1989. Leucocytes of rainbow trout (*Oncorhynchus mykiss*) pronephros: cell types producing superoxide anion. Dev. Comp. Immunol., 13: 217–224.

Rueda, A. and Coll, J.M., 1988. Cloning of myelomas and hybridomas in fibrin clots. J. Immunol. Methods, 114: 213–217.

Sanchez, M.C.T. and Coll, J.M., 1989. La estructura de las immunoglobulinas de los peces. Immunologia, 8: 47–54.

Suzuki, K., 1984. A light and electron microscope study of leucocytes in rock fish and rainbow trout. Bull. Jpn. Soc. Sci. Fish, 50: 1305–1315.

Warr, G.W. and Simon, R.C., 1983. The mitogen response potential of lymphocytes from the rainbow trout (*Salmo gairdneri*) reexamined. Dev. Comp. Immunol., 7: 379–384.

Williams, G.T., Smith, C.A., Spooncer, E., Dexter, T.M. and Taylor, D.R., 1990. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature, 343: 76–79.