Fish & Shellfish Immunology (1993) **3**, 371–381

Reduction of melanomacrophage numbers in stimulated trout kidney cell cultures

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(*Received 5 March 1992, accepted in revised form 17 July 1992*)

Cells with large cytoplasm (≥ 30 μm of diameter) and round and eccentric nuclei, were obtained in mitogen-stimulated rainbow trout kidney cells cultured in fibrin clots. These cells were adherent to plastic, phagocytised cell debris, stained with acridine orange and some had dense granules in their cytoplasm. These adherent cells were identified on the basis of these properties as macrophages, and those with cytoplasm granules as melanomacrophages. The percentage of melanomacrophages in the total adherent cell population decreased when the concentrations of the mitogens phytohaemagglutinin (PHA), Concanavalin A (Con A), and bacterial lipopolysaccharides (LPS) were increased in the cultures to the point where colonies of other cellular types began to appear. The addition of any three anti-trout serum immunoglobulin (Ig) monoclonal antibodies (MAbs) selected among a panel of 20 MAbs, was stimulatory for adherent cells and also decreased the percentage of melanomacrophages, in contrast to the other MAbs. The possible implications of melanomacrophages in the control of proliferation of other cellular types in trout kidney fibrin clot cultures are discussed.

Key words: melanomacrophage, macrophage, mitogens, trout.

I. Introduction

Both melanomacrophages (Plytycz *et al.*, 1989) and macrophages (Braun-Nesje *et al.*, 1982) have been described in rainbow trout, *Oncorhynchus mykiss* W., as in other teleost fish but there are still questions as to their function (Clem *et al.*, 1985; Chung & Secombes, 1987; Faisal & Ahne, 1990; Graham & Secombes, 1990; Vallejo *et al.*, 1991*a*).

To study this *in vitro* behaviour of trout melanomacrophages (as defined by Plytycz *et al.*, 1989) and macrophages, we chose the anterior kidney because it is the major haematopoietic tissue in fish (Bayne, 1986). An *in vitro* fibrin clot system has been described in which mammalian lymphocyte mitogens induced the proliferation (as measured by colony formation) of large-nucleated cells, eccentric-nucleated cells, multinucleated cells and small-lymphocytes (Coll, 1990; Estepa & Coll, 1992*b*). The use of fibrin clots for these studies allows not only the preservation of the morphology but also the possibility of studying both cell proliferation by colony formation and/or differentiation (Estepa & Coll, 1992*b*; Estepa & Coll, 1993).

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1050–4648/93/050371+11 $08.00/0

We report here that trout kidney adherent cells with all the characteristics of trout macrophages, respond to mitogens mainly with a decrease of their melanomacrophage component. In addition we try to correlate the melanomacrophages decrease in number with the proliferation of other trout kidney cell types.

II. Materials and Methods

PREPARATION OF RAINBOW TROUT HEAD KIDNEY CELLS

Anterior kidney cells were prepared as described previously (Coll, 1990; Estepa & Coll, 1992*b*). The trout (5–20 g body weight) were cooled to 4° C to reduce their activity and bled from the tail vein to reduce the red cell content of their kidney. Cultures were always prepared from individual fish to avoid any possible mixed leucocyte reactions (Stet & Egberts, 1991). The head kidney (pronephros) was removed and cut into pieces in a Petri dish. Clumps of cells were dissociated by passing the suspension through a 20-gauge needle. The cell suspension was decanted, centrifuged at 100 *g* for 10 min and washed once in cold cell culture medium (described below). The cell count was determined with a haemocytometer and the concentration adjusted to 2×10^5 round cells ml^{-1}. To isolate adherent cells, the anterior kidney cells were incubated in 25 cm^2 flasks for 7 days in the medium described below. The medium was then changed and the adherent cell cultures maintained at 14° C until used for the experiments, usually no more than a month.

CELL CULTURE IN FIBRIN CLOTS

The cell culture media used was RPMI-1640 (Flow Lab, Ayrshire, Scotland) (Dutch modification) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1·2 μg ml^{-1} anfotericin, 50 μg ml^{-1} gentamicin, 20 mM Hepes, 50 μM mercaptoethanol, 10% foetal calf serum (pretested for kidney leucocyte colony formation) 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 2 mg ml^{-1}.

Thrombin (Miles Lab, Elkart, Indiana) was added to the wells of a 96-well plate (Costar, The Netherlands) at 0·2–0·4 National Institutes of Health Units (NIH U) in 2 μl volume, and 100 μl of the cell suspension was pipetted into each well. After clotting occurred (in about 30 s), additives (mitogens and MAbs) in medium were pipetted in a maximum volume of 10 μl on the top of the clot. The plates were then placed in a plastic bag (Vaessen, Schoemaker Indtal, South Africa, Sant Boi de Llobregat, Barcelona, Spain), sealed and incubated at 20° C (Estepa & Coll, 1992*b*). After incubation for 7–14 days depending on the experiments, the clots were removed from the wells onto a 75 × 25 mm frosted-end glass slide with a spatula, squashed onto the glass with filter paper, fixed with 1·7% glutaraldehyde in 0·15 M sodium chloride 0·01 M sodium phosphate, pH 7·4, for 10 min, stained for 10 min with 0·025% Toluidine Blue in 0·01 M sodium borate, pH 8, and washed in running tap water for 10 min as described before (Rueda & Coll, 1988). Each well gave a fixed circle clot of about 6 mm diameter.

Cells were studied under an optical microscope at $\times 400$ magnification. Four wells were prepared for each experiment, one field was counted for each well and the average and standard deviations (S.D.) were calculated. The number of cells/well was calculated by the following formula; number of cells/field $\times$ total area per wall/area per field. The percentage of adherent cells was calculated by the following formula; number of adherent cells/total number of cells $\times 100$. The percentage of melanomacrophages was calculated by the following formula, number of melanomacrophages/number of adherent cells $\times 100$. A complete description of the technique has been published elsewhere (Coll, 1990; Estepa & Coll, 1992*b*). Tritiated thymidine incorporation by the whole trout kidney cells, adherent cells and non-adherent cells was measured as described before (Estepa & Coll, 1992*a*). Briefly, 1 μCi of methyl tritiated thymidine (60 Ci mmol^{-1}, Amersham, The Netherlands) was added in 25 μl of culture medium to 7-day cultures (200 000 cells ml^{-1}) and the cells cultured for 2 additional days. The cells were harvested with distilled water onto glass fibre filters with a Skatron cell harvester (Sterling, VA, U.S.A.). Samples were processed and averages $\pm$ S.D. from three different trout represented (Table 2).

CYTOLOGICAL STAINS

Staining for nonspecific esterase was made by the *a*-napthyl acetate method as described by Plytycz *et al.* (1989). Sudan Black B, nitroblue tetrazolium, and peroxidase staining, were carried out following the methods described by Estepa & Coll (1992*a*). 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 line (Bayne, 1986). Lysosomes appeared orange and nuclei green under the fluorescence microscope.

MONOCLONAL ANTIBODIES

Anti-trout serum immunoglobulin (Ig) monoclonal antibodies (MAbs) were used to stimulate head kidney cultures as described above. We used the panel of MAb described in Sanchez (1992) and Sanchez *et al.* (1989). Presence of MAbs in ascites was confirmed by ELISA titration (half maximal O.D. about 1/500 for most MAbs) and by the presence of a peak in the Ig region of cellulose acetate electrophoresis of the ascites (Sanchez *et al.*, 1991). The amount of MAb in the ascites was adjusted to 20–60 mg ml^{-1}. The characteristics of the MAbs used are given in Table 1. Ascites from mice containing anti-Ig MAbs were added to the fibrin clot cultures of head kidney trout cells to a final concentration of 2–6 mg of MAb ml^{-1} and cultured for 10 days at 20° C.

III. Results

PROPERTIES OF THE FLASK-ISOLATED ADHERENT TROUT KIDNEY CELLS AFTER FIXING THEM IN FIBRIN CLOTS

Kidney cells, after incubation in plastic flasks in liquid culture for 1 week, could be separated into adherent and non-adherent cells. The isolated adherent

Table 1. Characteristics of the anti-Ig MAbs used in this work

Clone	Isotype	ELISA	Blotting	Reference
1A6	IgG_1K	+	H	Sanchez *et al.*, 1989
1G7	IgG_1K	+	H	Sanchez *et al.*, 1991
1H2	IgG_1K	+	L	Sanchez, 1992
5C12	IgG_1K	+	H	Sanchez, 1992
1·14	IgG_1K	+	H	De Luca *et al.*, 1983

The isotype was determined by ELISA with a Biorad kit. H, heavy chains from trout Ig; L, light chains from trout Ig.

cells showed reaction with acridine orange (not shown) when stained in the flasks used for their isolation. For further characterisation, the adherent cells were washed with medium, removed by gentle agitation, centrifuged, placed onto a fibrin clot and immediately fixed. After fixing and staining, microscopically these cells possessed a rounded and eccentric nuclei and considerable cytoplasm (about 30 μm in diameter). Some of these cells were filled with brown granules. Only cells having a rounded and eccentric nucleus and abundant cytoplasm, possessed granules. Both small ($\leq 20\,\mu$m of diameter) and large ($\geq 20\,\mu$m of diameter) adherent cells showed granules. The amount of granules varied between none to a dense mass impossible to count. The average number of granules per adherent cell cytoplasm, was $54{\cdot}2 \pm 38$ ($N=30$). The majority of the granules were 0·1 μm in diameter with a range of $<0{\cdot}1$ to 1 μm. The adherent cells containing granules were found scattered throughout the clot and granules were visible in unstained clots both inside the adherent cells and free in the cell culture medium. The adherent cells in fibrin clots showed reaction with peroxidase and sometimes with non-specific esterase but not with Sudan-Black B or nitroblue tetrazolium. Their cell surface showed finger-like projections. Because of their morphology and staining characteristics shown by means of fixing in the fibrin clots, the adherent cells were considered to be either macrophages (no granules in their cytoplasm) or melanomacrophages (dense granules in their cytoplasm). The percentage of melanomacrophages in the total adherent cells from the kidneys of six trout was $44{\cdot}3 \pm 7$ (range 36–55%).

WHOLE TROUT KIDNEY CELLS CULTURED IN FIBRIN CLOTS IN THE PRESENCE OF MITOGENS

Based on their fixed morphology in fibrin clots before culture, all the kidney cells (adherent and non adherent) could be classified as belonging to one of the following types; lymphocytes, large-nucleated cells, multinucleated cells, eccentric nuclei cells and adherent cells (20–30 μm). The average number of the adherent cells in the whole kidney was 6·4% (range 2–11%, $N=9$).

The adherent cells appeared individually dispersed after 1 week of fibrin clot culture of the whole kidney cells in most of the mitogen containing cultures and only a few groups of three to four cells appeared in a few cultures. The percentage of melanomacrophages in the adherent cells increased slightly from 44·3 to 56% when the trout kidney cells were kept in fibrin clot culture for

1 week. However, as increasing concentrations of mitogens (PHA, Con A or LPS) were included in the cell culture medium, a decrease in the percentage of melanomacrophages could be measured [Fig. 1(a)] while an increase in the counts of total adherent cells [Fig. 1(b)], an increase in the counts of cells and an increase in the counts of colonies (Fig. 2) occurred in these same cultures (PHA or Con A). Five micrograms per millilitre of PHA, 75 $\mu g\ ml^{-1}$ of Con A and $\geq 100\ \mu g\ ml^{-1}$ of LPS was needed to reduce the percentage of melanomacrophages to $\leq 30\%$ [Fig. 1(a)].

From 1 to 2 weeks of fibrin clot culture in the presence of PHA or Con A, the percentage of melanomacrophages decreased (Fig. 3) while the number of colonies increased six and two-fold in cultures containing PHA and Con A, respectively (not shown).

PERCENTAGE OF MELANOMACROPHAGES IN ANTI-Ig MAb STIMULATED FIBRIN CLOT CULTURES

The addition of most of the anti-Ig MAbs to the fibrin clot cultures of whole trout kidney cells stimulated the number of eccentric nucleated and multinucleated cells. Three of the MAbs stimulated the number of adherent cells compared to the controls. To measure the percentage of adherent cells and melanomacrophages stimulated by anti-Ig MAbs, five MAbs were selected, two representing those which stimulated eccentric nucleated and multinucleated cells (MAbs 1A6 and 1G7) and three which specifically stimulated adherent cells (MAbs 5C12, 1H2 and 1·14).

The percentage of adherent cells was $\leq 25\%$ for control cultures, for cultures containing polyclonal anti-Ig, and for anti-Ig MAbs 1A6 and 1G7. The percentage of adherent cells was $> 30\%$ for anti-Ig MAbs 5C12, 1H2 and 1·14. In control cultures, $> 50\%$ of the adherent cells were melanomacrophages. In cultures containing PAbs or MAbs 1G7 and 1A6 20–30% of the adherent cells were melanomacrophages. However, in cultures containing anti-Ig MAbs 5C12, 1·14 and 1H2 the percentage of melanomacrophages was $\leq 10\%$ (Fig. 4).

REQUIREMENT OF ADHERENT CELLS FOR PHA-INDUCED PROLIFERATION

Since the increase in adherent cells and the decrease in the percentage of melanomacrophages in the fibrin clot cultures [Fig. 1(b)] was parallel to an increase in the number of colonies (Fig. 2) we decided to investigate a possible relationship between adherent cells and proliferation, and the effect of separating adherent cells and non-adherent cells upon PHA-induced cell proliferation.

Each of the whole kidney from three trout were independently fractionated into adherent (33%) and non-adherent (66%) cells. About 37% of the adherent cells were melanomacrophages. Thymidine incorporation in the presence of PHA was about the same for the adherent and non-adherent cell populations; however, it was five to ten-fold lower than the incorporation obtained when the unfractionated whole kidney was used (Table 2).

IV. Discussion

Previous studies had shown the importance of the adherent cells in the process of resistance to VHS disease since a specific memory response of these

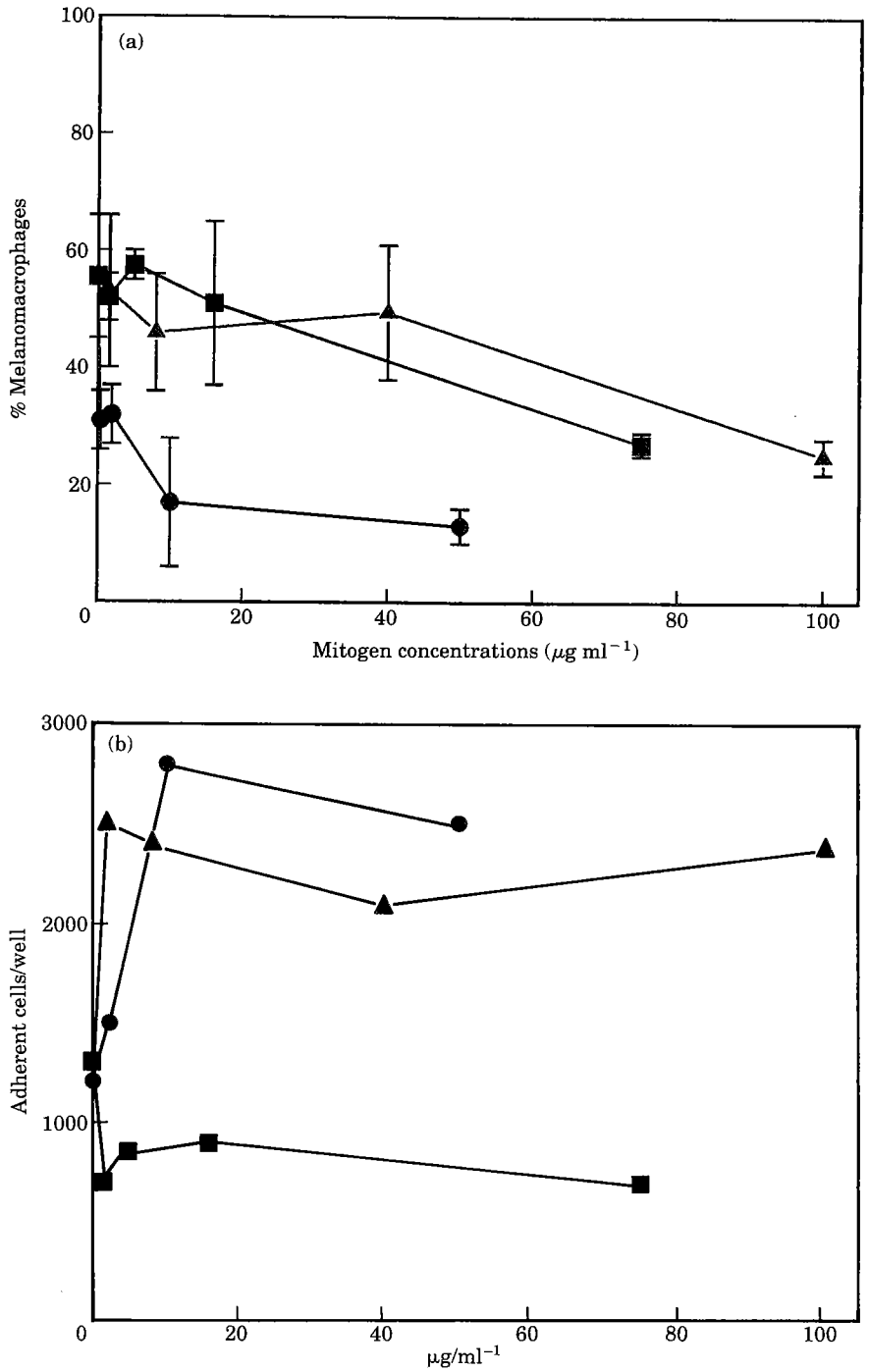

Fig. 1. Percentage of melanomacrophages (a) and number of adherent cells (b) in trout kidney cell cultures in the presence of increasing concentrations of mitogens. Trout kidney cells were cultured for 1 week in the presence of phytohaemagglutinin [PHA (●)]; Concanavalin A [Con A (■)], and lipopolysaccharide [LPS (▲)] from *E. coli*. Averages ± S.D. of duplicates are given in the figure.

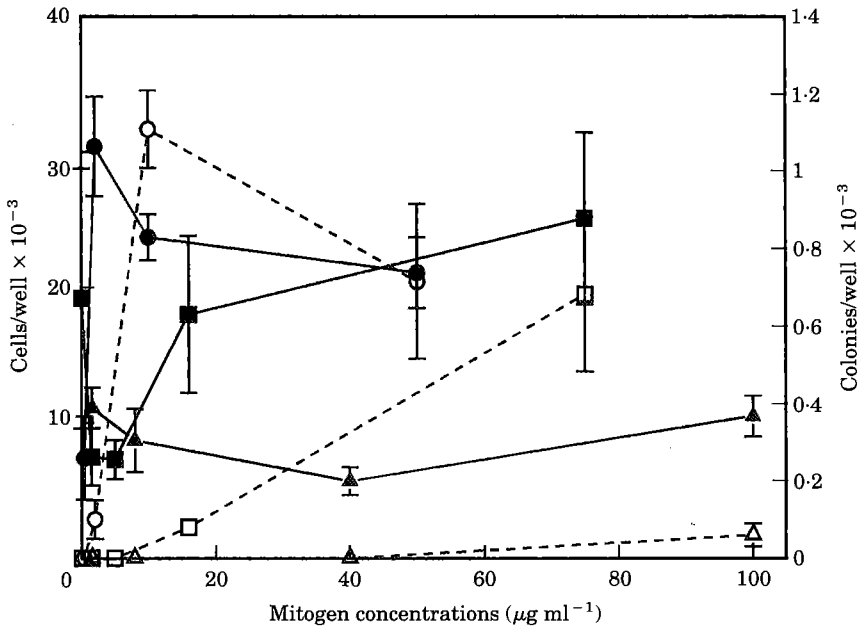

Fig. 2. Number of cells and colonies of adherent cells in trout kidney cell cultures in the presence of mitogens. PHA (●, ○); Con A (■, □); LPS (▲, △); (●, ■, ▲) number of cells; (○, □, △) number of colonies. Averages ± S.D. of tetraplicates (cells) or of duplicates (colonies) are given in the figure.

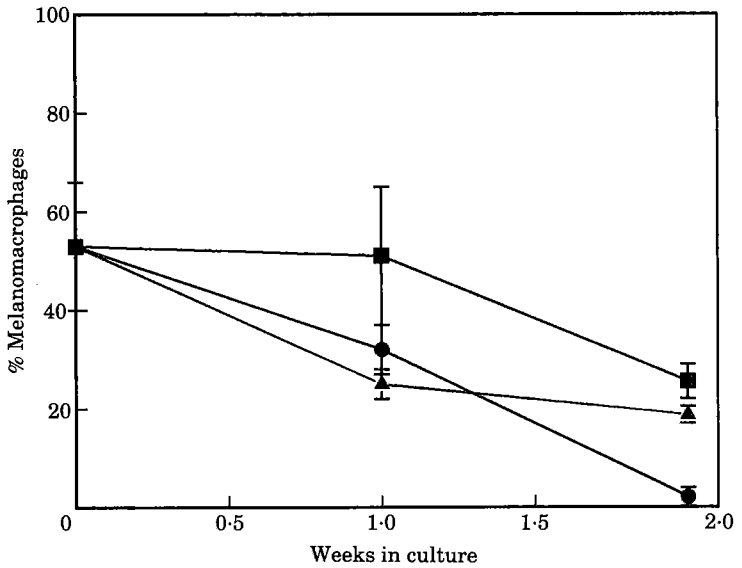

Fig. 3. Time course of the percentage of melanomacrophages in mitogen stimulated cultures. (●) 2 μg ml^{-1} of PHA; (■) 20 μg ml^{-1} of Con A; (▲) 100 μg ml^{-1} of LPS. Averages ± S.D. of duplicates are given in the figure.

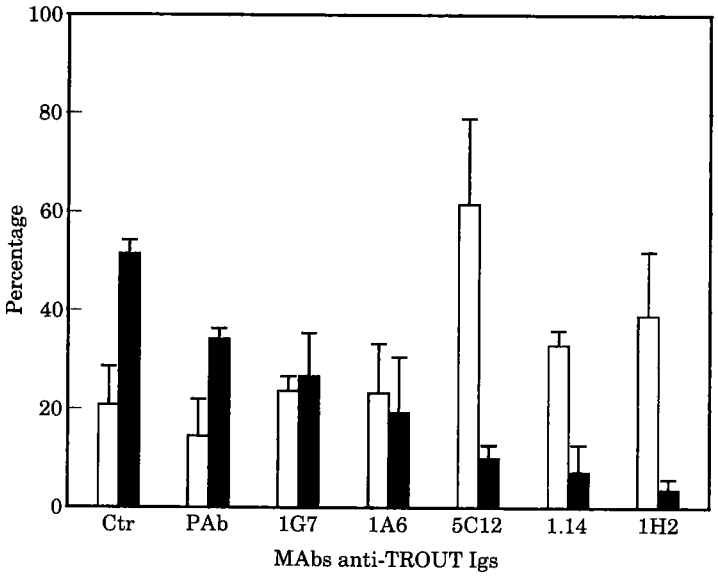

Fig. 4. Percentage of adherent cells and melanomacrophages cultured in the presence of selected anti-Ig MAbs. Average and S.D. from two different trout are represented in the figure. PAb, polyclonal Ab from the lymphocyte donor mouse used to make the hybridomas. Controls were made with ascites from uninjected mice. In these experiments the percentage of melanomacrophages for PHA (2 μg ml^{-1}), Con A (20 μg ml^{-1}) and LPS (100 μg ml^{-1}) stimulated cultures were 9·6 ± 5·3, 27·8 ± 12·1 and 29 ± 2, respectively. (□), % of adherent cells; (■), % of melanomacrophages.

Table 2. ^{3}H-thymidine incorporation by isolated adherent, isolated non-adherent and unfractionated whole trout kidney cells in the presence of PHA

Type of trout kidney cells	^{3}H-thymidine incorporation, cpm/well	
	control	+ PHA (2 μg ml^{-1})
Isolated adherent	600 ± 300	1650 ± 700
Isolated non-adherent	900 ± 400	1700 ± 600
Unfractionated whole	500 ± 100	26 000 ± 8000

Average ± S.D. from three different trout, duplicated.

cells from either trout kidney leucocytes immunised or resistant to VHSV 1 year after infection (Estepa *et al.*, 1991; Estepa & Coll, 1992*a*) was associated with the addition of nucleoproteins (Basurco *et al.*, 1991) and the glycoproteins of VHSV to their cultures. These results prompted further studies on the adherent cells.

It had been shown that the morphology and properties of the cells from healthy or VHSV immunised trout kidney, that responded to the N and G VHSV proteins (Estepa & Coll, 1992*a*; Estepa *et al.*, 1991) were the same as the adherent

cells isolated from healthy trout kidney (Coll, 1990). In this study, we have further characterised these adherent cells. Microscopically they showed granules in their cytoplasm, red blood cell phagocytosis (Coll, 1990) and were positive for acridine orange staining and peroxidase staining. They did not form colonies in response to PHA, Con A or LPS, and 10% of the cells showed fluorescence with a MAb against trout serum Ig (Sanchez *et al.*, 1991). Some of these properties have been described for fish macrophages (Chung & Secombes, 1987) and/or melanomacrophages (Clem *et al.*, 1985) even though the nature of the granules have not yet been investigated (Braun-Nesje *et al.*, 1982; Bayne, 1986) and our own findings agree with the definitions given by Plytycz *et al.* (1989). The importance of macrophages (monocytes) as accessory cells in higher vertebrate immune responses is well established, but it is only beginning to be known what function they perform in fish (Vallejo *et al.*, 1991*a*,*b*) by means of monocyte-like cell lines (Vallejo *et al.*, 1991*c*). Once activated by a variety of factors (White *et al.*, 1986), the adherent cells may exhibit increased metabolic activity (Graham *et al.*, 1988), phagocytosis (Olivier *et al.*, 1986), spreading (Estepa *et al.*, 1991) and adherence (Secombes, 1987). However, no studies have yet been reported to differentiate macrophages behaviour from that of melanomacrophages.

The trout macrophages/melanomacrophages appear to divide in the fibrin clot cultures. This is suggested by their increased counts [Fig. 1(b)] compared to their initial numbers at time 0 (maximal increments of about two-fold). However, none or very few small colonies were seen, and also none or very few (≤ 1 in about 1000 cells) metaphases could be detected, and never in melanomacrophages. Alternatively, the macrophages appearing in the culture could well be the result of the differentiation of the eccentric nucleated cells, since most of the macrophages are very similar to the eccentric nucleated cells except for their larger size.

All the mitogens tested (PHA Con A, or LPS) and the anti-Ig MAbs (1H2, 5C12, 1·14) which increased the percentage of adherent cells in culture did so by increasing the macrophages rather than the melanomacrophages, suggesting that the melanomacrophages could be the result of macrophage differentiation occurring after macrophage stimulation. Alternatively, macrophages may ingest granules (of melanin ?) *in vivo*, and this would not occur during our *in vitro* studies, so that if adherent cell percentages increase, it could only be of granule-free cells. The percentage of melanomacrophages was minimal in cultures containing other cell type colonies. Thus some relationship might be occurring between macrophage differentiation, malanomacrophages, release of cytoplasmic granules and colony formation of other cellular types, as suggested by the adherent cell requirement for proliferation (Table 2) and their apparent requirement for blast or large-nucleated cell colony formation (Estepa & Coll, 1993). Further fractionation experiments are needed to clarify the possible implications of adherent cells, macrophages, melanomacrophages and/or their products in trout kidney cell proliferation.

We appreciated the technical assistance of D. Frías and of J. Coll Perez in typing and drawing. This work was supported by Research Grant (8568) from the Instituto Nacional de Investigaciones Agrarias del Ministerio de Agricultura, INIA (Spain). E.A. is a recipient of a predoctoral fellowship from INIA.

References

Basurco, B., Sanz, F., Marcotegui, M. A. & Coll, J. M. (1991). The free nucleocapsids of the viral haemorrhagic septicaemia virus contain two antigenically related nucleoproteins. *Archives of Virology* **119,** 153–163.

Bayne, C. J. (1986). Pronephric leucocytes of *Cyprinus carpio*: isolation, separation and characterization. *Veterinary Immunology and Immunopathology* **12,** 141–515.

Braun-Nesje, R., Kaplan, G. & Seljelid, R. (1982). Rainbow trout macrophages in vitro: morphology and phagocytic activity. *Developmental and Comparative Immunology* **6,** 281–291.

Chung, S. & Secombes, C. J. (1987). Activation of rainbow trout macrophages. *Journal of Fish Biology* **31,** 51–56.

Clem, L. W., Sizemore, R. C., Ellsaesser, C. F. & Miller, N. W. (1985). Monocytes as accessory cells in fish immune responses. *Developmental and Comparative Immunology* **9,** 803–809.

Coll, J. M. (1990). Estimulación de colonias de células de riñón de trucha con fitohemaglutinina en cultivos de fibrina. *Immunología* **9,** 140–145.

De Luca, D., Wilson, M. & Warr, G. W. (1983). Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. *European Journal of Immunology* **13,** 546–551.

Estepa, A. & Coll, J. M. (1992*a*). *In vitro* immunostimulants for optimal responses of kidney leucocytes from trout surviving viral haemorrhagic septicemia virus disease. *Fish and Shellfish Immunology* **2,** 53–68.

Estepa, A. & Coll, J. M. (1992*b*). Mitogen induced proliferation of trout kidney leucocytes in fibrin clots. *Veterinary Immunology and Immunopathology* **32,** 165–177.

Estepa, A. & Coll, J. M. (1993). Properties of blast colonies obtained from head kidney trout in fibrin clots. *Fish and Shellfish Immunology* **3,** 71–75.

Estepa, A., Basurco, B., Sanz, F. & 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 Immunology* **4,** 43–52.

Faisal, M. & Ahne, W. (1990). A cell line (C2C) of adherent peripheral blood mononuclear leucocytes of normal common carp *Cyprinus carpio*. *Developmental and Comparative Immunology* **14,** 255–260.

Graham, S. & Secombes, C. J. (1990). Cellular requirements for lymphokine secretion by rainbow trout *Salmo gairdneri* leucocytes. *Developmental and Comparative Immunology* **14,** 59–63.

Graham, S., Jeffries, A. H. & Secombes, C. J. (1988). A novel assay to detect macrophage bactericidal activity in fish: factors influencing the killing of *Aeromonas salmonicida*. *Journal of Fish Diseases* **11,** 389–396.

Olivier, G., Eaton, C. A. & Campbell, N. (1986). Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). *Veterinary Immunology and Immunopathology* **12,** 223–234.

Plytycz, B., Flory, C. M., Calvan, I. & Bayne, C. J. (1989). Leucocytes of rainbow trout (*Oncorhynchus mykiss*) pronephros: cell types producing superoxide anion. *Developmental and Comparative Immunology* **13,** 217–224.

Rueda, A. & Coll, J. M. (1988). Cloning myelomas and hybridomas in fibrin-clots. *Journal of Immunological Methods* **114,** 213–217.

Sanchez, C. (1992). Analysis estructural y antigénico de las inmunoglobulinas de la trucha arcoiris, *Oncorhynchus mykiss*. PhD. Thesis. Universidad Complutense Madrid, Spain, 215 págs.

Sanchez, C., Dominguez, J. & Coll, J. M. (1989). Immunoglobulin heterogeneity in the trout *Salmo gairdneri* (Richardson). *Journal of Fish Diseases* **12,** 459–465.

Sanchez, C., Coll, J. M. & Dominguez, J. (1991). One step purification of rainbow trout immunoglobulin. *Veterinary Immunology and Immunopathology* **27,** 383–392.

Secombes, C. J. (1987). Lymphokine-release from rainbow trout leucocytes stimulated with Concanavalin A: effects upon macrophage spreading and adherence. *Developmental and Comparative Immunology* **11,** 513–520.

Stet, R. J. M. & Egberts, E. (1991). The histocompatibility system in teleostean fishes: from multiple histocompatibility loci to a major histocompatibility complex. *Fish and Shellfish Immunology* **1,** 1–16.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1991*a*). Phylogeny of immune recognition: role of alloantigens in antigen presentation in channel catfish immune responses. *Immunology* **74,** 165–168.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1991*b*). Phylogeny of immune recognition: Processing and presentation of structurally defined proteins in channel catfish immune responses. *Developmental Immunology* **1,** 137–142.

Vallejo, A. N., Ellsaesser, C. F., Miller, N. M. & Clem, L. W. (1992*c*). Spontaneous development of functionally active long term monocyte-like cell lines from channel catfish. *In Vitro Cell Developmental Biology* **27,** 279–283.

White, S. K., Chappell, L. H. & Secombes, C. J. (1986). Interaction between *Aeromonas salmonicida* and peritoneal macrophages of brook trout (*Salvelinus fontinalis*). *Veterinary Immunology and Immunopathology* **12,** 223–224.