Fish & Shellfish Immunology (1992) **2**, 53–68

In vitro immunostimulants for optimal responses of kidney cells from healthy trout and from trout surviving viral haemorrhagic septicaemia virus disease

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

The specific immune memory of trout surviving a double infection with viral haemorrhagic septicaemia virus (VHSV) was studied by culturing cells from the anterior kidney both in liquid and in a semisolid fibrin medium in the presence of purified proteins from VHSV. In addition, polyclonal stimulators of the immune response (phytohaemagglutinin, Concanavalin A, lipopolysaccharides and a panel of MAbs anti-trout serum immunoglobulin) were studied in healthy trout by culturing cells from the anterior kidney. Finally, an immunisation was stimulated by using simultaneous addition of both specific and unspecific immunostimulants to healthy trout kidney cells. The results suggest the necessity of employing both mitogens and viral nucleo and glycoproteins in subunit immunisation trials, and the opportunity to use this or similar *in vitro* assays to select for the proper epitopes in genetically engineered proteins during subunit vaccine development.

Key words: immunostimulants, trout kidney cells, viral haemorrhagic septicaemia, *in vitro*.

I. Introduction

Fish are the most primitive animals to have an adaptative immune system characterised by the existence of lymphocytes and immunoglobulins (reviewed by Sanchez & Coll, 1989). It is therefore of interest to study the immune system in this primitive model and whether or not it requires the cell types and interactions typical of mammalian immunity. To date, liquid culture and peripheral blood have been generally used (Faulmann *et al.*, 1983) to examine the response of fish lymphocytes to mammalian T and B lymphocyte mitogens (Blaxhall & Sheard, 1985; Kaattari & Yui, 1987) and to inactivated virus (Chilmonczyk, 1978). The few reports on fish lymphocyte proliferation in semisolid medium have been made by using the two-step agar cloning technique and peripheral blood lymphocytes either from carp (Caspi *et al.*, 1982) or from trout (Finegan & Mulcahy, 1987).

To study the immune response of trout to viral antigens, anterior kidney cells were chosen as the kidney is the major haematopoietic tissue in fish. An *in vitro*

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1050–4648/92/010053+16 $03.00/0

fibrin-clot system was developed in which mammalian lymphocyte mitogens induced the proliferation of four morphologically distinct cell types (described in Coll, 1990). Phytohaemagglutinin (PHA) was the best inducer of colony formation giving rise to at least four different types of colonies, formed either by large-nucleated cells, eccentric-nucleated cells, multinucleated cells or lymphocytes. The colonies grew in size with time of incubation, contained only one type of each cell and showed about 2% of metaphases. In addition, PHA also stimulated different types of adherent cells. In this *in vitro* system, none of these cells or colonies were the target for infectious pancreatic necrosis virus (IPNV), another virus of salmonids, whereas all of them were lysed by VHSV (Estepa & Coll, 1991; Estepa *et al.*, 1991*b*). Adherent cells were specifically stimulated by the glycoprotein of the virus spikes, and to a lesser extent by the nucleoproteins of the VHSV in healthy trout. In contrast, a specific memory response was associated more with the nucleoproteins rather than with the glycoprotein when kidney cells from trout immunised by injection with VHSV were employed (Estepa *et al.*, 1991*a*).

In this report, we extended those previous results to trout surviving VHS disease and also describe the use of mitogens, anti-trout serum Ig monoclonal antibodies (MAbs) and of purified viral proteins as stimulators of cells obtained from the anterior kidney of the trout. The results obtained suggest that all stimulants, the protein G, the protein N and a potent polyclonal mitogen like PHA, might be needed to obtain optimal cellular stimulation during immunisation.

II. Materials and Methods

CHEMICALS AND REAGENTS

Medium RPMI 1640 (Dutch modification, 290 mOsm kg^{-1} and 20 mM Hepes) and foetal calf serum were obtained from Flow Laboratories (Ayrshire, Scotland). Foetal calf serum batches (Flow) were selected by testing the growth of PHA induced colonies by the technique described below. Lyophilised human fibrinogen was obtained from A.B. Kabi (Stockholm, Sweden), it was reconstituted and dialysed extensively against distilled water, lyophilised and kept at $-20°$ C in aliquots. Other chemicals used were, gentamicin (Shering, Kenilworth, NJ, U.S.A.), thrombin (Miles, Elkhart, IN), phytohaemagglutinin (PHA), Concanavalin A (ConA) and amphotericin (Flow) and *Escherichia coli* 026: B6 lipopolysaccharide (LPS) (Difco, Detroit, MI).

PURIFICATION OF TROUT IMMUNOGLOBULIN (Ig)

Pooled trout serum dialysed against 10 mM Na_2PO_4 pH 7, was passed through a Trisacryl M DEAE (LKB) 4×5 cm column. After washing, the bound proteins were eluted with 100 mM of the same buffer. Fractions with Ig activity as assayed by enzyme immunoassay (described below) were pooled, concentrated by lyophilisation and applied to a $70 \times 2{\cdot}5$ cm Sephacryl S-300 column (Pharmacia, Uppsala, Sweden) equilibrated with 0·2 M NH_4HCO_3. Fractions with Ig activity

were pooled and concentrated. Purity was tested by 15–20% polyacrylamide gel electrophoresis with sodium dodecylsulfate and βmercaptoethanol (Sanchez *et al.*, 1989, 1991).

TROUT Ig ASSAY

Microwell plates (Dynatech, Plochingen, Germany) were coated with 10 mg ml^{-1} anti-trout Ig antisera (Seromed, Germany) diluted in distilled water. One hundred microlitres per well were incubated in a humid chamber for 3 h at 37° C. Plates were washed in 10-fold diluted dilution buffer (described below), dried for 2 h at 37° C and kept at 4° C in sealed boxes with dried silicagel. The assay procedure was as follows: the samples were diluted 1000-fold in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM NA_2HPO_4, 1·4 mM KH_2PO_4, 50 mg l^{-1} phenol red, 0·24 mM merthiolate, 5 g l^{-1} bovine serum albumin and 0·5 g l^{-1} Tween 20, pH 7·4), 100 μl of the diluted samples were added per well and incubated for 60 min at room temperature. The plates were then washed once with 10-fold diluted dilution buffer and incubated for 30 min at room temperature with 100 μl anti-trout Ig conjugated to peroxidase (EC 1.11.1.7), 1000-fold diluted in dilution buffer. After washing three times with the 10-fold diluted dilution buffer, 50 μl of 150 mM sodium citrate, pH 4·8 containing 3 mM H_2O_2 and 1 g of o-phenylenediamine per litre were added. Colour development was stopped by adding 50 μl of 4 M H_2SO_4 after 15 min. The results were read in a Titertek Multiskan at two wavelengths 492–620 nm. The A (absorbance) at 620 nm was used to correct for individual non-significant differences between wells (Martinez & Coll, 1988).

MONOCLONAL ANTIBODIES (MAbs) AGAINST TROUT Ig

Fusion and cloning were performed as described (Rueda & Coll, 1988; Sanchez *et al.*, 1989). Ascites were obtained as described previously (Coll, 1987) and pooled from at least three mice. Clones were tested for anti-trout Ig by using microwell plates (Dynatech) coated with 2 μg of purified trout Ig/well and developed with rabbit anti-mouse Ig conjugated to peroxidase (Sigma, Chemical Co., St Louis, MI). The procedure used was as described above. Presence of MAbs in ascites was confirmed by ELISA titration [half maximal O.D. (optical density) 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}.

PURIFICATION OF VHSV PROTEINS BY ELECTROPHORESIS AND ELECTROELUTION

The VHSV isolate 144 was isolated in Spain from rainbow trout and characterised as described (Basurco & Coll, 1989). The virus was grown in epithelial papilloma of carp (EPC) cells, assayed, concentrated by polyethyleneglycol (PEG) and purified by ultracentrifugation on sucrose gradients as described before (Basurco & Coll, 1989). The purified virus was analysed by electrophoresis and the protein content adjusted to 1 mg ml^{-1} by both absorbance at 280 nm ($\varepsilon = 1·4$) and Coomassie blue staining of the gel. VHSV proteins were isolated by

preparative gel electrophoresis of purified VHSV in the presence of SDS and βmercaptoethanol as described (Basurco *et al.*, 1991). After electrophoresis, the gel edges were stained with Coomassie blue and the unstained bands between the edges cut at approximately the following molecular weights: 60–80 kDa for G (glycoprotein), 40–45 kDa for N (nucleoprotein), 30–40 kDa for Nx (nucleoprotein), 20–25 kDa for M_1 (matrix protein) and 15–20 kDa for M_2 (matrix protein). The protein bands were then transferred to a dialysis bag and electroeluted in 0·05 M Tris/sodium acetate, pH 7·4 with 0·1% SDS at 100 volts 100 mA for 3 h. After electroelution the bags were dialysed against 0·2 M ammonium bicarbonate. The protein solution was extracted, lyophilised and resuspended in 50 μl of distilled water. Precipitates were discarded after centrifugation. Isolated proteins were re-electrophoresed and the gels were stained by silver nitrate (Biorad Silver staining kit, Richmond, VA). Protein content was adjusted to 200–300 $\mu g\ ml^{-1}$. Confirmation of the identity of the isolated viral proteins was made by enzyme immunoassay of 1 μg of protein bound per well (96-well plates, Dynatech, Plochingen, Germany), by using anti-N and anti-G specific MAbs against the VHSV proteins, and by following the procedures described by Basurco *et al.* (1991) and Estepa *et al.* (1991*a*).

RAINBOW TROUT

Trout (*Oncorhynchus mykiss*) were purchased from commercial farms after several annual tests indicated they were free of infectious pancreatic necrosis. The trout were held in 100 l aquaria with dechlorinated free-flowing water at 12–18° C until use. Thirty-six trout (0·2–1 g/fish) were infected with 10^5 $TCID_{50}$ VHSV ml^{-1} in 2 l of water cooled to 8–10° C during 2 h with strong aeration. Then they were put back into their aquaria and mortality recorded daily. After a month, they were given a second exposure to 10^6 $TCID_{50}$ VHSV ml^{-1}. About 30% of the trout survived, showing no signs of VHS disease. They were used for experiments 10–12 months after the last challenge, by which time their weight was 50–120 g.

CELLS FROM TROUT (*ONCORHYNCHUS MYKISS*) KIDNEY

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 cut into pieces 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 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 $2{\cdot}4 \times 10^5$ 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 $\mu g\ ml^{-1}$ amphotericin, 50 $\mu m\ ml^{-1}$ gentamicin, 20 mM Hepes, 50 μM mercaptoethanol, 10% pretested foetal 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 Units (NIH U) ml^{-1}.

Cultures of kidney cells were always prepared from individual fish to avoid any possible mixed lymphocyte reactions (Kaastrup *et al.*, 1988). One hundred μl of the cell suspension were pipetted into each well of a 96-well plate (Costar, The Netherlands). After clotting occurred (in about 30 s), the mitogens diluted in sterile water, were pipetted in a maximum volume of 10 μl on the top of the clot. 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, sealed and incubated at 20° C for 1–2 weeks.

After the incubation, the fibrin clots were removed from the wells onto a 75 × 25 mm frosted-end glass slide with a spatula. The clots were partially dehydrated by placing a rectangular piece of Whatman number 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 papers were removed, the fixed clots 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 Science Co., NY). Other details were as described (Rueda & Coll, 1988; Coll, 1990).

Mitogen-induced colonies were scored with the aid of an ocular eye piece at 100 ×. Cells were counted and identified at 400 ×. 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 (C.V.). Numbers were finally expressed per clot (per well), by the following formula, number of cells counted × total surface of the clot/surface of the clot counted.

PROLIFERATION ASSAYS

Trout kidney cells ($2·4 \times 10^5$ cells ml^{-1}) were pipetted in 96-well flat-bottom microtitre plates (Costar, The Netherlands) in 100 μl volume, different viral proteins were added in a maximum volume of 10 μl/well, the plates were sealed in plastic bags gassed with CO_2 in air and cultured for 10 days at 20° C. One μCi of methyl tritiated thymidine (60 Ci $mmol^{-1}$, Amersham, The Netherlands) was added in 25 μl of culture medium and cells cultured for 2 additional days. The cells were harvested with distilled water onto glass fibre filters with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with an aqueous counting scintillant cocktail (NCS Amersham) and counted on a Beckman liquid scintillation counter (Model EL 3800, Fullerton, CA). Duplicate cultures were averaged for every experiment.

CYTOLOGICAL STAINS

Staining for non-specific esterase was made by the σ-napthyl acetate method as described (Plytycz *et al.*, 1989). The following medium was prepared just before use; 10 mg of α-napthyl acetate were dissolved in 250 μl of acetone, diluted to 20 ml with 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, 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, diaminobenzidine tablets of 10 mg (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. Slides were held in the stain solution for 10 min, rinsed in water and mounted in glycerol/PBS (1:1).

Acridine orange (100 μl 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 appear orange and nuclei green under the fluorescence microscope.

To stain the cells with anti-trout Ig MAb 15 (Sanchez *et al.*, 1991), the cells were cultured for 1 week in the presence of 100 μg ml^{-1} of LPS in liquid medium, then resuspended in foetal calf serum at $0{\cdot}5 \times 10^6$ ml^{-1} and 200 μl were cytocentrifuged at 600 rpm for 10 min, onto slides previously washed and cleaned with ethanol/ether 1/1. Slides were fixed for 10 min at room temperature with 5% acetic acid in ethanol, and washed twice with PBS. Without allowing them to dry, 60 μl of MAb 15, 10-fold diluted ascites were added and incubated for 45 min in a humid chamber. After washing three times with PBS, 60 μl of anti-mouse IgG-FITC (Dakopatts, Denmark) diluted 50-fold in PBS were added and incubated for 30 min. After washing three times with PBS, haematoxylin was used for contrast staining. The slides were mounted in glycerol/PBS (1:1) containing 5% propylgalate and observed with an epifluorescence microscope.

III. Results

ADDITION OF VHSV TO KIDNEY CELLS FROM VHSV RESISTANT TROUT

The specific immunological memory response of kidney cells towards VHSV antigens was studied in trout 1 year after surviving a double infection with VHSV by thymidine incorporation and by culture in fibrin-clots.

Figure 1, shows that thymidine incorporation was highest in kidney cell cultures cultivated with either killed virus or nucleoprotein N. In parallel fibrin-clot culture adherent cells [Fig. 2(c)] were the most abundant when either killed virus or nucleoprotein N were added, however they were also abundant in the presence of glycoprotein G (Fig. 1). Only the fibrin-clot cultures containing nucleoprotein N showed some significative numbers of lymphocytes. The eccentric-nucleated, multinucleated [Fig. 2(a) and (b)] or large nucleated cells were found in small numbers in most of the fibrin-clot cultures (Fig. 1).

ADDITION OF POLYCLONAL IMMUNOSTIMULANTS TO KIDNEY CELLS FROM HEALTHY TROUT

To stimulate the non-specific immunological responses of trout kidney cells, PHA, ConA, LPS and a panel of anti-trout serum Ig MAbs were added to cultures of leucocytes from healthy trout.

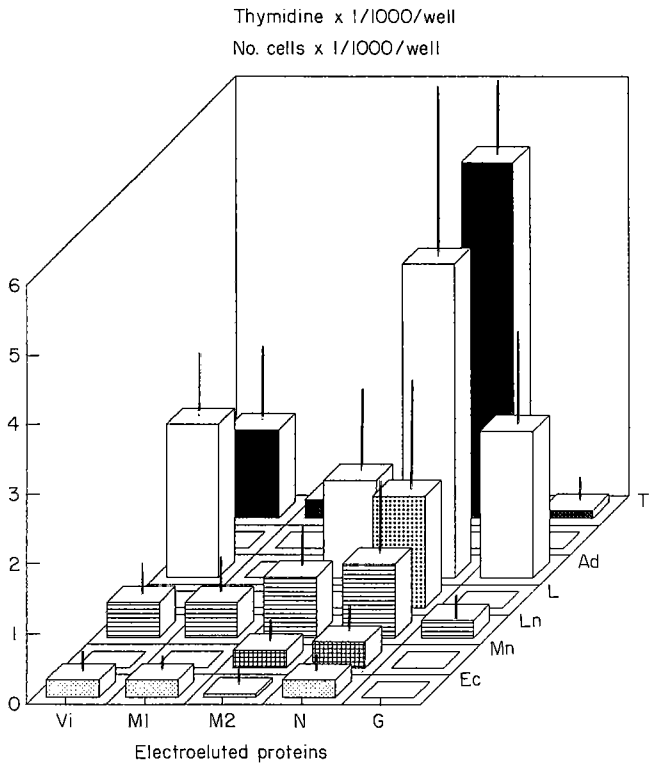

Fig. 1. Thymidine incorporation and kidney cells types stimulated by electroeluted proteins from VHSV. Cells ($2{\cdot}4 \times 10^4$/well in 100 μl) from VHSV resistant trout were cultured at 20° C for 10 days in the presence of about 10 $\mu g\ ml^{-1}$ of the VHSV viral proteins (M_1, M_2, N, and G). The results were averaged from N and Nx. The thymidine incorporation of M_2 was about the same as that of M_1. Averages of four different trout were used to obtain the results of thymidine incorporation (left scale in cpm $\times 10^{-3}$). Averages from two different trout were used to obtain the differential counts of kidney cell types (left scale in cells $\times 10^{-3}$). Vertical bars represent the CV, coefficient of variation. Control cultures (mean cpm of cells) in the absence of VHSV or viral proteins have been subtracted from the values shown. The number of cells in non-stimulated control cultures were 1250 ± 934/well eccentric nucleated cells, 200 ± 141/well large nucleated cells, 2425 ± 1460/well multinucleated cells, no lymphocytes, 1600 ± 353/well adherent cells and 1500 ± 1000 cpm/well of thymidine incorporation. Vi, purified heat-killed (100° C, 2 min) VHSV. M_1, M_2, N, and G, VHSV proteins purified by electroelution of preparative electrophoresis of purified VHSV. (▨), Ec, Eccentric nucleated cells; (▤), Ln, large nucleated cells; (▦), Mn, multinucleated cells; (▩), L, lymphocytes; (□), Ad, adherent cells; (■), T, incorporation of tritiated thymidine.

Both isolated cells and colonies were present in the cultures made in the presence of mitogens. In cultures containing PHA or ConA, large-nucleated cells appeared as dispersed individual cells and as colonies (not shown). Cells with eccentric nuclei formed colonies in large numbers in cultures made in the presence of PHA [Fig. 3(a)]. Multinucleated cells formed colonies both in PHA and ConA stimulated cultures [Fig. 3(b)]. Adherent cells did not form colonies [Fig. 3(c)]. Lymphocytes, present at the beginning of the cultures disappeared

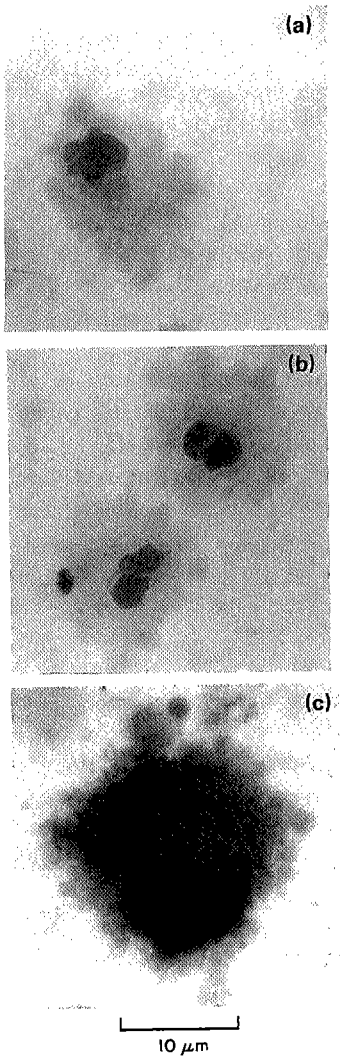

Fig. 2. Morphology of eccentric-nucleated cells (a), multinucleated cells (b) and adherent cells (c).

after 1 week in culture, except for a few colonies present in PHA containing cultures (not shown).

About 70% of the PHA or ConA-induced colonies were composed of eccentric nuclei, multinucleated or large-nucleated cells. PHA induced the highest number of colonies when compared with ConA since about 100 μg ml^{-1} of ConA was needed to obtain a comparable effect to the one obtained with 2 μg ml^{-1} of PHA (not shown).

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 seemed 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*

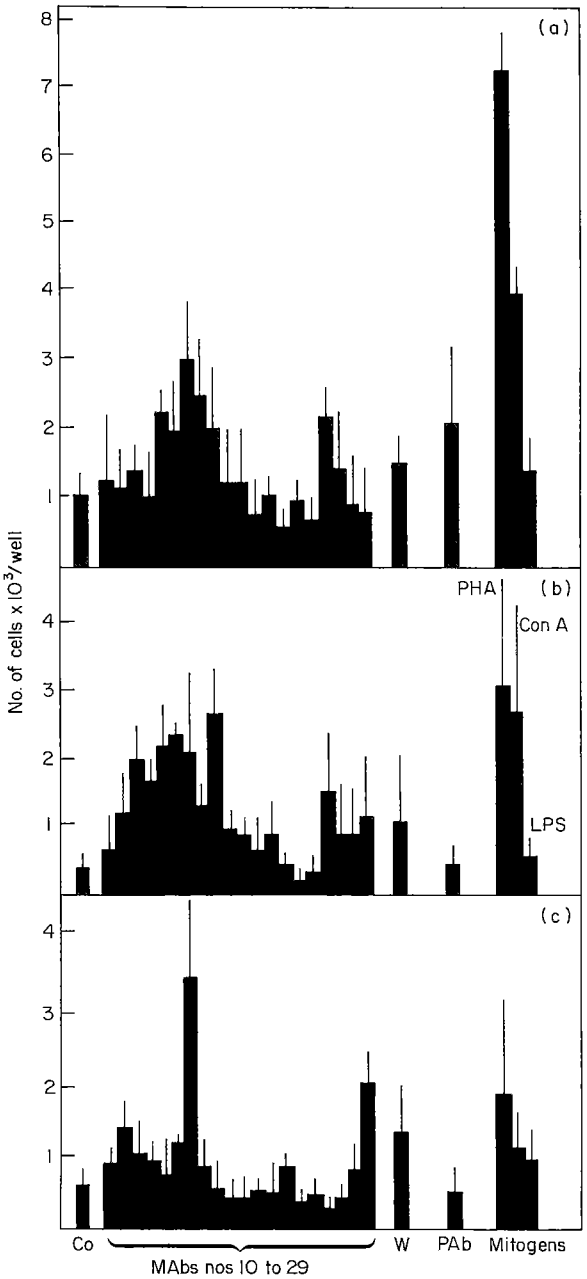

Fig. 3. Counts of trout kidney cells cultured in the presence of anti-Ig MAbs. Ascites from mice containing anti-trout Ig MAbs (nos 10–29) were added to the cultures at a final concentration of about 2–6 mg of antibody ml^{-1} PAb, polyclonal antibody from the lymphocyte donor mouse to make the hybridomas. MAb nos 10 and 15 have been described before (Sanchez *et al.*, 1989, 1991, respectively). W, was anti-trout Ig 1·14 from Dr Warr. Other Abs will be described elsewhere (Sanchez *et al.*, manuscripts in prep.). Controls were without any additives or with 5% ascites from uninjected mice. Mitogen concentrations were 2 $\mu g\ ml^{-1}$ PHA, 20 $\mu g\ ml^{-1}$ ConA and 200 $\mu g\ ml^{-1}$ LPS. The number of eccentric nucleated and multinucleated cells obtained with PHA and ConA represented in the figure, were obtained by counting both scattered cells and cells contained in the colonies. (a), Eccentric-nucleated cells; (b), multinucleated cells and (c), adherent cells. Average and S.D. from two different experiments are represented in the figure.

Table 1. Response of PHA and LPS stimulated trout kidney cells to anti-trout Ig MAbs

MAbs	Average ± S.D.	
	PHA-stimulated colonies/well	LPS-stimulated cells/well
—	475 ± 75	3600 ± 1721
10	440 ± 88	3750 ± 625
11	443 ± 101	3300 ± 471
15	662 ± 162	3150 ± 363
16	709 ± 164	3688 ± 664
23	496 ± 152	N.D.
29	468 ± 139	2600 ± 600

Trout kidney cells were incubated at 20° C for 1 week with either 5 μg of PHA ml^{-1} or 100 μg of LPS ml^{-1}. Then, MAbs (1 μg of ascites/100 μl/well) were added to the cultures and the cultures were incubated for an additional week. The PHA-stimulated cultures contained colonies of 20 cells per colony ($N=50$) whereas the LPS-stimulated cultures contained individual scattered cells. The PHA-stimulated colonies were composed of 85% eccentric-nucleated cells. N.D., Not determined. Averages and S.D. were calculated from the counts of four fields of view randomly selected from four different wells per each point. Total number of colonies or cells counted per point ranged from 44 to 70 colonies or from 100 to 150 cells.

Aeromonas sobria) induced some cell survival but no colony formation (not shown).

The addition of anti-trout serum Ig MAbs to the cultures of trout kidney cells stimulated the counts of eccentric-nucleated, multinucleated and adherent cells as compared to the controls. No lymphocytes and/or large nucleated cells and no colonies were stimulated by any of the MAbs used. The profiles of the responses obtained with the panel of 20 MAbs were similar for the eccentric-nucleated and the multinucleated cells [Fig. 3(a) and (b)]. The extent of stimulation of eccentric-nucleated cells was two to three-fold lower than with the mitogens [Fig. 3(a)]. The extent of stimulation of multinucleated cells was of about the same order of magnitude as with the mitogens [Fig. 3(b)]. The only MAbs that specifically stimulated adherent cells were the MAbs numbers 16 and 29.

The effect of prior stimulation of trout kidney cells with mitogens before the addition of MAbs was studied by first culturing the cells with PHA or LPS and then adding MAbs 10, 11, 15, 16, 23 or 29 to the same cultures. The PHA-stimulated cultures contained colonies of about 20 cells per colony ($N=50$) whereas the LPS-stimulated cultures contained individual scattered cells. Table 1 shows a small increase in the number of PHA-stimulated colonies with MAbs 15 and 16 but there was no increase in the number of LPS-stimulated cells with any of the MAbs used. Similar results were obtained by using the MAbs at a 10-fold higher concentration (not shown).

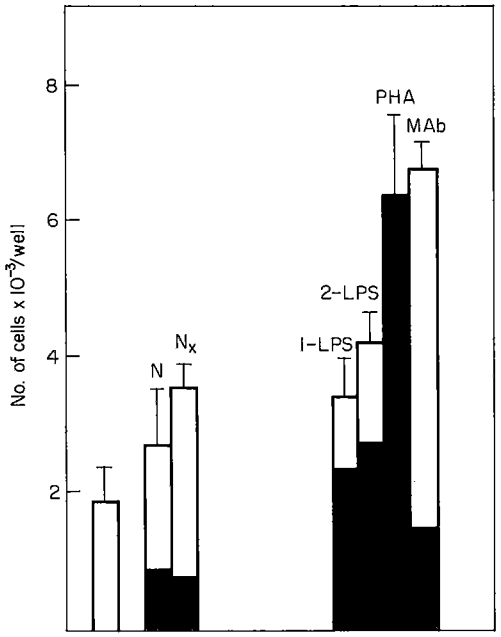

Fig. 4. Response of VHSV glycoprotein G stimulated trout kidney cells to mitogens, MAbs and nucleoprotein N/Nx. Healthy trout kidney cells were incubated at 20° C for 1 day in the presence of 15 μg ml^{-1} of VHSV glycoprotein G. Then, VHSV nucleoprotein N or Nx (15 μg ml^{-1}), LPS (batches 1 and 2), PHA (2 μg ml^{-1}) and MAb number 16 (10 μl of ascites) were added and incubation continued for a total of 3 weeks. Average and standard deviation from four clots are given in the figure. Control counts with no added substances was 150 $\pm$ 50 cells/well. (■ ■ ■), Counts without the VHSV glycoprotein; (□ □ □), counts with the VHSV glycoprotein.

ADDITION OF IMMUNOSTIMULANTS TO G-IMMUNIZED KIDNEY CELLS FROM HEALTHY TROUT

To mimic an immunisation *in vitro*, protein G was added to cultures of kidney cells from healthy trout kidney and then incubated with other viral proteins or mitogens. The addition of 15 μg ml^{-1} of glycoprotein G isolated from VHSV increased not only the number of cells about 10-fold with respect to the controls, but also their average diameter from 30 $\pm$ 12 μm to 54 $\pm$ 20 μm (N = 30). Increasing the concentration to 30 μg ml^{-1} did not increase the number of stimulated cells. Most of the stimulated cells were of the adherent type. The number of cells stimulated by G + N or G + Nx was about equal to the addition of the number of cells stimulated by each protein separately. A similar phenomena was observed by adding LPS to the G-stimulated cultures. However, the addition of PHA to the G-stimulated cultures stimulated a total number of cells that was similar to the number of cells stimulated by PHA alone. On the other hand, the addition of MAb 16 to the G-stimulated cultures, stimulated a number of cells two-fold higher than the number of cells stimulated by each protein separately (Fig. 4).

PROPERTIES OF THE *IN VITRO* STIMULATED KIDNEY CELLS

To further characterise the trout stimulated kidney cells, several staining procedures were applied to the cultures (Table 2). The kidney cells when incubated in plastic flasks in liquid culture for 1 week, could be divided into adherent

Table 2. Kidney cells type stimulated by mitogens, MAbs and viral antigens

Characteristics	L	Ln	En	Mn	Ad
Adherence to plastic surface	−	−	−	−	+
Granules in the cytoplasm	−	−	−	−	+
Trout red blood cell phagocytosis	−	−	−	−	+
Acridine orange staining	−	−	−	−	+
Peroxidase staining	±	−	−	−	+
Non-specific esterase staining	±	−	−	−	+
Sudan black B staining	−	−	−	−	−
Nitroblue tetrazolium staining	−	−	−	−	−
Fluorescence staining with anti-trout Ig 15†	−	−	+	+	+
Stimulation by panel of MAbs anti-trout Igs	−	−	+	+	+
Stimulation by VHSV nucleoprotein N	−	−	−	−	+
Stimulation by VHSV glycoprotein G†	−	−	−	−	+
Proliferation by PHA or ConA†	+	+	+	+	−
Stimulation by PHA or ConA	+	+	+	+	+
Stimulation by LPS	−	−	+	+	+
Susceptibility to VHSV infection *in vitro**	+	+	+	+	+

L, Lymphocytes; Ln, large nucleated cells; En, eccentric nucleated cells; Mn, multinucleated cells; Ad, adherent cells.

*Estepa & Coll (1991), Estepa *et al.* (1991*b*).

†Stimulation, increase in the number of scattered cells with respect to controls. +Proliferation measured by the presence of homogeneous colonies (Coll, 1990).

‡Fluorescence estimated in LPS stimulated cultures.

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 (30 μm in diameter) and a round nucleus. Some of these cells were filled with either brown corpuscles or the remains of red blood cells in older cultures. They were stained by acridine orange showing red fluorescent granules in their cytoplasm. Their morphology was identical to the cells stimulated by the N and G proteins from VHSV. They always appeared in the clots individually dispersed and occasionally in groups of three to four cells.

Peroxidase and non-specific esterase staining showed some positive small lymphocytes and adherent cells. No cells or colonies were stained by the Sudan Black B or the nitroblue tetrazolium staining. Most of the LPS stimulated cells that appeared fluorescent to the anti-trout Ig MAb number 15 belonged to the eccentric-nucleated or the multinucleated cells, but some small adherent cells were also positive.

IV. Discussion

The final goal of this study was to develop an *in vitro* immune response assay, to study the viral antigens and immunostimulants required to increase trout defensive mechanisms after VHSV infection.

The morphology and properties (Table 2) of the cells from trout kidney, 1 year after surviving a VHSV infection, and which responded to the N and G VHSV proteins were the same as the responding adherent cells described previously from a trout immunised against VHSV by injection (Estepa *et al.*, 1991*a*). These adherent cells were further characterised in this work; they showed granules in their cytoplasm, red blood cell phagocytosis, acridine orange staining, peroxidase staining and non-specific esterase staining. These properties have been described for fish macrophages and/or melanomacrophages (Braun-Nesje *et al.*, 1982; Bayne, 1986). In addition, some of these cells showed fluorescence (Table 2) with a MAb recognizing 85% of the trout serum Ig (Sanchez *et al.*, 1991). They did not proliferate in response to PHA, ConA or LPS and were susceptible to VHSV in *in vitro* infection (Estepa & Coll, 1991; Estepa *et al.*, 1991*b*). Because of the additive effects found in the experiments after *in vitro* 'immunisation' of the trout kidney cells with protein G and the later addition of other compounds, the cellular targets of protein G and protein N (or Nx) seem to be different (Fig. 4). Because of >two-fold synergistic (Fig. 4) rather than additive effects, the cellular targets of protein G and anti-Ig MAb seem to have something in common, suggesting that protein G is stimulating not only some cellular but also some humoral (Ig related cells) responses, as it has been shown earlier *in vivo* (Bernard *et al.*, 1983). The humoral immune response to VHSV seems to be necessary for resistance to the disease, since antibodies to VHSV are found in 54% of trout from previously infected farms, even though no anamnestic humoral immune responses were observed after reinfection (Olesen *et al.*, 1991). Protein G stimulates the adherent cells (macrophages) whereas protein N or Nx could also stimulate other cellular targets such as the small lymphocyte to proliferate (Fig. 1). The importance of macrophages (monocytes) as accessory cells in higher vertebrate immune responses is well-established, but it is not yet fully known what their function is in fish. The results obtained here together with the ones reported before (Estepa *et al.*, 1991*a*) suggest that both N and G proteins could be needed for a recombinant vaccine to be fully effective.

The multinucleated cells have some of the morphological properties of polymorphonuclear granulocytes (Bayne, 1986) but these cells formed PHA-induced colonies (Coll, 1990), had no granules in their cytoplasm, they were Sudan Black and nitroblue tetrazolium negative (Table 2), and were not observed to phagocytose bacteria (not shown). Furthermore, their staining with anti-Ig MAb (Table 2), and their parallel stimulation to the eccentric-nuclei cells when cultured with a panel of 20 different anti-Ig MAbs or LPS, suggest that these multinucleated cells may be related to the eccentric-nucleated cells rather than to the polymorphonuclear granulocytes. Both of these cells formed colonies when incubated with PHA (Coll, 1990) or ConA but not with LPS or anti-Ig MAbs (Fig. 3). As is the case of other haemopoietic colony stimulating factors, the LPS or the anti-Ig MAbs could only be promoting cell survival by inhibiting apoptosis (Williams *et al.*, 1990).

A noteworthy phenomenon in the trout was the polycellular response to some of the anti-Ig MAbs and its similarity to the LPS response (Fig. 3). The complete lack of stimulation by some of the MAbs could be due to the fact they were directed against buried determinants on the membrane Ig-bearing cells or to determinants only present in serum Ig (Emmrich *et al.*, 1975). Not one but two

morphological cell types were stimulated [Fig. 3(a) and (b)] and two MAbs (MAbs nos 16, 29) also stimulated adherent cells [Fig. 3(c)]. Only one of these cell types, the eccentric-nucleated cells [Fig. 2(a)] resembles morphologically the mammalian plasma cells (differentiated B cells). All these three types of cells were also found in cultures stimulated by LPS, PHA or ConA (Fig. 3). In addition, PHA (Coll, 1990) and ConA (Fig. 3) also induced the proliferation (as demonstrated by the presence of homogeneous colonies) of large nucleated, multinucleated, eccentric-nucleated cells and a few lymphocytes. No similar evidence of proliferation, however, could be demonstrated with MAbs, LPS or proteins G and N from VHSV.

Some work on fish lymphocytes (Ambrosius *et al.*, 1982; Caspi *et al.*, 1982; De Luca *et al.*, 1983) suggests that PHA/ConA and LPS responsive lymphocytes may be functionally equivalent to mammalian T and B cells, respectively. However, PHA has been shown to stimulate both Ig production and plaque-forming cells (Kaattari & Yui, 1987), both surface Ig+ and Ig− cells were LPS responders (De Luca *et al.*, 1983) and two to four morphological types of colonies were induced by PHA in semisolid medium (Caspi *et al.*, 1982; Coll, 1990). The functional equivalence of fish lymphocytes to mammalian T/B cells might not, therefore, be so simple. PHA may not be limited to T cell activation, or PHA may induce the production of lymphokines which activate B cells, or alternatively, T and B cells may not be distinct. Any of these hypotheses would explain the PHA-induced polycellular responses seen in the cultures described here. In any event, PHA was the strongest *in vitro* stimulator of non-specific cellular proliferation, and since peripheral blood lymphocytes respond to PHA *in vivo* (Bogner & Ellis, 1977), it could even be used as an adjuvant.

Large nucleated cells were abundant in fresh kidney (Coll, 1990) but they only responded to PHA and ConA. They did not respond to LPS, protein N or G from VHSV, MAbs to serum Ig nor specific stains. The large-nucleated cell forming colonies in the cultures were very similar to some of the cells appearing in the high cell density liquid cultures of PHA stimulated peripheral blood lymphocytes obtained from carp (Caspi *et al.*, 1982). These cells appeared in our cultures as ConA-induced colonies bursting from 3-day small groups of large-nucleated cells and macrophages (not shown). They might be the precursors of other cellular types found later in the cultures. According to the morphology of subpopulations of trout kidney cells defined by Plytycz *et al.* (1989) these cells might be called blasts.

Assuming that the requirements to stimulate specific cellular immunological memory (Fig. 1) would be similar to the requirements needed during immunization, this *in vitro* model was developed by ' immunising ' the cells with protein G and adding other substances to obtain maximal *in vitro* stimulation (Fig. 4). The results obtained suggest, but not prove, the necessity of employing protein G (to hypothetically increase humoral or Ig-related cellular responses), protein N (to increase memory as shown by Estepa *et al.*, 1991) and a potent polyclonal mitogen like PHA (to increase a non-specific response) to obtain immunisation of trout against VHSV. Preliminary *in vivo* results seem to confirm these assumptions. In addition, fractionation of the trout kidney cells into adherent, surface Ig+ and surface Ig− cells (De Luca, 1983; Secombes *et al.*, 1983) and the study of their responses towards purified viral antigens would help to better

explain the factors that regulate their growth and differentiation during viral infection and disease.

Thanks are due to Dr Agustín Zapata of the Department of Cell Biology of the Universidad Complutense de Madrid, for his help with the cytological stains. We appreciated the technical assistance of D. Frías, the help with the isolation of VHSV proteins from Dr B. Basurco and F. Sanz, and with the isolation of LPS from aquatic bacteria from J. Barrera. The hybridomas used in this work were obtained and grown *in vitro* by Dr J. Dominguez with the help of C. Hernandez. Purified monoclonal antibody 1.14 was a gift from Dr Warr. J. Coll Perez is thanked for his help 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).

References

Ambrosius, H., Fiebing, H. & Scherbaum, I. (1982). Phylogenetic aspects of fish immunoglobulins and lymphocyte receptors. *Development and Comparative Immunology* **2,** 3–13.

Basurco, B. & Coll, J. M. (1989). Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. *Bulletin of the European Association of Fish Pathology* **9,** 92–95.

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,** 141–151.

Bernard, J., De Kinkelin, P. & Bearzotti-Le Berre, M. (1983). Viral haemorrhagic septicaemia of rainbow trout: relation between the G polypeptide and antibody production on protection of the fish after infection with the F25 attenuated variant. *Infection and Immunity* **39,** 7–14.

Blaxhall, P. C. & Sheard, P. R. (1985). Preliminary investigation of the characteristics of fish lymphocytes separated on a Percoll discontinuous gradient. *Journal of Fish Biology* **26,** 209–216.

Bogner, K. H. & Ellis, A. E. (1977). Properties and functions of lymphocytes and lymphoid tissues in teleost fish. In *Contributions to the Histopathology of Fishes* (H.H. Reichenbach-Klinke, ed.) pp. 59–72. Stuggart: Gustav Fisher Verlag.

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

Caspi, R. R., Rozenszajn, L. A., Gothelf, Y., Pergamenikov-Litvak, T. & 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. *Developmental and Comparative Immunology* **6,** 683–692.

Chilmonczyk, S. (1978). Stimulation specifique des lymphocytes de truites arc-en-ciel (*Salmo gairdneri*) resistantes a la septicemie hemorragique viral. *Compte Rendu de l'Académie des Sciences. Paris* **287,** 387–389.

Coll, J. M. (1987). Injection of physiological saline facilitates recovery of ascitic fluids for monoclonal antibody production. *Journal of Immunological Methods* **104,** 219–222.

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.

Emmrich, F., Richter, R. F. & Ambrosius, H. (1975). Immunoglobulin determinants on the surface of lymphoid cells of carps. *European Journal of Immunology* **5,** 76–78.

Estepa, A. & Coll, J. M. (1991). Infection of mitogen-stimulated trout leucocytes with salmonid viruses. *Journal of Fish Disease* **14,** 100–108.

Estepa, A., Basurco, B., Sanz, F. & Coll, J. M. (1991*a*). Stimulation of adherent cells by the addition of purified proteins of viral haemorrhagic septicaemia virus to trout kidney cell cultures. *Viral Immunology* **4,** 43–51.

Estepa, A., Frías, D. & Coll, J. M. (1991*b*). *In vitro* infection of trout kidney cells with infectious pancreatic necrosis and viral haemorrhagic septicaemia viruses. *Bulletin of the European Association of Fish Pathologists* **11,** 101–104.

Faulmann, E., Cuchens, M. A., Lobb, C. J., Miller N. W. & Clem, L. W. (1983). An effective culture system for studying *in vitro* mitogenic responses of channel catfish lymphocytes. *Transactions of the American Fish Society* **112,** 673–679.

Finegan, C. B. & Mulcahy, M. F. (1987). Successful culture of rainbow trout lymphocytes in soft agar. *Developmental and Comparative Immunology* **11,** 419–425.

Kaastrup, P., Nielsen, B., Horlyck, V. & Simonsen, M. (1988). Mixed lymphocyte reactions (MLR) in rainbow trout (*Salmo gairdneri*) siblings. *Developmental and Comparative Immunology* **12,** 801–808.

Kaattari, S. L. & Yui, M. A. (1987). Polyclonal activation of salmonid B lymphocytes. *Developmental and Comparative Immunology* **11,** 155–156.

Martinez, J. A. & Coll, J. M. (1987). Preliminary clinical studies of C-reactive protein quantified by enzyme-linked immunoassay. *Clinical Chemistry* **33,** 2185–2190.

Martinez, J. A. & Coll, J. M. (1988). Selection and performance of monoclonal anti-C reactive protein in ELISA quantitative assay. *Clinica Chemica Acta* **176,** 123–132.

Olesen, N. J., Lorenzen, N. & Vestergaard-Jorgensen, P. E. (1991). Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). *Diseases of Aquatic Organisms* **10,** 31–38.

Plytycz, B., Flory, C. M., Galvan, 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 of myelomas and hybridomas in fibrin clots. *Journal of Immunological Methods* **114,** 213–217.

Sanchez, M. C. T. & Coll, J. M. (1989). La estructura de las immunoglobulinas de los peces. *Immunologia* **8,** 47–54.

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

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

Secombes, C. J., Van Groningen, J. J. M. & Egberts, E. (1983). Separation of lymphocyte subpopulations in carp *Cyprinus carpio L.* by monoclonal antibodies: immunohistochemical studies. *Immunology* **48,** 165–175.

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