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Viral-antigen dependence and T-cell receptor expression in leucocytes from rhabdovirus immunized trout

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Abstract

This work describes the characterization of trout haematopoietic in vitro long-term cell cultures showing specific viral antigen-dependent cell (ADC) proliferation. The ADC cultures were developed from outbreed trout after surviving viral hemorrhagic septicaemia virus (VHSV) infections or after immunization with purified VHSV. For in vitro long-term proliferation of the ADC cultures, adherent (Ad) cells obtained from autologous trout were pulsed with VHSV recombinant glycoprotein G4 (G4-pulsed Ad cells) and added periodically to the cultures. ADC did not proliferate in cultures obtained from non-infected control trout treated in parallel with G4 or from VHSV survivor/VHSV immunized trout kidney donors treated with non-viral proteins. After months in culture, the ADC acquired an increasingly homogeneous morphology compatible with that of mature trout lymphocytes, secreted supernatant 'factors', and were stained with rabbit antibodies to the ectodomain of recombinant trout T-cell receptor (TcR) β-chain. Together with all the above mentioned properties, the presence of TcR sequences in the ADC cultures confirmed by the expression of α- and β-chain TcR by nested PCR amplification and sequencing of the amplified bands, suggests that these ADC cultures contain trout T-like cells engaged in a VHSV response. These trout ADC cultures offer a first opportunity to further analyze fish anti-viral immunological responses.

Keywords: Trout; Rhabdovirus; Antigen-dependent cells; T-cell receptor; T-cells

1. Introduction

Short-term in vitro fish haematopoietic cell cultures have demonstrated the existence of functional T, B and antigen presenting cells (Vallejo et al., 1992). Long-term in vitro fish

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haematopoietic cell lines, however, remain as a few existing examples. Those include catfish antigen-presenting cells of the monocyte/macrophage series (Faisal and Ahne, 1990), B-cell lines maintained without restimulation, feeder cells or exogenous factors (Miller et al., 1994) and heterogeneous cell mixtures including putative T-cells (Clem et al., 1996; Lin et al., 1992).

On the other hand, fish anamnestic immune proliferative in vitro responses (T-like responses) to trout rhabdoviruses had been previously studied by using whole virus (Chilmonczyk, 1978), isolated viral proteins (Estepa and Coll, 1992b), recombinant viral protein fragments (Estepa et al., 1994b) and viral peptides (Lorenzo et al., 1995). The capacity of specific viral peptides to stimulate trout leucocyte proliferation together with the existence of protein processing and peptide presentation demonstrated in catfish cells (Vallejo et al., 1992), suggested that fish immune responses to virus might have similar characteristics as that of higher vertebrates. Confirming those observations, we recently reported an in vitro method to obtain antigen-dependent cell (ADC) lines from trout by using syngenic viral antigen presenting cells obtained from trout surviving rhabdoviral infections (Estepa et al., 1996; Estepa and Coll, 1997).

To perform the studies reported here, the viral hemorrhagic septicaemia virus (VHSV)/ trout model was chosen because of its important economic impact and because trout is one of the immunologically best studied fish. The glycoprotein G of VHSV was chosen as the antigen because it was expressed in the membrane of VHSV infected trout cells (Estepa et al., 1992a) and it was the strongest inducer of anamnestic trout lymphoproliferation (Estepa and Coll, 1992b). The yeast recombinant G4 form of the glycoprotein G was selected because it can be obtained in large supply and high purity. Moreover, it conferred some protection against in vivo VHSV challenge (Estepa et al., 1994a), it showed the highest stimulation index of anamnestic trout lymphoproliferation of all the recombinant G tested (Estepa et al., 1994b; Lorenzo et al., 1995) and it gave rise to ADC proliferation (Estepa and Coll, 1997).

This report characterizes trout long-term haematopoietic cell lines specifically dependent of viral antigen for proliferation. Considering the important economic impact of viral diseases in salmonid fish aquaculture (Leong et al., 1995), the opportunity to analyze anti-viral responses using an in vitro experimental model might be of great interest.

2. Materials and methods

2.1. Viruses

The VHSV 07.71 isolated in France (LeBerre et al., 1977) from rainbow trout *Oncorhynchus mykiss* (Walbaum) was grown in epithelial papillosum cyprine (EPC) cells and purified as described (Estepa et al., 1994b).

2.2. Recombinant G4 and N3 VHSV proteins

The recombinant fragment G4 (aa 9-443) and N3 (aa 1-404) were cloned and expressed in the yeast *Saccharomyces cerevisae* DC04 as reported before (Estepa et al., 1994b). G4

and N3 inclusion bodies with ~80% of purity were isolated from yeast French press homogenates by centrifugation through 20% sucrose and used after sonication and inactivation by γ-irradiation (7000 rads, Laboratory Irradiator IBL437C CIS-Biointernational).

2.3. Production of trout survivors of VHSV infection.

Outbreed rainbow trout (*Oncorhynchus mykiss* W.) (2 g per trout) were first infected during 2 h at 14°C with 10^6 $TCID_{50}$ ml of VHSV (Basurco and Coll, 1992). Under these conditions trout survival was 10% to 30% ($n = 4$, n = number of experiments). The trout surviving the infection were challenged 2 months later with 10^6 $TCID_{50}$ ml of VHSV (2 h, 10°C). Confirming previous observations, 5% to 24% ($n = 4$) of the initial number of trout survived both infections and showed no signs of VHS. Survivor trout (T24, T25 and T26) were used 4–6 months after the last VHSV challenge (Enzmann and Konrad, 1993).

2.4. Immunization of trout with purified VHSV

We used the procedure reported before (Estepa et al., 1991) which resulted in the production of anamnestic proliferation of haematopoietic cells from the kidney of immunized trout. Trout of about 1 kg of body weight purchased from commercial farms intraperitoneally injected with purified VHSV and trout T35 were used for experiments 4 months after last injection.

2.5. Establishment of antigen-dependent cell (ADC) cultures

Leucocytes were obtained from individual trout kidney as described previously (Estepa and Coll, 1997). The cell culture medium was RPMI-1640 (Dutch modification, 290 mosM kg) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1.2 μg ml^{-1} amphotericin, 50 μg ml^{-1} gentamicin, 20 mM Hepes, 50 μM mercaptoethanol, 10% pretested fetal calf serum and 2% pretested pooled rainbow trout serum. To start the ADC cultures, 3 ml (~3×10^6 cells ml) of the kidney leucocyte cell suspension were incubated with 18 μg ml^{-1} of G4 in 25 cm^2 plastic flasks at 20°C with 5% CO_2 in air. After 2 weeks of culture, the non-adherent (non-Ad) cell population was removed and used for the ADC cultures.

2.6. Preparation of G4-pulsed Ad cells

After removing the non-adherent (non-Ad) cells, the remaining adherent (Ad) cells (macrophages, dendritic-like cells, stromal cells, etc.) were used to prepare the G4-pulsed Ad cells. The Ad cells were incubated with 18 μg ml^{-1} of G4 during 1 h at 20°C. They were then either treated with mitomycin (Estepa and Coll, 1997) or irradiated with 7000 rads (Laboratory irradiator IBL437C, CIS Biointernational). They were harvested by mechanical shaking and frozen at −70°C in the presence of 90% trout serum and 10%

DMSO until used. For controls, some Ad cells were either not treated with G4 or treated with ovalbumin.

2.7. Addition of autologous G4-pulsed Ad cells to ADC cultures

The ADC population was distributed in 96-well plates (~25 000 cells/well) in 100 μl of culture medium and stimulated by the addition of ~5000 autologous G4-pulsed Ad cells per well in 50 μl of cell culture medium. Because of the present difficulty to interpret allogenic reactions (Rodrigues, 1996), experiments were performed only with autologous cells. After 5–10 days, colonies and scattered cells were observed throughout the wells. These proliferating non-Ad cells were called antigen-dependent cells (ADC) and could be maintained during more than a year by periodically adding G4-pulsed Ad cells.

2.8. Proliferation assays

Proliferation assays were used as described before (Lorenzo et al., 1995). Stimulation index (SI) was calculated by the formula, counts per minute (cpm) incorporated/ background.

2.9. Transmission electron microscopy

Cells were pelleted, by centrifugation (1000 × *g*, 10 min) washed in PBS and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 during 2 h at 4°C. Cells were postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in araldite (Serva). Thin sections were mounted on copper grids, stained with lead citrate and uranyl acetate and observed in a Jeol 100-B transmission electron microscope at 60–80 kV.

2.10. Preparation of antibodies anti-ADC and anti-TcR

Female BALB/c mice were first injected intraperitoneally with ~20 μg of protein from ADC cultures in Freund's complete adjuvant and then with four monthly injections in Freund's incomplete adjuvant. To obtain about 40 ml of pooled diluted ascites containing antibodies, ADC-immunized mice were each intraperitoneally injected with 2×10^6 myeloma X63/Ag 8653 cells. 2 ml of anti-ADC pooled ascites were mixed with extracts from 10^7 cells from each of RTG-2 and kidney cells from non-infected trout and overnight incubated at 4°C to decrease background. The extracts were prepared by homogenizing the cells in distilled water and removing the debris by centrifugation. The mixture was then centrifuged and the supernatant used for the assays.

The extracellular region of the T-cell receptor (TcR) Cβ fragment (aa 40 to 230) was cloned by Dr. J. Charlemagne (INRA, París, France) in the plasmid pGex 4T2 (Transgene) as an expression vector. After optimization of expression and purification (Dr. B. Rentier, University of Liege, Belgium), the fusion protein GST-TcR was used to immunize rabbits. The rabbit antiserum to recombinant TcR fragment (RartL) detects a protein of about 60 KDa by western blotting in a lysate of rainbow trout peripheral blood

leucocytes or spleen leucocytes while it fails to detect any protein in rainbow trout muscle. By flow cytometry, RartL labels about 20% of the trout PBL population (reagents and data kindly provided by Dr. Charlemagne and Dr. Rentier).

2.11. Flow cytometry analysis

Peripheral blood cells (PBL) were obtained from non-infected trout in 8 ml of RPMI centrifugated onto 10 ml of Ficoll at 3000 × *g* 40 min at 4°C. A band between the Ficoll and the medium containing the PBL was harvested and the cells washed twice in RPMI. Cells (ADC, PBL, and Ad cells) were centrifuged at 700 × *g* for 10 min and the pellet resuspended in PBS (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4) + 1% bovine serum albumin + 0.1% Na_3N (PBS-BSA-azide) containing 50-fold diluted either mouse ascites with anti-ADC antibodies or RartL. After 1 h at 20°C with occasional agitation the cell suspensions were centrifuged again, resuspended in 400-fold diluted rabbit antimouse IgG-FITC conjugate or goat anti-rabbit IgG-FITC conjugate (Nordic, Tilburg, The Netherlands) and incubated 30 min at 20°C. The cell suspensions were again centrifuged and washed twice. To assay for surface IgM, the pellet was resuspended in the PBS-BSA-azide buffer containing 20 μg ml^{-1} of anti-trout IgM 1G7 monoclonal antibody (Sanchez et al., 1993). After 1 h at 20°C with occasional agitation, the plates containing the cell suspensions were centrifuged again, washed with PBS, resuspended in 400-fold diluted rabbit anti-mouse IgG-FITC conjugate (Nordic, Tilburg, The Netherlands) and incubated 30 min at 20°C. The cell suspensions were again washed twice and then resuspended in PBS. The same day of the harvest, 5000 cells were analyzed in a Beckton–Dickinson (San José, CA) FACScan flow cytometer.

2.12. Primer design for TcR α- and β-chain messenger amplification

Two pairs of primers (Isogen, Bioscience) were selected for specific RT-PCR amplification, by using the nucleotide sequences of the constant region of the α- and β-chains of the TcR genes of trout, accession numbers U18122 and U50991 (GenBank data libraries) (Partula et al., 1994, 1995, 1996). The program PRIMER (version 0.5) of the Wisconsin package (version 9.0) was used to select the best 18–22 nucleotides primers with an optimal T_m of 57–70°C and a product size of 350–500 bp.

The primers selected for the α-chain were the #51 (forward ^{517}5ACTGATCATTGAGACAAGAGAG^{538}) and the #52 (reverse ^{936}5GAGATTCCAAAGTGCAATCTAC^{915}) amplifying a 472 bp DNA fragment. The primers #53 (forward ^{564}5TGAAGTCAAAGAAATACCACAGC^{585}) and the #54 (reverse ^{872}5TCTTCTGGAAGGATTTCTTGTG^{851}) internal to the first ones, were selected to confirm by nested PCR the specificity of the products obtained previously. The primers #53 and #54 amplified a 334 bp DNA fragment. The primers selected for the β-chain were the #37 (forward ^{100}5′TGTGTAGCCACCCGCTTC^{117}) and the #38 (reverse ^{593}5′CCTGACCCTTTACCCGTT^{576}) amplifying a 494 bp DNA fragment. The primers #39 (forward ^{116}5′TCTACCCCGACCACGTACC^{135}) and #40 (reverse ^{533}5′CTTATGTTCGCCCCCTTCCTC^{513}), internal to the first ones, were selected to confirm by nested PCR the specificity of the products obtained previously. The primers #39 and #40 amplified a 418 bp DNA fragment.

2.13. Isolation of RNA and RT-PCR

RNA from muscle or kidney cell cultures of non-infected trout and 10 month-old ADC cultures 20 days after the last addition of G4-pulsed Ad cells was isolated with RNAgents (Promega, Madison, USA). The cDNA were synthesized from 2–10 μg of RNA with 50 pg of primer #52 (α-chain) or primer #38 (β-chain), 10 mM DTT, 100 μM of each of the dNTPs (Promega), 5 units of HPRI (Boehringer-Manheim), 10 μl of reverse transcriptase buffer (BRL, Gaithersburg, USA) and 1 μl of MMLV reverse transcriptase (BRL) in a final volume of 100 μl, during 35 min at 37°C. After addition of 50 pg of primer #51 (α-chain) or primer #37 (β-chain), four units of Ampli-Taq polymerase (Perkin-Elmer, Weiterstadt, Germany), and water to 100 μl, the samples were amplified for 30 cycles of 1 min at 94°C, 1 min at 59°C and 2 min at 72°C followed by an extension step of 10 min at 72°C. 5 μl of the above amplified DNA products were reamplified by adding 50 pg of each of the internal primers #53 and #54 for the α-chain or #39 and #40 for the β-chain, four units of Ampli-Taq polymerase, 10 μl of 10 × Ampli-Taq buffer (Perkin Elmer), 1.5 mM Mg_2Cl and 100 μM of each of the $dNTP_s$. Reamplification was performed by 30 cycles of 2 min at 94°C, 1 min at 62°C and 2 min at 72°C followed by an extension step of 7 min at 72°C. Products were analyzed in 1% agarose gels stained with ethidium bromide.

3. Results

3.1. Stimulation of trout kidney cell cultures

The capacity of VHSV proteins to stimulate the in vitro proliferation of kidney leucocytes obtained from VHSV survivor trout (T24, T25 and T26) and VHSV immunized trout (T35) was estimated by using recombinant G4 and N3. The highest proliferative response (SI) to heat-killed VHSV (∼10 μg of VHSV protein/ml) varied between 25–50 depending on the trout. The proliferative response to G4 was dependent on the G4 concentration, T26 showed the highest SI (∼15) and T25 the lowest SI (∼5) (Fig. 1). The significant proliferative response to N3 was 2–4-fold lower than to G4 and thus served as a convenient control for specific G4 induced proliferation. In the absence of VHSV or VHSV proteins or in the presence of ovalbumin as an heterologous protein control, trout kidney cell cultures did not show any significant proliferation over background (results not shown) confirming previous observations (Estepa et al., 1994b; Lorenzo et al., 1995). Moreover, when kidney cells from non-infected trout were cultured with VHSV or with G4 or N3 recombinant VHSV proteins, they did not show any significative proliferation over background (not shown).

3.2. Generation of antigen-dependent cell (ADC) cultures

Kidney cells obtained from VHSV survivor trout (T24, T25 and T26) and trout immunized with purified VHSV (T35) were distributed in 25 cm^2 flasks at 6×10^6 cells per flask, four flasks per trout. One flask per trout was maintained in the presence of

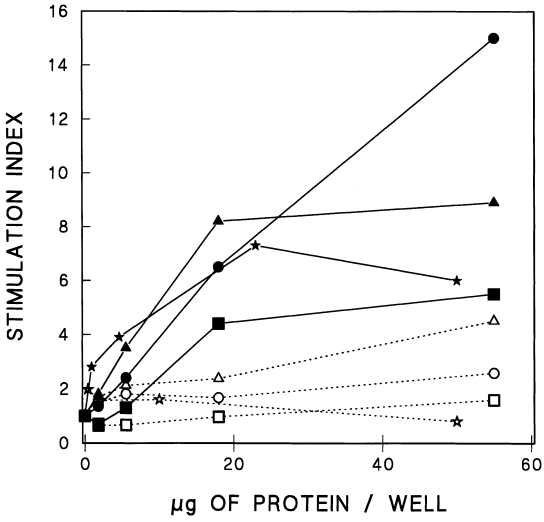

Fig. 1. Proliferation of trout kidney cells induced by G4 and N3. Trout (T) cells (10 000 cells/well) were obtained from kidney from trout survivors of VHSV (T24, T25 and T26) or from trout immunized with VHSV (T35) and incubated in the presence of recombinant G4 or N3 VHSV proteins. T24 kidney cells incubated with G4 (▲—▲) or N3 (Δ—Δ); T25 kidney cells incubated with G4 (■—■) or N3 (□—□); T26 kidney cells incubated with G4 (●—●) or N3 (○—○); T35 kidney cells incubated with G4 (*—*) or N3 (☆—☆). Cpm for controls without G4 or N3 were 435 ± 114 (T24), 737 ± 103 (T25), 579 ± 52 (T26) and 203 ± 40 (T35). Averages of duplicate wells in a representative proliferation assay are represented. So were omitted for clarity.

18 µg ml^{-1} of G4, the rest of the flasks maintained in the absence of G4 protein were used to obtain the Ad cells. In the cultures maintained in the presence of G4, cell proliferation could be visually observed after 15–20 days. Both colonies and scattered cells were present in the cultures. Cells continue to proliferate but only if G4 was added periodically to the cultures. In the cultures maintained in the absence of G4, none or very few cells were present 3 months later. The few cells surviving in those cultures showed signs of deterioration, pycnotic nuclei and lysis, and did not respond to further additions of G4.

To assess whether the recognition of G4 by the survivor trout kidney cells was mediated by a conventional cell presentation mechanism, we investigated the G4-specific proliferation requirement. With this purpose, the proliferative response obtained in reconstituted kidney cell cultures containing combinations of non-Ad cells, Ad cells and G4 was analyzed. Significant proliferative responses were only obtained when all, non-Ad cells, Ad cells and G4 were present in the cultures, suggesting that G4 presenting cells were required for the proliferation of the non-Ad cells. The proliferating non-Ad cells have been called antigen-dependent cells or ADC. The addition of Ad cells without G4 or of G4 without Ad cells to the ADC did not stimulate its proliferation (Fig. 2(A)). The same phenomenon was observed by using N3 as inducer of proliferation, but proliferation was much less intense (Fig. 2(A)).

To obtain long-term presenting cells, we then used autologous Ad cells pulsed with G4 but otherwise free of soluble G4 as presenting cells. The Ad cells were thus first

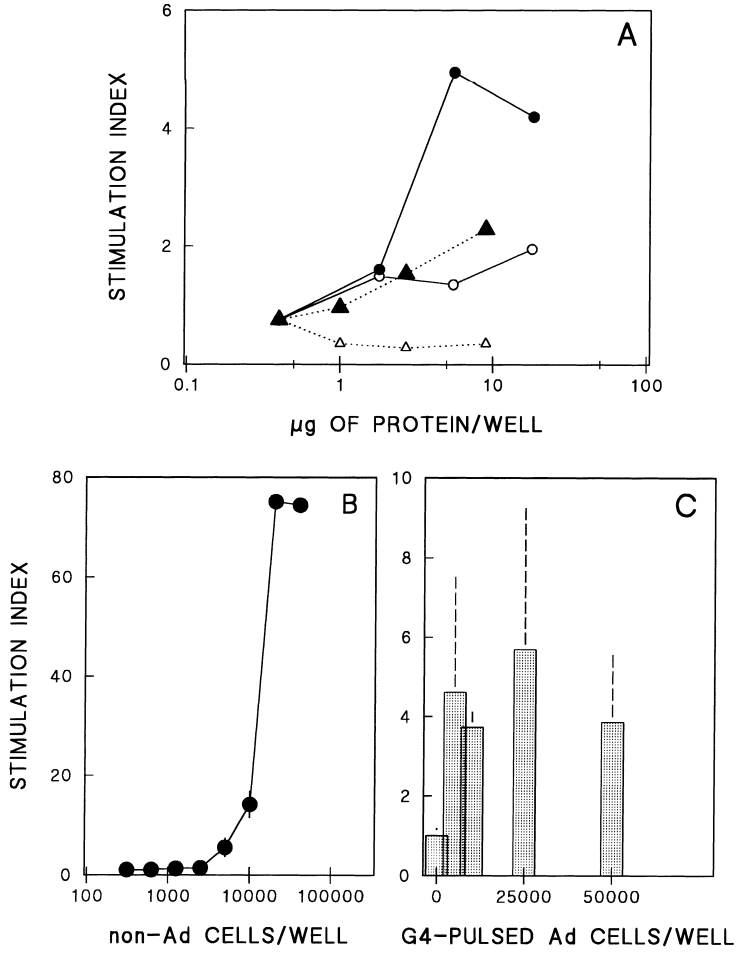

Fig. 2. Proliferation of separated and reconstituted trout kidney cells. Trout kidney cells were separated in Ad and non Ad cell populations by adherence to the flask plastic surface. (A) Proliferation of 10 000 non-Ad cells/well estimated after incubation ±G4 or ±N3 VHSV recombinant proteins in different concentrations and in the presence (●,▲) or absence (○, Δ) of Ad 10 000 cells. ●—●, G4 + Ad cells; ○—○, + G4; ▲....▲, +N3 +Ad cells; Δ.....Δ, +N3. (B) Proliferation of different concentrations of non-Ad cells in the presence of 25 000 G4-pulsed Ad cells. (C) Proliferation of 10 000 non-Ad cells in the presence of different concentrations of G4-pulsed Ad cells. Controls made by non-Ad cells + non-antigen-pulsed Ad cells, or non-Ad cells alone were not different (534 ± 102 cpm) from background. Averages and standard deviations are represented were indicated ((B) and (C)). Results obtained with T26 are shown here. Similar results were obtained with cultures from T24, T25 and T35 (not shown). Results obtained with non-immune (not challenged) trout were non significant.

incubated with G4, then the excess of G4 eliminated by centrifugation, and finally the G4-pulsed Ad cells treated with mitomycin or inactivated by irradiation to avoid its proliferation. In the presence of a constant number of G4-pulsed Ad cells (Fig. 2(B)), the ADC showed an initial cell-concentration dependence of a minimum of about 100 000

cells per ml for proliferation, confirming previous results (Estepa and Coll, 1997). Due to this cell concentration dependence and despite of many efforts, the ADC lines could not be cloned. The ADC showed an optimal concentration requirement of 250 000 G4-pulsed Ad cells/per ml for proliferation (Fig. 2C). The G4-pulsed Ad cells treated with either mitomycin or irradiation had the same effects (not shown). The addition of ovalbumin-pulsed, non-recombinant yeast-pulsed or non-pulsed Ad cells did not produce any detectable proliferation of the ADC cultures (not shown). Although Fig. 2(A)–(C) show the results obtained with cells from T26, similar results were also obtained with cells from T24 and T25 (not shown) and from trout immunized with purified VHSV (T35), confirming that ADC cultures could be developed from other VHSV resistant or immunized trout. The ADC lines could be maintained for more than a year with 1 month periodic restimulations of autologous G4-pulsed Ad cells.

Supernatants from proliferating ADC cultures were obtained and assayed in non-infected trout haematopoietic cells for the presence of secreted 'factors'. The G4-pulsed Ad cells stimulated ADC cultures to secrete 'factors' which enhanced non-infected trout kidney cell proliferative responses to suboptimal doses of PHA (Fig. 3).

3.3. Morphology of the cells in the ADC cultures

After the first addition of G4 to the kidney cell cultures, many morphological cell types seem to be proliferating in the flasks. These cell types were similar to the ones appearing after polyclonal mitogenic stimulation (Estepa and Coll, 1992b). Their detailed morphology (Estepa et al., 1996) and evolution (Estepa and Coll, 1997) has been reported in detail before. As more additions of G4-pulsed Ad cells were made, most of the cell types disappeared from the cultures except the ones with eccentric nuclei (Caspi et al., 1982) and the lymphocyte-like cells (Slierendrecht et al., 1995). Similar observations were made in cultures of ADC from any trout or each time the G4-pulsed

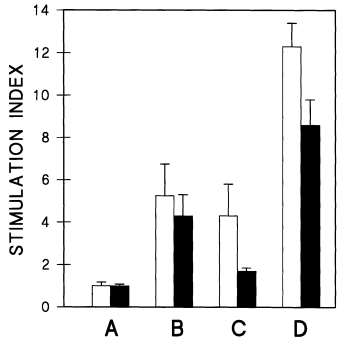

Fig. 3. Mitogenic activities of supernatants from ADC cultures. Kidney cells from non-infected trout were cultured in the presence of supernatants from ADC cultures and combinations with phytohemagglutinin (PHA). A, addition of 20 μl control cell culture medium. B, addition of 10 μl of cell culture medium and 2 μg ml^{-1} of PHA (10 μl). C, addition of 10 μl of cell culture medium 10 μl of supernatants from 20 day-old ADC cultures. D, addition of 2 μg ml^{-1} of PHA (10 μl) and 10 μl of supernatants from 20 day-old ADC cultures. Open bars, supernatants from T24. Solid bars, supernatants from T26.

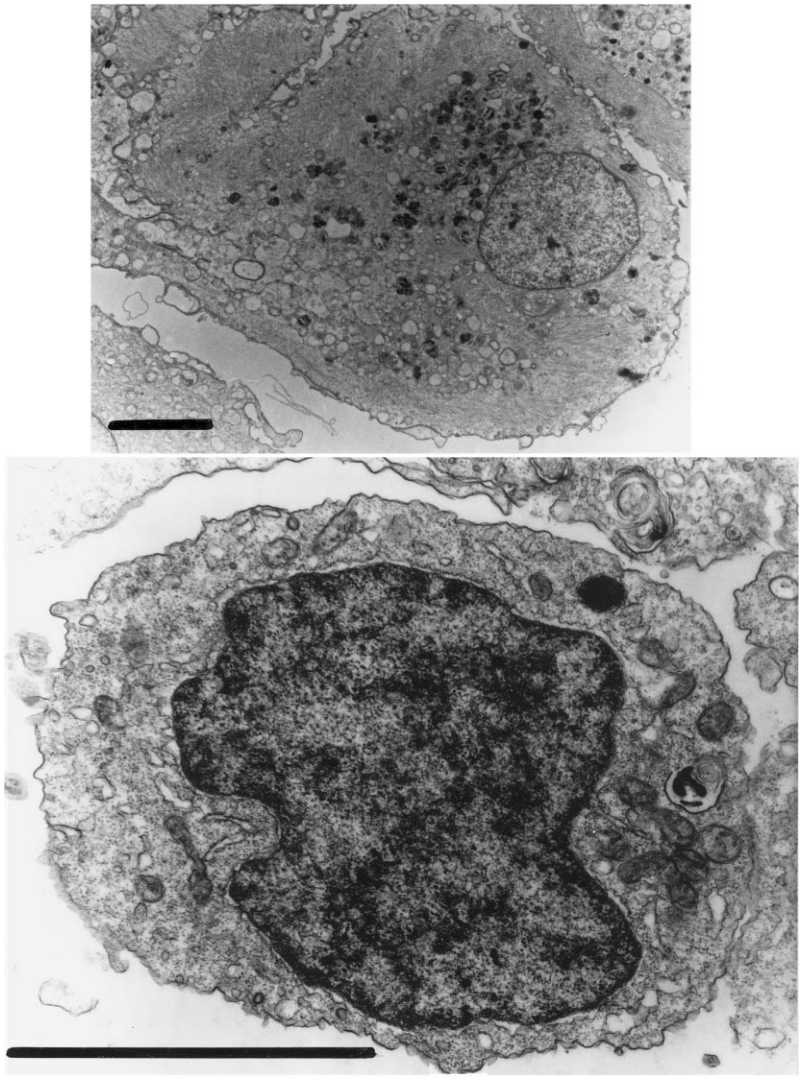

Fig. 4. Morphology of G4-pulsed Ad cells (up) and ADC (down). Twenty days after the last stimulation with G4-pulsed Ad cells 4 months-old ADC cultures were prepared for electron microscopic examination. The cells were pelleted, included, cut and examined by transmission electron microscopy. One of the largest G4-pulsed Ad cell, 20 days in the culture, is shown in the upper figure. Bars are 4 μ.

Ad cells were added. Transmission electron microscopy showed that the ADC had a lymphoid-like morphology with 5–10 μ of diameter and round nuclei with abundant heterochromatin (Fig. 4 down). Among the highly heterogeneous cell population of Ad cells, most of them were much larger (20–30 μ diameter) and showed an stromatic-like morphology (Diago et al., 1993), an irregular surface and eccentric nuclei with scarce heterochromatin (Fig. 4 up).

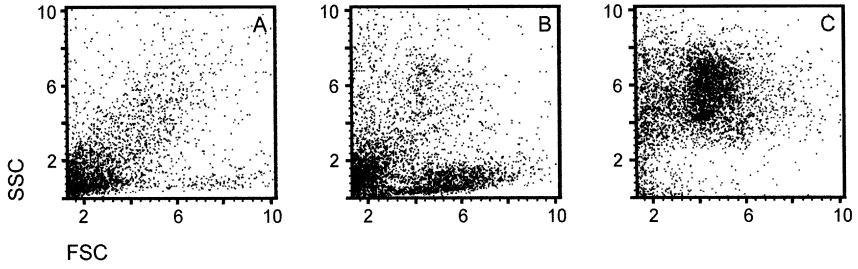

Fig. 5. Size and complex heterogeneity of ADC cultures by cytofluorometry. G4-pulsed Ad cells were defrozen, pelleted and prepared for cytofluorometry. Five and twenty days after the last stimulation with G4-pulsed Ad cells (25 000 cells/well), 4 months-old ADC cultures, were harvested and 5000 cells analyzed by cytofluorometry. Dot plots of G4-pulsed Ad cells (A), proliferating ADC cultures, 5 (B) and 20 (C) days after the addition of G4-pulsed Ad cells. FSC, forward scatter (proportional to cell size). SSC, side ward scatter (proportional to cell complexity).

3.4. Flow cytometry of ADC cultures

To independently assess the nature of the cell types in the ADC cultures after the addition of G4-pulsed Ad cells, a time course of dot plots of size versus granulometry were obtained by flow cytometry (Fig. 5). Five days after the G4-pulsed Ad cell addition, cells of intermediate size and low granulometry (FSC $\sim$ 6/SSC $\sim$ 1) appeared in the ADC cultures (Fig. 5(B)). Twenty days after the G4-pulsed Ad cell addition, the cell population slightly decreases in size and increases in granulometry to a highly homogeneous population at FSC $\sim$ 5/SSC $\sim$ 6 (Fig. 5(C)). Comparison of the dot plots obtained at 5 (Fig. 5(B)) and at 20 days (Fig. 5(C)) after the G4-pulsed Ad cell addition confirmed a transition to a more homogeneous cell population and the almost complete disappearance of the added G4-pulsed Ad cells (Fig. 5(A)). Under the gating conditions employed, thymocytes obtained from non-infected trout were homogeneously concentrated in an small region (FSC $\sim$ 2/SSC $\sim$ 0.5), kidney cells were heterogeneously distributed throughout all sizes, as reported before (Chilmonczyk et al., 1995; Estepa et al., 1992a, b) and spleen or peripheral blood leucocytes were concentrated around two main regions (FSC $\sim$ 2/SSC $\sim$ 1.5 and FSC $\sim$ 5/SSC $\sim$ 5.5).

Between 15.8 to 21.1% ($n = 3$) of the total ADC population 20 days after the last G4-pulsed Ad cells addition showed an increase in positive fluorescence when stained with RartL (Fig. 6). This low percentage of staining could be explained because of the existence of different ADC differentiation stages after being stimulated by G4 or to the heterogeneity of the cells in the cultures. Only 36.2% of the total ADC population could be stained with mouse antibodies to ADC cells (not shown) suggesting also that the rest of the ADC show no availability of its epitopes. Taking these former data as the maximal percentage of ADC to be stained under the conditions employed, between 43.6 to 58.2% of all the available cells in the ADC cultures could be stained with RartL. Under the same conditions, about 26% of non-infected trout PBL were also stained with RartL. In contrast, no increase in immunofluorescence could be observed in the G4-pulsed Ad cells when stained with either RartL (Fig. 6) or mouse antibodies to ADC (not shown). Membrane fluorescence of the ADC cultures was confirmed by observation with an

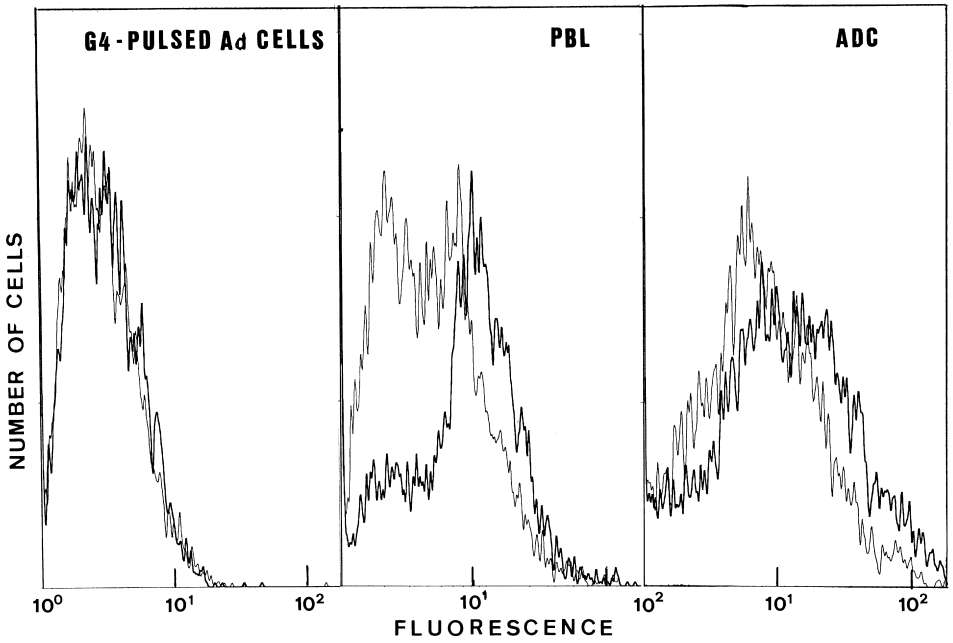

Fig. 6. Fluorescence at 515–545 nm by flow cytometry of RartL stained G4-pulsed Ad cells, PBL and ADC cultures. Cells were stained with preimmune serum (—·—) and with anti-recombinant TcR fragment antibodies RartL (—). Fluorescence intensity (*x*-axis) vs. number of cells (*y*-axis) were plotted.

immunofluorescence microscope by using both mouse antibodies to ADC or RartL (not shown). No positive staining of ADC or ADC cells for surface trout IgM could be detected by flow cytometry in the same ADC cultures by using MAb 1G7. MAb 1G7 stained 14% and 38.4% of the cells in the kidney and PBL, respectively (not shown).

3.5. Expression of TcR sequences in ADC cultures

To determine the possible expression of TcR genes by ADC cultures, we performed RT-PCR on their extracted RNA, using primers specific for the constant segment of the α- and β-chain of the trout TcR genes. To minimize the possible RNA contamination due to the presence of the G4-pulsed Ad cells, RNA was extracted from ADC cultures 20 days after its addition, when very few remaining Ad cells were present in the ADC cultures (Estepa and Coll, 1997). Primers specific for the α- and β-chain TcR trout genes generated DNA products of the expected size from the ADC RNA, indicating that these cells express α- and β-chain TcR genes. The specificity of the primer amplification was confirmed by reamplification of the DNA products by internal sequence primers or nested PCR (Fig. 7). Fig. 7 also shows that similar TcR bands were expressed in kidney leucocytes from non-infected trout cultured in the presence of Concanavalin A but with less intensity than in the ADC cultures when compared in a cell to cell basis. No similar band intensities were obtained by using cells from non-infected trout kidney, non-infected trout muscle or Ad-cells (not shown).

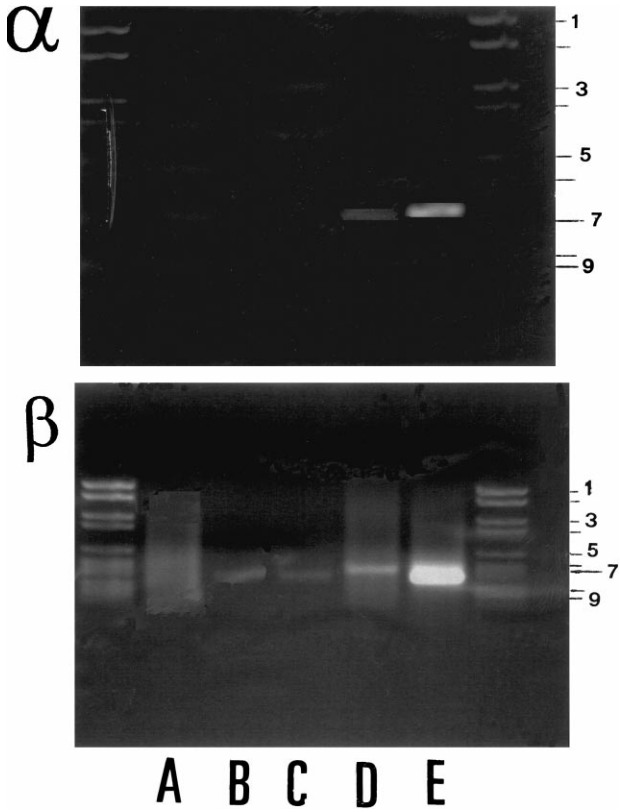

Fig. 7. Agarose gel electrophoresis of reamplified RT-PCR products from the RNA of ADC cultures. RNA from kidney cells and from 10 months-old ADC cultures 20 days after addition of G4-pulsed Ad cells were isolated and amplified by a first RT-PCR and then reamplified by a nested PCR. For the TcR α-chain (α), the RNA was first RT-PCR amplified with primers #51 and #52 and then the DNA with the internal primers #53 and #54. For the TcR β-chain (ß) the RNA was first RT-PCR amplified with primers #37 and #38 and then the DNA with the internal primers #39 and #40. The reamplified products were then analyzed in 1% agarose gels and stained with ethidium bromide. The figure shows one representative result from four experiments. (A) no DNA. (B) DNA from kidney cells from non-infected trout. (C) DNA from kidney cells from non-infected trout incubated for 2 days. (D) DNA from kidney cells from non-infected trout incubated for 2 days in the presence of 4 μg ml^{-1} of Concanavalin A. (E) DNA from 10 month-old ADC cultures 20 days after addition of G4-pulsed Ad cell. Numbers to the right, molecular weight DNA markers, 1, 2176 bp; 2, 1766 bp; 3, 1230 bp; 4, 1033 bp; 5, 653 bp; 6, 517 bp; 7, 453 bp; 8, 394 bp; 9, 280 bp.

Finally, sequencing of the reamplified DNA bands (not shown) confirmed that they correspond to the expected region of trout α and β TcR sequences (Partula et al., 1995).

4. Discussion

We have characterized the trout cell lines showing in vitro viral antigen-dependent cell (ADC) proliferation that were reported before (Estepa et al., 1996; Estepa and Coll,

1997). The ADC cultures could only be obtained from the kidneys of trout either surviving two consecutive viral hemorrhagic septicemia virus (VHSV) infections or hyperimmunized with purified VHSV but not from non-infected trout. These results confirmed the specificity of previous observations (Estepa et al., 1994b; Lorenzo et al., 1995). The long-term proliferation of the ADC cultures was dependent on the periodical restimulation with the G4 protein of VHSV expressed in yeast and presented by autologous Ad cells pulsed with G4 (G4-pulsed Ad cells). ADC cultures did not grow autonomously and ADC proliferation could not be obtained with ovalbumin-pulsed Ad cells or non-pulsed Ad cells. On the other hand, ADC cultures stimulated with N3-pulsed Ad cells (N protein of VHSV expressed in yeast) showed none or little proliferation depending on the trout used. These results suggest that the ADC cultures are derived from trout cells that specifically recognized the G4 antigen of VHSV and could be involved in the VHS disease resistance of the survivors.

To have enough supply of putative syngeneic antigen presenting cells through more than a year after the trout were killed, we have used individual trout haematopoietic cell cultures of Ad cells (Diago et al., 1993; Estepa et al., 1994a; Estepa and Coll, 1997). The individual Ad cell cultures were needed because of the difficulty to interpret results with trout allogenic cultures and the difficulty in obtaining some of the few existent inbred or syngeneic trout populations (Dorson et al., 1995; Ristow et al., 1995). Moreover, only in salmon (Rodrigues, 1996) there is some knowledge of the variability of the class I or of the class II histocompatibility genes (Grimholt et al., 1994).

The requirement of G4-pulsed Ad cells for ADC proliferation confirmed previous evidence of the need of 2 different cell populations for trout T-like cell proliferation. One of the cell populations is needed to present the G4 (Ad cells) and the other to proliferate (ADC) (Estepa and Coll, 1992a; Estepa et al., 1994b). Since there is no formal evidence that the Ad cells can present the G4 antigen to the ADC, the G4 protein could also be inducing the Ad cells to secrete the factors required for ADC proliferation. Conditioned medium from either G4-pulsed Ad cells or proliferating ADC cultures did not stimulate proliferation of the ADC cultures in the absence of G4-pulsed Ad cells (not shown). However, the ADC cultures secreted 'factors' which enhanced 2–3 fold the non-infected trout kidney cell proliferative responses to suboptimal doses of PHA (Fig. 3). Similar enhancement of suboptimally mitogen-stimulated proliferative responses have been reported for unspecific T-like cells derived from catfish (Clem et al., 1996) and for PBL from human rabies rhabdovirus vaccinees (Perrin et al., 1991). More experimentation is needed to clarify these aspects of the ADC proliferation.

To investigate whether the proliferating cells in the ADC cultures could be the trout equivalent to T-cells, the presence both of the β-chain TcR protein in their membranes and of the mRNA from the α- and β-chains of the TcR genes were studied.

Flow cytometry analysis revealed the expression in 15.8–21.1% ($n = 3$) of the cells from the heterogeneous mixture of the ADC cultures of some β-chain TcR epitopes (calculated from the data of Fig. 6(C)). This represents a lower level of staining than that shown by an early T cell Monoclonal antibody (MAb) marker reactive with 30-50% of carp young thymocytes (Rombout et al., 1997). About 26% of the leucocytes were also stained with RartL in non infected trout PBL (Fig. 6(B) and results not shown). However, no staining different from background could be obtained in cells from kidney, thymus and

spleen (not shown) or in G4-pulsed Ad cells (Fig. 6(A)). The low level of staining of the ADC could be due to the low capacity of the RartL reagent to distinguish T-cells, to the low proportion of TcR expressing cells in the population, or to the low exposition of the epitopes as suggested by the maximal 36.2% staining with anti-ADC antibodies. While questions still arise with respect to the specificity of the RartL reagent, till now this is the only reagent of this kind available in trout. The ADC cultures were also negative for the only trout lymphocyte marker presently available, the anti-IgM that identifies B-like cells (not shown). More studies should be made when better anti-trout TcR antibodies become available.

PCR amplified bands were present in the ADC that correspond to the expected size from the α- or β-chain trout TcR. The size and the sequence of the amplified bands, strongly indicate that the specific bands amplified from the mRNA rather than from any contaminating genomic DNA. Similar bands, but at lower intensity, appeared also when kidney cells were incubated with ConA (a specific T-cell mitogen) but not from muscle, Ad cells, or PBL. In the absence of any other specific markers, all these data strongly suggest an enrichment of ADC cultures in trout T-like lymphocytes.

Due to the high success rate of the new technique to develop ADC from trout (four ADC lines out of four VHSV trout donors), this approach should be easily repeated in trout as well as in other fish/antigen models, so that this type of responses can be further analyzed. Although the role of T cell-like fish lymphocytes in fish immunity is now well documented, its participation in disease resistance remain practically unknown. The availability of ADC cultures could also facilitate the production of antibodies specific for trout T-cells (T-cell marker) as an alternative to the use of recombinant proteins (Partula et al., 1995).

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