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An in vitro method to obtain T-lymphocyte-like cells from the trout

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Abstract

We describe a methodology to obtain from the trout T-lymphocyte-like cell cultures showing in vitro antigen-dependent cell (ADC) proliferation. ADC cultures were developed from each of 3 outbred rainbow trout that survived two consecutive viral haemorrhagic septicaemia virus (VHSV) infections (an important salmonid fish disease in Europe). As stimulating antigen, we used a yeast recombinant form of the glycoprotein G (G4) of VHSV. No similar ADC cultures could be obtained from non-infected control trout kidney cells incubated in the presence of G4, confirming previously published observations. For long-term culture, and to overcome the absence of inbred trout populations, autologous haematopoietic adherent (Ad) cells were obtained and maintained from each individual trout kidney and periodically used as viral antigen-presenting cells (called G4-pulsed Ad cells) to autologous ADC cultures. The methodology to obtain trout ADC lines constitutes a new tool that would facilitate further studies on the comparative and developmental immunology of lower vertebrates. It could also be applied to research on fish vaccination and viral antigen-presentation mechanisms.

Keywords: T-lymphocyte; Antigen-dependent cell proliferation; Viral hemorrhagic septicemia virus; Glycoprotein G4; In vitro method; Trout

1. Introduction

Long-term cell lines of various leucocyte cell types are valuable tools for the investigation of different aspects of the immune response in mammalians. However, there are few examples of such cell lines in fish and, therefore, the functional aspects of immunity in such animals are poorly understood. Cell lines which have been derived from fish include, antigen-presenting cells of the monocyte/macrophage series (Faisal and Ahne, 1990; Vallejo et al., 1991a,b) and B-cells (Miller et al., 1994). Both of these have been obtained in catfish. To the best of our knowledge, no T-lymphocyte-like cell lines have yet been reported from any fish.

Antigen-induced immunoproliferation assays of leucocytes obtained from virally immunized or vaccinated animals (anamnestic immunoproliferation) have been used as an assay for mammalian T-cell activities (Kontsekova et al., 1992; Kutubuddin et al., 1992). Some of those assays also included rhabdoviruses (MacFarlan et al., 1984; Burkhart et al., 1994). Thus, in mammals, in vitro proliferation of antigen-dependent cells (ADC) is considered to be

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an assay for T-cells. Anamnestic immune proliferative responses to rhabdoviruses in fish have been mostly studied in trout using whole virus (Chilmonczyk, 1978), isolated viral proteins (Estepa, 1992; Estepa and Coll, 1992a,b), recombinant viral protein fragments (Estepa et al., 1994b) and viral peptides (Lorenzo et al., 1995a,b). No leucocyte proliferation could be obtained from non-infected control trout kidney donors treated in parallel with all those VHSV antigens (Estepa et al., 1994b; Lorenzo et al., 1995a,b). Furthermore, trout individually restricted the selection of the rhabdoviral peptides stimulating the leucocyte anamnestic proliferation (Lorenzo et al., 1995b), suggesting the existence of peptide-presenting capacities and T-cell proliferation with similar characteristics to those of higher vertebrates (Burkhart et al., 1994; MacFarlan et al., 1984). However, to confirm this, ADC need to be isolated. To develop such ADC in trout, we have used the viral haemorrhagic septicaemia virus (VHSV), because of the important economic impact of these rhabdoviruses in the international salmoniculture industry. We have concentrated on the glycoprotein G of VHSV because trout neutralizing antibodies show exclusive specificity for this antigen (Bernard et al., 1983; Engelking and Leong, 1989; Estepa et al., 1994a,b; Gilmore et al., 1988; Lorenzen et al., 1990; Olesen et al., 1991), a recombinant form of G (G4) confers some protection against in vivo VHSV challenge (Estepa et al., 1994a,b), the G protein is expressed in the membrane of infected cells (Estepa et al., 1992) and the G protein and peptides derived from it are the strongest inducers of anamnestic lymphoproliferation (Estepa and Coll, 1992a,b; Estepa et al., 1994a,b; Lorenzo et al., 1995a,b).

Since neither inbred nor syngeneic trout populations are at present widely available (Ristow et al., 1995) and the histocompatibility groups of the trout are not yet well defined (Stet and Egberts, 1991), it was necessary to establish a primary individual trout haematopoietic cell culture from each donor trout in order to have enough supply of putative antigen-presenting cells during a year-long experimental period (Diago et al., 1993; Diago et al., 1991; Estepa et al., 1994a). The successful application of this technique has been the key to establishing the T-lymphocyte-like trout cell lines defined as ADC described in this report.

2. Materials and methods

2.1. Virus and recombinant G4 VHSV protein

The VHSV 07.71 isolated in France (LeBerre et al., 1977) from rainbow trout *Onchorynchus mykiss* (Walbaum) was grown in epithelial papillosum cyprine (EPC) cells and purified as described (Basurco et al., 1991). The recombinant fragment G4 (aa 9-443) devoid of the transmembrane region and part of the signal peptide, was cloned and expressed in the yeast *Saccharomyces cerevisiae* DC04 as previously described (Estepa et al., 1994b).

2.2. Production of trout survivors of VHSV infection

Outbred trout (0.5–2 g body weight) were first infected over 2 h at 12–14°C with 10^6 $TCID_{50}$/ml of VHSV attenuated by 10 passages on EPC cells (Basurco and Coll, 1992). Under these conditions, trout survival was 10–30% ($n = 4$, n = number of experiments). The trout surviving the infection were challenged 2 months later with 10^6 $TCID_{50}$/ml of VHSV (2 h, 10–11°C). As in previous studies (De-Kinkelin, 1988), 5–24% ($n = 4$) of the initial number of trout survived both infections and showed no signs of VHS. Survivor trout were used 4–6 months after the last VHSV challenge (Enzmann and Konrad, 1993; Lorenzo et al., 1995a,b).

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

Leucocytes from individual trout kidneys were obtained as described (Estepa and Coll, 1992a,b), to avoid any possible mixed leucocyte reactions (Stet and Egberts, 1991). Trout kidneys were chosen as a source of leucocytes because cells responding to the VHSV antigens (Estepa et al., 1994b) and to the VHSV peptides (Lorenzo et al., 1995a,b) were known to be present in this organ. Furthermore, large numbers of trout leucocytes could not be obtained from any other organ in mature trout (Chilmonczyk, 1978). Briefly, the kidney was removed from trout previously cooled down to 4°C, killed by cutting off the head and blood allowed to drain for ~5 min. The kidney was cut into pieces, dissociated with a Pasteur pipette, decanted and washed in cell medium.

The cell culture medium was RPMI 1640 (Dutch modification, 290 mOsm/kg) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 μg/ml amphotericin, 50 μg/ml 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 ($\sim 3 \times 10^6$ cells/ml) of the kidney leucocyte cell suspension was incubated with 18 μg/ml of G4 in 25-cm^2 plastic flasks at 20°C with 5% CO_2. After 2 weeks of incubation, the supernatants containing the non-adherent (non-Ad) cell population were gently removed and used as a source of T-lymphocyte-like cells for the ADC cultures. The Ad cell population remained attached to the surface of the flasks (Diago et al., 1991; Estepa et al., 1992).

2.4. Preparation of G4-pulsed Ad cells

Long-term haematopoietic cell cultures from each individual trout donor were obtained in parallel kidney cell cultures from the same leucocyte suspensions, after removing the non-Ad cells as described above (Diago et al., 1991; Estepa et al., 1992). The remaining adherent (Ad) cell population (macrophages, dendritic cells, stromal cells, etc.) were either used to prepare G4-pulsed Ad cells or maintained for long-term haematopoietic stromal cell cultures as described (Diago et al., 1991). To prepare G4-pulsed Ad cells, the Ad cells in the flasks were incubated with 18 μg/ml of G4 for 1 h at 20°C, washed 3 times with phosphate buffered saline (PBS), incubated with 30 μg/ml of mitomycin in cell culture medium for 1 h at 20°C, washed 3 times with PBS, harvested by mechanical shaking and frozen at −70°C in fetal calf serum and 10% DMSO. Some Ad cells were not treated with G4, but only with mitomycin, and were used as controls.

3. Results

Leucocyte cells from the kidneys of 3 VHSV survivor trout (T24, T25 and T26) were cultured in 25-cm^2 flasks in the presence of 18 μg/ml of G4 recombinant protein from VHSV. Cell proliferation could be observed after a few days in each of the cultures from the 3 VHSV survivor donor trout. Kidney leucocyte cells from non-infected trout when cultured with G4 recombinant VHSV protein did not show any proliferation, thus confirming previously published observations (Estepa et al., 1994b; Lorenzo et al., 1995a,b).

To assess whether the recognition of G4 by the leucocyte cells was mediated by a conventional T-cell receptor (TcR)–antigen interaction, we investigated the requirement for presenting or accessory adherent (Ad) cells for G4-specific proliferation. With this purpose, 2 weeks after initiation of the cultures mentioned above, each of the flasks containing kidney cell suspensions were separated into Ad and non Ad-cell populations. Then, the proliferation response obtained in cultures containing every possible combination of non-Ad cells, Ad cells and G4 were tested. The experiments were restricted to autologous cell cultures because of the present impossibility of correctly interpreting allogeneic cultures. Significant proliferative responses were only obtained in autologous combinations when non-Ad cells, Ad cells and G4 were all present, suggesting that presenting or accessory cells were needed for G4 induced proliferation of ADC.

To corroborate these results and derive long-term presenting cells, the Ad cells were incubated with

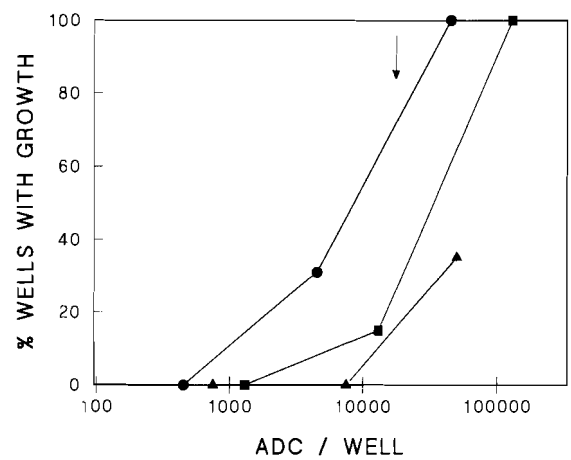

Fig. 1. Dependence of ADC proliferation on initial cell concentration. The experiment was performed in the presence of autologous 25000 G4-pulsed Ad cells per well (100 μl volume) for each of 3 trout surviving VHSV infection (T24, T25 and T26). Different initial concentrations of ADC were added to 36 wells at each concentration. After 2 weeks in culture, the number of wells with growth was counted and expressed as a percentage using the following formula: number of wells with growth at each concentration/36 × 100. The donor trout studied were T24 (▲), T25 (■), and T26 (●).

G4. After eliminating the excess of G4, the G4-pulsed Ad cells were treated with mitomycin to inhibit proliferation, and then frozen until use. The non-Ad cell population was distributed in 96-well plates containing 100 μl/well of culture medium (25 000 cells/well) and then stimulated by the addition of 5000 autologous G4-pulsed Ad cells in 50 μl of cell culture medium. After 5 days, colonies of dividing cells and scattered cells were observed throughout the wells. These proliferating cells were called antigen-dependent cells (ADC) and could be maintained for approximately 1 year by periodically adding G4-pulsed Ad cells. In the presence of a constant number of G4-pulsed Ad cells, the ADC showed a cell-concentration dependence for proliferation that differed with the various trout used (Fig. 1). The minimum proliferation requirement for an initial ADC concentration has not yet permitted the cloning of these cells, despite numerous attempts.

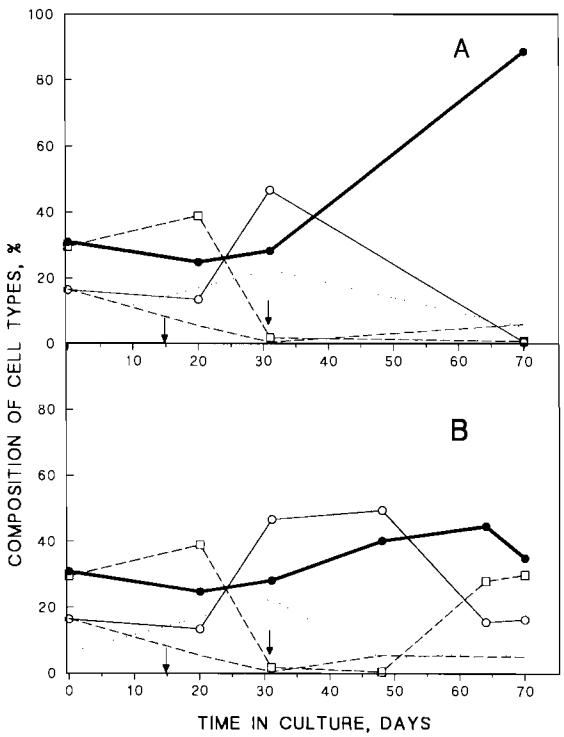

Fig. 2. Time course evolution of morphological cell types during the establishment of ADC cultures. The time course of the cellular types in the ADC cultures from T26 after the addition of its autologous G4-pulsed Ad cells was followed using cytocentrifuge preparations. After fixing and staining, the morphological cell types were classified using criteria defined previously (Estepa and Coll, 1992a,b): □--□, multinucleated; ○—○, eccentric nuclei; —, adherent cells; · · ·, large nucleated; ●—●, mature lymphocytes. About 200 cells were counted per slide and results were expressed as percentage of the total number of cells counted at each time point during culture. Time 0 and the vertical arrows indicate the time of addition of the G4-pulsed Ad cells. *A* and *B* show the time course evolution in two different experiments.

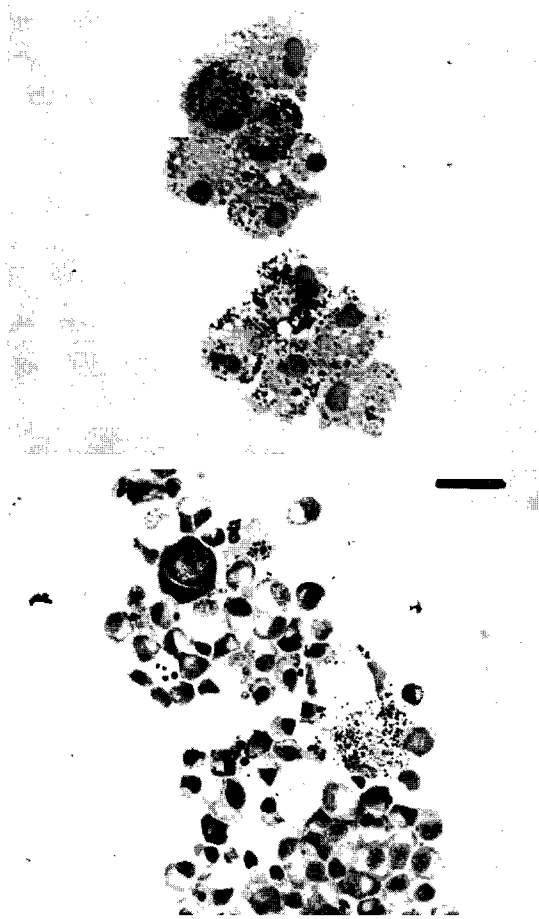

Fig. 3. Morphological appearance of G4-pulsed Ad cells (upper panel) and ADC (lower panel) in cytoslides. G4-pulsed Ad cells (25 000 cells/well) were added to resting ADC cultures (about 1 month after the last G4 stimulation). Cytocentrifuge preparations were obtained at 700 rpm (3 min), fixed for 10 min with 1% glutaraldehyde and stained with Toluidine blue in borate buffer. They were made permanent using Depex. Cytocentrifuge preparations of G4-pulsed Ad cells (upper panel) and proliferating ADC cultures, 15 days after the addition of G4-pulsed Ad cells (lower panel). Scale bar: ~ 10 μm.

After the first addition of G4 to whole kidney, many morphological cell types proliferated in the flasks (Fig. 2A,B). These cell types were of similar

heterogeneity as the ones appearing after polyclonal mitogenic stimulation of whole kidney cells, as previously reported (Estepa and Coll, 1992a,b). After further additions of G4-pulsed Ad cells (Fig. 3, upper panel) to ADC cultures, the proportion of multinucleated cells decreased and more and more eccentric nuclei cells and mature lymphocytes dominated in the ADC cultures (Fig. 3, lower panel). Large nucleated cells (morphologically resembling lymphoblasts) were also present in the cultures, but always at low frequency. Fig. 2 shows the evolution of cell types in two representative ADC cultures from T26. As more additions of G4-pulsed Ad cells were made, most of the cell types disappeared from the cultures except those cells having eccentric nuclei (Caspi et al., 1982) and the more mature lymphocytes (Slierendrecht et al., 1995) (Fig. 2A) although multinucleated cells were sometimes also present (Fig. 2B). The added G4-pulsed Ad cells could be detected for only a few days after addition to the cultures before disappearing over a few more days most probably because of the mitomycin treatment. Similar observations were made in cultures of ADC from the other trout or each time a cycle of addition of G4-pulsed Ad cells was started on resting ADC cultures.

4. Discussion

We have described a new method for the preparation of trout T-lymphocyte-like cell lines showing ADC proliferation in vitro. ADC cultures were developed from each of 3 individual rainbow trout belonging to an outbred population surviving VHSV infections but were not obtained from non-infected control trout (Estepa et al., 1994b). The ADC lines are presumed to have been derived from cells that specifically recognized the G4 antigen of the VHSV virus. Further evidence that most of the proliferating cells in the ADC cultures were trout T-lymphocyte-like cells including the following: (1) their homogeneous morphology by cytofluorometry; (2) the specific proliferation dependence of both G4 and G4-pulsed autologous cells, but not of other protein-pulsed autologous cells; (3) the lack of surface staining with trout anti-IgM antibodies and the high level of expression of TcR trout genes (Partula et al., 1994; Partula et al., 1995) (manuscript in preparation).

The requirement of G4-pulsed Ad cells for ADC proliferation confirmed previous evidence of the need for at least two cell populations in trout T-like cell proliferation. A requirement for Ad cells had also been found previously for concanavalin A-induced proliferation of large-nucleated cells to form colonies in fibrin-clot cultures (Estepa and Coll, 1992a,b). Moreover, the fractionation of kidney cells into Ad and non-Ad cells inhibited the proliferative response both to the polyclonal mitogen phytohaemagglutinin (mitogenic inespecific response) and to G4 (anamnestic specific response) (Estepa et al., 1994a,b).

The establishment of long-term haematopoietic cell cultures (Diago et al., 1991) to be used as a source of presenting/accessory cells was of key importance in developing the reported ADC cultures. There was only one other report of fish antigen-presenting cells available for long-term use, namely the spontaneous proliferating catfish leucocytes morphologically resembling mammalian monocytes or macrophages (Vallejo et al., 1991a,b). The present novel approach for developing ADC from trout can, most probably, be used in any outbred fish population to study many aspects of fish immunology. Due to the high success rate of the technique described here (3 ADC lines from 3 trout donors), the approach should be easily replicable in other fish/antigen systems, thus making it possible to analyze further this type of response. Although the participation of T-lymphocyte-like subpopulations (to date defined as surface Ig-negative cells) in fish immunity is now well documented (Desvaux and Charlemagne, 1981; Miller and Clem, 1984; Miller et al., 1986; Sizemore et al., 1984; Vallejo et al., 1991a,b), their relevance to disease remains unexplored. Therefore, the availability of ADC cultures should greatly facilitate such studies. For instance, the production of antibodies specific for fish T-cells (T-cell marker) from TcR-enriched ADC cultures could be an alternative to the use of recombinant α- or β-chain proteins for the same purpose (Partula et al., 1995, 1996).

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