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MORPHOLOGY OF ANTIGEN DEPENDENT HAEMATOPOIETIC CELLS FROM TROUT SURVIVING RHABDOVIRAL INFECTIONS

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Abstract

This report describes the morphology of the first fish haematopoietic cell lines obtained from trout surviving viral haemorrhagic septicemia (VHS) (an important fish disease in Europe) that show specific viral antigen-dependent proliferation in vitro. The in vitro growth of these cell lines (antigen-dependent cells or ADC) was dependent on the presence of VHSV recombinant glycoprotein G4 in the culture medium and/or autologous G4-pulsed adherent (Ad) cells. No similar cell lines could be developed from uninfected healthy trout. The ADC resembled lymphoid-like cells with an average diameter of 5-10μm and round eccentric nuclei. However, they showed a larger amount of cytoplasm than trout kidney or peripheral blood lymphocytes. The availability of these cell lines would be helpful for further in vitro studies of fish viral pathology and immunology.

Introduction

Trout possesses an immune system with largely unknown responses to rhabdoviral infections (Estepa *et al*, 1991; Leong *et al*, 1995). Trout has functional: T and B cells with families of rearranged genes encoding their antigen receptors, antibodies (primarily of the IgM class), phagocytic cells, MHC molecules, complement, interleukins, etc. (Warr, 1996). However despite the existence of B-cell lines (Miller *et al*, 1994), the existence of T-cells (except to a functional level) is not yet clear because the lack of appropriate T-cell markers.

Although trout anamnestic immune proliferative responses (T-like responses) to viruses (Chilmonczyk, 1978), isolated proteins (Estepa and Coll, 1992) recombinant proteins (Estepa, 1992; Estepa *et al*, 1994) and viral peptides (Lorenzo *et al*, 1995; Lorenzo *et al*, 1995) and the existence of both fish cell protein processing and membrane presentation of peptides (Vallejo *et al*, 1991) have been demonstrated, to our knowledge specific antigen-dependent cell lines (T-like cell lines) have not been yet obtained from trout or from any other fish.

We have developed viral antigen-dependent cell (ADC) lines that because they were obtained from trout surviving viral infections should be related to the viral immune resistance mechanisms (Estepa *et al*, 1996). Considering the important economic impact of viral diseases of salmonid fish, the opportunity to analyze rainbow trout anti-viral responses using an in vitro experimental model is of great interest.

Materials and Methods

Viruses. The VHSV 07.71 isolated in France (LeBerre *et al*, 1977) from rainbow trout *Onchorynchus mykiss* (Walbaum) was grown in the epithelial papillosum cyprine (EPC) cell line and purified as described (Basurco and Coll, 1991).

Recombinant G4 VHSV protein. Protein G4 (aa 9-443) was cloned and expressed in the yeast *Saccharomyces cerevisae* DCO4 as reported previously (Estepa *et al*, 1994; Thiry *et al*, 1991).

Survivor trout of VHSV infections. VHSV survivor trout were obtained as described before (Estepa *et al*, 1994; Lorenzo *et al*, 1995) from an outbred trot population and have been used 4-6 months after the last VHSV challenge.

Establishment of antigen-dependent cell (ADC) lines. Leucocytes from trout kidney

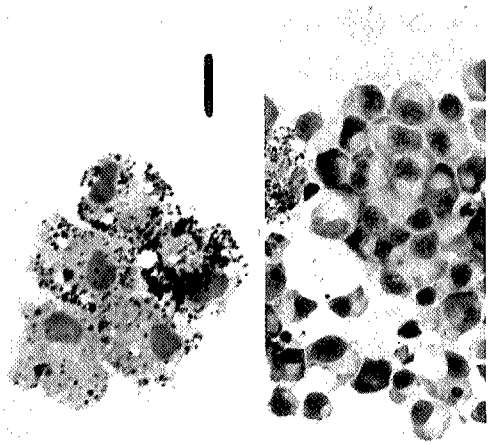

Figure 1.- Morphological appearance of G4-pulsed Ad cells (left) and proliferating ADC (right). ADC from VHSV resistant trout, 15 days after addition of autologous G4-pulsed Ad cells. Cytocentrifuge preparation of the cells fixed with 2% glutaraldehyde 10 min and stained with toluidine blue. Bar is 15µm.

were obtained and cultured as described (Estepa and Coll, 1992) from individual VHSV survivor trout to avoid any mixed leucocyte reactions (Stet and Egberts, 1991). G4 recombinant protein (18µg/ml) was added to the trout leucocyte cell suspensions ($3x10^6$ cell/ml) for starting the cultures. After 2 weeks the supernatants containing the non-adherent cell population were removed and used for the antigen-dependent cell (ADC) cultures (Estepa *et al*, 1996). The remaining adherent (Ad) cell population (macrophages, dendritic-like cells, stromal cells, etc.) were used to prepare the G4-pulsed presenting cells (Ad cells) (Diago *et al*, 1991). Ad cells were incubated with 18µg/ml of G4 during 1 h at 20°C, washed 3 times with phosphate buffered saline, 10 mM sodium phosphate 0.15 M NaCl, pH 7.4 (PBS), incubated with 30µg /ml of mitomycin during 1 h at 20°C, washed 3 times with PBS, harvested by mechanical shaking of the flask and frozen at -70°C in the presence of 50% fetal calf serum and 15% DMSO. Some Ad cells were not treated with G4 but only with mitomycin for control. Before use the G4-pulsed Ad cells wereunfrozen and pelleted.

ADC cultures were distributed in 96-well plates, 100µl of volume per well (25.000 cells/well) and stimulated with autologous G4-pulsed Ad cells (5000 cells/well). After 5 days colonies of dividing cells were observed only if the cultures contained both non-Ad cells and G4-pulsed Ad cells. The ADC lines were maintained by monthly stimulation (by adding G4-pulsed Ad cells).

Transmission electron microscopy. Fifteen days after addition of G4-pulsed Ad cells, ADC cultures were harvested and pelleted by low speed centrifugation, washed in PBS and fixed in 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 at 4 °C. Cells were postfixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Araldite. Thin sections (~ 80 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and were observed in a JEOL-100 B electron microscope at 60-80 Kv.

Results

Individual cell cultures established from the kidney of 3 VHSV survivor trout in the presence of 18µg/ml of G4 showed cell proliferation. After 2 weeks, the cell cultures were separated into Ad and non-Ad cell populations. Since neither inbred nor syngeneic trout are available at present, a long-term cell culture of Ad cells was established from each kidney donor (Diago *et al*, 1993; Diago *et al*, 1991; Estepa *et al*, 1994) to have enough supply of antigen presenting cells during the experimentation (more than a year after trout were killed). Non-Ad cells disappeared from the cultures ~2 months after the last G4 estimulation. The addition of Ad cells without being pulsed with G4 did not stimulate the proliferation of the non-Ad cells. The addition of Ad cells pulsed with G4 did stimulated the proliferation of the non-Ad cells. A minimum of 5000 of G4-pulsed Ad cells/well were required for the non-Ad cell proliferation.

After the first addition of G4 to whole kidney, many morphological cell types seem to be proliferating in the flasks. After additions of G4-pulsed Ad cells (Fig. 1 left) were made, most of the different cell types disappeared from the cultures except the eccentric nucleated cells and the more mature lymphocyte-like cells. Figure 1 (right) shows the aspect of a cytocentrifuge preparation containing proliferating non-Ad cells or ADC. The added G4-pulsed Ad cells could be detected during a few days after its addition to the cultures but were disappearing a few more days later, most probably because of the mitomycin treatment. Similar observations were made in cultures of ADC from the 3 trout.

Transmission electron microscopy showed that the ADC had a lymphoid-like morphology, 5-10µm in diameter and round nuclei with abundant heterochromatin (Fig. 2 right). In their cytoplasm numerous vesicles of different sizes containing heterogeneous materials, dense

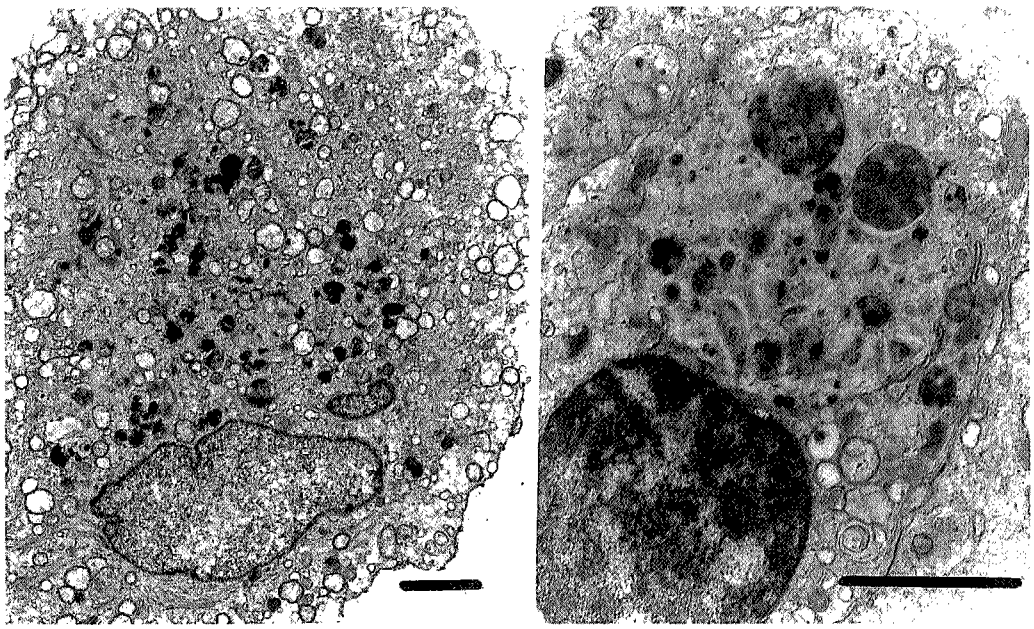

Figure 2.- Ultrastructure of G4-pulsed Ad cell (left) and proliferating ADC (right). Photomicrographs of ADC cultures 15 days after addition of autologous G4-pulsed Ad cells. The cells shown are representative of the two main cellular types observed. The bars are 2 µm.

inclusion bodies, some mitochondria and small profiles of rough endoplasmic reticulum were observed. However, they showed a lower ratio of nucleus/cytoplasm than the typical lymphocytes found in trout kidney (Alvarez *et al*, 1996). Much larger (20-30µm in diameter) appeared the Ad cells with an stromatic cell-like morphology (Fig. 2) (Diago *et al*, 1991), round-ovate and eccentric nuclei with scarce heterochromatin and visible nucleoli. The most noticeable features of their cytoplasm were the abundant tonofilament bundles and electrodense inclusion materials. Well developed Golgi and vesicles were also observed. These cells appeared joined together with desmosomes.

Discussion

We have obtained and morphologically characterized rainbow trout permanent cell lines showing specific antigen-dependent (ADC) proliferation in vitro. The ADC lines were developed from 3 individual trout belonging to an outbred population surviving viral hemorrhagic septicemia (VHSV) infection. The in vitro proliferation of ADC lines depends on the presence of viral recombinant glycoprotein G4 in the culture medium and/ or G4-pulsed autologous Ad cells. The requirement of G4-pulsed Ad cells populations for ADC cell proliferation confirmed previous evidence of the need of at least 2 cell populations for leucocyte trout proliferation (mitogenic inespecific responses and anamnestic specific responses) (Estepa and Coll, 1992; Estepa *et al*, 1994). We were not capable of developing similar ADC lines from non-infected trout as reported previously (Estepa *et al*, 1994; Lorenzo *et al*, 1995), strongly suggesting that the ADC lines came from cells that specifically recognized the G4 antigen of VHSV.

The establishment of long-term haematopoietic stomal cultures (Diago *et al*, 1991) to be used as a source of presenting/accessory cells (Ad cells) have been of key importance to develop the ADC lines. There is only another report of antigen presenting fish cells available for long-term use, the spontaneous proliferating catfish peripheral blood leucocytes morphologically resembling mammalian monocytes or macrophages (Vallejo *et al*, 1991).

Although the importance of T-like cell lymphocytes to 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*, 1991), its participation in viral diseases remain practically unknown. The availability of ADC lines should greatly facilitate those studies. Due to the high success rate of this technique (3 ADC lines out 3 VHSV survivor trout donors), this approach should be easily repetitive in the trout/VHSV model as well as in any other fish/pathogen systems, so that this type of responses can be further analyzed.

After the first addition of G4 to the whole kidney, many morphological cell types seem to be proliferating in the flasks. These cell types were of about the same heterogeneity than the ones appearing after polyclonal mitogenic stimulation of whole kidney cells, as reported before (Estepa and Coll, 1992). With more additions of G4-pulsed Ad cells, the cells appearing in the ADC cultures were more homogeneous resembling lymphocyte morphology respect to their size, round nuclei and abundant heterochromatin as observed both in the optical and in the electron microscope. However, there was a reduced nucleus/cytoplasm ratio when compared to that of the typical trout kidney or peripheral blood lymphocytes (Alvarez *et al*, 1996). These cells could be intermediate between the more immature lymphocytes and the completely mature lymphocytes but since no antigen-dependent fish cells have been observed before, it could also be the true morphological appearance of a T fish lymphocyte specifically involved in the specific reaction against a viral intruder. More studies are waiting to explore this and/or other possibilities, which are now open by this new model not only for VHS but probably for other fish diseases as well.

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