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NEUTRALISING EPITOPE(S) OF THE GLYCOPROTEIN OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS ARE EXPRESSED IN THE MEMBRANE OF INFECTED TROUT MACROPHAGES

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Introduction

To study the *in vitro* VHSV infection of trout macrophages we selected the anterior kidney because it is the major haematopoietic tissue in the rainbow trout, *Oncorhynchus mykiss* (Coll, 1990). Characterisation of the isolated kidney adherent cells as macrophages and melanomacrophages as described previously (Estepa and Coll, 1992a, 1992b). In the fibrin clot *in vitro* system (Coll, 1990; Estepa and Coll, 1992b), cells or mitogen induced colonies from trout kidney were the target of the viral haemorrhagic septicaemia virus (VHSV) (Estepa and Coll, 1991a; Estepa *et al,* 1991b). It was not clear from those studies whether or not the trout macrophages were also a lytic target for VHSV. Trout macrophages were stimulated by the glycoprotein of the spikes and by the nucleoproteins of the VHSV in cultures of cells from the anterior kidney of healthy trout and of trout immunised by injection with VHSV (Estepa and Coll, 1991c). These results were confirmed by using leucocytes from trout resistant to VHSV one year after infection (Estepa and Coll, 1992a), making clear the importance of the trout macrophages in resistance to VHS. Here we report the *in vitro* conditions under which trout macrophages, presented VHSV neutralising epitopes in their membranes after viral infection.

Material and Methods

Anterior kidney cells from rainbow trout - These cells were prepared as described previously (Coll, 1990; Estepa and Coll, 1992a) from trout of 5-20g body weight. The trout were cooled to 4°C and bled from the tail vein to reduce the blood content of the kidney. The head kidney (pronephros) was removed and cell suspensions obtained as described. To isolate adherent cells (macrophages), the head kidney cells were incubated in $25cm^2$ bottles during 10 days in the medium described below, then medium was changed and adherent cells kept at 14°C until used for the experiments. The cell culture medium (Flow Labs, Ayrshire, Scotland) was RPMI-1640 (Dutch modification) with 2mM L-glutamine, 1mM sodium pyruvate, 1.2µg/ml amphotericin, 50 µg/ml gentamicin, 20 mM Hepes, 50µM mercaptoethanol, 10% pre-tested foetal calf serum and 0.5% pooled rainbow trout serum.

Virus - The strain of virus used was VHSV 07.71 (gift of Dr.P.de Kinkelin, INRA, Jouy en Josas, France) isolated from rainbow trout. The virus was cultured in epithelioma papillosum cyprine (EPC) cells, and added to the kidney macrophage cultures as supernatant from infected EPC cell monolayers after complete cytopathic effect (Basurco and Coll, 1989).

Immunofluorescence by flow cytometry :- Immunofluorescence of VHSV-infected cells was carried out after macrophages were infected at 0.2 VHSV-07.71 $TCID_{50}$ per macrophage (cultures containing $1x\ 10^6$ macrophages/5 ml). Non-infected controls were included in parallel experiments. Incubation was at 14°C for 5 days without 5% CO_2 gassing (Estepa *et al,* 1991b). Macrophages were detached from the surface of the $25cm^2$ bottles by mechanical agitation and re suspended in PBS (0.05M sodium phosphate, 0.15M sodium chloride, pH7.4) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide. Mechanical agitation separated macrophages from other adherent cells of fibroblastic appearance.

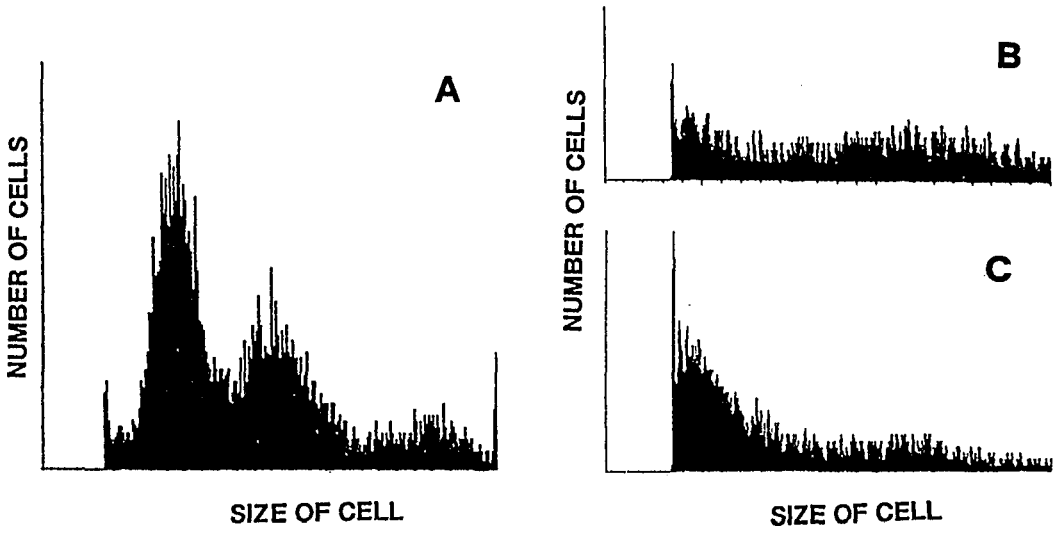

Figure 1.- Effect of VHSV infection on size distribution of macrophages obtained by plastic adherence of trout kidney cells The macrophages were isolated by adherence to the plastic surface of 25 cm^2 bottles (Costar) by incubation of 3 x 10^6 head kidney cells for 1 week at 14°C, then the non-adherent cells were washed with fresh medium, incubated 2 more days, washed again, and infected with about $0.2TCID_{50}$ VHSV per macrophage. (**A**), size distribution of anterior kidney cells from trout before cell culture. (**B**), size distribution of macrophages. (**C**), size distribution of macrophages 5 days after infection with VHSV.

Macrophage suspensions were centrifuged at 300g for 10 min and the macrophage pellet was then gently re suspended in PBS, BSA, azide containing 50-fold diluted mouse ascites with the anti-VHSV antibodies (Sanz and Coll, 1992a,b). After 1 hour at 20°C with occasional agitation, the cell suspensions were centrifuged again, re suspended in 400-fold diluted rabbit anti-mouse IgG-FITC conjugate (Nordic, Tilsburg, The Netherlands) and incubated for 30 min at 20°C. The macrophage suspensions were again centrifuged, washed twice and then re suspended in PBS containing 0.3% paraformaldehyde. On the day of harvest and staining, 5,000 macrophages were examined by flow cytometry in a Becton-Dickinson (San José, California) FACScan apparatus using the program LYSYS II version 1.0. Green fluorescence was measured at FL1 (514-545 nm).

Results

When the cultures of macrophages were infected with the appropriate titer of VHSV 07.71, total cell lysis occurred after 1 week or more in culture (n = 6). Higher multiples of infection resulted in delayed cell lysis or no lysis at all. The macrophage size profile as measured by flow cytometry was as shown in Fig. 1B. After 5 days of VHSV infection the number of larger macrophages decreased with an increase in the number of small macrophages (Fig. 1C). When these

macrophages were stained with neutralising polyclonal or monoclonal (lH10) antibodies, significant differences in fluorescence intensity were found between infected and non-infected macrophages (Fig. 2), whereas parallel controls with no antibodies present only showed background fluorescence. The infected-cell related immunofluorescence was localised in a macrophage population of small size (Fig. 2).

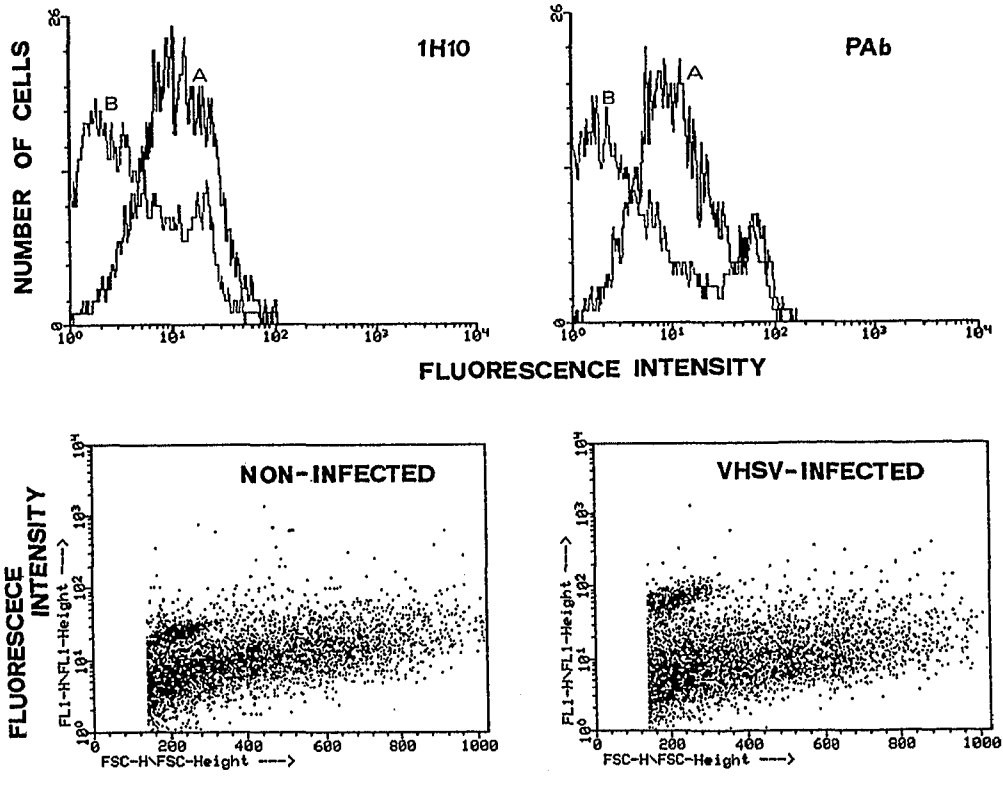

Figure 2. Flow cytometry of macrophages stained with MAb lH10 and with PAb. —A—, VHSV infected; —B—, non-infected. lH10, MAb anti-glycoprotein of VHSV with neutralising-enhancing activity (Sanz and Coll, 1992). PAb, polyclonal antibody obtained from the mouse used to make the anti-VHSV hybridomas.

Discussion

The importance of macrophages (monocytes) as accessory cells in higher vertebrate immune responses is well established, but their function is in fish is not yet completely understood (Estepa *et al*, 1991c; Estepa and Coll, 1992a). Because of the possibility of obtaining trout macrophage populations with a high degree of purification by plastic adherence, these could easily be infected *in vitro* with VHSV and their VHSV epitope expression studied by flow cytometry. Although the average size of the macrophage population decreased after infection, before complete lysis occurred, positive membrane fluorescence could be

demonstrated in that population. This showed that the epitopes defined by polyclonal antibodies containing neutralising activity and even more by MAb lH10 with neutralising-enhancing activity (Sanz and Coll, 1992a) are exposed in the membrane of the infected macrophages.

The findings reported here open the possibility of further studies into the recognition of infected macrophages by cells of the immune system of the trout. Most probably, some of the cells from VHSV resistant trout are active killers of any infected trout macrophage because, due to the fast development of the disease there is almost no time to elaborate antibodies which would also need the macrophages to be elaborated. Further experiments are in progress to investigate these and other possibilities.

Summary

Macrophages isolated from rainbow trout kidney and infected with viral haemorrhagic septicaemia virus (VHSV) showed positive membrane immunofluorescence with neutralising polyclonal and monoclonal antibody (MAb) anti G lH10. These findings open the possibility of using trout macrophages as presenting cells to study the epitopes relevant to the protection against VHSV.

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