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Susceptibility of Trout Kidney Macrophages to Viral Hemorrhagic Septicemia Virus

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ABSTRACT

Viral hemorrhagic septicemia virus (VHSV) lysed the macrophages from rainbow trout kidney cultures either isolated by plastic adherence or stimulated with purified glycoprotein G from VHSV. The trout macrophages supported the replication of VHSV as tested by cell culture and by sandwich ELISA of the supernatants from infected cultures. VHSV-infected macrophages showed a decrease in both acridine-orange fluorescence and average size. Immunofluorescence studies with flow cytometry showed positive membrane staining with monoclonal antibodies (MAbs) anti-N and anti-G VHSV. These findings open the possibility of using trout macrophages as presenting cells to study the possible existence of helper or cytotoxic epitopes relevant to the protection of trout against VHSV.

Adherent cells were specifically stimulated by the glycoprotein G of the spikes and by the nucleoproteins N/Nx (4) of the viral hemorrhagic septicemia virus (VHSV) in kidney cell cultures from control noninfected (immunologically naive with respect to VHSV) or VHSV-immunized trout (16). These results were confirmed by using trout resistant to VHSV 1 year after infection (17), suggesting an important role for the adherent cells in the process of resistance to the VHS disease. The morphology and properties of the cells from trout kidney that responded to the N and G VHSV proteins were the same as the adherent cells isolated from noninfected trout kidney described previously (16). These adherent cells showed granules in their cytoplasm, red blood cell phagocytosis, acridine-orange staining, peroxidase staining, and nonspecific esterase staining. In addition, these cells (trout kidney adherent acridine-orange staining cells) were tested against a panel of 20 monoclonal antibodies (MAbs) and it was found that only two MAbs against trout serum immunoglobulin stimulated the cells as might be expected if one were working with mammalian macrophages (17). On the other hand, they did not proliferate in response to PHA, Con A, or LPS (18). According to the morphology of the subpopulations of trout kidney cells defined by Plytycz et al. (19), Braun-Nesje et al. (6), or Bayne (5), the adherent cells can be identified as macrophages and melanomacrophages (Estepa et al., in press). The importance of macrophages as accessory cells in higher vertebrate immune responses is well established, but it is not yet completely known what their function is in fish.

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Increased susceptibility after irradiation (7) and partial destruction of melanomacrophage centers in the anterior kidney (1) have been related to the VHSV presence in the lymphoid organs of VHSV-infected rainbow trout, *Oncorhynchus mykiss*, W. To further investigate VHSV susceptibility of trout lymphoid cells, an *in vitro* fibrin-clot culture system was developed in which mammalian lymphocyte mitogens induced the proliferation (as measured by colony formation) of different morphological types of colonies (10,18), composed of large-nucleated cells, eccentric-nucleated cells, multinucleated cells, and small lymphocytes. In cultures of whole kidney cells all these cells or colonies were susceptible to VHSV infection (14,15). However, it is unknown whether the macrophages are or not susceptible, because of their very low counts in all the previously reported studies (14,15).

By using cultures highly enriched in macrophages, either by isolation or by stimulation with the protein G of VHSV, we report here that trout kidney macrophages supported the replication of VHSV and presented N and G VHSV epitopes in their plasma membranes after VHSV infection. It is now clear that trout macrophages are susceptible to VHSV infection.

MATERIALS AND METHODS

Isolation of macrophages from the kidney of rainbow trout (*Oncorynchus mykiss*, W.). Fingerling trout (*Onchorynchus mykiss*, W.) were purchased from a commercial farm (Uña, Cuenca, Spain) after several annual tests indicated the farm was free of infectious pancreatic necrosis. VHSV-resistant trout were obtained by two VHSV infections as described before (3,16,17) and used for the experiments 8–12 months after first VHSV infection. Noninfected trout were kept as controls during the same time.

Macrophages were prepared from trout whole head kidney cells obtained as described in detail previously (10,18). Briefly, the head kidney (pronephros) was removed from trout previously cooled down to 4°C, killed by cutting off the head and from which the blood was allowed to drain for about 5 min, cut into pieces, dissociated, decanted, and washed in cell culture medium. The cell concentration of the head kidney round cells was determined with a hemocytometer. To isolate their macrophage population, 3×10^6 round cells from the whole head kidney were incubated in 25-cm^2 plastic flasks in 5 ml of the medium described below. After 10 days, the medium was changed (to eliminate the nonadherent cell population) and the macrophages were kept at 14°C until used for the experiments. The yield was about 1×10^6 macrophages/flask.

Cell culture of whole kidney trout cells in fibrin clots. The cell culture medium (Flow Lab, Ayrshire, Scotland) was RPMI-1640 (Dutch modification) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1.2 μg/ml anfotericin, 50 μg/ml gentamicin, 20 mM HEPES, 50 μM mercaptoethanol, 10% pretested fetal calf serum, and 0.5% pooled rainbow trout serum. Fibrinogen (A.B. Kabi, Stockholm, Sweden) was added to the medium just before use to a final concentration of 2 mg/ml (10,18).

Whole head kidney cell suspensions were prepared as described above and adjusted to 160,000 round cells per ml. Thrombin (Miles Lab, Elkart, IN) was added at 0.2–0.4 NIH U/well in 2 μl volume, and 100 μl of the cell suspension was pipetted into each well of a 96-well plate (Costar, The Netherlands). After clotting occurred (in about 30 sec), purified G protein from VHSV, phytohemaglutinin (PHA) obtained from Flow, and/or VHSV suspended in medium were pipetted into each well in a maximum volume of 10 μl on the top of the clot. The plates were then placed in a plastic bag (Vaessen, Schoemaker Indtal, S.A., Sant Boi de Llobregat, Barcelona, España), sealed, and incubated at 14°C. After the incubation, the fibrin clots were removed from the wells onto a 75 × 25-mm frosted-end glass slide with a spatula, and stained with toluidine blue as described (21). Four slides were made for each experiment, one field was counted with an optical microscope at 400× for each slide (about 30 cells per field) and averages were calculated. A complete description of the technique has been published elsewhere (10).

Viruses. The strain of virus used was the VHSV 07.71, a gift of Dr. 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 cell monolayers after a complete cytopathic effect was noted. VHSV was generally used at a multiplicity of infection (moi) of about 0.2 $TCID_{50}$ of VHSV per macrophage.

TABLE 1. CHARACTERISTICS OF THE ABS ANTI-VHSV AND ANTI-IG USED[a]

Antigen	*Clone*	*Isotype*	*ELISA*	*IF*	*Blot*	*Neutralization*	*Reference*
VHSV	PAb	—	+	+	+	+	4
VSHV	1H10	IgG_1 K	+	+	G	+	25
VHSV	2C9	IgG_{2a} K	+	+	N + Nx	−	4
Trout Ig	1G7	IgG_1 K	+	−	H	−	22
Trout Ig	1H2	IgG_1 K	+	−	L	−	24

[a]The antigens used to immunize the mice to obtain the MAbs were 80% purified VHSV by concentration with polyethylenglicol (2) and 95% purified trout serum immunoglobulin (trout Ig) by DEAE and gel permeation chromatography (24). IF, immunofluorescence over EPC infected cells. Specificity was determined by immunoblotting. Only MAb 1H10 was neutralizing VHSV. G, Glycoprotein from VHSV (4); N + Nx, nucleoproteins from VHSV; H, heavy chains from trout Ig; L, light chains from trout Ig. MAbs 1G7 and 1H2 react with 84 and 15% of the total trout Ig of serum. 1H2 react with an L chain of 24 kDa (24). PAb, polyclonal antibody obtained from the mouse ascites used to obtain the anti-VHSV hybridomas (4).

To test for VHSV after the macrophage infection, a lower moi of 0.0001 was used to infect to minimize the interference with the tests for VHSV, after incubation for 10 days the supernatants from the cultures were titrated in EPC cell monolayers (or by ELISA as described below) for the presence of virus by the $TCID_{50}$ method using 5-fold series of dilutions (2). Parallel cultures to which VHSV was added immediately before processing were used as controls (15).

Enzyme-linked immunosorbent assay (ELISA). Supernatant from virus-infected macrophage cultures was examined for the presence of VHSV by the two MAb-based ELISA sandwich as previously described (25). The supernatants of the infected cultures were titrated by 2-fold dilutions in duplicates.

Acridine-orange stain. The acridine-orange at a final concentration of 100 μg/ml of culture medium was added to the cultures to stain macrophages for 1 min, followed by a brief rinse in medium. The stained cells remained adherent to the plastic. Acridine-orange is specific for lysosomes of the monocyte/macrophage cell lines (5). Lysosomes appeared orange and nuclei green under the fluorescence microscope. Flow cytometry was performed as described below.

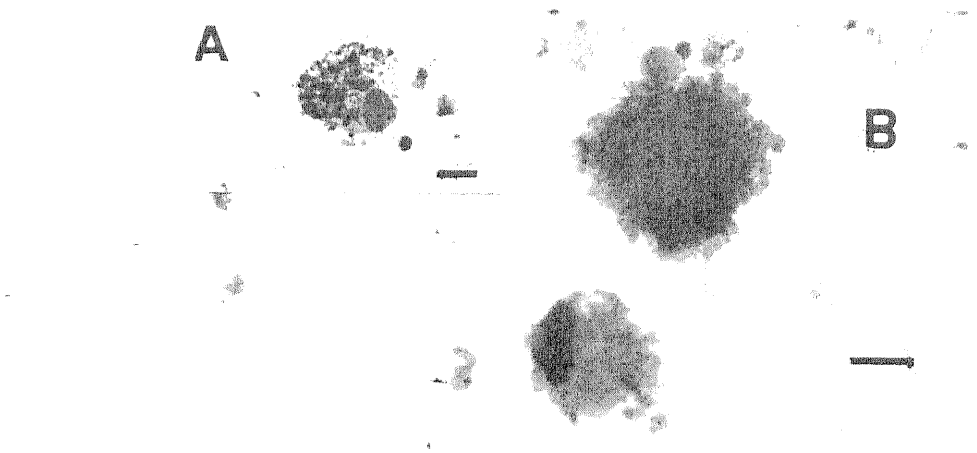

FIG. 1. Morphology of macrophages in fixed fibrin clots. The same morphology was obtained both if cells were isolated from head kidney cells by adherence to plastic and then cultured in fibrin clots or if cells were cultured in the presence of protein G from VHSV. A, melanomacrophages. B, macrophages. Bars = 10 μm.

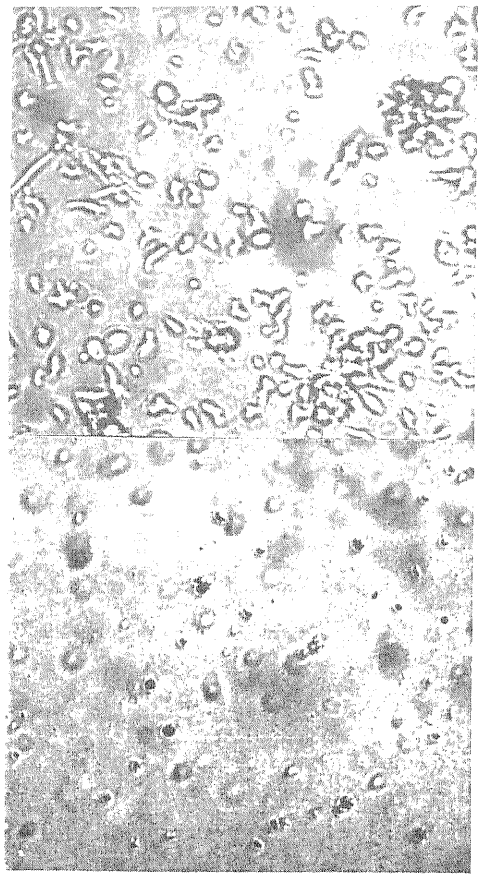

FIG. 2. Effect of VHSV infection of macrophages obtained by plastic adherence of head kidney cells from non-infected trout. The macrophages were isolated by adherence to the plastic surface of 25-cm^2 flasks by incubation of 3×10^6 head kidney cells during 10 days at 14°C, then macrophages were washed with fresh medium, incubated 2 more days, washed again, and infected with 0.2 $TCID_{50}$ of VHSV per macrophage. Up, macrophages before the infection photographed *in vivo* by phase contrast. Down, the same cultures 10 days after VHSV infection.

Monoclonal antibodies (MAbs). To obtain MAbs to trout serum immunoglobulins, mice were immunized with 95% purified trout serum immunoglobulins (24). To obtain MAbs to VHSV proteins, mice were immunized with concentrated virus of 80% purity (2).

Purification and ELISA assays of trout immunoglobulins (22,23) and of VHSV were performed as we described previously (25). Fusion, cloning, and ascites (pooled from at least three mice) were obtained as described (21). Presence of MAbs in ascites was confirmed by ELISA titration (half maximal OD about 1/500 for most MAbs) and by the presence of a peak in the Ig region of cellulose acetate electrophoresis of the ascites. The amount of MAb in the ascites was adjusted to 20–60 mg/ml. The characteristics of the MAbs used are given in Table 1. MAb 2C9 was purified by chromatography over protein A-Sepharose as described (25).

Purification of VHSV protein G by gel electrophoresis and electroelution. The virus was grown in EPC cells, assayed, concentrated by polyethyleneglycol (PEG), and purified by ultracentrifugation on sucrose gradients as described before (2). The purified virus was analyzed by electrophoresis and the protein content adjusted to 1 mg/ml by both absorbance at 280 nm ($\epsilon = 1.4$) and Coomassie blue staining of the gel. VHSV protein G was isolated by preparative gel electrophoresis of purified VHSV in the presence of SDS and 2-mercaptoethanol as described (4,16).

Flow cytometry. Macrophages were obtained after 10 days incubation of head kidney trout cells at 20°C and kept at 14°C for 1 month (Fig. 5). Cultures containing 1×10^6 macrophages per 5 ml per flask were

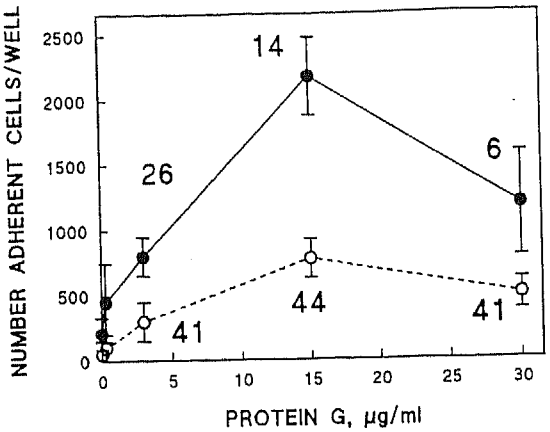

FIG. 3. Effect of VHSV infection of macrophages obtained by stimulation of head kidney cells from VHSV-resistant trout with protein G from VHSV. Head kidney cells (16,000 cells/well containing 600 macrophages/well at time 0) were cultured at 20°C during 11 days in the presence of several concentrations of protein G. Then 0.2 $TCID_{50}$ of VHSV per cell were added to the cultures, incubated at 14°C during 14 days without 5% CO_2 gassing, and harvested. (●) Counts of macrophages in noninfected cultures; (○) counts of macrophages in VHSV infected cultures. The numbers inside the figure are the percentage of macrophages that were melanomacrophages (n = 100).

infected at 0.2 VHSV–07.71 $TCID_{50}$ per macrophage. Noninfected controls were included in every experiment. Incubation was at 14°C for 5 days. Macrophages were detached from the surface of the 25-cm^2 bottles by mechanical agitation in order to separate them from adherent fibroblastic cells and resuspended in PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide.

Macrophage suspensions were centrifuged at 300 g for 10 min and the pellet was gently resuspended in PBS + BSA + azide containing 50-fold diluted mouse ascites with anti-VHSV antibodies. After 1 h at 20°C with occasional agitation, the macrophage suspensions were centrifuged again, resuspended in 400-fold diluted rabbit antimouse IgG-FITC conjugate (Nordic, Tilburg, The Netherlands), and incubated 30 min at 20°C. The macrophage suspensions were again centrifuged and washed twice and then resuspended in PBS + 0.3% paraformaldehyde (13,20).

The same day of the harvest and staining, 5000 cells were analysed by flow cytometry in a Beckton-Dickinson (San José, CA) FACScan apparatus using the program LYSYS II version 1.0. Fluorescence was measured at FL1 (514–545 nm, green) or FL2 (564–606 nm, orange).

TABLE 2. REPLICATION OF VHSV IN MACROPHAGES ISOLATED FROM NONINFECTED TROUT KIDNEY[a]

Method	*Time of Virus Addition*	*VHSV*
EPC monolayers	After incubation	≤20
	Before incubation	2500
ELISA	After incubation	0.12
	Before incubation	0.23

[a]Macrophages were incubated for 10 days at 14°C. Viruses were added before the incubation or after the incubation (0.0001 $TCID_{50}$ per macrophage). Results are expressed as $TCID_{50}$/ml after titration on EPC monolayers or as the absorbance at 492–620 nm after sandwich ELISA.

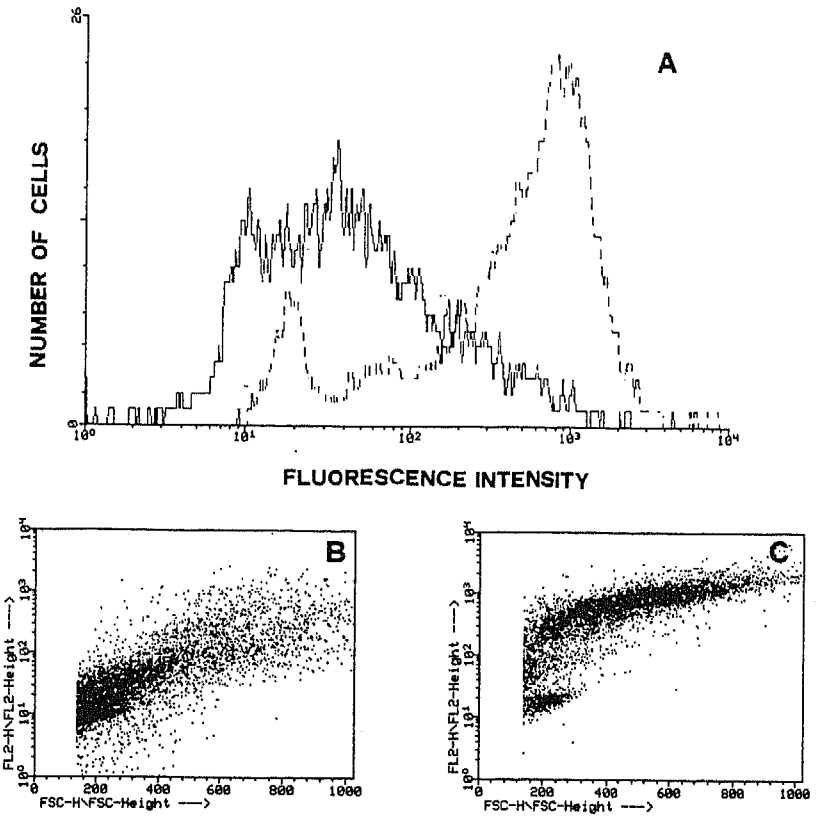

FIG. 4. Fluorescence at 564–606 nm measured by flow cytometry of macrophages stained with acridine-orange. (A) (——) VHSV-infected macrophages; (– – –), noninfected macrophages. (B) Fluorescence (FL2-FL2-Height) versus size (FSCH/FSC-Height) of VHSV infected macrophages. (C) Fluorescence versus size of noninfected macrophages.

RESULTS

When macrophages (Fig. 1) isolated from noninfected trout were infected with VHSV (0.2 $TCID_{50}$ per macrophage) they exhibited a total cell lysis after 7–10 days in culture (Fig. 2). These results were confirmed by using macrophages isolated from six different trout. The extent of this lysis could be controlled by the experimental conditions of the infection. For example, when the cultures were infected and maintained in an atmosphere of 5% CO_2 in air during infection, there was a decrease in the count of cells, but by using a higher pH (discharging the CO_2 after 4–5 days of infection or not gassing) no surviving macrophages could be found in the cultures 7–10 days after infection. Similar cultures to which VHSV heated at 100°C for 10 min was added, did not show any effect. If a $\geq$ 4-fold higher multiplicity of infection (moi = 0.8 $TCID_{50}$ per macrophage) was used, the *in vitro* infection was not apparent or was delayed several weeks (not shown).

To expose macrophages to VHSV in the conditions less favorable for VHSV infection, purified protein G was added to cultures of whole kidney cells from VHSV-resistant trout to stimulate macrophages, prior to the *in vitro* challenge with VHSV at the same moi than above. The addition of 15 μg/ml of glycoprotein G isolated from purified VHSV increased not only the number of macrophages about 10-fold with respect to the controls, but also their average diameter from 30 ± 12 to 54 ± 20 μm (n = 30). Increasing the concentration to 30 μg/ml did not increase the number of stimulated macrophages. Most of the stimulated cells were of the adherent macrophage type. Infection with VHSV produced also the same macrophage lysis as observed with the unstimulated macrophages; however, because of the possibility to quantify the fibrin clots, the lysis could be measured as cell count (Fig. 3). Infection with VHSV, therefore, decreased the number of macrophages, and the ones that could be detected showed a lighter staining than the uninfected controls, indicating that most probably they were either dead or dying.

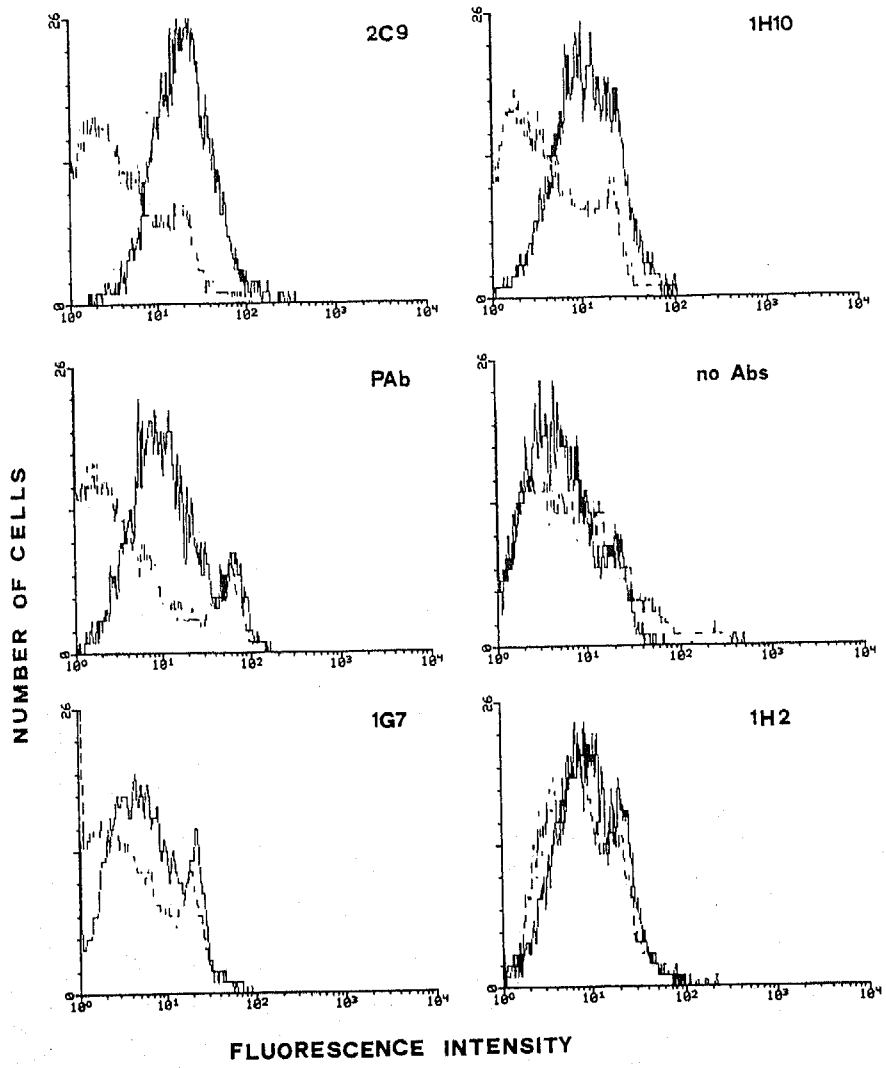

FIG. 5. Fluorescence at 515–545 nm measured by flow cytometry of VHSV-infected macrophages stained with MAbs anti-VHSV (2C9, 1H10, PAb) and anti-trout Ig (1G7, 1H2). Fluorescence intensity (*X* axis) versus number of cells (*Y* axis); (——) VHSV-infected macrophages; (– – –) noninfected macrophages. Abs as described in Table 1.

To investigate the possible replication of VHSV in the trout macrophages, the viral titer was determined 7 days postinfection. The titer of VHSV increased ≥ 100-fold or 2-fold by $TCID_{50}$ or by ELISA, respectively (Table 2).

The average macrophage size obtained from a noninfected trout as measured by flow cytometry was greater than the average size of the whole kidney cells from which the macrophages were isolated (not shown). The size/acridine-orange fluorescence profile of the isolated macrophages showed at least two populations of small and larger macrophages, respectively (Fig. 4C) differing also in fluorescent intensity. After VHSV infection, the larger cells decreased both in number and acridine-orange fluorescence intensity (Fig. 4B). Independently of the size, the high fluorescence intensity profile of the whole macrophage population stained with acridine-orange decayed after the VHSV infection (Fig. 4A).

VHSV infected macrophages showed positive fluorescence by flow cytometry before complete cell lysis (5 days after infection) as compared with non-infected macrophages when stained with purified MAb anti-N VHSV (2C9), MAb anti-G VHSV (1H10), and/or mouse polyclonal anti-VHSV (Fig. 5). No significative

TABLE 3. CHARACTERISTICS DESCRIBED TO DATE FOR THE MACROPHAGES ISOLATED FROM TROUT KIDNEY CELLS[a]

Characteristics	*Result*	*Reference*
Adherence to plastic surface	+	5
Granules in the cytoplasm	+	10
Trout red blood cell phagocytosis	+	6, 11, 15
Acridine-orange staining	+	5, 10
Stimulation by VHSV nucleoprotein N	+	15, 17
Stimulation by VHSV glycoprotein G	+	15, 17
Spreading after stimulation	+	8, 17
Peroxidase staining	+	18
Superoxide production	+	19
Nonspecific esterase staining	+	18
Sudan black B staining	−	18
Nitroblue tetrazolium staining	−	18
Proliferation by PHA or Con A	−	10, 18
Stimulation by PHA or Con A	+	10, 18
Stimulation by LPS	+	10, 18
Required for thymidine incorporation	+	9
Fluorescence with anti-Ig	±	17
Stimulation by MAbs anti-Igs	±	15
Susceptibility to VHSV *in vitro*	+	This work
Membrane fluorescence with anti-N	+	This work
Membrane fluorescence with anti-G	+	This work

[a]Stimulation, increase in the number of scattered cells respect to controls. Proliferation, as measured by the presence of colonies.

differences between infected and noninfected macrophages were obtained if the staining step with anti-VHSV antibodies was omitted. A small difference between VHSV infected and noninfected macrophages was found when staining with MAb anti-trout Ig 1G7 but none with MAb 1H2 (Fig. 5).

DISCUSSION

Because of the possibility of obtaining macrophage populations with a high degree of purification, either by plastic adherence or by culture with purified protein G from VHSV (Table 3), the question about their *in vitro* susceptibility to VHSV could be easily studied. In both cases macrophages supported VHSV replication and as a result were lysed after infection. By using fibrin clots we could easily quantify the macrophage lysis (Fig. 3) but it was the same lysis phenomenon in both unstimulated and stimulated macrophages (Fig. 2).

The complete lysis of macrophages when infected at an moi of 0.2 but no (or greatly delayed lysis) at an moi of 0.8 is probably due to the presence of interference particles that arise at high moi in this rhabdovirus strain (12). The same behavior was obtained when EPC cell lines were infected with the same inoculum of VHSV 07.71 used in these studies (not shown).

Both average cell size and the intensity of acridine-orange fluorescence decreased in the isolated macrophages shortly after VHSV infection. Both types of results were as expected. Cell lysis would cause cellular fragments and since the flow cytometry will detect those fragments resulting from the lysis of the larger cells, the overall result will decrease the average size of the population before a complete cell lysis. On the other hand, the macrophage lysis will provoke the release of the lysosomes from the macrophage cytoplasm to the medium where they will be removed by the centrifugation steps thus causing a decrease in the fluorescence intensity. Acridine-orange stain could thus be used as a marker of trout macrophage lysis to be used in further studies.

Since the macrophages were a VHSV target, the possibility of using trout kidney adherent cell cultures for infection with VHSV to investigate VHSV antigen presentation was further studied. Before their complete lysis, the surviving cell population showed membrane fluorescence with MAb anti-G VHSV (neutralizing MAb) and with MAb anti-N VHSV, showing that the epitopes defined with these MAbs are exposed in the membrane of these infected cells. A small subpopulation of the macrophages seems to contain surface Ig confirming previous studies [17].

The findings reported here open the possibility of using trout macrophages as presenting cells to study helper and cytotoxic epitopes relevant to the protection of trout against VHSV. Since specific cytotoxicity has been recently demonstrated in fish (26) and trout macrophages seem to recognize specific antibody-coated pathogens (27) there will be an interesting point to study some of these possibilities in the VHSV/trout model.

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