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► **To cite this version:**

S Chilmonczyk, I Voccia, D Monge. Pathogenesis of viral haemorrhagic septicaemia virus: cellular aspects. *Veterinary Research*, 1995, 26 (5-6), pp.505-511. hal-00902382

HAL Id: hal-00902382

<https://hal.science/hal-00902382>

Submitted on 11 May 2020

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Pathogenesis of viral haemorrhagic septicaemia virus: cellular aspects

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Summary — Leucocyte populations from rainbow trout subjected to experimental viral haemorrhagic septicaemia virus (VHSV) infections under *in vivo* and *in vitro* conditions were analysed by flow cytometry. Quantitative analysis shows that only a low percentage of the leucocytes support viral replication. Lymphocytes, compared with monocytes granulocytes and macrophages, are the least susceptible sub-population. A decrease in the amount of the phagocytic activity was observed after the cells were infected with VHSV. Obvious modifications were observed through dot plot profiles in the composition of the cell sub-populations and in the cell morphology. In addition to a direct effect of VHSV on the macrophages, a systemic effect is produced. This could also be related to the stress induced by the experimental infection process.

rainbow trout / rhabdovirus / leucocyte / phagocytosis

Résumé — **Pathogénie de la septicémie hémorragique virale : aspects cellulaires.** Les populations leucocytaires de truites arc-en-ciel infectées par le virus de la septicémie hémorragique virale (SHV) selon des conditions expérimentales *in vivo* et *in vitro* ont été analysées principalement en cytométrie de flux. Une étude quantitative a montré que le virus de la SHV ne se répliquait que dans une faible proportion des leucocytes. Les lymphocytes ont été les cellules les moins sensibles à l'infection virale. Suite à l'infection on a constaté une réduction de l'activité phagocytaire. La composition des sous-populations leucocytaires a également été changée (modification des profils cellulaires "dot plot"). En plus d'un effet direct du virus de la SHV sur les macrophages, une modulation systémique, qui pourrait être liée au stress induit par les conditions expérimentales de l'infection, est possible.

truite arc-en-ciel / rhabdovirus / leucocyte / phagocytose

INTRODUCTION

Haematopoietic tissues are particularly susceptible to fish rhabdoviruses. Cytologic studies have demonstrated that virus replication generally occurs in the non-lymphoid cells,

but the leucocyte population could also be involved in rhabdovirus pathogenic processes. To date the mechanisms involved in rhabdoviruses penetration into fish are not understood. Leucocytes may serve as the target cells for the initial phase of infec-

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tious haematopoietic necrosis virus (IHNV) infection (Chilmonczyk and Winton, 1994). Trout macrophages support the replication of viral haemorrhagic septicaemia virus (VHSV) (Estepa *et al*, 1992). Many methods for virus detection and the diagnosis of viral diseases have been developed and were recently reviewed (Sanz and Coll, 1992). So far, the detection of either low levels of virus in fish organ samples or virus-infected cells soon after an experimental infection requires time-consuming virus amplification steps such as co-cultivation assays. This work reports the preliminary results of experiments performed to develop efficient flow cytometric assays which permitted the rapid evaluation of virus-infected samples.

MATERIALS AND METHODS

Fish

The experimental infections and leucocyte collection were performed using VHSV-free rainbow trout (RBT) with a mean weight of 20 g for *in vivo* infections and 80 g for *in vitro* infections.

Cell line and virus

Epithelioma papulosum cyprini (EPC) cells were grown at 14°C in minimum essential medium (MEM) supplemented with 10% fetal calf serum and 12 mM Tris buffer. The INRA VHSV isolate 07-71 was used in the experimental infections.

Co-cultivation

Leucocytes collected from RBT following the experimental infections were plated onto monolayers of EPC cells. Co-cultivation assays were performed according to a 2-step procedure as previously described (Chilmonczyk and Winton, 1994) and the virus titer in the co-culture supernatants was determined by plaque assays.

Plaque assays

The VHSV was quantified by plaque assay using EPC cells incubated at 14°C for 3 d.

Leucocyte populations

The cells were collected from the blood, pronephros and thymus and were isolated after centrifugation through a Ficoll cushion as previously described (Chilmonczyk 1978).

Experimental infections

In vivo infections

Water-borne (WB) or intraperitoneal (IP) procedures were used to infect the fish. The WB infections were induced by exposing the fish to 1×10^5 plaque-forming units (pfu) / ml water. The RBT were kept for 1 h in aerated static water and then held in pathogen-free water until sampling. The fish were sacrificed at 6, 12, 24 and 72 h post-exposure. The IP infections were performed by injecting 1 to 100 pfu / fish.

In vitro infections

Cells were inoculated at a multiplicity of infection (MOI) ranging from 1 to 5 and incubated for 1 h at 14°C. The cells were then washed with MEM and were resuspended with sufficient anti-VHSV monoclonal antibody (MAb) A17 to neutralize the unabsorbed virus. After 2 h at room temperature, the cells were washed and suspended in MEM-10 to await the assays.

Flow cytometry

A standard flow cytometer instrument (FACScan-Becton Dickinson) was used in the study of cell samples with an excitation at 488 nm and detection filter for FITC. The data was analysed with the program LYSIS II version 2.0.

Flow cytometric measurement of phagocytosis

The leucocyte suspensions were incubated for 18 h at 14°C with fluoresceinated (FITC) latex beads (diameter = 1.7 µm). Non-ingested beads were separated from the phagocytes by centrifuging (100 *g* x 10 min) over a cushion of 5% BSA in PBS. The cell pellet was then resuspended in PBS for flow cytometric measurement. The fluorescence setting (FL1) was established using a suspension of FITC-latex beads in PBS. Only the cells displaying a fluorescence intensity corresponding to the cumulative fluorescence of at least 3 FITC-latex beads were considered positive for phagocytic activity. The fluorescence histograms of cell number *versus* fluorescence intensity were analysed. For each sample, 10 000 events were recorded and the phagocytosis was expressed as the percentage of cells that had ingested at least 3 FITC-latex beads.

RESULTS

Co-cultivation assays

The leucocyte susceptibility to VHSV was demonstrated in this work. After experimental WB infections, the percentage of fish from which VHSV could be detected increased steadily with the post-infection time. The leucocyte populations from the blood, pronephros and thymus were analysed. At 6, 12 and 18 h post WB infection 17% (5 out of 29), 32% (8 out of 25) and 72% (13 out of 18) of the fish, respectively, harboured VHSV. A very low number of infectious virus particles (5–20 pfu) was detected in the VHSV-positive samples at 6 and 12 h post infection.

Flow cytometry analysis

Detection of VHSV antigens

The cells expressing VHSV-N or -G proteins were determined by flow cytometry.

At 6 and 12 h post-infection, only a low number of VHSV-positive cells (2–10 out of 10 000) were detected. In addition to flow cytometric analysis, the cells collected from each organ were processed for virus titration by plaque assay. Table I indicates a good correlation between co-cultivation assays and flow cytometric analysis. The samples in which the VHSV proteins were detected 6 h post WB infection were also found to be positive for infectious VHSV by plaque assay.

Flow cytometry allows the visualization of the leucocyte heterogeneity based on the cell size (forward scatter parameter = FSC) and cell complexity (side scatter parameter = SSC). A gate corresponding to the lymphocyte population was made and analysed. VHSV-G or -N proteins were found in only 1 to 3 percent of the lymphocytes.

Phagocytic activity

Phagocytosis assays were carried out *in vitro* directly on the leucocytes collected from the RBT experimentally infected with

Table I. Detection of VHSV-infected fish. Number of VHSV-positive samples out of the total number of assayed fish.

	Flow cytometry	Plaque assay
Kidney	3/12	4/12
Spleen	1/8	2/8
Blood	1/9	1/9
Thymus	2/12	3/12

Data from flow cytometry and plaque assay techniques were compared at 6 h post VHSV waterborne infection. Cells collected from the kidney, spleen, blood and thymus were either labelled with monoclonal antibody F5 (flow cytometry) or co-cultivated for 8 d with EPC cell monolayers prior to virus titration (plaque assay).

VHSV or on leucocytes collected from the control fish and subsequently infected under *in vitro* conditions (5 pfu/cell).

In vivo infections

The phagocytic activity was compared between VHSV-infected leucocyte populations from the blood, pronephros and thymus and the control samples from normal fish. Due to the large interfish variability, the average number of phagocytes was determined for each organ from the control fish data. As early as 6 h post-infection, a noticeable decrease in the number of phagocytic cells was observed in the pronephros while no obvious change occurred in the blood or thymus. A gradual and consistent decrease in the number of phagocytes was observed from 6 to 72 h post WB or IP infections.

In vitro infections

The number of phagocytes increased significantly from the initial number when the leucocyte populations were maintained under *in vitro* conditions. Depending on the fish, after 2 d of leucocyte culture, the phagocyte population can be 3 to 10 times higher than the initial number (on day 0). After an *in vitro* infection with VHSV, the phagocytic function was observed to be inhibited (fig 1). It took at least 2 d post-infection to obtain a noticeable decrease in the number of phagocytes. As the number of phagocytes was greater under the culture conditions, the reduction was more especially obvious in the *in vitro* experiment.

Cellular dot plot profiles

The profile pattern of cell size and cell complexity according to FSC and SSC parameters (dot plot representation) was compared between the control and VHSV infected fish. The flow cytometric profiles of unlabelled (autofluorescence) cells are rep-

resentative of the organ from which the leucocytes originated. Each organ displayed a characteristic dot plot pattern for their respective leucocyte population. We observed that 25 out of the 32 RBT displayed a similar organ-specific pattern for the cell dot plots. VHSV infection affected the FSC/SSC pattern of the leucocyte populations in the blood and lymphoid organs. A large proportion of the leucocytes progressively displayed a higher cell complexity and reduced cell size (fig 2) as time passed post-infection. The lymphocytes appeared to be less affected than the monocyte and granulocyte populations. As early as 6 h post WB or IP infection, a slight modification had already occurred in the course of the disease. Extensive modifications of the dot plot profiles were sometimes observed. Such modifications occurred more frequently following IP injections. A modified dot plot pattern was observed in a majority of the IP infected RBT (10 out of 12) while only 9 out of 20 WB infected fish displayed a similarly modified pattern.

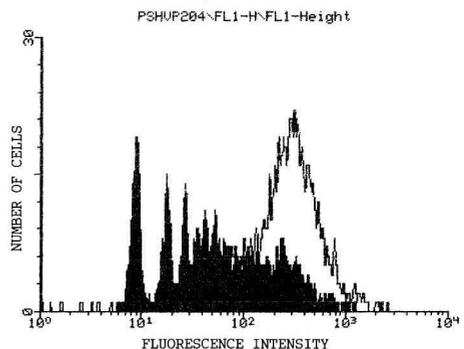


Fig 1. Phagocytosis of FITC conjugated latex beads. Flow cytometric analysis of the phagocytic activity of pronephros phagocytes collected from control rainbow trout. The fluorescence intensity is directly correlated to the phagocytic activity. Black curve: day 4 VHSV-infected cells *in vitro*. Open curve: non-infected cells.

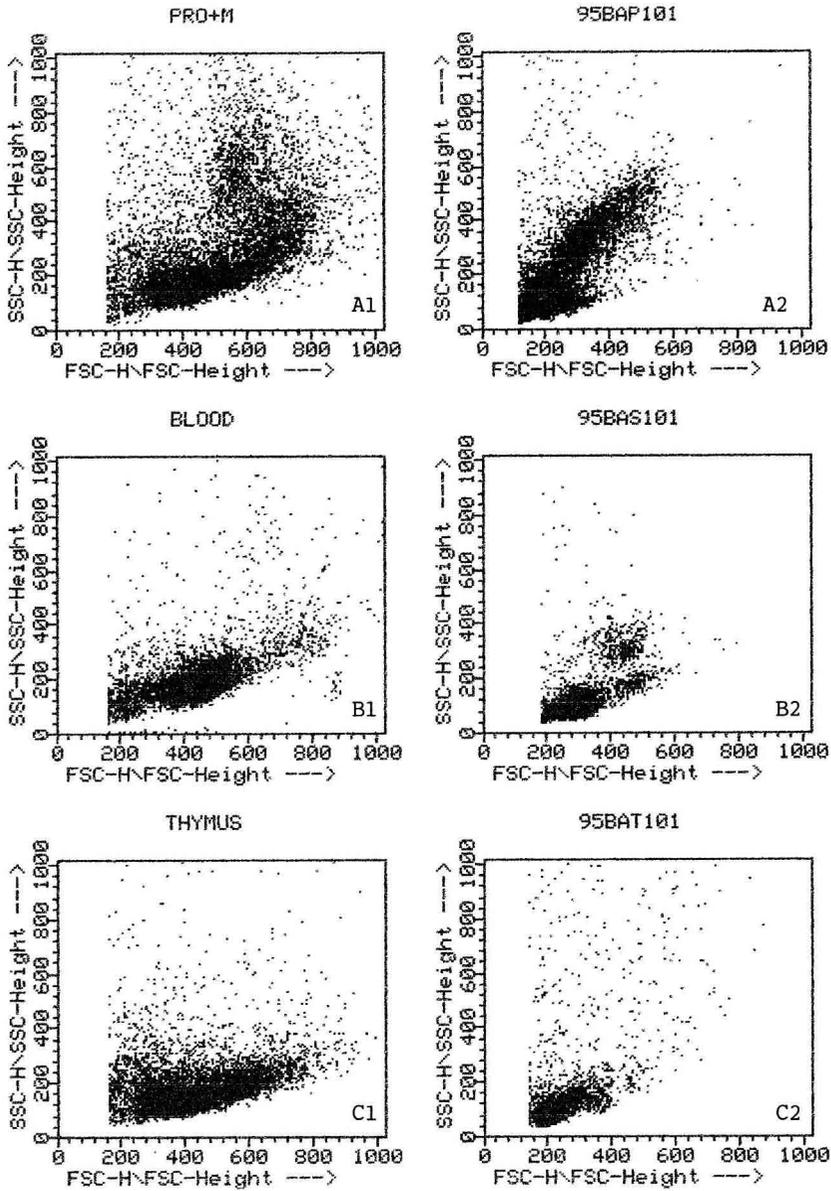


Fig 2. Cytograms differentiating unlabelled leucocyte populations (cell autofluorescence). Dot plot profiles are compared between cells collected from control fish (1) and cells collected from VHSV water-borne infected fish 12 h post-exposure (2). A = pronephros; B = blood; C = thymus; SSC = side scatter parameters (cell complexity); FSC = forward scatter parameters (cell size).

DISCUSSION

The results obtained in this study showed that VHSV replicated in leucocyte populations derived from the kidney, spleen, thymus and blood of RBT. Among the various cell types which composed the leucocyte population it appeared that lymphocytes are the least susceptible leucocyte population to VHSV.

The mechanisms involved in the establishment of rhabdovirus infection in fish are not well understood. The data obtained for VHSV corroborated with those previously obtained with IHN (Chilmonczyk and Winton, 1994) and confirmed that leucocytes represent one of the cell types involved in both rhabdovirus infections. The detection of virus particules associated with leucocytes as early as 6 h post experimental infection, suggested that leucocytes are among the initial targets for fish rhabdoviruses. Comparative analysis of the initial phases of VHSV infection showed a good correlation between the flow cytometry and plaque assay techniques. Samples in which the VHSV antigens were detected by flow cytometry were also demonstrated positive for infectious VHSV (table I). Our preliminary experiments indicate that flow cytometry is a fast and reliable technique for studying the ability of VHSV to infect and replicate in RBT leucocytes. Flow cytometric analysis provides some advantages over the techniques of co-cultivation, plaque assay or the cell count method for cells expressing VHSV specific fluorescence. It is sensitive, and not time consuming. It provides quantitative data and discriminates between the virus-sensitive cell subpopulations.

It has been previously shown that VHSV replicates within the macrophages (Estepa *et al*, 1992). In this work we clearly demonstrated a suppressive effect of VHSV on the phagocyte populations. This was either

due to a cytopathic effect resulting in the reduction of the number of phagocytes or to an impairment of phagocytic functions. Disease may affect phagocyte functions, and particularly the respiratory burst activity (Angelidis *et al*, 1987). Our results demonstrate that VHSV can also induce a systemic modulation of the defense mechanisms. This was illustrated by the suppressive effect observed with phagocytosis and the modifications in the dot plot profiles of the leucocyte populations after IP or WB infections. These effects began as early as 6 h post- WB VHSV infection. The first virus replication cycle was not completed at 6 h post-infection and virus particles or virus antigens were only demonstrated in a few cells. In addition to its well-known cytopathic effect, VHSV may induce non-specific factors producing a rapid response from the defense mechanisms. Stressful conditions have been shown to modulate phagocytic activity (Secombes and Fletcher, 1994). Corticosteroid treatments have been shown to result in leucopaenia in fish (Ball and Hawkins, 1976; Johansson-Sjöbeck *et al*, 1978). In fish subjected to a cold shock stress, leucopaenia followed by leucocytosis occurred within 3 min (Pickford *et al*, 1971). So, in addition to a direct VHSV effect, the impairment of phagocytic activity and the modifications of the dot plot profiles could also be related to the stress induced by the experimental infection procedures. The exact nature and mechanisms of the effectors involved in these modulations of the defense system are not yet understood. This preliminary work presents evidence which suggests that VHSV could induce such modulations.

Even though few phenotypical markers are available to discriminate between the leucocyte populations of RBT, flow cytometer emerges as a useful tool to study the early phases of rhabdovirus pathogenic processes.

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