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Effect of the turbot aquareovirus on fish macrophages using an *in vitro* model

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ABSTRACT: The turbot aquareovirus TRV has an invasive behaviour, as the virus spreads throughout most fish organs and tissues. In the present study the presence of TRV inside macrophages of both naturally and experimentally infected turbot was demonstrated, and the ability of the virus to resist killing and to replicate within turbot macrophages *in vitro* was evaluated. The virus survived and replicated in the phagocytic cells, as demonstrated by electron microscopy and growth curve. Superoxide anion (O_2^-) production of phorbol 12-myristate 13-acetate (PMA)-stimulated macrophages infected with TRV was similar to that of normal macrophages. However, the levels of O_2^- produced by macrophages infected with a non-virulent *Aeromonas salmonicida* strain lacking the A-layer were clearly reduced when phagocytic cells had been previously incubated with TRV, but this reduction did not modified the ability of infected macrophages to kill the bacteria. These results indicate that the viral agent eludes the recognition system of the macrophages and the bactericidal capacity of these phagocytic cells is not affected by the presence of the virus, although the interaction with the virus must affect the mechanism of reduction of molecular oxygen into O_2^-

KEY WORDS: Aquareovirus · Turbot macrophages · Killing · Survival

INTRODUCTION

In fish, as in other vertebrates, phagocytes are a major defense against infection (Avtalion & Shahrabani 1975, MacArthur & Fletcher 1985, Olivier et al. 1986). In fact, it has been demonstrated that pathogenic organisms and particulate antigens are rapidly removed from the blood by tissue-fixed macrophages (Rubin et al. 1987).

Macrophages play an essential role in resistance to viral infections (Mogensen 1979, Haller 1981, Mogensen & Virelizier 1987). They can restrict viral replication by inactivation of extracellular viruses, lysis of virus-infected cells, secretion of enzymes competing for virus-dependent substrates, or production of defective interfering virus (Morahan et al. 1985). Nevertheless, a variety of viral pathogens have chosen precisely this type of cell as their preferred habitat (Burstin et al. 1983, Stoddart & Scott 1989). In this sense, several studies have demonstrated the important role of macrophages in the infectivity of reoviruses (Papadimetriou 1965, 1968).

Our previous studies involving *in vitro* infection of turbot tissues with the turbot aquareovirus TRV have shown that this virus replicates mainly in the kidney, but also, to a lesser extent, in the liver and heart. In addition, infection trials indicated that this viral agent spreads quickly within the organism, maintaining relatively high viral titers in most fish tissues and finally developing a persistent infection in the host. One possible route for the rapid dissemination of the virus throughout all fish organs and tissues could be the circulatory system. However, no virus was detected in the serum nor in red cells from either naturally or experimentally infected turbot (Rivas et al. 1993, in press).

Therefore, we decided to investigate the role of macrophages in TRV infection. The interaction be-

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tween the viral agent and fish macrophages could be of crucial importance in explaining some aspects of the TRV invasion process, including penetration into the host and infection of target tissues.

In the present study, the presence of TRV within macrophages from naturally and experimentally infected turbot was assessed and the capacity of the virus to enter macrophages and to replicate within them was evaluated using an *in vitro* model. Moreover, the effect of the virus on superoxide anion production, an important defence mechanism in macrophages (Secombes & Fletcher 1992), was also tested.

MATERIALS AND METHODS

Isolation of resident trout and turbot macrophages. Macrophages were obtained from the head kidney of turbot *Scophthalmus maximus* basically as previously described (Graham & Secombes 1988). Briefly, the head kidney was aseptically removed and disrupted through a 100 µm nylon mesh, with 5 ml Leibovitz' medium (L-15) containing 2% foetal calf serum (FCS), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}) and 10 IU ml^{-1} heparin. The cell suspension was layered onto a 34 to 51% Percoll (Sigma) discontinuous density gradient and centrifuged at 400 × *g* for 30 min at 4°C. Cells separated in the interface were collected, centrifuged at 400 × *g* for 5 min and resuspended in L-15 supplemented with 1% FCS. Viable cell concentration was determined by trypan blue exclusion, and 2×10^7 cells ml^{-1} were allowed to adhere to 24- or 96-well plates. After 3 h at 15°C, unattached cells were discarded and the remaining adhering cells on the plates were covered with L-15 supplemented with 5% FCS. About 95% or greater of these cells were macrophages as judged by morphology.

Virus and cells. The aquareovirus TRV isolated in the northwest of Spain from turbot suffering from a mixed bacterial and viral infection (Lupiani et al. 1989) was used in this study. The virus was propagated in monolayers of chinook salmon embryo cells (CHSE-214) at 15°C, by inoculation at a multiplicity of infection (MOI) of 0.1. Eagle's Minimal Essential Medium (EMEM) supplemented with 2% FCS, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin was used. When the cytopathic effect was extensive, the cells were scraped off and subjected to freezing-thawing. The suspensions were clarified by low-speed centrifugation (2000 × *g* for 10 min at 4°C) and stored at −80°C until use.

All virus infectivity assays were carried out as triplicate microtitrations on CHSE-214 cells in 96-well culture plates. Results were recorded after 14 d incubation at 15°C, and 50% infectivity end-points calculated according to Reed & Müench (1938), and expressed as average $TCID_{50}$ ml^{-1}.

Bacterial strains. *Aeromonas salmonicida* strain MT004 (Marine Laboratory, Aberdeen, Scotland), a non-virulent strain lacking the A-layer and susceptible to killing by normal trout macrophages (Graham et al. 1988), was cultured in tryptic soy broth (TSB) for 24 h at 22°C.

Detection of TRV in macrophages from naturally and experimentally infected turbot. A group of 5 turbot averaging 1 kg were intraperitoneally injected with 1 ml of TRV (10^5 $TCID_{50}$ ml^{-1}). A second group of 5 fish were injected with 1 ml of EMEM and used as controls. Fish were held in 20 l aquaria at 15°C with aeration. After 2 d the macrophages were isolated as described above, diluted to a concentration of 2×10^7 cells ml^{-1}, lysed by freezing-thawing, and intracellular virus titrated as described above.

In addition, macrophages isolated from turbot obtained from a fish farm known to have TRV carrier fish were processed in the same way.

Electron microscopy. Macrophage monolayers on plastic coverslips in 24-well tissue culture plates were inoculated with TRV at a MOI of 10. After a 1 h adsorption, monolayers were washed 3 times with phosphate buffered saline (PBS) covered with 200 µl of EMEM containing penicillin and streptomycin, and incubated at 15°C. At different times post-infection, coverslips were removed, washed, and infected cells fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 1 h at 4°C. Cells were scraped off, washed, and rinsed overnight at 4°C in the same buffer. Post-fixation was performed in 2% osmium tetroxide for 1 h. Cells were then dehydrated and embedded in Epon. Serial ultrathin sections were collected on 150-mesh grids, counterstained with uranyl acetate and lead citrate and then observed on a Philips CM-12 electron microscope.

Viral invasion and intracellular survival assays. Monolayers of untreated and cytochalasin-D (a potent inhibitor of actin-dependent phagocytosis) treated (0.5 µg ml^{-1}, 1 h before infection) macrophages, in 24-well tissue culture plates, were inoculated with viral suspensions at a MOI of 10. After 1 h of adsorption at 4°C, infected cells were washed 3 times with PBS and covered with 200 µl of EMEM containing penicillin and streptomycin. Infected monolayers were incubated at 15°C, and at different times the supernatant was completely recovered and titrated to determine viable extracellular viruses. The monolayers were washed 3 times with EMEM, and macrophages resuspended in 1 ml of distilled water and lysed by 3 freezing-thawing cycles. After low-speed centrifugation, the supernatant was titrated to determine viable intracellular virus. The course of the infection was followed over a maximum

period of 24 h. Simultaneously, macrophages infected with different MOI (0.1 to 10) were observed at different times to follow the appearance of cytopathic effects.

Superoxide anion detection. For the detection of superoxide anion a method based on the reduction of Nitroblue tetrazolium (NBT, Sigma) was employed (Rook et al. 1985). This study was carried out using (1) uninfected macrophages; (2) macrophages infected with TRV for 5 and 30 min, 1, 5, 7 and 24 h, and 7 d; (3) macrophages infected with TRV for 5 and 30 min, and 5 h, and then infected with 20 µl of *Aeromonas salmonicida* (2.4×10^9 cells ml^{-1}), and (4) macrophages infected only with 20 µl of *A. salmonicida*. Thirty minutes after the infection macrophages were washed 3 times with phenol red-free Hanks' balanced salt solution (HBSS). The macrophage monolayers were then incubated at room temperature for 30 min in medium containing 1 mg ml^{-1} NBT in the presence or absence of 100 ng ml^{-1} of phorbol 12-myristate 13-acetate (PMA, Sigma), an activating agent of the respiratory burst. The optical density (OD) was read after 15 min in a multiscan spectrophotometer (Titertek Multiskan Plus, Flow) at 600 nm. Assays were performed in quadruplicate for each of 3 individual fish. These data were analysed by Student *t*-test and 1-way analysis of variance (ANOVA).

Bactericidal activity of TRV-infected macrophages against *Aeromonas salmonicida*. The assay was performed essentially as previously described (Graham et al. 1988). Monolayers of macrophages, in 96-well plates, were inoculated with TRV (10^5 $TCID_{50}$ $well^{-1}$), and incubated at 15°C. At different times (5, 30 min; 4, 19 h), half of the monolayers on each plate were inoculated with different dilutions of *A. salmonicida* MT004 in TSB (from 2.5×10^7 to 9.6×10^4 cells ml^{-1}), centrifuged and incubated for 5 h at 15°C. The supernatant fluids were removed and all the monolayers lysed with 50 µl of 0.2% Tween 20 (Sigma). At this point, those wells which had not been infected with bacteria were inoculated with *A. salmonicida*, representing the 'zero time bactericidal determination'. Then, 100 µl of TSB was added to each well to support an overnight growth at 18°C of the surviving bacteria. After this time, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) (5 mg ml^{-1}) was added per well, and after exactly 15 min the optical density was read at 600 nm in a multiscan spectrophotometer (Flow). A bactericidal assay with uninfected macrophages was performed in parallel for comparative purposes. Assays were performed in quadruplicate and repeated 3 times. These data were statistically examined by a 2-tailed Student *t*-test at a 5% level of significance.

RESULTS

Detection of TRV in macrophages obtained from infected fish

The presence of TRV within turbot macrophages was assessed in both naturally and experimentally infected turbot. Virus isolated after 2 d of the experimental fish infection showed titers between 10^3 and 10^4 $TCID_{50}$ per 2×10^7 phagocytic cells. The viral titer detected in phagocytic cells from naturally infected turbot at different times was slightly lower (10^2 $TCID_{50}$ ml^{-1}).

Electron microscopical examination of TRV-infected macrophages

Electron microscopy was performed to verify the intracellular presence of TRV in fish macrophages during the infection assay. At different times post-infection (p.i.) cell structures associated with viral replication were observed inside infected macrophages. Fig. 1A shows the cytoplasm of TRV-infected phagocytic cells containing small viroplasmic areas, associated with rough endoplasmic reticulum (RER) cisternae, containing 45 to 50 nm subviral particles, which may represent partially formed virus. Incomplete viruses measuring from 25 to 45 nm were also observed scattered through the cytoplasm of infected cells (Fig. 1B).

Entry of TRV into macrophages and intracellular growth

The capacity of TRV to enter into macrophages and to grow intracellularly was evaluated by an *in vitro* model of infection over 24 h, and the results are shown in Fig. 2. As observed, both intracellular (I.V.) and extracellular (E.V.) viruses were detected 2 h p.i. at relatively high levels (around 5×10^4 $TCID_{50}$ ml^{-1}). Titer of I.V. showed an exponential increase from 6 to 12 h p.i., reaching a maximum of 5.6×10^6 at 12 h p.i., which represents a total increase of 2 logs. Except for a small peak at 6 h p.i., the level of E.V. was relatively constant (5×10^4 $TCID_{50}$ ml^{-1}) throughout the experiment.

Development of syncytia is the typical cytopathic effect that aquareoviruses produce in continuous cell lines (Winton 1989). Although no syncytia appeared in TRV-infected macrophages, morphological changes in phagocytic cells were observed from the first hours post-infection (Fig. 3). However, changes in cell morphology were never followed by total destruction of the monolayers. Similar alterations were observed in macrophages previously incubated with cytochalasin-D.

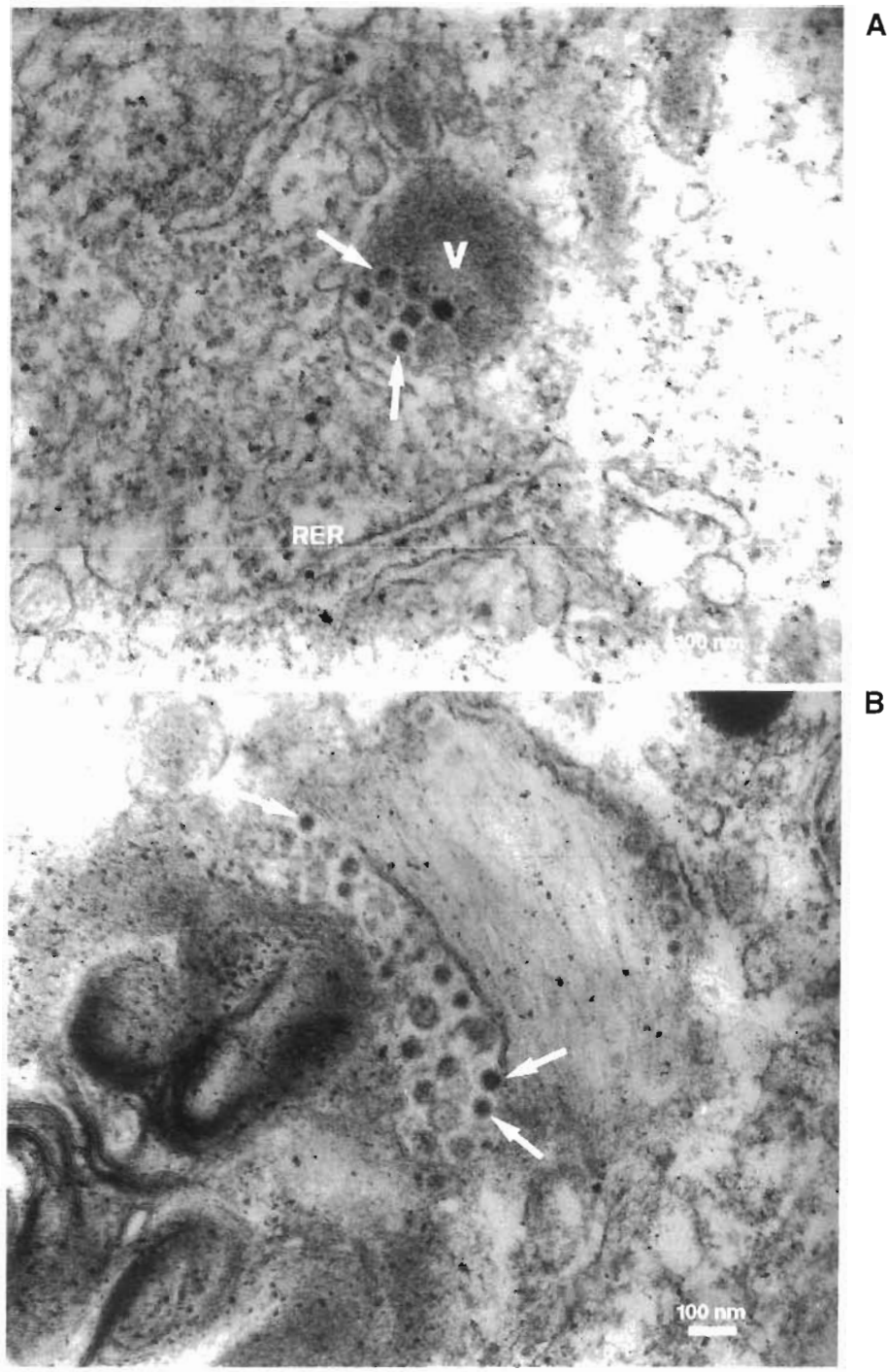

Fig. 1 Electron microscopy of turbot macrophages at 24 h after TRV infection. (A) A small area of viroplasm (V) which contains 45 to 50 nm virus particles (arrows) is located near vesicles of the RER. (B) Subviral structures 25 to 45 nm diameter (arrows) scattered through the cytoplasm of infected macrophages

Superoxide anion detection

This assay was performed in order to study the effect of TRV on the production of superoxide anion by PMA-stimulated macrophages infected with TRV and/or *Aeromonas salmonicida*. No significant differences ($p < 0.05$) in O_2^- production were observed between non-infected macrophages and fish macrophages infected with TRV for different periods of time (5, 30 min; 1, 5, 7, 24 h; 7 d), as shown in Fig 4A. However, incubation of phagocytic cells with TRV did reduce significantly ($p < 0.05$) the activation of the respiratory burst produced by the combination of PMA and *A. salmonicida* in macrophage monolayers (Fig. 4B).

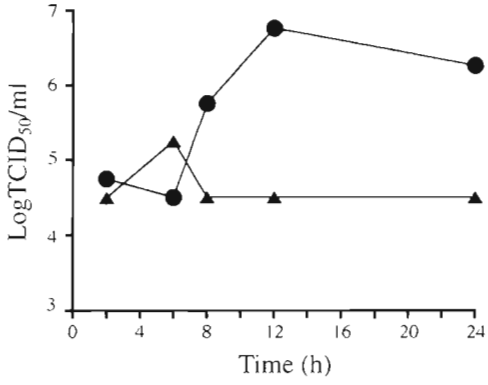

Fig. 2. Survival of TRV in turbot-derived macrophages cultures. Titer of viable extracellular virus (▲); titer of viable intracellular virus (●)

Bactericidal capacity

In order to determine if the interaction between TRV and fish macrophages could affect the killing ability of phagocytic cells against another microorganism, a bactericidal assay against *Aeromonas salmonicida* was performed using macrophages previously incubated with TRV for 5 and 30 min, and for 4 and 19 h. Our results showed that the capacity of TRV-infected macrophages to kill *A. salmonicida* (at a conc. of 2.5×10^7 cells ml^{-1}) was virtually unaltered with respect to uninfected macrophages (Fig. 5). *A. salmonicida* was killed by both TRV-infected and uninfected macrophages after 5 h incubation, since a significant decrease ($p < 0.05$, as determined by Student *t*-test) in the number of viable bacteria was

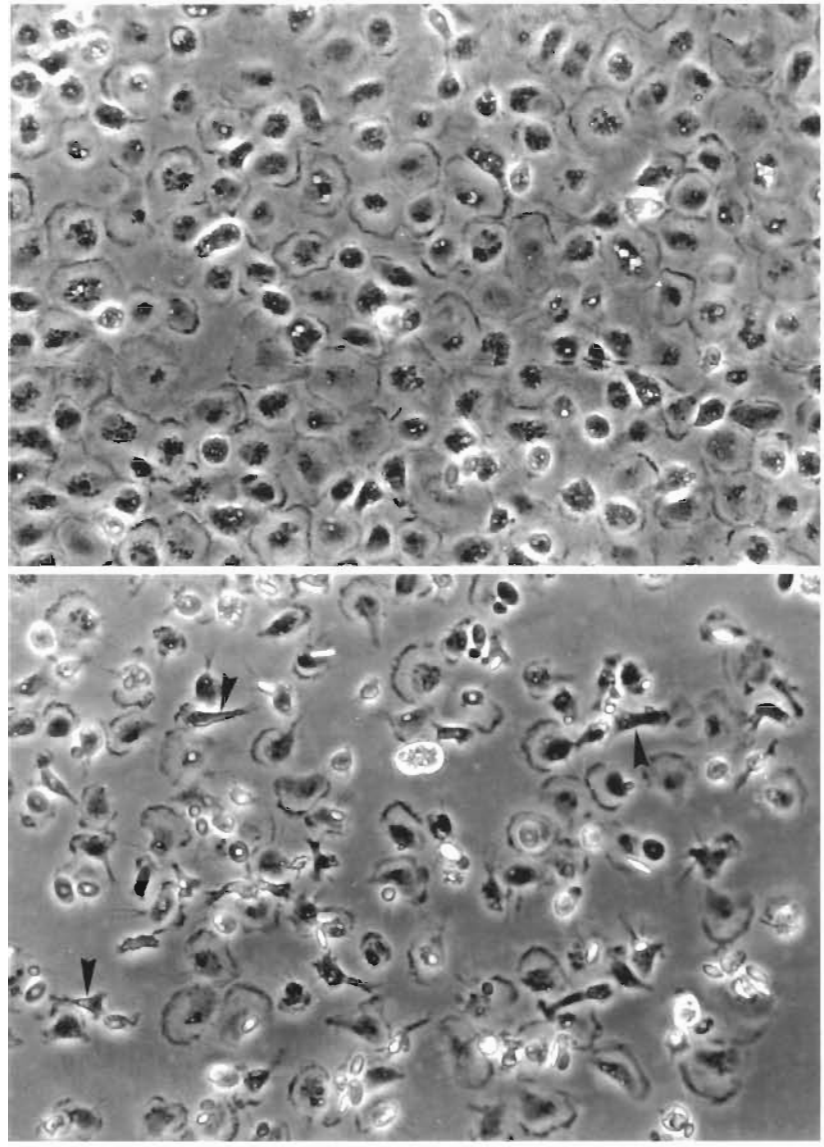

Fig. 3. Monolayers of TRV-infected and non-infected macrophages. (A) uninfected turbot-derived macrophages; (B) cell alterations observed in turbot-derived macrophages infected with TRV for 8 h

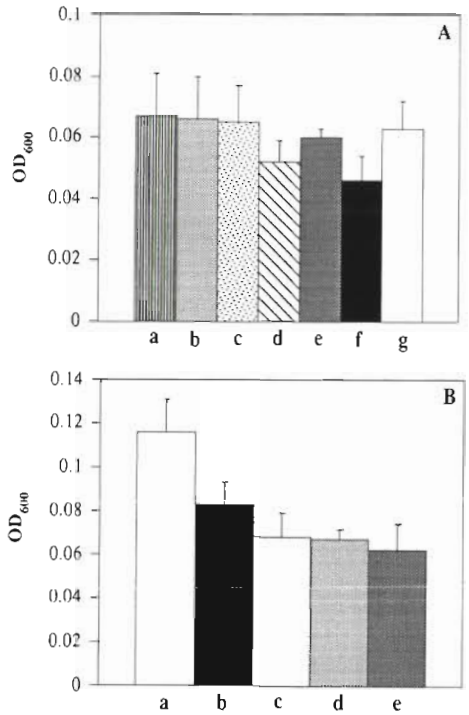

Fig. 4. Superoxide anion production by turbot macrophages. (A) Superoxide anion production, in the presence of PMA, by turbot macrophages infected with TRV for: (a) 5 min; (b) 30 min; (c) 1 h; (d) 5 h; (e) 24 h; (f) 7 d. (g) Non-infected macrophages. (B) Superoxide anion production, in the presence of PMA, by turbot macrophages infected with *Aeromonas salmonicida* for 30 min and previously incubated with TRV for: (b) 5 min; (c) 30 min; (d) 5 h. (a) Non-viral infected macrophages. (e) Non-bacterial or -viral infected macrophages

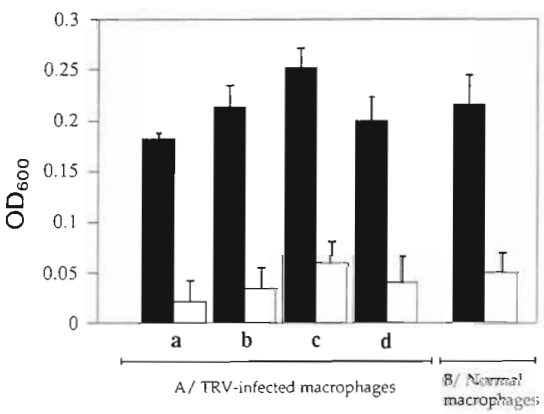

Fig. 5. Effect of TRV infection on the bactericidal activity of turbot macrophages against *Aeromonas salmonicida*. (A) Macrophage monolayers in 96-well plates were incubated with TRV (10^5 $TCID_{50}$ $well^{-1}$) for: (a) 5 min; (b) 30 min; (c) 4 h; or (d) 24 h. Half of the wells were then employed for the 'zero time bactericidal determination' (black bars), and the remaining wells used to determine the effect of TRV infection on the bactericidal activity of macrophages (open bars). (B) Macrophages not infected with virus were used as controls. Results are given as bacterial growth after incubation at 18°C overnight, as determined by optical density readings at 600 nm

observed compared with the 'zero time bactericidal determination' in all cases.

DISCUSSION

Since the isolation of the turbot aquareovirus TRV (Lupiani et al. 1989) we have focused on the characterization of this virus under a wide range of circumstances. One topic studied was the route of entry of the viral agent into the host and its dissemination throughout the fish organs and tissues. In the course of such studies it has been surprising to observe the rapid spreading of the virus within the organism (Rivas et al. in press). The hypothesis first considered was that the virus could be transported by the blood, but the virus was not detected either in sera or in erythrocytes obtained from naturally or experimentally infected turbot. However, the possibility that other kinds of blood cells could be involved in the rapid spreading of this virus could not be ruled out. In the present work the presence of TRV inside monocytes/macrophages of both naturally and experimentally infected fish has been demonstrated, and the role of fish macrophages in the maintenance of the virus inside the host, as well as the effect of the virus on these cells, has been studied. To our knowledge this study represents the first report in which the ability of an aquareovirus to resist killing by macrophages as well as the effect of the viral agent on the phagocytic cells has been assessed.

One of the most hostile environments encountered by an invading microorganism is the intracellular environment of macrophages, because to survive within them a pathogen must be resistant to several antimicrobial factors. Therefore, the ability of microorganisms to survive within phagocytic cells is a potent method of preventing clearance from the host. This association has been reported to exist between phagocytic cells and viruses from both fish and mammals (Yu et al. 1982, Burstin et al. 1983, Panuska et al. 1990).

Viroplasms are structures typically associated with reovirus replication. We have demonstrated by electron microscopy the presence of viroplasms containing subviral particles, as well as viral structures of different sizes scattered throughout the cytoplasm of TRV-infected macrophages, which suggests that the virus is replicating within these cells. Moreover, the results of the growth curve not only confirmed the intracellular location of TRV but also demonstrated the replication of the virus within the phagocytic cells. In addition, the capacity of TRV to infect fish macrophages treated with cytochalasin-D indicates that the virus can enter into the macrophages without being phagocytized.

On the other hand, the appearance of an undefined cytopathic effect, as well as no destruction of the

monolayer, is in concordance with the results obtained by McChesney & Oldstone (1987), who indicated that viruses replicating in lymphocytes are not lytic, and cytopathic effects are not often seen. Furthermore, it has been reported that the lymphocytic choriomeningitis virus infects mononuclear phagocytes and lymphocytes *in vivo* during both acute and persistent infection, but the virus does not kill these cells (McChesney & Oldstone 1987). Similarly, the respiratory syncytial virus infecting alveolar macrophages does not produce cytolysis, loss of cell viability or syncytia up to 25 d p.i. (Panuska et al. 1990).

The production of microbicidal oxygen radicals by phagocytic cells is regulated by the process of activation *in vivo*, or by what is known as priming *in vitro*. Activated or primed monocytes produce large amounts of oxygen radicals when they phagocytize microbes, or are triggered by chemical stimuli like PMA. The non-activation of the respiratory burst during the course of the experiment suggests that the viral agent is not scanned by the macrophages. Similar results were obtained by others (Abramson et al. 1982, Rager-Zisman et al. 1982), who reported that the vesicular stomatitis virus and the influenza virus either did not influence the respiratory burst or reduced it. In contrast, during infection of macrophages with herpes simplex virus type 2, cells acquired an increased capacity to react with a respiratory burst after membrane triggering (Mogensen et al. 1989, Giridhar et al. 1991).

It was interesting to observe that the production of O_2^- radicals by macrophages infected with *Aeromonas salmonicida* was reduced significantly when the phagocytic cells had previously been incubated with TRV. This reduction was first detected 5 min p.i., and the OD values decreased progressively until reaching similar levels to those of non-infected macrophages. These results indicate that viral internalization and subsequent replication may interact with the mechanisms involved in the reduction of molecular oxygen into the superoxide anion radical.

The bactericidal assay performed with *Aeromonas salmonicida* showed that the killing ability of macrophages against this microorganism was still present after incubation with TRV. It has been previously reported that superoxide anion is a potent bactericidal agent for several *A. salmonicida* strains (Karczewski et al. 1991). However, our findings suggest that under these circumstances alternative mechanisms to respiratory burst activity may be involved in the killing of *A. salmonicida* MT004, since the O_2^- production was clearly reduced in macrophages incubated with TRV whereas the bactericidal activity was still present.

The results obtained in the present study clearly demonstrate that TRV has the ability to enter, survive, replicate and persist inside turbot macrophages *in vitro* without apparently affecting them, but inhibits O_2^- production in the presence of *Aeromonas salmonicida*. These observations suggest that macrophages may be a site for viral persistence in fish and that these cells may act to transport the virus to fish organs and tissues, without being affected by the viral agent. Further studies are needed to understand the mechanisms involved in the interaction between TRV and fish macrophages *in vivo*, as well as the potential role that macrophages may play in the immunity of the host against aquareovirus.

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