Pathogenesis and immune response in Atlantic salmon (Salmo salar L.) parr experimentally infected with salmon pancreas disease virus (SPDV)

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Atlantic salmon parr were injected intraperitoneally with salmon pancreas disease virus (SPDV) grown on CHSE-214 cells. The viraemia, the histopathological changes in target organs and some immune parameters were taken at intervals up to 30 days post-infection (dpi). The earliest kind of lesion was necrosis of exocrine pancreas, appearing as soon as 2 dpi. It progressed towards complete tissue breakdown at 9 dpi before resolving gradually. Concurrent to this necrosis, a strong inflammatory response was in evidence from 9 dpi in the pancreatic area for a majority of fish. A necrosis of the myocardial cells of the ventricle occurred in infected fish mainly at 16 dpi and it faded thereafter. The monitoring of the plasma viral load showed a rapid haematogenous spreading of SPDV, peaking at 4 dpi, but also the absence of a secondary viraemia. No interferon (IFN) was detected following the infection of parr with SPDV, probably owing to an IFN activity in Atlantic salmon below the detection level of the technique. Neutralising antibodies against SPDV were in evidence from 16 dpi and they showed a time-related increasing titre and prevalence. The phagocytic activity in head-kidney leucocytes was always significantly higher in the infected fish than in the control fish, being particularly high by 9 dpi. Lysozyme and complement levels were both increased and they peaked significantly in the infected fish at 9 and 16 dpi respectively. These results demonstrated that an experimental infection of Atlantic salmon parr with SPDV provoked a stimulation of both specific and non-specific immunity with regards to the viraemia and the histopathology.

Key words: salmon pancreas disease virus (SPDV), Atlantic salmon, histopathology, viraemia, neutralising antibodies, interferon, lysozyme, complement, phagocytic activity.

I. Introduction

Pancreas disease (PD) was first recorded in Scotland in 1976 [1]. It has since been diagnosed in countries where Atlantic salmon (Salmo salar L.), the
target species, is intensively reared, including the U.S.A. [2], Norway [3] and Ireland [4]. Some cases were also reported in France and Spain [5]. It affects Atlantic salmon smolts during their first year at sea and causes significant economic loss, with up to 50% mortality in Ireland [6].

The clinical symptoms of PD are sluggish swimming behaviour and anorexia, which leads to slow growth and emaciation [1]. The histopathology of PD includes an acute haemorrhagic necrosis of the exocrine pancreas, which causes an inflammatory response and subsequent fibroplasia [1, 7]. A focal to diffuse necrosis of the myocytes in the heart ventricle and in the skeletal muscle were also described [4, 8, 9] and they are now considered as a strong indication of PD when observed [10].

The virus responsible for the disease has been isolated and named salmon pancreas disease virus (SPDV) [11]. Weston et al. [12] have recently identified it as an alphavirus. The difficulties associated with its isolation [13] and its culture in cell lines [10, 14] may have limited the work devoted to the immunity of Atlantic salmon against PD. However, some authors have demonstrated an acquired immunity to experimental PD by performing passive immunisation experiments [15, 16], and the presence of neutralising antibodies after an infection with SPDV has also been reported [17].

The present study was undertaken to reproduce the typical histopathological changes of PD in Atlantic salmon parr after an experimental infection with cell cultured SPDV, and then to study the evolution of the viraemia and some of the immune parameters of the host.

II. Materials and Methods

CELLS AND VIRUS

Chinook salmon embryo (CHSE-214) cells [18] were grown at 20° C in Eagle’s minimum essential medium (MEM) with McPherson-Stocker modification (BHK21, Eurobio, France), supplemented with 1% of 200 mM L-glutamine (Eurobio), 10% foetal bovine serum (DAP, Australia), 100 I.U. of penicillin and 0·1 mg of streptomycin per ml (Eurobio). This supplemented medium is subsequently referred to as MEM. The cells were cultured according to Wolf & Quimby [19] in 75 cm² plastic cell culture flasks or 96 well microtitration plates (Corning, U.S.A.).

The agent of PD used in this experiment was the P42p isolate of SPDV [20]. The virus was propagated by inoculating confluent 24 h old CHSE-214 cells with SPDV at a multiplicity of infection (MOI) of 0·01. After 8 days of incubation at 14° C, the virus was harvested. On this occasion, the cells were disrupted by freezing and thawing once and the cell debris removed by centrifugation at 1300 × g for 15 min at 4° C. To obtain the virus for the present experimental infection, three passages of the P42p isolate on CHSE-214 cells were performed under the conditions described above. In spite of adaptation to cell culture, P42p isolate often fails to produce a complete and reliable cytopathic effect (CPE). The titration of SPDV production was therefore performed by a reverse technique adapted from Payment & Trudel [21]. Briefly, the infection of a cell monolayer with a primary non-cytopathic virus prevents
the over-infection of cells with a secondary cytopathic virus. This heterologous interference is used in a 50% endpoint dilution assay [22] to calculate the infectious dose of non-cytopathic virus ‘protecting’ 50% of the tissue cultures from the CPE induced by a constant amount of the secondary virus. This ‘protective’ dose is expressed in tissue culture infectious dose 50% (TCID_{50}) per ml. In the present experiment, viral haemorrhagic septicaemia virus (VHSV) was used as the secondary virus. In practice, 10-fold dilutions of SPDV-infected cell culture supernatant, starting from 1/100 up to 1/10^{10}, were prepared in MEM. Fifty μl of each dilution was inoculated in four replicates on to 24 h old confluent CHSE-214 cell monolayers in 96 well microtitration plates and incubated for 1 h at 14°C. Then, (MEM 150 μl) was added to each well and the plates were maintained at 14°C for 8 days. The medium was then removed from the plates and cells were over-infected with 500 TCID_{50} of VHSV per well in 50 μl of MEM. After one hour at 14°C, each well received 150 μl of MEM and the plates were incubated at 14°C for a further 4 days. Negative and positive control cell monolayers for VHSV CPE were included in each plate, consisting of wells receiving no SPDV and no VHSV or no SPDV and 500 TCID_{50} of VHSV, in 50 μl of MEM. Control cell monolayers only infected with SPDV dilutions in four replicates were also included. Cell layers were finally fixed and stained by addition of 100 μl per well of 1% crystal violet in 20% ethanol and the titre was expressed in TCID_{50} per ml, as described above. According to this reverse technique, the titre of the final production of SPDV was found to be 10^{6} TCID_{50} ml^{-1}.

FISH

One year old Atlantic salmon parr, with a mean weight of 40 ± 10 g and originating from a local farm, were used. Their disease-free state, i.e. the absence of virus, was determined on cell culture prior to transfer to experimental facilities. The fish were fed daily with dry commercial pellets (Le Gouessant, France) at 2% body weight.

EXPERIMENTAL DESIGN

After being anaesthetised with 2-phenoxyethanol, 75 parr were intra-peritoneally (i.p.) injected with 100 μl containing an infectious dose of 10^5 TCID_{50}. The control group consisted of 75 fish injected i.p. with 100 μl of CHSE-214 cell culture supernatant free from virus. Control and infected groups were each separated in three replicates and held in covered 50 l tanks, on the basis of 25 fish in each tank. Fish were maintained in filtered and oxygenated freshwater at 14 ± 1°C. Sampling was carried out 2, 4, 9, 16 and 30 dpi from five fish in each tank.

SAMPLING

Blood was collected from the caudal vein into heparinised vacutainers and centrifuged at 1200 × g for 10 min at 4°C. After collection, the plasma of each fish was split into 5 aliquots of 60 μl and stored at −80°C. The pancreas
associated with pyloric caeca, the heart and a portion of the red skeletal muscle beneath the lateral line were sampled from these fish and fixed in Bouin’s fixative.

Anterior kidneys were collected, briefly wiped on paper and pushed through a 100 μm sterile steel mesh with 3 ml of RPMI 1640 medium with Dutch modification (Eurobio). The resultant cell suspensions were layered on to a 34/51% v/v pre-formed Percoll gradient (Sigma, U.S.A.) and centrifuged at 400 × g for 30 min at 4°C. Leucocyte enriched suspensions (10 ml) were collected at the 34/51% interface and centrifuged at 1200 × g for 5 min at 4°C. Cells were thoroughly washed twice, by pouring off the supernatant, re-suspending the pellet with Cortland saline [23] and centrifuging at 1200 × g for 5 min at 4°C. After the last washing, cells were resuspended in 100–400 μl of Cortland saline, depending on the amount of harvested cells. Cell viability was assessed using the trypan blue exclusion method and enumeration was performed with a Thomas’ haemocytometer. The number of viable leucocytes was finally adjusted to 10^7 cells ml^−1 in Cortland saline.

HISTOLOGY

After 48 h in Bouin’s fixative, tissues were dehydrated through graded ethanol baths and embedded in paraffin wax. Sections (6 μm) were cut and stained with haematoxylin and eosin or Masson’s trichrome. For each fish, the histopathological changes were graded using ad hoc scales. Briefly, the abundance of healthy exocrine pancreas was graded as none, scarce, moderate or abundant. In the same way, the severity of the exocrine pancreas necrosis, the pancreas inflammation, the cardiomyocytic and the skeletal muscle necrosis were graded as none, mild, moderate and severe.

VIRAEMIA

The viraemia in the control and in the SPDV-infected fish was quantified according to the reverse technique described above, the plasma samples being used as the source of SPDV instead of the cell culture supernatant.

IMMUNE PARAMETERS

Interferon

The titre of plasma interferon (IFN) was determined according to the spectrophotometric method described by Renault et al. [24]. After the inactivation of SPDV by heating at 50°C for 30 min [11], the plasma samples were diluted two-fold in MEM, from 1/10 to 1/10240, and 100 μl of each dilution was inoculated in duplicate on to confluent 24 h old CHSE-214 cell monolayers in 96 well microtitration plates. After 20 h of incubation at 20°C, the medium was poured off, replaced by 200 μl of MEM containing 4000 TCID_{50} of VHSV and the microplates were incubated for 4 days at 14°C. After fixation and staining of the monolayers with 1% crystal violet in 20% ethanol, the microplates were washed in tap water and dried.
A standard plasma IFN was produced in rainbow trout (Oncorhynchus mykiss W.) according to Secombes [25] by an i.p. injection of $2 \times 10^7$ TCID$_{50}$ of VHSV for each fish. The titre of this standard IFN was 4500 U ml$^{-1}$ as determined by a spectrophotometric assay. Four wells of each plate were treated with 100 µl of this trout standard IFN, i.e. 450 U well$^{-1}$, after it had been heated at 50°C for 30 min. A control of the VHSV CPE was also included, consisting of 4 wells per plate receiving MEM instead of plasma. Finally, 4 wells of each plate received no VHSV and no IFN treatment and they were considered as control cell monolayers.

The optical density (O.D.) of wells was read at 600 nm in a multiscan spectrophotometer (iEMS, Labsystems, France). The O.D. values obtained for the VHSV CPE control and for the control cell monolayers corresponded to 0 and 100% protection respectively. The O.D. 50% was then calculated and used in the formula established by Renault et al. [24]. The IFN titre was finally expressed in units (U) per ml of plasma, one unit being defined in such a way as it corresponds to the tenth of the reciprocal of plasma dilution which led to O.D. 50%.

**Neutralising antibodies**

Neutralising activity in plasma from infected and control fish was determined by the plaque neutralisation test described by Olesen & Jørgensen [26] with the following adaptations to SPDV. Individual plasma samples were diluted two-fold in MEM, from 1/5 to 1/2560, in 96 well microtitration plates in order to get a final volume of 50 µl. SPDV culture supernatant (100 µl), with a titre of 500 TCID$_{50}$, was added to each dilution of plasma and plates were incubated at 14°C. After 30 min, 50 µl of fresh rainbow trout serum, diluted 1/10 in MEM, were added to each well as a source of complement and plates were re-incubated for another 30 min at 14°C. Fifty µl of each mixture of plasma, virus and complement was then adsorbed on 24 h old confluent CHSE-214 cell monolayers in 96 well microtitration plates for 1 h at 14°C. Cells were subsequently overlaid with 150 µl MEM for each well. The remaining SPDV was then titrated as described above. The neutralising antibody titre was taken as the highest plasma dilution which neutralised 50% of SPDV.

**Leucocyte phagocytic activity**

Phagocytosis in the head-kidney was assayed by chemiluminescence [27], using zymosan particles (Sigma) opsonised with trout serum and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) as the substrate of the reaction. Opsonised zymosan and luminol solution were prepared as 1 mg ml$^{-1}$ and 17.5 µg ml$^{-1}$ in Cortland saline, respectively. For each infected or control fish, 100 µl of leucocyte suspension, i.e. $10^6$ cells, were mixed with 200 µl of opsonised zymosan and 700 µl of luminol in a polystyrene vial. Immediately after mixing, vials were placed in the carousel of an automated luminometer (1251 Luminometer, BioOrbit) at 20°C. The time-course of the chemiluminescent response was followed for 2 h at constant time intervals. Results
are expressed as the area under the curve of chemiluminescence \( v \) time (mV s).

**Lysozyme**

Plasma lysozyme activity was determined using a turbidimetric assay [28], adapted to microtitration plates. Briefly, a bacterial suspension of *Micrococcus lysodeikticus* (Sigma) was prepared at a concentration of \( 1 \cdot 25 \text{ mg ml}^{-1} \) in a \( 0 \cdot 05 \text{ M} \) sodium-phosphate buffer pH 6.2. Fifty \( \mu l \) of the individual plasma samples were plated in 96 well microtitration plates. The reaction was initiated in a multiscan spectrophotometer, by addition of \( 160 \mu l \) well \(^{-1} \) of *M. lysodeikticus* suspension, using an automatic dispenser. Readings of O.D. at a wavelength of 450 nm were performed every min for 20 min, the plate being shaken for 10 s before each reading. Using a standard hen egg white lysozyme (Sigma) in sodium-phosphate buffer, the concentration of lysozyme in Atlantic salmon plasma was expressed in \( \mu g \text{ ml}^{-1} \).

**Complement**

Classical pathway of plasma complement activity was assessed by a haemolytic assay [29] with sheep red blood cells (sRBC), performed in 96 well microtitration plates. For sRBC sensitisation, the anti-sRBC serum, or haemolytic serum, was raised in rainbow trout. Briefly, after being diluted at 1/15 in EDTA–gelatin veronal buffered saline (EDTA–GVB), to avoid natural haemolytic activity, and de-complemented at 45°C for 30 min, trout haemolytic serum was mixed with an equal volume of a 2% sRBC (BioMérieux, France) suspension in the same buffer. The mixture was incubated for 30 min at 30°C. Sensitised sRBC suspension was finally adjusted to 2% in a glucose gelatin veronal buffer saline (GGVB).

Atlantic salmon plasma, diluted at 1/128 in GVB to avoid natural haemolytic activity, was added in increasing amounts, from 10 to 90 \( \mu l \) well \(^{-1} \), in 96 well microplates filled with GVB, to get a final volume of 100 \( \mu l \). Fifty \( \mu l \) of 2% sensitised SRBC suspension was finally added to each well. Control values of 0 and 100% haemolysis were obtained using, respectively, 100 \( \mu l \) of GVB or 100 \( \mu l \) of non-decomplemented trout haemolytic serum at 1/50 in ultrapure water, instead of diluted plasma samples.

After an hour of incubation at 20°C, microplates were centrifuged at 200 \( \times \) g for 5 min at 4°C and 75 \( \mu l \) of each well supernatant were transferred to 96 well microplates filled with 75 \( \mu l \) of GVB per well. The O.D. was finally measured at 540 nm in a multiscan spectrophotometer. The number of complement units per ml of plasma was determined in reference to the 50% haemolysis (CH\(_{50}\) units ml\(^{-1}\)), using the formula established by Sakai [30].

**STATISTICAL ANALYSIS**

At each sampling day, plasma lysozyme, complement activity and leucocyte phagocytic activity of the three replicates of the infected fish were compared to the values of the three replicates of the control fish by one-way ANOVA. Significantly different groups were finally identified using a Newman and Keuls test.
II. Results

No mortality was recorded throughout the experiment.

HISTOPATHOLOGY OF PANCREAS DISEASE

No histopathological change was recorded in the skeletal muscle of any control or SPDV inoculated fish.

Control fish

Throughout the experiment, an abundant amount of healthy exocrine pancreas, characterised by dense sheets of well-organised acinar cells (Fig. 1a) was observed in a majority of control fish (Fig. 2). On each sampling day, one to four control fish out of 15 were found to have moderate amounts of healthy exocrine pancreas (Fig. 2), usually scattered at the periphery of an abundant

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**Fig. 1.** (a) Normal appearance of the pancreas area in Atlantic salmon parr, showing abundant acinar cells a, with zymogen granules, and pyloric caeca c. An islet of endocrine cells e is observed. (b) Acute pancreatic injury in a fish 9 days after the inoculation with SPDV. Various stages of acinar cell necrosis (arrowed) can be seen, with a swollen appearance, basophilia and pyknotic nuclei. (c) Inflammation i and early fibrosis in the pancreatic area of a fish 9 days after the injection of SPDV. Some intact acinar cells remain a but necrosis (arrowheads) can be seen. Collagen deposition (arrowed) is revealed by a green staining. (d) Necrosis in the ventricular myocardium from a fish 16 days after the inoculation with SPDV. Several shrunken, eosinophlic cardiomyocytes with pyknotic nuclei (arrowed) are observed. (a), (b), (d): Haematoxylin and eosin. Magnification × 400. (c): Masson’s trichrome. Magnification × 400.
amount of adipose tissue or surrounding islets of endocrine cells. Among the fish with abundant amounts of healthy exocrine pancreas, some individuals presented a necrosis of the tissue. Thus, from 2 to 9 dpi, one to five control fish had a mild necrosis of acinar cells and one fish displayed a moderate affection of the tissue on day 4 (Fig. 3). There was no sign of subsequent progression of this exocrine pancreas necrosis, apart from the three fish still presenting a mild affection by 30 dpi. No control fish displayed any sign of inflammation or fibrosis in the pancreas area (Fig. 4) and no histopathological change was recorded in the heart (Fig. 5).
By 2 dpi, 11 out of 15 SPDV inoculated fish had abundant amounts of healthy exocrine pancreas (Fig. 2) and 5 of these had a mild necrosis of acinar cells (Fig. 3), being the only lesion recorded at that time.

By 4 dpi, nine fish out of 15 had abundant amounts of healthy exocrine pancreas (Fig. 2) but with mild necrosis (Fig. 3). Fish with a scarce or moderately abundant healthy exocrine pancreas presented for the first time a mild necrosis of acinar cells together with the first signs of a mild necrosis.

**SPDV inoculated fish**

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inflammatory reaction in the pancreas area (Fig. 4). No histopathological change was noticed in the heart of any infected fish by 4 dpi.

By 9 dpi, 12 SPDV inoculated fish displayed severe necrosis of the tissue (Fig. 3). This was characterised by the presence of numerous rounded basophilic acinar cells with pyknotic nuclei (Fig. 1b) and the complete absence of, or very scarce amounts of intact exocrine pancreas (Fig. 2). All sampled fish had inflammatory cells within the pancreas area (Fig. 4) and for a majority, nine fish out of 15, this infiltration was severe, associated with early fibrosis and collagen deposition (Fig. 1c). Six fish displayed a mild or moderate necrosis of the ventricular myocytes (Fig. 5) and a combination of severe exocrine pancreas necrosis, scarce amounts of healthy acinar cells and severe fibroinflammatory infiltration.

By 16 dpi, 10 fish out of 15 had no or scarce amounts of intact exocrine pancreas (Fig. 2). There was no or mild amounts of acinar cell necrosis (Fig. 3) and a severe infiltration of the pancreatic area with inflammatory and fibrotic cells (Fig. 4). In four of these fish, a severe cardiomyocytic necrosis of the ventricle was observed (Fig. 1d). Regardless of the severity, 12 out of 15 fish displayed necrosis of the ventricular myocardium at that time (Fig. 5).

On the last sampling day, 12 fish had a scarce to moderate amount of healthy exocrine pancreas (Fig. 2). Among these, 11 were found to have no necrotic acinar cells (Fig. 3) including eight fish with no or mild infiltration of inflammatory cells (Fig. 4). Only two fish displayed a mild necrosis of ventricular cardiomyocytes by 30 dpi, the rest had no histopathological lesion in the heart (Fig. 5).

VIRAEMIA (TABLE 1)

No SPDV was isolated from the control fish at any sampling date. In the infected group, the virus was found in all sampled fish by 2 dpi and the average viraemia was $3 \times 10^5$ TCID$_{50}$ ml$^{-1}$ of plasma. A peak value of $3 \times 10^6$ TCID$_{50}$ ml$^{-1}$ was reached on the fourth day after the infection, the virus being re-isolated from all sampled fish. Although all fish were also found to be infectious by 9 dpi, a drop of the viral titre was observed. By 16 dpi, for the first time, five fish were found to have no detectable SPDV in their plasma. By 30 dpi, SPDV was re-isolated only from three fish out of 15, leading to a low average viraemia of $10^2$ TCID$_{50}$ ml$^{-1}$.

IMMUNE PARAMETERS

Interferon

No significant protection of the cell monolayers against the VHSV CPE was observed with any of the plasma samples taken from the control fish. By 4 dpi, only three plasma samples of the SPDV-infected fish led to a very low level of protection of the cell monolayers. Although the O.D. values obtained with the plasma of these fish were above the 0% protection value, they were too low to calculate the 50% protection point. As a consequence, it was impossible to quantify the plasma IFN.
Neutralising antibodies (Table 2)

No neutralisation of SPDV occurred with any of the control plasma. In the infected group, neutralising antibodies were first recorded by 16 dpi in 11 out of 15 fish and the average titre was 1/40. At day 30, all infected fish displayed neutralising antibodies to SPDV and the average titre was 1/60.

Phagocytic activity (Fig. 6)

Due to problems with leucocyte isolation on the Percoll gradient, no phagocytic activity was recorded on day 2 and 4 in both groups. On the other sampling days, the phagocytic activity of SPDV-infected fish leucocytes was always significantly higher than in controls. A peak value was recorded by 9 dpi in both groups, 5·5 × 10^6 mVs for infected fish v. 2·7 × 10^6 mVs for the controls. On the subsequent days, this activity declined, remaining at the same level of 2·5 × 10^6 mVs in SPDV inoculated fish and varying from 0·5 to 1·2 × 10^6 mVs in controls.

Lysozyme (Fig. 7)

The plasma lysozyme level in control fish gradually increased from the first to the last sampling day, from 0·7 to 1 μg ml⁻¹. In the SPDV inoculated fish,
the lysozyme level was always higher than in the controls and by 9 dpi, a significant difference was observed. The average concentration of lysozyme was then 1.4 µg ml⁻¹. After this peak, the parameter decreased and the last measurement at 30 dpi indicated a lysozyme level close to the control values.

**Complement activity (Fig. 8)**

In control fish, classical activity of the complement varied from 13 to 23 CH50 units ml⁻¹ of plasma depending on the sampling day. Throughout the experiment, this parameter was always higher in infected fish for a given day. It increased from 2 to 16 dpi, but the average value of 34 CH50 units ml⁻¹ recorded at 16 dpi was the only one to be significantly higher than the controls. On the last sampling day, the average complement activity within the plasma of infected fish had returned to a lower value of 19 CH50 units ml⁻¹, close to the control value at that time.
IV. Discussion

In this study, the severe histopathology of the pancreas and the heart of Atlantic salmon inoculated with SPDV was very similar to the condition described in field outbreaks of PD [4, 8]. Our results also confirmed the existence of intermediate degrees of severity in the necrosis of the exocrine pancreas and the ventricular myocardium, as previously observed [15, 17] in experimental infections. In this respect, the grading of the lesions associated to PD was particularly useful to take into account all these degrees of severity and it was performed using a scale designed for all our experimental PD trials.

By contrast, pancreatic lesions in control fish remained mild in most cases, with no sign of progression to other organs and no SPDV re-isolation, ruling out the possibility of cross contamination. The cell damage may be due to a degree of toxicity of the cell culture supernatant or to fixation artefact. In the infected fish, this side effect may have somehow interfered in the estimation of the SPDV induced injuries and makes their interpretation difficult in the first 4 days after the infection. However, it should be noticed that the prevalence of the exocrine pancreas necrosis by 4 pdi was markedly higher in the SPDV inoculated group.

The 9 days period required to reach the maximum of pancreatic destructions after the inoculation of cell cultured SPDV is in accordance with the findings of McLoughlin et al. [17]. Other reports of experimental infections mentioned earlier [15, 31] or later [32] peaks of pancreatic acinar cell necrosis but after the inoculation of infective tissue homogenates. These discrepancies may therefore result from a relationship between the infectious dose and the time-course of the pancreatic histopathology in experimental PD, as already demonstrated by Raynard & Houghton [33]. Regardless of the kinetics, the severe breakdown of the exocrine pancreas observed in our study in almost all the infected fish was consistently reached in previous PD trials [15, 17, 31]. In addition to the direct action of SPDV, the nature of the pancreas itself may explain this severity. Indeed, Karne & Gorelick [34] observed in mammals that

Fig. 8. Complement activity in Atlantic salmon 2, 4, 9, 16 and 30 days after the i.p. injection of 10⁵ TCID₅₀ per fish of SPDV or virus-free cell culture supernatant. *Significant difference between infected (■) and control (□) fish. Results are means (n=15)±s.e.
the action of viruses, which selectively injure the pancreatic acinar cells, can be potentiated by the premature activation of pancreatic enzymes and their leakage after the cell death. These authors also stressed the involvement of the inflammatory cells in the increase of cell damage, in particular by secreting reactive oxygen species. In our study, the inoculation of Atlantic salmon with SPDV provoked a massive infiltration of the pancreas area with mononuclear cells and this inflammation was found to be well correlated to the acinar cell necrosis. A similar inflammation was described by Boucher et al. [31] but Murphy et al. [15] and McLoughlin et al. [17] reported more limited infiltrations of the pancreas area. This situation may result from the difficulty of appraising the inflammatory infiltrate, in particular within the neoplastic fibrosis that consistently occurs after the pancreas destruction.

As evidence of a recovery, the inflammation and the exocrine pancreas necrosis were greatly reduced by 30 dpi and an increase in the number of fish with higher amounts of healthy exocrine pancreas was also observed. The transience of the pancreatic pathological changes is a frequent feature of PD but McLoughlin et al. [17], as several authors, questioned the existence of a regenerative process of the pancreatic acini. However, Houghton [32] already described recovery in parr and post-smolts after an experimental infection, with low levels of non-recovery in parr.

The cardiomyopathy during PD has already been described and it is now accepted as a reliable feature of the condition [10]. The one week delay that we observed between the peak pathology in the pancreas and in the heart is somewhat different from the report of Murphy et al. [15] who reported a shift of only 2 to 4 days and McLoughlin et al. [17] who described a parallel onset and evolution of the necrosis in both organs. In addition to a possible variation of the infectious dose, this might be due to some influence of the viral isolate as demonstrated by Gomez et al. [35] for the B3 strain of coxsackievirus. This virus infects mammals and provokes a necrosis of the exocrine pancreas and the ventricular myocardium, which is very similar to the damage that is described in PD. In fact, some variants of this B3 strain show a weaker ability to replicate in the heart. A similar difficulty of replication of our P42p isolate of SPDV in the cardiac tissue may account for the delayed appearance of necrosis. The absence of any previous quantitative study of the histopathology of PD makes it impossible to be certain if this replication peculiarity also has consequences for the severity of this cardiomyopathy.

Skeletal muscle necrosis did not occur in the present study. This seems to be a feature of experimental PD, compared with the naturally occurring condition, as Murphy et al. [15] and McLoughlin et al. [36] also failed to reproduce the skeletal myopathy. McLoughlin et al. [17] obtained some degenerative changes in the skeletal myofibres and observed that the lesions remained less severe than those reported in the field. According to these authors, the less intense muscular activity of fish in experimental conditions may explain this difference. It should be noticed that the fish in our study were kept in 50 l tanks where the swimming activity is highly reduced. Murphy et al. [15] also suggested that the use of a freshwater rather than a seawater model may influence the onset of the skeletal myopathy in experimental PD. Interestingly, McLoughlin et al. [7] reported that peak mortalities in field
outbreaks of PD in Ireland were closely related to severe skeletal myopathy. The absence of lesions in the skeletal muscle or limited injuries may also explain the absence of mortality in experimental PD.

Concerning the viraemia, SPDV was re-isolated from the plasma of all the infected fish as soon as 2 dpi and the average titre reached a maximum by 4 dpi, both of which suggest a rapid haematogenous spreading of the virus throughout the fish and, in particular, towards the target organs. By 9 dpi, SPDV was still found in all the inoculated fish, coinciding with the peak of the exocrine pancreas necrosis and the appearance of the first cardiomyocytic necrosis. However, a decrease in the average viraemia was observed from that time. The histopathological damage did not therefore lead to a marked release of viral progeny into the bloodstream. An elimination of the viral particles released from the injured cells may have been mediated by inflammatory cells. From 16 dpi, the plasma of some SPDV inoculated fish was found to be non-infective. A similar trend was reported by Houghton [37] and McLoughlin et al. [17]. This transitory viraemia differentiates SPDV from the infectious pancreatic necrosis virus (IPNV) which is usually associated with a long-lasting viraemia, resulting in chronically infected fish [38].

Type I IFN are considered to be one of the earliest anti-viral immune factors to be induced. Dorson et al. [39] showed that the production of IFN in salmonids occurs in the first 5 days after viral infection. In the present experiment, it was not possible to quantify any anti-viral activity in plasma consistent with IFN. To the best of our knowledge, no study of IFN production in Atlantic salmon following an experimental viral infection has ever been published. It is unlikely that there is no IFN synthesis in this species as IFN-induced Mx proteins and their related genes have been found by Robertsen et al. [40]. Possibly, the amount of plasma IFN in Atlantic salmon may be too low to be detected using conventional techniques.

The anti-viral response in fish is also known to be partly effected by the induction of neutralising antibodies [41]. We first detected them in the plasma of infected fish 16 days after the inoculation of SPDV and both the antibody titres and their prevalence among the fish increased until the last sampling day. This timing of appearance is late compared with the reports of McLoughlin et al. [17] who demonstrated neutralising antibodies as soon as 10 dpi. Slight differences in the experimental conditions, i.e. stage of fish maturity, weight, salinity and a lower infectious dose in the present experiment, may explain this delay. However, the titres and the subsequent evolution of neutralising antibodies were similar in both experiments. In any case, the neutralising antibodies appear too late to play any role in protection against the early acute pancreas necrosis. However, they may control the viral spreading in the later stages of the disease and account for the clearance of SPDV in the plasma. Thus, the presence of high titres of antibodies was positively correlated to a weaker viraemia, suggesting the neutralisation of SPDV by antibodies had occurred in vivo. This protective role of neutralising antibodies against PD has already been demonstrated by passive immunisation experiments [15, 16].

In contrast to these humoral factors, little is known about cell-mediated anti-viral defence mechanisms in salmonids. In the present experiment, the
phagocytic activity of head-kidney leucocytes was significantly higher in the SPDV-infected fish from 9 dpi. The enhancement of chemiluminescence would therefore reflect the production of oxidising agents by cells phagocytising viral particles, which are present in the kidney at that time, as demonstrated by Houghton [37]. This synthesis of oxidising agents by head-kidney macrophages in response to a viral infection has recently been stated in turbot by Tafalla et al. [42]. Despite this, a systemic stimulation of the leucocytes should be considered, as the high phagocytic activity in the head-kidney by 9 dpi coincided with the onset of the inflammatory reaction in the pancreas. This stimulation might be mediated by circulating cytokines, which are produced in large amounts by leucocytes during acute pancreatitis in mammals [43].

Lysozyme, in addition to its antibacterial properties [44], is also a major secretory product of neutrophils and macrophages and it can be considered as a reliable systemic indicator of their stimulation [45]. In this study, the plasma lysozyme levels were significantly increased by 9 dpi in response to the SPDV infection. The amount of plasma lysozyme would therefore reflect the recruitment of the phagocytic cells in the pancreatic inflammation. The role of this secretion of lysozyme in the impairment of SPDV infection remains to be proved but Siwicki et al. [46] demonstrated that a treatment with exogenous lysozyme leads to a significant protection of salmonids against a viral infection by stimulating lymphocyte proliferation and antibody secretion.

The increase in plasma complement activity following the SPDV infection is in contradiction with the complement consumption model [47]. According to this model, the mobilisation of the complement components in the course of an infection is usually accompanied by a decrease in the CH50 values. On the contrary, in the first 9 days following the inoculation of SPDV, no significant difference in the complement activity was observed between the control and the infected fish and, by 16 dpi, the infected fish even displayed a significantly higher CH50 value. These results suggest that high amounts of complement components were secreted, in particular in the late phase of the viral infection, mirroring the inflammatory process in the pancreas, as for lysozyme. Indeed, mononucleated phagocytes are known to be an important source of these components in mammals [48]. The 7 day delay between the onset of the inflammation and the peak of complement activity may result from the time required for the synthesis of these components and their secretion into the general circulation. This significant production of complement by 16 dpi indicates that high amounts of components are available in the blood and that they may therefore contribute to the reduction of the viraemia through the envelopment of SPDV in a coating of proteins, which is considered as a major mechanism of viral neutralisation [49]. Thus, complement may act in combination with the neutralising antibodies to facilitate the clearance of SPDV in the blood.

In conclusion, the most reliable features of PD were produced in Atlantic salmon parr using the P42p isolate of SDPV. In parallel to the well-known pathological changes in the target organs, the strong pancreatic inflammation was shown to be accompanied by a more systemic stimulation of immunity, both non-specific and specific. The results also suggest that this overall immune response is correlated to the resolution of the histopathology and also
to the reduction of the viraemia. An eventual vaccine against PD may be
designed not only to induce the production of neutralising antibodies but also
to stimulate the cell-mediated anti-viral mechanisms of the fish.

References
An exocrine pancreas disease of farmed Atlantic salmon in Scotland. Helgoländer
Meeresuntersuchungen 37, 571–586.
(Salmo salar) postsmolts infected with Infectious Pancreatic Necrosis Virus (IPNV).
Bulletin of the European Association of Fish Pathologists 9, 83–85.
(1992). The sequential pathology of pancreas disease in Atlantic salmon farms in
Ireland. Journal of Fish Diseases 15, 401–408.
salmon: proceedings of a European Commission Workshop. Scottish Office
Aquaculture report 1, 2–4.
Development of a computerized information retrieval system for Atlantic salmon,
7 McLoughlin, M. F., Nelson, R. T., McCoy, M. A., Kennedy, D. G., Rice, D. A.,
Atlantic salmon—Current state of knowledge in Ireland. Fish Veterinary Journal 1,
21–27.
Severe degenerative cardiomyopathy associated with pancreas disease in Atlantic
salmon, Salmo salar L. Journal of Fish Disease 20, 95–98.
disorders of salmonids, a diagnostic challenge. Bulletin of the European Association
of Fish Pathologists 17, 205–208.
(1995). Isolation of a toga-like virus from farmed Atlantic salmon Salmo salar with
Disease Virus, an alphavirus infecting farmed Atlantic salmon, Salmo salar L.
Virology 256, 188–195.
of salmon pancreas disease virus (SPDV) from farmed Atlantic salmon, Salmo salar
L., in Scotland. Journal of Fish Disease 21, 469–471.
14 Welsh, M., Weston, J., Borghmans, B. J., Mackie, D., Rowley, Nelson, R.,
model for pancreas disease of Atlantic salmon Salmo salar L. Aquaculture Research
26, 861–874.
neutralisation and passive immunisation. Fish & Shellfish Immunology 6, 465–472.
Experimental pancreas disease in Atlantic salmon Salmo salar post-smolts induced
by salmon pancreas disease virus (SPDV). Disease of Aquatic Organisms 26, 117–
124.


