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Pathology of Atlantic salmon *Salmo salar* intraperitoneally immunized with oil-adjuvanted vaccine. A case report

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ABSTRACT: Gross and histopathological lesions in farmed Atlantic salmon *Salmo salar* L. immunized by intraperitoneal injections of oil-adjuvanted vaccines are reported. A few fish farms report serious side effects leading to poor feed uptake and conversion, retarded growth and downgrading at slaughtering due to severe adhesions between abdominal organs and body wall. The lesions consist of granulomatous tissue adhering to and embedding different visceral organs in such a way that normal functions may become severely affected. Histologically, the tissue is characterized by granulomas embedded in a fibrous granulation tissue with high numbers of eosinophilic granule cells. Possible explanations for the occurrence of severe lesions at some farms are discussed.

KEY WORDS: Oil-adjuvanted vaccine side effects · Adhesions · Histopathology · Vaccination · *Salmo salar*

INTRODUCTION

Severe disease outbreaks, primarily caused by severe bacterial septicaemias like cold-water vibriosis *Vibrio salmonicida* and furunculosis *Aeromonas salmonicida* subspecies *salmonicida*, caused considerable losses in Norwegian fish farming throughout the 1980s. Since the early 1990s, the significance of these diseases has dramatically reduced. As a consequence the percentage of fish treated with antibiotics in Norwegian fish farms has been reduced from 60% in the years 1981–1988, to 2.3% in 1994 (Grave et al. 1996). Several factors have contributed to this positive development, e.g. segregation of year classes, mandatory health control and certification and disinfection of processing water from slaughtering plants. There can be little doubt that the most significant factor in reducing outbreaks is the extensive vaccination programs that have been carried out. In particular, the introduction of oil-adjuvanted vaccines for immunization against furunculosis and other septicaemic diseases caused by Gram-negative bacteria has shown good effect and reduced the occurrence of furunculosis both experimentally (Erdal & Reitan 1992, Midtlyng et al. 1996) and in the field (Midtlyng 1996).

In a few cases, the use of oil-adjuvanted vaccines has also led to considerable side effects, as some farms report up to 50% downgrading at slaughter due to extensive adhesions between individual visceral organs or between visceral organs and the body wall and to indications of retarded growth (Breck unpubl. data). Although visceral adhesions associated with vaccination are well known and accepted in fish (Horne et al. 1984, Lillehaug et al. 1992, Mulvey et al. 1995), the lesions reported here are far more extensive and severe than those hitherto described (Midtlyng 1994, Midtlyng et al. 1996). In this study, gross and histopathological appearance of these lesions in fish from an affected farm are described.

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MATERIALS AND METHODS

Preliminary studies indicated that at least 3 sea farms had experienced severe side effects with almost identical lesions. One of those, located on the west coast of Norway, was chosen for this study. This farm had reported retarded growth and unacceptable lesions in the abdominal cavity in approximately 60% of the fish vaccinated with a particular commercial vaccine. A total of 183 000 fish had been intraperitoneally injected (i.p.) with 0.2 ml multivalent vaccine (*Aeromonas salmonicida* subspecies *salmonicida*, *Vibrio anguillarum* and *Vibrio salmonicida*) adjuvanted with a mixture of vegetable and animal oils from February 7 to March 10, 1995, a minimum 8 to 9 wk prior to sea-transfer. The weight of the fish at the time was from 44 to 144 g (average 95 g), and the fish were vaccinated on an automatic vaccination table using 0.7 mm diameter needles. The temperature at the time of immunization was 2.6 to 3.0°C, while the sea temperature varied from 8.5°C at sea-transfer in May to a maximum of 14°C in September until a minimum of 2°C was reached in February. The fish were fed a standard, commercial dry pellet feed. The population had experienced an outbreak of infectious pancreatic necrosis (IPN) with a 7% mortality the first summer in sea water and a moderate infestation of salmon lice *Lepeophtheirus salmonis* in the autumn but had not been treated.

For this study 270 fish were examined in March 1996, approximately 10 mo after sea-transfer. Samples of fish were randomly netted from 5 cages with different size gradings, all fish of same origin, and killed by a blow to the head followed by severance of the gill arteries. The length and weight of each fish were recorded before the fish were opened for necropsy and the side effects scored on a scale from 0 to 6, where 0 represents fish with no visible lesions, and 6 represents fish with severe and widespread adhesions (Midtlyng et al. 1996). In 10 fish, tissue from areas with extensive adhesions was fixed in a solution of methanol, formalin and acetic acid in the volume ratio 85:10:5 (MFAA) (Mayrhofer 1980, Reite 1996) for 72 h. Internal organs (kidney, liver, spleen, heart, gills) were fixed in 10% buffered formalin. All fixed samples were subjected to standardized histological techniques and stained with haematoxylin and eosin (H&E) and van Gieson.

RESULTS

Gross pathology

The weight of the fish was 1085 g ± 635 SD (range 200 to 3255 g). Most fish appeared normal in shape, but some of the smaller individuals developed a down-pouching of the anterior part of the abdomen which was firm at palpation (Fig. 1).

Adhesions

Of the fish examined 253 (93.7%) showed grossly visible adhesions in the abdominal cavity (Table 1). A

Fig. 1. *Salmo salar*. Vaccinated fish showing extensive fusions between visceral organs and discolouration. Length of fish is 30 cm

total of 171 fish (63.3 %) had lesions that were regarded as serious (a score of 3 or higher). As shown in Table 2, the lesions could be found in several locations in the abdominal cavity, but occurred most frequently in the following 3 locations: (1) dorso-anteriorly near the septum transversum and oesophagus; (2) around the spleen and pyloric caecae (near the injection site); (3) in the caudal portion, along the decending intestine and near the urinary bladder.

On all locations, the organs were attached to the neighbouring organs and to the abdominal wall by strong strands. In some cases, granulomatous response was up to 20 mm in thickness, which made it difficult to physically separate the various organs from the abdominal wall. Although the colour of the adhesions varied, most were black and typically a 'print' of the lesions could be found on the peritoneal wall (Fig. 2). Lesions in the dorso-anterior portion of the abdominal cavity usually adhered to the septum transversum, swimbladder and body walls and often engulfed the oesophagus, stomach, liver and gonads into one solid mass. In a few cases, remnants of whitish oil leaked from the cut surface of the adhesions.

Table 1. *Salmo salar*. Adhesion score for 270 vaccinated Atlantic salmon. Scale ranges from 0 (fish with no visible lesions) to 6 (fish with serious and widespread lesions). No fish with the score 6 were found

	0	1	2	3	4	5	Total
No. of fish	17	27	55	85	74	12	270
Percent	6.3	10.0	20.4	31.5	27.4	4.4	100

Table 2. *Salmo salar*. Location of adhesions in 270 vaccinated Atlantic salmon. DW: dorsal wall, LW: lateral wall

Location of lesion	Not present N	%	Present N	%	Total N	%
Stomach/DW	267	98.9	3	1.1	270	100
Stomach/LW	269	99.6	1	0.4	270	100
Liver/DW[a]	165	61.1	105	38.9	270	100
Liver/LW	259	95.9	11	4.1	270	100
Pancreas/DW	253	93.7	17	6.3	270	100
Pancreas/LW[b]	82	30.4	188	69.6	270	100
Spleen/DW	270	100.0	0	–	270	100
Spleen/LW[b]	89	33.0	181	67.0	270	100
Posterior gut/DW	269	99.6	1	0.4	270	100
Posterior gut/LW	225	83.3	45	16.7	270	100

[a]Including oesophagus and/or gonads; [b]Near injection site

Organs

Fish with none or few lesions had normal gut contents consisting of more or less digested pellets. Fish with severe lesions, especially in the anterior part of the abdominal cavity, had fine-granular contents, often mixed with considerable amounts of small, orange-coloured or pink crustaceans 1 to 2 mm in length. The contents in the posterior portion of the gut tended to be more yellowish or whitish than in fish feeding normally. The colour of the flesh was normal in all investigated fish. The swimbladder was frequently constricted in one or several locations, making the diameter of the organ uneven.

Fig. 2. *Salmo salar*. Severe adhesions with discolouration in the anterior and posterior portion of the abdominal cavity

In fish where the lesions were extensive in the dorso-anterior portion of the abdominal cavity, the gonads were usually involved in the lesions, being more or less engulfed by fibrous tissue and impossible to separate from the oesophagus and stomach (Fig. 3).

In the liver and kidney of a few fish, multiple light grey or greenish nodules up to 1 mm in diameter could be seen on the surface and also to some extent penetrating deeper into the organ. In several fish, the pyloric caecae were embedded into a compact dark mass of hard tissue.

Fish with extensive lesions in the posterior abdominal cavity typically had thickened and sometimes cystic ureters.

Histopathology

Adhesions

Histologically, the adhesions were characterized by extensive proliferation of fibroblasts, forming a dense, tumorous-like, well-vascularized fibrous matrix. This tissue frequently was arranged in sworls or loose granuloma-like formations with margins which stained red with van Gieson. Large empty vacuoles with an even margin were freqently found in the centre of these loose granulomas (Fig. 4). The dominating cell types, besides the fibroblasts, were lymphocytes and oval eosinophilic cells with a granular cytoplasm and eccentric nucleus. In some cases, the granular contents of the cell had coalesced, making the cytoplasm of the cell a homogenous, eosinophilic mass. In some areas, these eosinophilic cells constituted up to 50% of the total cell area (Fig. 5). In addition, macrophage activity, including melanomacrophages, was extensive in some areas.

Organs

In fish with macroscopically visible liver lesions, a multiple, granulomatous response was seen scattered throughout the hepatic parenchyma. The majority of the granulomas contained several multinucleated giant cells, frequently with an amorphous yellowish/brownish content. The central veins and sinusoids appeared dilated and congested in most fish.

Pancreatic tissue was partly surrounded by and infiltrated with fibrous tissue, macrophages and eosinophilic granular cells, quite similar to that described for the larger fibrous adhesions. In some fish, there were signs of pancreatic regeneration with small islets of pancreatic tissue in the middle of large, fibrous masses (Fig. 6). Infiltrative growth with fibrous tissue and granulomatous response was seen in the muscle and kidney of a few fish, adjacent to lesions in the abdominal cavity. The gonads were frequently surrounded by fibrous tissue that usually also infiltrated into the organ. No lesions were found in the gills, heart and spleen of investigated fish.

DISCUSSION

Adhesions in the abdominal cavity of mammals after injury or irritation of foreign particles is well-known and the mechanisms for such formations have been

Fig. 3. *Salmo salar*. Cut surface of ovaries adherent to masses of granulomatous tissue

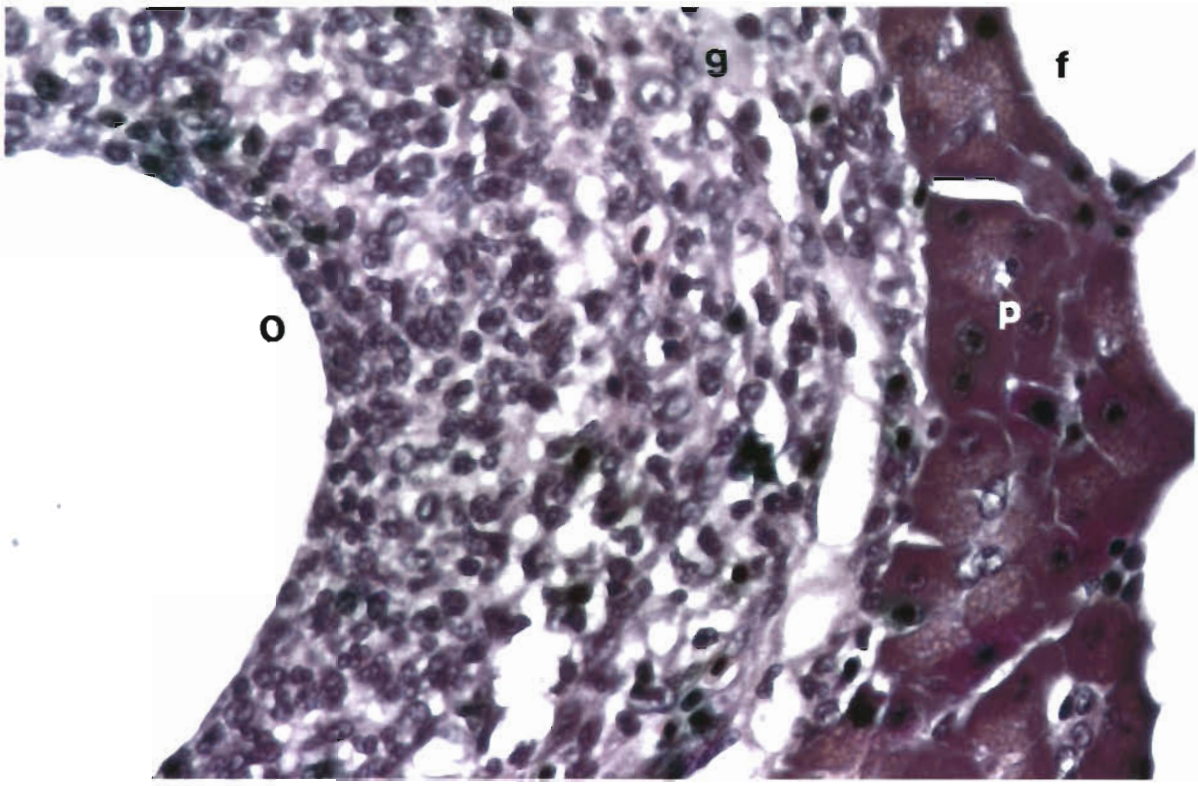

Fig. 4. *Salmo salar*. Fibrous tissue surrounding an oil droplet in vaccine lesion adjacent to the pancreatic area. H&E, ×400. o: oil droplet, p: pancreatic tissue, f: perivisceral fat, g: granulation tissue

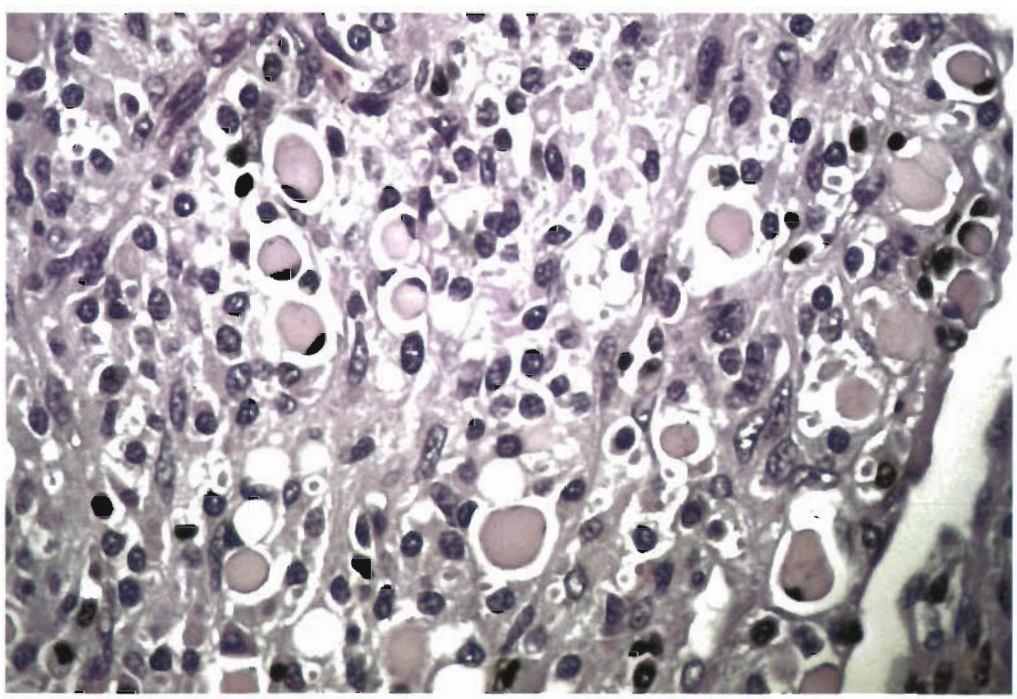

Fig. 5. *Salmo salar*. Eosinophilic cells in granulation tissue adjacent to the pancreatic area. H&E, ×400

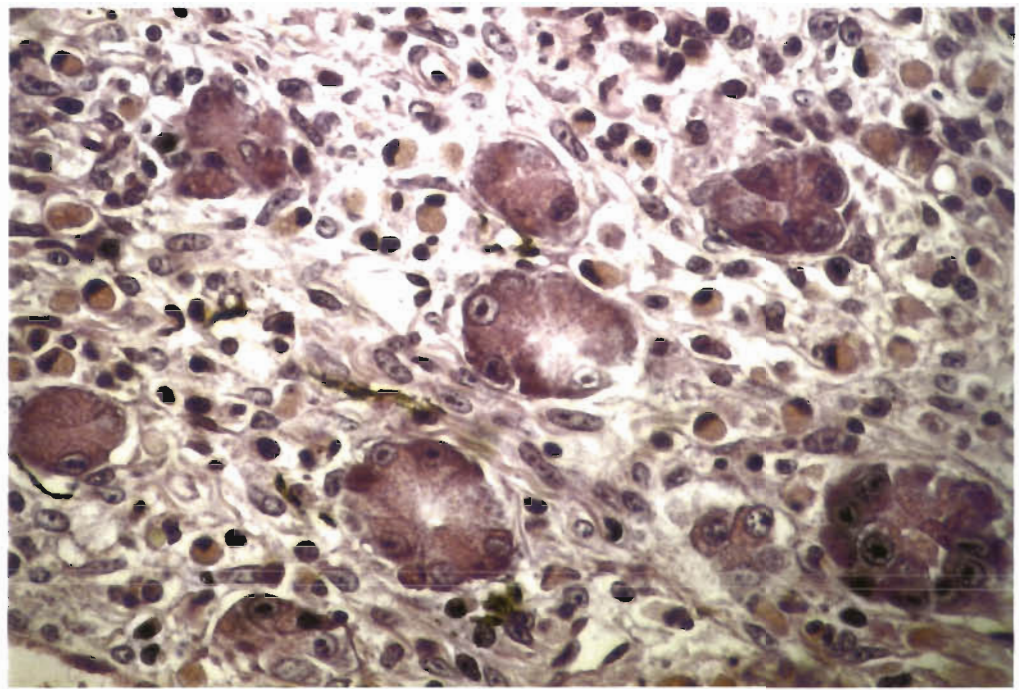

Fig. 6. *Salmo salar*. Islets of pancreatic tissue in fibrous masses. Numerous eosinophilic granular cells are also present. H&E, ×100

described (Menzies 1992). In recent years, post-vaccination fibrosarcomas in cats immunized with adjuvanted rabies- and feline leukemia virus has received considerable attention (Hendrick et al. 1994, Doddy et al. 1996). Thus, it is hardly surprising that adjuvanted vaccines may cause different negative side effects in fish (Horne et al. 1984, Lillehaug et al. 1992, Midtlyng 1994, Mulvey et al. 1995). The combination of bacterins and oils used in modern fish vaccines for i.p. injection initiate a local inflammatory response with protective effects concurrently with a prolonged stimulation of the immune system due to the slow release of antigens (depot effect) from the oil (Midtlyng et al. 1996). Adhesions due to inflammation after i.p. immunization with oil-adjuvanted vaccines is now a consistent finding in farmed salmon and can therefore be used as one of several criteria for differentiation between wild salmon and salmon escaped from fish farms (Lund et al. 1995). What is surprising in the above-mentioned cases, is the extent and severity of the lesions. Until recently, the side effects of this particular vaccine have been acceptable and similar to other oil-adjuvanted fish vaccines. During the past year, reports of serious side effects like growth retardation, increased unspecific mortality and downgrading at slaughtering due to extensive adhesions have increased. Interestingly, only a few farms report these serious side effects, in spite of the fact that a large number of farms have used the same vaccine; most report good protection and very limited side effects.

In this respect, this condition has many similarities to the postvaccinal fibrosarcomas in cats mentioned above (Hendrick et al. 1994, Doddy et al. 1996).

The severity of the lesions raises the question of the function of the organs affected. There can be little doubt that the lesions seen in the most severely affected fish interfered to a considerable extent with normal feed uptake and gastrointestinal motility. The lesions in the anterior part of the abdominal cavity compressed the oesophagus and made the passage of feed pellets difficult or impossible. This is also reflected in the stomach contents of these fish, which usually contained only fine-granular particles and planktonic crustaceans in contrast to normal fish where more or less intact feed pellets filled the stomach. This alone can probably explain the growth retardation reported by the farmers in these fish. To what extent the lesions in the middle and posterior portion of the abdominal cavity physically interfere with digestion is harder to acertain, but the normal motility of the stomach and gut must be severely compromised in some cases. The dorso-anterior location of the lesions has an obvious potential for interference with cardiovascular and swimbladder function as discussed by Mulvey et al. (1995). In a separate study on a similar material (Poppe unpubl. data), 50 out of 97 fish had severe lesions in the dorso-cranial portion of the abdominal cavity with the gonads uni- or bilaterally embedded in a fibrous matrix making normal gonadal developement impossible. These lesions will without

doubt make these fish unsuitable as broodfish. This location also shows that the vaccine is transported from the injection site to different parts of the peritoneal cavity and that the 'depot effect' of the vaccine may last up to 1 yr.

Lesions of a type similar to those described in this paper are known from early trials, but the extent and severity of these lesions may seem difficult to explain. The growth of the fibrous granulation tissue is rather aggressive and may almost seem tumorous as is reflected by the fact that lesions also occur in kidney, liver and muscle. Their disseminated occurrence indicates spread through growth or via blood and/or macrophages, rather than from injection of the vaccine into the organ per se. This is in accordance with the results obtained by Press et al. (1996), where the presence of lipopolysaccharides (LPS) in the bacterin was demonstrated in macrophages in the liver, kidney and spleen. The abundant eosinophilic granule cells in the fibrous matrix may be mast cells. There is evidence that mast cells of many teleosts, including Atlantic salmon, have both eosinophilic and basophilic components in their granules, and the nature of the fixative, embedding and staining procedures will determine whether these cells turn out to be 'mast cells' or 'eosinophilic granule cells' (Reite & Evensen 1994, Reite 1996). The alcohol-based fixative (MFAA) used for the granulation tissue in the present study preserves the structure of the granule cells better than formalin (Reite & Evensen 1994). Although the function and significance of these multipotent defence cells is not fully known, their abundant occurrence in the lesions clearly indicate that they play an important role in the inflammatory process in the lesions (Sveinbjørnsson et al. 1996). In some of the affected farms, including the one described in this paper, the fish developed extensive black lesions, while in other farms they were typically off-white or light grey. The explanation for this great difference in melanomacrophage activity is not known.

The intriguing fact that the same vaccine results in dramatically different side effects, both between farms and within the same population, is yet to be explained. The size of the fish and the temperature at the time of vaccination and thereafter are factors known to influence the development of protective immunity (Lillehaug et al. 1993). The importance of these variables for protective immunity has diminished with the introduction of oil-adjuvanted vaccines that provide a slow release of antigens over a prolonged period. The presence of oil droplets in the lesions more than 1 yr after immunization clearly shows that the vaccine components may provoke an ongoing granulomatous process. However, the importance of these factors for the possible development of side effects is not known. The occurrence of severe lesions at only a few farms could also indicate alterations in the composition of the adjuvant or bacterin of a particular batch of vaccine, as all the affected farms have used the same batch of vaccine. Finally, a concurrent latent infection could enhance the response of the fish causing unpredictable lesions. No bacteriological tests were done in this study, but former attempts to culture microorganisms from the lesions in severely affected fish in this and other farms have failed to show any bacterial involvement. The possible role of a concurrent viral infection (e.g. IPN) is yet to be elucidated.

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