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CpG DNA Induces Protective Antiviral Immune Responses in Atlantic Salmon (*Salmo salar* L.)

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Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides within specific sequence contexts (CpG motifs) are detected, like bacterial or viral DNA, as a danger signal by the vertebrate immune system. CpG ODN show promise as vaccine adjuvants and immunoprotective agents in animal models. Here we report that pretreatment with CpG ODN in animals induces nonspecific protection against viral infection. A panel of different synthetic CpG ODN was tested for the in vitro effects in Atlantic salmon (*Salmo salar* L.) leukocytes. The ODN were tested for their capacity to stimulate proliferation of peripheral blood leukocytes and to induce production of interferon-like factors in head kidney leukocytes. These studies revealed that the sequence and number of the CpG motifs as well as the lengths of the ODN contribute to their stimulatory activity. ODN with the 6-mer CpG motif (5′-GTCGTT-3′) showed the highest stimulatory activity and were shown to induce protection against infectious pancreatic necrosis virus when injected in Atlantic salmon. Expression of the Mx transcript, as an indicator of alpha/beta interferon induction, was induced in the CpG-injected fish. These results suggest that CpG DNA in fish induces early, nonspecific antiviral protection.

Unmethylated cytosine-guanidine dinucleotides within the context of certain flanking sequences (CpG motifs), as originally defined for bacterial DNA, have diverse stimulatory effects on the innate and adaptive immune systems (reviewed in reference 31). In mammals several of these effects contribute to strong Th1-type adjuvant activity for antigen-specific responses. CpG DNA triggers B cells to proliferate and secrete immunoglobulins and cytokines, both of which contribute to stronger humoral responses (30, 34). CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete various Th1 cytokines (55–57), which in turn activate T and NK cells to secrete a broad range of cytokines (2, 6, 60). The recognition of CpG motifs by the innate immune system requires engagement of Toll-like receptor 9, which induces cell signaling and subsequently triggers an immune response (17). Several studies have documented the potent adjuvant activity of CpG DNA in vaccines with various antigens, including viral antigens (8, 19, 20, 36, 47), and with DNA vaccines (48, 53). Additionally, CpG DNA has been shown to provide nonspecific protection and induce resistance to lethal challenges with the intracellular bacteria *Listeria monocytogenes* (33) and *Francisella tularensis* (11). CpG oligodeoxynucleotides (ODN) have been shown to be locally protective against herpes simplex virus type 2 in mice (14), but there are no published reports of the systemic protective effects of CpG ODN against viral infections in animals.

Recently published studies have shown that CpG DNA activates fish leukocytes. Synthetic ODN containing CpG motifs induce production of interferon (IFN)-like cytokine activity in salmonid leukocytes (24, 25), stimulate interleukin-1 production in rainbow trout (*Oncorhynchus mykiss*) macrophages (25), and activate nonspecific cytotoxic cells from channel catfish (*Ictalurus punctatus*) (46). The strong activating effect of CpG DNA on fish leukocytes suggests the use of CpG ODN as an immunostimulant in fish.

The economically important pathogen infectious pancreatic necrosis virus (IPNV), an aquabirnavirus, was chosen as the model virus, because its replication has previously been shown to be inhibited by IFN (22, 41). IPNV causes a serious disease, acute infectious pancreatic necrosis (IPN), in salmonids (54). IPNV has a naked capsid that encloses two segments, A and B, of double-stranded RNA (9). Segment A encodes three known gene products within one large open reading frame, including the two major capsid proteins VP2 and VP3 (9). For salmonids, mortality due to IPNV is generally considered to be a fry disease, but natural outbreaks of IPN in older fish are also reported (54). Considerable efforts have been made to develop traditional vaccines based on killed virus as well as vaccines based on recombinant DNA technology. Recombinant segment A and VP2 have been shown to induce protective immunity when injected in fish (7, 42). A successful prophylactic strategy to combat viral diseases like IPN in fish farming relies both on innate immune responses, like cytokines and natural killer cells, and on specific responses, like antibodies and cytotoxic T cells.

In the present study, we tested whether defense mechanisms against viral infections could be up-regulated by in vivo treatment of fish with CpG ODN. The optimal CpG motif which activates mouse or rabbit immune cells has the general formula purine-purine-CG-pyrimidine-pyrimidine, and the best CpG motif is found to be GACGTT (34, 49), while for activating human and primate cells, the optimal motif is GTCGTT (13, 15, 16, 32). Since fish cells may respond to CpG motifs different from those of previously tested animals, a panel of synthetic ODN was examined for their ability to stimulate Atlantic salmon leukocytes in vitro. The ODN giving the strongest in vitro stimulatory effect was injected into fish, and IPNV resistance was tested. Furthermore, we tested whether this resis-

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tance correlated with expression of the Mx gene, which is suggested to be a marker for IFN-α/β (45).

MATERIALS AND METHODS

Materials. Phosphothiorate-modified ODN were purchased from MedProbe (Oslo, Norway). The ODN sequences used are provided below (see Fig. 2). The ODN were dissolved in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]). The synthetic double-stranded RNA poly(I-C) was purchased from Pharmacia Biotech; lipopolysaccharide (LPS; from *Escherichia coli* O111:B4), bacterial DNA (from *E. coli* strain B), and calf thymus DNA were from Sigma.

Fish for in vitro studies. Two-year-old nonvaccinated Atlantic salmon, *Salmo salar* L. strain NLA (Norwegian Salmon Breeding, Sunndalsøra, Norway), weighing approximately 0.5 kg, were obtained from the Norwegian Institute of Fisheries and Aquaculture (Aquaculture Research Station, Tromsø, Norway). The fish were kept at 10°C in tanks supplied with running fresh, filtered water from a local river and fed commercial dry feed (Skretting, Stavanger, Norway).

Proliferation. Atlantic salmon peripheral blood leukocytes (PBL) from six fish were used for cell proliferation studies, and the cells were isolated as previously described (50). Aliquots (100 μl) of RPMI medium (Gibco) with 5% fetal calf serum (FCS; Gibco) containing 8×10^5 leukocytes were added to 96-well tissue culture plates (Nunc) in the presence of an additional 100 μl of synthetic ODN, LPS, bacterial DNA, or calf thymus DNA at different concentrations; cultures were prepared in triplicate for each treatment. The cell cultures were incubated for 6 days in 5% CO_2 at 14°C. The cells were pulsed with [^{3}H]thymidine 18 h prior to harvest, and the uptake of radioactivity was determined by a liquid scintillation harvester. The stimulation index was determined as the ratio of [^{3}H]thymidine incorporation in CpG ODN-stimulated cells to that in cells incubated in medium alone. A Student's two-tailed *t* test was used to determine the statistical significance of the results. Differences were considered statistically significant when P was <0.05.

Production of IFN-containing supernatants. Atlantic salmon HK leukocytes were obtained as described previously (12), with modifications of the Percoll densities. Briefly, the head kidneys were removed from the fish and passed through a 100-μm-pore-size cell strainer (Falcon) in L-15 medium containing penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}), 2% FCS, and heparin (20 U ml^{-1}). The resulting cell suspensions were placed on a 25 to 54% Percoll gradient and centrifuged at $400 \times g$ for 40 min at 4°C. The interface was collected and washed twice in L-15 medium. The leukocyte suspensions were seeded at a density of 1.5×10^6 cells per well in 96-well microtiter plates. Leukocytes from six fish were stimulated with ODN (2 μM) in L-15 medium containing penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and 5% FCS. Controls were leukocytes incubated with medium alone. At 2 days poststimulation, the supernatants were collected individually, pooled, and centrifuged at $400 \times g$ for 30 min.

IFN assay. IFN was assayed by inhibition of the cytopathic effect of IPNV on Chinook salmon embryo-214 (CHSE-214) cells. CHSE-214 cell monolayers in 96-well tissue culture plates (Nunc) were incubated for 24 h with various dilutions of IFN supernatants in Eagle's minimum essential medium (Gibco) containing 2% FCS and then challenged with IPNV (strain Sp) at a multiplicity of infection of 0.01. Controls included CHSE-214 cells exposed to the medium throughout (cell control) or exposed to the medium and then challenged with IPNV (virus control). All supernatants were tested in six replicates. After 3 days, a cytopathic effect was evident in the infected, untreated control cells, and the cells were fixed and stained with 1% crystal violet in 20% ethanol. The cell monolayer was then washed with tap water three times and eluted in 0.05 M sodium citrate–0.05 M citric acid–50% ethanol. The extent of virus-induced cytolysis was quantified by reading the optical density at 560 nm (OD_{560}) in a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corporation). IFN-like activity in the supernatants was calculated as previously described (51), with 1 U of IFN-like activity defined as the reciprocal dilution of leukocyte supernatant per milliliter at which 50% protection against virus infection was obtained.

Treatment of Atlantic salmon with ODN and poly(I-C). Nonvaccinated Atlantic salmon, *Salmo salar* L., strain NLA, reared in a hatchery at the Aquaculture Research Station, Tromsø, Norway, and weighing approximately 50 g, were used in two experiments. The fish were kept in tanks supplied with running fresh water at 10°C, exposed to continuous light, and fed commercial dry feed at the onset of the experiments. Head kidney material from 20 fish was tested for the presence of carrier IPNV as described by Hetrick (18) and found to be virus negative prior to treatments and challenges. Treatments were done with fish in the presmolt stage 1 week before transfer to seawater. In experiment 1, duplicate groups of 65 fish were injected intraperitoneally (i.p.) with 0.2 ml of poly(I-C) (100 μg per fish), non-CpG ODN control (100 μg per fish), or CpG ODN at concentrations of 1, 10, 50, and 100 μg per fish. Saline-injected fish were used as controls. In experiment 2, groups of fish were injected i.p. with 0.2 ml of poly(I-C) (100 μg per fish; 150 fish), non-CpG ODN control (100 μg per fish; 150 fish), or CpG ODN (50 μg per fish; 83 fish). Saline-injected fish (150 fish) were used as controls.

IPNV challenge of Atlantic salmon. Groups of Atlantic salmon smolts treated with CpG ODN and poly(I-C) were challenged by allowing them to bathe in water infected with IPNV (bath challenged) 1 week after treatment, at the time of transfer to seawater, as described by Johansen and Sommer (23). An IPNV isolate of the Sp serotype, passed once in the CHSE-214 cell line, was used for the challenges. In experiment 1, duplicate groups of about 65 fish were bath challenged for 3.5 h at virus concentrations of $10^{5.46}$ 50% tissue culture infective doses ($TCID_{50}$) per milliliter. In experiment 2, 70 fish from each group in duplicate tanks (A and B) were bath challenged, except for the CpG ODN group, in which 73 fish were put into tank A only. At the same time, 10 fish from each group to be used for sampling were bath challenged in a third tank, tank C. The challenge dose in experiment 2 was $10^{5.31}$ $TCID_{50}$ ml^{-1}, and the challenge lasted for 3.5 h. After being challenged, the fish in both experiments were held in tanks receiving UV-treated seawater (at 10°C) and fed commercial dry feed. Mortalities were monitored daily for about 5 weeks. An IPNV rapid agglutination kit was used on head kidney samples from dead fish to verify IPN as the cause of death (58). Mortality results from replicates with no statistically significant differences were pooled. A chi-square test was used to identify differences among replicates and treatments. The relative percent survival (RPS) was calculated by the following formula: [1 − (% cumulative mortality of the treated group/% cumulative mortality of the control group)] × 100.

IPNV titration. In experiment 2, five fish from each group in tank C were sacrificed 7 and 21 days after IPNV challenge and head kidneys were sampled and frozen at −80°C for later IPNV titer tests. The samples were homogenized in Eagle's minimum essential medium with 2% FCS, and titers were determined by end-point dilutions carried out in four replicates on CHSE-214 cells in 96-well plates (Nunc). The lowest detectable level of IPNV was $10^{2.25}$ $TCID_{50}$ g^{-1} of tissue.

RNA extraction and Mx-specific reverse transcription-PCR. The spleens and head kidneys were removed from the fish at 24 to 96 h after injection with poly(I-C), non-CpG ODN control, or CpG ODN 1681, each at a concentration of 100 μg per fish (three or four fish per group). The organs were stored in RNAlater (Ambion, Austin, Tex.) until RNA isolation. Total RNA was extracted with Trizol reagent (Gibco BRL, Roskilde, Denmark), quantified by measuring the absorbance at 260 nm, and then treated with RNase-free DNase I to remove all contaminant DNA. Synthesis of cDNA was made from 5 μg of total RNA in a 20-μl volume by using Superscript II reverse transcriptase (Gibco BRL). Eighty microliters of water was then added, and the samples were stored at −20°C until PCR analysis. The primers, Mx-forward (5′-TACATGATAGTCAAGTGCAG-3′) and Mx-reverse (5′CCTGGGAGCTCCCTTCCACG-3′), were designed to specifically amplify a 514-bp Mx fragment. Primers specific to rainbow trout β-actin, actin forward (5′-AGA GCT ATG AGC TGC CTG ACG GAC 3′) and actin reverse (5′-CAG GGC TGT GATY CTC CTT CTG C-3′), were used as internal PCR controls and gave a 247-bp fragment. Amplification was performed in 25-μl reaction volumes containing the REDTaq ReadyMix PCR reaction mix (Sigma), with $MgCl_2$ (2.5 mM), 2.5 μl of template, and reverse and forward primers (0.1 μM). Briefly, PCRs were performed at 94°C for 3 min and then at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min for 25 to 30 cycles, with a final extension at 72°C for 7 min. PCRs for quantification purposes were stopped in the exponential phase of amplification, and tests were performed on serial dilutions of templates as previously described (4). The total amount of cDNA was calibrated on the basis of the amplification of actin cDNA from the same template, and PCR products were analyzed by agarose gel electrophoresis and quantified by image analysis (Image Station; Kodak). Mx expression data were analyzed using a two-way analysis of variance, followed by a Scheffé post hoc hypothesis test, as described by Zar (61). The distribution of residuals was used to evaluate the applicability of statistical methods.

RESULTS

Specific ODN induce proliferation of Atlantic salmon leukocytes. In initial experiments, the mitogenic effects on Atlantic salmon PBL of ODN 1668 (the optimal murine CpG motif) and ODN 1681 (the optimal human CpG motif) and the reversed GpC ODN (ODN 1740) as a control were examined. Stimulation of the PBL with CpG DNA led to a dose-depen-

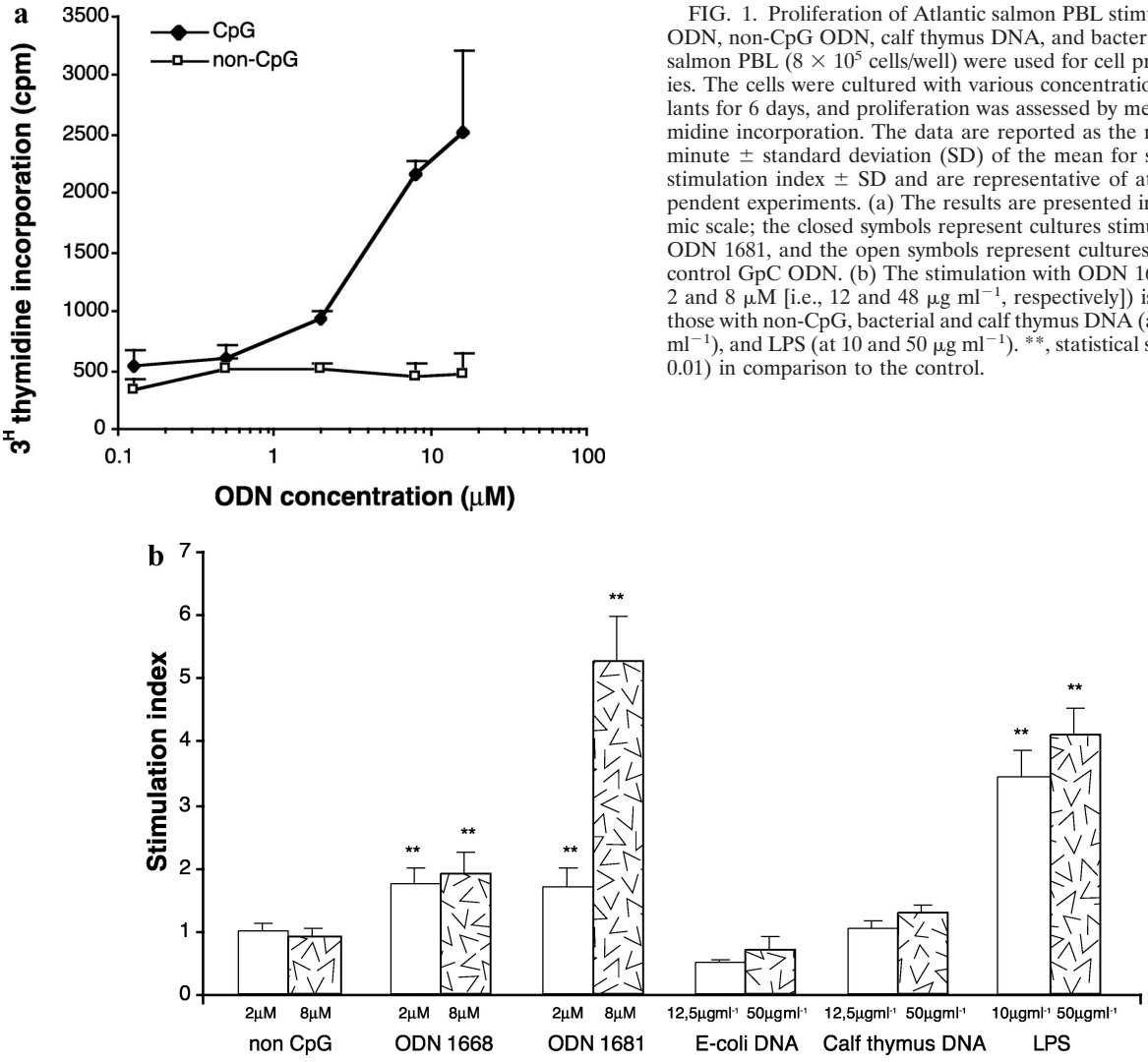

FIG. 1. Proliferation of Atlantic salmon PBL stimulated with CpG ODN, non-CpG ODN, calf thymus DNA, and bacterial LPS. Atlantic salmon PBL (8×10^5 cells/well) were used for cell proliferation studies. The cells were cultured with various concentrations of the stimulants for 6 days, and proliferation was assessed by measuring [^{3}H]thymidine incorporation. The data are reported as the mean counts per minute ± standard deviation (SD) of the mean for six fish or as the stimulation index ± SD and are representative of at least two independent experiments. (a) The results are presented in a semilogarithmic scale; the closed symbols represent cultures stimulated with CpG ODN 1681, and the open symbols represent cultures stimulated with control GpC ODN. (b) The stimulation with ODN 1681 and 1668 (at 2 and 8 μM [i.e., 12 and 48 μg ml^{-1}, respectively]) is compared with those with non-CpG, bacterial and calf thymus DNA (at 12.5 and 50 μg ml^{-1}), and LPS (at 10 and 50 μg ml^{-1}). **, statistical significance ($P < 0.01$) in comparison to the control.

dent proliferation (Fig. 1a), whereas the non-CpG control DNA was not stimulatory. ODN 1681 at 8 μM had a significantly better stimulatory ($P < 0.01$) effect for fish PBL than did ODN 1668 (Fig. 1b). Neither calf thymus DNA nor bacterial DNA at concentrations of 12.5 and 50 μg ml^{-1} stimulated proliferation of Atlantic salmon blood leukocytes. The stimulatory effects of LPS (at 10 and 50 μg ml^{-1}) were comparable to those of CpG DNA (Fig. 1b).

Screening of different ODN for the ability to stimulate IFN production and cell proliferation in Atlantic salmon leukocytes. Previous studies have tested ODN 1668 and 1670 with the murine motif (GA/AA)CGTT, and these ODN stimulated IFN-like cytokine activity in Atlantic salmon leukocytes (24, 25). In the present study a panel of different ODN was tested for both PBL proliferation and induction of IFN-like activity in HK leukocytes.

HK leukocytes were incubated with CpG ODN of various overall lengths and containing different combinations and numbers of CpG motifs. The data presented in Fig. 2 confirm that CpG motifs are the essential elements in ODN that activate leukocytes in fish, since elements in which the CpG are inverted to GpC lose stimulatory activity (for example, compare ODN 1720 and 1740 to ODN 1668 and 1681, respectively). In ODN 1670 there is a central core element, AAGCTT, but additionally there is one distal CG in the 5′ end and two distal CGs in the 3′ end. When the bases CG are inverted to GC, a reduction in stimulation is seen (Fig. 2, compare ODN 1670 to ODN 1682, 1671, 1673, and 1672). When the lengths of the ODN were reduced below 13 bp (compare ODN 1670 to 1675 and ODN 1660 to 1659), they lost their stimulatory effect. ODN with lengths of 16 to 17 bp showed a reduction in stimulatory capacity compared to the capacity of ODN with lengths of 20 to 22 bp (compare ODN 1670 to 1679).

Optimal activation of Atlantic salmon leukocytes, measured by both antiviral activity and proliferation, was seen with ODN 1669 and 1681 containing the 6-mer core element GTCGTT. When these ODN were compared to ODN with similar lengths and flanking regions but different 6-mer core motifs, reductions in stimulatory capacities were seen (Fig. 2, compare ODN 1681 to ODN 1670, 1680, 1674 and 1683 or ODN 1669 to ODN 1651, 1652, 1660, and 1668). The motif GTCGTT (present in ODN 1669 and 1681), previously reported to be

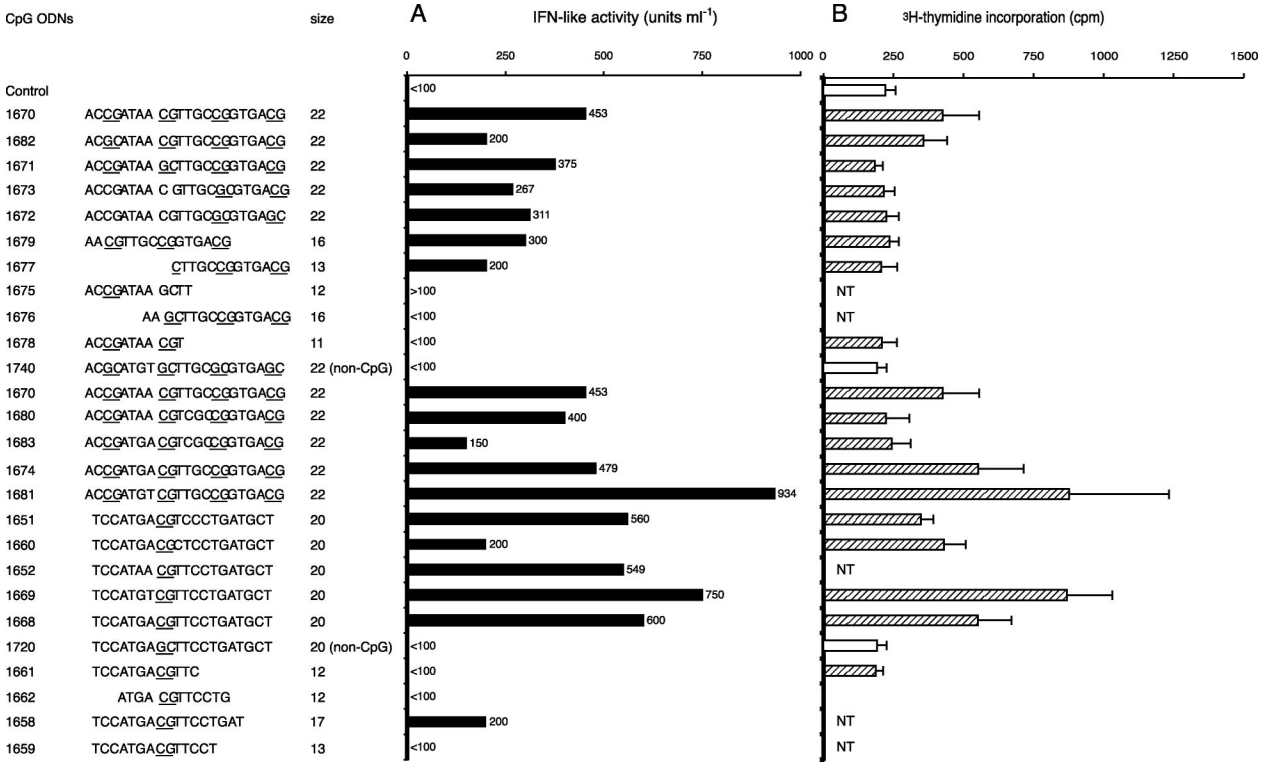

FIG. 2. Screening for the optimal sequence of phosphothiorate ODN to activate Atlantic salmon leukocytes. Leukocytes were incubated with different ODN (2 μM) with the sequences indicated (CG or GC dinucleotides are underlined). (A) Supernatants were harvested from HK leukocytes 48 h poststimulation, and antiviral activity was detected. The results are given as the mean IFN-like activity for pooled supernatants from six different fish and are representative of at least four different experiments. (B) Cell proliferation of Atlantic salmon PBL assessed by thymidine incorporation. The data are reported as the mean counts per minute ± standard deviation of the mean for six fish and are representative of at least two independent experiments.

optimal for human leukocytes (15), has a better stimulatory effect in fish than do the optimal murine motifs (GA/AA)C GTT.

Resistance to IPNV infection in Atlantic salmon injected with CpG ODN. To analyze whether immunostimulatory ODN such as ODN 1681 could induce protection against viral infections in fish, challenge experiments with IPNV were chosen as an experimental test system. In experiment 1, fish were injected i.p. with different doses of ODN 1681, and injections of poly(I-C), a known IFN inducer in fish, and saline were included for comparison. The fish, in two parallel tanks (A and B), were bath challenged with IPNV 1 week after injection and monitored daily for signs of disease and mortality. The fish started to die at day 10, and the experiment was stopped at day 34, about 1 week after the fish in the control group had ceased to die (Fig. 3). In the CpG ODN-treated groups, the fish started to die at days 10 and 11, while the poly(I-C)-injected fish showed a delay in onset of mortality and started to die at day 13. There were no statistically significant differences in mortality between the replicates of the saline group and groups of fish that had received 1, 50, and 100 μg of CpG ODN. Thus, results for these groups were pooled (Table 1). Fish which had received 50 and 100 μg of CpG ODN were significantly more resistant to IPNV infection than the saline-injected controls ($P < 0.05$, chi-square test), and the RPS values for these groups were 21 and 20%, respectively (Table 1). The lowest concentration of CpG ODN, 1 μg per fish, offered no significant protection compared to results for the control group, while the injection of 10 μg of CpG ODN and 100 μg of poly(I-C) gave significant protection in tank A ($P < 0.05$ and 0.001, respectively) but not in tank B.

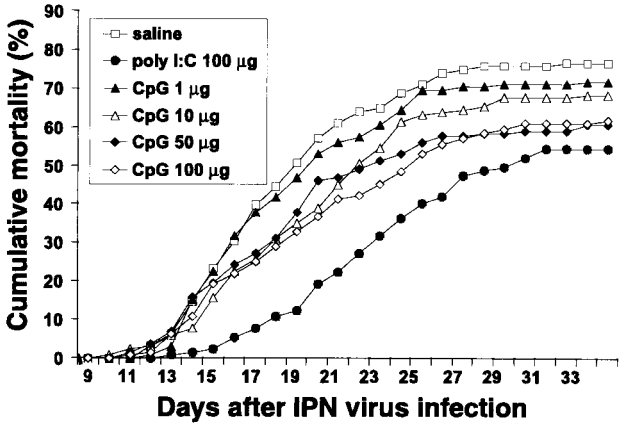

FIG. 3. Cumulative mortality for CpG ODN-injected Atlantic salmon challenged with IPNV at 7 days postinjection. The average mortality for duplicate tanks ($n = 70$ fish) is shown on the ordinate and is plotted against the number of days postchallenge.

TABLE 1. Dose-response study[a]

Treatment and dose	Tank	Cumulative mortality (%)	Mean cumulative mortality (%)	RPS	Mean RPS	Statistical significance (χ^2 test)[b]
Saline	A	75	77			
	B	78				
Poly(I-C), 100 μg	A	43	55	43	28	
	B	66		12		
CpG						
1 μg	A	67	72	11	6	n.s.
	B	77		1		
10 μg	A	58	69	23	11	
	B	79		−1		
50 μg	A	56	61	25	21	$P < 0.01$
	B	65		17		
100 μg	A	55	62	27	20	$P < 0.05$
	B	69		12		

[a] Cumulative mortality was calculated following IPNV challenge of CpG ODN- and poly(I-C)-injected fish 7 days postinjection. RPS = {1 − [% mortality of fish given CpG ODN or poly(I-C)/% mortality of fish given saline]} × 100.
[b] Statistical significance is based on a comparison to results for the saline group. n.s., not significant.

In experiment 2, groups of fish were injected i.p. with ODN 1681 and with non-CpG ODN 1720, poly(I-C), or saline as a control. The fish were placed in three tanks before challenge with IPNV. Tanks A and B with 70 fish per group were used to monitor mortality, while 5 fish from each group in tank C were sampled at days 7 and 21 postinfection and virus titers in the head kidneys of these fish were assessed. At day 7 postinfection, there were no mortalities in any of the groups in tank C (Table 2). No virus was detected in the CpG ODN- and poly(I-C)-injected fish, while a low virus titer could be detected in one fish in the saline group and in two fish in the non-CpG ODN-injected group (Table 2). At day 21 there were still no mortalities registered in the CpG ODN- and poly(I-C)-injected groups in tank C, while one out of five fish and two out of five fish were dead in the saline and non-CpG groups, respectively. The viral titers in the dead fish were not determined, but all the fish had clinical signs of IPN mortality and were found to be IPNV positive according to the IPNV agglutination kit. No virus could be detected in samples from the poly(I-C) group, while in the CpG group three out of five fish had no virus and only low virus titers could be detected in the remaining two fish. For the saline and non-CpG groups, all the surviving fish had high virus titers. In tanks A and B mortalities were monitored for 37 days. No statistically significant differences in mortalities were registered between replicates. Table 2 shows the cumulative mortalities for the groups in tanks A and B at 21 and 37 days postchallenge. Fish treated with CpG ODN or poly(I-C) showed a delayed onset of mortality compared to those of the saline and non-CpG groups and reached final cumulative mortalities of 37 and 41%, respectively, at day 37. Mortalities in the saline and non-CpG groups were 55 and 52%, respectively, at day 37. No protection was obtained after treatment with non-CpG ODN, while treatment with poly(I-C) significantly protected the fish against IPNV infection ($P < 0.05$; chi-square test). Furthermore, significant protection was obtained after treatment with CpG ODN ($P < 0.05$) in tank A.

Mx expression. To determine the possible role of IFN induction in the protective effect obtained by CpG ODN and poly(I-C) treatment, the expression of Mx genes was detected by semiquantitative reverse transcription-PCR. So far, zebra fish is the only fish species for which an IFN gene has been cloned and characterized (1), and there are no available re-

TABLE 2. Cumulative mortality and viral titers following viral challenge of CpG-, non-CpG-, and poly(I-C)-injected Atlantic salmon on indicated days postinjection

Treatment	Fish in tank C								Fish in tanks A and B		
	Day 7			Day 21					Mean cumulative mortality (%)[b]		Statistical significance (χ^2 test)[c]
	No. of virus negative/total no.	No. of dead/total no.	No. with titer of $>10^{2.40}$/total no.[a]	No. of virus negative/total no.	No. of dead/total no.	No. with indicated titer/total no.[a]			Day 21	Day 37	
						$>10^{2.40}$	$>10^{4.00}$	$>10^{6.00}$			
Saline	4/5	0/5	1/5	2/5	1/5			2/5	17	54 (76/140)	
Non-CpG	3/5	0/5	2/5	0/5	2/5		1/5	2/5	14	55 (77/140)	
CpG	5/5	0/5		3/5	0/5	1/5	1/5		8	37 (27/73)	$P < 0.05$
Poly(I-C)	5/5	0/5		5/5	0/5				5	41 (57/140)	$P < 0.05$

[a] Titer measured in $TCID_{50}$ per gram of tissue.
[b] Mean results (number of dead/total number of fish) for tanks A and B are given except for the results from the CpG-infected group. which are for tank A only.
[c] Statistical significance is based on a comparison between results for fish on day 37 postinjection and results for the saline group.

agents that will detect IFN directly in salmonid fish. However, the Mx gene of Atlantic salmon has been cloned and characterized (52) and has been shown to be an IFN-α/β-induced gene in fish, as in mammals (45). Our results showed that both CpG DNA and poly(I-C) injection induce Mx expression in head kidney and spleen (Fig. 4), and a two-way analysis of variance showed significant effects of both time and treatment in the spleen and head kidney. In both organs, an induction of transcripts, although not significant, could be detected at 24 h postinjection. At 48 h postinjection, a significant Mx induction was seen in these organs for both CpG ODN- and poly(I-C)-injected fish, while at 96 h the induction decreased. In the poly(I-C)-treated fish, a strong Mx induction was still detected in both organs, but in CpG ODN-treated fish, no Mx expression was detected in the head kidney and a low, not significant expression was detected in the spleen.

DISCUSSION

Previous studies have demonstrated that CpG ODN are efficient vaccine adjuvants in mice and may also act as immunoprotective agents (28, 38). Although the adjuvant effect of DNA has been demonstrated in goldfish (26), the potential for using CpG DNA as a prophylactic treatment in fish has not been thoroughly investigated. The present work was undertaken to examine whether CpG ODN could induce resistance to viral infections in fish. We were primarily interested in the identification of a CpG motif that would give optimal stimulation in fish. It was therefore of interest to identify in vitro test systems that could predict in vivo efficacy in fish. In earlier studies it has been shown that salmonid cells secrete antiviral components upon stimulation with certain CpG ODN (24, 25), but a screening for maximal responsiveness has not been conducted. In other species it has been shown that CpG DNA are mitogenic for PBL, and assays to measure proliferation have been used to screen for activity (16, 34). In an introductory experiment, we examined the proliferative response of fish PBL to CpG ODN. Our results showed that Atlantic salmon PBL respond to CpG ODN in a sequence-specific manner, since non-CpG ODN and calf thymus DNA had no stimulatory effects. These studies also showed that proliferative responses varied among different CpG ODN, so this type of assay was found to be suitable for activity screening and it was combined with the IFN assay.

Based on previous results in which ODN 1668 and 1670, with the murine motif (GA/AA)CGTT, was shown to stimulate antiviral activity in Atlantic salmon leukocytes (24, 25), we designed a panel of different CpG phosphothiorate ODN. In this panel were ODN containing various combinations in the 6-mer core motif, and variations in the numbers and total lengths of CpG motifs were also tested. Both human and mouse motifs were included in this screening, and both stimulated fish leukocytes, although the human motifs had a higher stimulatory capacity in both assays. This result verifies what is described in other studies, i.e., that different species differ in their responses to specific CpG motifs and that the motif GT CGTT is the most effective in most species, including cattle, primates, cats, and dogs (reviewed in reference 44). Another similarity shared by fish and humans is that bacterial DNA fails to stimulate proliferation of their PBL (37), while *E. coli* genomic DNA is reported to induce mouse B cells to proliferate both in vivo and in vitro (34). Our study also showed that additional CpG motifs outside the 6-mer core contribute to the stimulatory effects. Reduction in the lengths of the ODN below 18 bp reduced or eliminated activity.

Herein we demonstrated for the first time that injection of CpG ODN in animals increases resistance to viral infections. Fish that were treated with CpG DNA prior to challenge with IPNV had reduced viral titers and mortality compared to those of control fish given either saline or control ODN. Optimal activation was seen with CpG ODN at doses of 50 and 100 μg per animal, while 1 μg gave no protection. The protection against infection obtained with CpG DNA was as efficient as injection with the IFN inducer poly(I-C). The induction of an IFN-α/β response in the CpG ODN- and poly(I-C)-injected fish was confirmed by semiquantitative PCR, which detected increased expression of the IFN-inducible Mx transcript. Earlier studies have shown that salmonids are protected from infectious hematopoietic necrosis virus (IHNV) (10) and from infectious salmon anemia virus infections when treated with poly(I-C) (21). Furthermore, Jensen et al. (21) showed that poly(I-C) injection in Atlantic salmon induces Mx protein expression in different organs, including head kidney and spleen, which is also documented in our study. Although Mx expression was higher and more sustained in the poly(I-C)-injected fish than in the CpG ODN-injected fish, there was no significantly higher protection of the poly(I-C)-treated fish in the challenge experiments. However, a more delayed onset of mortality was seen in the poly(I-C)-treated fish than in the CpG ODN-treated fish, and all poly(I-C)-injected fish were found to be virus negative at day 21 postchallenge, while IPNV was detected in two of five CpG ODN-treated fish. This difference may be due to the earlier and higher IFN induction in poly(I-C)-injected fish. Distribution studies of fluorescence-labeled CpG have shown that CpG ODN can be found both in head kidney and spleen 4 and 24 h after i.p. injection (unpublished data), which indicates that IFN induction most probably occurs locally in these organs, followed (possibly) by a systemic distribution.

Recently, it has become clear that IFN-α/β represents key molecules in the protective immune response to many virus infections not only by directly and indirectly interfering with replication of intruding virus but also by paving the way for subsequent specific responses (3). DNA vaccination studies with fish have indicated that IFNs may play a role in the protection provided by these vaccines. DNA vaccines based on glycoproteins of salmonid fish rhabdoviruses induce protection as early as 7 to 8 days postvaccination (35, 40). The timing of this response is probably too early for a specific immune response in fish and indicates that nonspecific mechanisms are involved. A study by Kim et al. reported that all rainbow trout fry which received DNA vaccines containing G genes of three different fish rhabdovirus species, IHNV, snakehead rhabdovirus, and spring viremia of carp virus, were protected against IHNV challenge 30 days postvaccination, thus indicating nonspecific antiviral protection (27). The induction of IFN-α/β in the vaccinated animals was confirmed by detection of increased Mx production. In the challenge, the fish received two different doses of virus (10^3 and 10^5 PFU), and for the lower challenge dose, lower mortality was found for the group in-

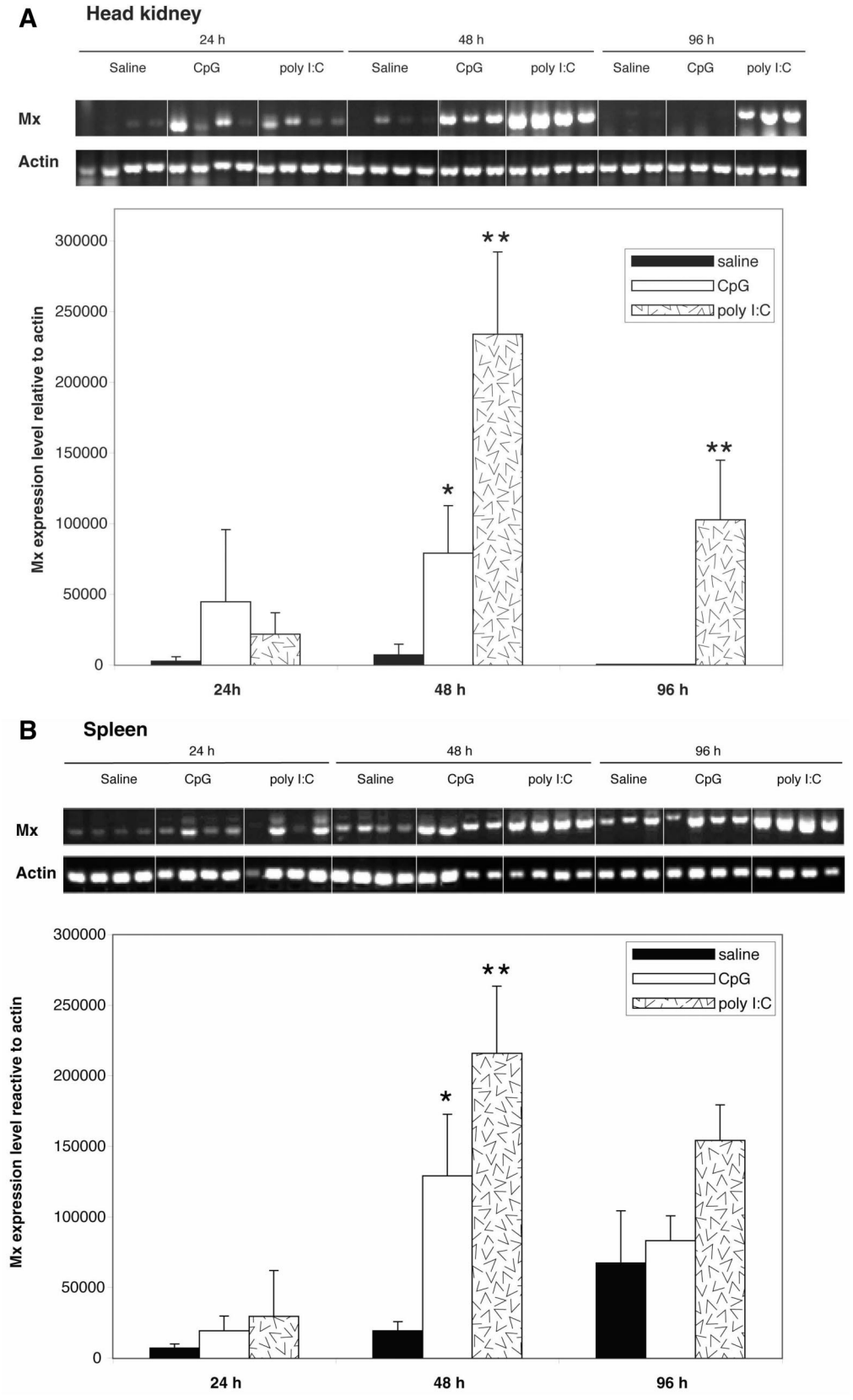

FIG. 4. (A) Effects of CpG ODN and poly(I-C) injection on Mx expression in Atlantic salmon head kidney. PCR was performed as described in Materials and Methods. The level of expression was calculated relative to the actin expression level. Data are the means ± standard deviations of results for three to four fish. (B) Effects of CpG ODN and poly(I-C) injection on Mx expression in Atlantic salmon spleen. PCR was performed as described in Materials and Methods. The level of expression was calculated relative to the actin expression level. Data are the means ± standard deviations of results for four fish. ** and *, statistical significance in comparison to the control at a P value of < 0.01 and < 0.05, respectively.

jected with plasmid alone than for control fish that had received phosphate-buffered saline (27). This result indicates that immunostimulatory DNA and CpG sequences in the plasmid backbone may contribute to protection at a low challenge dose.

CpG DNA is known to be an excellent immune adjuvant in various murine disease models and can augment both humoral and cellular immune responses (5, 8, 28, 38, 47). Also in naked DNA vaccines, it is reported that vaccination is much more efficient when the plasmid vector for mammalian DNA contains unmethylated CpG motifs (29, 48, 53). Whether the protection obtained by DNA vaccination in fish can be explained by adjuvant-like activities of CpG motifs in the plasmid backbone is uncertain, since studies performed so far have shown that injection with vector alone or with other genes inserted gives little or no protection (39). Further analysis is needed before it can be concluded that the effects of such motifs contribute to the efficacy of DNA vaccines in fish. Most probably, future fish virus vaccines will be based on recombinant viral proteins, and since these antigens have poor immunogenicity, they will require adjuvants to elicit an adequate immune response. Many of the adjuvants used in fish vaccines, and in particular oil-based adjuvants, contribute to good protection, but at the same time they give serious side effects (43). CpG DNA used as an adjuvant is reported to induce stronger immune responses with less toxicity than other adjuvants according to test results with murine models (59). The potent immunostimulatory activities of CpG DNA reported for fish in this and other studies point to CpG DNA as an interesting adjuvant to be tested in fish vaccines.

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