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Protection of Balb/c mice against infection with FMDV by immunostimulation with CpG oligonucleotides

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Abstract

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs are potent stimulators of the innate immune system, and are capable of aborting several infections in a non-specific manner. We here report studies of the capacity of such ODN to protect mice against infection with foot and mouth disease virus (FMDV). Susceptibility of Balb/c mice to infection with isolates from the different serotypes of FMDV was investigated, and, at the same time, the capacity of CpG ODN to modulate the infection was evaluated. Treatment with CpG significantly reduced viremia, disease and death in five of six serotypes, when compared to no treatment or treatment with a control ODN. The effect was observed when ODN was administered simultaneously with, or up to 12 h after, infection with FMDV, and lasted for 14 days post treatment. The potential application of CpG ODN for control of FMDV during an outbreak is discussed.

Keywords: Foot and mouth disease virus; CpG ODN; Protection

1. Introduction

Foot and mouth disease (FMD) is one of the most contagious and economically devastating diseases of husbandry animals. The disease is caused by foot and mouth disease virus (FMDV), a member of the family Picornaviridae. It infects a range of production animals including cattle, sheep, goats and pigs, and a range of wild animals. Although eradicated in most of Europe and North America, the virus remains a threat to food production. In case of an outbreak, several measures are taken including movement restrictions, culling of infected herds, and occasionally ring vaccination to limit further spread from infected premises. The highly contagious nature of this virus makes rapid intervention essential.

The virus spreads by contact, by fomites, and perhaps most importantly in terms of effective disease control, by aerosols over long distances. In this context, controlling infection of pigs is particularly important because pigs excrete large amounts of virus (Alexandersen and Donaldson, 2002). In addition, pigs generally do not respond as well to FMD vaccination as do ruminants (Doel et al., 1994). To overcome this, high potency vaccine formulations containing oil adjuvants have been developed, which induce protection in pigs as early as 4 days post vaccination (Barnett et al., 2002; Salt et al., 1998). Interestingly, this early protection occurs before protective levels of neutralizing antibodies are induced, suggesting that other mechanisms may contribute to protection during the first day after vaccination. In these studies, nonvaccinated animals were used as controls, whereas animals vaccinated with adjuvant alone, or animals infected with heterologous virus, were not included. Thus, it is not known whether the early protection is related to vaccine components other than antigen, or whether it is serotype-specific. If the effect were solely mediated by innate effector mechanisms, the protection would be expected to be independent of presence of antigen in the vaccine, and to protect against different types of FMDV.

However, even with high potency emergency vaccines, a time window of 4–5 days remains, during which animals are not protected against disease and infection. An approach to induce more rapid protection was recently reported by Grubman and colleagues (Chinsangaram et al., 2003; Moraes et al., 2003). When pigs were infected with a recombinant adenovirus expressing porcine interferon-alpha, they were subsequently protected against infection with FMDV (Chinsangaram et al., 2003; Moraes et al., 2003). Protection lasted 1–3 days after infection with the cytokine inducing adenovirus, and declined

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gradually after 5–7 days. When cytokine induction was performed 1 day after FMDV inoculation, no protection was observed. The adenovirus could be administered simultaneously with FMDV vaccination, and thereby supplement the long-term protection afforded by vaccination.

Mice can be used as infection models for FMDV, although different factors such as serotype, strain, passage history and mouse strain all affect susceptibility to and outcome from infection (Fernandez et al., 1986; Salguero et al., 2005; Subak-Sharpe, 1961; Subak-Sharpe et al., 1963).

Protection against FMDV infection by innate immune stimulation has been studied in mice as well. Pyran – a divinyl ether-maleic anhydride copolymer – as well as poly I:C have been studied for their protective effects in mice (Richmond, 1971; Richmond and Hamilton, 1969), and diacetylsplenopentin was reported to have an effect on FMDV infection in guinea pigs (Liebermann et al., 1993). Treatment of mice with sub-immunosuppressive doses of cyclophosphamide also was able to protect mice against FMDV infection (Portiansky et al., 1989). In all cases, protection was rapid and relatively short-lived, but of sufficient duration to protect during the first day after treatment. In natural hosts of FMDV, the treatments were, however, without effect on subsequent challenge infection (McVicar et al., 1973).

We have studied the use of CpG based stimulation to protect against infection with FMDV in mice. Treatment with CpG containing oligonucleotides (CpG ODN) induces a complex state of activation of innate immune functions, including cytokine synthesis and cellular activation. Stimulation with CpG ODN has been shown to protect against a number of bacterial and viral infections, including herpesviruses (Harandi et al., 2003), orthopoxvirus (Rees et al., 2005), influenza virus (Dong et al., 2003) and birnavirus (Jørgensen et al., 2003). Although the core sequence should always be an unmethylated CG dinucleotide, the stimulatory effect also depends on the sequences flanking the CG core. The flanking sequence is to some degree species-specific, but knowledge regarding stimulatory sequences in production animals is gradually becoming available (Kamstrup et al., 2001; Mutwiri et al., 2003; Rankin et al., 2001). Thus, CpG ODNs for treatment in natural hosts of FMDV infection can be envisaged.

2. Materials and methods

2.1. Virus and cells

The FMDV plaque isolate C-S8c1 (kindly donated from Dr. Francisco Sobrino, CISA-INIA, Valdeolmos, Spain), originally described by Sobrino and colleagues were cultured twice on ST cells and once on BHK-21 cells. The supernatant from BHK-21 cells was used for inoculation of mice. The virus titer ($CCID_{50}$) in the supernatant was determined by titration on BHK-21 cells. All other FMDV isolates were received from The Institute for Animal Health, Pirbright Laboratory, Pirbright, UK. These were: A-22 Iraq 24/64 August 96 (serotype A), O-Manisa Turkey 8/69 (serotype O), Asia 1 Shamir, Israel 3/89 (serotype Asia 1), SAT 1 Bot 1/68 (serotype SAT1), SAT 2 Zim 5/81 (serotype SAT2), and finally SAT 3 Zim 4/81 (serotype SAT3). Serotypes A, O, and Asia were propagated on calf kidney cells. All other isolates were propagated in BHK-21 cells. Titration of stock virus was performed on the same cell type as used for propagation. Titration for viremia was in all cases performed in BHK-21 cells, and all titers are given as $\log_{10}$ $CCID_{50}$, and averages are given as average($\log_{10}(CCID_{50})$).

2.2. Animal experiments

Female Balb/c mice (Taconic Europe, Denmark) aged 7–9 weeks were used in all experiments. All animal experiments were performed according to national and institutional guidelines. Mice were kept in isolation facilities at DFVF, Lindholm, and fed commercial diets and water ad libitum. In all experiments, mice were inspected twice a day until day 5 p.i. for emergence of clinical signs. Moribund mice were euthanized. Mortality was calculated at 2 weeks p.i.

2.3. Administration of oligonucleotides

Oligodeoxynucleotides (ODNs) were synthesized by DNA Technology (Aarhus, Denmark). For increased stability in vivo, ODNs with phosphorothioate backbone were used. Administration of CpG was done by subcutaneous injection of 100 μg CpG in 100 μl sterile PBS per mouse. The ODN "1826" (sequence: 5′-TCCATGACGTTCCTGACGTT-3′), previously reported to have an immunostimulatory effect in mice (Jakob et al., 1999) was used for stimulation. As controls, mice were injected with the non-stimulatory GpC ODN designated "1826inv", in which the two CpG motifs were inversed to a GpC, while the flanking nucleotides were kept unchanged (sequence: 5′-TCCATGAGCTTCCTGAGCTT-3′), or by use of PBS only.

2.4. Infection with FMDV

In all experiments, FMDV inoculation was carried out by i.p. injection of 100 μl of virus diluted in sterile PBS. Dosage of virus varied between experiments and is given in Section 3. Blood samples were taken by tail vein puncture for detection of viremia and virus-specific antibodies. Virus load present in serum was estimated by titration on BHK-21 cells. The presence of serum antibodies to FMDV was determined by either ELISA based on the method described in Have et al. (1984), or by immuno peroxidase monolayer assay (IPMA) based on BHK-21 cells infected with the homologous virus isolate (Sobrino et al., 1983). All mice showing viremia on one or more days and/or seroconversion, or died before blood sampling was carried out, were scored as infected.

2.5. Statistical analysis

All statistical calculations were performed using GraphPad Instat for Windows, ver. 3.00. Viremia averages were calculated as average(log(viremia)). Comparison of viremia between groups was done using one-way analysis of variance, and using

a parametric test. Mortality was compared using Fischer's exact test.

3. Results

Three separate experiments were carried out; SEROTYPE, DURATION (DUR), and RAPIDITY (RAPID). In the SEROTYPE experiment, the effect of CpG oligonucleotide on infection with six different serotypes was tested. Both the DUR and RAPID experiment focused on the kinetics of the CpG-induced response against FMDV. The RAPID experiment was set up to investigate the speed of the response, i.e. how fast the anti-FMDV effect developed, while the DUR experiment focused on the duration of protective state, i.e. longevity of the response.

3.1. SEROTYPE experiment

This experiment served a dual purpose. Only sparse information is available on FMDV infection in adult mice, as compared to newborn mice. It is known, that the outcome of infection depends on mouse strain, virus strain, inoculation dose, and, to some extent, also passage history of virus. Since susceptibility of the mouse strain used (Balb/c) towards the virus strains used was not known (and, even less so, the mouse infectious dose 50%), this experiment probed for susceptibility of the mouse using a fixed amount of virus. Preliminary experiments with serotype C virus had shown that a dosage of 10^3 $CCID_{50}$ led to consistent infection in Balb/c mice, and therefore this dosage was used for inoculation with representative isolates of the seven different serotypes of virus. In parallel with the infection of naïve mice, the immunostimulatory ODN 1826 (CpG ODN) was tested for effect on infection. One group of control mice was injected with the non-stimulatory GpC ODN (GpC group). Mice were treated with ODNs (or PBS) 4 days prior to inoculation (day 4 p.i.) with the relevant isolate of FMDV (day 0 p.i.). All mice in this experiment were tested for viremia on days 1, 2, and 3 p.i., and for seroconversion on days 7 and 14 p.i. Seroconversion on days 7 and 14 was tested by IPMA using the relevant isolate.

Inoculation with serotype A resulted in infection of all mice of the GpC group and 78% of mice in the PBS group, respectively, while 50% of the CpG ODN-treated mice became infected (Table 1). No clinical disease was noted in any of the mice. Not only was the number of viremic mice reduced by CpG treatment; among those mice that developed viremia, the average maximum virus load in serum (MVLS) was more than 100 times lower in mice treated with CpG ODN. Together, these observations show that inoculation of mice with CpG ODN at 4 days prior to infection with an FMDV serotype A markedly reduced the virus infection.

Inoculation of Balb/c mice with 10^3 $CCID_{50}$ of serotype C virus resulted in severe disease and death in unstimulated or GpC-treated groups. At this virus dose, 90% of mice in the two control groups either died or were euthanized in a moribund condition. All 10 mice of the CpG group survived throughout without signs of disease throughout the experiment. Of these mice, 20% were infected, while the remaining mice were neither viremic nor seroconverted. Average MVLS (calculated for infected mice only) was approximately 100 times lower in the CpG group than in the control groups (Table 1). Thus, CpG ODN treatment markedly reduced the number of mice, which developed viremia, and viral load in those that did. In addition, pretreatment with CpG ODN dramatically reduced mortality. These results clearly show that the immunostimulatory ODN 1826 induced protection against infection with this serotype C isolate.

Essentially the same was observed following inoculation with the FMDV serotype Asia 1, where the mortality was 100%, 89%, and 0% for the GpC, PBS, and CpG group, respectively. Only one out of 10 mice was viremic and had seroconverted in the CpG group, and the MVLS of this mouse was 56 times lower than that of the control groups (Table 1).

Neither viremia nor seroconversion was detected following inoculation with 10^3 $CCID_{50}$ of serotype O virus. This indicates that either Balb/c mice are not susceptible to infection by this virus, or the inoculation dose of 10^3 $CCID_{50}$ was not sufficient for infection. For this reason, the effect of CpG ODN treatment towards infection with a serotype O isolate could not be followed in this experiment.

Table 1
Groups of 10 mice were inoculated with CpG ODN, GpC ODN, or PBS only, and subsequently inoculated with 10^3 $CCID_{50}$ of each of the seven FMDV serotypes

Serotype	Mortality[a]	Infected[b]	ISR[c]	Average MVLS[d]	MVLS-SR[e]
	CpG/GpC/PBS	CpG/GpC/PBS		CpG/GpC/PBS	
A Iraq	0/0/0	50/100/78	1.8	3.6/5.7/5.9	158
C-S8c1	0/90/90	20/90/90	4.5	3.1/5.3/4.8	89
Asia 1	0/100/89	10/100/89	4.7	5.0/6.9/6.6	56
SAT1	0/80/50	10/80/50	6.5	4.7/6.8/6.6	100
SAT2	0/40/60	90/100/100	1.1	5.0/6.5/6.2	22
SAT3	80/100/100	80/100/100	1.3	7.6/7.8/7.7	1

Inoculations with FMDV serotype A, C, Asia 1, SAT1, SAT2, and SAT3 all resulted in infected mice in the control groups. Inoculation with the O serotype did not result in productive infection, for which reason this serotype is not included in the table.

[a] Mortality was calculated at 14 d.p.i.

[b] Infected mice included both viremic mice, seroconverted mice, and mice that died before blood sampling for viremia measurements.

[c] ISR: infection suppression ratio = % infected mice in control groups/% infected in CpG group.

[d] Average MVLS (maximum virus load in serum) is given as $\log_{10}(CCID_{50})$/ml serum, and is calculated from viremic mice only on blood samples from days 1, 2, and 3 p.i.

[e] MVLS-SR: maximum virus load in serum suppression ratio = $10^{((\text{average MVLS in GpC group} + \text{average MVLS in PBS group}/2) - \text{average MVLS in CpG group})}$.

Inoculation with the SAT1 serotype isolate resulted in 80% and 50% infected mice in the GpC and PBS groups, respectively, all of which died. Thus, for this virus the mouse infectious dose-50% is approximately equal to 10^3 $CCID_{50}$. In the CpG-treated group all mice survived, and only one mouse was viremic and found to be seropositive (Table 1). Again, these results show that mice treated with CpG ODN are almost fully protected against infection with the SAT1 isolate and fully protected against mortality induced by this isolate.

In mice inoculated with the SAT2 isolate 100% of the control groups mice, and 90% of the CpG mice were infected, respectively. While all mice survived in the CpG group, 40% and 60% of GpC- and PBS-treated mice, respectively, died. Furthermore, the average MVLS for the CpG treated group was lower than the control group (Table 1). Thus, even if 90% of CpG-treated mice were infected following inoculation with SAT2, the course of the infection as observed by mortality and average MVLS showed that CpG treatment markedly reduced the level of viremia and completely protected against mortality (Table 1).

The last group of mice in this experiment was inoculated with SAT3. All mice in the control groups died, while 80% of the CpG-treated mice were infected and died (Table 1). Thus, the two surviving mice were completely protected. No clear difference could be observed between the groups concerning the average MVLS. However, mice treated with CpG showed a delay in disease onset and death (Fig. 1), indicating that the CpG treatment did, in fact, influence the infection with SAT3. The SAT3 serotype induced the most rapid disease progression of all serotypes, with most mice dead or moribund within 24 h after inoculation, while other serotypes in general required 2–4 days to reach maximum severity of disease (data not shown).

In conclusion, the SEROTYPE experiment showed, that pre-treatment of Balb/c mice with the immunostimulatory ODN 1826 four days before inoculation with an infectious dose of FMDV, induced full or partial protection against disease and death, and drastically reduced the average virus load in serum for five out of six serotypes. The effect on infection with the highly lethal serotype SAT3 was less prominent, but there was a moderate decrease in mortality and a delay in onset of disease and death in the CpG treated group, indicating that the infection in these mice was to some extent modulated.

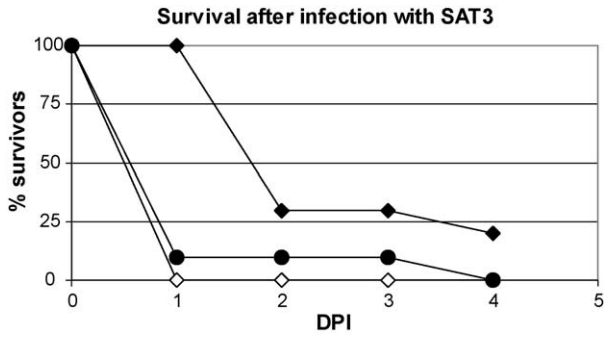

Fig. 1. Percent survival in groups of mice ($n = 10$) inoculated with 10^3 $TCID_{50}$ FMDV serotype SAT3 4 days after treatment with CpG ODN (♦), GpC ODN (◇) or PBS (●), respectively. DPI: day post infection.

3.2. DURATION experiment

In the DURATION (DUR) experiment, seven groups of mice ($n = 5$) were given CpG ODN on day 0, and subsequently inoculated with 10^4 $CCID_{50}$ serotype C virus at different time points after CpG inoculation (time points given in Table 2). Furthermore, one group ($n = 5$) was treated with GpC ODN and inoculated with 10^4 $CCID_{50}$ C-S8c1 immediately after GpC treatment (control group). The virus inoculum in DUR experiment was increased compared to the SEROTYPE experiment, since only approximately 90% of mice were infected. All mice were tested for viremia on day 2 p.i., and for serotype C antibodies on day 14 p.i. All mice in the control group were infected and died, and the average virus load in serum (VLS) was $10^{6.5}$ $CCID_{50}$/ml. In the groups treated with CpG ODN, mice were fully protected against infection (i.e. no detectable viremia and no seroconversion), when inoculation with virus was carried out on day 0

Table 2
In the duration experiment, groups of mice ($n = 5$) were treated with CpG or GpC ODN and subsequently inoculated with FMDV serotype C on different time points

Day of FMDV inoculation[a]	ODN	Mortality[b]	Infected[c]	ISR[d]	Average VLS 2 d.p.i.[e]	VLS-SR[f]
0	CpG	0	0	Not applicable	<1.0	>316228
2	CpG	0	20[$]	5	<1.0	>316228
4	CpG	0	60	1, 7	4.5	100
7	CpG	0	80	1, 25	5.0	32
14	CpG	0	80	1, 25	4.3	158
21	CpG	40	100	1	5.7	6
29	CpG	20	100	1	5.2	20
35	CpG	40	100	1	4.5	100
0	GpC	100	100	1	6.5	1

[a] Time points are given as days post inoculation with ODNs.
[b] Mortality was calculated at 14 days post virus inoculation.
[c] Infected mice includes both viremic mice, seroconverted mice, and mice that died before blood sampling for viremia measurements.
[d] ISR: infection suppression ratio = % infected in control groups/average % infected in CpG group.
[e] Average VLS (virus load in serum) is given as $\log_{10}(CCID_{50})$/ml serum, and is calculated from viremic mice only.
[f] VLS-SR: virus load in serum suppression ratio = $10^{((\text{average VLS in GpC group} + \text{average VLS in PBS group}/2) - \text{average VLS in CpG group})}$.
[$] This mouse seroconverted, but no viremia was observed.

(Table 2). In groups inoculated with virus on day 2 or later, one or more mice were viremic. No clinical symptoms were observed in groups inoculated on days 0, 2, 4, 7, and 14 post CpG ODN treatment. Mice inoculated with FMDV on days 21, 29, and 35 were all viremic and seroconverted and had up to 40% mortality (Table 2). Thus, in conclusion we observed full protection against mortality in groups infected up to 14 days after treatment with ODN 1826. This observation was statistically significant from the control group ($p < 0.01$). Mice inoculated with virus on day 21 also showed a mortality that was significantly different from the control group ($p < 0.05$). Mice inoculated with virus immediately after CpG treatment, were completely protected against infection with the dose of virus used. This observation was also significantly different from that of the control group ($p < 0.01$). The average VLS was lower in all CpG ODN-treated groups than in the group treated with GpC ODN (Table 2). However, this observation was not statistically significant.

3.3. RAPIDITY OF IMMUNITY experiment

In the RAPIDITY experiment six groups of mice ($n = 5$) were inoculated with 10^4 $CCID_{50}$ of the serotype C (same dosage as in the DUR experiment) on day 0 followed by stimulation with CpG ODN at different time points thereafter (time points given in Table 3). One group of mice ($n = 6$) was inoculated with virus but did not receive CpG ODN. Mice were tested for viremia on days 1, 2, and 3 p.i., and for seroconversion on day 14 p.i. In the control group, 83% of the mice were infected, showing relatively high MVLS (up to 10^9 $CCID_{50}$/ml). Two out of six control mice died. All mice survived in the groups treated with CpG ODN 1826 at 0 or 12 h post infection (h.p.i.), with 40% or 80% of the mice being infected, respectively (Table 3). Maximal VLS was relatively low in infected mice in the group treated at 0 h as compared to the control group (>60,000 times on average). In the group treated 12 h post FMDV inoculation average MVLS that was approx. thousand times lower than that of the control group. In groups treated with ODN 1826 at 24, 36, or 48 h.p.i., 14 out of 15 mice were infected, with an average mortality of 64% (Table 3).

4. Discussion

The SEROTYPE experiment served a dual purpose. Representative isolates from the seven serotypes of FMD virus were tested for their ability to infect mice, and the effect of immunostimulation with CpG ODN on the course of infection was studied.

Large variations in susceptibility, pathogenicity, and mortality were observed after inoculation of a fixed dose of virus of different serotypes. Virus of serotype C, Asia1 and SAT3 induced rapid disease and death in all infected animals, with SAT3 being the most rapidly lethal infection. In contrast, the serotype A virus used in this study produced no overt disease, in spite of high viremia and seroconversion. Pathology from FMDV infection in mice includes pancreas lesions, lymphopenia, as well as effects on heart, lungs and other organs (Fernandez et al., 1986; Platt, 1959; Salguero et al., 2005). The mechanisms leading to different pathology for different virus strains remain unknown. The level of viremia (maximal virus load in serum) did not correlate with the mortality, so other differences in tissue and organ destruction must be present which were not addressed during the present study.

The relative pathogenicity/mortality observed between A, C, and SAT1 serotypes corresponds to that reported for C57/Bl6 mice (Salguero et al., 2005), suggesting that the difference in pathogenesis may be consistent in different mouse strains. Since we have only tested infection of mice with a single representative strain of each serotype, we cannot make general statements concerning the different serotypes. In fact, different strains within a serotype may behave very differently (data not shown), as also published for two different passage levels of a serotype C virus (Salguero et al., 2005). All virus isolates used in this study had been passaged in cell culture before inoculation into mice.

Pretreatment of mice with a single injection of an oligonucleotide containing a potent CpG stimulatory motif (CpG-ODN) 4 days prior to virus inoculation, resulted in complete abrogation of mortality for all virus types, except for SAT3, and complete or partial suppression of viremia and seroconversion. In gen-

Table 3
In the immunity experiment, groups of mice ($n = 5$) were inoculated with 10^4 $CCID_{50}$ FMDV serotype C, and treated with CpG ODN at different time points

Time point for CpG treatment[a]	Mortality[b]	Infected[c]	ISR[d]	MVLS[e]	MVLS-SR[f]
0	0	40	2	<1.0/<1.0/3.3/1.8/<1.0	63096
12	0	80	1	<1.0/2.2/3.9/6.1/5.6	794
24	40	100	0.8	5.6/6.1/5.6/7.2/5.6	25
36	80	100	0.8	4.4/8.0/8.0/6.7/5.1	10
48	60[**]	80	1	6.1/6.7/6.7/<1.0/6.0	100
Not treated	33	83	1	9.0/8.0/8.0/<1.0/6.8/5.0	1

One group ($n = 6$) was not treated with CpG ODN and served as control group.
[a] Time points for CpG treatment are given as hours post FMDV inoculation.
[b] Mortality was calculated at 14 d.p.i.
[c] Infected mice include both viremic mice, seroconverted mice, and mice that died before blood sampling for viremia measurements.
[d] ISR: infection suppression ratio = % infected in control groups/average % infected in CpG group.
[e] MVLS (maximum virus load in serum) is given as $\log_{10}(CCID_{50})$/ml serum for each individual animal.
[f] MVLS-SR: maximum virus load in serum suppression ratio = $10^{((\text{average MVLS in GpC group} + \text{average MVLS in PBS group}/2) - \text{average MVLS in CpG group})}$.
[**] Three mice died before treatment with CpG ODN, while the remaining two mice survived to the end of the experiment. Of these two mice, one was not infected.

eral, we found that viremia above a certain level always lead to seroconversion, while seroconversion was sometimes observed without detectable viremia. The latter observation may be due to the viremia being of very short duration, leading to inadequacy of the sampling scheme to detect such low-level, short duration viremia. The possibility that CpG-ODN could have a direct inhibitory effect on virus replication was studied in vitro (data not shown). Virus was cultivated with or without the presence of CpG ODN in the culture medium. No effect was observed, leading us to believe that the effect on virus replication in vivo, is immune system-mediated. Since CpG ODN is known to induce a complex state of activation of innate immune mechanisms, these are most likely responsible for the protection observed. Induction of cytokines could be a plausible explanation, since interferon-alpha has been reported to protect pigs against infection with FMDV (Chinsangaram et al., 2003; Moraes et al., 2003). CpG ODN has been reported to induce interferon (IFN)-alpha, but also other cytokines of relevance, most notably IFN-gamma (Klinman et al., 1996; Krieg et al., 1995; Yamamoto et al., 1994).

However, while cytokines usually are considered short-lived (days) and CpG treatment induces only transient increases in key cytokines (Krieg et al., 1998), protection in our experiments was observed over a period of weeks. This suggests that protection is not solely mediated by a burst of cytokine induced upon injection of CpG ODN, but may rely on stimulation of other immune mechanisms.

CpG ODN stimulates monocytes to proliferate and mature into tissue macrophages (Sparwasser et al., 1998), which could favor immune control of infection. In pigs infected with FMDV, macrophages have been implied to play an important role in carrying infectious virus for several hours, subsequently releasing it at other sites (Rigden et al., 2002). If activation of macrophages by CpG ODN will induce virus destruction rather than transportation, such an effect could help controlling the virus infection. Other cell types may be involved as well. Natural killer cells are also activated by CpG ODN, and could be involved in elimination of cells infected by FMDV, which downregulates MHC class I expression in vitro (Sanz-Parra et al., 1998) and possibly also in vivo. If so, FMDV-infected cells would become targets for NK-mediated killing.

The experimental conditions used in the SEROTYPE experiment were not optimal for protection against viremia. Firstly, this experiment tested protection 4 days after administration of CpG. As shown in the DUR experiment, the protective effect of CpG stimulation was optimal immediately after administration. Secondly, the fixed dosage of 10^3 $CCID_{50}$ of virus may not have been optimal for all serotypes. The DUR experiment showed a two-phase level of protection. Initially, both viremia and mortality were abrogated, while at later stages, mortality was abrogated but viremia only partly or not suppressed. This could be related to different mechanisms being active at different stages of immune activation after CpG ODN stimulation. The results of the RAPID experiment further demonstrated that CpG ODN induced mechanism are indeed rapid. Treatment at 12 h post infection abrogated mortality, even if not capable of protecting against viremia (Table 3). This further points to a lack of correlation between level of viremia and mortality, and suggests that organ or tissue damage leading to disease and death are reached at a time point after viremia. Clearly, CpG stimulation is capable of preventing this destruction when administered 12 h post infection.

We have only presented data on protection of Balb/c mice, but have evidence that a protective effect is also exerted in C57/Bl6 mice infected with the serotype C virus [Sevilla, personal communication].

Thus, CpG stimulation seems effective against different viruses, as well as in different strains of mice. So far, we have not been able to identify the effector mechanism leading to protection. Transfer of either serum or immune cells from CpG stimulated to naïve recipient mice has not given conclusive results, and the volume of serum obtained so far has precluded systematic analysis for presence of cytokines. Further studies to address this issue are in progress.

Taken together, our findings show that the innate immune system of mice may be activated to control infection with FMDV. If such an effect can be reproduced in natural hosts of FMDV infection, this finding may be relevant in at least two different contexts. Firstly, in case emergency vaccination is to be applied, CpG treatment may be used as a supplement to achieve immediate protection, while the vaccine-induced adaptive response will provide longer term immune protection. Thus, the time window before vaccine-induced protection is obtained may be covered by CpG treatment. In addition, CpG ODN has a potent adjuvant effect, augmenting the adaptive response (Brazolot Millan et al., 1998; McCluskie and Davis, 1998; Moldoveanu et al., 1998). Secondly, if vaccination is not applied, CpG may still be applied as a means controlling the infection, provided that efficacy and sufficient longevity of response can be obtained. Both factors will be influenced by the choice of ODN sequence and type, dosage, and formulation. CpG stimulation does not need to be matched to the strain of virus causing an outbreak, which is a major practical complication and time consumer for intervention strategies based on vaccination. CpG based drugs are safer to produce, and less complicated to store, distribute, and formulate than FMDV vaccines. Furthermore, treatment with CpG ODN will not induce FMDV-specific antibodies, so surveillance based on antibody detection will not be compromised.

From a practical perspective, the major criteria would not be viremia, but disease and virus secretion. These issues cannot be addressed using our infection model, but should be done in natural hosts of FMDV. A possible protective capacity of CpG based drugs on FMDV in natural hosts can only be studied using infection experiments in these species, such as pigs, cattle or small ruminants. Such studies are currently in preparation.

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