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CpG-DNA protects against a lethal orthopoxvirus infection in a murine model

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Abstract

CpG-DNA has been described as a potent activator of the innate immune system, with potential to protect against infection caused by a range of pathogens in a non-specific manner. Here two classes of CpG-DNA (CpG-A and CpG-B) have been investigated for their abilities to protect mice from infection with an orthopoxvirus (vaccinia virus). Dosing with either CpG-A or B by the intraperitonal or intranasal route protected mice against a subsequent intranasal challenge with vaccinia virus. To our knowledge, this is the first time CpG-mediated protection has been demonstrated at the lung surface. The level of protection was greater when CpG-DNA was administered intranasally demonstrating a clear relationship between the route of CpG dosing and infection route. Treatment with CpG-B reduced viral titer in the lung by 10,000-fold at day 3 post-infection. The CC chemokines RANTES and MIP-1β were elevated in the broncho-alveolar lavage from animals treated intranasally with CpG-B compared to untreated and intraperitoneally dosed controls, and it is possible that these chemokines play a role in the clearance of intranasally delivered vaccinia virus.

Keywords: CpG-DNA; Orthopoxvirus; Innate immunity

1. Introduction

Smallpox is caused by variola virus, a member of the orthopoxvirus family. The disease is host-restricted to humans and the virus does not have the ability to naturally infect other mammalian hosts. Whilst the last naturally occurring smallpox infection was in Somalia in 1977, there is concern that illicit stocks of the virus may exist and that the organism may be used in a bioterrorism attack.

Whilst rodents cannot be infected with variola virus they can be infected with other related orthopoxviruses, and provide models of disease in humans. The mouse model of orthopoxvirus infection was first reported in the late 1950s (Fenner, 1958) and later refined by Turner (1967). These studies showed that neurotropic strains of vaccinia virus, such as WR and IHD, were able to cause a lethal pneumonia in mice when given intranasally (i.n.). When delivered subcutaneously, intravenously or intraperitoneally (i.p.), there was no evidence of disease. More significantly, the prior immunisation of mice with a live smallpox vaccine provided protection against a subsequent intranasal challenge with vaccinia virus. This finding suggests that this model of disease can provide meaningful insight into the efficacy of prophylaxis for orthopoxvirus infections.

The only currently available prophylaxis for orthopoxvirus infection in humans known to be effective is the smallpox vaccine (vaccinia virus). This vaccine is administered by scarification and provides excellent protection from disease. However, it is known to cause side effects in some

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vaccinees, some of which are classified as very severe. In addition, there are a number of pre-existing medical conditions which contra-indicate vaccination, including skin conditions and immunocompromised status. Against this background there is an urgent need to devise both improved vaccines and alternative prophylactics.

Multi-cellular organisms have a number of defense systems which enable them to resist infection by pathogens. One of these, the innate immune system, is a highly conserved system that has been described in species as diverse as insects and humans (Hemmi et al., 2000; Medzhitov et al., 1997). In this study we have considered the possibility that activation of the innate immune system can provide protection against orthopoxvirus infection.

Activation of the innate immune system is a consequence of the recognition of pathogen-specific molecules, and it then provides protection in a non-specific manner. The Toll-like receptors (TLRs) on host cells have been shown to play a key role in the recognition of these pathogen-specific molecules. To date 10 TLRs have been identified in humans and mice, and TLR9 has been shown to recognize unmethylated cytosine and guanosine dinucleotides (CpG) and their flanking regions of DNA (Poltorak et al., 1998; Tokunaga et al., 1984). Such CpG motifs are frequently abundant in the DNA of pathogens but suppressed in eukaryotes.

Three classes of synthetic CpG-DNA have been described based on their structure and immunostimulatory properties. CpG-A DNA has a structure with phosphorothioate (PS) guanine rich sequences called "polyG motifs" at the 5′ and 3′ ends, and a palindromic phosphodiester core containing one or more CpG motifs (Ballas et al., 1996; Krieg, 2002; Krug et al., 2001). CpG-A DNA induce the highest production of IFN-α, marked NK cell activation, but relatively low levels of B cell activation. CpG-B DNA in contrast, has a fully PS-modified backbone and induce modest levels of IFN-α, weaker NK cell activation, but excellent B cell activation (Ballas et al., 1996; Boggs et al., 1997). CpG-C, the most recently described oligodeoxynucleotide (ODN) class, have been designed to combine the immune effects of A and B class ODN by combining strong B cell, type 1 IFN-α and natural killer cell activation (Vollmer et al., 2004) (for further information on CpG ODN consult http://www.coleypharma.com).

The activation of the innate immune systems that is triggered after exposure to CpG-DNA has been reported to protect against lethal challenge with a number of viral, bacterial and protozoal intracellular pathogens (Elkins et al., 1999; Gramzinski et al., 2001; Krieg et al., 1998; Zimmermann et al., 1998). Klinman et al. (1999) demonstrated that CpG-DNA administered by a number of different routes can provide partial protection against challenge with both *Bacillus anthracis* and Ebola virus. In addition, CpG-DNA delivered intravaginally has been shown to protect against genital herpes infection (Harandi et al., 2003). This finding suggests that the delivery of CpG-DNA to the mucosal surface through which the pathogen enters, is able to induce protective responses. However, to date there are no published reports on ability of i.n. delivered CpG-DNA to provide protection against pathogens delivered to the respiratory tract.

In this study Type A (CpG 1585) and B (CpG 2006) ODNs were tested for their protective efficacy towards an otherwise lethal vaccinia virus i.n. challenge. CpG-DNA was administered either systemically or intranasally.

2. Materials and methods

2.1. Mice

Six to eight-week old female BALB/c mice (Charles River UK, Ltd.) were used in all experiments. Animals were specific pathogen free and were housed and treated according to the guidelines specified under the Animals (Scientific Procedures) Act. The median lethal dose 50 (MLD_{50}) for the IHD strain of vaccinia virus was 1×10^5 plaque forming units (pfu) by the intranasal route (data not shown). After challenge, animals were monitored twice daily and weighed each day (early morning) when clinical signs of disease were also noted. A weight loss of 30%, or severity of clinical signs was used to determine the humane end-point, at which mice were culled. Control animals were typically culled by day 10 post-challenge.

2.2. Growth and purification of virus

Vaccinia virus strain IHD (ATCC VR-156) was obtained from the American Type Culture Collection (ATCC). This strain was originally derived from the New York City Department of Health strain of vaccinia virus. RK13 rabbit kidney cells were grown to approximately 80% confluence in DMEM (Gibco) supplemented with 10% FCS, 0.01% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin The medium was removed and the cells infected with 0.1 pfu vaccinia IHD/cell in 5 ml of DMEM with 2% FCS, and the flasks incubated at 37 °C for 1 h to allow adherence of virus to the cells. The inoculum was discarded and replaced with 2% FCS DMEM and flasks incubated at 37 °C for 48 h. Half the medium was removed from each flask for purification of cell free virus and the adherent cells were scraped into the remaining media. Cell-free virus was pelleted by centrifugation at 72,000 × *g* for 1 h, the supernatant removed and the pellet resuspended. Harvested cells were homogenized using a pestle and mortar to release the cell-associated virus. Virus was pelleted by sucrose density ultracentrifugation through a 36% sucrose solution in PBS and centrifugation at 33,000 × *g* for 1.5 h at 4 °C. The supernatant was removed, the virus resuspended in PBS and stored at −80 °C. Virus titer was determined by serial dilution onto RK-13 cells for 72 h at 37 °C, the cells were fixed in formal saline, and stained with crystal violet. The numbers of plaques at each dilution were counted and the virus titer determined.

2.3. CpG treatment and vaccinia virus infection

Synthetic oligonucleotides containing CpG motifs were designed and synthesized by Coley Pharmaceutical Group. The class A CpG 1585 (5′ GGG GTC AAC GTT GAG GGG GG 3′) and the class B CpG 2006 (5′ TCG TCG TTT TGT CGT TTT GTC GTT 3′) were used as described below in all experiments.

Groups of animals ($n = 10$, 6 or 5 depending on experiment) were given two doses of CpG-DNA 10 days and 3 days before pathogen challenge. In duration of protection experiments a single i.n. dose of CpG-DNA was given on a range of different days before challenge. CpG administration was either intraperitoneally (i.p.) in 100 μl volume or intranasally (i.n.) in 50 μl volume with light halothane anaesthesia. Doses of CpG 1585 were either 100 or 300 μg, whereas CpG 2006 was used at 75 or 150 μg. Control animals were untreated. On the day before virus challenge blood was collected by vene puncture of a marginal tailvein and allowed to clot on ice. Sera were prepared by centrifugation for 5 min at 13,000 rpm, collected and frozen at −80 °C for immune analysis. Virus challenge was by the intranasal route in 10 μl volumes.

2.4. Bronchoalveolar lavage

Animals dosed as described were humanely culled by cervical dislocation. An incision was made in the neck of the animal and the thymus removed to reveal the trachea. A sterile 19-gauge butterfly needle (Venisystems™) was inserted via the trachea into the lungs. The lungs were filled with 2 ml ice-cold sterile phosphate-buffered saline (PBS), using a 5 ml syringe. The bronchoalveolar lavage (BAL) fluid was slowly aspirated back into the syringe, returned to ice, and then stored frozen at −80 °C for later analysis.

2.5. Cytokine assays

The Th1/Th2 cytokine bead analysis system (CBA™, BD Biosciences) was used to assay for tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-5 (IL-5), interleukin-4 (IL-4) and interleukin-2 (IL-2). Murine chemokines RANTES, MIP-1α and MIP-1β were assayed according to the Manufacturer's instructions using appropriate ELISA kits (R&D Systems Ltd.).

2.6. Organ virus titration

On days 1, 3, 5, 7 and 9 post-challenge one group of animals was humanely culled by cervical dislocation and brain, lungs and spleen were removed under aseptic conditions and stored at −80 °C for virus titration. In addition, at least 14 days post-challenge, surviving animals were humanely culled by cervical dislocation and the spleens were removed under aseptic conditions and stored at −80 °C for virus titration. Organs were homogenized in sterile PBS using an organ sieve and particulate tissue removed by centrifugation at 3000 × *g* for 5 min. Organ homogenates were serially diluted and overlaid onto RK13 cells in a 96-well plate. Plates were incubated for 72 h at 37 °C, the medium removed and the cells fixed by the addition of 10% formal saline prior to staining with alcoholic crystal violet solution. Virus titers were calculated using the method of Reed and Muench.

2.7. Plaque reduction assay

Plaque reduction assays were performed by infecting RK-13 monolayers with 50–100 pfu of virus per well in 24-well cell culture plates, in DMEM supplemented as before. Various concentration of CpG 2006 were added to the culture medium and the infected cell cultures were incubated for 2 days at 37 °C with 5% CO_2. CpG concentrations were 10, 5, 2.5, 1.25 and 0.625 μg/ml. The monolayers were fixed and stained as previously described. Visible plaques were counted and the IC_{50} values calculated.

3. Results

3.1. CpG-DNA protective effects to vaccinia virus challenge

When mice were challenged with 10 MLD_{50} of vaccinia virus IHD by the i.n. route clinical signs of disease became apparent in control animals by day 4 post-challenge with weight loss in all animals. By day 6 post-challenge control animals demonstrated severe clinical signs of disease including piloerection, hunching and respiratory problems. In the animal model development stages, lethal experiments were performed. These revealed that 98% of animals with a 30% weight loss went on to die, therefore, together with clinical signs of disease this was taken as the humane end-point in all experiments. In most experiments all control animals were culled between day 7 and day 10 at the humane end-point described.

Female BALB/c mice were dosed with CpG-A (1585) or CpG-B (2006) by either the i.n. or i.p. route, 10 days and 3 days before challenge with 10 MLD_{50} of vaccinia virus IHD by the i.n. route. CpG dosing by the i.n. route was performed under light anaesthesia in a relatively large (50 μl) volume, as this has been demonstrated to target delivery to the deep lung specifically, whereas smaller volumes tended to deposit in the upper respiratory tract (Eyles et al., 1999). In the control group (untreated with CpG), mice showed marked weight loss and clinical signs of severe infection and 9/10 mice were culled by day 10 having reached the humane end-point in this study. The surviving control animal had signs of infection throughout the duration of the experiment, and failed to regain the weight lost but this was not considered severe enough to warrant culling. In contrast, mice which had been treated (i.n. or i.p.) with 75 or 150 μg of CpG 2006 survived challenge with vaccinia virus and had returned to within 5% of their initial weight by day 19 post-challenge (Table 1).

Table 1
Survival of BALB/c mice treated with CpG 1585 or CpG 2006 intranasally or intraperitoneally 10 days and 3 days before intranasal challenge with 10 MLD_{50} vaccinia virus IHD

Treatment	Number of survivors	
	i.n.	i.p.
100 μg CpG 1585	10/10	3/10
300 μg CpG 1585	10/10	7/10
75 μg CpG 2006	10/10	10/10
150 μg CpG 2006	10/10	10/10
Untreated controls	1/10	

Mice which had been dosed i.n. with CpG 1585 (100 or 300 μg) also survived a subsequent challenge with 10 MLD_{50} of vaccinia virus IHD by the i.n. route. However, when CpG 1585 was administered by the i.p. route, complete protection against a subsequent challenge with vaccinia virus was not observed; only 3/10 mice which had been dosed with 100 μg CpG 1585 and 7/10 mice which had been dosed with 300 μg CpG survived challenge. In all the groups of mice dosed with CpG 1585 the mean weight had returned to within 5% of their initial mean weight by day 19 post-challenge.

3.2. Intranasal CpG 2006 protects against 400 MLD_{50} challenge with vaccinia IHD

Since dosing of mice with 75 μg CpG 2006 by the i.n. route provided good protection against i.n. challenge with 10 MLD_{50} of vaccinia virus, we next investigated whether protection could be demonstrated against higher challenge doses of vaccinia virus, and whether protection could be demonstrated after a single dose of CpG 2006. Groups of six BALB/c mice were dosed i.n. with either 75 μg CpG 2006 given 10 and 3 days before challenge, or given 3 days before challenge. Irrespective of the CpG dosing regime, all of the mice challenged with 10 MLD_{50} of vaccinia virus IHD survived (Fig. 1). In comparison, all of the control (undosed) animals had been culled by day 9 due to the severity of the clinical signs of disease. When other groups of mice were challenged with 100 MLD_{50} of vaccinia virus IHD, 5/6 animals, which had received a single dose of CpG 2006 3 days before infection survived. All of the mice, which had received two doses of CpG 2006 at 10 and 3 days before challenge survived this challenge. In both treatments groups, all of the surviving treated animals returned to within 10% of their starting weight by day 14 post-challenge. In groups of mice challenged with 400 MLD_{50} vaccinia virus (Fig. 2) 5/6 survived irrespective of the CpG dosing regime.

At day 21 post-challenge all surviving animals were humanely culled. Spleens were removed from three animals in each dosing and challenge group, homogenized, diluted and dilutions added to cultures of RK13 cells. No cytopathic effects were observed in any of the cells, indicating that the viral load, if any, was below detectable levels. This suggests that all surviving animals had cleared the vaccinia virus challenge.

3.3. Dissemination of virus in CpG-treated mice following intranasal challenge

Groups of mice ($n = 5$) were treated with CpG 2006 (75 μg i.n.) at 10 and 3 days before challenge with 10 MLD_{50} vaccinia virus IHD by the i.n. route. On days 1, 3, 5, 7 and 9 post-challenge animals were humanely culled and brains, lungs and spleens were removed from one group of CpG-treated animals and one group of untreated controls. Titration of organs for virus load revealed that in animals treated with CpG, virus was at undetectable levels from any of the organs tested on day 1 post-challenge, whereas virus was detectable in the lungs of untreated controls at this time-point (Fig. 3). On day 3 post-infection virus load was heavy in the brains, lungs and spleens of untreated animals; however, in CpG-treated animals virus was undetectable in brains, and was up to 500-fold and 10,000-fold reduced in spleens and lungs, respectively. Mean viral count in the brain of untreated animals was approaching 10^5 pfu on day 5 post-challenge, whereas in CpG-treated animals only 1/5 had detectable virus in the brain and this was at very low levels. However, as virus was undetectable in the brain of any other CpG-treated animal at this or any of the other time-points it seems probable that this individual mouse could have received a sub-optimal dose of CpG.

3.4. Direct effect of CpG-DNA on vaccinia virus replication

CpG-DNA (two separate batches) was added to infected RK-13 monolayers as described to determine if there was any direct effect on viral replication. No reduction in viral plaque count was observed after CpG treatment. This suggests that the CpG is not exerting a direct antiviral effect and that the protective effects observed in vivo are a result of activation of the host immune system.

3.5. Duration of protection in CpG-DNA treated animals

Groups of five BALB/c mice were dosed with a single 75 μg dose of CpG 2006 on day 21, 14, 7, 3 or 1 before i.n. challenge with 10 MLD_{50} vaccinia virus IHD. In the group of mice treated with a single dose of CpG-DNA on the day before vaccinia virus challenge, 80% survived. All of the mice dosed with CpG 2003 at 3 or 7 days before challenge survived challenge. However, the mean weight loss from mice dosed 3 days before challenge reached a maximum of 15% of the starting body weights, whereas mice dosed 7 days before challenge lost a maximum of 21% of their starting weights. In the group of mice dosed with CpG 14 days before challenge, 40% survived and none of the mice dosed with CpG 21 days before challenge survived after challenge with vaccinia.

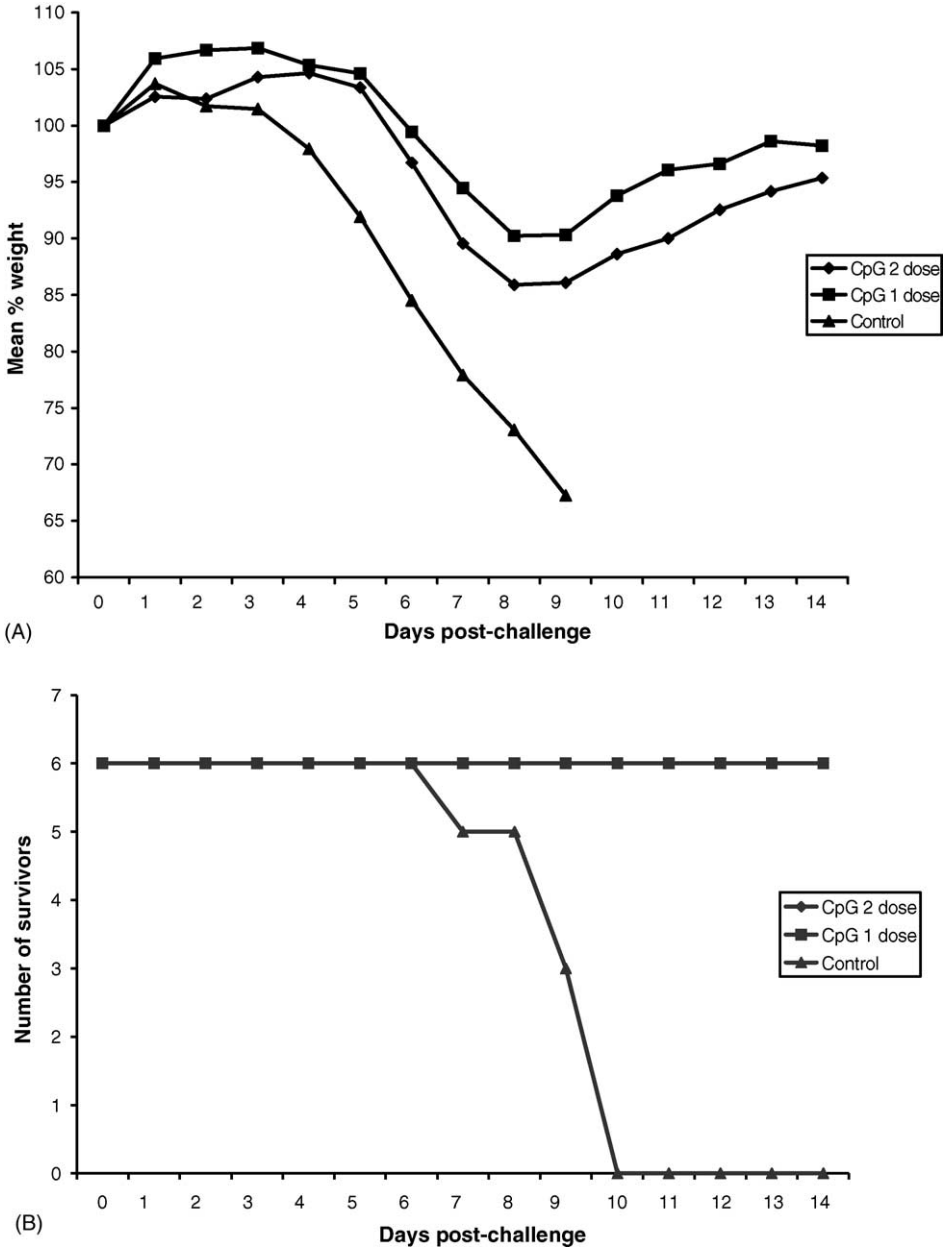

Fig. 1. (A) Mean percentage weight loss in groups of BALB/c mice treated with CpG 2006, and challenged intranasally with 10 MLD_{50} vaccinia virus IHD. (B) Survival of BALB/c mice treated intranasally with CpG 2006 and challenged intranasally with 10 MLD_{50} vaccinia virus IHD.

These findings indicate that CpG 2006 is most effective in preventing disease caused by vaccinia virus when given between 3 and 7 days before challenge.

3.6. Systemic cytokine production in CpG-DNA treated animals

Sera were collected from mice by vene puncture on the day before challenge and stored at −80 °C until analysis. In animals treated with CpG1585 the levels of IFN-γ, TNF-α, IL-2, IL-5 or IL-4 in sera were not significantly different from the levels of cytokines in naïve animals (data not shown). In contrast, sera from animals dosed i.p. CpG 2006 had levels of IFN-γ and TNF-α which were significantly higher ($p = 0.03$ and 0.00345, respectively, unpaired test) than those in naïve animals (Fig. 4).

3.7. Localized (lung) cytokine and chemokine production in CpG-DNA treatment groups

On the day before parallel groups of dosed animals were challenged, broncho-alveolar lavage (BAL) was performed on mice which had received two doses of 75 μg of CpG 2006 DNA by the i.n. or i.p. route. BAL fluid was assayed for IFN-γ, TNF-α, IL-2, IL-5, IL-4, RANTES, MIP-1α and MIP-1β. IL-2, IL-4 and IL-5 and were not detected in BAL from CpG-

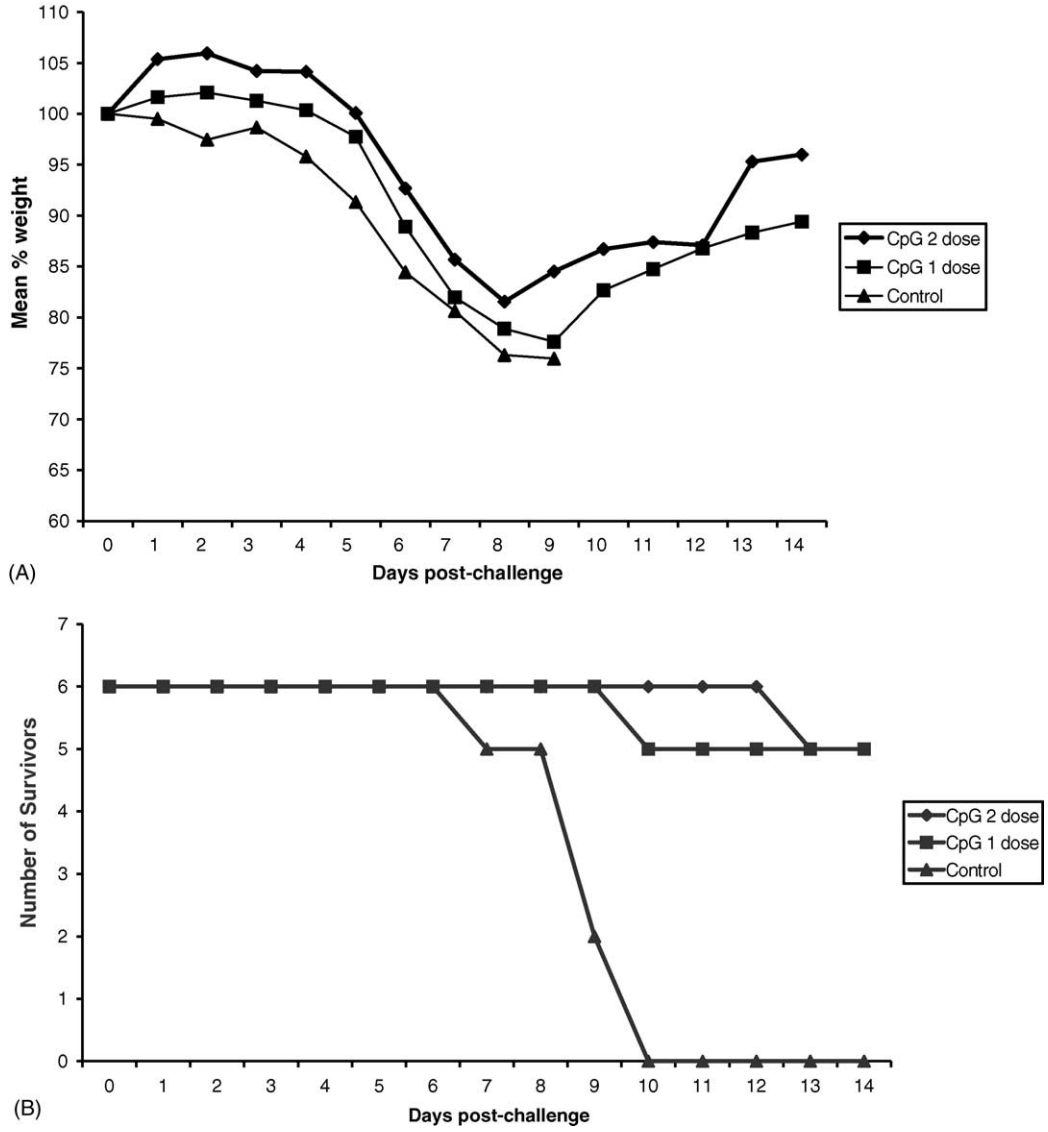

Fig. 2. (A) Mean percentage weight loss in groups of BALB/c mice treated with CpG 2006, and challenged intranasally with >400 MLD_{50} vaccinia virus IHD. (B) Survival of BALB/c mice treated intranasally with CpG 2006 and challenged intranasally with >400 MLD_{50} vaccinia virus IHD.

DNA treated animals or from control animals. In comparison with BAL from control mice or from mice dosed with CpG by the i.p. route, the levels of TNF-α, IFN-γ and MIP-1α in BAL were significantly higher in 3/5 mice which had been dosed with CpG by the i.n. route. However, the mean concentrations of 15.64, 10.44 and 18.84 pg/ml, respectively, were not considered to be biologically significant. In comparison with control and i.p. dosed mice, the concentrations of the chemokines RANTES and MIP-1β, were elevated in 3/5 mice dosed with CpG 2006 by the i.n. route (Fig. 5). The mean levels of RANTES and MIP-1β were 206.2 and 115.4 pg/ml, respectively, and were considered to be biologically significant. Whilst animals treated i.p. with CpG were not found to have significantly increased BAL cytokine or chemokine levels, their systemic TNF-α and IFN-γ were significantly elevated. This suggests that, whilst control of the virus at the lung surface may not have been achieved, systemic dissemination may have been partially controlled by the elevated cytokines observed in the serum.

4. Discussion

The data presented here demonstrate the protective efficacy of CpG-DNA against lethal orthopoxvirus challenge in a murine model. To our knowledge, this is the first time CpG-DNA mediated protection has been demonstrated to a pathogen at the lung surface. In this model CpG-B DNA produced enhanced levels of protection compared to CpG-A DNA. The reasons for this are not clear. Both types of CpG induce NK cell activation and IFN-α, although these responses are preferentially induced by CpG-A (Krug et al., 2001). Also CpG-B is able to activate B cells (Ballas et al., 1996; Boggs et al., 1997). The studies with CpG-A revealed a clear link between the site of activation of the innate immune system

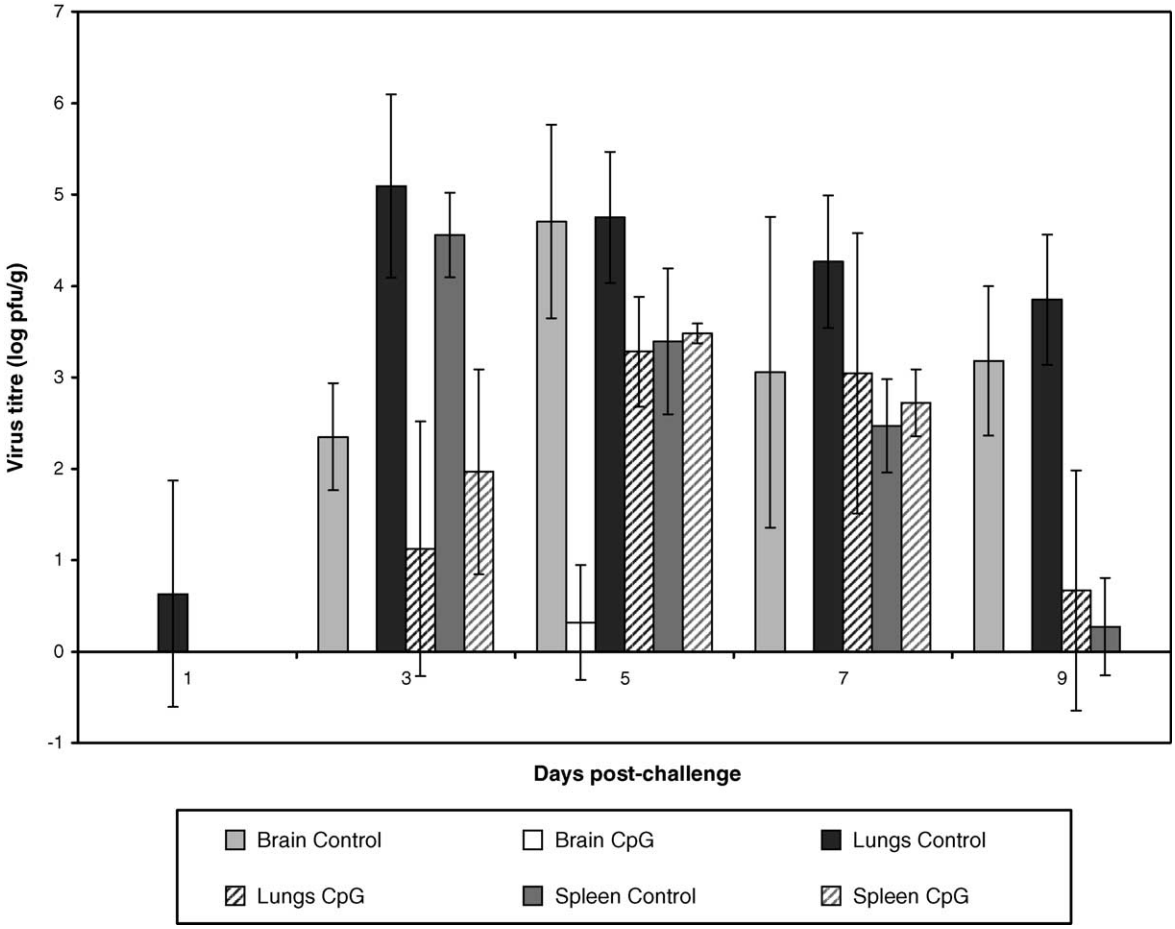

Fig. 3. Dissemination of virus in CpG-treated mice and untreated controls following intranasal challenge with vaccinia virus IHD.

and the degree of protection afforded to vaccinia virus challenge. The duration of protection extended to at least 14 days after dosing with CpG-DNA but was maximal at between 3 and 7 days after dosing.

The analysis of virus dissemination in the lungs, brain and spleen of CpG-treated animals compared to untreated controls suggests that CpG treatment markedly alters the pathology of disease. There is little evidence of viral dissemination to the brain in CpG-treated animals, whereas high levels of virus were detected in untreated controls. If dissemination of virus to the brain is direct via the olfactory nerve, the results suggest that the treatment of the respiratory tract with CpG-DNA is effective at blocking this. Alternatively, if dissemination to the brain is via the blood, as the total viral load is reduced in CpG-treated animals, it would suggest viral load in blood would show a concomitant reduction, with a subsequent reduction in dissemination to the brain. Viral plaque reduction studies demonstrated that CpG-DNA had no direct effect on viral replication, this suggests that protective effects observed in vivo are a result of immune system activation and cytokine and chemokine cascade.

Many organisms have evolved mechanisms to evade or overcome the host immune system (Haig, 1998; Lalani and McFadden, 1999) and many poxviruses encode homologues of host cytokines and chemokines and their receptors which modulate the host response to infection. For example, orthopoxviruses including vaccinia, express a protein which binds CC chemokines with high affinity (Alcamí et al., 1998). The elevated levels of the CC chemokines RANTES and MIP-1β in broncho-alveolar lavage fluid from animals treated intranasally with CpG-B DNA suggest a role for these chemokines in CpG-DNA mediated protection to vaccinia virus challenge. In general, CC chemokines have been shown

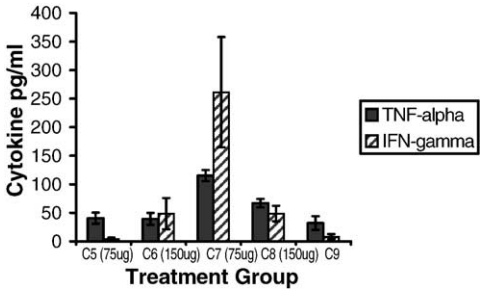

Fig. 4. Levels of cytokines in sera of mice treated with CpG 2006 intranasally (C5 and C6) or intraperitoneally (C7 and C8).

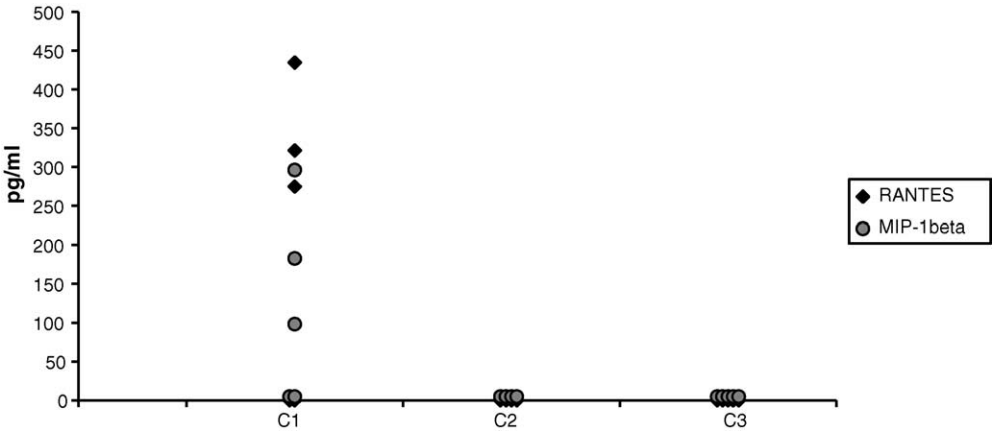

Fig. 5. Levels of RANTES and MIP-1β in BAL of mice treated with CpG 2006 intranasally (C1) or intraperitoneally (C2).

to attract macrophages and T cells and RANTES, MIP-1α and MIP-1β have been implicated in the clearance of a number of pathogens. For example MIP-1α is required for the clearance of acute *Klebsiella pneumoniae* infection (Lindell et al., 2001). The level of the vaccinia chemokine binding protein may not have been sufficient to bind all of the CC chemokines produced after CpG dosing of mice, thereby allowing the clearance of vaccinia virus.

It is also possible that some CC chemokines modulate the binding of vaccinia virus to host cells. Both RANTES and MIP-1β are agonists for the CCR5 receptor and there are some reports that CCR5 may act as a receptor for poxviruses. Notably, myxoma virus (a member of the poxvirus family) can infect cell lines transfected with CCR5, where it is unable to infect the wild type cells (Lindell et al., 2001). In the context of the work reported here it may be significant that the infection of CCR5-transfected cells by myxoma virus was inhibited by RANTES. In studies with both vaccinia virus and myxoma virus, McFadden et al. (2000) reported that the viral binding and internalization steps were unaffected by CCR5 expression. Instead, it appeared that the CCR5 effects occurred prior to DNA replication at the switch from early to late viral gene expression and subsequent virion production. This would in turn reduce viral dissemination though the host. Furthermore, this may represent a novel post-entry strategy for the exploitation of cell surface receptors to determine cellular tropisms by viruses. The upregulation of the CCR5 agonists, in particular RANTES by the pre-treatment of animals with CpG-DNA, could interfere with viral replication within the host cells. This occurs in SIV infection, where the upregulation of CC chemokines MIP-1α, MIP-1β and RANTES is significantly associated with protection against infection (Ahmed et al., 2002; Bergmeier et al., 2002). This may allow host cells to control the infection and prevent replication. Alternatively, as suggested by McFadden et al. (2000), the utilization of CCR5 as some kind of co-receptor or post-entry strategy for poxviruses may be blocked by the upregulation of "natural" CCR5 agonists.

In addition to the effects described on host chemokines and their receptors, vaccinia virus also has a number of effects on the function of interleukins and interferons. The C12L protein of vaccinia virus inhibits murine IL-18 and subsequently the IL-12 induced production of IFN-γ, thus promoting increased virus virulence in the murine intranasal model (Smith et al., 2000; Symons et al., 2002). In these studies, the observed slight elevation in IFN-γ production in animals pre-treated with CpG-DNA may have been sufficient to overcome the binding effects of viral proteins which inhibit IL-18 and subsequent IFN-γ production by a cascade effect. Recent data reported by Liu et al. (2004) suggest that intranasal administration of IFN-α or IFN-γ immediately prior to and post-infection with vaccinia virus resulted in 100 and 90% survival, respectively (in a murine model). Virus titers in the lungs of treated animals were reduced by 1000–10,000-fold, as compared to controls. This correlates with the data presented here, and would suggest that pre-existing interferon at the site of infection (produced by CpG administration) can protect animals against lethal challenge.

CpG-DNA activates the immune system via Toll-like receptor 9 (Hemmi et al., 2000; Poltorak et al., 1998; Tokunaga et al., 1984) leading to activation of NF-κB. Vaccinia virus encodes a protein (A52R) which down-regulates the activation of NF-κB by the TLR family members IL-1R, IL-18R and TLR4 (Bowie et al., 2000). More recently, the vaccinia virus protein A52R has been found to block the activation of NF-κB by multiple TLRs (Harte et al., 2003); however, data were not shown to demonstrate its effects on TLR9 (the receptor for CpG-DNA). This suggests that to be effective, CpG-DNA must be administered before challenge with vaccinia virus.

These preliminary results suggest that CpG-DNA delivered directly to the lung mucosal surface offers potential for the prevention of disease caused by orthopoxviruses delivered by the inhalation route. Taken together with published reports of the protective effect of similar CpG-DNA to other organisms as diverse as Ebola virus (Klinman et al., 1999), *B. anthracis* (Klinman et al., 1999), *Listeria monocytogenes*

(Elkins et al., 1999; Krug et al., 2001), *Francisella tularensis* (Elkins et al., 1999) and *Plasmodium yoelli* (Gramzinski et al., 2001), these results suggest that CpG therapy has great potential as a generic immunomodulator, against a range of infectious agents. Further studies will be required to determine the protective efficacy of CpG-DNA delivered to the lung mucosal surface against other pathogens delivered to the respiratory tract. This may lead to the development of a generic immunomodulator, which may be delivered to the lung surface shortly before infection takes place.

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