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Protective immunity against acute phleboviral infection elicited through immunostimulatory cationic liposome-DNA complexes

Brian B. Gowen [a,*], Jeff Fairman [b], Donald F. Smee [a], Min-Hui Wong [a], Kie-Hoon Jung [a], Anne M. Pace [a], Matthew L. Heiner [a], Kevin W. Bailey [a], Steven W. Dow [c], Robert W. Sidwell [a]

[a] *Institute for Antiviral Research, Utah State University, Logan, UT 84322, United States*
[b] *Juvaris BioTherapeutics Inc., 6200 Stoneridge Mall Road, Pleasanton, CA 94588, United States*
[c] *Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523, United States*

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Abstract

Cationic liposome-DNA complexes (CLDC) have been demonstrated to induce potent antitumor activities. The ability of these complexes to elicit protective immunity against viral infections has not been fully explored. Here we report findings on the use of CLDC as an antiviral agent in a mouse model of acute phleboviral (Punta Toro virus) disease. CLDC treatment of mice challenged with Punta Toro virus (PTV) resulted in dramatic increases in survival and reduced viral burden and other parameters indicative of protection against disease. CLDC were effective when administered by intraperitoneal and intravenous routes and elicited protective immunity when given within 1 day of virus challenge. Treatments administered 36 h or longer after challenge, however, were not effective in preventing mortality or disease. CLDC treatment induced release of a number of potential antiviral cytokines including IFN-γ, IL-12, and IFN-α. Taken together, our findings indicate that non-specific immunotherapy with CLDC appears to be an effective treatment for blocking PTV-induced disease and suggests that further exploration in other viral disease models may be warranted.

Keywords: Liposome; Plasmid DNA; Innate immunity; Phlebovirus

1. Introduction

Viruses that cause hemorrhagic fever are among the deadliest and most feared due to the devastating effects they can have on both human and animal populations. The potential threat of weaponization has led to their classification as category A highest priority pathogens (NIAID, 2002) underscoring the importance of identifying prophylactic and therapeutic interventions in the event of intentional release as well as natural outbreaks. Included in this group of viruses is Rift Valley fever virus (RVFV) which is endemic to the African continent. RVF is an acute bunyaviral disease that affects humans and domesticated animals. More recent outbreaks affecting humans have caused considerable morbidity and mortality and indicate geographic spread to areas outside of sub-Saharan Africa (Ahmad, 2000; CDC, 1994; Meegan, 1979).

The requirement for maximum-level containment facilities and greatly restricted access associated with the select agent status of RVFV creates a major challenge for the development of antiviral drugs for treatment of RVFV infection. Closely related to RVFV, Punta Toro virus (PTV) is a member of the Bunyaviridae family, genus *phlebovirus*, that produces a fatal hepatic disease in mice similar to that caused by RVFV in humans and livestock (Pifat and Smith, 1987). Safe to work with under biosafety level 2 (BSL-2) containment, this animal model of acute phleboviral disease facilitates investigations of promising antivirals and has served as a highly predictive substitute for the more biohazardous phleboviruses such as RVFV (Sidwell et al., 1994).

More than a decade ago, studies were undertaken investigating a gene delivery system that employed the use of liposomes complexed to plasmid DNA with the goal of eliciting expression of the delivered gene product in target tissues (Zhu et al., 1993). It became apparent that the injection of the complex of plasmid DNA and liposomes resulted in a profound activation of innate host immunity. This activation occurred whether or

* Corresponding author. Tel.: +1 435 797 3112; fax: +1 435 797 3959.
E-mail address: bgowen@cc.usu.edu (B.B. Gowen).

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not the plasmid DNA component was coding or non-coding (empty vector), indicating that gene expression was not required. The response was substantially dependent upon formation of a cationic lipid DNA complex (CLDC), since neither entity alone had significant stimulatory properties except at exceedingly high in vivo doses. With these early observations, it has become recognized that the stimulation of innate immunity, triggered by CLDC, was due in part to a liposome-mediated potentiation of the inherent responsiveness of the mammalian immune system to non-methylated CpG motifs within the bacterial DNA of the plasmids (Krieg, 2002). Recently it has been demonstrated that CpG motifs are recognized by Toll-like receptor 9 (TLR9) (Hemmi et al., 2000), an interaction requiring internalization that is facilitated by the lipid component. Liposomes have been shown to enhance the immunostimulatory activity of CpG oligodeoxynucleotides by 15–40-fold (Gursel et al., 2001). This route-sensitive and dose-dependent effect has been recognized in multiple species including rodents, rabbits, dogs, and non-human primates (S.W. Dow, unpublished results) and is characterized by an immediate up-regulation of a broad-array of soluble and cellular host defenses. Intravenous (i.v.) administration of CLDC also resulted in release of IL-12, IFN-γ, IFN-α, TNF-α and recruitment and activation of macrophages and NK cells in vivo (Dow et al., 1999a,b; Freimark et al., 1998). Here we report our findings evaluating the potential antiviral activity of CLDC in a mouse model of acute phleboviral infection.

2. Materials and methods

2.1. Animals

Female 12–14 g C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). All animals were held for a minimum of 48 h prior to experimentation. Animal procedures used in this study complied with guidelines set forth by the USDA and Utah State University's Institutional Animal Care and Use Committee.

2.2. Virus

The Adames strain of PTV was provided by Dr. Dominique Pifat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). The virus used in these experiments was from a stock prepared following four passages of the original virus stock through LLC-MK_2 (rhesus monkey, kidney) cells. PTV was inoculated into mice via the subcutaneous route.

2.3. Test materials

Sterile 10 mM solutions of the cationic liposome DOTIM {octadecenoyloxy{ethyl-2-heptadecenyl-3-hydroxyethyl} imidazolinium chloride and cholesterol were prepared in a 1:1 molar ratio as described previously, except that the liposomes were extruded through a final filter diameter of 200 nm rather than 100 nm (Dow et al., 1999a). Liposome-DNA complexes were formed just prior to injection by gently mixing cationic liposomes with plasmid DNA at a ratio of 16 nmol lipid per 1 μg DNA in 5% dextrose in water at room temperature. The plasmid (pMB75.6 empty vector; 3 mg/mL) used for these studies contained a human cytomegalovirus enhancer/promoter but lacked a gene insert downstream of the promoter. The plasmid also contained a kanamycin resistance gene, an IXX8 intron, a poly adenylation site from the human growth hormone gene, a bacterial origin of replication derived from ColEl, and a bacteriophage f1 origin of single replication. The final plasmid DNA concentration in the complexes was 100 μg DNA per ml. Experimental doses are indicated in μg amounts, referring to the total DNA content complexed with the cationic lipid. Ribavirin, known to be active against PTV (Sidwell et al., 1988), was supplied by ICN Pharmaceuticals Inc. (Costa Mesa, CA) and served as the positive control for challenge studies. Ribavirin was dissolved in sterile saline and given i.p. in all experiments.

2.4. PTV challenge studies

A total of 15–21 mice from each group (25–31 for the placebo group) were treated with a single i.p. injection of 10% sucrose vehicle (placebo) or various doses of CLDC, at indicated times, pre- or post-infectious challenge with 2×10^4 cell culture 50% infectious doses ($CCID_{50}$) of PTV. Ribavirin was administered twice a day for 5 days with treatment starting 4 h pre-inoculation. Five to 10 animals from each group were sacrificed on day 3 or 4 of infection and their livers were removed, weighed, and scored visually on a scale of 0–4 for hepatic icterus; zero being normal and four being maximal yellow coloration. Serum was collected from survivors for serum alanine aminotransferase determinations and virus titers were obtained for both liver homogenates and serum samples. The remaining animals in each group were observed for death out to 21 days. To assess potential toxicity associated with test drugs, three uninfected mice from each treatment group were observed for any visible signs of toxicity and normal weight gain. For comparison, three sham-infected animals were included as normal controls in order to establish baselines for all parameters tested. The experiment evaluating efficacy by i.v. route of administration was performed similarly.

2.5. Liver and serum virus titers

Virus titers were determined using an infectious cell culture assay as previously described (Sidwell et al., 1988). Briefly, specific volumes of liver homogenate or serum were serially diluted and added to triplicate wells of LLC-MK_2 cell monolayers in 96-well microplates. The viral cytopathic effect (CPE) was determined 5 and 6 days post-virus exposure and the 50% endpoints were calculated as described (Reed and Muench, 1938). The assay detection limit was 2.8 $\log_{10}$ $CCID_{50}$/g of liver or ml of serum. In samples presenting with no detectable virus, a value of <2.8 logs was assigned. Therefore, a mean virus titer value preceded by "<" indicates that at least one of the samples had undetectable levels of virus and is likely an over-

estimate of the actual mean viral load. For statistical analysis, a value of 2.8 logs was used for samples with undetectable virus.

2.6. Serum alanine aminotransferase (ALT) determinations

Detection of ALT in serum serves as an indirect method for evaluating liver damage. Serum ALT levels were measured using the ALT (SGPT) Reagent Set purchased from Pointe Scientific Inc. (Lincoln Park, MI) following the manufacturer's recommendations. The reagent volumes were modified for use with 96-well microplates.

2.7. Cytokine profiling

Mice were treated as indicated with various dose amounts of CLDC and serum was collected after 4, 8 and 16 h. Systemic gamma interferon (IFN-γ), interleukin-12 (IL-12), and alpha interferon (IFN-α) levels were assayed using ELISA reagents purchased from R&D Systems (Minneapolis, MN).

2.8. Statistical analysis

The Fisher's exact test (two-tailed) was used for evaluating increases in total survivors. The log-rank test was used for time-to-death survival analysis and was performed using JMP statistical software (SAS, Cary, North Carolina). The Mann–Whitney test (two-tailed) was performed to analyze the differences in mean day to death, virus titers, and serum ALT levels. Wilcoxon ranked sum analysis was used for mean liver score comparisons.

3. Results

3.1. Prophylactic and therapeutic intraperitoneal CLDC treatment of PTV infection in mice

Due to the nature of CLDC as an immunostimulant, we tested whether it could elicit protective antiviral activity against PTV by single dose injection. CLDC were initially evaluated at various doses pre- and post-PTV challenge. As summarized in Table 1, all doses and schedules were highly effective as only three animals were lost out of a total of 42. In the 0.5 μg, −24 h pre-treatment group, the sole mouse that died survived significantly longer than placebo-treated animals. The 20 μg treatments given at 4 h pre-infection and 24 h post-infection were equally effective protecting 90% of the mice from a lethal challenge dose of PTV (Table 1). The doses tested did not appear to have any overt toxic effects as a parallel toxicity study indicated normal weight gain and healthy appearance (data not shown). Although three of the four CLDC treatments resulted in significant reductions in liver scores, they were not as effective as the positive control drug, ribavirin (Table 1). No virus was detected in liver homogenates from the CLDC 20 μg therapeutic treatment group or the ribavirin group, however, due to overall low liver virus titers and the sensitivity limitations of the cell-based infectious assay used, the results were not significant by the statistical test employed (Table 1). In contrast, all CLDC treatments were found to produce significant reductions in serum viral loads, with the 20 μg therapeutic CLDC dose having the most dramatic effect. In addition, CLDC-treated mice had significantly reduced serum concentrations of ALT, indicative of reduction in liver injury. It should be noted that due to mortality prior to the time of sacrifice (day 4), serum could only be

Table 1
Protection of mice against lethal PTV infection with prophylactic and therapeutic i.p. CLDC treatment

Treatment[a]/time (h)	Dosage	Number of surv/total	Mean day of death[b] ± S.D.	Survival log-rank prob > χ^2	Disease parameter[c]			
					Mean virus titer[d] ± S.D.		ALT[e] ± S.D.	Liver score[f] ± S.D.
					Liver	Serum		
CLDC/−24	5 μg	11/11***		<0.0001	<2.9 ± 0.6	<3.0 ± 0.6***	139 ± 155***	2.9 ± 0.4
CLDC/−24	0.5 μg	10/11***	12.0	<0.0001	<3.0 ± 0.6	<3.4 ± 1.4**	367 ± 1059**	2.1 ± 0.8**
CLDC/−4	20 μg	9/10***	6.0	<0.0001	<3.6 ± 0.7	<5.1 ± 1.3**	144 ± 336***	2.3 ± 0.8*
CLDC/24	20 μg	9/10***	5.0	<0.0001	<2.8	<2.8***	40 ± 48***	2.5 ± 0.4*
Ribavirin	75 mg/kg/day	10/10***		<0.0001	<2.8	<2.8 ± 0.1***	16 ± 15***	0.6 ± 0.4***
10% Sucrose		0/20	5.3 ± 0.5		<3.2 ± 0.8	7.1 ± 0.4	2678 ± 550	3.0 ± 0.7
Sham-infected		3/3			<2.8	<2.8	32 ± 24	0.0 ± 0.0

[a] Single dose CLDC treatment given at the indicated times. Ribavirin started at −4 h and was given twice per day for 5 days. Placebo 10% sucrose solution was administered at −24 h.
[b] Mean day of death of mice dying prior to day 21.
[c] Determined 4 days post-virus challenge. Ten mice per group (due to mortality prior to time of sacrifice, serum titers and ALT were determined for only five mice from the placebo group).
[d] Log_{10} cell culture 50% infectious dose $CCID_{50}$/g of liver or ml of serum. The assay detection limit was 2.8 log_{10} $CCID_{50}$/g or ml; a value of <2.8 was assigned for samples presenting with no detectable virus.
[e] ALT, alanine aminotransferase; measured in international units per liter.
[f] Score of zero (normal liver) to four (maximal discoloration).
* $P < 0.05$ compared to 10% sucrose placebo-treated controls.
** $P < 0.01$ compared to 10% sucrose placebo-treated controls.
*** $P < 0.001$ compared to 10% sucrose placebo-treated controls.

obtained for 5 of the 10 animals in the placebo group. Therefore, it is likely that the mean serum virus titer and ALT levels observed for the placebo group are an underestimate of the actual disease severity. Overall, the initial study with CLDC indicated remarkable protection against lethal PTV challenge and reduction of disease manifestations associated with infection.

3.2. Intraperitoneal CLDC dose effect on treatment of PTV infection in mice

Having seen remarkable efficacy following single dose CLDC administration 24 h post-infection, additional experiments were undertaken to determine the effective dose range of CLDC. In the first trial, doses spanning four orders of magnitude were evaluated. As shown in Table 2, Trial 1, the 0.3 μg dose (100% survival) appeared to demarcate the cut-off for the amount of CLDC needed to elicit significant protection. Unexplainably, the 3 μg dose seemed to drop-off slightly to 80% survival in contrast to the 30 and 0.3 dose treatments where no mice were lost. Although not to the degree seen with ribavirin, highly significant reductions in liver discoloration were observed with CLDC (Table 2, Trial 1). Consistent with the dose-dependent decrease in liver scores, marked declines in serum ALT levels were seen. The two higher CLDC doses prevented ALT levels from rising substantially above the baseline identified in normal mice and the reduction seen with the 0.3 μg group was also impressive. As the results demonstrate in Table 2, Trial 1, liver virus titers were found to be unremarkable as even the placebo group presented with animals for which virus could not be detected by infectious cell culture assay. In contrast, high levels of systemic virus were present in nine of 10 placebo-treated animals and titers were reduced by greater than three logs with the two highest doses of CLDC, comparable to that seen with ribavirin.

A second trial was conducted to better resolve the efficacy limits of CLDC. In this evaluation, which examined half-log dilutions of drug starting with 3 μg of DNA, a more robust placebo die-off was seen (Table 2, Trial 2). The highest doses of 3, 1, and 0.3 μg protected 90, 80, and 70% of infected animals, respectively, from an LD_{85} inoculum of virus. At the lowest dose of 0.1 μg, only 30% of the animals survived. This was consistent with the data from Trial 1 where 0.3 μg was the lowest effective dose. As reflected in Table 2, Trial 2, good correlation was also evident in the evaluation of hepatic icterus where the two highest doses produced significant reductions in liver discoloration. Notably, PTV could not be detected in any of the five livers examined in the 3 μg CLDC treatment group; however, as before, due to low virus titers and variability with the placebo group, no statistical significance was achieved. All other treatments, including ribavirin, had detectable liver virus in at least one of the five animals (Table 2, Trial 2). As seen with liver virus titers, no detectable serum virus was found in the 3 μg CLDC group. Ribavirin treatment also resulted in undetectable levels of serum virus on day 3 of the infection. All other CLDC treatments

Table 2
Dose effect of i.p. CLDC treatment on PTV infection of mice

Treatment[a]	Dosage	Number of surv/total	Mean day of death[b] ± S.D.	Survival log-rank prob > χ^2	Disease parameter[c]			
					Mean virus titer[d] ± S.D.		ALT[e] ± S.D.	Liverscore[f] ± S.D.
					Liver	Serum		
Trial 1, CLDC	30 μg	11/11***		0.0009	<2.8	<3.5 ± 1.1**	57 ± 74***	1.5 ± 0.7***
	3 μg	8/10	5.5 ± 0.7	0.0249	<2.9 ± 0.6	<3.3 ± 0.9**	12 ± 12***	1.8 ± 0.9***
	0.3 μg	11/11***		0.0009	<2.9 ± 0.3	<5.2 ± 1.6	333 ± 704**	3.2 ± 0.3***
	0.03 μg	2/11	4.4 ± 0.5	0.2107	<3.1 ± 0.7	6.7 ± 0.9	3872 ± 1158	3.6 ± 0.3*
Ribavirin	75 mg/kg/day	10/10***		0.0014	<2.8 ± 0.1	<3.7 ± 1.4*	19 ± 4***	0.0 ± 0.0***
10% Sucrose		7/20	5.0 ± 1.2		<3.2 ± 1.0	<6.5 ± 1.6	4424 ± 1811	3.9 ± 0.2
Sham-infected		3/3			<2.8	<2.8	45 ± 11	0.0 ± 0.0
Trial 2, CLDC	3 μg	9/10***	4.0	0.0005	<2.8	<2.8***	35 ± 13***	1.1 ± 1.1**
	1 μg	8/10***	9.5 ± 4.9**	0.0007	<3.3 ± 0.7	<5.5 ± 1.6	1946 ± 2553	2.1 ± 1.1**
	0.3 μg	7/10**	4.0 ± 0.0	0.0228	<3.5 ± 1.0	5.4 ± 0.5**	438 ± 555**	3.0 ± 1.2
	0.1 μg	3/10	4.8 ± 0.8	0.8072	<3.3 ± 1.1	6.3 ± 0.7	3248 ± 1913	3.6 ± 0.4
Ribavirin	75 mg/kg/day	10/10***		<0.0001	<.2 ± 0.9	<2.8***	15 ± 6***	0.4 ± 0.5**
10% Sucrose		3/20	5.7 ± 1.3		<3.8 ± 1.4	6.8 ± 0.7	2939 ± 1322	3.5 ± 0.4
Sham-infected		3/3			<2.8	<2.8	20 ± 10	0.0 ± 0.0

[a] Single dose CLDC treatment 24 h post-challenge. Ribavirin given twice per day for 5 days.
[b] Mean day of death of mice dying prior to day 21.
[c] Determined 3 days post-virus challenge. Ten mice per group in Trial 1 and 5 per group (10 for the placebo) in Trial 2.
[d] Log_{10} cell culture 50% infectious dose $CCID_{50}$/g of liver or ml of serum. The assay detection limit was 2.8 log_{10} $CCID_{50}$/g or ml; a value of <2.8 was assigned for samples presenting with no detectable virus.
[e] ALT, alanine aminotransferase; measured in international units per liter.
[f] Score of zero (normal liver) to four (maximal discoloration).
* $P < 0.05$ compared to 10% sucrose placebo-treated controls.
** $P < 0.01$ compared to 10% sucrose placebo-treated controls.
*** $P < 0.001$ compared to 10% sucrose placebo-treated controls.

Table 3
Outcome of delayed i.p. CLDC treatment on PTV infection of mice

Treatment[a]	Initiation of treatment (h)	Number of surv/total	Mean day of death[b] ± S.D.	Survival log-rank prob > χ^2	Disease parameter[c]			
					Mean virus titer[d] ± S.D.		ALT[e] ± S.D.	Liver score[f] ± S.D.
					Liver	Serum		
CLDC, 1 μg	24	9/11*	4.5 ± 0.7	0.0165	<2.8	<3.6 ± 1.1**	125 ± 186**	1.9 ± 0.2**
	36	4/11	4.1 ± 0.9	0.7911	<3.2 ± 0.9	6.1 ± 1.1	3591 ± 1781	3.6 ± 0.4
	48	1/11	4.5 ± 1.2	0.0578	<3.7 ± 1.3	7.0 ± 0.5	4381 ± 895	3.2 ± 0.6
	60	0/11	5.0 ± 1.3	0.0312	<3.6 ± 1.2	7.1 ± 0.9	3722 ± 1176	3.0 ± 0.4
	72	0/11	4.7 ± 1.0	0.0166	<3.1 ± 0.8	6.3 ± 0.5	3021 ± 903	3.2 ± 0.3
Ribavirin	−4	10/10***		0.0007	<2.8	<2.8**	15 ± 6**	0.2 ± 0.3**
10% Sucrose	24	7/21	5.0 ± 1.3		<3.7 ± 1.4	6.6 ± 0.1	3845 ± 727	3.2 ± 0.7
Sham-infected		3/3			<2.8	<2.8	9 ± 4	0.0 ± 0.0

[a] Single dose CLDC treatment. Ribavirin given twice per day for 5 days.
[b] Mean day of death of mice dying prior to day 21.
[c] Determined 3 days post-virus challenge. Five mice per group.
[d] Log_{10} cell culture 50% infectious dose $CCID_{50}$/g of liver or ml of serum. The assay detection limit was 2.8 log_{10} $CCID_{50}$/g or ml; a value of <2.8 was assigned for samples presenting with no detectable virus.
[e] ALT, alanine aminotransferase; measured in international units per liter.
[f] Score of zero (normal liver) to four (maximal discoloration).
* $P < 0.05$ compared to 10% sucrose placebo-treated controls.
** $P < 0.01$ compared to 10% sucrose placebo-treated controls.
*** $P < 0.001$ compared to 10% sucrose placebo-treated controls.

had 5.4–6.3 logs of systemic virus. Nonetheless, compared to the placebo-treated group that had a mean viral load of 6.8 logs, the decrease seen in the 0.3 μg CLDC treatment group was found to be highly significant (Table 2, Trial 2). Concordant with the serum virus data, baseline levels of ALT were observed in the 3 μg CLDC and ribavirin groups. Also, the animals in the 0.3 μg group presented with reduced liver disease as indicated by the significant abrogation of serum ALT activity. Interestingly, the 1 μg dose presented with greater levels of ALT activity (Table 2, Trial 2). Due to the acute nature of the infection in mice, this result was spurred by two of the five animals in the 1 μg group having very high ALT levels at the time of sacrifice, while levels for the other three were relatively low. These two mice also had higher systemic virus resulting in large standard deviations for this and ALT disease parameters.

3.3. Effects of delayed CLDC therapy on disease outcome

Based on the results from the initial studies, the 1 μg CLDC dose was evaluated for therapeutic efficacy in more advanced states of PTV infection. As previously seen in Table 2, when treatment was administered at 24 h, a high percentage of animals survived infectious challenge (Table 3). By delaying treatment an additional 12 h the survival rate fell precipitously to 36%, comparable to the level observed with the placebo-treated animals (33%). Further delay in the time of administration out to 48 h or longer increased detrimental effects associated with delayed therapy as nearly all animals died in those treatment groups (Table 3). Compared to the placebo group, the reduced survival in the 60 and 72 h groups was found to be statistically significant by log rank survival analysis and the 48 h group ($P = 0.0578$), although not considered significant, was noteworthy (Table 3). There was no detectable liver virus found in the 24 h CLDC or ribavirin groups, whereas all other treatments had measurable virus similar to that observed for the placebo-treated animals. A similar profile was obtained when analyzing systemic virus with only the 24 h CLDC and ribavirin groups significantly abrogating serum virus titers. As expected, concomitant drop-offs in serum ALT levels were also observed in these two groups, while the treatments delayed 36 h or more presented with ALT activity analogous to that seen in the placebo group (Table 3). These data were also consistent with the significant reduction in liver scores seen only in 24 h CLDC and ribavirin treatment groups and suggest that timely CLDC treatment may be required for limiting virus-induced liver damage and decreasing virus titers in this disease model.

3.4. CLDC induction of lFN-γ, IL-12, and lFN-α

IFN-γ, IL-12, and IFN-α are essential components for the establishment of antiviral host defenses (Romani et al., 1997; Samuel, 2001). The ability of CLDC to induce these cytokines in 4-week old mice used in the PTV disease model was evaluated by ELISA. As shown in Fig. 1A, in a dose-dependent manner, serum IFN-γ levels were found to be highest at the 8 h time point and waned moderately by 16 h. In contrast, 8 h exposure to 3 μg CLDC administered by i.v. route resulted in an IFN-γ response more than 25-fold greater than the equivalent dose given i.p. (Fig. 1A, inset). Moreover, the kinetics were noticeably different between the treatment routes as i.v. exposure dropped-off dramatically (98%) following the 8 h sampling, whereas, by i.p., a gradual decrease (45%) was observed. In the case of IL-12p70, as revealed in Fig. 1B, levels seemed to peak between the 4–8 h times and drop-off significantly by 16 h when dosed i.p. Interestingly, the 0.3 μg dose, which offered protection in challenge studies (Table 2), only elicited low levels of IL-12p70.

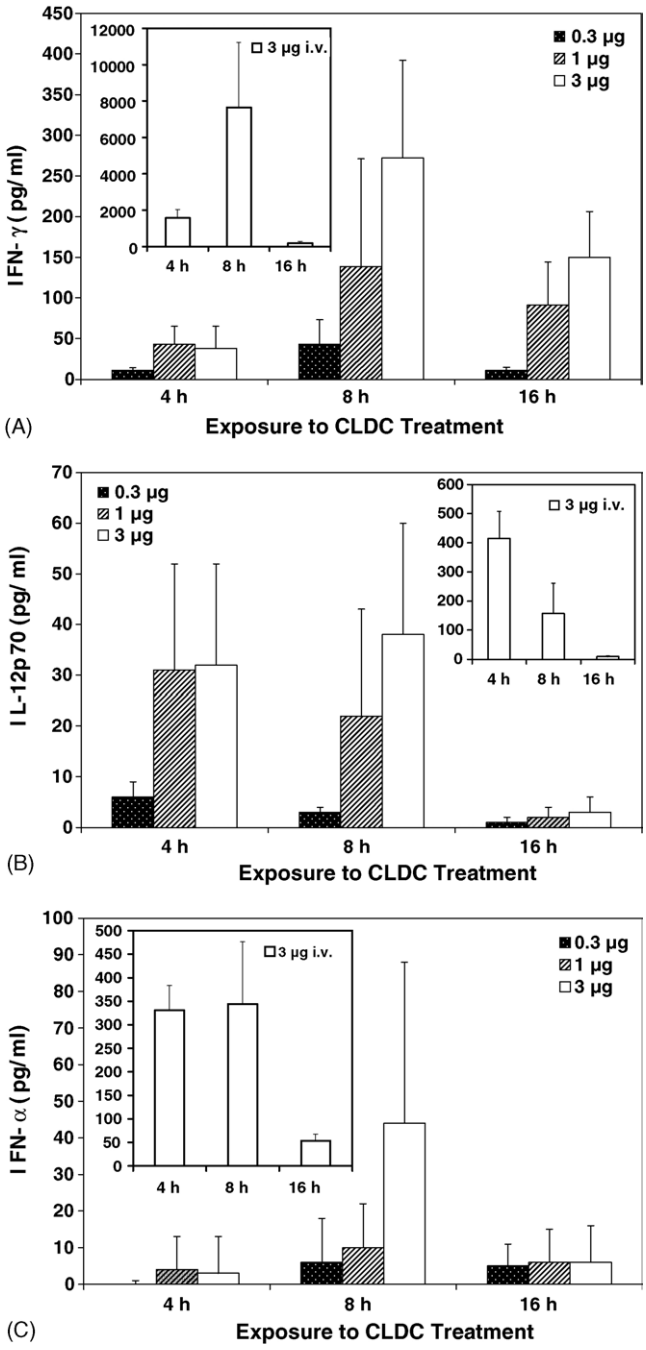

Fig. 1. Effect of CLDC on IFN-γ, IL-12p70, and IFN-α induction in uninfected mice. Four-week old mice were injected i.p. with 0.3, 1, or 3 μg of CLDC and serum was collected at the indicated exposure times. Animals treated by intravenous (i.v.) route with 3 μg of CLDC were also included for comparison (insets). The samples were analyzed by ELISA for systemic (A) IFN-γ, (B) IL-12p70, and (C) IFN-α production and the data represent the means and standard deviations of groups of five animals.

As before, different kinetics were observed for i.v. dosing as IL-12p70 levels peaked at 4 h and decreased rapidly thereafter (Fig. 1B, inset). At the 4 h exposure time, IL-12p70 levels were more than 12-fold higher than that observed with i.p. dosing (Fig. 1B). As for IFN-α, only low-level production was elicited by i.p. CLDC, with the exception of the 3 μg dose where the levels in one animal approached 90 pg/ml (Fig. 1C). As with IFN-γ and IL-12p70, the levels of IFN-α were much greater when CLDC were administered by the i.v. route (Fig. 1C, inset). When comparing profiles, there was a 110-, eight-, and nine-fold increase in IFN-α levels at the 4, 8, and 16 h time points, respectively, when CLDC were dosed i.v. In contrast to that seen by i.p., i.v. delivery resulted in peak levels of IFN-α at both the 4 and 8 h exposure times (Fig. 1C, inset). Collectively, the data indicate that i.p. CLDC can elicit the production of IFN-γ, IL-12p70, and IFN-α in weanling mice; however, i.v. delivery was much more efficient in stimulating elevated levels of these cytokines with distinctly different kinetics.

3.5. Intravenous CLDC treatment of PTV infection in mice

In light of the observed cytokine profiles, an experiment assessing i.v. CLDC delivery was conducted starting with 10-fold less material than used for i.p. in the previous challenge study. As indicated in Table 4, treatment with the top dose of 300 ng (0.3 μg) resulted in 70% protection against an LD_{90} challenge dose. Significant reductions in systemic virus load and liver disease were evident as well at this dose. A 60% survival rate was achieved with the 30 ng dose (Table 4), which was not efficacious when given i.p. (Table 2). Despite significant protection from death at this dose, very little impact was seen on other disease parameters evaluated with the exception of reduced ALT levels. As in the first experiment, due to the mortality of many of the animals in the placebo group prior to sacrifice, serum could not be obtained; therefore, the indicated serum virus titer and ALT values are likely an underestimate of the magnitude of disease. The 3 ng dose was not effective at protecting mice from death nor reducing virus burden or liver damage and discoloration (Table 4). The antiviral activity of i.v. CLDC was less than expected and may have been influenced by stress associated with tail vein delivery of CLDC in 4-week old animals.

4. Discussion

Robust in vivo immunological stimulus and antitumor activity elicited by CLDC have been previously reported (Dow et al., 1999a). More recently, CLDC have also been shown to be potent inducers of type I interferon production in vivo (Sellins et al., 2005). The aim of this work was to evaluate CLDC for antiviral activity in a model of acute phleboviral disease. Collectively, in the PTV mouse model, a single dose of 0.3 μg of CLDC delivered i.p. was found to protect more than 86% of challenged animals, compared to 25% for the placebo group, when treatment was initiated 24 h post-virus inoculation. However, this dose was not as effective as quantities of 3 μg or more at reducing the other disease parameters evaluated, which was reflective of the cytokine profiles obtained for these doses. The 3 μg dose was comparable to the positive control drug ribavirin in limiting viral burden and liver disease. This is remarkable considering that ribavirin treatment was initiated prior to infectious challenge and was given twice a day for 5 days compared to single dose treatment with CLDC.

In mice, PTV infection and disease manifestations progress rapidly with the majority of animals succumbing to lethal infec-

Table 4
Dose effect of i.v. CLDC treatment on PTV infection of mice

Treatment[a]	Dosage	Number of surv/total	Mean day Survival of death[b] ± S.D.	Survival log-rank prob > χ^2	Disease parameter[c]			
					Mean virus titer[d] ± S.D.		ALT[e] ± S.D.	Liver score[f] ± S.D.
					Liver	Serum		
CLDC	300 ng	7/10**	4.3 ± 2.5	0.0025	<3.0 ± 0.4	<3.0 ± 0.4	189 ± 363*	2.7 ± 0.8*
	30 ng	6/10**	4.3 ± 0.5	0.0124	<2.8	<5.4 ± 1.5	1379 ± 777	4.0 ± 0.0
	3 ng	1/10	4.8 ± 0.8	0.7534	<3.9 ± 1.4	5.5 ± 1.4	1589 ± 927	3.5 ± 0.6
Ribavirin	75 mg/kg/day	9/10***	4.0	<0.0001	<2.8	<3.3 ± 1.1	17 ± 10*	0.0 ± 0.0***
10% Sucrose		2/20	4.7 ± 1.0		<3.1 ± 0.8	<5.7 ± 2.0	2439 ± 896	3.8 ± 0.3
Sham-infected		3/3			<2.8	<2.8	32 ± 24	0.0 ± 0.0

[a] Single dose CLDC treatment 24 h post-challenge. Ribavirin given twice per day for 5 days.
[b] Mean day of death of mice dying prior to day 21.
[c] Determined 4 days post-virus challenge. Five mice per group (nine for the placebo). Due to mortality prior to day of sacrifice, serum titers and ALT were determined for only two and four mice from the 3 ng CLDC and placebo group, respectively.
[d] Log_{10} cell culture 50% infectious dose $CCID_{50}$/g of liver or ml of serum. The assay detection limit was 2.8 $\log_{10}$ $CCID_{50}$/g or ml; a value of <2.8 was assigned for samples presenting with no detectable virus.
[e] ALT, alanine aminotransferase; measured in international units per liter.
[f] Score of zero (normal liver) to four (maximal discoloration).
* $P < 0.05$ compared to 10% sucrose placebo-treated controls.
** $P < 0.01$ compared to 10% sucrose placebo-treated controls.
*** $P < 0.001$ compared to 10% sucrose placebo-treated controls.

tion by 96 h, but occasionally as early as 48–72 h post-virus inoculation. If CLDC therapy was delayed until 36 h after viral challenge, the protective effect was lost. Treatment of mice with more advanced stages of disease was found to be deleterious as groups of mice treated at or after 48 h had a collective survival rate of 3% (1/33), compared to 33% (7/21) for placebo-treated animals. The data suggest that once PTV infection has reached a certain point, the immunostimulatory activity elicited by 1 μg quantity of CLDC may exacerbate the disease. This is in contrast to several other immune modulators previously tested against PTV which showed efficacy out to 36–48 h; notably, however, many doses were evaluated in these studies (Sidwell et al., 1992; Smee et al., 1991). A more comprehensive multi-dose investigation into this matter is necessary as a lower effective dose such as 0.3 μg, which is three-fold less than the amount used in the delayed administration study, may yield more encouraging results. It should also be noted that handling stress associated with treatment likely contributes to the observed increase in mortality as the combination of late stage infection and elevated stress due to treatment may worsen the prognosis considerably. Taking this into consideration, future investigations into this matter should include placebo groups for each timepoint. Collectively, our findings with i.p. delivery of CLDC suggest that protective immunity against PTV disease is elicited when administration occurs within 24 h of initiation of infection; however, immunotherapy at more advanced stages of infection may be disadvantageous. Experiments to establish the relationship between dosage of CLDC, cytokine response, extent of viremia, and efficacy are currently being designed in order to better understand the therapeutic window of CLDC treatment.

IFN-γ, IL-12, and IFN-α are Th1 response cytokines that promote activation of cell-mediated immunity critical to combating infections of viral origin (Romani et al., 1997; Samuel, 2001). The cytokine profiles obtained for groups of mice treated i.p. with CLDC correlated well with the in vivo challenge studies. At the 8 h exposure time, IFN-γ, IL-12, and IFN-α levels were all nearly 2–3 times greater in the 3 μg dose group as compared to the lower doses and it was the only dose capable of eliciting notable amounts of IFN-α. This treatment dose was found to be optimal for survival, reduction of viral load, and limitation of liver disease. Remarkably, the 0.3 μg dose induced only minimal levels of cytokines, yet in the context of survival it was equally as effective as the higher dose in providing a significant level of protection. It would appear that the lack of a vigorous Thl cytokine response observed following treatment with 0.3 μg of CLDC is not critical to the survival outcome. Nevertheless, in the context of infectious challenge, the combination of the native antiviral defenses triggered by exposure to the virus and low-dose CLDC, likely stimulates a sufficient Th1 response to overcome the infection.

Potent and comparable immune activation resulting from systemic i.v. administration of CLDC has been demonstrated in various strains of mice (Dow et al., 1999a). By comparison with i.p. dosing, i.v. delivery of 3 μg of CLDC resulted in dramatically elevated cytokine levels demonstrating far more immunostimulatory capacity by this route. Moreover, considerable differences in kinetics profiles for all three cytokines evaluated were discovered when comparing the two routes. Intraperitoneal delivery of CLDC was initially selected as the route of choice since systemic treatment through tail vein injection in weanling C57BL/6 mice would have been a daunting task considering the large number of animals used in these studies. However, based on the results of our cytokine profiling efforts comparing i.p. and i.v. dosing in 4-week old animals, additional exploration evaluating systemic delivery was necessary. Although significant protection was observed at the 300 and 30 ng doses, as well as reduction in disease parameters (mostly with the 300 ng dose), the results were less than expected considering the cytokine profiles

seen with the 3 μg dose. It is conceivable that the additional stress associated with the challenges of tail vein dosing of PTV-infected weanling mice was deleterious to survival. Further, it is plausible that too strong a response or differences in cytokine kinetics may not have been optimal for treating this particular viral disease.

Although there is some indication as to the basis of the immunostimulatory properties of CLDC, the precise mechanism by which the complexes contribute to heightened antiviral host defense remains unclear. Collectively, our data suggest that CLDC are an effective therapeutic for the treatment of PTV infection by i.p. and i.v. routes of administration. Further investigation exploring efficacy by other routes of administration, the mechanism by which CLDC induces protective immunity against PTV, and its potential use as an immunotherapeutic in other viral disease models is currently underway.

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