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Sterically Stabilized Cationic Liposomes Improve the Uptake and Immunostimulatory Activity of CpG Oligonucleotides[1]

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Immunostimulatory CpG oligonucleotides (ODN) show promise as immune adjuvants, anti-allergens, and immunoprotective agents. Increasing the bioavailability and duration of action of CpG ODN should improve their therapeutic utility. Encapsulating ODN in sterically stabilized cationic liposomes provides protection from serum nucleases while facilitating uptake by B cells, dendritic cells, and macrophages. In a pathogen challenge model, sterically stabilized cationic liposomes encapsulation doubled the duration of CpG ODN-induced immune protection. In an immunization model, coencapsulation of CpG ODN with protein Ag (OVA) magnified the resultant Ag-specific IFN-γ and IgG responses by 15- to 40-fold compared with Ag plus CpG ODN alone. These findings support the use of sterically stabilized cationic liposomes to significantly enhance the therapeutic efficacy of CpG ODN. ***The Journal of Immunology,*** **2001, 167: 3324–3328.**

Bacterial DNA contains unmethylated CpG motifs that strongly stimulate the mammalian immune system. Synthetic oligonucleotides (ODN)[3] expressing CpG motifs patterned after those found in bacteria trigger cells of the immune system to proliferate, mature, and produce cytokines, chemokines, and Ig (1–4).

These immunostimulatory activities are being harnessed therapeutically. CpG ODN show promise as immune adjuvants, significantly improving the immune response to coadministered Ags (5–9). The strong Th1 response elicited by CpG ODN down-regulates Th2-mediated IgE and cytokine production, thereby interfering with allergic asthma (10–12). Finally, by preactivating the innate immune system, CpG ODN can protect naive animals against a variety of microbial and parasitic pathogens (13–17) .

Prolonging the bioavailability and duration of action of CpG ODN may improve their therapeutic efficacy. Unfortunately, phosphorothioate CpG ODN used in vivo are rapidly eliminated from the circulation due to adsorption onto serum proteins and degradation by serum nucleases (18–20). One potential method for protecting ODN from degradation while increasing their uptake by cells of the immune system involves liposome encapsulation (5, 20–22). A series of first and second generation lipid vesicles was synthesized that varied in composition, size, and charge. Sterically stabilized cationic liposomes (SSCL) contain positively charged and hydrophilic elements that efficiently encapsulate CpG ODN and increase their uptake by cells of the immune system. The immunostimulatory activity of SSCL-encapsulated ODN significantly exceeded that of free ODN in vitro and in vivo.

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[3] Abbreviations used in this paper: ODN, oligonucleotides; SSCL, sterically stabilized cationic liposomes; PEC, peritoneal exudate cells; LN, lymph node.

Materials and Methods

Mice

Specific pathogen-free BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed in sterile microisolator cages.

Oligonucleotides

Immunostimulatory ODN_{1555} (GCTAGACGTTAGCGT) and ODN_{1466} (TCAACGTTGA), and control ODNs in which the CpG motif was methylated or inverted, were synthesized by the Center for Biologics Evaluation and Research core facility. All ODN were free of endotoxin and protein contamination.

Liposome preparation

Cholesterol and various phospholipids (Avanti Polar Lipids, Alabaster, AL) were combined in different ratios to form liposomes that varied in charge, stability, lamellarity, and fluidity (Table I). Lipid stocks were prepared in chloroform and were stored at 10 mg/ml under argon at −20°C until use.

Liposomes were generated by evaporating phospholipid mixtures in a round-bottom flask using a rotary evaporator (Büchi, Flawil, Switzerland). The solvent-free dry lipid film was purged with argon to eliminate residual chloroform and oxygen, thereby preventing lipid peroxidation. To generate empty multilamellar vesicles, 1 ml PBS was added to each 20 μmol dried lipid film. These were sonicated five times for 30 s each time at 4°C using a Vibra Cell Sonicator (Sonics and Materials, Danbury, CT). The small unilamellar vesicles were then mixed with 1 mg/ml ODN, frozen on dry ice, and freeze-dried overnight (Flexi-Dry; Kinetics Group, Santa Clara, CA) (22).

ODN encapsulation was achieved during rehydration. Sterile dH_2O (100 μl) was added to the dehydrated liposome/ODN powder and vortexed for 15 s every 5 min for 30 min at room temperature. PBS (900 μl) was added to the mixture, yielding a final liposome concentration of 20 μM lipid/mg DNA. Vesicles <150 nm in diameter were produced by 20–30 cycles of extrusion through polycarbonate filters using a Liposofast extruder (Avestin, Ottawa, Canada) (23). Liposome formulations were stored at 4°C until use.

Assays

RAW 264.7 mouse macrophages were transiently transfected with an IL-12 p40 promoter-luciferase gene vector (pGL3 basic vector) as previously described (24). Cells (10^6) were transfected with 5 μg plasmid DNA. After 24 h cells were pooled, washed, and cultured for 18 h with 1 μM ODN in 12-well macroplates. Cells were harvested and monitored for luciferase activity as recommended by the manufacturer (Promega, Madison, WI).

BALB/c spleen cells were prepared in RPMI 1640 supplemented with 5% FCS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.11 mg/ml sodium pyruvate, and 0.5 mM 2-ME. The cells were cultured at 37°C in a 5% CO_2 incubator. Single-cell suspensions were serially diluted in flat-bottom 96-well microtiter plates that had been precoated with anti-cytokine Abs as previously described (25). Cells were incubated with 1 μM ODN (unless otherwise stated) at 37°C for 8 h, and their secretion of cytokine was detected colorimetrically (26). ELISAs for quantifying cytokines were

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Table I. *Efficiency of ODN encapsulation by different types of liposomes*[a]

Liposome Type	Liposome Composition (molar ratio)	% ODN Encapsulation	
		OD_{260}	^{32}P
Neutral	PC[b]:Chol (1:1)	46.2 ± 2.3	44.7 ± 4.2
Anionic	PC:DOPE:PS (1:0.5:0.25)	33.5 ± 4.8	38.1 ± 2.5
Cationic	DC-Chol:PC:DOPE (4:6:0.06)	63.5 ± 3.6	61.0 ± 4.2
Stealth	Chol:DOPE:PEG-PE (4:6:0.06)	51.7 ± 6.6	53.6 ± 3.7
Cationic-stealth	DC-Chol:DOPE:PEG-PE (4:6:0.06)	88.5 ± 5.3	90.1 ± 3.4

[a] Results represent the mean ± SD of three to five independent tests of each preparation. Percent ODN encapsulation was calculated based on the amount of ODN remaining in the supernatant using UV spectroscopy (OD_{260}) and on ^{32}P incorporation of ODN in liposome pellets.
[b] PC, phosphatidylcholine; Chol, cholesterol; DOPE, dioleylphosphatidylethanolamine; PS, phosphatidylserine, DC-CHOL, dimethylaminoethane-carbamol-cholesterol; PEG-PE, polyethylene $glycol_{2000}$-phosphatidylethanolamine.

performed as described above, except supernatant rather than cells were added to anti-cytokine-coated plates.

In vivo CpG ODN binding and uptake

BALB/c mice were injected i.p. with 50 μg free or liposome-encapsulated FITC-labeled CpG ODN. Mice were sacrificed 2–48 h later, and single spleen cell suspensions (2×10^6/ml) were immediately prepared. One aliquot of cells was fixed and analyzed for FITC-ODN content by FACS (BD Biosciences, San Jose, CA). Another aliquot was stained with PE-labeled phenotype-specific Abs (BD PharMingen, San Diego, CA). These included CD11c for dendritic cells, CD11b for macrophages, and B220 for B cells. The autofluorescence of untreated cells was used to establish assay background.

Ag-specific immune responses

Mice were immunized i.p. with liposome (0.2 μM lipid/mouse) containing OVA (2 μg; Sigma, St. Louis, MO) and/or CpG ODN (10 μg/mouse) on days 0 and 14. Serum was collected on days 14 and 28. IgG, IgG1, and IgG2a anti-OVA titers in serum were determined by ELISA (25). Mice were sacrificed on day 28, and a single spleen cell suspension (2×10^5 cells/well) was prepared in RPMI 1640 supplemented with complete medium. Cells were stimulated in vitro with 5 μg OVA for 36 h. IFN-γ levels in culture supernatants were monitored by ELISA (25).

Bacteria and growth conditions

Listeria monocytogenes strain EGD (ATCC 15313, American Type Culture Collection, Manassas, VA) was grown in modified Mueller-Hinton broth (Difco, Detroit, MI) (14). Mice treated with 50 μg ODN (alone or liposome incorporated) were challenged i.p. 2 or 4 wk later with 10^3 LD_{50} of *L. monocytogenes* in 500 μl PBS. Survival was monitored for 3 wk.

Statistical analysis

All cytokine and Ig assays were conducted at least twice on at least three independently studied mice per group. All immunization and bacterial challenge experiments were performed on a minimum of 5–10 mice/group. Statistical significance was evaluated using Student's *t* test.

Results

Effect of liposome formulation on CpG ODN incorporation

Initial experiments evaluated the efficiency with which ^{32}P-labeled CpG ODN were incorporated into various types of lipid vesicle. Anionic liposomes interacted poorly with CpG ODN, attaining encapsulation efficiencies of <40% (Table I). Neutral and cationic liposomes showed progressively higher levels of encapsulation. SSCL composed of a positively charged derivative of cholesterol, a hydrophilic derivative of phosphatidylethanolamine, and the neutral lipid dioleylphosphatidylethanolamine were the most successful in incorporating anionic CpG ODN (Table I). Incorporation efficiency was influenced by the charge, but not by the size or sequence of the ODNs studied (data not shown).

In vitro activity of liposome-encapsulated CpG ODN

The ability of CpG ODN to stimulate cells and up-regulate cytokine expression was monitored by transiently transfecting RAW264.7 murine macrophages with an IL-12 p40/luciferase construct. It was previously shown that CpG ODN increase IL-12 p40

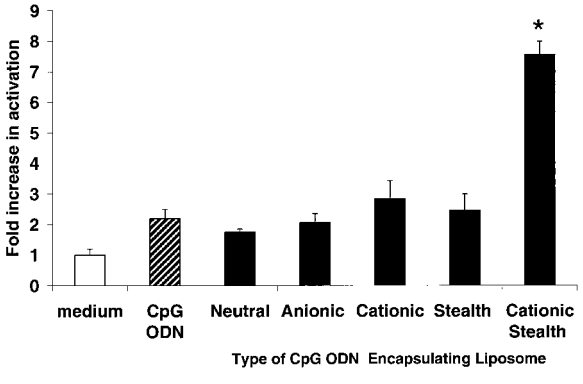

FIGURE 1. IL-12 p40 expression is induced by CpG ODN-encapsulated liposomes. RAW 264.7 cells were transfected with an IL-12 promoter-luciferase gene vector (pGL3 basic vector). Transfected cells (10^6) were stimulated for 24 h with 0.5 μM free or liposome-encapsulated CpG ODN and monitored for luciferase activity. Data show the fold increase in luciferase activity at 24 h compared with that with medium alone and represent the mean ± SD of three independent experiments. *, $p < 0.01$ when compared with free CpG ODN.

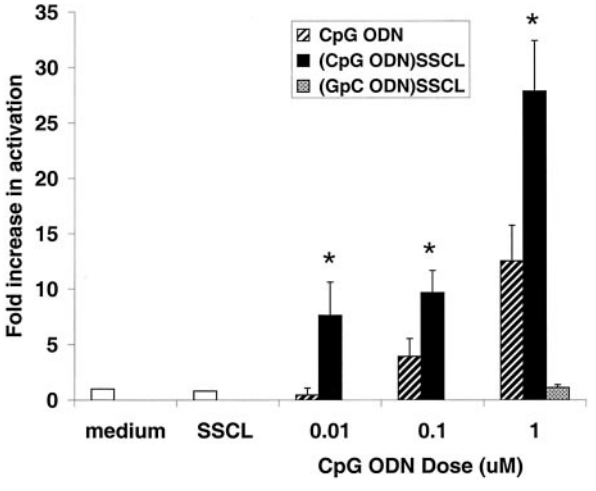

FIGURE 2. Effect of CpG ODN dose on IL-12 p40 expression. RAW 264.7 cells were transfected as described in Fig. 1 and stimulated with 0.01–1.0 μM free or SSCL-encapsulated CpG ODN. Data show the fold increase in luciferase activity at 24 h compared with that with medium alone and represent the mean ± SD of three independent experiments. *, $p < 0.01$ compared with free CpG ODN.

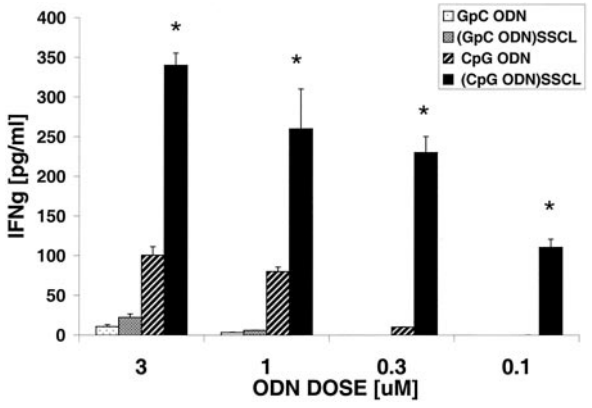

FIGURE 3. Effect of liposome encapsulation on CpG ODN-mediated spleen cell activation. BALB/c splenocytes (2×10^5/well) were stimulated with 0.1–3.0 μM ODN. Culture supernatants were collected after 36 h and assayed for IFN-γ by ELISA. Results represent the average $\pm$ SD of three independent experiments, with each assay performed in triplicate in each experiment. $*$, $p < 0.01$ compared with free CpG ODN.

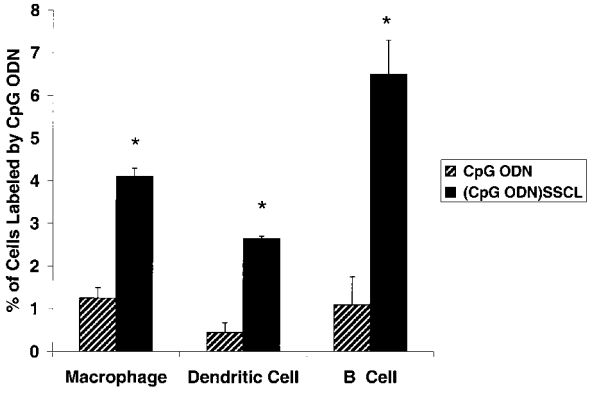

FIGURE 4. Uptake of $(CpG\ ODN)_{SSCL}$ by spleen cells. Mice (three per group) were injected i.p. with 50 μg FITC-labeled free or SSCL-encapsulated CpG ODN. After 48 h, spleen cells were isolated and stained with PE-labeled phenotype-specific mAbs. Data show the percentage of phenotype-positive ODN-labeled cells identified by FACS. Note that the uptake of $(CpG\ ODN)_{SSCL}$ by $CD11b^+$ macrophages, $CD11c^+$ dendritic cells, and $B220^+$ B cells was significantly higher than that of free CpG ODN for all cell types. $*$, $p < 0.01$)

promoter activity in this cell line (24). As shown in Fig. 1, RAW264.7 cells treated with SSCL-encapsulated CpG ODN (hereafter $(CpG\ ODN)_{SSCL}$) expressed significantly more luciferase activity than cells treated with an equal amount of free ODN (or ODN in other types of liposome; $p < 0.01$). Moreover, stimulation by $(CpG\ ODN)_{SSCL}$ persisted at concentrations below the effective range of free CpG ODN ($p < 0.001$; Fig. 2). The stimulation observed in these experiments was CpG motif dependent, since empty SSCL or SSCL containing control ODN did not increase luciferase expression (Fig. 2).

The activity of $(CpG\ ODN)_{SSCL}$ was confirmed using freshly isolated BALB/c spleen cells. Consistent with previous reports (3, 27), free CpG ODN elicited a dose-dependent increase in IFN-γ production by normal murine spleen cells (Fig. 3). $(CpG\ ODN)_{SSCL}$ stimulated significantly more IFN-γ production at all concentrations examined ($p < 0.001$). This stimulation was CpG specific, since control ODN did not induce IFN-γ (Fig. 3). CpG ODN encapsulated in other types of liposomes were less stimulatory (data not shown).

Uptake of $(CpG\ ODN)_{SSCL}$ in vivo

Normal BALB/c mice were injected i.p. with 50 μg free or SSCL-encapsulated FITC-ODN. Uptake of these ODN by spleen and peritoneal exudate cells (PEC) 2 h postinjection was significantly higher in mice treated with $(CpG\ ODN)_{SSCL}$ than in free ODN ($p < 0.05$ for both sites; Table II). This difference persisted at 24 h, with 3- to 6-fold more cells incorporating label ($p < 0.001$).

To determine the phenotype of the cells that bound and internalized $(CpG\ ODN)_{SSCL}$, spleen cells from these mice were treated with PE-labeled phenotype-specific mAbs. The results indicate that $(CpG\ ODN)_{SSCL}$ was taken up by significantly more $CD11b^+$ macrophages (1 vs 4%; $p < 0.01$), $CD11c^+$ dendritic cells (0.5 vs 3.0%; $p < 0.01$), and $B220^+$ B cells (1 vs 6%; $p < 0.01$) than free CpG ODN (Fig. 4).

In vivo effects of $(CpG\ ODN)_{SSCL}$: immune cell activation

Immune activation manifest by increased cytokine production and the up-regulation of CD40 expression was examined in BALB/c mice injected i.p. with 50 μg free or SSCL-encapsulated CpG ODN. Results show that spleen cells from $(CpG\ ODN)_{SSCL}$-treated mice produced significantly more IL-6, IL-12, and IFN-γ ($p < 0.001$; Table III) and expressed higher levels of CD40 ($p < 0.001$; Table III), than those from mice treated with free CpG ODN. This enhanced immune activation persisted for at least 48 h (Table III; $p < 0.001$ for all cytokines). Similar findings were obtained when lymph node (LN) cells and peritoneal exudate cells (PEC) from these animals were analyzed (data not shown). These findings indicate that liposome encapsulation increased both the magnitude and duration of CpG-induced immune activation in vivo.

In vivo effects of $(CpG\ ODN)_{SSCL}$: adjuvant activity

CpG ODN can act as immune adjuvants, boosting the immune response to coadministered protein Ags (16, 21). For example, coadministering free CpG ODN with OVA increases the resultant IgG2a anti-OVA response of BALB/c mice by 6-fold ($p < 0.01$) and IFN-γ production by 1.5-fold (Table IV).

If CpG ODN plus OVA were coencapsulated in SSCL, a 40-fold increase in the IgG2a anti-OVA response was obtained ($p < 0.01$). This treatment also increased Ag-specific IFN-γ production by 10-fold ($p < 0.01$; Table IV).

Optimal immunogenicity was observed when OVA and CpG ODN were encapsulated in the same liposome. Administering free

Table II. *SSCL encapsulation enhances CpG ODN uptake in vivo*[a]

	Spleen		PEC		LN	
Treatment	2 h	24 h	2 h	24 h	2 h	24 h
PBS	0.38 ± 0.23	0.40 ± 0.13	0.47 ± 0.21	0.68 ± 0.19	0.15 ± 0.06	0.25 ± 0.16
CpG ODN	0.63 ± 0.11	1.12 ± 0.33	23.02 ± 8.42	29.86 ± 6.19	0.18 ± 0.07	0.83 ± 0.34
$(CpG\ ODN)_{SSCL}$	06.43 ± 1.48*	8.66 ± 2.30*	88.76 ± 7.17*	84.81 ± 10.41	0.19 ± 0.08	4.69 ± 1.10*

[a] BALB/c mice were injected i.p. with 50 μg FITC-labeled free or liposome-encapsulated CpG ODN. Animals were sacrificed after 2 or 24 h, and cells from the spleen, PEC, and LN cells were analyzed for ODN uptake by FACS. Results represent the mean $\pm$ SD of three independently studied mice/group and experiments were repeated with similar results. $*$, $p < 0.01$ comparing the effect of $(CpG\ ODN)_{SSCL}$ to that of free CpG ODN.

Table III. *Liposome-encapsulated CpG ODN enhance cytokine production in vivo*[a]

Time Post-Treatment (h)	Treatment	IL-6 (ng/ml)	IL-12 (ng/ml)	IFN-γ (pg/ml)	% CD40-Positive Cells
2	PBS	0.08 ± 0.02	0.21 ± 0.02	3.08 ± 0.22	ND
	CpG ODN	0.31 ± 0.01	0.58 ± 0.28	27.28 ± 11.02	ND
	$(CpG\ ODN)_{SSCL}$	1.87 ± 0.12*	6.43 ± 1.19*	178.42 ± 12.32*	ND
24	PBS	0.08 ± 0.02	0.21 ± 0.02	3.08 ± 0.22	0.00
	CpG ODN	0.16 ± 0.05	0.45 ± 0.11	13.16 ± 9.65	0.01
	$(CpG\ ODN)_{SSCL}$	1.43 ± 0.42*	7.69 ± 0.86*	224.30 ± 16.33*	0.78
48	PBS	0.08 ± 0.02	0.21 ± 0.02	3.08 ± 0.22	ND
	CpG ODN	0.18 ± 0.07	0.32 ± 0.23	5.31 ± 2.07	ND
	$(CpG\ ODN)_{SSCL}$	1.32 ± 0.35*	5.69 ± 1.26*	186.12 ± 19.85*	ND

[a] BALB/c mice were injected i.p. with 50 μg ODN. Spleen cells were isolated 2–48 h later and cultured in vitro for 36 h. Ongoing cytokine production was determined by ELISA. At 24 h, spleen cells were also fixed and stained with PE-labeled anti-CD40 mAb and analyzed by FACS. Results represent the mean ± SD of three mice/group independently studied, and experiments were repeated with similar results. *, $p < 0.01$ for $(CpG\ ODN)_{SSCL}$ vs CpG ODN.

OVA with encapsulated $(CpG\ ODN)_{SSCL}$ elicited immune responses of significantly lower magnitude (Table IV). These findings are consistent with the results of previous studies showing that optimal immunogenicity requires that the CpG ODN be kept in close physical proximity to Ag (6, 16).

In vivo effects of $(CpG\ ODN)_{SSCL}$: immunoprotective activity

CpG ODN stimulate an innate immune response that promotes host survival following pathogen challenge (14, 28). While this response protects the host from a broad array of pathogens, it persists for only a short period (14, 16, 28). Since SSCL encapsulation increases the magnitude and duration of CpG-induced cellular activation, its ability to extend the duration of CpG-induced protection was examined.

BALB/c mice were injected i.p. with 50 μg CpG ODN or $(CpG\ ODN)_{SSCL}$. Two weeks after treatment all animals survived infection by 10^3 LD_{50} of *L. monocytogenes* (data not shown). However, when challenge was delayed until 30 days, only those animals treated with encapsulated ODN survived ($p < 0.0001$; Fig. 5).

Discussion

CpG ODN stimulate an innate immune response characterized by the activation of lymphocytes and professional APC. Clinical trials are underway to examine the ability of these molecules to act as immune adjuvants, anti-allergens, and immunoprotective agents (29). Yet, rapid in vivo clearance limits the uptake and shortens the therapeutic half-life of free ODN. Encapsulation in sterically stabilized cationic liposomes improves the uptake of CpG ODN by cells of the immune system, concomitantly increasing the magnitude and duration of their in vivo activity.

Multiple different liposome formulations were tested. SSCL were the most efficient at encapsulating CpG ODN. This is consistent with earlier studies showing that sterically stabilized liposomes protect and improve the uptake of antisense ODN and plasmid DNA during cancer chemotherapy (22, 30–33). SSCL are composed of three distinct phospholipid elements. Dimethylaminoethane-carbamol-cholesterol increase liposome membrane stability while improving the uptake and encapsulation of DNA (34, 35). Dioleylphosphatidylethanolamine is a pH-sensitive neutral lipid that improves the cytosolic delivery of ODN following internalization (36, 37). Polyethylene glycol-PE stabilizes the liposome and also facilitates cellular uptake (37–39). Preliminary experiments showed that this three-component combination was more effective than multiple other combinations at ODN uptake and delivery (Table I and Fig. 1, and data not shown).

The uptake of CpG ODN by spleen, PEC, and LN cells was significantly enhanced by SSCL encapsulation (Table II). In addition, the magnitude and duration of immune cell activation induced by $(CpG\ ODN)_{SSCL}$ significantly exceeded those of free ODN, as measured by cytokine production and the up-regulation of CD40 expression (Table III). This translated into improved in vivo activity. Consistent with this finding, Ignatius et al. (37) recently demonstrated that dendritic cells more effectively take up and present protein encapsulated in a polyethylene glycol-stabilized liposome.

Normal BALB/c mice immunized with CpG ODN coencapsulated with OVA generated Ag-specific IgG responses far greater than mice immunized with Ag alone or Ag plus nonencapsulated ODN (Table IV). In particular, coencapsulation increased Ag-specific IFN-γ production (10-fold) and IFN-γ-dependent IgG2a anti-OVA Ab production (40-fold), consistent with the preferential induction of a Th1-biased immune response. It is well established that optimal immunogenicity is achieved by maintaining Ag and ODN in close physical proximity (16, 40, 41). Liposome coencapsulation provides an effective method for ensuring the simultaneous presentation of Ag plus adjuvant to relevant APC, while

Table IV. *Coencapsulation of OVA plus CpG ODN in SSCL improves immunogenicity*[a]

Treatment	IgG	IgG1	IgG2a	IFN-γ (pg/ml)
PBS	35 ± 5	62 ± 15	11 ± 5	29 ± 2
OVA	530 ± 50	320 ± 20	72 ± 20	47 ± 5
OVA + CpG ODN	1,650 ± 350	200 ± 5	450 ± 90	56 ± 5
$(CpG\ ODN)_{SSCL}$ + OVA	9,300 ± 3,100	4,600 ± 700	6,200 ± 1,740	386 ± 40
$(CpG\ ODN + OVA)_{SSCL}$	19,000 ± 2,300*†	2,220 ± 750*	29,000 ± 4,600*†	700 ± 90*†

[a] BALB/c mice were injected i.p. with 2 μg OVA plus 10 μg CpG ODN. They were boosted on day 14 and sacrificed on day 28. Serum anti-OVA titers at day 28 were determined by ELISA. Ag-specific cytokine production was detected by stimulating spleen cells (2×10^5/well) with OVA (5 μg/ml) for 36 h in vitro. IFN-γ levels in culture supernatants were determined by ELISA. Results represent the mean ± SD of three studied mice/group independently. The experiment was repeated with similar results. *, $p < 0.01$ for $(CpG\ ODN + OVA)_{SSCL}$ vs OVA + CpG ODN; †, $p < 0.01$ for $(CpG\ ODN + OVA)_{SSCL}$ vs $(CpG\ ODN)_{SSCL}$ + OVA.

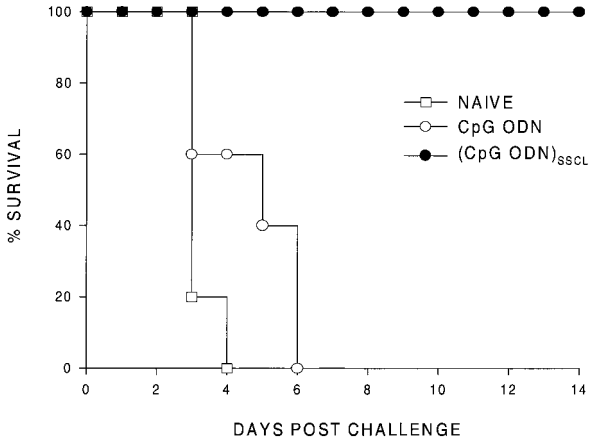

FIGURE 5. $(CpG\ ODN)_{SSCL}$ provide protection against lethal pathogen challenge. BALB/c mice were injected i.p. with 50 μg free or $(CpG\ ODN)_{SSCL}$. Four weeks later animals were challenged i.p. with 10^3 LD_{50} of *L. monocytogenes* and monitored for survival. Results represent the total experience from two experiments involving 10 mice/group/experiment.

providing protection against serum proteases and nucleases and eliminating the need to covalently couple ODN to Ag.

Immunoprotection experiments provided additional evidence of the in vivo activity of $(CpG\ ODN)_{SSCL}$. Previous studies showed that the innate immune response elicited by CpG ODN protected normal mice from a variety of bacterial, viral, and parasitic pathogens for approximately 2 wk (13–15). When $(CpG\ ODN)_{SSCL}$ were administered, the duration of protection doubled, with 100% of mice surviving 10^3 LD_{50} of *L. monocytogenes* 4 wk post-treatment (Fig. 5). Thus, by facilitating the uptake of CpG ODN, SSCL encapsulation increases the magnitude and duration of their in vivo activity. These findings suggest that the immunotherapeutic potential of CpG ODN may be enhanced by SSCL incorporation.

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