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Original Research

Cationic Liposome–Oligonucleotide Complex as an Alternative Adjuvant for Polyclonal Antibody Production in New Zealand White Rabbits (*Oryctolagus cuniculus*)

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Because of their ideal size and temperament, rabbits are commonly used in polyclonal antibody production. Immunostimulatory adjuvants—such as Freund complete and incomplete adjuvants as well as various proprietary products—trigger a robust immune response, which increases antibody concentrations. However, these adjuvants can cause excessive soft tissue reactions, prompting concerns regarding animal wellbeing. This study assessed the safety and efficacy of cationic liposome–oligonucleotide complexes (CLDC) as an alternative adjuvant to conventional adjuvants. On days 0 and 14, 15 female New Zealand white rabbits were vaccinated subcutaneously with 15 μg ovalbumin mixed with either CLDC, Freund adjuvant (day 0, complete; day 14, incomplete), or a proprietary adjuvant (*n* = 5 per group). Antibody titers were measured by direct ELISA on days 0, 14, and 28. Rabbits were palpated daily for lesion development, and all lesions were measured. Rabbits in all groups developed a significant antibody response to ovalbumin over 28 d. However, the differences between groups were not statistically significant. No rabbits in the CLDC group developed skin lesions, whereas 80% of rabbits that received Freund adjuvant and 100% of those that received the proprietary product developed skin lesions. This study demonstrates that CLDC may be a valuable and effective alternative adjuvant for polyclonal antibody production in rabbits—one that avoids the palpable injection-site lesions often seen with other adjuvants.

Abbreviations: APC, antigen-presenting cells; CLDC, cationic liposome oligonucleotide complexes; FCA, Freund complete adjuvant; FIA, Freund incomplete adjuvant; TLR, toll-like receptor

Polyclonal antibodies are used in an expansive array of diagnostic assays and therapeutics worldwide.[12] A variety of mammalian species are commonly immunized to produce these antibodies, including rabbits, sheep, goats, and rodents.[6,12] Rabbits are an ideal model because of their small size, even temperament, ease of venipuncture, decreased housing costs, and single IgG type.[6,12]

To create polyclonal antibodies, a vaccine consisting of an antigen mixed with an adjuvant is administered to the host species.[4] Depending on the antigen used, vaccination protocols for these adjuvants are similar, with booster vaccines given 2 to 6 wk after the initial vaccine.[6] Various types of adjuvants are used to stimulate a robust host antibody response to a given antigen and include water-in-oil emulsions, immune-stimulating complexes, and aluminum salt adjuvants.[11,14] The most commonly used adjuvants for polyclonal antibody production in animals are Freund complete adjuvant (FCA), Freund incomplete adjuvant (FIA), and a proprietary formulation (TiterMax Gold, TiterMax, Norcross, GA).[14,18] These antigens use water-in-oil emulsions to create an antigen depot effect within the skin, stimulating humoral and cellular immune responses. However, animal wellbeing concerns are associated with these adjuvants,[6,10,14,18] and as such, the USDA has considered their use to cause more than momentary pain when used.[17] These adjuvants may cause malaise, pain or distress, or other toxic effects because of the depot mechanism of immune responses.[6,9,14,18] Common lesions are focal necrosis and granulomas at the site of injection, which may extend to local lymphatics.[14]

Cationic liposome oligonucleotide complexes (CLDC) were developed as a nonviral gene delivery vector; they also potentiate immune responses.[4] The cationic lipid capsule bearing the antigen or DNA of interest effectively binds negatively charged molecules on the cell surface, increasing cytoplasmic delivery.[8,11] Studies indicate that CLDC triggers toll-like receptor (TLR) and liposomal activity to stimulate inflammatory cascades, especially after repeated exposures.[4,16] CLDC preferentially targets antigen-presenting cells (APC), such as dendritic cells.[7,11] CLDC can be delivered intravenously, regionally, or locally with minimal side effects[11,16] and have been used effectively in several vaccines[1,3,7,15] as well as other immunotherapies.[4] The CLDC form potent vaccine adjuvants for protein antigens and result in antibody responses equivalent to those due to FCA.[4]

We hypothesized that CLDC can be used as an alternative adjuvant in polyclonal antibody production, in which ovalbumin was used as a model antigen, with fewer associated adverse effects than the commonly used Freund and proprietary

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adjuvants. Compared with the proprietary formulation and Freund adjuvant, CLDC elicited an equivalent antibody response with no lesion development, suggesting that CLDC may be a suitable alternative adjuvant for polyclonal antibody production.

Materials and Methods

Animals and housing. Adult female New Zealand White rabbits (n = 15; age, 3 to 4 mo) were purchased from Western Oregon Rabbit Company (Philomath, OR) and housed in an AAALAC -accredited animal facility. Rabbits were allowed to acclimate for 1 wk after their arrival at the facility. These animals were SPF for *Pasteurella multocida*, *Eimeria steidae*, and *Psoroptes cuniculi*. Rabbits were individually housed (Allentown, Allentown, NJ) under standard conditions with enrichment toy and food items. They had unrestricted access to Teklad Global High-Fiber Rabbit Diet (no. 2031, Envigo, Huntingdon, England, United Kingdom) and sterile water. Supplemental alfalfa hay was given 3 d each week. Room environments were maintained at 18.8 to 20 °C, 20% to 40% humidity, and a 12:12-h light:dark cycle. All procedures and care were approved by the IACUC.

Vaccine preparation. CLDC was created by mixing 300 µL 2-dioleoyl-3-trimethylammonium-propane (DOTAP) cationic lipid with 150 µL polyinosinic-polycytidylic acid (poly[I:C]) in 3 mL 5% dextrose, and adding 15 µg ovalbumin. FCA, FIA, and proprietary adjuvants (catalog nos. F5881, F5506, and T2684, respectively; Sigma-Aldrich, St Louis, MO) were mixed according to the manufacturers' instructions. By using sterile 3 mL-syringes connected with double female connectors, 500 µL each adjuvant was mixed 1:1 with 15 µg ovalbumin (concentration, 30 µg/mL) such that each vaccine contained 15 µg ovalbumin antigen. Total volumes for the Freund and proprietary-formulation vaccines were 1 mL, whereas that for the CLDC vaccine was 3 mL.

Vaccine inoculation. Each rabbit was randomly placed into 1 of the 3 adjuvant groups (n = 5). The dorsum of each rabbits was shaved on days 0 and 14 by using a #40 clipper blade, from the level of the scapula to the sacrum and approximately 3 in. (7.6 cm) to either side of midline. The vaccination site was further prepared by using a chlorhexidine scrub prior to each vaccination. The skin of the back was gently tented to create a subcutaneous pocket for injection. Vaccines were administered subcutaneously on days 0 and 14, divided into 8 to 10 sites, 3 cm apart, and 5 cm lateral to the spine. In the Freund group, FCA was used on day 0, and FIA was used on day 14.

Vaccination site evaluation. Throughout the 28-d study, rabbits were monitored daily for overall health as well as lesion formation. Rabbit health was assessed according to the following inhouse ABCD scale: A) well-groomed rabbit that is eating, drinking, urinating, and defecating normally and showing normal behavior; B) rabbit shows subtle behavioral changes, mild lethargy, poor grooming, lack of interest in environment, or an unusual condition (for example, skin trauma, eye lesion, lameness, hair loss); C) rabbit is reluctant to move, is not eating or drinking normally, or has profound bleeding, lethargy, hunched posture, or pale mucous membranes; and D) rabbit is moribund. Per this scale, any animals that received scores of C or D are immediately seen by a veterinarian. Any lesion sites were noted and gently palpated, and each lesion's maximal diameter was measured daily by using calipers (Bel-Art Products, Pequannock, NJ). Rabbits were weighed on days 0, 14, and 28.

Blood collection and ELISA. Prior to vaccination on day 0, 10 mL of blood was collected from the auricular artery of all rabbits and centrifuged for serum collection; subsequent blood samples were collected on days 14 and 28. Serum was stored at –20 °C until analysis. Serum was analyzed by using direct ELISA. Antigen solution was prepared by dissolving 0.5 mg ovalbumin in 500 mL bicarbonate buffer (1.515 g Na_2CO_3, 3.0 g $NaHCO_3$, 500 mL deionized H_2O); 100 µL of antigen solution was placed in each well of a 96-well plate and incubated at 4 °C overnight. Wells were washed twice with washing solution (0.05% Tween 20 in PBS); 200 µL blocking solution (0.5% dried milk in PBS) then was added to each well and incubated for 2 h at room temperature. The wells were washed 3 times with washing solution. Each well was filled with diluted rabbit serum (1:50 in blocking solution) and incubated at 37 °C for 90 min. Wells were washed 3 times, after which 100 µL of 1:500 goat antirabbit antibody conjugated with horseradish peroxidase (AIDS Vaccine Program, National Cancer Institute, Frederick, MD) was added to each well and incubated at 37 °C for 90 min. Wells were washed 5 times with 100 µL washing buffer; then 100 µL of substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and allowed to incubate for 30 min at room temperature. To stop reactions, 100 µL H_3PO_4 (provided by the kit manufacturer) was added to each well. The optical density of each well was read on a microplate spectrophotometer (Multiskan Spectrum, Thermo Fisher Scientific, Waltham, MA) using manufacturer-supplied software (SkanIt Software version 2.4.4 RE, Thermo Fisher Scientific).

Statistics. Statistical analysis was performed by using Vassar Stats (http://www.vassarstats.net). ELISA results (optical densities of antibody samples) were compared by using 3×3, 2-factor ANOVA with repeated measures. One-way ANOVA was used to compare lesion number and size between groups. For all analyses, posthoc pairwise comparisons were performed by using the Tukey Honest Significant Difference test. A P value less than 0.05 was considered statistically significant.

Results

Analysis of antibody production. Blood was collected on day 0 prior to initial vaccination, on day 14 prior to booster vaccination, and again on day 28. The baseline optical density was very low on day 0, with no significant difference between groups (P = 0.123; Figure 1). At day 14, all rabbits displayed a similar increases in optical density (P = 0.942). In all rabbits, optical density on day 28 was greater than the baseline value (P = 0.07), but the day 28 value did not differ between any of the groups. Each group showed a significant (P < 0.01) increase in optical density between day 0 and day 28, indicating a significant antibody response to ovalbumin in all adjuvant groups.

Lesions. One rabbit in the CLDC group had mild bruising noted on day 15, after administration of the booster vaccine on day 14, but none of the rabbits in the CLDC group developed skin nodules or other abnormalities (Figure 2). In contrast, 4 of the 5 rabbits receiving that received Freund adjuvant displayed palpable subcutaneous nodules, with lesion formation beginning on day 5. These lesions ranged in size from less than 1 cm to 3.3 cm, with an average maximal diameter of 2.0 cm and an average of 2.6 lesions per rabbit. All 5 rabbits that received the proprietary formulation developed palpable subcutaneous nodules, with lesion formation beginning on day 5. These lesions ranged in size from less than 1 cm to 1.8 cm, with an average diameter of 1.4 cm and an average of 1.8 lesions per rabbit. Most nodules were rounded and moderately well demarcated; they began as soft nodules and became progressively firmer before healing (Figure 3). In addition, 4 large areas of diffuse soft tissue swelling were noted in rabbits that received Freund adjuvant, with 2 noted among the rabbits injected with the proprietary

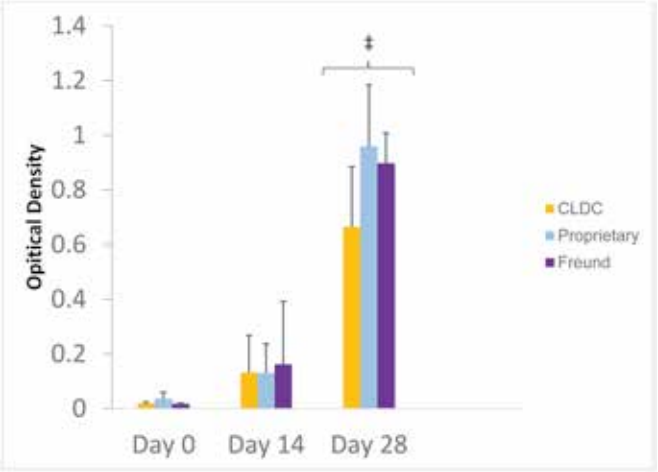

Figure 1. Antibody concentrations as represented by optical density obtained by using ELISA. Antibody responses did not differ between groups at any time point. ‡, Value significantly ($P < 0.001$) greater than that on day 0 for the same group, indicating a significant antibody response to ovalbumin across all groups.

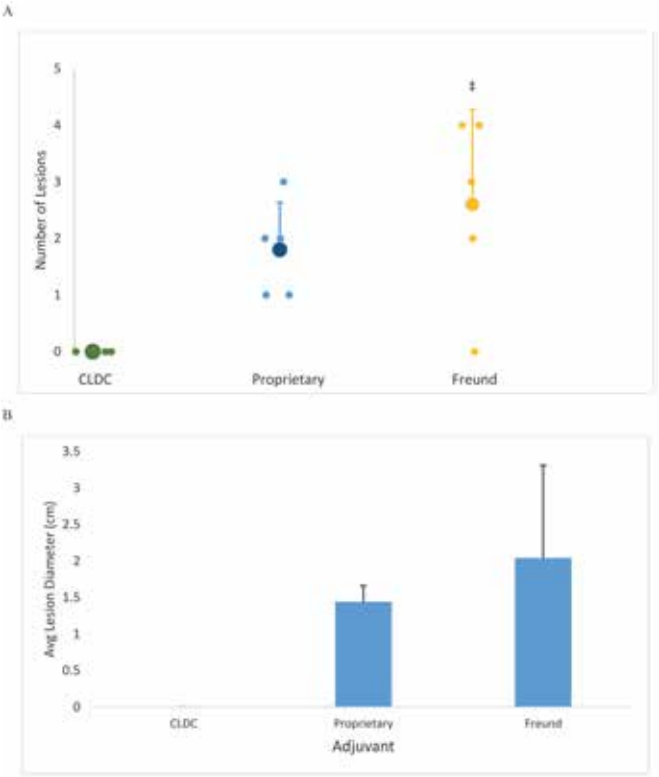

Figure 2. (A) The average number of lesions did not differ significantly ($P = 0.368$) between the groups treated with Freund adjuvant or the proprietary formulation. However the average number of lesions was greater ($P = 0.006$) in the Freund group than in rabbits that received CLDC. The average number of lesions in the rabbits given the proprietary formulation was nearly significantly ($P = 0.052$) different from that in the CLDC group. The largest circle for each group denotes its average number of lesions (bar, 1 SD). (B) The average size of lesions did not differ between rabbits given Freund adjuvant compared with the proprietary formulation ($P = 0.436$).

formulation, but these areas were not measured. One rabbit in the proprietary adjuvant group developed mild ulceration of a booster injection nodule, noted on day 20. Another rabbit from that group developed erythematous wheals (approximate diameter, 0.5 to 1.5 cm) at the 10 initial injection sites. The wheals were first noted at day 14, after the rabbits were shaved a second time to prepare for booster vaccines. No rabbits in any group demonstrated a painful response on palpation, and all remained systemically healthy. Overall, animals in the CLDC group were scored as A according to on the inhouse scoring system, whereas those in the other 2 groups were scored as B because of the skin lesions but were otherwise considered normal. All rabbits gained weight throughout the study (Table 1).

Overall, the average number of lesions did not differ significantly between the Freund and proprietary adjuvant groups ($P = 0.368$), but the Freund group had more ($P = 0.006$) lesions than the CLDC group, and the difference between the proprietary adjuvant and CLDC groups approached significance ($P = 0.052$). The average size of lesions did not differ between the Freund and proprietary adjuvant groups ($P = 0.436$).

Discussion

The adjuvant CLDC has been used effectively in several vaccines[1,3,7,15] and other immunotherapies[4] and can be delivered intravenously, regionally, or locally with minimal side effects.[11,16] In the current study, we assessed the antibody response in rabbits vaccinated with CLDC as an adjuvant and ovalbumin as the model antigen. CLDC has been assessed with ovalbumin in previous studies evaluating vaccines. During the formation of a intranasal mucosal vaccination against *Burkholderia pseudomallei*,[7] the CLDC–ovalbumin adjuvant–antigen combination increased levels of serum IgG1 and IgG2; IgA levels within saliva and bronchoalveolar lavage fluid; and $CD8^+$ T-cell responses in bronchoalveolar lavage fluid and blood, compared with ovalbumin alone.[7,13] The CLDC–ovalbumin combination did not inhibit TLR activation and was more efficient at shifting the IgG1/IgG2a response toward IgG2a than was the TLR ligand Pam_3CysSK_4;[15] and CLDC–ovalbumin induced comparable antibody responses regardless of the charge on the liposome.[15] These experiments demonstrate that, although each adjuvant–antigen combination may need to be optimized, CLDC has the potential to promote an antibody response to ovalbumin. However, other antigens may not produce a sufficiently robust antibody response when used with CLDC adjuvant and must be investigated.

The common adjuvants used to produce polyclonal antibody typically form an antigen depot and stimulate the immune response. Freund adjuvants are the most effective adjuvants currently used for polyclonal antibody production.[14] FCA includes heat-killed whole *Mycobacterium* cells in mineral oil. Although FIA is very similar in composition to FCA, FIA lacks mycobacterial cells thus making it less reactive than FCA and an ideal adjuvant for booster immunizations.[14] The mineral oil component of FCA and FIA has a 3-pronged function: it forms an antigen depot for slow release, acts as a vehicle for antigen transport to immune cells, and interacts with APCs such as phagocytes, macrophages, and dendritic cells.[14] The mycobacterial cells in FCA stimulate TLR2, TLR4, and cytokine responses, creating a delayed hypersensitivity response.[14] The proprietary adjuvant we tested contains copolymer CRL89-41 mixed with squalene.[15] These block copolymers have high molecular weights and function as adjuvants by stimulating APC and the complement cascade.[4] When used with some antigens, the tested proprietary formulation stimulates a slightly lower antibody response than those after FCA and FIA and is associated with fewer adverse lesions.[9,10,14]

Both FCA and FIA are viscous when mixed and injected and form nodules at the injection site, which occasionally ulcerate.

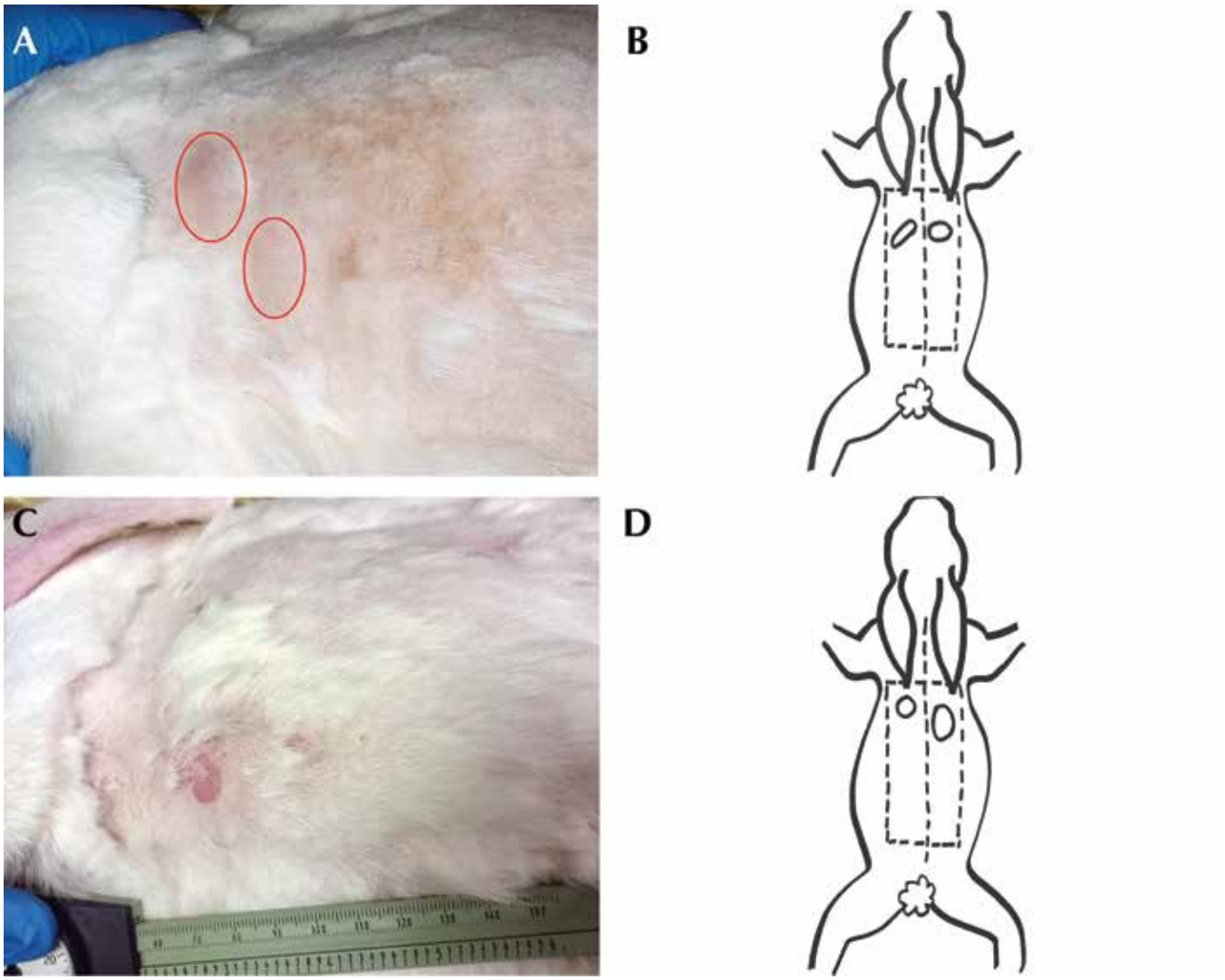

Figure 3. (A) Lesions due to FCA, day 5. The lesions (red circles) are swollen and slightly erythematous. (B) Locations of lesions due to FCA, day 5. (C) Lesions due to proprietary formulation, day 28. One lesion is swollen with mild ulceration; the second soft swelling is obscured by hair regrowth. (D) Locations of lesions due to proprietary formulation, day 28.

Table 1. Weights (kg) of rabbits over time

Rabbit	Day 0	Day 14	Day 28
CLDC 1	3.88	4.23	4.60
CLDC 2	3.76	4.20	4.57
CLDC 3	3.78	4.04	4.42
CLDC 4	3.42	3.53	3.70
CLDC 5	3.31	3.85	4.08
Proprietary 1	3.90	3.90	4.04
Proprietary 2	3.93	4.51	4.91
Proprietary 3	3.55	4.02	4.33
Proprietary 4	3.87	4.02	4.55
Proprietary 5	3.85	4.13	4.74
Freund 1	3.72	4.03	4.35
Freund 2	3.90	4.23	4.71
Freund 3	3.86	4.18	4.68
Freund 4	3.32	3.62	3.70
Freund 5	3.78	4.01	4.14

All rabbits maintained normal appetites and gained weight throughout the course of the study.

These nodules have previously been characterized as focal granulomas.[9,14] We injected each rabbit in 8 to 10 sites, and lesions formed in 1 to 4 of the injection sites. Although every effort was made to ensure that the amount of adjuvant-antigen mixture was consistent between sites, the viscosity of the mixture may have led to small changes in the amount given at each site, creating slight variability between injections. This possible variability in injection volume might explain why some lesions were larger and more apparent than others. In addition, some of the emulsion might have been deposited intradermally along the needle tract during withdrawal, increasing the potential for lesion formation. Intradermal injection is another common administration route for FCA and the proprietary formulation for polyclonal antibody production, and, given the area into which inflammatory cells can infiltrate, might result in more clinically significant granulomas than occur when these adjuvants are given subcutaneously.[6,9,14] We did not specifically look for intradermal tracts after the administration of adjuvant–antigen mixture. However, given the viscosity of these 2 adjuvants, inadvertent intradermal injection and subsequent nodule formation is likely going to occur when either of these products is administered. Regardless of any variability in adjuvant volume between injection sites or

deposition of adjuvant in the dermis, rabbits vaccinated with either FCA or the proprietary formulation developed palpable nodules and robust antibody responses.

CLDC combines the immunostimulatory effects of oligonucleotides and liposomes. The liposomes facilitate uptake of the oligonucleotide by APC and protect the oligonucleotide from nuclease activity. The uptake by APC stimulates TLR4, as do Freund adjuvants, and results in IFNγ production and a subsequent antibody response.[3,5] These outcomes are achieved without the notable inflammatory responses seen with oil emulsion-based adjuvants. Perhaps, due to the decreased viscosity of CLDC compared with FCA and the proprietary formulation, CLDC inoculations were completely subcutaneously, with no dermal tracts, decreasing the opportunity for lesion formation. Because no gross lesions were apparent, we did not pursue histologic evaluation of the injection site. However, the fact that CLDC did not incite any gross lesions is noteworthy in itself.

All rabbits developed a strong antibody response to ovalbumin by day 28 (Figure 1). No rabbits in the CLDC group developed skin lesions or adverse side effects, whereas 80% of the rabbits in the FCA group and 100% of the rabbits given the proprietary antigen developed skin lesions. Although the CLDC group had the lowest antibody response, our results suggest that CLDC is an effective adjuvant for ovalbumin polyclonal antibody production that is associated with fewer side effects than the other common adjuvants we tested. Our standard protocol for polyclonal antibody production typically includes an initial vaccination of the relevant antigen with Freund adjuvant or the proprietary product, followed by boosters at 2-wk intervals. Therefore we evaluated antibody response at 14 and 28 d. It may be beneficial to compare the efficacy of CLDC as an adjuvant when booster vaccines are given after 4 or 6 wk[3] or when more boosters are given,[14] both to refine the best immunization and collection schedule for this specific adjuvant–antigen combination and to determine the effects of continuous booster immunization on lesion development and antibody titers. Although 3- to 6-wk boosters are prevalent, all of the adjuvants in our study resulted in increased antibody titers after the 2-wk booster injections, thus demonstrating their effectiveness as adjuvants.

Other alternatives to producing polyclonal antibodies without the use of adjuvants include a surgical, noninflammatory model whereby a sterilized 'whiffle ball' is surgically implanted in the subcutaneous space.[2,6] This method creates a chamber for inoculation and fluid retrieval, eliminating the need for blood or ascites collection.[2] However, the model does require surgical implantation followed by several weeks of recovery from the procedure. Although an alternative method of polyclonal antibody production, this surgical method is not as common as using Freund or other adjuvants, according to the number of literature citations. Therefore we sought to evaluate a nonsurgical model with an alternate adjuvant that may not form the lesions associated with the common proprietary and Freund adjuvants.

A potential shortcoming of the use of CLDC as an adjuvant is the increased technical skill required to create the vaccine compared with using other adjuvants. The CLDC vaccine readily precipitates out of solution when a DNA:lipid (DOTAP) imbalance occurs within the complex.[16] However, this aggregation of positively charged cationic liposomes and negatively charged plasmid DNA of the CLDC may increase the host reaction to the vaccine.[16] To combat excessive precipitation, we increased the amount of 5% dextrose diluent used in the vaccine, added ovalbumin last, and administered the vaccine promptly after mixing.[16]

An advantage of the CLDC vaccine was its ease of injection despite its increased volume (3 mL CLDC vaccine compared with 1 mL of the others). The larger volume may allow increased interaction with the host, recruiting more APC to uptake CLDC. However, the use of CLDC as an adjuvant may be limited to larger species than rabbits when increased volumes are needed to generate a sufficiently large immune response.

The primary benefit of CLDC as an adjuvant, as shown in our current study, is to generate an immune response with no to minimal side effects. This alternative adjuvant offers a refinement to the current practices of polyclonal antibody production in rabbits by decreasing the potential for lesion formation, which is commonly seen when Freund adjuvants are used.[6,10,14,18] In turn, this decreased lesion formation may improve overall animal wellbeing.

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