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Immunostimulatory lipid implants containing Quil-A and DC-cholesterol

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ABSTRACT

Biocompatible lipid implants which promote the sustained release of antigen have potential as novel vaccine delivery systems for subunit antigen as they may reduce or remove the requirement for multiple administrations. Of particular interest are sustained release systems that release antigen incorporated into particles. Previous work has demonstrated that lipid implants prepared from phosphatidylcholine, cholesterol, the adjuvant Quil-A, and ovalbumin as the model antigen could stimulate an immune response equivalent to that induced by a prime and boost with a comparable injectable vaccine. However, entrapment of antigen into particles released from the implant was low. Therefore the aim of this study was to firstly determine if the inclusion of a cationic derivative of cholesterol, DC-cholesterol, into the implants increased antigen entrapment and immunogenicity, and secondly, if a cationic implant could induce at least a comparable immune response as compared to a prime and boost with an injectable vaccine. The inclusion of DC-cholesterol had only a minor effect on antigen entrapment into particles released from the implants and the implants did not stimulate cellular responses as effectively as the comparable injectable vaccine or the unmodified implant containing Quil-A and cholesterol, although the vaccine did induce stronger responses than either soluble protein alone, or protein co-delivered in alum.

1. Introduction

The successful development of vaccine formulations that are able to stimulate cell-mediated immunity still poses a challenge in vaccinology. Research has resulted in the use of subunit antigens as they provide a safer alternative compared to vaccines prepared from attenuated or heat-killed pathogens. It is well known that the delivery of soluble subunit antigen is associated with low levels of immunogenicity, and poses the danger of tolerance induction towards the weakly immunogenic antigen. Therefore, attempts have been made to incorporate the subunit antigen into a particulate carrier that can mimic a pathogen due to its small size (Bramwell and Perrie, 2005a,b, 2006; Copland et al., 2005; Friede and Aguado, 2005; Storni et al., 2005). Ideally, an adjuvant is incorporated into such particulate carriers, allowing for the simultaneous delivery of antigen and adjuvant to the same antigen-presenting cell (APC) (Bramwell and Perrie, 2005a,b). However, even after incorporation of subunit antigens into immunogenic particulate carriers, multiple doses of vaccine have to be given in order to stimulate effective immune responses. This increases costs, and often also results in a decrease in patient compliance with the multi-dose vaccination regimes (Friede and Aguado, 2005). Therefore, the development of a system that allows for sustained release of the antigen over a prolonged period of time is desirable. We have previously shown that the incorporation of antigen into implants that release colloidal particles such as liposomes is beneficial in terms of immune stimulation (Myschik et al., 2008b). However, incorporation of antigen into particles released by the implants was low and this may have impacted on the efficacy of this approach. One strategy to improving the incorporation of antigens into particles is to formulate particles with a positive charge. This approach was successfully utilised by McBurney et al. (2008) who reported that cationic immune stimulating complexes (PLUSCOMs) entrapped significantly more antigens than did traditional anionic ISCOMs. The cationic charge in PLUSCOMs was achieved through the incorporation of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol, or DC-cholesterol (DCCHOL), which is a cationic derivative of cholesterol (CHOL) (Fig. 1). This molecule was first reported by Gao and Huang (1991), who prepared cationic liposomal preparations from DCCHOL and dioleoylphosphatidylethanolamine (DOPE), and found the cationic liposomal formulation was as effective with regards to transfection as the commercially available product "Lipofectin". The authors also reported a reduced toxicity compared to the commercial preparation (Gao and Huang, 1991). The main use of DCCHOL is still in the formulation of DNA vaccines, when DCCHOL is often used in combination with a helper lipid such as DOPE (Hui et al., 1994; Sternberg et al., 1994; Goyal and Huang, 1995; Perrie et al., 2003). The use of DCCHOL for peptide or protein vaccines remains less common. Nonetheless, Brunel et al. (1999) utilised DCCHOL in a

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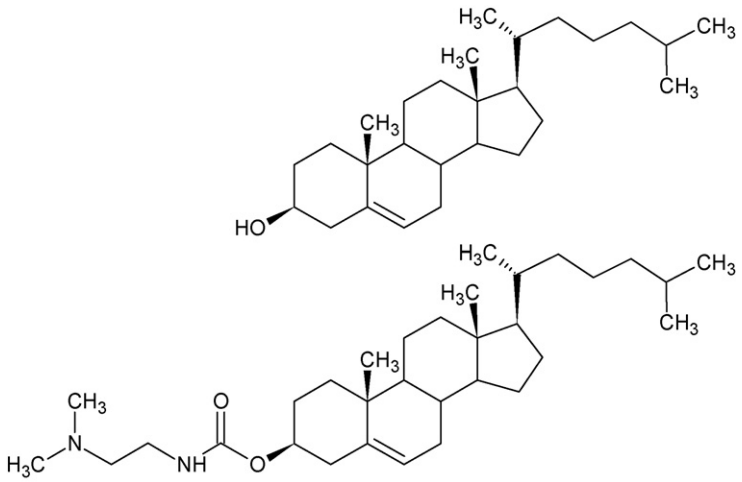

Fig. 1. Schematic representation of the chemical structures of (A) CHOL and (B) its cationic derivative DCCHOL. CHOL has a polar hydroxyl group attached to the steroid backbone, whereas DCCHOL contains a tertiary amino group linked to the steroid backbone via a carbamoyl linker.

hepatitis B vaccine and reported that DCCHOL functioned as an immunomodulator, where it stimulated balanced antigen-specific Th1 and Th2 type immune responses, and resulted in a greater increase in antibody responses towards the vaccine in BALB/c mice than alum. In addition, Guy et al. (2001) reported that DCCHOL-containing influenza split vaccines tested in their study showed an adjuvant effect in terms of primary and secondary antibody responses. Therefore, in addition to its cationic charge, DCCHOL appears to have some adjuvant effect.

In this study, we report on the development and *in vivo* investigation of sustained release lipid implants prepared from phosphatidylcholine, cationic DC-cholesterol, and Quil-A as the adjuvant. It was our hypothesis that these lipid implants would release cationic liposomal structures upon hydration with buffer that would entrap high amounts of antigen and therefore improve the efficacy of sustained release particulate delivery systems.

2. Materials and methods

2.1. Materials

Purified Quil-A™ (QA) (lyophilised powder) was obtained from Brenntag Biosector, Frederikssund, Denmark. Phosphatidylcholine from egg yolk (PC) was sourced from Northern Lipids Inc., Vancouver, Canada. Cholesterol (purity approximately 95%), albumin from chicken egg (OVA), fluorescein isothiocyanate (FITC), and phosphate buffered saline (PBS) sachets pH 7.4 (0.01 M) were purchased from Sigma–Aldrich, MO, USA. Chloroform (purity 99-99.4%) was obtained from Merck, Darmstadt, Germany. Triton® X-100 (octylphenol-polyethyleneglycolether) was obtained from SERVA Feinbiochemica, Heidelberg, Germany. Distilled deionised water (Milli-Q Water systems, Millipore, MA, USA) with a conductivity of less than 0.1 μS was used throughout the study. 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DCCHOL) was obtained from Avanti Polar Lipids, Alabaster, USA. All other chemicals used in this study were of analytical grade.

2.2. Preparation of lipid implants

Liposomal dispersions were prepared using the lipid-film hydration method (Copland et al., 2000), which is a modification of the Bangham method used for the preparation of liposomes (Bangham et al., 1962). The ratio of QA:DCCHOL:PC was 0.2:2:7.8 (w/w of total lipids). The compression of the lyophilised powder into implant matrices was achieved using a Carver Laboratory Press (Model 3392, Fred S. Carver Inc., WI, USA) at a mass of 0.2 tons. The lipid powders were admixed with 40% (w/w) crystalline CHOL to achieve sustained release as described before (Demana et al., 2005; Myschik et al., 2008a). The punch and die system had a diameter of 2 mm, and the compressed implants had a cylindrical shape and a weight of approximately 9 mg.

2.3. Preparation of injectable formulations

The QA-containing liposomal formulations for injection were prepared using the lipid-film hydration method. 500 μl of formulation was carefully layered on 1 ml of a 10% (w/w) sucrose in PBS cushion in microtubes and spun for 30 min at approximately 20,200 × *g*. The supernatant containing unbound FITC–OVA was removed and the remaining formulation spun for another 2 min to produce a pellet. The pellet was resuspended in 500 μl of 5% Triton® X in PBS to allow for the determination of incorporated FITC–OVA. For *in vivo* administration, liposomal preparations were spun through a sucrose cushion for 30 min at approximately 20,200 × *g* as described above. The supernatant containing unbound FITC–OVA was removed and the remaining formulation spun for another 2 min to produce a pellet, which was subsequently resuspended in sterile PBS to obtain a dose of 50 μg QA/10 μg OVA. If necessary, liposomal formulations which contained either no OVA or no QA were admixed to give a final concentration of 50 μg QA and 10 μg OVA in the injectable formulation.

2.4. Characterisation of formulation

The presence of colloidal structures upon hydration was investigated by placing implants into vials containing 400 μl of PBS buffer and withdrawing samples at set time points. Colloidal structures present in the samples were investigated using TEM (Philips CM 100 microscope) at an acceleration voltage of 100 kV and a magnification of 66,000× or 93,000×, after negative staining with 2% phosphotungstic acid pH 5.2. Micrographs were obtained using a MegaView III digital camera (Soft Imaging Systems, Muenster, Germany). Different areas of the grids were viewed in order to obtain a representative impression of the sample.

The size and zeta potential of the colloidal structures was determined using a Malvern Zetasizer Nano-ZS (Malvern Instruments, UK).

Table 1
Immunisation protocol and composition of the different formulations used for the assessment of the quality of immune response evoked by the formulations

Group	Formulation	Adjuvant	Amount of antigen	Immunisation time points
A	QA OVA CHOL implant	100 μg	20 μg OVA	Day 0
B	QA OVA DCCHOL implant	100 μg	20 μg OVA	Day 0
C	QA OVA DCCHOL injectable formulation	50 μg	10 μg OVA	Day 0, 14
D	OVA DCCHOL implant	–	20 μg OVA	Day 0
E	Alum + OVA	200 μl	10 μg OVA	Day 0, 14
F	PBS + OVA	–	10 μg OVA	Day 0, 14

2.5. Entrapment of fluorescently labelled protein

Studies were performed to determine the amount of protein entrapped into colloidal particles which were formed upon hydration of implants. Slight modifications to the method published by Demana et al. (2005) were applied. Implants without the addition of extra CHOL were placed into vials and 750 μl of PBS buffer (0.01 M, pH 7.4) was added. Preparations were stirred at room temperature using a magnetic stirrer and protected from light for 12 h (Bibby Sterlin Ltd., Stone Staffordshire, UK) to allow for formation of colloidal particles. The resulting dispersions were placed onto 1 ml of a sucrose cushion (10%, w/w) and centrifuged at 20,200 × *g* for 30 min (Centrifuge 5417C, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The supernatant containing unbound OVA was discarded. 1 ml PBS buffer containing 5% (w/v) Triton® X-100 was added to the remaining pellet. Samples were gently vortexed to ensure sufficient mixing and then subjected to fluorescence analysis. The amount of fluorescently labelled model antigen was quantified using fluorescence spectrophotometry. Fluorescence was analysed (Shimadzu FR 540, excitation 495 nm, emission 520 nm) and the protein content was calculated using calibration curves. The entrapment of fluorescently labelled model antigen into colloidal structures was expressed as a percentage of the original amount of protein present in the implant and was analysed in triplicate.

2.6. Release of fluorescently labelled protein

In vitro release of model antigen from the freeze–dried lipid implants was investigated over a period of 10 days. Implants were placed into vials containing 400 μl of PBS and incubated at 37 °C in a water bath. All samples were protected from light throughout the experiment. At predetermined time intervals (5 min to 240 h), 50 μl samples of supernatant were withdrawn and the volume replaced by the same volume of fresh PBS buffer. Samples were then dispersed in 1 ml of 5% Triton® X-100, and gently vortexed to ensure complete mixing. The amount of fluorescently labelled model antigen was quantified using fluorescence spectrophotometry. Fluorescence was analysed (Shimadzu FR 540, excitation 495 nm, emission 520 nm) and the protein content was calculated using calibration curves for the respective antigen. The total amount of protein present in the implant was calculated for each implant and all samples were analysed in triplicate.

2.7. Mice

Male C57Bl/6 mice and male OT-I and OT-II transgenic mice were bred and maintained under specific pathogen-free (SPF) conditions at the HTRU, Dunedin, New Zealand. OT-I transgenic mice express predominantly H-2K^b restricted CD8$^+$ T cells specific for the OVA peptide SIINFEKL ($OVA_{257–264}$), whereas OT-II transgenic mice have a high proportion of CD4$^+$ T cells with a T cell receptor (TCR) which recognises $OVA_{323–339}$. All mice were used from 6 to 8 weeks of age, and had access to water and food *ad libitum*. All experiments were approved by the University of Otago Animal Ethics Committee.

2.8. Immunisation protocol

Adoptive transfer of OT-I and OT-II transgenic T cells into C57Bl/6 mice was performed on day −1 of experimentation as previously described (Myschik et al., 2008b, 2008). Briefly, the spleen, axial and brachial lymph nodes were harvested from OT-I and OT-II transgenic mice. Single-cell suspensions (8×10^6 cells/ml) were prepared in sterile PBS and 500 μl of mixed OT-I and OT-II cells were injected via the tail vein into C57Bl/6 mice. Mice received either a QA OVA CHOL, QA OVA DCCHOL, or OVA DCCHOL implant on day 0, or were injected subcutaneously with QA OVA DCCHOL injectable formulations, OVA in alum or OVA in PBS on day 0 and 14. Implants contained 20 μg of OVA whereas the injectable formulations which were given two times contained 10 μg of antigen. In order to administer the implants, mice were anaesthetised using halothane, and a small cut was made on the shaven back through which a trochar was inserted. The implants were placed at the back of the neck at the site of administration of the injectable vaccines. The incision was subsequently closed with a Michel clip. Mice which received the injectable formulation or OVA in PBS also received a blank implant consisting of CHOL and PC (Table 1).

2.9. Analysis of cellular and humoral responses

All mice were sacrificed on day 28 of the experiment using a lethal dose of anaesthetic. Spleens, axial and brachial lymph nodes and blood were harvested. Spleens were pooled from mice receiving the same vaccine, whereas lymph nodes were analysed separately for each mouse. Cells were isolated and stained for analysis by flow cytometry. Cells were incubated firstly with anti-CD16/CD32 antibody (to block non-specific binding to cell surfaces), followed by antibodies directed against either the cytotoxic T cell marker CD8 or the helper T cell marker CD4, as well as the transgenic T cell markers Vα2 and Vβ5.1 (all BD Pharmingen, San Diego, CA, USA). Cells were analysed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). OVA-specific IgG titres were determined by ELISA (Myschik et al., 2008b, 2008).

2.10. Statistical analysis

Where applicable, results are expressed as mean ± S.D. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) with a confidence interval of 95% using SPSSR 14.0, Release 14.0.0 (SPSS Inc., Chicago, IL, USA) followed by post hoc analysis using Tukey's pairwise comparison.

3. Results

3.1. Entrapment efficiency of FITC–OVA into liposomal formulations

The entrapment efficiency of FITC–OVA into colloidal particles released from the lipid implants upon hydration and into the injectable vaccine was analysed. Due to the cationic charge of the CHOL derivative, the formation of positively charged colloidal

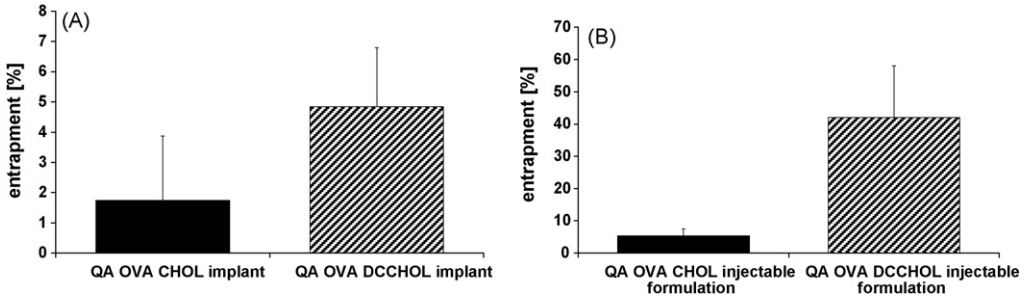

Fig. 2. (A) Comparison of the entrapment efficiency of FITC–OVA in colloids released from QA OVA implants containing CHOL (black bar) or DCCHOL (dashed bar). (B) Comparison of the entrapment efficiency of FITC–OVA into injectable liposomal formulations containing CHOL (black bar) or DCCHOL (dashed bar). Data represents the results from three independent replicates + S.D.

particles was expected, resulting in charge–charge interactions with the negatively charged antigen OVA. It was hypothesised that larger amounts of OVA may be attached and/or incorporated into the positively charged liposomal particles. Indeed the entrapment efficiency of FITC–OVA in colloids released from QA OVA DC-CHOL implants was higher as compared to FITC–OVA entrapment/adsorption to colloids released from QA OVA CHOL implants (Fig. 2A). However this increase in entrapment/adsorption was not statistically significant. On the other hand, the average entrapment of OVA into QA DCCHOL liposomes in the injectable vaccine was approximately 8-fold higher than the entrapment efficiency for the OVA-containing QA CHOL liposomes (Fig. 2B).

3.2. Morphology of colloidal structures

An examination of the release of colloidal particles upon hydration with buffer from the cationic lipid implants was carried out. Colloidal particles were analysed using TEM. Liposomal structures were observed in samples taken from the 1 h time point onwards, and most particles were multilamellar. As shown in Fig. 3A liposomes were found in the 4 h sample, with some liposomes displaying an irregularity in their bilayer structures (as shown by the arrow). These may be defect structures in the liposomal bilayers. Eight hours after the start of the release study, liposomes of different sizes, but predominantly displaying multiple layers were observed. However, some smaller colloidal particles (as shown by the arrow in Fig. 3B) were found as minor structures. An increase in the number of such particles was evident in the 48 h sample (Fig. 3C). Additionally, structures displaying helical or cylindrical features as observed in Fig. 3D were common. These structures have an interesting similarity to the self-assembled cylindrical micelles of neat DCCHOL in aqueous media which were described by Wu et al. (2004). The helical micelles were found on various positions of the TEM grid, and could indicate that some DCCHOL was not completely incorporated into liposomes, but formed cylindrical structures independent of the liposomes. On day 5 of the release study, large multilamellar liposomes surrounded by small spherical structures were observed (Fig. 3E). Large liposomes in addition to some DCCHOL cylinders were also seen on day 9 (Fig. 3F).

The morphology of colloidal particles present in the QA OVA DCCHOL injectable formulations was also examined. As depicted in Fig. 3G, large multilamellar liposomes were visible after negative staining. Additionally, some smaller liposomal structures were also observed in the same samples (Fig. 3H), indicating that at least two distinct particle sizes were present in the formulation. Small colloidal structures resembling ISCOMs were also observed, and are indicated by the arrows in Fig. 3H. Interestingly, in comparison to the TEM samples obtained from the DCCHOL-containing implants, no cylindrical DCCHOL helices were observed in the injectable vaccine formulations.

The heterogeneity of the injectable samples is also reflected in the polydispersity index (PDI) which was obtained in the PCS measurements. The zeta potential of all formulations was positive due to the cationic nature of DCCHOL. The size of the injectable formulation differed depending on whether the colloidal structures were loaded with OVA or not. An increase in size upon loading was observed (Table 2).

3.3. Kinetics of antigen release

The impact of the inclusion of DCCHOL on the sustained release of antigen from the implants was investigated and compared to lipid implants prepared from cholesterol (Myschik et al., 2008a). As apparent from the graph presented in Fig. 4, the release of FITC–OVA from the QA OVA DCCHOL implants (grey circles) was slightly, but significantly, faster compared to implants prepared from QA OVA CHOL (black squares). Nonetheless, release was still sustained over 7 days (168 h) at which time complete release from the QA OVA DCCHOL implants was achieved.

3.4. Investigation of cellular immune responses in vivo

Analysis of the expansion of $CD8^+$ T cells in response to the different vaccine formulations was undertaken because an expansion of $CD8^+$ T cells is a necessary requirement for the activation of cytotoxic T lymphocytes (CTL). These T cell subsets have the ability to kill pathogen-infected cells and tumours. The flow cytometry data obtained from the lymphocytes from all three independent experiments confirmed a strong expansion of $CD8^+$ T cells in the group of mice that received the QA OVA CHOL implant, that was significantly different to all other vaccine formulations tested in this study (Fig. 5A). This expansion is in concordance with the results obtained from a previous study for the QA OVA CHOL implants (Myschik et al., 2008b). The administration of the QA OVA DCCHOL implant did not result in a significant expansion of $CD8^+$ T cells. In fact the

Table 2
Size (*z*-average), polydispersity index (PDI), and zeta potential (ZP) of injectable QA DCCHOL, QA OVA DCCHOL formulations, and liposomal DCCHOL-containing formulations for the administration *in vivo*

Formulation	*z*-Ave (nm)	PDI	ZP (mV)
QA DCCHOL liposomes	1223 ± 306	0.54 ± 0.08	18.58 ± 9.78
QA OVA DCCHOL liposomes	3744 ± 712	0.34 ± 0.14	22.37 ± 9.76
QA OVA DCCHOL liposomes (*in vivo* mix)	1191 ± 336	0.52 ± 0.17	22.35 ± 8.75

Data represents the average of five independent measurements ± standard deviation.

expansion of CD8 T cells was significantly lower in mice given the QA OVA DCCHOL implant as compared to mice given the QA OVA CHOL implant suggesting the inclusion of DCCHOL reduces vaccine efficacy. Transgenic $CD8^+$ T cells numbers in mice given the QA OVA DCCHOL implant were higher than those in mice given the cationic implant without QA adjuvant (OVA DCCHOL) but the increase was not significant.

The $CD8^+$ T cell expansion induced by the QA OVA DCCHOL injectable vaccine was interesting in that while the expansion of $CD8^+$ T cells in the spleens was comparable to that achieved by the QA OVA CHOL implant, the expansion of transgenic cells in the lymph nodes was small. Low levels of expansion were observed for the OVA DCCHOL implant vaccine, alum + OVA, and the negative control PBS + OVA.

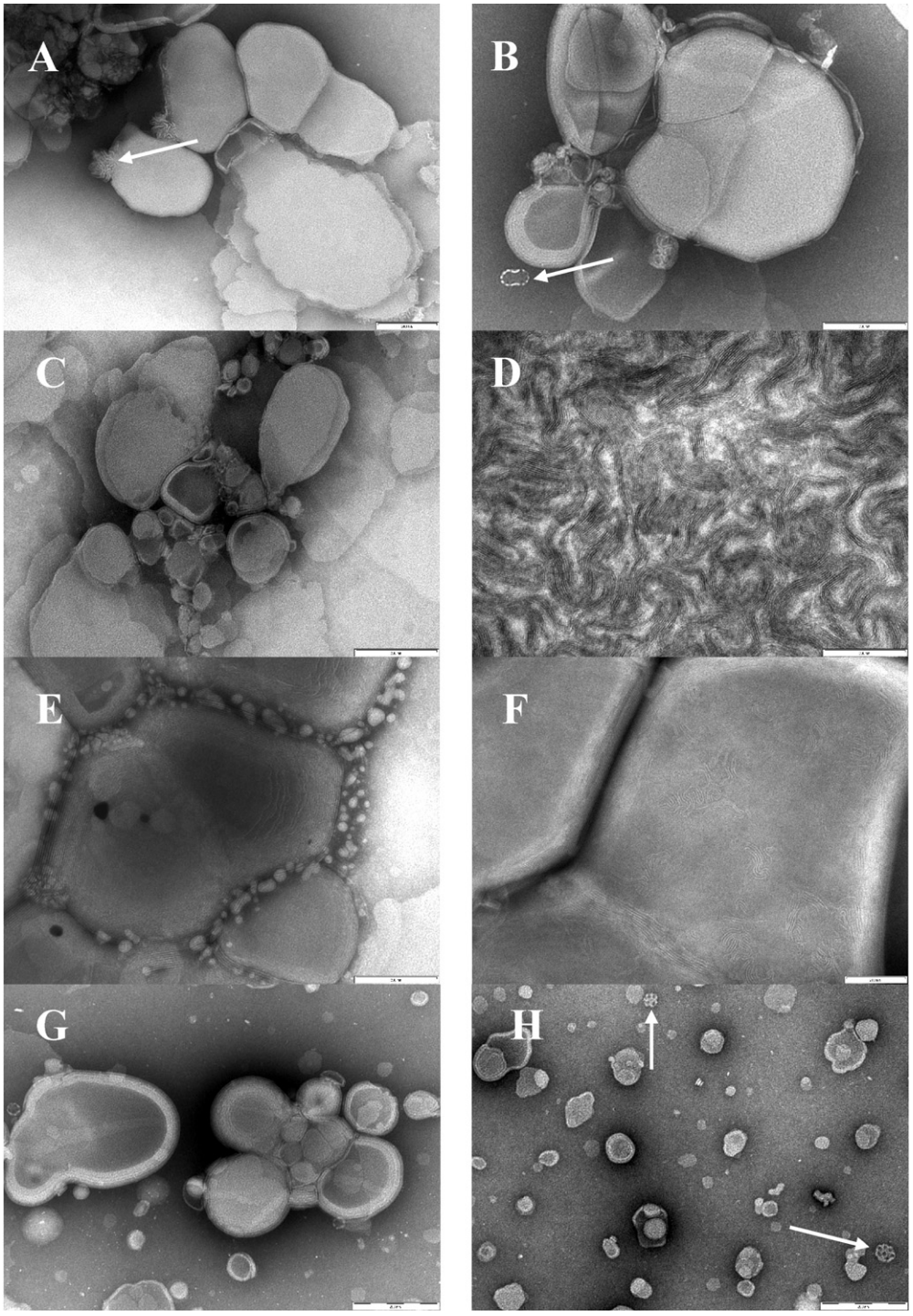

Fig. 3. Transmission electron micrographs of colloidal structures released by implants (A–F) and produced for the injectable vaccines (G and H). Implants containing DCCHOL were prepared with an excess of 40% CHOL to achieve sustained release and contained FITC–OVA as the model antigen. Implants were placed into PBS buffer pH 7.4 at 37 °C, and buffer samples were withdrawn after (A) 4 h, (B) 8 h, (C) 48 h, (D) 48 h, (E) 5 days, and (F) 9 days. Samples were negatively stained with 2% phosphotungstic acid pH 5.2. (G) Injectable vaccines were also negatively stained and analysed by TEM. Multilamellar liposomes of different sizes were observed. (H) Smaller liposomal structures, including some ISCOM-like structures (arrows), were also observed. Bar = 200 nm.

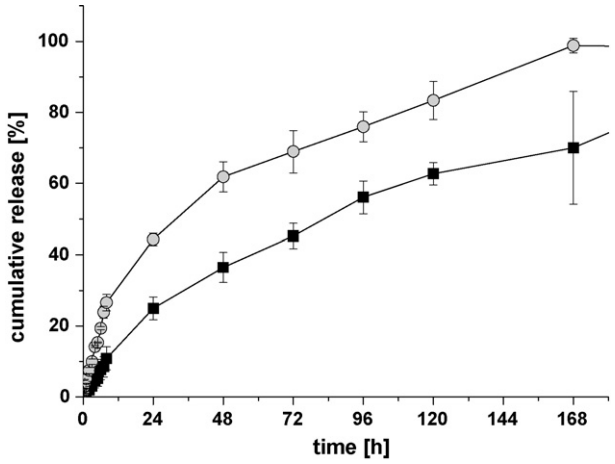

Fig. 4. Cumulative release of FITC–OVA from implants prepared with CHOL (squares) or DCCHOL (circles). Release data of the first 7 days of the release study is shown. Data represents the mean value of three independent replicates ± S.D. obtained from one of two independent release studies. The first 7 days of the release study are depicted.

3.5. Investigation of expansion of transgenic CD4⁺ T cells

Since CD4⁺ T cells may play an important role as helper cells for the activation of immune responses towards intracellular pathogens or tumours, the expansion of this T cell subset was investigated by flow cytometry (Fig. 5B). Significant differences were observed between the individual vaccine groups in this study, and a significantly larger expansion of CD4⁺ transgenic T cells was observed for mice immunised with alum + OVA. This immune response is expected because alum has been described to successfully activate CD4⁺ T helper responses, in addition to its ability to stimulate humoral immunity (Gupta, 1998). The response towards alum + OVA was significantly different to all other vaccine groups in two out of three experiments, however in one of the three repetitions an expansion of CD4⁺ T cells was also observed for the QA OVA DCCHOL injectable formulation. Overall, the expansion of CD4⁺ T cells was low considering the percentage of total CD4⁺ T cells.

3.6. Analysis of OVA-specific antibody titres in serum

The production of OVA-specific IgG antibodies in sera obtained from mice on day 28 of the study was analysed using an ELISA (Fig. 6). A stronger antibody production was found for all vaccines compared to the negative control of PBS + OVA. Interestingly the QA OVA DCCHOL injectable vaccine stimulated the highest antibody titres which were significantly higher than those stimulated by the OVA CHOL implant. The presence of antibodies in response to the alum-containing vaccines correlates with previous observations in our lab (Myschik et al., 2008b, 2008). In general, alum-based vaccines are known to produce high antibody titres (Gupta, 1998). There was no significant difference in antibody titres in mice immunised with any of the implants.

4. Discussion

The inclusion of cationic lipids into liposomes allows the possibility of adsorbing negatively charged antigen onto the surface of colloidal particles due to charge–charge interactions. The presence of charged lipids has been reported to alter the distances between the bilayers (Gregoriadis, 1990) and may allow for a higher entrapment efficiency of soluble antigen in addition to the negatively charged antigen being adsorbed onto the particle surface. Lipid implants and injectable formulations containing cationic DCCHOL were prepared and analysed with regard to their physico-chemical characteristics. In terms of antigen release, this was slightly faster from the QA OVA DCCHOL implants compared to the QA OVA CHOL implant. One reason for this faster release may lie in the chemical nature of DCCHOL. DCCHOL possesses a tertiary amino group, which results in the positive charge of the polar head group. Since cationic lipids generally show swelling behaviour in buffer because of the presence of salts (Hauser, 1984), it is possible that DCCHOL also may show some degree of swelling (Wu et al., 2004). This may have resulted in a stronger penetration of buffer into the lipid implant matrix, and a subsequently higher release of OVA antigen. The TEM investigation of the colloidal structures released from the QA OVA DCCHOL implants revealed the formation of liposomes. However, helical structures resembling DCCHOL worm-like micelles that have been reported by Wu et al. (2004) were also observed. This finding suggests that DCCHOL may not have been readily incorporated into the colloidal particles, and may have been released as free DCCHOL. In comparison, when the injectable QA OVA DCCHOL samples were analysed by TEM, DCCHOL worm-like helices were not observed in these samples. This may be due to differences in the preparation of the formulations. With the cationic liposome formulations, stirring and therefore input of energy takes place, presumably resulting in a more homogenous distribution of the DCCHOL molecules within the bilayers. This may reduce the release of free DCCHOL molecules.

The entrapment efficiency of OVA into liposomes released from QA OVA DCCHOL implants was low and only slightly higher than that achieved in the QA OVA CHOL implant. In comparison, the overall loading of OVA in the injectable liposomal formulations made with DCCHOL (QA OVA DCCHOL injectable formulation) was about 8-fold higher than that of QA OVA CHOL liposomes. The discrepancy between entrapment of OVA into QA DCCHOL injectable liposomes and QA DCCHOL implants is most likely due to the different production techniques as already mentioned. DCCHOL aggregates released from the implants may have simply bound OVA and possibly also the negatively charged QA, as a result of charge interactions making them unavailable for incorporation into liposomal structures. With regard to immune stimulation, this could mean that the antigen and/or adjuvant may have not been as effectively presented to APCs in particulate form.

The cationic DCCHOL-containing implants were then assessed with regard to their ability to stimulate immune responses *in vivo* as compared to CHOL implants. The cationic implants stimulated a weaker CD8⁺ T cell response and comparable CD4⁺ T cell and antibody responses as compared to the anionic implants. This is perhaps not unexpected due to the very modest change in antigen entrapment that occurred when DCCHOL was included in the formulation and the tendency of the DCCHOL to form helices. It has been reported in the literature that helper lipids such as DOPE are required for the formation of stable liposomes containing cationic cholesterol derivatives (Farhood et al., 1992; Goyal and Huang, 1995). Therefore, it would be of interest to investigate whether the addition of a helper lipid such as DOPE would lead to a more homogenous distribution of DCCHOL in the implants, more stable liposomal structures, increased antigen entrapment and improved immunogenicity. Contradictory results have also been reported as regards the efficiency of cationic liposomes in stimulating immune responses. In early studies, Allison and Gregoriadis (1974) compared the efficiency of subcutaneous administration of diphtheria toxoid (DT) entrapped into negatively or positively charged liposomes containing stearylamine to the administration of soluble, free DT antigen. The cationic liposomes elicited weaker primary and secondary antibody responses towards DT than the same dose of free antigen (Allison and Gregoriadis, 1974). In contrast, the group

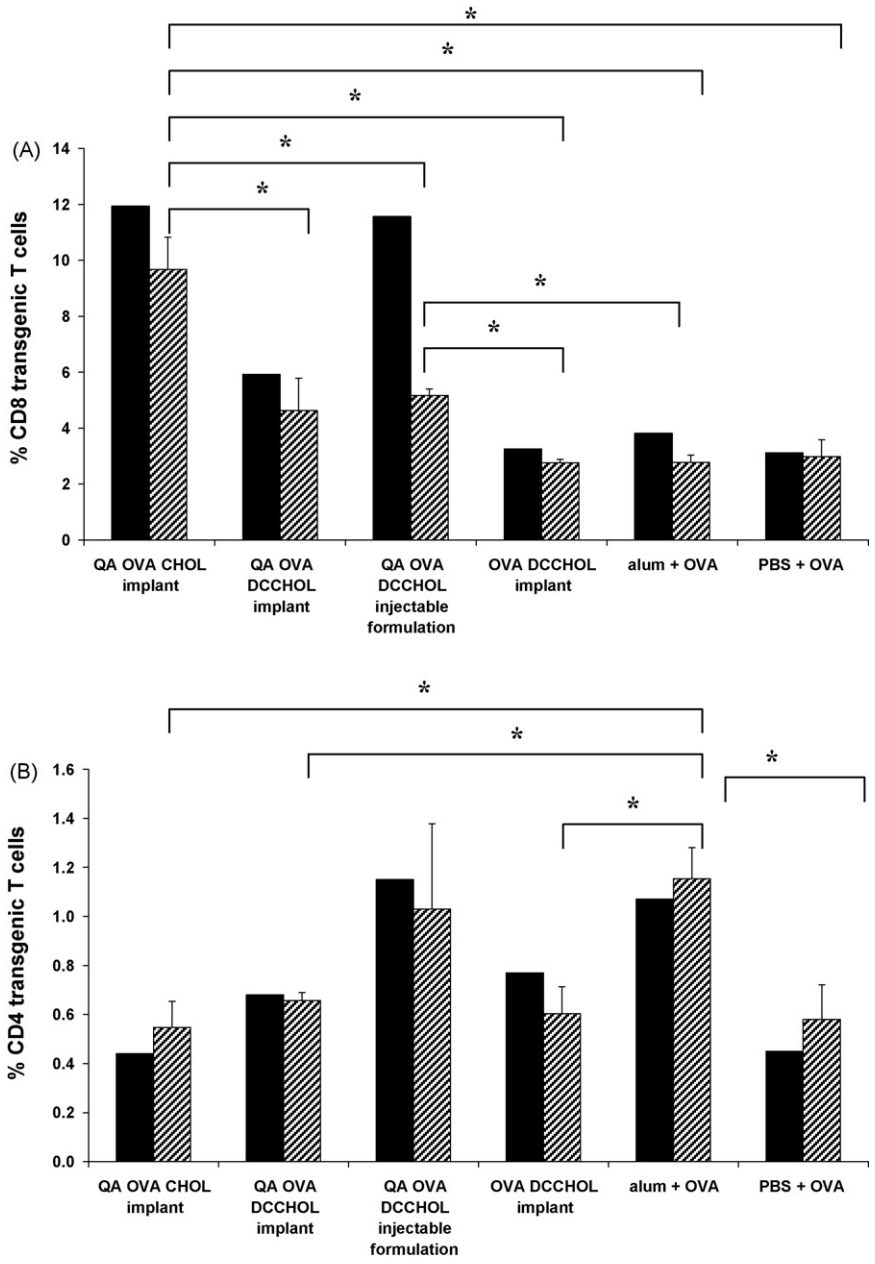

Fig. 5. (A) Expansion of transgenic $CD8^+$ T cells, as a percentage of total CD8 T cells, in lymph nodes and spleens obtained from C57Bl/6 mice on day 28 of the study. Black bars represent the results obtained from the splenocytes (cells were pooled 2–3 mice); dashed bars represent the results obtained from lymph nodes of individual mice. Results for the lymph nodes are expressed as mean ± S.D., and significant differences are indicated by an *. (B) Expansion of transgenic $CD4^+$ T cells, as a percentage of total CD4 T cells obtained from C57Bl/6 mice on day 28 of the study. Black bars represent the results obtained from splenocytes (cells were pooled from 2 to 3 mice); dashed bars represent the results obtained from lymphocytes obtained from individual mice. Results for the lymph nodes are expressed as mean ± S.D., and significant differences are indicated by an *.

of Nakanishi reported stronger immunogenicity of cationic stearylamine liposomes compared to anionic liposomes for the delivery of OVA (Nakanishi et al., 1997, 1999). Therefore, the effectiveness of immune stimulation may also be antigen dependent, and the best charge of the liposomal formulation must be investigated on a case-by-case basis.

A second aim of this study was to determine if the cationic implant could stimulate a comparable response to an injectable cationic vaccine given multiple times. In terms of both the cellular and humoral immune responses, the mice given a prime and boost with the cationic injectable vaccine had a superior immune response as compared to mice receiving the implant. As regards the antibody response this improvement was significant, however, this may be an experimental artefact due to the time point at which antibody titres were determined, which was 28 days after the implant had been administered but only 14 days after boosting with the injectable vaccine. Interestingly in all three repetitions of this experiment the cationic QA OVA DCCHOL injectable formulation stimulated a significant expansion of $CD8^+$ T cells in the spleens as compared to the lymph nodes. The reason for this may perhaps be explained by antigen from the injectable vaccine distributing to the spleen rather than to the draining lymph nodes. This difference in immune responses in the spleen and lymph nodes was also seen in the previous experiments which

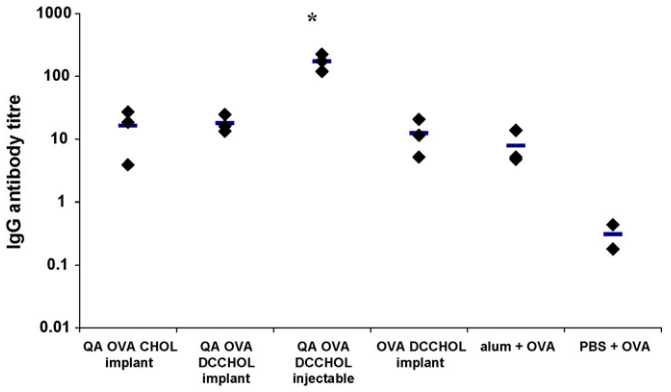

Fig. 6. OVA IgG antibodies determined using an OVA-specific ELISA. The diamonds represent the values obtained for each mouse; the lines represent the mean values for each group. The graph shows results obtained from one of three independent experiments with 2–3 mice per group per experiment. Statistical analysis was performed using ANOVA followed by Tukey's pairwise comparison. Significant results ($p \leq 0.05$) are indicated by an *.

utilised a QA OVA CHOL injectable vaccine (Myschik et al., 2008b, 2008).

Previous work had demonstrated the importance of including a strong adjuvant in the implant formulation such as QA (Myschik et al., 2008). In the current study two adjuvants were in fact included in the formulation as DCCHOL, as well as imparting a positive charge, has been reported to have adjuvant activity (Brunel et al., 1999; Guy et al., 2001). In the previous study, the administration of OVA CHOL implants did not result in antibody production (Myschik et al., 2008b, 2008), whereas in this study OVA DCCHOL implants stimulated significant levels of antibody as compared to mice immunised with OVA in PBS. Therefore, the adjuvant effect must be a result of DCCHOL, since no other adjuvant was present in that particular formulation. Guy et al. (2001) have also reported on an adjuvant effect of DCCHOL on antibody responses. Here, neutral CHOL/DOPC liposomes were compared to liposomes containing DCCHOL, and it was concluded that the adjuvant effect was due to the presence of DCCHOL in the formulations investigated.

In conclusion, QA DCCHOL implants did not stimulate $CD8^+$ T cell responses as effectively as the comparable injectable vaccine or an implant containing QA and CHOL. Therefore, the cationic charge of DCCHOL added no value to the formulation in terms of T cell expansion. However, some adjuvant effect on total IgG antibody responses was observed and the vaccine did induce stronger responses than either soluble protein alone, or protein co-delivered in alum.

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