G Dennis Sprott1,3, Chantal J Dicaire3, Jean-Philippe Côté2,3, and Dennis M Whitfield3

1Present address: Université de Montréal, Faculté de Médecine Vétérinaire, St. Hyacinthe, 3200 Sicotte, Québec J2S 7C6, Canada.
2National Research Council of Canada, Institute for Biological Sciences, 100 Sussex Drive, Ottawa, Ontario K1A 0R6, Canada.
3National Research Council of Canada, Institute for Biological Sciences, 100 Sussex Drive, Ottawa, Ontario K1A 0R6, Canada.

Received on February 28, 2008; revised on April 4, 2008; accepted on April 28, 2008

Subunit vaccines capable of providing protective immunity against the intracellular pathogens and cancers that kill millions of people annually require an adjuvant capable of directing a sufficiently potent cytotoxic T lymphocyte response to purified antigens, without toxicity issues. Archaeal lipid vesicles, prepared from isoprenoid lipids extracted from archaea, are one such adjuvant in development. Here, the stability of an archaeal core lipid 2,3-di-O-phytanyl-sn-glycerol (archaeol) is used to advantage to synthesize a series of glycosylarchaeols and show that subtle variations in the carbohydrate head group alters the type and potency of immune responses mounted in a mammal. Critically, a glycosylarchaeol was required to elicit high cytotoxic CD8+ T cell activity, with highest responses to the antigen entrapped in archaeosomes containing disaccharides of glucose in α- or β-1–6 linkage (β-gentiobiose, β-isomaltose), or of β-lactose. This first study on synthetic archaeal lipid adjuvants reveals potential for this class of regulatory friendly, easily scalable, inexpensive, and potent glyco-adjuvant.

Keywords: adjuvant/archaea/disaccharides/glycosylarchaeols/synthesis

Introduction

The causative agents for many of the most devastating human diseases, including malaria, tuberculosis, and HIV, are classified as intracellular pathogens. Considerable literature suggests that vaccine development for these agents, and for cancers, is likely to require a strong cell-mediated, particularly cytotoxic CD8+ T cell (CTL), response (Rappuoli 2007). As vaccineology advances from the use of whole attenuated pathogens toward safer, defined subunit vaccines, an adjuvant system becomes critical to direct and elevate the immune response to co-delivered purified antigens (Petrovsky and Aguilar 2004). Alum, while currently approved for human vaccines, is primarily an antibody rather than a cell-mediated adjuvant. One of several adjuvant systems in development to address the need for a safe effective CTL adjuvant (Aguilar and Rodriguez 2007) is archaeosomes.

Polar lipids that are novel to the domain of life Archaea are characterized as isoprenoid glyco- and phospho-ether lipids with stereochemistry (sn-2,3) opposite to that in glycerolipids of Bacteria or Eucarya (Kates 1992). Natural archaeal total polar lipid (TPL) extracts can be hydrated to form extremely stable, nanosized vesicles (archaeosomes) that target entrapped antigens to antigen-presenting cells (Tolson et al. 1996) and upregulate the expression of their co-stimulatory proteins (Krishnan et al. 2001), without any associated toxicity observed in mice (Patel et al. 2002). Adjuvant studies using TPL archaeosomes have determined that the polar lipids extracted from Methanobrevibacter smithii are especially promising. These membrane lipids consist of 60 wt% archaeols and 40 wt% membrane-spanning bipolar caldarchaeols with head groups of primarily phosphoserine and gentiobiose, and lesser amounts of phosphoinositol and phosphate (Sprott et al. 1999). The scheme in Figure 1 summarizes what is known regarding the mechanism for M. smithii archaeosome adjuvants. As described in detail in a recent review (Krishnan and Sprott 2008), M. smithii archaeosomes promote both MHC class I and class II responses to an entrapped antigen, where the latter MHC class II response is both cell mediated (Th1) and antibody (Th2) directed. Archaeosome TPL vaccines with striking protective ability have been demonstrated for the intracellular pathogen Listeria monocytogenes (Conlan et al. 2001) as well as for solid and metastatic tumors (Krishnan et al. 2003).

Archea are nonpathogenic microbes (Cavicchioli et al. 2003) and presumably would not have pathogen-associated molecular patterns to serve as danger signals that activate the innate immune system (Pulendran et al. 2001). Indeed toll-like receptors 2 and 4 do not appear to be activated by our lead archaeosomes composed of the polar lipids extracted from the commensal human gut methanogen, M. smithii (Krishnan et al. 2007). Despite the recent push toward the development of toll-like receptor agonists as adjuvants, questions remain regarding safety and efficacy (van Duin et al. 2006), and the question whether a toll-free adjuvant would lead to better vaccines remains unanswered (Ishii and Akira 2007). Archaeosomes appear to be quite novel among adjuvants by apparent inability to activate toll-like receptors, yet exhibiting long-lasting (Krishnan et al. 2007) vaccine efficacy.

Many of the mechanistic details for immune activation have yet to be determined (Medzhitov 2007) which complicates establishing structure-activity relationships for the design of directed adjuvant activity. Here we describe immunological results based on a series of novel synthetic archaeal glycolipids where some of these details can be ascertained. The presence and structural detail of the carbohydrate head group of a
glycosylarchaeal lipid mimetic is shown herein to be important in the adjuvanting process.

Results and discussion

Use of archaean total polar lipid natural mixtures as an adjuvant system limits the adjuvant composition to only those lipids, and in the proportions, that are extracted from archaean species. Our initial assumption was that natural mixtures of archaean polar lipids in an extract would consist of immunostimulatory, immunotargeting, immunoinactive, and immunodepressing species; the latter partially based on downregulation of the adaptive immune response associated with liposomes containing phosphatidylserine (Hoffmann et al. 2005). Theoretically then, more potent archaean lipid adjuvants than found in extracts could be designed and synthesized chemically as simplified and defined archaosome compositions.

Our synthetic approach was to first obtain the archaean lipid core by methanolic-HCl hydrolysis of the polar lipids extracted from *Halobacterium salinarum*, chosen because this archaean has only one core lipid, namely fully saturated archaeol. Upon head group hydrolysis the mixture of natural polar lipids is converted to a single lipid, namely archaetidylglycerol, which is known (Rotzschke et al. 1991), was entrapped within archaosomes from a synthetic glycosylarchaeol combined with synthetic phosphatidylglycerol (PG) and cholesterol. PG chains were dipalmitoyl to mimic the C-16 (plus 4 methyl branches) of the C-20 phytanyl chains. Upon subcutaneous injection at 0 and 3 weeks, measuring antigen-specific CD8$^+$ CTL activity in splenic cells and titrating anti-OVA (ovalbumin) antibody in mouse sera assessed adjuvant activity for both MHC class I and class II (Th2) pathways. Results are compared with archaosomes consisting of total polar lipid extracts from *M. smithii*, as these are known to promote high CTL activity to an entrapped antigen (Krishnan et al. 2003). Other adjuvant comparisons are not shown because *M. smithii* archaosomes have outperformed Alum, Freund’s complete adjuvant, liposomes, and live *Listeria* recombinant for both antibody and CTL responses (Sprott et al. 1997; Krishnan, Dicaire, et al. 2000; Krishnan, Sad, et al. 2000; Krishnan et al. 2007). PG/cholesterol liposomes or the archaean analog of PG, archaetidylglycerol, combined with cholesterol had very low adjuvant activity (Figure 3A and B). Clearly then, the mere presence of the archaean isoprenoid lipid, archaetidylglycerol, was insufficient to promote strong immune responses of either MHC class I or class II, and indicates that this phospholipid may be classified as immunoinactive. Critically, we observed a dramatic increase in both CTL activity and antibody titers upon addition of glycosylarchaeols to PG/cholesterol documenting the importance for a glycosylarchaeol in the adjuvant process (Figure 3A and B). Since we are unaware of a specific diglucose-binding lectin on antigen-presenting dendritic or macrophage cells (APCs) it was surprising to us that 45 mol% $\alpha$-tetraMannosylarchaeol (Figure 3C) and shorter variations of it (not shown) designed to target the mannose receptors on APCs (East and Isacke 2002) were less effective CTL adjuvants than 15–45 mol% $\beta$-gentiobiosylarchaeol. In fact maximal CTL activity was required in excess of 15 mol% $\beta$-gentiobiosylarchaeol, but this activity declined when the $\beta$-gentiobiosylarchaeol was increased beyond 45 mol% to 60 mol% (Figure 3C). This drop in activity was explained by instability at 60 mol% $\beta$-gentiobiosylarchaeol, as aggregation and settling of the archaosomes occurred. This result also explains a previous erroneous conclusion that $\beta$-gentiobiosylarchaeol purified from a methanogen had little adjuvant potential (Sprott et al. 1999), as in that study the archaosomes tested contained about 60 mol% $\beta$-gentiobiosylarchaeol. CTL adjuvant effects were antigen-specific as little response occurred for the nonspecific EL-4 target cells (Figure 3D).

We proceeded by synthesizing a series of the diglycosylarchaeols to test if linkage configuration and sugar type were important for adjuvant activity (Figure 2). Disaccharides were...
chosen rather than longer oligosaccharides to minimize both the cost of synthesis and of any possibility of raising an anti-glycolipid antibody response. For all disaccharide lipids synthesized, archaosomes could be formed readily using 25 mol% diglycosylarchaeol mixed with DPPG/cholesterol in mol% ratio of 55/20. Because archaosomes formed less well at higher mol% ratios (35% or more) for certain of the diglycosylarchaeols to be compared, all formulations of diglycosylarchaeols with DPPG/cholesterol were tested only at 25/55/20 mol%. These preparations entrapped OVA similarly at close to 40 μg mg⁻¹ archaosomes, were comparable in size within the range of 50–150 nm diameter, and remained stable as judged by phase-contrast microscopy prior to each injection. CTL assays using splenic cells from immunized mice revealed a striking difference in antigen-specific adjuvant activity of archaosomes dependent on the structural details of the disaccharide head group used (Figure 4A). Diglycosylarchaeols with either an α- or β-1,4 linkage were relatively inactive compared to 1,6 linkages. β-Gentiobiosylarchaeol consistently gave best activities, as shown by highest % lysis of target cells at a low effector/target ratio, but a change of the β-1,6 linkage between glucose residues to α-1,6 (isomaltose) or change to a Gal-Glc disaccharide-linked β-1,4 (lactose) resulted in similarly high CTL adjuvant activity. Configuration of the linkage was important in the case of Gal-Glc-archaeols as the α-1,6 linkage of meliobiose was less active than β-1,4 of lactose. A similar pattern of antigen-specific
Fig. 3. Antigen-specific immune responses generated in mice immunized with various liposomes and archaeosomes are dramatically enhanced by the addition of a glycosylarchaeol. The antigen ovalbumin (OVA) was entrapped in DPPG/cholesterol (80/20, mol%) liposomes, archaeosomes composed of arachaidylglycerol (AG)/cholesterol (chol) (80/20, mol%), or β-gentiobiosylarchaeol/DPPG/chol, where chol was 20 mol% and the mol% changes of β-gentiobiosylarchaeol were compensated by changes in mol% DPPG. Mice were injected subcutaneously (15 µg OVA) a t0 a nd 3 w e e k s .

(A) CTL responses in splenic cells are high when the adjuvant included β-gentiobiosylarchaeol. Essentially no CTL response occurs in mice immunized with OVA in the absence of any adjuvant (OVA). Immune responses for the M. smithii total polar lipid (TPL) archaeosomes were included for comparison. Lysis of nonspecific EL-4 targets was always less than 3% (not shown).

(B) Means of anti-OVA antibody responses in blood taken from groups of five mice were significantly higher for β-gentiobiosylarchaeol/DPPG/chol than either DPPG/chol (P value 0.0006) or AG/chol (P value 0.0445), but not compared to TPL (P value 0.2170). (C) Representative CTL responses at the effector:target ratio of 11:1 show that the CTL activity varies directly with the mol% increase in β-gentiobiosylarchaeol from 0 to 45 mol%, and thereafter declines. Means are significant (P < 0.05) between 0 and 15 mol%, 15 and 45 mol%, but not between 0 and 5 mol% (P values were 0.0021, 0.0001 and 0.0596, respectively). At 45 mol% α-tetraMannosylarchaeol is an effective CTL adjuvant, but the means are significantly less compared to β-gentiobiosylarchaeol also at 45 mol% (P value 0.0083). (D) Controls for the CTL assay using nonspecific EL-4 targets reveal little to no lysis. Error bars represent s.e.m. These data are consistent with at least one other animal study, and with antigen-specific CD8+ T cell frequencies in the same splenic cells assayed by Elispot (data not shown).

Responses is seen by the Elispot analysis of the same splenic cell populations (Figure 4B).

Dectin-1 binds exclusively to β-1,3 oligomers of glucose where the minimum number of residues for activity is a 10- or 11-mer (Palma et al. 2006). Thus, the known specificity of this recently discovered β-glucan receptor excludes it as an explanation for the boost in adaptive immune responses by β-gentiobiosylarchaeol. Other known receptors will require careful scrutiny. However, the presence of novel undiscovered receptors on APCs that recognize the diglycosylarchaeols reported herein are likely.

Since the β-linkage of the monosaccharide 6-deoxyquinovose to a lipid core results in a more tightly packed bilayer membrane than its α-isomer, with potential for accompanying changes in biological activity (Matsumoto et al. 2005), we tested whether this feature would influence CTL adjuvancing. Both α- and β-anomers of gentiobiosylarchaeol were synthesized and used to generate antigen-loaded archaeosomes for the assessment of adjuvant activity. Significantly better activity was found for the β-anomer (Figure 4C), supporting the role of the disaccharide to engage receptors by better recognition of a more extended orientation from the surface of the archaeosome, as reported for packing of other β-glycolipids (Jarrell et al. 1987). Similar data for antigen-specific CD8+ T cell frequencies were obtained by the Elispot assay of splenic cells, where the β-gentiobiosylarchaeol adjuvant resulted in significantly higher frequencies than α-gentiobiosylarchaeol (P = 0.0037) (Figure 4B).

Perhaps indicative of a selective MHC class I glycolipid adjuvant mechanism, there were no significant differences among the means of anti-OVA antibody titers in blood for any of the diglycosylarchaeols (Figure 4D), suggesting that all proceed by a similar MHC class II antigen processing pathway.

In conclusion, we have shown that a weak CTL adjuvant consisting of DPPG/cholesterol liposomes can be converted to a strong adjuvant by the addition of a synthetic diglycosylarchaeol. CTL adjuvant activity is high with gentiobiose, with linkage to the archaeol in a preferred β rather
Archaeal synthetic glycolipid mimetics

Fig. 4. MHC class I immune responses in mice immunized with various synthetic diglycosylarchaeols depend on the carbohydrate head group structure of the adjuvant. OVA-loaded archaeosomes were prepared from various diglycosylarchaeols mixed with DPPG and cholesterol (25/55/20 mol%). Immunization of mice and assays were conducted as in Figure 3. (A) CTL response comparing a sequence of diglycosylarchaeols with head groups linked β to the sn-1 hydroxyl of archaeol revealed variations in activity. Controls for the CTL assay using nonspecific EL-4 targets never exceeded 7% lysis (not shown). (B) Elispot assays showing CD8⁺ T cell frequencies for the same splenic cell populations used in (A) and (C). Antigen-specificity is shown by comparing assays in the absence (−) or presence (+) of SIINFEKL peptide. (C) Ag-specific CTL responses in mice immunized with the gentiobiosylarchaeol/DPPG/cholesterol adjuvant where gentiobiose is linked either α or β to archaeol show a stronger immune response for the β configuration. The letter c refers to the control lysis obtained for the nonspecific EL-4 target. In all E:T ratios the means of α configuration were significantly less than β (P values were 0.0001, 0.0001, and 0.0051 for the increasing E:T ratios shown, respectively). Error bars represent s.e.m. These data are consistent with at least one repeated animal study. (D) Means of anti-OVA antibody responses in blood taken from groups of five mice were not significantly higher (P < 0.05) for any of the diglycosylarchaeols.

than α configuration. We conclude that β-gentiobiosylarchaeol and β-gentiobiosylcaldarchaeol lipids (Sprott et al. 1999) account, at least in part, for the adjuvant activity of M. smithii TPL archaeosomes. However, strong activity also noted with β-lactosylarchaeol is encouraging for vaccine development in view of the low cost of lactose to serve as a readily available synthetic precursor. Also encouraging is the relatively simple synthesis reported herein using biosynthetic archaeol and carbohydrate precursors, compared to the multistep synthesis of other adjuvants such as glycosphingolipids (Long et al. 2007), QS-21A (Wang et al. 2005), or lipidA mimetics (Bazin et al. 2006). β-Lactosylarchaeol, for example, is synthesized from archaeol and lactose precursors in four synthetic steps.

We anticipate future design of a synthetic archaeosome adjuvant to include one or combination of glycosylarchaeol(s) combined with a synthetic anionic archaeal lipid. As a further design feature, the stability of the archaeosome may be altered as required to achieve directional preference toward either MHC class I or II responses by the addition of a caldarchaeol membrane-spanning lipid (Choquet et al. 1996), and avoid using stabilizing cholesterol that may promote membrane peroxidation (Schnitzer et al. 2007). Although the structural possibilities of archaeal glycolipids for synthesis and adjuvant testing are numerous and therefore somewhat daunting, the reward may be to develop a new class of synthetic adjuvant designed to achieve the type of immune response required for protection against specific human diseases.

Materials and methods

Preparation of archaeol

Halobacterium salinarum ATCC 33170 was grown aerobically and the biomass extracted with chloroform/methanol/water ratios to obtain the total lipids. Neutral lipids were removed by precipitating the polar lipids with acetone. 3 g of polar lipids in a 500-mL round bottom flask were refluxed in 150 mL of 2.5% methanolic-HCl at 64–65°C for 2 h, while stirring magnetically. Archaeol in the methanolic-HCl was partitioned into petroleum ether by mixing methanolic HCl/water/petroleum
ether (30–65°C fraction) in the ratio 93 mL/9.3 mL/93 mL. The ether was evaporated to yield the archaeol oil, further purified by passing through a silica gel G (EMD Chemicals, Inc., Gibbstown, NJ) column (bed 20 cm × 1.8 cm). Any traces of neutral lipids present were eluted with hexane prior to recovering pure archaeol by elution with hexane/ethylacetate = 9:1 (v/v) with an overall yield of 41–45% of the starting polar lipids (wt basis).

**Glycosylarchaeol synthesis**

Details of the chemical synthesis will be presented in a separate communication. All oligosaccharides were synthesized from commercially available monosaccharide and disaccharide precursors. For example, lactose was acetylated with acetic anhydride and sodium acetate and purified by crystallization. The resulting peracetate was converted to its known thiophenol glycoside (Tropper et al. 1991) by the action of BF$_3$OEt$_2$/PhSH. This was in turn activated by N-iodosuccinimide and silver trifluoromethanesulfonate in the presence of archaeol to yield the glycoside which after purification was deacetylated to yield the β-lactosylarchaeol. All compounds were characterized by 1H and 13C NMR as well as positive ion MALDI MS of lipid-containing species which gave the expected molecular ions, typically (M+Na)$^+$. Representative data for β-lactosylarchaeol are provided in Supplementary Figures 1A–C. Arachadiylglycerol was purified from _Halofex volcanii_ (Sprott, Larocque, et al. 2003).

**Archaeosome preparation and characterization**

Liposomes were prepared by hydrating 20–30 mg lipids at 40°C in 2 mL of PBS buffer (10 mM sodium phosphate, 160 mM NaCl, pH 7.1) with the test antigen OVA dissolved at 10 mg mL$^{-1}$. In some cases cholesterol (Sigma) or DPPG (Sigma) were mixed in chloroform/methanol with the synthetic glycosylarchaeols. These were dried to remove all traces of solvent and hydrated in PBS, as above. The size of the vesicles in the preparations was decreased by sonication in a sonic water bath (Fisher Scientific) at 40°C. Antigen not entrapped was removed by centrifugation at 200,000 × g (R$_{max}$) and pellets washed thrice with 12-mL volumes of PBS. In the case of TPL archaeosomes 82 ± 7% of protein antigen was protected from protease action, indicating entrapment within the vesicles (Krishnan, Dicaire, et al. 2000). Quantification of antigen loading by SDS polyacrylamide gel electrophoresis was as described (Sprott, Patel, et al. 2003). Antigen loading was based on salt-corrected dry weights. Average diameters were determined by the particle-size analysis using a 5 mW He/Ne laser (Nicomp 370).

Lipid vesicle vaccines were prepared and diluted just prior to each injection solely as a precaution to ensure stability. Storage of the OVA-archaeosomes shown in Figure 4 (various diglycosyl-archaeols/DPPG/cholesterol) for 16 months at 4°C resulted in no change detected upon microscopic examination or by average diameter measurements.

**Animal usage**

To determine adjuvant activity, OVA entrapped in archaeosomes (OVA-archaeosomes) were used to immunize female C57BL/6 mice on days 0 and 21 (8 weeks old on first injection). Injections were subcutaneous at the tail base with 0.1 mL PBS containing 15 µg OVA entrapped in 0.2–0.63 mg lipids. Blood samples were collected on week 6 from the tail vein for anti-OVA antibody titration done by ELISA (Krishnan, Dicaire, et al. 2000). Spleens were collected from duplicate mice to determine CTL activity using the Cr$^{51}$-assay done in triplicate with specific and nonspecific targets EG.7 and EL-4, respectively (Krishnan, Sad, et al. 2000). In addition to CTL measurements, Elispot assays performed as previously described (Sprott et al. 2004) in triplicate on the same splenic cells used for CTL assays determined antigen-specific CD8$^+$ T cell frequencies. All protocols were approved by the Institutional Animal Care Committee and were in compliance with guidelines set by the Canadian Council on Animal Care.

**Statistical analysis**

Means are reported as means ± s.e.m. and significance between means (P < 0.05) compared by the two-tailed t test.

**Acknowledgements**

The authors thank Mr. John Shelvey for growing *Halobacterium salinarum* biomass in our cell culture facility. Mrs. Eva Eichler expertly synthesized α-tetraMannosylarchaeol in the laboratory of D.M.W. This article is publication 42524 of the National Research Council of Canada.

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/

**Conflict of interest statement**

None declared.

**Abbreviations**

APC, antigen-presenting dendritic or macrophage cell; CTL, cytotoxic T lymphocyte; OVA, ovalbumin; PG, phosphatidylglycerol; TPL, total polar lipid.

**References**


