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# Adjuvant properties of a simplified $C_{32}$ monomycolyl glycerol analogue

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ABSTRACT

A simplified $C_{32}$ monomycolyl glycerol (MMG) analogue demonstrated enhanced immunostimulatory activity in a dioctadecyl ammonium bromide (DDA)/Ag85B-ESAT-6 formulation. Elevated levels of IFN-γ and IL-6 were produced in spleen cells from mice immunised with a $C_{32}$ MMG analogue comparable activity to the potent Th1 adjuvant, trehalose 6,6′-di-behenate (TDB).



Tuberculosis (TB) in man has for many years been combated by vaccination with an attenuated strain of *Mycobacterium bovis*—the *M. bovis* BCG vaccine developed more than 80 years ago. However, although more than 3 billion doses of BCG have been administered, it does not always provide satisfactory resistance in every population. Modern approaches use well-defined vaccine formulations to minimize side-reactions.[1] However, in order to induce a sufficient protective immune response, adjuvant potentiating agents may be required. An adjuvant is any substance that serves to direct, accelerate, prolong and/or enhance the specific immune response of a vaccine. The development of a specific kind of immune response (humoral or cell-mediated) can be determined by the choice of adjuvant. For example, protective immunity against intracellular pathogens like *Mycobacterium tuberculosis* requires a cell-mediated immune response, and a suitable adjuvant for a subunit vaccine directed against TB, should enhance a Th1 response.[2]

Mycobacteria have long been known to exert a number of immunomodulatory effects and are a good source of adjuvant preparations. Freund's complete adjuvant, consisting of a paraffin oil emulsion and heat killed mycobacteria,[3] is best-known, but live *M. bovis* BCG has also been used as an immunotherapeutic agent.[4,5] Similarly, purified components of mycobacteria have been shown to have immunostimulatory activity. Wax D (a complex of peptidoglycan, arabinogalactan and mycolic acids) was found to possess strong adjuvant activity and trehalose 6,6-dimycolate (or synthetic analogues thereof) has been included in various adjuvant formulations.[6–8] Rosenkrands et al.[9] investigated the immunostimulatory capacity of a total lipid extract of *M. bovis* BCG Copenhagen. The tuberculosis subunit vaccine candidate, Ag85B-ESAT-6 (H1) was administrated in combination with BCG lipids in cationic liposomes formed of either dimethyl dioctadecyl ammonium bromide (DDA), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTAP) or cholesteryl 3β-*N*-(dimethylaminoethyl) carbamate hydrochloride (DC-Chol); neutral liposomes were formed by 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE) DOPE-phosphatidylcholine (PC) and anionic liposomes formed by DOPE-PC-phosphatidylglycerol (PG). Immune responses were monitored by in vitro restimulation of peripheral blood mononuclear cells purified 1 week after the last immunisation. Rosenkrands et al.[9] showed that apolar BCG lipids, together with DDA, elicited the most pronounced levels of IFN-γ released. DDA-BCG lipids only gave minimal levels of IL-5. Analysis of antigen-specific antibodies demonstrated an efficient induction of both IgG1 and IgG2a antibodies by the combinations of DDA-BCG lipids. The IgG1 titre was 2.6-fold higher than that when the antigen was administrated in alum. The ratio of IgG1:IgG2a was increased with the DDA-BCG lipids compared to the alum control.

Further studies have shown that in subcutaneous immunisation of mice with 10 μg of the total, polar or apolar lipids incorporated into DDA liposomes and administrated in conjunction with the recombinant fusion protein Ag85B-ESAT-6 (H1), the apolar fraction was found to induce IFN-γ release of almost four times that recorded with polar lipids.[10] The apolar components were

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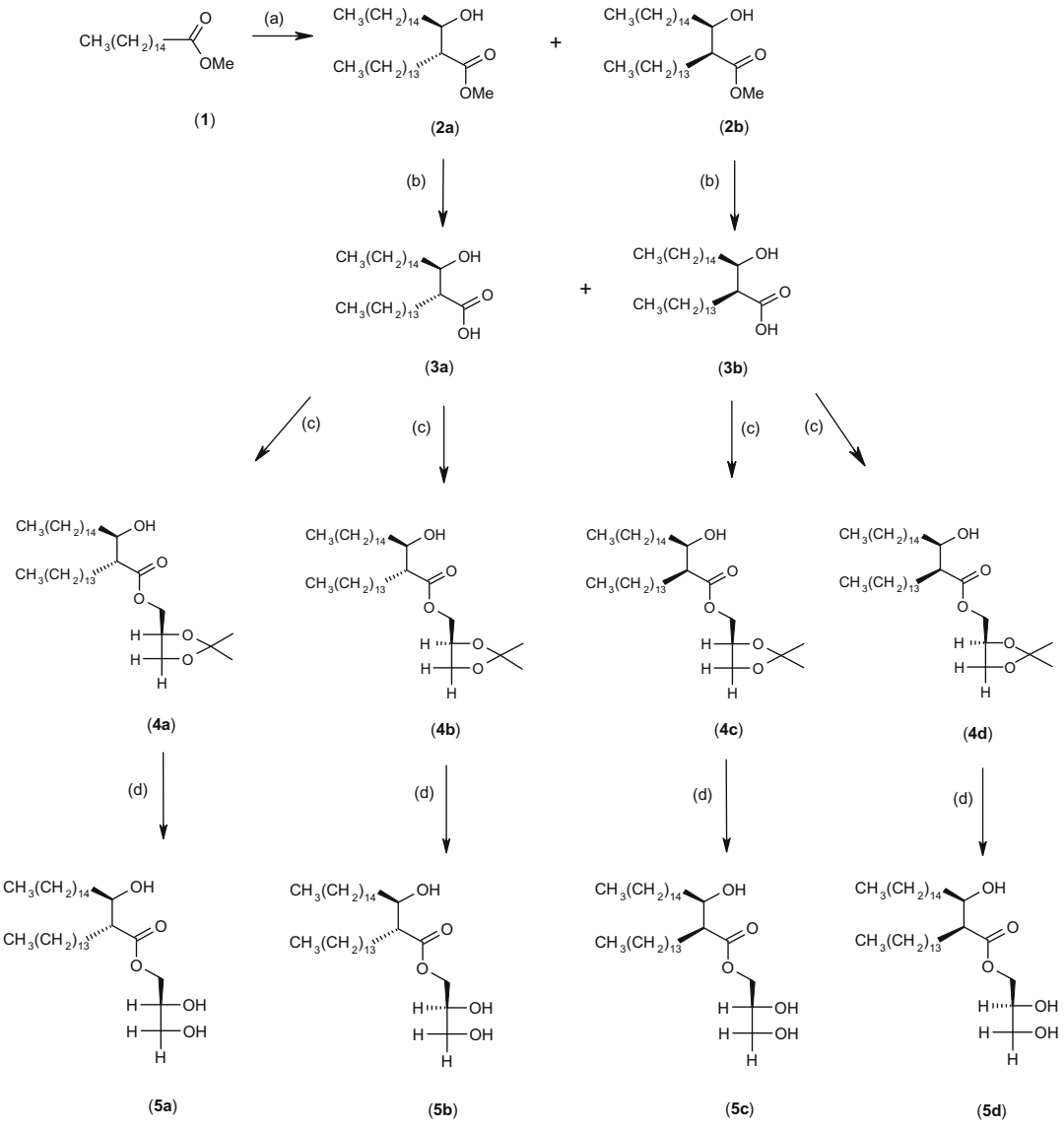
separated according to the procedures outlined by Dobson et al.,[11] to give four principal lipid fractions, phthiocerol dimycocerosates (PDIMs), triacylglycerols (TAGs), phenolic glycolipids (PGLs) and $C_{70-90}$ monomycolyl glycerol ($C_{70-90}$ MMG). $C_{70-90}$ MMG induced extremely high levels of all three cytokines IL-12, TNF-α and IL-6 with values of >250 pg/ml, 3500 pg/ml and 5000 pg/ml, respectively. Herein, we report the synthesis of four structural isomers of a simplified $C_{32}$ MMG analogue and immunological evaluation. In order to generate a series of MMG analogues, the core structures were broken down into two entities, the lipid tail and the glycerol unit. The *rac* or *sn* isopropylidiene-protected glycerol units were purchased from Sigma Aldrich. The $C_{32}$ corynomycolate isomers were synthesised according to Datta et al.[12] Briefly, the $C_{32}$ corynomycolic acid was synthesised by sodium hydride promoted self-Claisen condensation of methyl palmitate (**1**) followed by sodium borohydride reduction of the resulting keto ester to give the diastereomeric hydroxyesters (**2a**) and (**2b**) (Scheme 1). Flash column chromatography of the hydroxyesters mixture gave first **2b,** followed by **2a** (TLC $R_f$ 0.55 and 0.40 in chloroform, respectively). Hydrolysis with 5% KOH in 1:1 biphasic butanol:water released the racemic corynomycolic acid (**3a**) and its diastereoisomer (**3b**). Coupling of the glycerol unit with the mycolic acids (**3a** and **3b**) was achieved using dicyclohexylcarbodiimide (DCC) and 4-pyrrolidinopyridine (PYP).

The 1,2-isopropylidiene-protected glycerol (**4a**–**d**) preferentially coupled with the carboxyl terminus of the unprotected mycolic acid, however a doubly coupled mycolate with a glycerol unit was identified as a minor side-product (<5%) by mass spectrometry. The TLC $R_f$ values of the product and side-product were 0.20 and 0.40, respectively (hexane/ethyl acetate 95:5) and were separated by column chromatography. Deprotection of the isopropylidiene was achieved with trifluoroacetic acid (TFA)/tetrahydrofuran (THF)/water (8:17:3) generating *erythro* and *threo* $C_{32}$ *sn* and *rac* glycerol coupled products (**5a**–**d**). NMR and mass spectral analysis of **5a** is outlined in Andersen et al.[10]

Ten groups of three BALB/c mice received three doses of 2 μg H1 with 250 μg DDA and 50 μg of trehalose 6,6′-di-behenate (TDB), a potent Th1 adjuvant or quantities of lipid adjuvant as described in Table 1 at days 0, 14 and 28.[13] Blood samples were taken via the tail vein at regular intervals to monitor H1 specific antibody and antibody isotype titres using standard enzyme linked immunosorbent assay (ELISA) techniques. The groups were as outlined in Table 1. Analysis of H1 specific cell proliferation was carried out using 100 μl volumes of spleen cells from individual mice as




**Scheme 1.** Reagents and conditions: (a) (i) NaH, dry xylene, reflux, 5 h (82 %); (ii) NaBH4, 3 h, rt (95 %); (b) 5 % KOH, 1:1 butanol/water, reflux (90%); (c) DCC, PYP, L-α-β-isopropylidene glycerol (45 %); (d) 8:17:3 (TFA/THF/$H_2O$), rt, overnight (49%).

**Table 1**
Dosage groups used to analyse $C_{32}$ mycolic acid variants

| Group | Dose |
|---|---|
| 1 | MMG [50] |
| 2 | **5a**[a] [50] |
| 3 | **5c**[a] [50] |
| 4 | **5b**[a] [50][b] |
| 5 | **5d**[a] [50] |
| 6 | **5a**[a] [5][b] |
| 7 | **5a**[a] [0.5][b] |
| 8 | Incomplete Freund's (ICF)(without DDA) |
| 9 | TDB [50] |
| 10 | Naïve control |

Key: [micrograms per dose of lipid adjuvant].
[a] GLYCEROL $C_{32}$ (**5a–d**).
[b] Expected immunoactive agent.

previously described.[14] These were seeded onto 100 μl volumes of media in sterile 96-well suspension culture plates containing various concentrations of H1 antigen. After 72 h incubation, 0.5 μCi of [$^3$H]thymidine (Amersham, UK) was added to each of the microcultures, and the incubation continued for a further 24 h. The well contents were harvested onto glass filter mats using a cell harvester (Titertek). The discs representing each well were punched from the filter mats into 5 ml volumes of scintillation fluid (Optiphase Hisafe, Fisher, UK) to measure incorporation of [$^3$H]thymidine into the cultured cells using standard scintillation counting procedures. Concomitant H1 specific cytokine production was analysed by incubation of similar spleen cell microcultures with a concentration of 5 μg/ml H1 antigen. Supernatants obtained from these microcultures were assessed for IFN-γ and IL-6, IL-5, IL-10 and IL-2 using DuoSet® capture ELISA kits (R&D Systems Europe, Oxfordshire, UK) according to the manufacturer's instructions. From the adjuvants tested, it could be hypothesized that the synthetic analogue that corresponds with the natural MMG might be of most interest in mimicking the adjuvant effect of this mycobacterial agent. Interestingly, it could be seen that this synthetic agent was more effective than the optimized dose of the TDB adjuvant in the elicitation of H1 specific spleen cell proliferation following restimulation of spleen cells from immunised mice with antigen in vitro (Fig. 1). However, this was only seen when the dose of the adjuvant was 100-fold reduced in comparison to the dose of TDB. The dose of TDB has previously been optimized to 50 μg in 250 μg DDA in order to provide the best adjuvant activity. It is clear from these results that from the adjuvants tested, the most effective dose or concentration of the MMG analogue is 0.5 μg with 250 μg DDA. H1 specific cytokine production showed that comparatively more IFN-γ and IL-6 was produced by spleen cells from mice immunised with the low dose synthetic adjuvant corresponding to the natural MMG structure (Fig. 2).

From the other cytokines assessed, most groups generated elevated IL-2 indicative of a T-cell driven component to the adjuvant effect of these analogues (data not shown). Figure 3 shows that H1 specific IgG2a titres were also high for the low dose analogue. All of the formulated analogues were able to engender enhanced immune responses at the 50 μg dose (Figs. 1 and 3) and these were comparable to the potent Th1 adjuvant TDB. This observation was corroborated by isotype specific antibody responses detected in blood samples taken throughout this study (IgG1 and total IgG not shown). The immunogenicity studies in mice showed that a glycerol based non-polar mycobacterial lipid analogue adjuvant formulated in DDA vesicles can give IgG2a mediated anti H1 antibody titres at least as high as DDA/TDB with 100-fold less of the adjuvant moiety present compared to TDB ($P = 0.867$ after the third dose).

The IgG2a antibody titre delineates a Th1 type response in BALB/c mice, thought to correlate strongly with protective immune responses. The BALB/c model is usually predisposed to Th2 type responses and therefore the ability to engender a Th1 type response here, supported by the antibody subtype and cytokine data, is worthy of note. Whilst it remains to be seen whether alternative synthetic analogues behave in the same way as **5a** that is representative of the mycobacterial MMG in their activity at very low dose, the data collated from this study strongly support the use of synthetic MMG as a potent adjuvant with excellent potential. These results also show the potential for DDA vesicles as a platform for the delivery of lipid based co-adjuvants in order to elicit potentially effective Th1 biased immune responses against H1 and other protein subunit vaccines. This study also emphasizes the importance of optimization of adjuvant dose or adjuvant ratio in particulate delivery platforms containing co-adjuvants. The inverse dose-response adjuvant activity of the **5a** isomer was highly

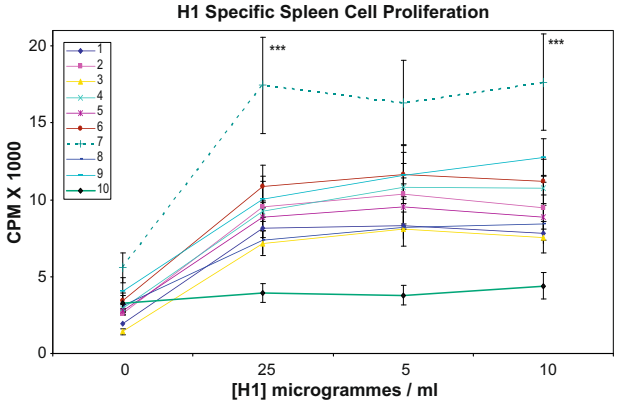



**Figure 1.** Spleen cell proliferation following restimulation with H1 antigen. *** Significantly greater than all other groups ($n = 3$, $P < 0.05$). Legend Key: [micrommes per dose of lipid adjuvant]; *GLYCEROL C32 (**5a–d**); **Expected immunoactive agent. Treatment groups are delineated in Table 1.

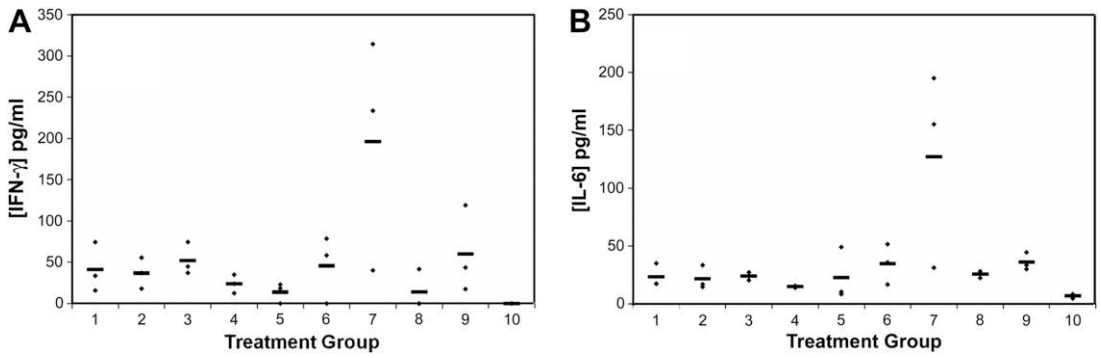



**Figure 2.** H1 specific cytokine production following restimulation of spleen cells with antigen. (A) Interferon gamma (IFN-γ); (B) Interleukin-6 (IL-6). Horizontal bars represent the average cytokine production. Treatment groups are delineated in Table 1.

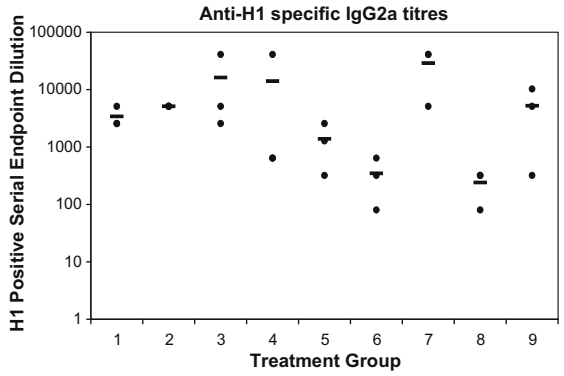



**Figure 3.** Serum anti-H1 specific IgG2a antibody titres following immunisation with the various formulations including the $C_{32}$ MMG analogues. Horizontal bars represent the average antibody titres. Treatment groups are delineated (Table 1).

unexpected. Speculation as to how this occurs may require investigation into physicochemical aspects as well as possible biological mechanisms of action—such as interaction with Toll-like receptors and antigen presenting cells. Such possible interactions are reviewed elsewhere.[15] Delineation of the exact mechanisms that underpin this inverse dose-response adjuvant activity provides the basis for interesting research from a biological as well as a pharmaceutical perspective.

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