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The ability of heat-killed *Mycobacterium vaccae* to stimulate a cytotoxic T-cell response to an unrelated protein is associated with a 65 kilodalton heat-shock protein

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SUMMARY

Exogenous antigens are generally presented by Class II major histocompatibility (MHC) molecules. When administered with an adjuvant, however, they are capable of inducing a $CD8^+$ T-cell response where antigen recognition is associated with Class I MHC. Accordingly, immunization with soluble ovalbumin (OVA) alone does not activate $CD8^+$ cytotoxic T cells (CTL) but when given in complete Freund's adjuvant (CFA), or in formulations of a number of novel adjuvants, an OVA-specific $CD8^+$ CTL response can be detected. We show in this report that immunization with soluble OVA mixed with heat-killed *Mycobacterium vaccae*, but not with other common pathogenic and saprophytic mycobacteria, can activate OVA-specific $CD8^+$ CTL. An OVA-specific CTL response is detected when mice are immunized by either the intraperitoneal or intranasal route and their spleen cells are re-stimulated *in vitro*. Adjuvant activity of heat-killed *M. vaccae* is present in *M. vaccae* culture filtrate, in soluble protein components of whole *M. vaccae* and in the 65 kDa heat-shock protein (hsp) of *M. vaccae*. *Mycobacterium vaccae* has previously been shown to have no adverse side-effects in humans. The current results suggest that *M. vaccae* may be useful as an adjuvant for vaccines and other immunotherapies where $CD8^+$ CTL responses to exogenous proteins are crucial.

INTRODUCTION

New generation vaccines are being developed which are better defined and offer advantages, such as lower toxicity, but they are often poorly immunogenic. Consequently, there is a need for new potent safe adjuvants or immunomodulators that are compatible with subunit or DNA vaccines. There are several classes of adjuvants, including aluminium salts, surface active reagents, polyanions, bacterial products and slow-release compounds. Aluminium adjuvants, together with calcium phosphate and a squalene formulation are the only adjuvants approved for human vaccine use. These approved adjuvants are not effective in stimulating cell-mediated immunity but rather stimulate a good antibody response.[1] Successful vaccination against a number of infectious diseases requires both humoral and cell-mediated immunity and for some diseases, such as tuberculosis, protective immunity appears to be strongly associated with a T helper type 1 (Th1) response.[2] In recent years there has been a search for adjuvants that may selectively promote a Th1 response and there is evidence that infectious organisms such as Leishmania[3] and some mycobacteria[4] can contain such adjuvant components.

Mycobacterium vaccae is a non-pathogenic mycobacterium found in the soil. It has been considered as a possible vaccine or immunotherapeutic agent for human tuberculosis[5] although its efficacy, at present, is controversial.[6,7] It is proposed that *M. vaccae* promotes a Th1 response, overcoming the pathogenic Th2 response of individuals with tuberculosis while providing cross-reactive epitopes to stimulate a protective immune response.[7] In the mouse, immunization with a low dose of heat-killed *M. vaccae* reduces the mycobacterial load after challenge with *M. tuberculosis*.[8] Both $CD4^+$ and $CD8^+$ T cells appear to be important in the protective immune response against *M. tuberculosis*.[2,9] Immunization with heat-killed *M. vaccae* leads to, amongst other things, the stimulation of $CD8^+$ T cells. These produce interferon-γ (IFN-γ) in response to macrophages infected with *M. tuberculosis*, stimulate the production of interleukin-12 (IL-12) and specifically lyse

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Abbreviations: BCG, *Mycobacterium bovis* bacillus Calmette–Guèrin; CFA, complete Freund's adjuvant; CTL, cytotoxic T lymphocytes; DD, delipidated and deglycolipidated; DMEM, Dulbecco's modified Eagle's medium; hsp, heat-shock protein; IL-5, interleukin-5; IL-12, interleukin-12; IFN, interferon; kDa, kilodalton; MHC, major histocompatibility; OVA, ovalbumin; PBS, phosphate-buffered saline.; TCA, trichloroacetic acid.

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M. tuberculosis-infected macrophages.[10] Thus *M. vaccae* is capable of stimulating a cytotoxic response associated with type 1 cytokines (TC1) immune response to antigens shared with *M. tuberculosis*.

Exogenous antigens, such as those present in the heat-killed *M. vaccae* preparation, are usually not presented via the major histocompatibility complex (MHC) Class I pathway; however, there are exceptions.[11] Macrophages can regurgitate processed antigen as peptides[12] or transfer antigens from phagosomes into the cytosol so that endogenous and exogenous antigens use a final common pathway for Class I presentation.[13,14] Exogenous antigens can prime $CD8^+$ cytotoxic T cells (CTL) when they are administered with an adjuvant[15–20] although stimulation of CTL by native protein appears to depend on the characteristics of the protein as well as the adjuvant.[21–23] Heat-killed *M. vaccae* may contain adjuvants together with antigens common to *M. tuberculosis* which together stimulate the production of *M. tuberculosis*-specific $CD8^+$ CTL.

In this report we have used the CTL response to ovalbumin (OVA) to determine whether *M. vaccae* can function as an adjuvant to stimulate $CD8^+$ CTL responses to other protein antigens. We observe that heat-killed *M. vaccae*, but not other heat-killed mycobacteria such as *M. bovis* bacillus Calmette–Guèrin (BCG) or *M. tuberculosis* and other pathogenic and saprophytic mycobacteria, can induce a $CD8^+$ CTL response when given to mice in conjunction with OVA. Furthermore, heat-killed *M. vaccae* can function as an adjuvant for stimulation of an immune response through the mucosa of the airways. The significance of these findings to current concepts in immunology and to the development of a more effective adjuvant or immunotherapeutic agent suitable for use in humans is discussed.

MATERIALS AND METHODS

Antigens and adjuvants

Purified chicken ovalbumin (OVA Grade VI), muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) and trehalose 6, 6′-dimycolate from *M. tuberculosis* were purchased from Sigma Chemical Co. (St. Louis, MO). Complete Freund's adjuvant (CFA) was purchased from Difco (Detroit, MI). OVA was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) for low molecular weight impurities, which were found only to be present in trace amounts.

Mycobacterium vaccae (ATCC 15483), *M. smegmatis* (ATCC 27199), *M. phlei* (ATCC 11758), *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* BCG were obtained from AgResearch Wallaceville, New Zealand and grown by standard procedures. Culture filtrates of *M. vaccae* were supernatants from cultures of *M. vaccae* in 7H9 medium passed through a 0·45-μm filter. Heat-killed *M. vaccae* was prepared from the mycobacterial pellet resuspended in phosphate-buffered saline (PBS) at 10 mg/ml (equivalent to 10^{10} organisms per ml) and autoclaved for 15 min at 120°. To produce *M. vaccae* components, *M. vaccae* culture filtrate was concentrated by ultrafiltration [10 kDa molecular weight cut off (MWCO)], precipitated in 10% trichloroacetic acid (TCA), redissolved in 0·1 M Tris buffer (pH 8), re-precipitated with acetone, dissolved in water and finally re-precipitated in chloroform/methanol (2 : 1) at a ratio of 6 volumes chloroform/methanol to one volume of water. A delipidated and deglycolipidated form of *M. vaccae* (DD-*M. vaccae*) was prepared by sonicating autoclaved *M. vaccae*, and resuspending the pellet after centrifugation in 100 ml chloroform/methanol (2 : 1). After incubation at room temperature for 1 hr the material was centrifuged, re-extracted in chloroform/methanol, re-centrifuged and the dried pellet was resuspended in PBS. Glycolipids were removed by refluxing in 50% v/v ethanol for 2 hr and collecting the insoluble material by centrifugation. This extraction was repeated twice. Protein extracts were made by treating DD-*M. vaccae* with 2% SDS in PBS for 2 hr at 56°. The protein extraction was also repeated twice and SDS was removed from the extract by precipitation at 4°. After removal of the SDS precipitate the extracted proteins were precipitated with acetone, incubated at −20° for 2 hr, collected by centrifugation, dried and re-dissolved in PBS.

Recombinant *M. vaccae* heat-shock protein 65 (hsp 65) was obtained as follows. Serum, from *M. vaccae*-immunized cynomolgus monkeys, was used to probe a *Bam*HI lambda-ZAP (Stratagene, La Jolla, CA) genomic library of *M. vaccae*. An hsp 65 clone was verified as plaque pure at the tertiary screen, excised to a phagemid clone and sequenced to reveal a 2·2-kilobase (kb) partial clone of a hsp 65 homologue containing the 3′ end of the *M. vaccae* hsp 65 gene. To obtain the full-length clone, two primers were designed, one complementary to the 5′ end of the partial sequence, the other complementary to the 5′ end sequence of hsp 65 from *M. tuberculosis*, *M. paratuberculosis* and *M. leprae*. These primers were used to amplify a 642-nucleotide fragment which was cloned into Bluescript (Stratagene, La Jolla, CA), linearized and ligated to the partial *M. vaccae* hsp 65 2·2-kb *Bam*HI fragment to assemble the full-length hsp 65 gene in PBS. The full-length gene was cloned directionally and in frame into pET16 with a modified polylinker and expressed as a 6xHis–hsp 65 fusion protein. The protein was produced from recombinant *Escherichia coli* both as a soluble protein and as insoluble inclusion bodies. These were combined, after denaturing in 6 M guanidine (Gdn)HCl, and purified by chromatography on nickel-chelating Sepharose FF. The column was washed with binding buffer (6 M urea/0·5 M NaCl/20 mM Tris–HCl pH 8·0), with binding buffer containing 0·5% sodium deoxycholate, and with binding buffer containing 20 mM imidazole. The hsp 65 was eluted with binding buffer containing 300 mM imidazole, and was refolded by dialysis against decreasing concentrations of urea in 20 mM Tris–HCl pH 7·5. More than 90% of the protein was shown, by analytical SDS–PAGE, to be present as a single band of apparent MW 61 kDa.

The expression of genes in recombinant bacteria may lead to contamination of the purified proteins with bacterial endotoxin (lipopolysaccharide; LPS). In this case, the histidine-tagged recombinant-derived protein was purified by affinity chromatography on nickel-chelating Sepharose. While bound to the column, the protein was washed with buffer containing sodium deoxycholate to remove bacterial contaminants, including LPS. Residual endotoxin levels were assayed by the colorimetric *Limulus* amoebocyte lysate assay and were found to be less than 100 U/mg protein. A gel of the purified *M. vaccae* hsp is shown in Fig. 1. Other recombinant proteins of *M. vaccae* including the homologues of the antigen 85 (Ag 85) A, B and C complex of *M. tuberculosis* were derived as

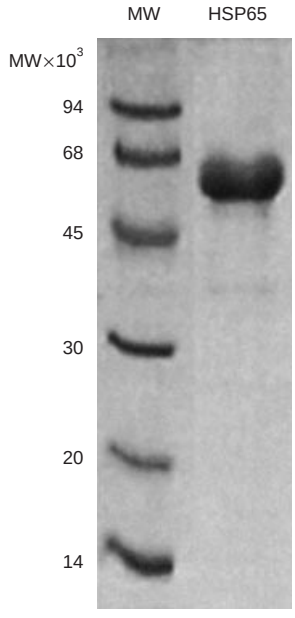

Figure 1. Analytical SDS–PAGE of purified *M. vaccae* HSP65**.** A sample of purified *M. vaccae* HSP65 (approximately 5 μg) was analysed by SDS–PAGE (12·5% acrylamide/0·33% Bis) and the gel was stained with Coomassie blue R250.

detailed elsewhere[24] and purified in the same way. Endotoxin levels in these preparations were also less than 100 U/mg protein.

Protease treatment

Soluble protein extracts of DD-*M. vaccae* were treated with Pronase E (Merck, Darmstadt, Germany). The equivalent of 20 μg protein was incubated with 20 μg Pronase E at 37° for 4 hr. The enzyme was heat-inactivated at 95° for 10 min before samples were mixed with OVA for injection into mice.

Animals and immunization

Specific pathogen-free female 7–10-week-old C57BL/6J (H-$2^{b)}$ mice were obtained from the Department of Laboratory and Animal Science, University of Otago, Dunedin, New Zealand, or from the animal facility at Genesis R & D Corp. Ltd, Auckland, New Zealand. Groups of mice (three to five mice per group) were immunized intraperitoneally with 100 μg OVA in PBS, adjuvant, or mixed with mycobacterial preparations. For heat-killed mycobacterial preparations, 1 mg wet weight was mixed with 100 μg OVA as this dose of heat-killed *M. vaccae* had been shown previously to stimulate a CTL response to mycobacterial antigens[10] and natural killer cell activity.[25] For intranasal immunizations mice were anaesthetized with 4–6 mg ketamine/0·025% xylazine per 20 g mouse. OVA or OVA mixed with 500 μg autoclaved *M. vaccae* in a volume of 40 μl was delivered one drop at a time to each nostril. This dose of *M. vaccae* was more convenient to administer by the intranasal route.

In vitro *stimulation of CTL*

Spleens were removed 9–10 days after immunization, the cells of each group were pooled and $3{\cdot}5 \times 10^7$ spleen cells were cultured with 3×10^6 E.G7-OVA[11] which had previously been γ-irradiated (20 000 rads) or treated with mitomycin C (Sigma Chemical Co.) at 20 μg/ml for 2 hr. Cultures were set up in a total volume of 10 ml Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 110 mg/l sodium pyruvate, 36 mg/l L-arginine, 116 mg/l folic acid, 60 mg/l penicillin, 100 mg/l streptomycin and 2 mM glutamine (tissue culture medium). After 6 days at 37° in 5% $C0_2$ in air, the cytotoxic activity of effector cells was assayed.

Cytotoxicity assay

EL4 and E.G7-OVA target cells were labelled with 100–200 μCi ^{51}Cr-labelled sodium chromate at 37° for 45 min. After washing, 5×10^3 ^{51}Cr-labelled target cells were incubated with effector cells at graded dilutions in 96-well V-bottomed plates. Supernatants were harvested after 5 hr and radioactivity was determined in a Micro Beta Plus liquid scintillation counter (Wallac, Finland). The percentage specific lysis was calculated according to the formula: [(c.p.m. released by CTL − c.p.m. released spontaneously) / (total c.p.m. taken up − c.p.m. released spontaneously)] × 100, where c.p.m. are counts per minute.

Spontaneous release was less than 14% of total c.p.m. taken up. Standard deviations of triplicate estimations did not exceed 4% specific lysis. Lytic units (LU) were calculated from the concentration of effector cells which gave 50% specific lysis and were expressed as $\log_{10}$. For example if the fraction of a culture containing effector cells that resulted in 50% specific lysis was 0·033, there were 30·3 lytic units generated per culture (1/0·033) which is 1·48 $\log_{10}$ LU. Lytic units were only calculated from experiments where a least three data points were on the straight line part of the plot of percentage specific lyis against $\log_{10}$ concentration of effector cells. Geometric means of lytic units from replicate experiments were expressed with standard deviations.

$CD8^+$ T-cell depletion

Spleen cell cultures were depleted of $CD8^+$ T cells by antibody-mediated complement lysis or by passage through a T-cell separation column. Cells were treated with 1 μg/ml anti-CD8 (clone 536.72, Pharmingen, San Diego, CA) for 30 min on ice, washed once and incubated with low toxicity rabbit complement (Serotec, Oxford, UK) at a final dilution of one in five. After incubation at 37° for 45 min, dead cells were removed by centrifugation over Ficoll and washed three times. Alternatively, cells were loaded onto a T-cell separation column which depleted $CD8^+$ T cells (R & D Systems, Minneapolis, MN) and run according to the manufacturer's instructions. After depletion, cells were tested for residual $CD8^+$ cells by flow cytometry and assayed for cytotoxic activity.

RESULTS

Induction of OVA-specific CTL by OVA delivered with *M. vaccae*

Spleen cells from C57BL/6J mice immunized with OVA in various formulations were examined for the development of OVA-specific CTL responses. The spleen cells were cultured with irradiated E.G7-OVA stimulator cells for 6 days and then assayed for cytotoxic activity on E.G7-OVA and EL4 target

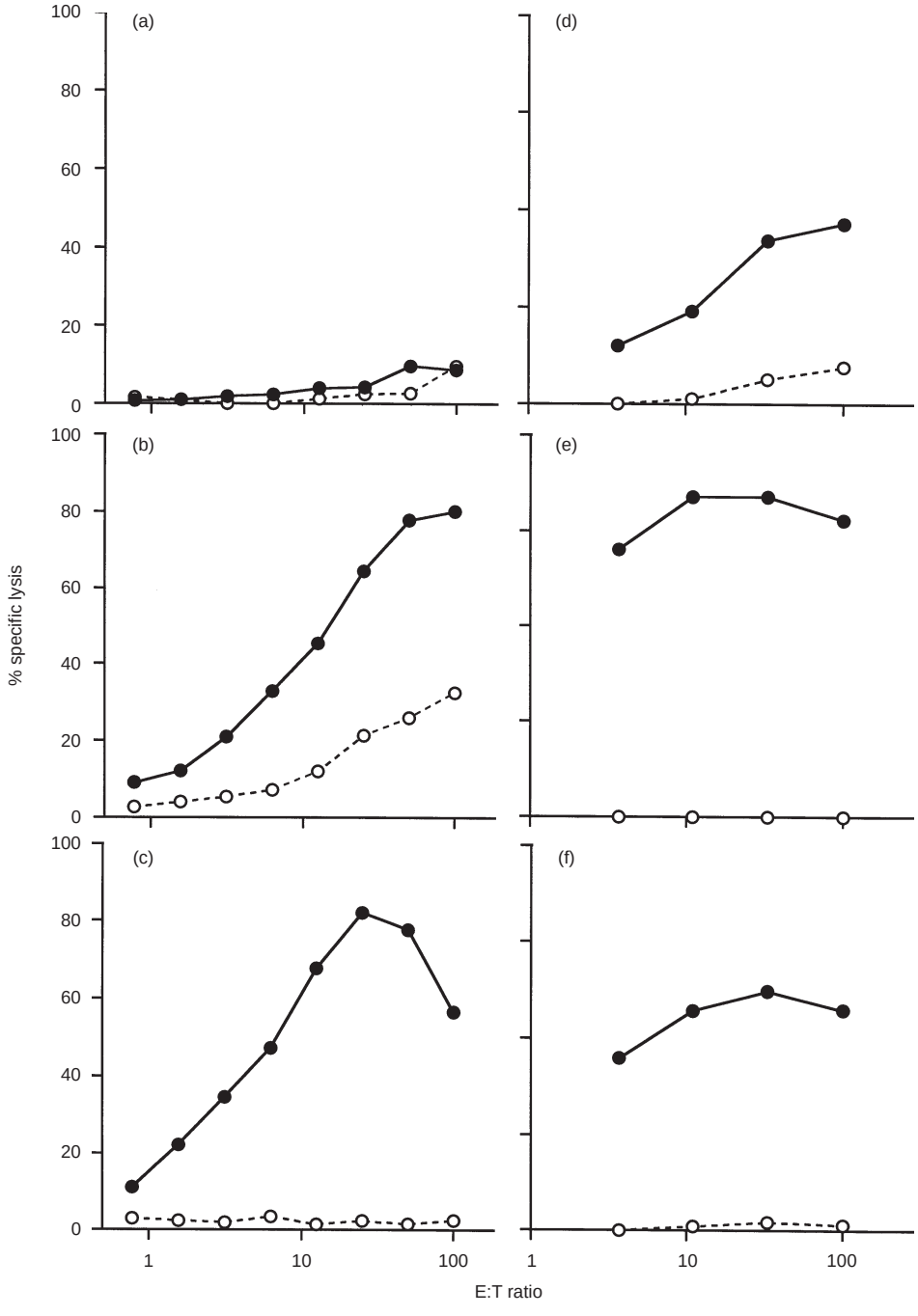

Figure 2. Induction of OVA-specific CTL response by OVA delivered with *M. vaccae*. C57BL/6J mice were immunized i.p. with (a) OVA alone, (b) OVA in CFA, (c) OVA plus 1 mg heat-killed *M. vaccae*; or with (d) OVA plus 50 μg muramyl dipeptide, (e) OVA plus 50 μg trehalose 6, 6′-dimycolate, or (f) OVA plus 1 mg heat-killed *M. vaccae*. Spleens were removed 10 days later and splenocytes were stimulated *in vitro* with E.G7-OVA cells for 6 days. The cytotoxic activities were measured using ^{51}Cr-labelled E.G7-OVA (●), or EL4 (○). Per cent specific lysis at various effector to target ratios are shown and represent two separate experiments.

cells. The results shown in Fig. 2 are from two separate experiments. In the first experiment mice immunized with OVA mixed with 1 mg heat-killed *M. vaccae* generated a highly specific OVA CTL response (Fig. 2c), whereas a CTL response was not detectable when mice were immunized with native OVA (Fig. 2a). Treatment with heat-killed *M. vaccae* alone did not stimulate a CTL response (data not shown). As expected, immunization with OVA in CFA generated OVA-specific CTL but there was some nonspecific lysis of EL4 target cells (Fig. 2b). No response was detected after immunization with OVA in IFA (data not shown). In a second experiment two other known adjuvants derived from mycobacteria, muramyl dipeptide and trehalose 6, 6′-dimycolate also stimulated an OVA-specific CTL response (Fig. 2d,e). In the second experiment, in which heat-killed *M. vaccae* (Fig. 2f) was used as the positive control, the maximum percentage specific lysis

plateaued at 60% whereas in the results of the experiment shown in Fig. 2(c) it reached nearly 80%. To demonstrate that heat-killed *M. vaccae* does give reproducible adjuvant activity, even though the percentage specific lysis sometimes reaches a plateau at different levels, the results of 10 additional separate experiments were analysed and results expressed as mean lytic units are shown in Table 1. These results confirm that although exogenous OVA primes CTL when injected in a water-in-oil emulsion containing *M. smegmatis* (CFA), OVA mixed with *M. vaccae* or known mycobacterial adjuvant components in the absence of oil is sufficient for the development of OVA-specific CTL.

CTL induced by OVA and *M. vaccae* are CD8$^+$

To establish whether the CTL response stimulated by *M. vaccae* was mediated by CD8$^+$ CTL, spleen cell cultures from immunized mice were treated with anti-CD8 antibody and complement prior to assay for CTL activity. After CD8$^+$ T-cell depletion, which did not totally deplete all CD8$^+$ T cells, OVA-specific CTL activity was reduced from 2·72 to 1·48 $\log_{10}$ LU per $3{\cdot}5 \times 10^7$ cultured spleen cells. Spleen cells, when passed through a column that removed >95% CD8$^+$ T cells, lost all detectable OVA-specific CTL activity (Table 2).

Other killed mycobacteria do not induce OVA-specific CTL

To investigate whether other common heat-killed mycobacteria alone can contribute to the development of an OVA-specific CTL response, C57BL/6J mice were immunized with OVA mixed with 1 mg heat-killed *M. tuberculosis*, *M. bovis* BCG, *M. phlei*, or *M. smegmatis*. Only mice immunized with OVA in combination with various doses of *M. vaccae* or *M. vaccae* culture filtrate developed OVA-specific CTL (Fig. 3). A dose of 1 mg heat-killed *M. vaccae* gave 2·10 $\log_{10}$ LU per culture whereas although 10 μg heat-killed *M. vaccae* was sufficient to

Table 1. Adjuvant activity of different components of *M. vaccae*

Adjuvant	Lytic units (mean ± SD)§
1 mg heat killed *M. vaccae* ($n=10$)*	2·36 ± 0·26
100 μg *M. vaccae* culture supernatant ($n=4$)†	2·27 ± 0·30
M. vaccae hsp65 ($n=3$)‡	1·48 ± 0·03

* Number of experiments using four different batches of heat-killed *M. vaccae*; † number of experiments using four different batches of *M. vaccae* culture supernatant; ‡ number of experiments using two different batches of *M. vaccae* hsp65; § geometric mean of $\log_{10}$lytic units per culture using EG.7-OVA target cells with standard deviation.

Table 2. Effect of depletion of CD8$^+$ cells on CTL activity after immunization with OVA mixed with *M. vaccae*

		Lytic units per culture	
Treatment*	% CD8$^+$ cells†	EG.7-OVA targets	EL4 targets
C′	17·2	2·72 ± 0·02‡	<0·1
Anti-CD8 + C′	9·6	1·48 ± 0·03	<0·1
Column depletion	<5	<0·1	<0·1

*Spleen cells from immunized mice were challenged *in vitro* with irradiated EG.7-OVA cells and 6 days later were depleted of CD8$^+$ T cells before assay for CTL activity; †percentage of CD8$^+$ T cells determined by flow cytometry; ‡geometric means and standard deviation of $\log_{10}$ lytic units from triplicate assays.

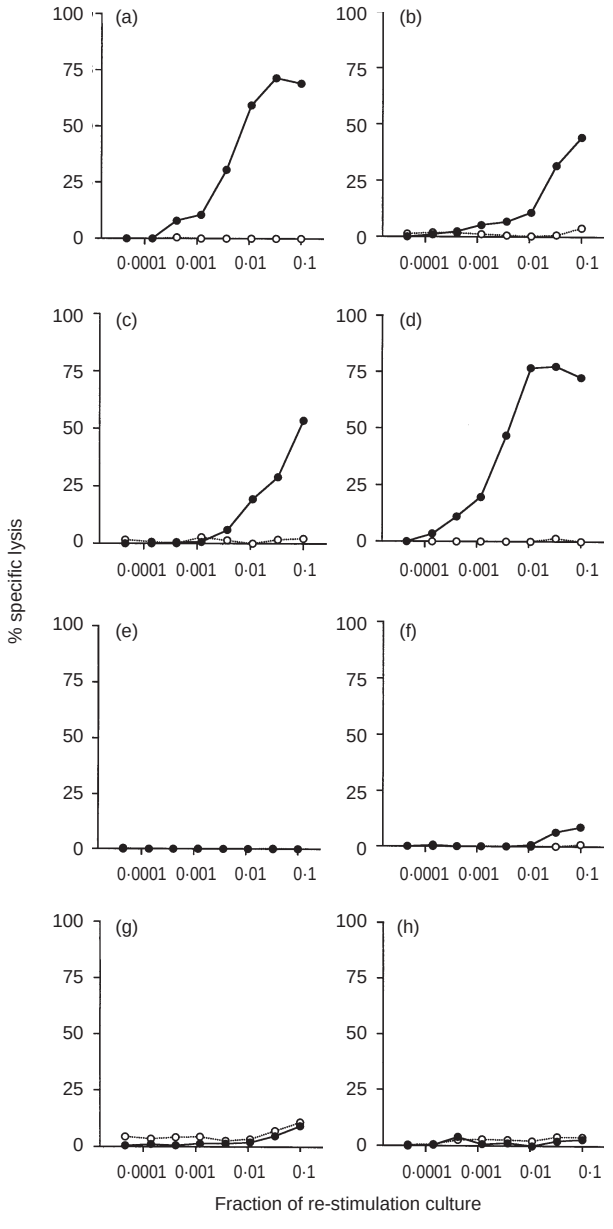

Figure 3. Heat-killed *M. tuberculosis* does not induce OVA-specific CTL. C57BL/6J mice were immunized i.p. with OVA mixed with (a) 1 mg heat-killed *M. vaccae*, (b) 100 μg heat-killed *M. vaccae*, (c) 10 μg heat-killed *M. vaccae*, (d) 100 μg *M. vaccae* culture supernatant, (e) 1 mg heat-killed *M. tuberculosis*, (f) 1 mg heat-killed *M. bovis* BCG, (g) 1 mg heat-killed *M. phlei*, or (h) 1 mg heat-killed *M. smegmatis*. Spleens were removed 10 days later and splenocytes were stimulated *in vitro* with E.G7-OVA cells for 6 days. The cytotoxic activities were measured using ^{51}Cr-labelled E.G7-OVA (●), or EL4 (○). The results shown are the per cent specific lysis at various dilutions of effector cells. One representative experiment of two is shown.

stimulate a specific CTL response (Fig. 3c) only 1·09 $\log_{10}$LU per culture were generated. Heat-killed *M. leprae* was also unable to induce an OVA CTL response (data not shown). In a repeat experiment spleen cells from mice immunized with OVA administered with 1 mg heat-killed *M. vaccae* generated 2·05 $\log_{10}$LU/culture whereas spleen cells from mice immunized with OVA administered with other heat-killed mycobacteria did not generate a CTL response.

M. vaccae components

To determine which components of *M. vaccae* were involved in the adjuvant effect and to eliminate known lipid mycobacterial adjuvants, lipids and proteins were extracted from autoclaved *M. vaccae*. DD-*M. vaccae* elicited a CTL response comparable to that caused by the amount of heat-killed whole *M. vaccae* from which it was derived (data not shown). Protein from DD-*M. vaccae* was also highly active in eliciting a CTL response to soluble OVA and this was considerably reduced by treatment with Pronase E (Fig. 4b,c). A major component of the soluble *M. vaccae* protein ran at 65 kDa on a gel and protein sequence data showed homology to the 65 kDa hsp of *M. tuberculosis*. Consequently, the 65 kDa hsp from *M. vaccae* was cloned, sequenced, expressed in *E. coli* and tested for its ability to provide adjuvant activity for the stimulation of OVA-specific CTL *in vivo*. This protein displayed adjuvant activity that resulted in the generation of a highly specific CTL response to OVA (Fig. 5d) (1·48 $\log_{10}$LU per culture against EG.7-OVA target cells). Another recombinant protein from *M. vaccae*, the homologue of the *M. tuberculosis* Ag 85A protein, did not induce a cytotoxic response to OVA (Fig. 5a). Two further experiments were carried out using hsp 65 and mean LU/culture from the three experiments are shown in Table 1. In these experiments, seven other recombinant proteins from *M. vaccae* were shown not to exhibit adjuvant activity for CTL responses to OVA. They included the homologues of Ag 85B and Ag 85C of *M. tuberculosis* and GV7, GV14, GV22, GV28 and GV29.[24]

Immunization at mucosal surfaces

The ability of *M. vaccae* to elicit a CTL response to OVA after immunization by the intranasal route was investigated. Mice were immunized intranasally with 100 μg OVA mixed with 500 μg heat-killed *M. vaccae* and their spleen cells were re-stimulated *in vitro* with mitomycin C-treated E.G7-OVA cells. Neither OVA nor *M. vaccae* alone could induce an OVA-specific CTL response, whereas when they were combined, a strong CTL response was detected (Fig. 6). The specificity of the response as indicated by the lysis of EL4 target cells was slightly less than that observed when immunization was via the intraperitoneal route. The results demonstrate that *M. vaccae* can provide adjuvant activity for immunization at mucosal surfaces.

DISCUSSION

An adjuvant is required for inducing a CTL response to peptides or proteins. Consequently, they have been incorporated into adjuvant vehicles such as liposomes,[16,19] DNA[26], formulated with or without oil-based adjuvants,[17,18,20] or fused with hsp 70.[27] The results of this study show that by simply mixing OVA with heat-killed *M. vaccae,* it is possible to stimulate an OVA-specific $CD8^+$ CTL response.

It is well known that mycobacteria contain compounds with immunonstimulant activity. The first microbial products to be systematically investigated as adjuvants were components of *M. tuberculosis*. The trehalose 6,6′-dimycolate glycolipid or cord factor and forms of muramyl dipeptide are two that have been extensively studied.[28] Two such compounds were shown to act as adjuvants in the stimulation of an OVA-specific CTL response by soluble OVA (Fig. 2). Live intact mycobacteria can act as highly effective adjuvants and recombinant BCG, expressing human immunodeficiency virus (HIV) genes, is currently being tested as a potential vaccine for HIV.[29] Live BCG can stimulate a CTL response[30] but when killed appears to be inactive both in stimulating $CD8^+$ CTL specific for *M. tuberculosis*-infected macrophages (M. Skinner unpublished) and as an adjuvant for the induction of OVA-specific CTL (Fig. 3). Similarly, live *M. tuberculosis* can stimulate $CD8^+$ CTL[2] but killed *M. tuberculosis* can neither induce *M. tuberculosis*-specific $CD8^+$ CTL (M. Skinner unpublished) nor act as an adjuvant for the generation of OVA-specific CTL[18] (Fig. 3). Although purified components of *M. tuberculosis* do have adjuvant activity (Fig. 2) they may not be present in high enough concentrations or in an appropriate form in the

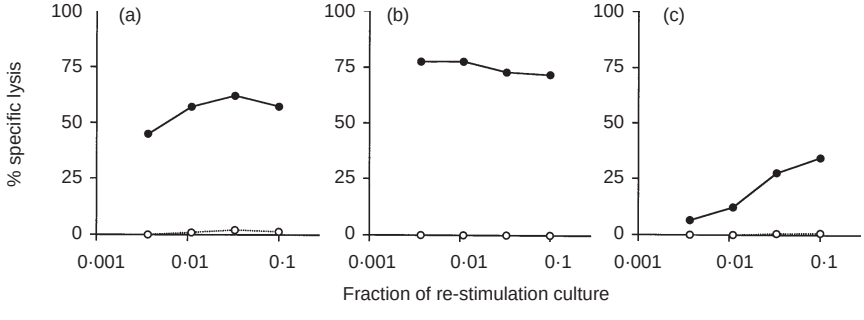

Figure 4. Adjuvant activity is present in soluble proteins extracted from *M. vaccae* C57BL/6J mice were immunized i.p. with OVA mixed with (a) 1 mg heat-killed *M. vaccae*, (b)10 μg soluble protein extracted from *M. vaccae*, or (c) 10 μg soluble protein extracted from *M. vaccae* and treated with Pronase E. Spleens were removed 10 days later and splenocytes were stimulated *in vitro* with E.G7-OVA cells for 6 days. The cytotoxic activities were measured using ^{51}Cr-labelled E.G7-OVA (●), or EL4 (○). The results shown are the per cent specific lysis at various dilutions of effector cells. One representative experiment of two is shown.

heat-killed preparations of whole mycobacteria used in this study. A number of other common slow- and fast-growing heat-killed mycobacteria, were also inactive as adjuvants for induction of OVA-specific CTL responses (Fig. 3). In contrast, heat-killed *M. vaccae* stimulates CTL which will specifically lyse *M. tuberculosis*-infected target cells[10] and can act as an adjuvant for a CTL response to an unrelated protein, OVA. (Table 1, Figs 2, 3).

In order to determine which components of *M. vaccae* contain adjuvant activity, *M. vaccae* was treated to remove

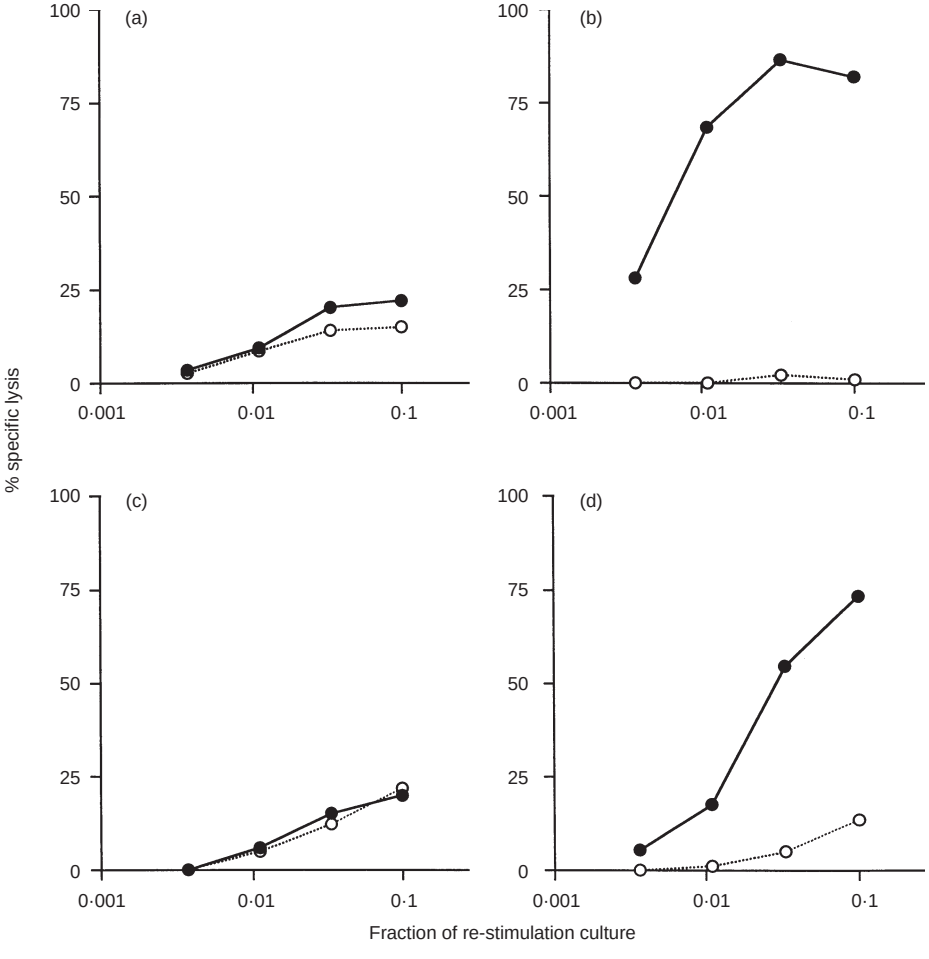

Figure 5. *Mycobacterium vaccae* hsp65 has adjuvant activity. C57BL/6J mice were immunized i.p. with (a) OVA mixed with 50 μg *M. vaccae* Ag 85A, (b) OVA mixed with 1 mg heat-killed *M. vaccae*, (c) 50 μg *M. vaccae* hsp65, or (d) OVA mixed with 50 μg *M. vaccae* hsp65. Spleens were removed 10 days later and splenocytes were stimulated *in vitro* with E.G7-OVA cells for 6 days. The cytotoxic activities were measured using ^{51}Cr-labelled E.G7-OVA (●), or EL4 (○). The results shown are the percent specific lysis at various dilutions of effector cells. One representative of three is shown.

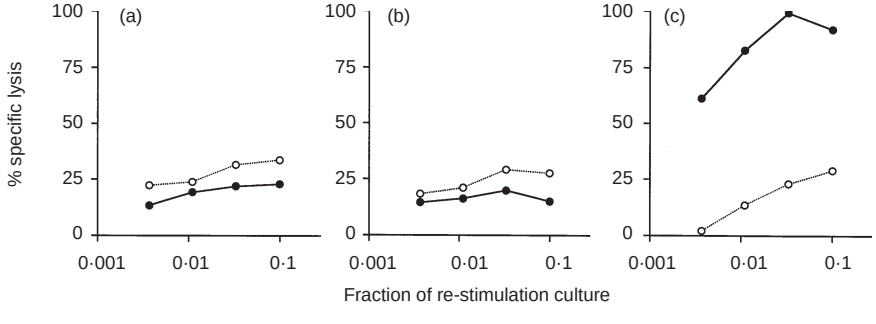

Figure 6. Heat-killed *M. vaccae* as a mucosal adjuvant. C57BL/6J mice were immunized intranasally with (a) OVA (b) 500 μg heat-killed *M. vaccae*, or (c) OVA mixed with 500 μg heat-killed *M. vaccae*. Spleens were removed 10 days later and splenocytes were stimulated *in vitro* with E.G7-OVA cells for 6 days. The cytotoxic activities were measured using ^{51}Cr-labelled E.G7-OVA (●), or EL4 (○). The results shown are the per cent specific lysis at various dilutions of effector cells. One representative experiment of three is shown.

lipids, glycolipids and soluble proteins. Although there was activity in insoluble components, it was clear that activity was also present in one or more proteins (Fig. 4). Adjuvant activity was almost entirely depleted from the soluble proteins of *M. vaccae* after pronase treatment. Protein adjuvants are uncommon, with the exception of hsp, which can bind small peptides and stimulate tumour-specific[31] and viral-specific CTL.[32] CTL responses to a whole protein, for example OVA, have been generated only when a fusion protein between OVA and a hsp is used for immunization.[27] In this work *M. vaccae* was simply mixed with OVA immediately prior to immunization. Under these conditions of association it was not possible to generate a CTL response with the CTL immunodominant peptide SIINFEKL ($OVA_{257-264}$) recognized in association with $H2K^b$ (data not included), as CTL responses generated with hsp–peptide combinations require special conditions of association.[32] Two recombinant polypeptides from *M. vaccae* were tested for adjuvant activity and a CTL response was detected using hsp 65 but not using Ag 85A. Both polypeptides had less than 100 U/mg protein LPS and seven other recombinant polypeptides from *M. vaccae* prepared in the same way did not exhibit this type of adjuvant activity (data not included). Consistent results were obtained using the purified recombinant hsp 65 as an adjuvant with less variability between replicate experiments than obtained using whole heat-killed *M. vaccae* or *M. vaccae* culture supernatant (Table 1).

IL-12 can enhance priming of CTL to exogenous antigens.[33] Both *M. vaccae* hsp 65 and Ag 85A were good stimulators of IL-12 production from macrophages (data not shown), but they may have other differential effects on macrophages or other antigen-presenting cells. It has recently been reported that hsps can activate monocyte-derived macrophages through a CD14-dependent pathway, which results in an increase in the inflammatory cytokines IL-1β, IL-6 and tumour necrosis factor-α (TNF-α).[34,35] Preliminary studies on human peripheral blood mononuclear cells indicate that *M. vaccae* hsp 65, like other Th1-inducing adjuvants including CpG oligodinucleotides and Monophosphoryl lipid A (MPL), stimulates production of IL-1β and TNF-α as well as that of IL-12.[24] The pathway by which hsps stimulate cytokine production presumably involves Toll-like receptors (TLR) but this has yet to be shown. Activation via a CD14 independent pathway can leads to an increase in TNF-α alone.[35] Although not yet demonstrated it is likely that a set of inflammatory chemokines are also stimulated which could modulate the migration of antigen-presenting cells and T cells. Thus hsps may have a dual role as both chaperone and cytokine and could have an important function in initiating immune responses.

It is unlikely that hsp 65 is the sole component of the protein adjuvant activity of *M. vaccae* as preliminary data suggests that there are additional polypeptides derived from *M. vaccae* that can function as adjuvants for CTL responses.[24] There are to date six known members of the TLR family of receptors which may recognize a family of cytokine-like ligands that participate in host immune responses.[36] Gram-negative LPS uses TRL-2 while the mycobacterial product lipoarabinomannan utilizes TLR-4 to initiate intracellular signals.[37] Other mycobacterial components, may also mediate cellular activation via TLRs. It is likely that proteins as well as lipid-containing molecules may be ligands for TLR as the Toll ligand in the fly is a protein. Further studies are underway to investigate the effect of *M. vaccae* and recombinant polypeptides from *M. vaccae* on antigen-presenting cells.

Mycobacterium vaccae also has the ability to elicit an immune response when immunization is via the intranasal route (Fig. 5) demonstrating that it can act at a mucosal surface. There are few mucosal adjuvants and none at present registered for human use. The cholera toxin B subunit can induce mucosal CTL immune responses[38] but is toxic. *Mycobacterium vaccae* is a potent immunomodulator, inducing strong Th1-type immune responses[39] even during an established allergic Th2 response, leading to the inhibition of IL-5 and immunoglobulin E (IgE) production.[40] It is a good inducer of IL-12 production,[10,24] a property which may contribute to its ability to induce both Th1 and TC1 immune responses. Thus, in addition to providing a new mucosal adjuvant, the observation that heat-killed *M. vaccae* can act at respiratory surfaces makes it a good candidate for the development of new treatments for asthma and allergic rhinitis.

There are a number of novel adjuvants that have the ability to induce Class I-restricted $CD8^+$ CTL responses after immunization with new generation vaccines such as proteins. However, some of these may be toxic or cause adverse reactions in humans. Heat-killed *M. vaccae* is safe, easy to produce and well-tolerated in humans.[41,42] Although killed *M. vaccae* was originally proposed for tuberculosis immunotherapy it may prove to be a more useful treatment for other diseases such as prostate cancer[42] and psoriasis[43] and for new generation vaccines intended to stimulate cytotoxic responses mediated by $CD8^+$ T cells.

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