A New Oil-Based Antigen Delivery Formulation for both Oral and Parenteral Vaccination

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Abstract: The ability of an oil-based carrier vehicle to act as an antigen delivery system *via* the oral and/or parenteral routes was investigated. The formulation consists of hydrophilic macromolecules (antigens) solubilised in oil phase, in the absence of water, by virtue of being wrapped in a sheath of phospholipid amphiphile. Results obtained demonstrate that the level of mucosal IgA antibodies detected in the stools of mice immunised orally with cholera toxin B fragment (CTB) or *E. coli* heat-labile toxin (LT) in oil is much higher than the level of IgA produced by mice immunised with CTB or LT alone. In addition, mice immunised orally with *Y. pestis* antigens (F1 and V) and CTB as immunostimulant in oil produce a significantly increased ($p<0.02$) systemic IgG response against both antigens (F1 and V) than mice orally immunised with F1, V and CTB without oil. Six out of ten mice immunised with F1 and V antigens in oil survived an aerosol challenge of 100 LD_{50} doses of virulent *Y. pestis*. Furthermore, animals immunised sub-cutaneously with the HIV antigen (HGP-30) in oil induced much stronger humoral and cellular responses against the antigen than mice immunised with the antigen alone. Taken together, these findings indicate that oil can be used successfully as an antigen delivery system in vaccine formulations without the necessity of an aqueous phase or an emulsification process. This greatly enhances the stability and ease of production of a formulation manufactured for commercial use.

INTRODUCTION

Oil-based emulsion formulations have had a long history of use as antigen carriers in the field of vaccines, the most well-known class of which are the Freund's adjuvants – both complete and incomplete. Although such adjuvants are highly effective inducers of immune responses, their use in humans is not recommended because of the occurrence of unacceptable local reactions, including granuloma formation. Multiple emulsions have been described [1] which do not give rise to such effects, but these formulations remain in liquid form, and are generally unstable, both by virtue of the need for antigen to be in aqueous phase, in close contact with surfactants for extended time periods, as well as the possibility of the two phases in the emulsion separating over time. Water-in-oil emulsions typically function as vaccine carriers by (i) acting as a slow-release depots for soluble antigen, and prolonging exposure to immune cells and (ii) providing a focus to attract phagocytic and other cells into a localised area where antigen and oil are ingested concomitantly, and where cell-cell interactions can take place. Such interactions include recruitment of inflammatory cells, and local tissue damage can occur as a result.

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Oils have also been used in oil-in-water liquid microemulsions, where the oil is usually employed as a carrier of immunostimulant. While these formulations disperse rapidly from the injection site, the association between oil droplet and antigen is not always very strong, and the same concerns regarding stability arise as for water-in-oil preparations.

Oil-based emulsions are particularly suited to subcutaneous delivery, and this route has been successfully used to deliver vaccines [2]. Oral immunisation with emulsions has also been shown to be effective [3-6] in animal studies, but none of these approaches has resulted in a commercial product for clinical use. Indeed, with notable exceptions e.g. the polio vaccine, immunisation *via* the oral route is a procedure yet to be employed widely in humans.

To overcome these problems, a new approach to formulating antigens in oil vehicles has been developed [7], in which the antigen is solubilised directly in the oil phase as a result of being wrapped up in an amphiphile sheath, creating a structure akin to a reverse micelle (see Fig. **1**). The advantage of this type of formulation is that the preparation is essentially anhydrous, with no free water being present, so that problems relating to protein denaturation and phase separation are minimal. Secondly, the oil, by virtue of the amphiphile contained (principally phospholipid) is self-emulsifying, so that after ingestion or injection it breaks

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down into small droplets which can readily disperse through the lymphatics, reducing the opportunity for adverse local reactions to be mounted. In contrast to conventional micro-emulsions, however, the antigen is still entrapped within the oil phase in these droplets, and is thus taken up and processed by phagocytic cells at the same time as the oil droplets are engulfed.

The ability to deliver vaccines by the oral route would be highly desirable and convenient, allowing non-invasive self-administration, and although much work has recently been directed towards this aim [8], this approach has been stymied by multiple difficulties. These have included damage to the vaccine in transit through the stomach and gut, rendering it non-efficacious, the induction of oral tolerance, and the requirement for multiple doses to establish immunity. If these difficulties could be overcome, oral immunisation has the added advantage of priming the mucosal immune system including the lungs and the respiratory, gastro-intestinal, rectal and genito-urinary tracts. The latter are all targets for various microbial pathogens.

In this paper, we have used antigens from two serious human pathogens HIV and *Yersinia pestis*, the plague-causing bacterium, which target the rectal and respiratory tracts, respectively. We describe the successful use of oily emulsions for immunisation with antigens from these pathogens by the sub-cutaneous and oral routes and we describe the efficacy of oral immunisation to protect mice against aerosolised *Y. pestis.*

MATERIAL AND METHODS

Animals

Female BALB/c strain albino mice (6-9 weeks old) were bought from B and K Ltd, Harlan, Charles River Laboratories, UK, or Taconic Farms NY. The animals were allowed to acclimatise to the animal housing unit before immunisation was started.

Antigens

CTB was purchased from Sigma. This material was prepared from whole cholera toxin (CT) by physical separation, but retains trace amounts of CT-A (less than 0.5%). *Escherichia coli* heat labile toxin (LT) was kindly donated by Intervet BLV. *Yersinia pestis* antigens (F1 and V) were prepared as previously described [9, 10]. Briefly, the F1 antigen was precipitated from the supernatant of *Y. pestis* grown at 37ºC by addition of 40% (w/v) ammonium sulphate and purified by repeated resuspension and centrifugation of the pellet in 20mM Tris-HCl at pH 8. The V antigen was produced as a recombinant fusion protein with glutathione-s-transferase in *Escherichia coli*, cleaved with factor Xa (Boehringer Manheim UK Ltd) for 18 hr at 22ºC and purified by affinity absorption. Recombinant HGP-30 was supplied by Cel-Sci Inc. Phosphate-buffered saline (PBS) used for dispersion of the antigen prior to lyophilisation was a standard physiological isotonic saline (100 mOsm) buffered with 0.05 M phosphate. Unless otherwise stated, all other standard chaemicals and reagents were purchased from Sigma UK Ltd.

Preparation of Oil Formulations

The amphiphile, soya phosphatidyl choline (Sigma) was dispersed in distilled water by probe sonication for 10 minutes with cooling at a concentration of 100 mg/ml. In the case of formulations containing HGP-30, phospholipid concentrations of 75 and 150 mg/ml were also employed. A volume of 100 µl of this solution was dispensed into glass screw-capped 2 ml vials. Subsequently, either 100 µg of CTB, 100 µg of LT, 200 µg of HGP-30, or 2500 µg of F1 and V protein antigens from *Yersinia pestis* were added to individual vials of the amphiphile with mixing. In the case of the *Y. pestis* antigens, the formulations were prepared with or without addition of 100 µg of CTB. No additional immunostimulants were added to any other of the antigens. The mixture was lyophilised overnight at 4°C under a vacuum of less than 1mbar, and to the dry residue 1 ml of different oils of approved for human use was added. Mineral oil, squalene

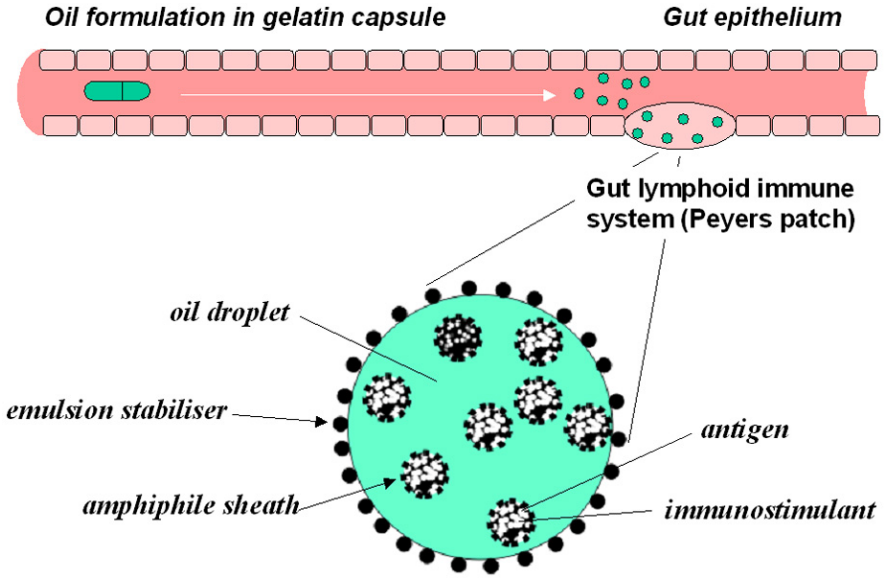

Fig. (1). Internal structure of oil droplet, after self-emulsification in the intestine.

and phytol were obtained from Sigma UK Ltd. The synthetic medium-chain triglycerides M818 (triglyceride of fractionated vegetable fatty acids C_8 and C_{10} and contains about 4-5% linoleic acid) and M840 (propylene glycol ester of saturated fractionated vegetable fatty acids C_8 and C_{10}) were purchased from Huls AG, Germany. The mixtures were left on a roller mixer at room temperature until crystal clear solutions were obtained. The formulations were stored at –20ºC until required for use.

Retention of Protein in Oil Dispersion

400 µl of PBS and 20 µl of oil formulation were added to each other in 2 ml glass vials. The samples were mixed vigorously on a vortex mixer until fully dispersed (thirty seconds) and then spun down in a centrifuge at 2000g for 10 minutes. The oil formed a small annulus within the meniscus at the top of each tube, giving direct access to the aqueous phase in the centre of the ring. 200 µl of the aqueous phase of each sample was transferred into a separate well of a microplate, and the protein concentration was measured using a Biochemika quantification kit based on binding to Coomassie Brilliant Blue (Sigma UK) Absence of interference due to scattering was confirmed by measuring OD at off-peak wavelength (492nm).

Immunisation

Groups of 6 mice were either dosed orally by gavage or subcutaneously immunised with their respective oil formulation or free antigen in PBS (+ 3% sodium bicarbonate to neutralise gastric acid in the case of oral immunisation). All formulations were prepared such that individual mice received either 10 µg of CTB, 10 µg of LT, 20 µg of HGP-30 or 250 µg of F1 and V proteins (with or without 10µg of CTB) where indicated, in a total volume of 100 µl per mouse. Groups of mice immunised with F1+V ± CTB, were boosted exactly as for the priming immunisation on days 10 and 31.

Collection of Stool Samples

Stools were collected 1 day before immunisation and at different times after the first, second or third immunisation. The stools collected from each group before and after immunisation were either pooled, or weighed individually and added to 5 ml of a solution of PBS 3% BSA, with 1mM phenylmethylsulfonyl fluoride (PMSF) a protease inhibitor. The samples were allowed to stand for 10 minutes at room temperature and then vortexed and incubated overnight at 4ºC. After incubation they were centrifuged for 4 minutes at 4,000 rpm and the supernatants were collected and analysed for antibody by ELISA. One gram of stools in 5 ml was considered as 1/5 dilution and the concentrations of the stool supernatants were normalised accordingly.

Collection and Analysis of Blood Samples

Blood samples were collected by tail vein puncture into 75 µl of heparinised capillary tubes. The samples were added to 1.425 ml of a 0.02% Tween 20 solution in PBS to give a final dilution of plasma in PBS of approximately 1/100. Samples were collected at different times after immunisation and stored immediately at -20ºC until required.

Detection of Antibodies Against F1 and V Antigens

The measurement of IgG titre was performed on individual separated serum samples by means of a modified ELISA [11], in which serum samples were diluted in duplicate in a two-fold dilution series on a microtitre plate (Titertek) which had been pre-coated with F1 or rV antigens (5 µg/ml in PBS), washed and incubated with 1% skimmed milk powder in PBS to remove non-specific binding. After washing, peroxidase-conjugated anti-mouse IgG (Sigma, Poole UK), and anti-mouse IgG1 or IgG2a (Sera Lab) were used as required at dilutions of 1:2000, prior to washing and developing the plate with azinobis (3-ethylbenzthiozolinesulfonic acid) (ABTS). The IgG titration was performed on individual serum samples, whilst the isotyping was carried out with a pooled sample representative of the treatment group. Antibody titre was estimated as the maximum dilution of serum giving an absorbance 414 nm reading 0.1 units over background and was presented as $\log_{10}$ antibody and from this, group mean titres ± standard error of mean (SEM) values were derived per treatment group.

Detection of Anti-CTB Antibodies

Antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Plates (COSTAR, 96 wells, flat bottom) were coated overnight at 4ºC with 5 µg/ml of CTB in carbonate buffer pH 9.6 (0.1 ml per well). The following day the plates were emptied and blocked for 1 hour at 37ºC by incubating the wells with a solution of 1% casein in buffer pH 9.6 (0.2 ml/well). The plates were then washed three times with PBS 0.05% Tween 20 in a Dynatech manual plate washer. After washing, stool or serum samples were dispensed in duplicates into individual wells of the plates and diluted in doubling dilution starting from 1/100 (serum) or 1/5 (stools). The samples were washed again and incubated with goat anti-mouse IgG (1/4000) or goat anti-mouse IgA (1/8000) antibodies (Sigma, UK) for 3 hours at 37ºC. After another sequence of washing, rabbit anti goat IgG conjugated with alkaline phosphatase (1/2000) from Sigma was added to the plates (100µl/well) and incubated for 90 minutes at 37ºC. The plates were washed once more, and then the enzymatic reaction was developed with 5 mg/ml of *p*-nitrophenyl phosphate in diethanolamine buffer (0.1 ml/well). The optical density was read at 405 nm in a Titertek plate reader after 15 and 30 minutes of incubation at room temperature.

ELISA data for IgG anti- CTB activity were analysed using a parallel line assay method. Briefly, in this method the background activity is subtracted from the reference and test samples. This value was plotted against the logarithm of the serum sample dilutions and a least square regression line plotted through the points on the linear part of the curve. For each sample in turn a combined "pooled" line is then derived using the regression line for the reference sample on the sample plate. Lines are plotted through the reference and test dilutions using the pooled regression equation and the difference in the X-axis intercept of these is used to calculate the potency of the test serum relative to the reference. The process was conducted automatically using a computer linked directly to the plate reader. The reference serum (A) was obtained from mice immunised orally with 10 µg of CTB.

After a previous replicate analysis that showed a mean X-axis intercept at 3744 for IgG1 and IgG. The ELISA calculation programme not only gave the activity but also indicated whether the comparison of the ELISA units of the samples to the ELISA units of the reference serum was significant by performing a t-test on the two slopes. The test slope was discarded where the slope was too shallow or comparability was less than 0.05.

The activity of IgA antibodies against CTB and LT and IgG1 antibodies against CTB in the stools could not be calculated accurately by this process, therefore, IgA antibodies against CTB and LT and IgG1 antibodies against CTB detected in the stools were expressed as optical density at (1/10) dilution.

DTH Assay with HGP-30

Animals previously immunised with HGP-30, either alone or in oil, were anaesthetised with Metofane™ and 25 µl of a saline solution as a control (right ear) or containing 5 µg of HGP-30 (left ear) was administered. Then at 24 hours the swelling in the ears was measured with a digital readout micrometer (Mitoya, sensitivity to 0.001 mm). The percent increase of control and experimental ears was calculated and a group mean determined.

Statistical Analysis

A Student's t-test was applied to determine the significance of the difference between treatment group means.

RESULTS

Humoral and Cellular Response of Mice Immunised Subcutaneously with HGP-30 in Oil

To investigate the influence of oil on humoral and cellular responses against the HIV antigen HGP-30 after parenteral immunisation, mice were immunised three times with HGP-30 either free or incorporated in oil. Briefly, mice were injected subcutaneously three times with 5 µg of HGP-30 either free in saline or incorporated in the synthetic triglyceride Miglyol 818 (M818) without the presence of any immunostimulant. Fourteen days after the last immunisation the level of IgG1, IgG2a, IgG2b, IgG3 and IgA in the blood was measured by ELISA. In addition, fourteen days after the second immunisation the change in ear thickness was measured, after topical administration of free antigen, to determine the T cell response to the antigen.

The results obtained showed that mice immunised with HGP-30 incorporated in M818 produced a much better antibody response against the antigen for all immunoglobulin sub-classes (Fig. **2a**) compared with HGP-alone, which gave levels no higher than background. In addition, the delayed hypersensitivity response against the antigen (Fig. **2b**) was also higher in the groups immunised with HGP-30 in M818, regardless of the concentration of the phospholipid in the M818.

IgA Response Against LT of Mice Immunised with Different Oils

To investigate the influence of formulation in oil on oral administration of LT, mice were immunised orally on two occasions with antigen either in aqueous solution or incorporated in oil. Several different oils were employed for comparison, namely mineral oil (MO), squalene (SQ), phytol (PH), Miglyol M818 (M818), and Miglyol M840 (M840). These oils were chosen because they are all oils which are of biological origin, and/or are commonly used in pharmaceutical practice as vehicles for drugs, vitamins etc. The formulation method used was identical in each case, except that a different oil was added at the final stage to solubilise the lyophilised residue. Mice were gavaged twice with 10 µg of LT either free or incorporated in oil. The second dose was administered twenty one days after priming. Stool samples were collected prior to and at different times after immunisation. The samples were then pooled and processed for analysis by ELISA for determination of the presence of IgA antibodies against LT. The results show that different oils differ

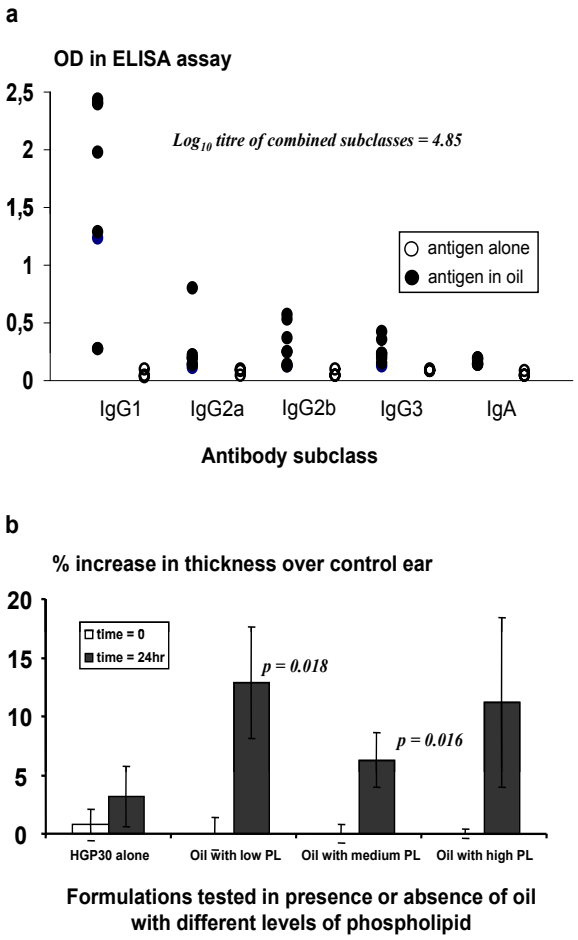

Fig. (2). Influence of oil on the humoral and cellular response against HGP-30 antigen after subcutaneous immunisation. Mice (seven per group) were subcutaneously immunised three times on days 1, 14 and 49 with 20 µg of HGP-30 either free in PBS or incorporated in M818 without the presence of any immunostimulant. (**a**) Fourteen days after the last immunisation the level of IgG1, IgG2a, IgG2b, IgG3 and IgA in the blood was measured by ELISA. (**b**) Fourteen days after the second immunisation the percentage of ear thickness change was measured to determine the T cell response to the antigen. Points in chart (a) represent values from individual animals.

in their ability to generate strong IgA responses, and the mineral oil and M818 are particularly effective (Fig. **3a**). In particular, oral priming with LT in mineral oil induces a much better response than antigen alone at three weeks, and this difference is maintained after boosting (Fig. **3b**).

Although the efficiency of incorporation of antigen in the oil is 100%, there is the possibility that antigen may be lost during the process of emulsification of the oil in the stomach and intestine, with the antigen leaking out of the oil droplets into the surrounding aqueous medium. In light of the marked differences in efficacy between different oils tested, a study was conducted to determine the retention of encapsulated material within the droplets formed after these self-emulsifying oils were dispersed in aqueous media, in order to determine whether there was a correlation between retention and strength of immune response. Two proteins were tested as described in the Materials and Methods section, and with the exception of squalene, the percentage retention of material in each of the oils is very similar for the two proteins. Leakage from the oils ranged between 5 and 60%, and there is little evidence that high efficacy in generation of an immune response is related to high retention, since the mineral oil, which induces the highest IgA levels had a low retention of both proteins after dispersion (Fig. **4**).

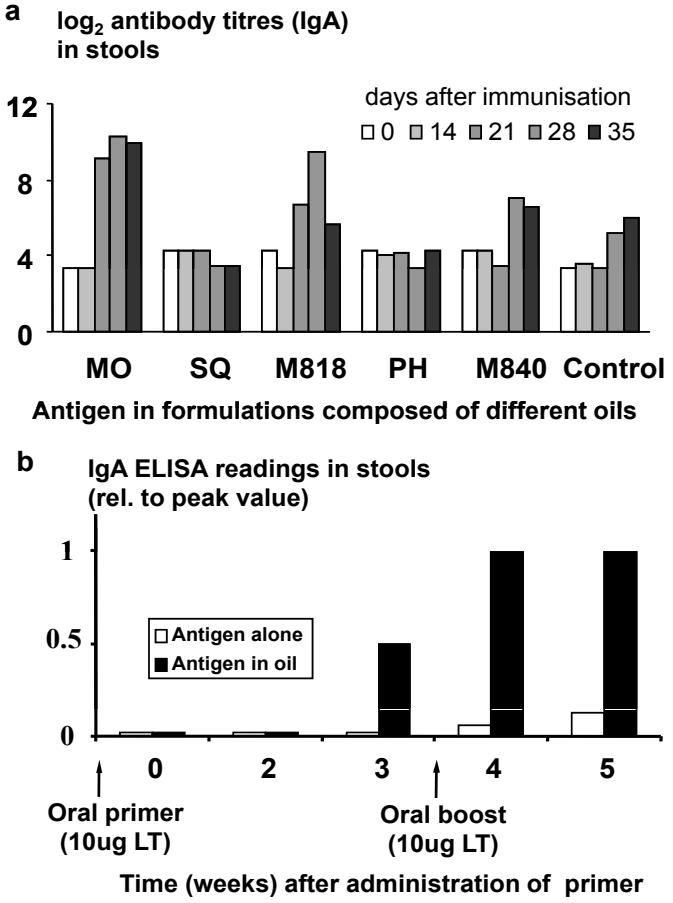

Fig. (3). IgA response of mice orally immunised with LT incorporated in different oils (see text). Mice were gavage twice with 10μg of LT either free in saline (control) or incorporated in different oils. Stool samples were collected prior to immunisation and at different times after the first and second gavage. (**a**) shows comparison between different oils, and (**b**) shows the time course for mineral oil.

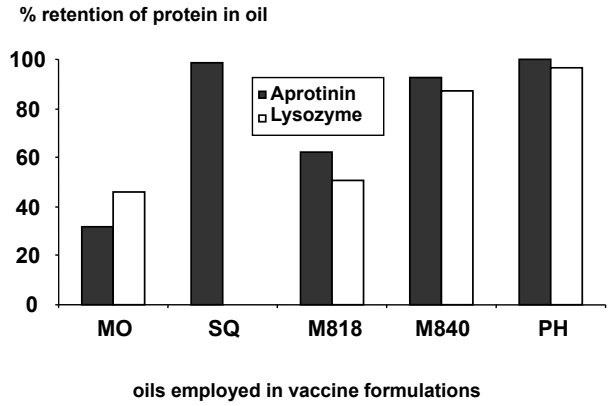

Fig. (4). Retention of proteins in oils (see text) after dispersion in aqueous media. 20 μl of different oils, into which either aprotinin or lysozyme had been incorporated, were dispersed vigorously in PBS for 30 seconds, then separated by centrifugation, in order to measure the quantity of protein leaking from each oil into the aqueous phase by the Coomassie Blue binding assay.

Influence of Oral Priming on Subsequent Parenteral Challenge

To investigate the influence of oral priming with CTB before subcutaneous challenge, mice were orally immunised with CTB (with or without oil) before subcutaneous challenge with free CTB. Two groups of six mice each were primed orally by gavage with 10 μg of CTB either in oil or in 0.1 ml of PBS + 3% sodium bicarbonate. Sixty days after priming, a second gavage with a dose identical to that received in the priming dose was given. Two weeks after the second gavage, the mice received a subcutaneous challenge of 3 μg/0.1 ml of CTB in PBS. A control group received subcutaneously 3 μg of CTB in 0.1 ml of PBS without oral priming. Blood and stool samples were collected one day before and 2 weeks after the subcutaneous challenge. The samples were analysed for IgG1 and IgA antibodies against CTB by ELISA.

The results showed that oral priming with 10 μg/0.1 ml of CTB in oil produced a much better IgA response in the mucosa than did oral priming with CTB in PBS + 3% sodium bicarbonate (Fig. **5a**). The mucosal IgA response obtained with oral priming with 10 μg/0.1 ml of CTB either in PBS/bicarbonate or oil was not affected by subcutaneous challenge. However, priming of CTB in oil induced a much better IgG1 response in the blood after subcutaneous challenge than priming with free CTB in PBS+ 3% sodium bicarbonate ($p \leq 0.015$) (Fig. **5b**). This experiment was repeated on two subsequent occasions and similar observations were made.

IgG Response Against F1 and V Antigens of Mice Immunised with Oil

In order to determine whether an oil-based vehicle could be used for oral delivery of the protein antigens F1+V with or without the immunostimulant CTB, groups of 10 mice were dosed by oral gavage with 25 μg of each antigen ± 10 μg CTB, in 0.1 ml volume. The control groups received orally 25 μg of F1 and V antigens (with or without CTB) in PBS or only 10 μg of CTB in PBS. Groups of animals were

boosted by oral gavage with exactly the same formulations as for the primary immunisation at days 10 and 31. Twenty-one days after the boost at day 52, blood samples were collected by tail vein puncture from 10 animals per group, allowed to clot and the sera collected and individually assayed for IgG antibodies against F1 and V antigens by ELISA.

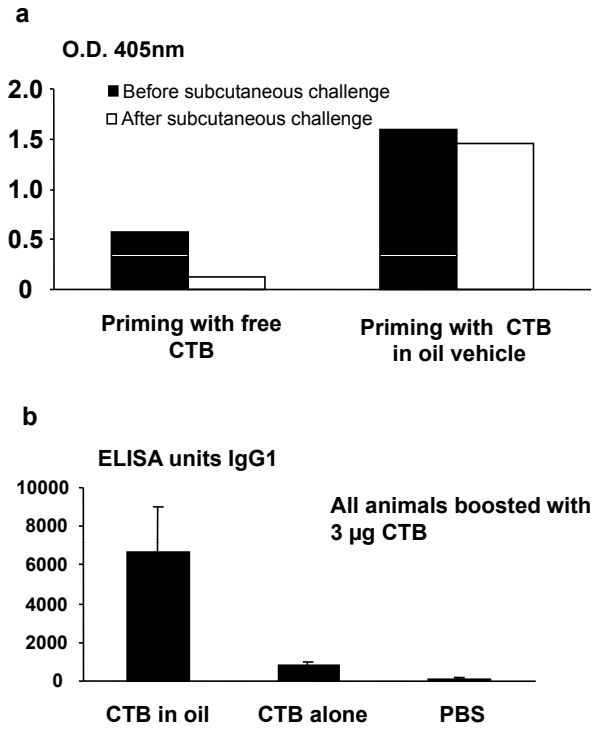

Fig. (5). Influence of oral priming with 10 µg of native derived CTB (either in PBS + 3% sodium bicarbonate or in oil) before subcutaneous challenge with native derived CTB. (**a**) IgA antibody response in the stool and (**b**) IgG1 in the blood. Error bars indicate Standard deviation. For the stool results, error bars could not be plotted because the samples of each group were pooled.

The results showed that immunisation with F1 + V antigens incorporated in oil in the presence of CTB induced a significantly increased serum IgG response ($p<0.02$)against the V antigen compared to the groups immunised with free molecules (Fig. **6a**). Serum antibody titres to F1 were also high at day 31, although in this case the use of the oil vehicle to deliver F1+V+CTB did not significantly enhance titres over those observed for animals receiving F1+V+CTB without oil (Fig. **6b**). This is explained in the context of previous data [12] where the anti-F1 response in the mouse develops more rapidly than the anti-V response. Therefore any enhancement of titre is likely to significantly impact the anti-V response rather than the anti-F1 response.

Protection Against Aerosol Challenge of Virulent *Y. pestis* in Mice Immunised Orally with F1 and V Antigens in Oil

On day 91, 39 days after completion of the immunisation schedule, animals were exposed to an aerosol challenge with virulent *Y. pestis* in order to determine whether the immune responses generated by the oil-based formulations of F1+V ± CTB were protective. Animals were challenged with an aerosol dose of 10^4 CFU (Colony Formation Units) of virulent *Y. pestis*. This level of challenge represents 100 median lethal doses (MLD) in the mouse model, as previously described [13]. Mice were contained in an isolator within Advisory Committee on Dangerous Pathogens (ACDP) Category 3 containment guidelines throughout the challenge event and for fourteen days thereafter. The animals were challenged in a retaining device allowing nasal-only exposure system for a ten-minute period to an aerosolised stream of *Y. pestis* strain GB. The inhaled dose per mouse was estimated by comparison of the number of colony forming units delivered in the aerosol stream with the number of CFU recorded on impingers appropriately placed within the exposure equipment, as described [13].

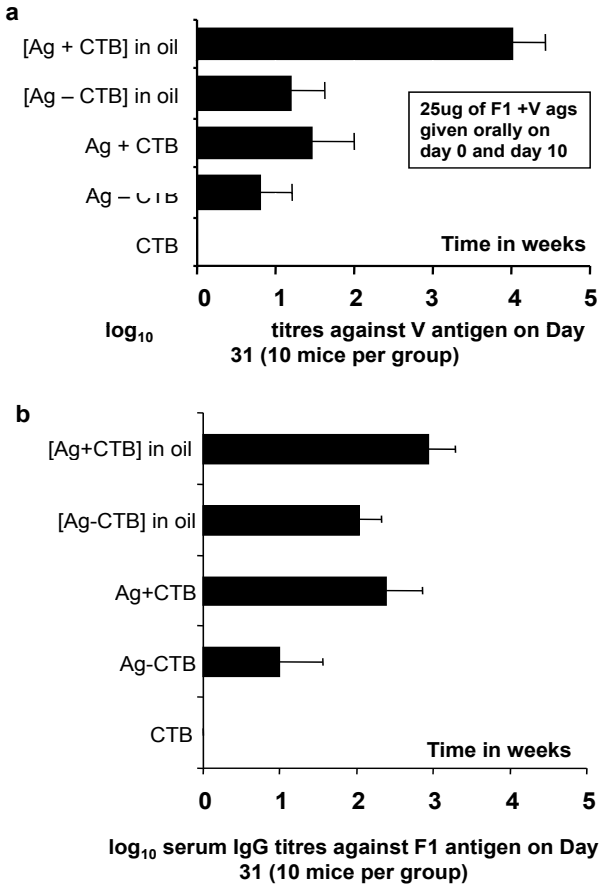

Fig. (6). Systemic IgG antibody response against F1 and Vag antigens of mice orally immunised with oil. BALB/c female mice (9 weeks old) were gavaged twice with either 25 µg of (**a**) F1 and (**b**) V antigens with or without 10 µg of CTB in M818 or in PBS. Twenty-one days after the second gavage, blood samples were collected by tail vein puncture and individually analysed for the presence of IgG antibodies against F1 and V by ELISA. Error bars indicate standard errors of the mean.

Following exposure, animals were returned to their cages and were observed over the subsequent fourteen days for the development of symptoms. In this period, any animal displaying a collection of symptoms pre-determined to represent humane end-points, was promptly euthanased. Time-to-

death was carefully recorded. Animals completing the fourteen-day observation period without the development of symptoms, were recorded as survivors and were humanely killed prior to autopsy and the major organs (spleen, liver, lungs) were examined for gross morphological changes.

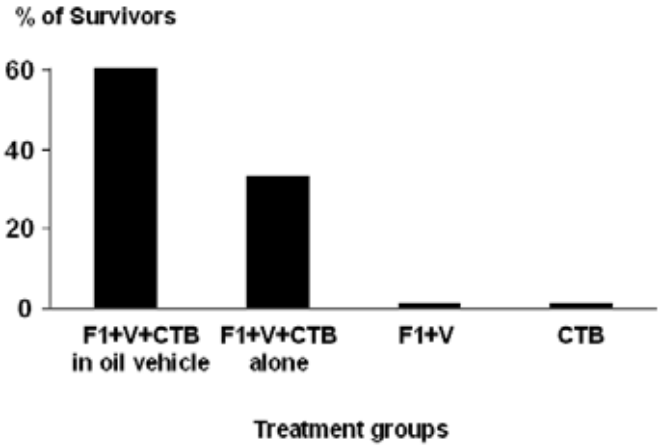

Fig. (7). Protection against aerosol challenge of virulent *Y. pestis* in mice immunised orally with F1 and V antigens in oil. BALB/c female mice (9 weeks old) were gavaged three times with 25 μg of F1 and V antigens with or without 10μg of CTB in M818 or in PBS.

The results show that 6 of 10 mice immunised with F1 and V antigens + CTB in oil survived a 100 MLD challenge with virulent *Y. pestis* (Fig. **7**). The only other group which showed some protection was the F1 + V + CTB group, in which 3 of 10 survived. Of the surviving mice in the group immunised with F1 + V + CTB in oil which were autopsied, no gross morphological changes were noted in the major organs examined.

DISCUSSION

The ability of the oil-based formulations employed here to enhance markedly the immune response to parenterally administered antigen is demonstrated in the first experiment reported with HGP-30. When administered s.c. as a solution in buffer, no response was seen, while administration in oil (medium-chain triglyceride) with no added immunostimulant, generated a strong, rapid and principally antibody-mediated response, although a small but measurable T-cell-mediated DTH response was also obtained, as evidenced by a contact sensitivity assay. Comparison with experiments conducted at the same time indicates that stronger and more rapid responses were elicited with the oil-based formulation than with the antigen conjugated co-administered with alum, or in liposomes (personal communication – data not shown).

The observation that antigen in an oil-based vehicle gives rise to a strong immune response even in the absence of other carrier proteins or immunostimulants, suggests that administration of this formulation *via* routes which have traditionally been anergic may induce an immune response and could be particularly advantageous in situations where the integrity of other carriers may be prejudiced. One such route is oral, where the objective is to access the Peyer's patches *via* the intestinal tract.

In initial experiments to investigate the efficacy of orally-administered materials in eliciting immune responses, antigens were chosen for which immunity, if generated, could be physiologically relevant in terms of protection against an infectious disease. For the first experiments the antigen employed was whole *E. coli* heat-labile toxin (LT) containing both A and B subunits. Antigen-specific IgA was detected in the stools of mice three weeks after receiving LT *via* the oral route in oil, but not when the antigen was administered in free form. IgA generated in response to LT alone was seen after a booster was given at the end of week 3, but the levels were still much lower than those obtained with the oil formulation. This is all the more remarkable since LT is already known to be a powerful mucosal adjuvant when administered orally, and may be considered to be an antigen which incorporates its own immunostimulant. The observation that incorporation in the oil enhances the anti-LT response demonstrates that the oil does not interfere with the mechanisms coming into play when LT acts on immune cells in the gut, and may also have a role in protecting it during its passage along the gastro-intestinal tract.

That this is not the only effect exerted by the oil, however, is indicated by experiments comparing the efficacy of different oils in generating the immune response, where there is little correlation to be seen between antibody levels induced and antigen retention within dispersed oil droplets, suggesting the possibility that the oil might itself be exerting an immunostimulatory effect on immune cells. This is particularly the case with mineral oil and the medium chain triglyceride Miglyol M818. The findings here are in accord with recent reports demonstrating that, when administered parenterally, antigens contained in water-in-oil emulsions generate the strongest responses when the oil is a non-metabolisable one, such as mineral oil [14].

Similar results have been observed using cholera toxin B fragment (CTB; data not shown). CTB was also employed to investigate whether oral administration could induce a serum antibody response in the blood stream. A single dose of CTB antigen, either free or in oil, produced no discernible change in antibody levels in the bloodstream. A subsequent challenge with sub-cutaneously administered antigen generated antibodies, whose titre was markedly higher in the group receiving antigen in oil, compared with antigen alone. This demonstrates that exposure to CTB is probably producing memory cells located in a site which has access to antigen regardless of the route of administration. Upon introduction of antigen parenterally, these memory cells are recruited to mount a response in the bloodstream, rather than their sphere of activity being limited solely to the gut mucosa. The inclusion of CTB in these formulations has clearly enhanced the antibody responses achieved. CTB is a potent mucosal adjuvant, through its ability to bind mucosal antigen presenting cells [15-17] and thus to enhance antigen presentation by macrophages. The CTB used in this study contains traces of CTA, and the resultant small amount of holotoxin may confer adjuvanticity through a sequence of events involving GM_1 receptor binding of macrophages, followed by ADP-ribosylation and increased intracellular cAMP [18]. The combination of CTB and oil has been demonstrated here to be a potent oral delivery system.

The ability of the oil formulation to enhance the generation of antibody levels in the bloodstream after oral immunisation was further demonstrated using antigens F1 and V from *Yersinia pestis*, in combination with CTB employed as

an immunostimulant. Co-incorporation of antigen and immunostimulant in the carrier significantly increased the serum IgG titres compared with proteins administered free in the absence of oil. Subsequently, animals immunised with 3 oral doses of the oil-based formulation of F1+V+CTB, were 60% protected against a stringent aerosol challenge with virulent *Y. pestis*. This is the first time to our knowledge that an oral formulation of F1+V has been demonstrated to confer some protection against multiple lethal doses of aerosolised plague bacteria in the mouse model.

The ability of the oil formulation to enhance the generation of antibody levels in the bloodstream after oral immunisation was further demonstrated using antigens F1 and V from *Yersinia pestis*, in combination with CTB employed as an immunostimulant. Co-incorporation of antigen and immunostimulant in the carrier significantly increased the serum IgG titres compared with proteins administered free in the absence of oil. Subsequently, animals immunised with 3 oral doses of the oil-based formulation of F1+V+CTB, were 60% protected against a stringent aerosol challenge with virulent *Y. pestis*. Whilst we have previously demonstrated that salmonella-delivered F1 antigen [19, 20] or Salmonella-delivered V antigen [21] or salmonella-delivered F1-V [22] was highly protective in the mouse model of bubonic plague, there have been fewer reports of immunogenic [23] non-living oral formulations of F1+V being protective, particularly against multiple lethal doses of aerosolised plague bacteria in the mouse model, as demonstrated here.

In this work, CTB has been employed to demonstrate how incorporation of agents into the novel oil formulation described here can improve their immunogenicity, even when they can act as adjuvants in their own right. CTB was also used in this study as an adjuvant for the plague antigens. While concern has been raised about the use of the whole Cholera holotoxin (CTA and CTB) as an adjuvant on safety grounds, many clinical trials of oral cholera vaccines (both whole and subunit) have already been conducted successfully. Studies in humans employing CTB and LTB as adjuvants in nasal formulations have also been carried out [24, 25], and Non-toxic variants of these adjuvants are currently being developed [26, 27] which should be perfectly acceptable for administration to humans, especially *via* the oral route. It should be recognised, however, that the flexibility of the formulation process means that a wide range of other agents can be incorporated into the oil – including CpG constructs, MPL, chitosan particles, bacterial fragments and whole viruses etc – which can also contribute adjuvanticity to the whole preparation.

CONCLUSIONS

In this paper, the ability of oil-based formulation to enhance immune responses (particularly humoral) to antigens has been clearly demonstrated. In the case of the formulation of the particular composition employed here, this is brought about because of (1) the stimulatory effect on macrophages of engulfment of oil droplets, (2) targeting of antigen within oil droplets to phagocytic cells and (3) the ability of the formulation to co-present antigen and immunostimulant with the same droplet to the same cell. The formulations are efficacious *via* parenteral routes even without co-administration of additional immunostimulant, and have the advantage over current oil formulations in that their self-dispersing properties allow them to diffuse rapidly from the site of injection, thus avoiding the possibility of inciting local tissue reactions. The same formulations are also effective when administered to the gastro-intestinal tract, presumably targeting antigen to the Peyer's patches.

Various multiple emulsion formulations are currently being tested in humans [28-34], using new oil adjuvants such as Montanide ISA [35]. Multiple emulsions, if prepared fresh, can be very strong candidates for new vaccines administered parenterally by injection. Such preparations are not so suitable for oral administration, however, since passage through the human stomach can damage them easily. In order to avoid the stomach, the formulations will need to be encapsulated, and the presence of significant quantities of water in the emulsions may introduce insurmountable incompatibilities, leading to very short half-lives, even with soft-gelatin capsules. The formulation approach described here [36], where no internal aqueous compartment is required, overcomes these problems, and provides, with its high reproducibility and efficiency of incorporation, a very promising solution to administration of oil-based vaccines *via* the oral route.

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ABBREVIATIONS

BSA	=	Bovine serum albumin
CFU	=	Colony-forming units
CTB	=	Cholera toxin B fragment
DTH	=	Delayed-type hypersensitivity
ELISA	=	Enzyme-linked immunosorbent assay
LD	=	Lethal dose
LT	=	Heat-labile toxin from *E. coli*
M818	=	Miglyol 818
M840	=	Miglyol 840
MO	=	Mineral oil
PBS	=	Phosphate-buffered saline
PH	=	Phytol
PMSF	=	Phenyl methyl sulphonyl fluoride
SEM	=	Standard error of the mean
SQ	=	Squalene

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