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Studies on the Co-encapsulation, Release and Integrity of Two Subunit Antigens: rV and rF1 from *Yersinia pestis*

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Abstract

In the development of combination or multiple sub-unit vaccines, determination of the encapsulation, release and integrity of two or more proteins co-encapsulated within microspheres is an important issue. A new extraction method, which exhibits excellent protein recovery, has been developed which enables samples to be used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent measurement of individual antigens encapsulated within microspheres. Using the new method, the protein loading of poly-(L-lactide) microspheres co-encapsulating two plague sub-unit antigens was found to be 1·22% (w/w) for recombinant V antigen (rV) and 1·24% (w/w) for recombinant F1 (rF1) by SDS-PAGE. The total protein loading was 2·49% (w/w) by bicinchoninic acid assay. The individual release of the two subunit antigens from the co-encapsulated microspheres was determined by SDS-PAGE analysis and rF1 was found to have a higher burst release than rV. The integrity and immunological activity of both rF1 and rV antigens was shown to be unaffected by the microencapsulation process.

This study shows that encapsulation of more than one antigen within poly-(L-lactide) microspheres is a viable method for the delivery of intact proteins.

Current immunization procedures for plague involve the parenteral administration of killed whole-cell preparations such as the Cutter USP Vaccine. Despite their widespread use, killed whole-cell vaccines for plague have an unsatisfactory incidence of transient local and systemic side effects (Perry & Fetherston 1997). Moreover, it is apparent that killed whole-cell vaccines are unable to induce appropriate immunity to counteract the pneumonic form of the disease, which is transmissible via airborne droplets (Meyer 1970). The development of vaccines which can induce protective levels of immunity against both bubonic and pneumonic plague is therefore a prime goal.

Fraction 1 (F1) is the purified sub-unit from the protein–polysaccharide complex of the capsule of *Yersinia pestis* (Baker et al 1952) with a molecular mass of 17·5 kDa (Bennett & Tornabene 1974) and an isoelectric point, pI = 4·1 (Friedlander et al 1995). The F1 sub-unit monomer contains 149 amino acids (Zav'yalov et al 1995) of which 45, 20 and 8 mol% are hydrophobic, acid and basic amino acids, respectively (Vorontsov et al 1990). Recombinant F1 (rF1) has been reported to have a molecular mass of 16 kDa (Titball et al 1997). F1 is believed to confer resistance to phagocytosis, possibly by forming aqueous pores in the membranes of phagocytic cells (Rodrigues et al 1992) or by interfering with complement-mediated opsonization (Williams et al 1972).

V antigen is a secreted protein which acts as a regulator of the low calcium response and may also act as a virulence factor (Skrzypek & Stanley 1995) that reduces local expression of the host cytokines, tumour necrosis factor α and γ interferon, in response to *Yersinia* infection (Nakajima et al 1995). Native V antigen has a molecular mass of 37 kDa (Brubaker et al 1987) and recombinant V antigen (rV) has a molecular mass of 36 kDa (Leary et al 1995).

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Both F1 and rV antigens are protective when injected with Freund's incomplete adjuvant (Williamson et al 1995). Protective efficacy is enhanced when F1 and rV are administered in combination showing a synergistic effect (Williamson et al 1995, 1996). Thus development of a combined sub-unit vaccine is a prime objective. Micro-encapsulation of the *Y. pestis* sub-units confers an adjuvant effect, thus abrogating the requirement for aluminium based adjuvants, and also facilitates mucosal immunization (Eyles et al 1998a, b). In light of the rationales for a microsphere-encapsulated combined rF1 and rV vaccine, co-micro-encapsulation of the recombinant antigens is an attractive approach.

Quantification of the antigen loading, immunological integrity and release profile of individual proteins in co-encapsulated systems is important in the development of combination or multiple sub-unit vaccines and is often overlooked. Current methods, bicinchoninic acid assay (Singh et al 1997) or amino acid analysis (Sharif & O'Hagan 1995), are used to determine small quantities of total protein concentrations in single encapsulated or co-encapsulated microspheres. High-performance liquid chromatography has also been used to study the integrity and release of proteins (Cleland et al 1997), although this method requires longer development times. Previously, radiolabelled protein has been employed to determine individual protein release from co-encapsulated microspheres (Conway & Alpar 1996a) but this method is not always feasible. In this study, the amount of individual protein encapsulated in and released from co-encapsulated poly-(L-lactide) microspheres has been determined by a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) assay (sensitive to 100 ng of each antigen). Detection was enhanced by using a combination of Coomassie Brilliant Blue and silver stain for the visualization of gel protein bands. To measure the amount of individual antigens encapsulated in microspheres, a new extraction method has been developed where the resulting extracts can be directly loaded onto PAGE gels. The integrity of both rF1 and rV antigens in the release medium and after co-encapsulation has also been studied.

Materials and Methods

Preparation of sub-units

The V antigen was expressed as a fusion protein with glutathione-S-transferase (GST) in *Escherichia coli*. The V protein was cleaved from the fusion with Factor Xa (Boehringer Mannheim U.K. Ltd) and rV protein was purified by affinity adsorption, as previously described (Leary et al 1995). rF1 was expressed in *E. coli*, harvested by ammonium sulphate precipitation and purified by fast protein liquid chromatography, as previously described (Titball et al 1997).

Microencapsulation of sub-units

Poly-(L-lactide) of molecular weight 2000 Da (resomer L104; Alpha Chemicals, Preston, UK) was used to encapsulate either rF1 (F2K) or rV (V2K) antigens, or co-encapsulate both antigens (FV), by a modification of a double emulsion solvent evaporation method (Conway & Alpar 1996b). Briefly, lyophilized antigens (3·5 mg rV (V2K), 9·6 mg rF1 (F2K), 2·5 mg rV, and 2·5 mg rF1 (FV)) were dissolved in 1·25 mL aqueous solution of 7·5% (w/v) polyvinyl alcohol (PVA; 88% hydrolysed, 13–23 kDa) and 0·25% (w/v) methylcellulose (2% solution 400 counts s^{-1}) and added into 5 mL dichloromethane containing 250 mg of polymer. The mixture was emulsified using a homogenizer (Silverson Machines, UK) for 4 min. The resultant emulsion was then added dropwise into 1·5% (w/v) PVA (150 mL) and homogenized for 8 min. The resultant w/o/w emulsion was gently stirred until the dichloromethane had evaporated. The particles were collected by centrifugation (100 000 *g*, 25 min), washed three times with distilled water and freeze-dried.

SDS-PAGE

For SDS-PAGE of standard, released and extracted proteins, 20 μL sample and 20 μL loading buffer (16·4% (v/v) 0·5 M Tris(hydroxymethyl)–aminomethane (Tris)–HCl pH 6·8, 16·4% (v/v) glycerol, 3·2% (w/v) sodium dodecyl sulphate (SDS), 1·6% (v/v) 2-β-mercaptoethanol, 0·001% (w/v) bromophenol blue) were vortexed, heated to 100°C for 30 min, centrifuged and 20 μL loaded onto discontinuous SDS-PAGE gels (15% (w/v) separating gel, 5% (w/v) stack gel). Electrophoresis of samples was performed at constant voltage (60 V) in a Tris/glycine/SDS buffer for 200 min. For non-denatured (native) PAGE of proteins, 40 μL sample and 10 μL ($\times$ 5) loading buffer (3·1% (v/v) 1 M Tris–HCl pH 6·8, 50% (v/v) glycerol, 0·05% (w/v) bromophenol blue) were vortexed, centrifuged and 25 μL loaded onto discontinuous native-PAGE gels (12% (w/v) separating gel, 5% (w/v) stack gel). Electrophoresis of samples was performed at constant voltage (60 V) in a Tris/glycine buffer for 200 min. Protein bands on PAGE

gels were visualized with either silver staining (Silver Stain Plus Kit, Bio-Rad Laboratories Ltd, Herts, UK) or Coomassie Brilliant Blue R-250 or a combination of both stains using a modification of the method described by De Moreno et al (1985). The optical density of bands on PAGE gels was measured by scanning densitometry (UVP Gel Scanner, UVP Ltd, Cambridge, UK) and calibrations were determined from known protein standards on each gel.

Western blotting
SDS-PAGE gels were transferred onto nitrocellulose (Bio-Rad Laboratories Ltd, Herts, UK) in ice-cold transfer buffer (Tris 0·3% (w/v), glycine 1·44% (w/v), methanol 20% (v/v)) for 1 h at constant voltage (100 V). The nitrocellulose was blocked for 1 h with Tris-buffered saline (pH 7·4) containing 3% (w/v) bovine serum albumin (BSA) and washed three times with Tris-buffered saline containing 0·05% (v/v) Tween 20. The nitrocellulose was probed with 5 mL mouse anti-V or anti-F1 monoclonal antibody (1 in 50) in Tris-buffered saline for 1 h and washed three times with Tris-buffered saline containing 0·05% (v/v) Tween 20. The nitrocellulose was coated with 5 mL anti-mouse peroxidase conjugated antibody (1 in 1000) in Tris-buffered saline for 1 h and washed three times with Tris-buffered saline containing 0·05% (v/v) Tween 20. Finally, the bands on the nitrocellulose were developed with 3,3′-diaminobenzidine tetrahydrochloride.

Antigen loading
Three different methods were used for recovery of antigens from V2K and F2K microspheres (performed in triplicate). For method 1, microspheres (approx. 2 mg) were digested in 1 mL 0·1 M sodium hydroxide (NaOH) containing 5% (w/v) SDS at 37°C until the medium lost all turbidity. The solution was neutralized to pH 7 with 1 M HCl. For method 2, microspheres (approx. 2 mg) were dissolved in 0·3 mL dichloromethane and were vortexed, centrifuged and extracted three times with phosphate buffered saline (PBS; pH 7·4) containing 5% (w/v) SDS ($2 \times 0{\cdot}3$ mL + $1 \times 0{\cdot}4$ mL). The PBS extractions were pooled. For method 3, microspheres (approx. 2 mg) were dissolved in 0·3 mL dichloromethane and 0·7 mL hexane, vortexed, centrifuged and extracted three times with PBS containing 5% (w/v) SDS ($2 \times 0{\cdot}3$ mL + $1 \times 0{\cdot}4$ mL). The PBS extractions were pooled. Antigen loadings for V2K and F2K microspheres by all three recovery methods were determined using the bicinchoninic acid assay procedure (Smith et al 1985) with appropriate antigen standards treated in the same manner as recovery samples. rV and rF1 antigens were recovered from FV microspheres with method 3 and quantified by SDS-PAGE 15% (w/v) and the bicinchoninic acid assay procedure (50% rV and 50% rF1 were used as standards for total protein concentrations).

Antigen integrity
rV and rF1 antigens were incubated at 37°C in PBS for 14 days and analysed at different time intervals by SDS-PAGE 15% (w/v), native PAGE (12% (w/v), rV antigen only) and indirect enzyme-linked immunosorbent assay (ELISA).

Indirect ELISA
Indirect ELISA was performed on the antigen integrity samples. Microtitre plates (96 well, Dynatech) were coated with each sample (200 μL) and left overnight at 4°C. Plates were washed once with PBS containing 0·02% (v/v) Tween 20 before addition of 4% (w/v) BSA in PBS containing 0·02% (v/v) Tween 20. After incubation for 1 h at 37°C, plates were washed once with PBS containing 0·02% (v/v) Tween 20. Plates were probed with mouse anti-V or anti-F1 monoclonal antibody (1 in 60) in PBS for 1 h at 37°C and washed once with PBS containing 0·02% (v/v) Tween 20. The signal from bound monoclonal was quantified by addition of anti-mouse peroxidase conjugated antibody (1 in 1000) in PBS for 1 h at 37°C, washing three times with PBS containing 0·02% (v/v) Tween 20, and colour development with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid. Absorbance at 405 nm was determined by spectrometry.

In-vitro release studies
Release of rV and rF1 antigens from FV microspheres was determined by incubation of spheres (approx. 10 mg) in 1 mL PBS/sodium azide 0·01% (w/v) in a shaking water bath at 37°C. At periodic intervals, the suspension was centrifuged and 100 μL of the supernatant was removed for analysis. Fresh buffer (100 μL) was added, the pellet was resuspended by gentle vortexing and returned to the shaking water bath. Release samples were analysed by SDS-PAGE (15% (w/v)) and bicinchoninic acid assay (50% rV and 50% rF1 used as standards for total protein concentrations). Two release samples were also used for Western blotting.

Results

Recovery of antigens from microspheres
To determine the best method for recovering the antigens from microspheres, the protein loading of

Table 1. Protein loading of single encapsulated microspheres measured by bicinchoninic acid assay using three different extraction methods.

Extraction method	F2K microsphere loading (% w/w)	V2K microsphere loading (% w/w)
Digestion with 0·1 M NaOH and 5% SDS	3·33 ± 0·42	0·97 ± 0·15
Extraction in PBS and 5% SDS/dichloromethane	3·22 ± 0·28	0·75 ± 0·02
Extraction in PBS and 5% SDS/dichloromethane and hexane	3·46 ± 0·20	0·96 ± 0·12

Values are mean ± s.d., n = 3.

two microsphere batches encapsulating either rV (V2K) or rF1 (F2K) were analysed by bicinchoninic acid assay. Three different methods (Table 1), including a new method involving the addition of hexane to the dichloromethane extraction method, were used. Recovery of rF1 antigen from F2K microspheres was unaffected by the method used whereas extraction of rV antigen from V2K microspheres by our new method gave loadings similar to NaOH/SDS digestion and higher values than standard dichloromethane extraction. The antigen loadings of the co-encapsulated FV microspheres using the new extraction method estimated by the bicinchoninic acid assay was 2·49 ± 0·12% (w/w).

SDS-PAGE of antigens

Silver staining of SDS-PAGE gels allowed detection of as little as 0·5 μg rV and 1 μg rF1. SDS-PAGE gels treated with Coomassie Brilliant Blue R-250 facilitated detection to as low as 1 μg protein whether rV or rF1. A combination of both stains using a modification of the method described by De Moreno et al (1985) allowed visualization of proteins to as low as 0·1 μg protein.

Antigen integrity at 37°C

The rV antigen supplied for this study existed as monomer (MW 36 kDa) and breakdown products (Vbps) on SDS-PAGE (Figure 1). Over a 14 day period at 37°C in PBS (Figure 2), the antigen concentrations remained constant as measured by the total optical density on SDS-PAGE of both rV and Vbps bands. The ratio of the intensity of Vbps to rV bands increased at later time intervals suggesting conversion of rV to Vbps. rV antigen exists as a dimer and higher oligomers on native-PAGE (result not shown) and over a 14 day period at 37°C the dimer band intensity decreased with the appearance of a higher molecular weight band (which did not run on gel) suggesting aggregation of rV antigen.

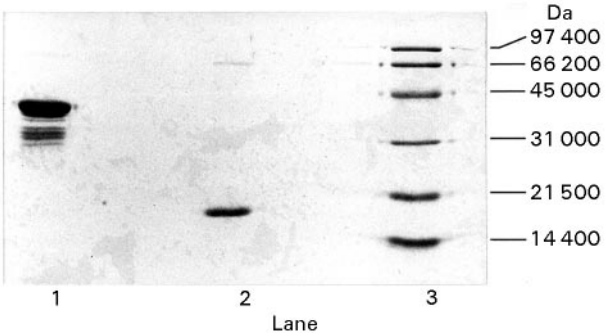

Figure 1. SDS-PAGE gel of molecular weight markers and sub-unit antigens. Lane 1, rV antigen 100 μg mL^{-1}; lane 2, rF1 antigen 100 μg mL^{-1}; lane 3, Bio-Rad low range markers (phosphorylase B 97·4; bovine serum albumin 66·2; ovalbumin 45; carbonic anhydrase 31; trypsin inhibitor 21·5; lysozyme 14·4 kDa).

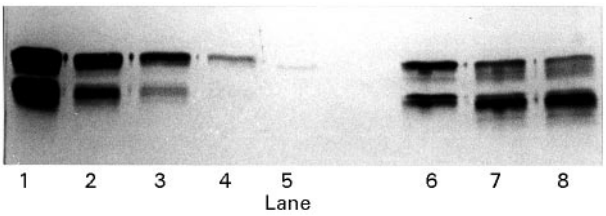

Figure 2. SDS-PAGE gel (15%) of the integrity of rV antigen in PBS at 37°C. Standard lanes (1–5): 1, rV 200 μg mL^{-1}; 2, rV 100 μg mL^{-1}; 3, rV 50 μg mL^{-1}; 4, rV 25 μg mL^{-1}; 5, rV 12·5 μg mL^{-1}. Sample lanes (6–8): 6, rV at start of experiment; 7, rV after 7 days; 8, rV after 14 days.

F1 exists predominately as an aggregate of 2000 kDa and little, if any, undenatured F1 enters native 4–20% (w/v) polyacrylamide gradient gels (Andrews et al 1996), which was also found when using a 12% (w/v) native gel to assess rF1 in this study. Heating at 100°C for 3 min causes the F1 oligomer to dissociate to a monomer and F1 remains as monomer in the presence of SDS and urea (Vorontsov et al 1990). rF1 could be assessed as its monomer by SDS-PAGE (Figure 1) and over a 14 day period at 37°C in PBS, rF1 antigen concentration remained constant as measured by the

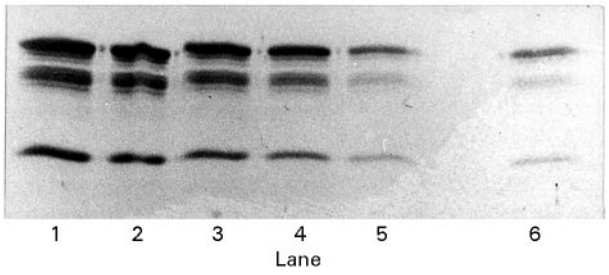

Figure 3. SDS-PAGE gel (15%) analysis of the sub-unit loadings in FV microspheres. Standard lanes (1–5): 1, rV + rF1 (250 μg mL^{-1} of each); 2, rV + rF1 (200 μg mL^{-1} of each); 3, rV + rF1 (150 μg mL^{-1} of each); 4, rV + rF1 (100 μg mL^{-1} of each); 5, rV + rF1 (50 μg mL^{-1} of each). Sample lane 6, FV microsphere loading.

optical density of the rF1 band (result not shown). Both rV and rF1 antigens remained at a constant immunological activity over the assessed time period at 37°C as determined by indirect ELISA (result not shown).

Release of rV and rF1 from FV microspheres

The antigen loadings of the co-encapsulated FV microspheres as determined by SDS-PAGE/densitometry were rV 1·22% (w/w) and rF1 1·24% (w/w) (Figure 3). Release of proteins from FV microspheres in PBS/sodium azide 0·01% (w/v) at 37°C were analysed by SDS-PAGE 15% (w/v) by the combined staining technique (Figure 4) and by bicinchoninic acid assay (release results shown in Figure 5, the concentration of rV antigen was determined using both rV and Vbps bands). rF1 antigen was shown to have a higher burst release than rV antigen. Western blots of release samples (results not shown) showed that the

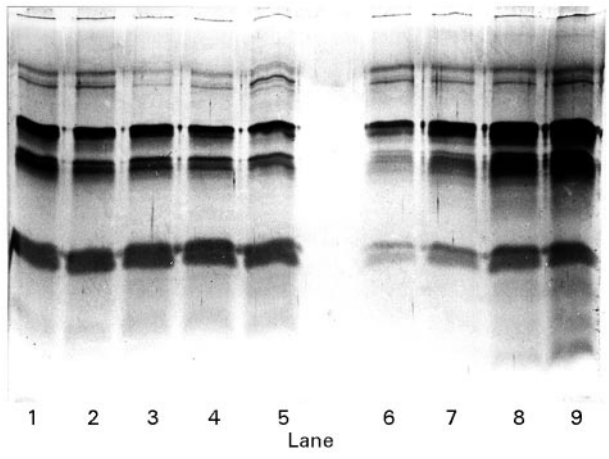

Figure 4. SDS-PAGE gel (15%) of the release samples from FV microspheres. Release sample lanes (1–5): 1, 48 h; 2, 24 h; 3, 6 h; 4, 3 h; 5, 1 h. Standard lanes (6–9): 6, rV + rF1 (5 μg mL^{-1} of each); 7, rV + rF1 (10 μg mL^{-1} of each); 8, rV + rF1 (25 μg mL^{-1} of each); 9, rV + rF1 (50μg mL^{-1} of each).

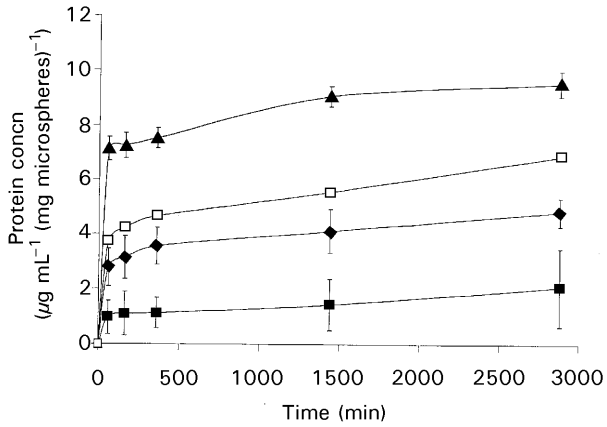

Figure 5. Release profiles for rV (■), rF1 (◆) and total protein (□) by SDS-PAGE and total protein (▲) by the bicinchoninic acid assay from FV microspheres (mean ± s.d., n = 3).

immunological activity of both antigens was retained after encapsulation and release.

Discussion

Logistical and immunological rationales which favour the development of a microsphere co-encapsulated rF1 and rV sub-unit vaccine have prompted the investigation and optimization of existing and new procedures to enable the status of multiple antigens entrapped within microspheres to be assessed. Several methods have been used to determine the incorporation or loading of proteins in microspheres. The most commonly used method involves alkaline hydrolysis of microspheres with NaOH and SDS, which has been shown to be very efficient in recovering proteins (Sharif & O'Hagan 1995). This digestion method was questioned, although the authors did admit microspheres were not completely digested after 4 h, which is most likely the reason for poor recovery yields (Gupta et al 1997). A pre-requisite for reliable and accurate protein determinations when using the digestion method is to treat the protein standards to the same conditions as the samples, which will compensate for any change to the protein structure during digestion (standard curves of treated and untreated protein by bicinchoninic acid are different, result not shown). A disadvantage with the alkaline hydrolysis method is that it can not be used for SDS-PAGE as the protein integrity is lost. Organic solvent extraction of microspheres with dichloromethane and PBS is a method which would allow samples to be used on SDS-PAGE gels. The efficiency of extraction of proteins by this method has been also doubted (Sharif & O'Hagan 1995; Gupta et al 1997), although neither group used SDS in the

aqueous phase which would result in a more complete recovery of protein, most likely by displacing protein molecules from the aqueous/organic solvent interface. In this study all three recovery methods used gave similar loadings for rF1 antigen from F2K microspheres. Extraction of rV antigen from V2K microspheres by our new method gave loadings similar to NaOH/SDS digestion and higher values than standard dichloromethane extraction. This is possibly due to the fact that hexane makes the organic phase more hydrophobic, enhancing the recovery of proteins. The new extraction method has an advantage over NaOH digestion in that its samples can be used for PAGE. Also, the new method was used to determine the antigen loadings of the co-encapsulated FV microspheres by SDS-PAGE/densitometry because of the good recovery of both antigens under investigation.

Enhanced visualization of protein bands for rV and rF1 antigens was achieved with a combination of Silver Stain (Bio-Rad Silver Stain Plus) and Coomassie Brilliant Blue R-250. Coomassie Brilliant Blue R-250 is a sulphonic acid dye which ionically interacts with the basic amino acid groups of proteins and may have secondary interactions including hydrogen bonding, van der Waals attraction and hydrophobic bonding between the dye and the protein, and between free dye and dye already associated with protein (Wilson 1983). Silver staining of proteins is probably due primarily to physical effects, which is supported by the fact that both negative-image and positive-image silver stains exist (Merril et al 1984). The mechanism of the increased sensitivity with the combined Coomassie Brilliant Blue–Silver Staining may be due to site-enhanced silver nucleation caused by the sulphonic acid dye (De Moreno et al 1985).

Retention of the integrity of proteins is an important issue when studying the release of antigens from encapsulated microspheres. rF1 antigen was assessed as its monomer by SDS-PAGE and over a 14 day period at 37°C in PBS, rF1 antigen concentration remained constant as measured by the optical density of the rF1 band. rV antigen showed evidence of aggregation over a 14-day period at 37°C in PBS on native-PAGE and showed a shift of intensity in protein bands from the monomer to breakdown products on SDS-PAGE. Despite this, rV and rF1 antigen retained their antigenic activity as determined by indirect ELISA. Antigens released from the microspheres also retained their antigenic activity as determined by Western blots, which shows that the encapsulation process used was not detrimental to the activity of the antigens. This is supported by the fact that co-encapsulation of these sub-units into microspheres elicits excellent immune responses in mice (Eyles et al 1998a, b). The proteins associated with rV antigen supplied were identical before and after encapsulation and therefore the optical density of all the associated bands were used in determining rV antigen release (all bands were immunologically active as determined by Western blots and therefore all are important when studying release). rF1 antigen released from the co-encapsulated FV microspheres had a higher burst effect than rV, which may be explained by rF1 antigen's hydrophobic nature leading to its location at or near the surface domains of the microspheres. Total protein release from the co-encapsulated microspheres was different depending on whether bicinchoninic acid or SDS-PAGE assays were used in the determination of protein concentrations, although the release profiles did not look dissimilar. Optical density of the bands for antigen standards used in the SDS-PAGE analysis consistently gave good correlation with the protein concentration loaded on gels and therefore the SDS-PAGE assay gave reliable results. In comparison, the bicinchoninic acid assay is dependent on a complexation method which can be effected by formulation excipients. Another problem with using the bicinchoninic acid assay for determining total protein concentrations of two or more proteins is that it is dependent on using a calibration curve reflecting the relative amounts of individual proteins in the protein solution under assessment. Protein to protein variation of the bicinchoninic acid assay is known to occur (Protein Assay Technical Handbook 1996) and so different proteins will give different calibration curves. As the relative amounts of individual proteins in a release sample is unknown and can vary over the release period, it is difficult to use the correct calibration. In this study, 50 : 50 rV : rF1 composition was used for the bicinchoninic acid standards which could have added to the discrepancy in total protein concentrations.

In conclusion, this study shows that the new extraction method involving the addition of hexane to dichloromethane, benefited from higher protein recovery efficiencies from microspheres and enabled analysis by SDS-PAGE. The concentration (determined by SDS-PAGE) and immunological activity (determined by indirect ELISA) of free rF1 and rV antigens at 37°C in PBS was maintained over a two-week period. SDS-PAGE is a suitable method for the determination of individual protein release from co-encapsulated microspheres. rV and rF1 antigens co-encapsulated within microspheres retained their immunological integrity which supports our findings that encapsulation of antigen sub-

units into microspheres is a viable delivery method for mucosal and parenteral vaccination.

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