On Technological and Immunological Benefits of Multivalent Single-Injection Microsphere Vaccines

Gérard Boehm,[1] Marisa Peyre,[2] Dorothea Sesardic,[2] Rachel J. Huskisson,[2] Fatme Mawas,[2] Alexandra Douglas,[2] Dorothy Xing,[2] Hans P. Merkle,[1] Bruno Gander,[1] and Pål Johansen[1,3]

Received April 18, 2002; accepted May 31, 2002

Purpose. With the aim of developing multivalent vaccines for single-injection, we examined the feasibility of combining antigens in biodegradable microspheres. Such vaccines are expected to improve vaccination coverage by reducing the number of vaccination sessions required to generate immunity.

Methods. Mono- and multivalent vaccines of *Haemophilus influenzae* type b (Hib) conjugate, diphtheria toxoid (DT), tetanus toxoid (TT), and pertussis toxin (PT) in poly (lactic acid) and poly(lactic-coglycolic acid) microspheres were prepared by spray drying, and the influence of coencapsulated antigens and excipients on antigen loading, release, and stability was examined. Two tetravalent formulations were tested in guinea pigs.

Results. Monovalent Hib and PT vaccines showed loading efficiencies of 10% (Hib) and 30% (PT) in both polymers. The loading efficiencies increased upon addition of trehalose and, even more, when the antigens were coencapsulated in di- and trivalent combinations. Highest loading efficiencies (>80%) were achieved with trivalent formulations (DT + PT + Hib) that also contained coencapsulated albumin. The percentage of antigen released during 24 h of incubation was typically 10–40% and decreased as loading efficiency increased. Enzyme-linked immunosorbent assay (ELISA) data revealed that TT, DT, and PT remained antigenic throughout the encapsulation and subsequent release processes. Finally, all antigens maintained their immunogenicity, since strong and sustained antibody responses were elicited after a single injection of tetravalent microsphere vaccines (DT + TT + PT + Hib) in guinea pigs.

Conclusions. This study reveals technologic benefit as well as an immunological potential of multivalent single-injection microsphere vaccines. The results support our hypothesis that coencapsulation of several antigens may intrinsically improve entrapment of antigenic and immunogenic antigen probably by virtue of increased protein concentration during microencapsulation leading to mutual stabilization of the components.

KEY WORDS: microspheres; antigens; protein stability; vaccination.

INTRODUCTION

The number of administrations required for most vaccines to induce protective immunity limits the vaccination coverage and success especially in countries with insufficient medical logistics. According to the World Health Organisation (WHO), operational costs of vaccination campaigns contribute to almost 90% of the total costs, and dropout rates from the first to the consecutive doses of vaccines of up to 70% have been registered. The availability of multivalent vaccines already improved vaccination campaigns in the past, but the development of single-injection (or single-dose) vaccines may further simplify vaccination. Indeed, antigens embedded in biodegradable poly (lactic acid) (PLA) and poly(lactic-coglycolic acid) (PLGA) microspheres (reviewed in [1]) have been shown to stimulate long-lasting antibody responses (2–4) as well as cytotoxic T lymphocytes (5,6).

While multivalent combined vaccines based on diphtheria (DT), tetanus (TT), pertussis (PT), *Haemophilus influenzae type b* (Hib), polio, and hepatitis B or measles, mumps, and rubella antigens are already in use, limited data on multivalent vaccines based on polymeric microspheres is available (2,3) as previous studies were almost exclusively restricted to the encapsulation of a single antigen type. Nevertheless, the coencapsulation of multiple antigens may raise specific constraints concerning the biophysical stability and the immunogenicity of the vaccines, which may not be predictable from the properties of the individual vaccine components but which may result from interactions between different components (7). The combination of antigens in conventional formulations either decreases (8,9) or increases (10) the immunogenicity of individual components, and similar features may be expected in microsphere-based multivalent vaccines.

Nevertheless, previous studies have demonstrated the stabilising effect of proteins (e.g., albumin and gelatine) on microencapsulated antigens. Therefore, the main objectives of this investigation were to study whether successful technologic concepts for TT and DT (4,11) could be transferred to Hib and PT antigens. Furthermore, we aimed at testing the hypothesis that coencapsulation of several antigens into PLGA microspheres may improve the encapsulation efficiency of intact material by virtue of increased total protein content during encapsulation. Our results show that multivalent microsphere vaccines are technologically feasible. Coencapsulation of multiple antigens can even enhance the loading efficiency of antigenic protein in the microspheres. Furthermore, tetravalent single-injection microsphere vaccines containing DT, TT, PT and Hib were highly immunogenic for all antigens as measured by induction of antibodies in guinea pigs.

EXPERIMENTAL

Materials

Hib conjugated to TT, genetically attenuated PT, DT, and column-purified TT were from Berna, the Swiss Serum and Vaccine Institute (Berne, Switzerland), Chiron Vaccines (Siena, Italy), Aventis Pasteur (Lyon, France), and Massachusetts Public Health Biologic Laboratories (Boston, MA, USA), respectively. PLGA 50:50 and PLA with intrinsic viscosities of approximately. 0.2 dL/g were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Trehalose was from Quadrant (Ruddington, United Kingdom) and bovine serum albumin from Fluka (Buchs, Switzerland). The immunologi-

[1] Institute of Pharmaceutical Sciences, ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.

[2] Division of Bacteriology, National Institute for Biologic Standards and Controls, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom.

[3] To whom correspondence should be addressed. National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. (e-mail: pjohans@nimr.mrc.ac.uk)

0724-8741/02/0900-1330/0 © 2002 Plenum Publishing Corporation

cal adjuvant Alhydrogel®, an aluminium hydroxide (alum), was from Superfos (Vedbaek, Denmark). All other substances were of pharmaceutical or analytical grade.

Methods

Preparation of Microspheres

Volumes of 2.0–2.5 mL of aqueous antigen or antigen mixtures were mixed with 20 mL polymer solution in dichloromethane (10%, w/v). In certain experiments, albumin and trehalose were also added to the aqueous phase. The W/O mixture was homogenised on ice by ultrasonication (2 × 10 s, 20 kHz) and spray-dried with a Büchi 190 Mini Spray Dryer (Flawil, Switzerland) at a rate of 2 mL/min and an inlet temperature of 38–39°C. The microspheres were collected and washed in aqueous poloxamer 188 (0.1%), passed through a 50 μm mesh sieve, collected on a 0.8 μm cellulose acetate filter, washed with heptane, and dried at room temperature and 50 mbar for 12 h. All formulations (n = 1–2) showed a regular and spherical microsphere morphology, with particle sizes in the range of 0.5 to 5 μm as measured by visible light microscopy and laser light scattering.

Antigen Loading and in Vitro Release

For determination of protein and antigen loading, the antigens were extracted from 100 mg microspheres as previously described (11), and the results were expressed in percent relative to the nominal antigen loadings. Antigen burst release from 80 mg microspheres in 4 mL PBS was analyzed after 24 h, (11), and the results expressed relative to the determined antigen loadings. Both loading and release determinations were performed in triplicate from one single batch of microspheres.

Determination of Hib and PT Content by Fluorimetry and Bial Assay

When encapsulated individually or together with trehalose, Hib, and PT were assayed fluorimetrically using an excitation wavelength of 280 nm and emission wavelengths of 320 (Hib) and 340 (PT) nm. In combination with other antigens or albumin, ribosyl content of Hib was quantified with the orcinol reaction as proposed by *Bial* (12); due to interference, the *Bial* method could not be applied to vaccine formulations containing trehalose. Briefly, 0.6 mL 0.1% $FeCl_3$ in concentrated hydrochloric acid and 60 μl of 100 mg/mL orcinol in 95% ethanol were added to 0.6 mL of the Hib solution. The samples were heated 40 min at 95°C and cooled to room temperature before measured photometrically at 670 nm.

Determination of DT, TT and PT Antigenicity by ELISA

In combination with other antigens or albumin, DT, TT, and PT were quantified by ELISA. Flat-bottom microtiter 96-well plates were used, which were washed thrice with 0.05% polysorbate 20 in 0.15 M phosphate-buffered saline of pH 7.4 (PBS) after each incubation step. DT (13) and TT (14) content were measured by ELISA as previously described. For PT analysis, the plates were coated with an anti-PT monoclonal antibody (NIBSC; Potters Bar; UK) in 0.05 M carbonate buffer of pH 9.6 by overnight incubation at 4°C. After 1 h at 37°C with PBS containing 0.5% albumin (PBS-BSA), the plates were incubated at 37°C for 2 h with serial dilutions of standard and test solutions of PT. Further incubations with sheep anti-PT antiserum (NIBSC code no. 97/572) in PBS-BSA (37°C, 2 h), alkaline phosphatase conjugated donkey antisheep IgG (The Binding Site; Birmingham, UK) in PBS-BSA (37°C, 2 h), and 1 mg/mL p-nitrophenyl phosphate (Sigma Chemical; St. Louis, MO, USA) in 10% diethanolamine buffer of pH 9.6 (20°C, 40 min) were conducted before the optical density was measured at 405 nm (Thermomax, Molecular Devices; Menlo Park, CA, USA). No analytical interference in the specific ELISA reactivity for PT, DT, or TT was observed in the presence of any other antigen or the additives trehalose and albumin at relevant concentrations (data not shown).

Immunization of Guinea Pigs and Determination of Antibodies by ELISA

Groups of female guinea pigs (250–300 g, strain DH) were immunized subcutaneously with either of two tetravalent microspheres vaccine formulations dispersed in 0.5 mL aqueous alum (0.035 mg Al^{3+} per dose per animal). The two formulations tested differed in their antigen loading (high vs. low nominal antigen loading) as well as in their relative content, i.e., different actual DT/PT, DT/Hib, TT/PT, TT/Hib, or PT/Hib ratios caused by different encapsulation efficiencies of the individual antigens (Fig. 1 and Fig. 2). An alum-adsorbed commercial tetravalent vaccine was used as positive control (50 and 20 Lf/mL DT and TT, respectively; 8 μg/mL chemically detoxified PT, but the vaccine also contains filamentous hemagglutinin and pertactin; 30 μg/mL Hib conjugated to TT), but at a higher alum dose (0.06 mg Al^{3+}). Animals were bled by cardiac puncture under anesthesia 2–16 weeks post immunization, and serum was prepared from clotted blood and stored at −20°C.

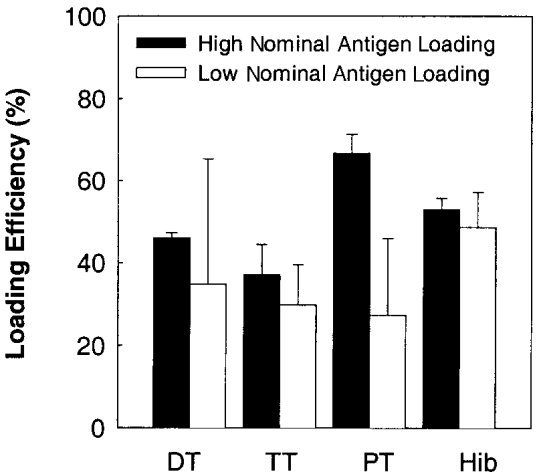

Fig. 1. Loading efficiency of antigens in two tetravalent poly (d, l-lactic-coglycolic acid) 50:50 microsphere vaccines with high (filled bars) and low (open bars) nominal antigen loadings. Microspheres were prepared with nominally 0.46 or 0.19% diphtheria toxoid (DT) (1.25 or 0.5 Lf/mg microsphere), 0.15 or 0.06% tetanus toxoid (TT) (0.5 or 0.2 Lf/mg microspheres), 0.05 or 0.02% pertusiss toxin (PT), and 0.1 or 0.04% *Haemophilus influenzae type b (*Hib) as well as 5% albumin. DT, TT and PT contents were determined by ELISA and Hib by the *Bial* method.

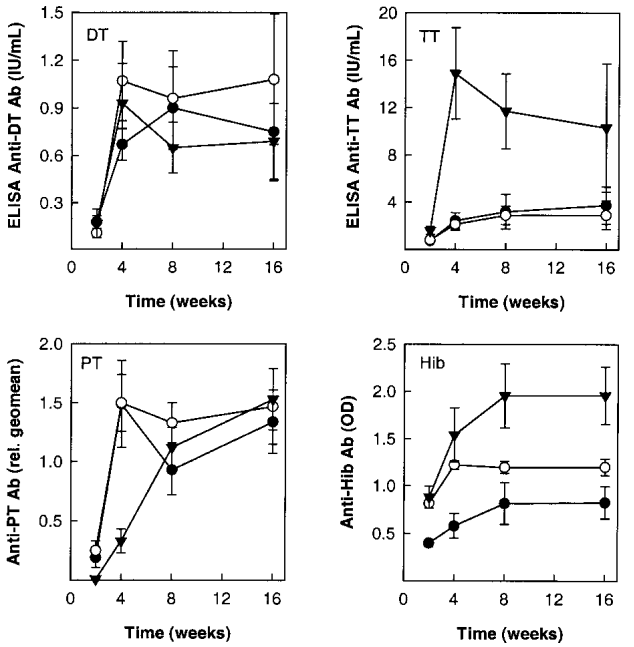

Fig. 2. Anti-diptheria toxin (DT), anti-tetanus toxin (TT), anti-pertussis toxin (PT) and anti-*Haemophilus influenzae type b (*Hib0 (d) antibodies in sera of guinea pigs after single injection of either of two tetravalent microsphere-based vaccines (see Fig. 1 for further details on vaccine formulations). The vaccination doses were 2.5 Lf DT, 0.8 Lf TT, 0.8 μg PT and 3.0 μg Hib (when low nominal loading) or 2.5 Lf DT, 0.8 Lf TT, 1.4 μg PT and 2.1 μg Hib (when high nominal loading), as determined *in vitro*. For control of specific immunogenicity, 2.5 Lf DT, 1 Lf TT, 1 μg PT and 2 μg Hib adsorbed on alum was given once. The results illustrate the arithmetic means ± standard errors (n = 8–10). ▼: alum-based control vaccine; ○; microsphere-based vaccine with high nominal antigen loading; ●: microsphere-based vaccine with low nominal antigen loading.

Anti-DT and -TT antibodies were determined by ELISA using coating antigens from Welcome (Beckenham, UK: lots BJGL 1004/F and MWC S208/A/F-6 and for DT and TT, respectively). The results expressed in terms of an inhouse guinea-pig reference standard (NIBSC code 98/572: 3.5 and 3.0 IU/mL nAb potency *in vivo* for DT and TT, respectively). Geometric means were calculated from two independent ELISA experiments on individual serum from each vaccine group. Anti-PT antibodies were determined by an inhouse method after coating plates with a PT from Smithkline Beecham (Uxbridge, UK: lot PAC057). The results were expressed as the geometric mean relative to an inhouse reference serum assayed in the same plate. Finally, antibodies against Hib polysaccharide polyribosyl ribitol phosphate (PRP) were determined using, as coating antigen, oligosaccharide conjugated human serum albumin (HbO-HA from Wyeth Lederle; Pearl River, NY, USA). The results were expressed as mean optical density (OD) from two sets of experiments.

RESULTS

Hib, PT, and DT Exerted a Mutual Synergistic Influence on Loading Efficiency When Coencapsulated. In monovalent PLGA 50:50 microsphere preparations, PT (30%) was more efficiently entrapped than Hib (10%) (Table I). The loading efficiency of Hib was improved when coencapsulated with PT (16% Hib), with DT (29% Hib), or with both PT and DT in a trivalent vaccine (59%Hib), the PT loading remained unchanged with DT (31% PT), but increased to 75% in the trivalent vaccine. By the same token, DT entrapment was 10 and 13% when coencapsulated with Hib and PT, respectively, but rose to 59% when all three antigens were coencapsulated. Hence, the coencapsulation of Hib, DT, and PT exerted a synergistic effect on the loading efficiency of the individual antigens.

Addition of Albumin to Multivalent Microsphere Vaccines Further Increased Antigen Loading. Albumin, which has been reported to stabilise microencapsulated proteins (11,15), was coencapsulated with selected antigen combinations to yield a total nominal protein loading of 5%. Coencapsulation with albumin increased the entrapment efficiency of Hib to approximately 80% in both divalent (with DT) and trivalent (with DT and PT) vaccines (Table I). The effect was even more striking for the loading of DT. Albumin improved DT-loading efficiency to approximately, 90% for all three combinations tested. The effect of albumin coencapsulation on PT loading could not be assessed. The described method for extracting PT from albumin-containing multiva-

Table I. Antigen Loading Efficiency and Burst Release (Measured after 24 h) of *Haemophilus influenza* Type b (Hib), Pertussis (PT), and Diphtheria (DT) Antigens Encapsulated Individually or in Combinations into PLGA 50:50 Microspheres

Encapsulated antigen	Loading efficiency (%)			Burst release (%)		
	PT	Hib	DT	PT	Hib	DT
PT	30 ± 2	—	—	8 ± <1	—	—
Hib	—	10 ± <1	—	—	41 ± 6	—
PT + Hib	n.d.	16 ± 2	—	n.d.	63 ± 2	—
PT + DT	31 ± 8	—	13 ± 3	19 ± 3	—	11 ± 2
PT + DT + BSA	n.d.	—	92 ± 15	n.d.	—	8 ± 3
Hib + DT	—	29 ± 1	10 ± 2	—	51 ± 8	49 ± 6
Hib + DT + BSA	—	78 ± 18	90 ± 18	—	37 ± 5	5 ± 2
PT + Hib + DT	75 ± 3	59 ± 9	59 ± 7	8 ± 2	62 ± 6	12 ± 3
PT + Hib + DT + BSA	n.d.	83 ± 14	89 ± 8	n.d.	14 ± 5	39 ± 8

Note: Loading efficiency represents the percentage (mean ± SD) of determined antigen content relative to the nominal loading, and the release data represent the percentage of released antigen relative to the determined antigen loading. Nominal antigen loadings were 0.037% Hib, 0.050% PT, 1.48% DT, and 5% BSA. For Hib, ribosyl content was analyzed by the Bial assay, whereas PT and DT antigenicity were analyzed by ELISA. n.d.: not detected.

lent microspheres, for yet unknown reasons, was not applicable to vaccines fabricated from the end-group capped PLGA 50:50 polymer (as described below, this problem did not appear when using the end-groups uncapped quality of PLGA 50:50).

The Polymer Type Had Little Influence, Whereas Trehalose Improved Antigen Loading in Monovalent Vaccines. To study the influence of other excipients on antigen loading, we prepared microspheres with different polymer types that contained trehalose (11,15) as stabilizer for the antigens. PLGA 50:50 and the more hydrophobic PLA were individually loaded with PT and Hib. The polymer type did not affect the encapsulation efficiency (Fig. 3). Moreover, coencapsulation of 5% trehalose enhanced the entrapment of PT only slightly from 30% to 35% for PLA, but from 30% to 53% for PLGA 50:50 microspheres. This amount of trehalose had no notable effect on the loading efficiency of Hib in PLA but increased the entrapment from 10 to 14% in PLGA 50:50 microspheres. Higher trehalose concentrations (10–20%) did neither improve further the loading efficiency of Hib in PLGA 50:50 nor affect the loading in PLA microspheres (data not shown).

Burst release of Antigens from Microspheres Generally Depended on How Efficiently the Antigens were Encapsulated. As a measure for the *in vitro* product quality, the amount of antigen released during 24 h incubation in buffered saline and 37°C was determined. Ideally, a limited fraction of the antigen should be released from the microspheres immediately for immunologic priming, whereas residual amounts should be retained for later boosting. In this study, an increase in the loading efficiency was typically related to a decrease in the burst release of both Hib and PT antigens. This relationship showed inverse linear correlation with $r^2 = 0.7$ and $r^2 = 0.9$ for monovalent Hib ($n = 5$) and PT ($n = 6$), respectively. The percentages of antigen released within the initial 24 h from mono-, di- and trivalent preparations were approximately 10–20% for PT, 40–60% for Hib, and 10–50% for DT (Table I), and the coencapsulation of either albumin (Table I) or trehalose (Fig. 3) lowered the burst release for all three antigens.

Tetravalent Vaccines Were immunogenic in Guinea Pigs. Two tetravalent PLGA 50:50 microsphere vaccines with different concentrations of DT, TT, PT, Hib, and 5% albumin were prepared and tested both *in vitro* and *in vivo*; the nominal antigen loadings were lower than in the aforementioned mono-, di- and trivalent preparations. Furthermore, since the 14 kDa end-group capped PLGA 50:50 (Resomer® RG502) is no longer available from Boehringer-Ingelheim, the tetravalent vaccines were prepared with the corresponding end-group uncapped PLGA 50:50 (Resomer® RG502H). Although the antigens were entrapped less efficiently in the tetravalent Resomer® RG502H than in the di- and trivalent Resomer® RG502, the loading efficiencies of all antigens generally increased with higher nominal antigen content (Fig. 1). The effect was most evident for PT (66 vs. 27% for high and low nominal antigen loading, respectively) and least for Hib (53 vs. 49%) and resembled that observed for di- and trivalent vaccines. The loading efficiencies for DT were 46 and 35%, whereas those for TT were 37 and 29%.

Encouragingly, all antigens remained immunogenic, because high titers of persistent antibodies were raised in guinea pigs after a single administration of either of the two tetravalent microsphere vaccines, as compared with the combined formulation based on alum. The antibody kinetics followed a similar pattern for all four antigens with a sharp increase in antibodies the first 4 weeks after immunization, after which antibodies slightly increased or persisted at a constant level (Fig. 2). Both microsphere types with low and high nominal content produced comparable results in terms of both kinetics and maximum titers ($p > 0.05$ as analyzed on antibody titers at 16 weeks). Whereas the anti-DT and anti-TT antibodies typically declined after 4 weeks in animals that received the control vaccine, the kinetics of anti-PT antibodies unexpectedly increased throughout the test period. The increase of anti-Hib antibodies after 4 weeks was not significant ($p > 0.05$).

DISCUSSION

The availability of efficacious multivalent single-injection vaccines would have a great impact on vaccination programs.

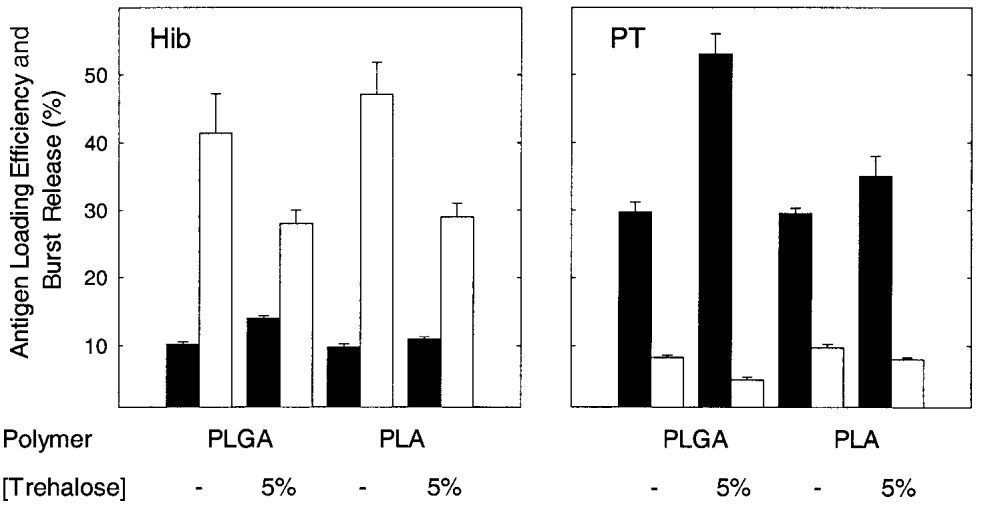

Fig. 3. Influence of polymer type (poly [d,l-lactic-coglycolic acid] vs. poly [d,l-lactic acid]) and the presence of the protein stabilising additive trehalose (0 vs. 5%) on *Haemophilus influenzae type b* (*left*) and pertussis toxin (*right*) loading efficiency (filled bars) and burst release (open bars) from monovalent microspheres. Hib content was determined by the *Bial* method and pertussis toxin by fluorimetry.

We therefore examined the technological feasibility of using polymeric microspheres for such vaccines by microencapsulating Hib, PT, DT, and TT by spray-drying. The results supported our initial hypothesis that coencapsulation of several antigens intrinsically improves loading efficiency of antigenically reactive antigens. Our hypothesis is based on the interfacial activity of proteins especially of albumin. At higher protein concentration, a relatively smaller fraction of the total protein is sufficient to saturate the W/O-interface during microencapsulation. This interfacial protein adsorption improves the physical stability of the W/O-dispersion so that the bulk fraction of protein is not exposed to the organic phase and should remain intact. Most promising, tetravalent antigen combinations were strongly immunogenic in guinea pigs.

Because little information is available on the microencapsulation of Hib and PT into polyester microspheres, we initially studied these two antigens individually in PLA and PLGA 50:50 polymers. Taking into account that Hib is conjugated to TT (approximately 1:3 ratio by weight), the loading efficiency determined for Hib (10–15%) was surprisingly low, because previous experiments had shown that up to 70% of the nominal TT loading could be expected in PLGA 50:50 microspheres (11). This suggests a negative influence of the polyribosyl-ribitol moiety on the encapsulation of Hib. However, it remains unknown whether this influence is linked specifically to Hib or more generally to polysaccharide moieties of proteins, because data on glycoprotein microencapsulation is unavailable. With respect to PT, filamentous hemagglutinin (FHA), pertactin, and PT have previously been microencapsulated by solvent evaporation and tested for oral and mucosal immunization (16,17), although only few details were provided on the *in vitro* characteristics. For FHA-containing microspheres, a loading efficiency of 60% (0.4% nominal loading by weight), linear antigen release kinetics, and a burst release of 10% were reported (17). More importantly also here, the microencapsulation process did not impair the immunogenicity of the antigens, and the vaccines were recently shown to protect mice against a respiratory challenge with *Bordetella pertussis* (18). Because PT is considered the most important pertussis antigen for vaccination, we choose it for inclusion in the multivalent microsphere-based vaccines studied here. The results showed that PT was more efficiently encapsulated than Hib (Fig. 3), but the two polymer types did not differ in terms of antigen loading and release.

The loading efficiencies of Hib and PT were improved by coencapsulating trehalose (Fig. 3), which has been shown to exert a positive effect on the microencapsulation of other proteins (11). Trehalose may physically stabilise the W/O emulsion during processing (19) and also hydrate preferentially the protein, thereby increasing its conformational stability (20). Indeed, Hib and PT appeared physicochemically intact within the given experimental parameters by assaying encapsulated and released antigen. The Hib-toxoid conjugate was assayed for its protein (fluorimetry) and polysaccharide (*Bial* method) moieties, whereas the amount of fluorimetrically detectable PT protein was compared with its antigenicity in ELISA. Because the determined amounts of encapsulated and released antigens did not vary with the assay method, both Hib and PT most likely remained stable during the processes (results not shown). However, for Hib the agreement between the fluorimetric and colorimetric (*Bial*) data only indicates that the protein and the polysaccharide moieties were encapsulated and released to a comparable extent; whether the two moieties were still conjugated remains unknown. Although the *in vivo* data advocates for a stable Hib conjugate, more discriminative assays are required for quantification of the stability.

The main goal of this investigation was to study the feasibility of coencapsulating several antigens in a single microsphere formulation. Previously, proteinaceous additives, such as albumin or gelatine, have been coencapsulated to improve the biologic response of bioactive compounds including antigens (4,15,21). Coencapsulation of several biologically active compounds, such as cytokines or sub-unit antigens, provided a tool to direct the immune response towards either a humoral or cellular-type response (22,23) or to achieve a broader immune response against a single infectious agent (24,25). Our approach was to prepare a vaccine formulation appropriate for concomitant stimulation of specific immune responses against antigens derived from different pathogens. The study provided encouraging data on the technological feasibility of combining antigens in a microsphere vaccine, because coencapsulated antigens remained antigenic and generally exerted a synergistic effect on loading efficiency. No single antigen was superior to the others in improving total antigen loadings. In addition, the coencapsulation of albumin further increased antigen loadings (Table I). Proteinaceous additives have previously been described to stabilise antigens during microencapsulation and upon exposure to aqueous solutions and elevated temperatures (11,14). We believe that the improved entrapment of antigenic antigen was an unspecific effect related partly to increased physical stability of the W/O emulsion during encapsulation and partly to the higher fraction of bulk antigen in the water phase that remained protected from contact with the organic medium by the interfacially adsorbed protein layer. Hence, the coencapsulated proteins mutually stabilised each other and, thereby, enhanced the loadings of the individual antigenic proteins in the microspheres as ascertained for DT, TT, and PT by antigenicity testing.

Despite different views as to which type of release kinetics is most preferable, antigen-burst release is an important aspect in the development of microspheres. Extracellular antigens in solution are normally unavailable for uptake and presentation by antigen-presenting cells after parenteral administration and may even induce tolerance. Hence, less efficient priming might be anticipated from antigen released extracellularly during the priming phase. It is also a notion that the number of surviving memory cells is directly proportional to the number of precursor cells activated during the priming phase of the immune response (26). Hence, one might prefer low-burst release to facilitate intracellular antigen release after phagosomal particle uptake and degradation in the phagolysosomal fusion product. Furthermore, long-term release of antigens from microspheres is difficult to monitor and even harder to interpret in terms of biological significance. Therefore, we decided to analyze the release behavior of the microspheres only in the initial phase. The amounts of released antigen determined after 24 h for the mono- and multivalent microspheres depended on both antigen and excipients. Burst-release values were in the range of 10–50% of the actual antigen content and considered acceptable in view of estimated amounts necessary for priming vs.

boosting *in vivo*. We observed a trend towards lower burst release with increasing loading efficiency for Hib in multivalent microspheres. However, it occurred that multivalent microspheres showed higher initial release rates than monovalent formulations. A possible explanation for this effect might be the higher nominal protein loadings of the multivalent microspheres creating a more porous microsphere matrix: the higher the protein concentration in the microspheres, the larger the absolute amount of protein near the surface available for release. Upon release of molecules located near the particle surface, pores are formed which may favor the release of additional fractions of protein.

Although the present study did not directly compare mono- and multivalent vaccines *in vivo*, we clearly demonstrated that a tetravalent vaccine based on biodegradable microspheres is feasible. After single administration of microspheres loaded with DT, TT, PT, and Hib, high titers of lasting antibodies against all four antigens were detected in guinea pig sera. The kinetics and magnitude of the immune response against the individual antigens differed only slightly as a function of formulation, i.e., alum vs. microspheres, low- vs. high-antigen loading. Because the purpose of the present study was to test the technological properties and the immunological feasibility of multivalent vaccines based on biodegradable microspheres, this primary experimental design did not permit us to study whether these differences in immunogenicity were significant or whether they were related to differential antigen stability or release characteristics, to the number of particles injected or to the differences in absolute and relative antigen contents. Also, to what extent these vaccines can generate protective immune responses is presently unknown and should be addressed in further testing of multivalent microsphere vaccines. Nevertheless, the levels of anti-TT and anti-DT antibodies suggest protective immunity, because the ELISA antibodies detected with the described method normally correlates well with neutralising antibodies (M.P. and D.S., unpublished observations) for which protection levels are 0.1 IU/mL for both TT and DT.

In a previous investigation with a divalent tetanus-diphtheria microsphere vaccine in rats, monovalent DT (2) or TT (3) microspheres produced stronger immune responses than did the divalent vaccine. This phenomenon was ascribed to antigenic competition between the two antigens, resulting in reduced antigen presentation. However, no immunological interactions were observed when guinea pigs were immunized with mixtures of DT and TT embedded individually in different microsphere populations, which were mixed prior to immunization (M.P. and D.S., unpublished observations). It has also been reported that the presence of more than one antigen in a conventional multivalent vaccine may result in reduced immunogenicity for all of the antigens (8) and that the capacity of the organism to deal efficiently with several antigens at the same time might be limited (27). The present study demonstrated that both the encapsulation efficiency and the correlation between antigen content, determined *in vitro,* and the immunological reactivity in animals depended on the specific antigen. Hence, combination of antigens requires careful and systematic monitoring of both *in vitro* and *in vivo* parameters, and an objective of future immunization tests should be made to reevaluate the optimal antigen doses required to induce adequate immune responses against the coencapsulated antigens.

Summing up, in this investigation, we discuss potential technological and immunological benefits of multivalent microsphere vaccines. Evidence for the feasibility of the encapsulation of Hib and PT in polymeric microspheres as well as of multivalent combinations of Hib, PT, DT, and TT was provided. The coencapsulation of antigens was particularly beneficial as it improved the entrapment efficiency of the individual antigens in a physicochemically and antigenically stable form, which was further improved by coencapsulating albumin. The fact that all antigens were strongly immunogenic in guinea pigs is encouraging in view of simplifying and improving vaccination procedures. It is our hope that this study may contribute to bias this technology towards perhaps clinical evaluation.

ACKNOWLEDGMENTS

This work was carried out in partial fulfilment of G. B.'s diploma in pharmaceutical sciences at ETH Zurich and was supported in part by the Department of Applied BioSciences and the Vice-president for Research at ETH Zurich. P. J. was supported by a grant (No. 4140.1) from the Swiss Federal Commission for Technology and Innovation, Berne, Switzerland. We also acknowledge support from the Swiss Serum and Vaccine Institute, Berne, Switzerland); Chiron Vaccines, Siena, Italy; and the WHO, Geneva, Switzerland.

REFERENCES

1. P. Johansen, Y. Men, H. P. Merkle, and B. Gander. Revisiting PLGA microsphere-based vaccines: an analysis of their potential in parenteral vaccination. *Eur. J. Pharm Biopharm.* **50**:129–146 (2000).
2. M. Singh, X. M. Li, H. Wang, J. P. McGee, T. Zamb, W. Koff, C. Y. Wang, and D. T. O'Hagan. Controlled release microparticles as a single dose diphtheria toxoid vaccine: immunogenicity in small animal models. *Vaccine.* **16**:346–352 (1998).
3. M. Singh, X. M. Li, H. Wang, J. P. McGee, T. Zamb, W. Koff, C. Y. Wang, and D. T. O'Hagan. Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine. *Infect Immun.* **65**:1716–1721 (1997).
4. P. Johansen, L. Moon, H. Tamber, H. P. Merkle, B. Gander, and D. Sesardic. Immunogenicity of single-dose diphtheria vaccines based on PLA/PLGA microspheres in guinea pigs. *Vaccine.* **18**: 209–215 (1999).
5. M. L. Hedley, J. Curley, and R. Urban. Microspheres containing plasmid-encoded antigens elicit cytotoxic T- cell responses. *Nature Med.* **4**:365–368 (1998).
6. Y. Men, H. Tamber, R. Audran, B. Gander, and G. Corradin. Induction of a cytotoxic T lymphocyte response by immunization with a Malaria specific CTL peptide entrapped in biodegradable polymer microspheres. *Vaccine.* **15**:1405–1412 (1997).
7. M. J. Corbel. Control testing of combined vaccines: a consideration of potential problems and approaches. *Biologicals.* **22**:353–360 (1994).
8. J. D. Clemens, C. Ferreccio, M. M. Levine, I. Horwitz, M. R. Rao, K. M. Edwards, and B. Fritzell. Impact of Haemophilus influenzae type b polysaccharide-tetanus protein conjugate vaccine on responses to concurrently administered diphtheria-tetanus-pertussis vaccine. *JAMA.* **267**:673–678 (1992).
9. S. A. Halperin, J. M. Langley, and B. J. Eastwood. Effect of inactivated poliovirus vaccine on the antibody response to Bordetella pertussis antigens when combined with diphtheria-pertussis-tetanus vaccine. *Clin Infect Dis.* **22**:59–62 (1996).
10. K. Redhead, D. Sesardic, S. E. Yost, A. M. Attwell, J. Watkins, C. S. Hoy, J. E. Plumb, and M. J. Corbel. Combination of DTP and Haemophilus influenzae type b conjugate vaccines can affect

laboratory evaluation of potency and immunogenicity. *Biologicals.* **22**:339–345 (1994).
11. P. Johansen, Y. Men, R. Audran, G. Corradin, H. P. Merkle, and B. Gander. Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives. *Pharm Res.* **15**:1103–1110 (1998).
12. G. Ashwell. Colorimetric analysis of sugar. *Meth Enzymol.* **3**:73–105 (1957).
13. P. Johansen, H. Tamber, H. P. Merkle, and B. Gander. Diphtheria and tetanus toxoid microencapsulation into conventional and end-group alkylated PLA/PLGAs. *Eur J Pharm Biopharm.* **47**: 193–201 (1999).
14. P. Johansen, H. P. Merkle, and B. Gander. Physico-chemical and antigenic properties of tetanus and diphtheria toxoids and steps towards improved stability. *Biochim Biophys Acta.* **1425**:425–436 (1998).
15. A. C. Chang and R. K. Gupta. Stabilization of tetanus toxoid in poly(DL-lactic-co-glycolic acid) microspheres for the controlled release of antigen. *J Pharm Sci.* **85**:129–132 (1996).
16. D. H. Jones, B. W. McBride, H. Jeffery, D. T. O'Hagan, A. Robinson, and G. H. Farrar. Protection of mice from Bordetella pertussis respiratory infection using microencapsulated pertussis fimbriae. *Vaccine.* **13**:675–681 (1995).
17. R. Shahin, M. Leef, J. Eldridge, M. Hudson, and R. Gilley. Adjuvanticity and prrotective immunity elicited by Bordetella pertussis antigens encapsulated in poly(D,L-lactide-co-glycolide) microspheres. *Infect Immun.* **63**:1195–1200 (1995).
18. M. A. Conway, L. Madrigal-Estebas, S. McClean, D. J. Brayden, and K. H. G. Mills. Protection against Bordetella pertussis infection following parenteral or oral immunization with antigens entrapped in biodegradable particles: effect of formulation and route of immunization on induction of Th1 and Th2 cells. *Vaccine.* **19**:1940–1950 (2001).
19. P. Johansen, H. P. Merkle, and B. Gander. Technological considerations related to the up-scaling of protein microencapsulation by spray-drying. *Eur J Pharm Biopharm.* **50**:414–417 (2000).
20. S. N. Timasheff and T. Arakawa. Stabilization of protein structure by solvents. In T. E. Creighton (ed.), Protein structure: a practical approach, Oxford, U.K. IRL Press, 1989, pp 331-345.
21. P. Johansen, F. Estevez, R. Zurbriggen, H. P. Merkle, R. Glück, G. Corradin, and B. Gander. Towards clincial testing of a single-administration tetanus vaccine based PLA/PLGA microspheres. *Vaccine.* **19**:1047–1054 (2000).
22. K. F. Griffin, B. R. Conway, H. O. Alpar, and E. D. Williamson. Immune responses to V antigen of Yersinia pestis co-encapsulated with IFN-gamma: Effect of dose and formulation. *Vaccine.* **16**:517–521 (1998).
23. D. E. Rafferty, M. G. Elfaki, and P. C. Montgomery. Preparation and characterisation of a biodegradable microparticle antigen/cytokine delivery system. *Vaccine.* **14**:532–538 (1996).
24. I. D. Spiers, H. O. Alpar, J. E. Eyles, A. Bozkir, J. Miller, and E. D. Williamson. Studies on the co-encapsulation, release and integrity of two subunit antigens: rV and rF1 from *Yersinia pestis*. *J Pharm Pharmacol.* **51**:991–997 (1999).
25. J. E. Eyles, E. D. Williamson, I. D. Spiers, A. J. Stagg, S. M. Jones, and H. O. Alpar. Generation of protective immune responses to plague by mucosal administration of microsphere co-encapsulated recombinant subunits. *J. Controlled Release.* **63**: 191–200 (2000).
26. S. M. Kaech and R. Ahmed. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nature Immunol.* **2**:415–422 (2001).
27. P. Kourilsky and A. A. Lindberg. Immunoprevention and therapy, *Nature Biotechnol.* **17**:Suppl. BV21-22 (1999).