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Comparison of biodegradable nanoparticles and multiple emulsions (water-in-oil-in-water) containing influenza virus antigen on the *in vivo* immune response in rats

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Multiple water-in-oil-water (w/o/w) emulsion and polymeric nanoparticle formulations containing influenza virus surface antigen hemagglutinin (HA) are thought to be suitable carriers for a vaccine delivery system. The multiple emulsion technique leads to high entrapment of HA, while the solvent evaporation technique encapsulates and adsorbs HA within the nanoparticle. Immune responses of these formulations were investigated in rats and compared with the immune response raised against the conventional vaccine. The responses were detected with the hemagglutinin inhibition (HAI) assay. A single administration of multiple emulsion (F1, F2, F3) and nanoparticle (F4) formulations proved to stimulate a more effective immune response in rats than conventional vaccine.

The effectiveness of influenza vaccine in reducing morbidity and mortality in children, elderly or debilitated patients has been estimated at 30–90% in several studies (Lemonie and Preat 1998; Podda 2001). Inactivated hemaglutinin (HA) is poorly immunogenic and usually requires several (booster) injections to confer adequate protection. To ensure reliable protection additional features are required to increase the immunogenicity. The search for effective vaccines for influenza focused on improving new adjuvants or vaccine delivery systems (Podda 2001; O'Hagan et al. 2001). There have been several reports on oil-in-water (o/w) and water-in-oil (w/o) emulsions (Hilgers et al. 1994a, 1994 b), microspheres (Coombes et al. 1998; Chattaraj et al. 1999) and liposomes (Hilbert et al. 1999) for influenza immunization. W/o/w multiple emulsions and polymeric nanoparticles which could be suitable delivery systems for influenza virus antigens, have not been studied in detail. The principal objective of this study was to investigate the immune response in rats against influenza antigen-loaded with both multiple emulsion and polymeric nanoparticle in comparison to the conventional vaccine.

A w/o/w multiple emulsion technique most suitable for achieving high entrapment of hydrophilic HA was used for encapsulation. Also an alternative approach was used, which would encapsulate and adsorb the antigen to the surface of a biodegredable polymer, and might be a more suitable delivery system for influenza vaccination. Trivalent influenza vaccine, Fluarix (a gift from Glaxo Smith Kline) recommended for the 2001–2002 season, each 0.5 mL containing 15 μg each of hemagglutinin antigens of A/Moscow/10/99 (H_3N_2), A/New Caledonia/20/99 (H_1N_1) and B/Sichuan/379/99 were used for preparation of the formulations.

The split influenza vaccine contained thiomersal which interferes in the bicinchoninic acid analysis of protein (Chattaraj et al. 1999). Hence, the vaccines were dialyzed and concentrated ten-fold using a Vivaspin 20 (10.000 MWCO Vivascience, Sartorius) at 2–4 °C. Concentrated antigen dispersions were washed with sterile phosphate buffered saline (PBS; pH 7.4) and again concentrated in order to remove the other ingredients.

Multiple emulsions were prepared as described previously (Bozkir et al. 2003). Firstly, homogenization is used to obtain a fine, homogeneous and stable w/o emulsion. Secondly, a continuous phase is poured into a dispersed phase in order to prepare a w/o/w emulsion. In general, a dispersed phase is poured into a continuous phase. Three stable and effective formulations were selected for this study (Bozkir et al. 2003), F1: purified antigen solution (PAS)/soybean oil, Cremophor RH-40 (HCO-40) and span 80/pluronic F-68, F2: PAS and hydroxypropyl-β-cyclodextrin/soybean oil, HCO-40 and span 80/pluronic F-68, F3: PAS/squalane, HCO-40 and span 80/pluronic F-68.

Several studies have shown the importance of the particle size for uptake. Jani et al. (1989) showed that an optimal particle uptake was observed with particles below 1 μm. Thus, a nanoparticle formulation (F4) was prepared by solvent evaporation technique with the modifications previously described (Bozkir and Saka 2002). The solvent evaporation technique has been used succesfully by several groups to entrap proteinic materials into PLGA nanoparticles and microparticles (Bozkir and Saka 2002, Coombes et al. 1998). Briefly, poly-(D,L-lactide-co-glycolide) (PLGA) was dissolved in dichloromethane 1% (w/v). This was emulsified in an aqueous phase containing poly(vinylalcohol) 0.4% (w/v) and HA (7.4 μg/mL) with a homogenizer at 10.000 rpm. The organic solvent was evaporated under reduced pressure. All preparation processes were carried out under aseptic conditions.

Randomly selected wistar albino rats (200–250 g) were pooled into 4 groups of 8 animals. Three groups were vaccined with emulsion formulations and one group with nanoparticle, while the other group was treated as a control group with a conventional vaccine. Each immunization dose contained 45 μg of total viral protein. Blood was collected (~ 1 mL) from the tail veins of rats before and at selected times (28 and 56 days) after immunization. The blood samples were centrifuged at 3000 rpm at room temperature for 15 min. Serum hemagglutination inhibition (HAI) assay were performed according to standard procedure (U.S. Department of Health, 1975). HAI titres were determined in order to identify the serum that inhibits the agglutination completely.

HAI test datas were evaluated by considering the 4 times titre increase of the immune response produced by F1, F2, F3 and F4 formulations and conventional vaccine on week 4 and week 8 significant, the following percent values were calculated for the strains in the Table and the Figure.

The Wilcoxon test was used to determine whether there were any statistically significant differences within each formulation between pre-immunization, week 4 and week

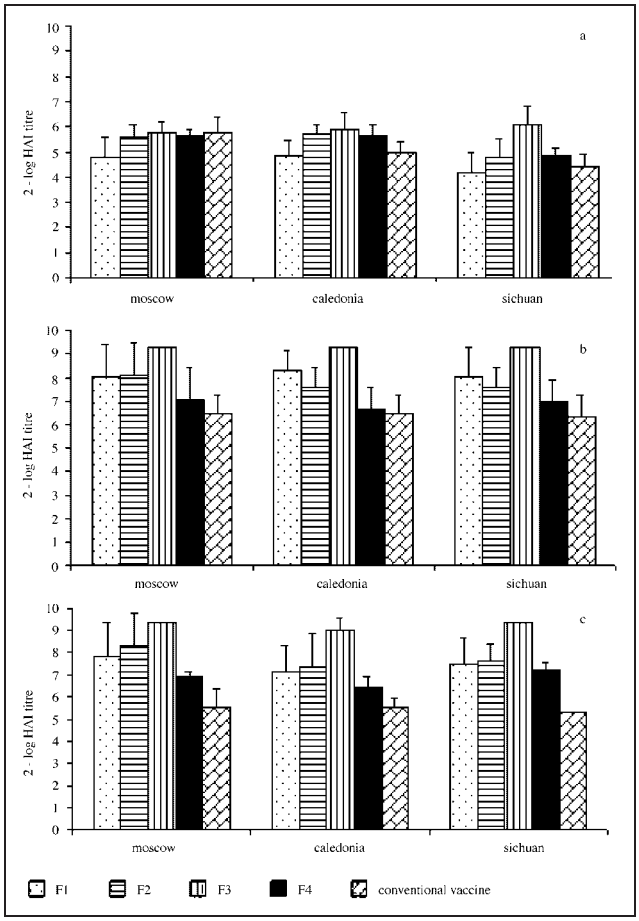

Fig.: IgG titres of formulations a: before vaccination, b: vaccination after 28 days, c: vaccination after 56 days

8 HAI data. The presence of significant differences in all 3 formulations between pre-immunization – week 4 and pre-immunization – week 8 data shows that there was a change in antibody titres formed against influenza as a result of immunization ($p < 0.05$). On the other hand there is a stastistical difference between pre-immunization – week 8 data for nanoparticles, indicating that PLGA proved suitable for the encapsulation of hydrophilic antigen leading to a continuous release from the nanoparticle. However, the immunogenicity level was lower than with multiple emulsions (Table). This situation could be explained according to Hilbert et al. (1999) by the fact that small particles prepared by an emulsion-based solvent evaporation procedure failed to generate an adjuvant effect after immunization. In addition, nanoparticles (0.650 ± 0.076 μm in diameter) are taken up by macrophages more easily than multiple emulsion formulations (8.38 ± 0.92, 7.92 ± 0.71, 17.84 ± 0.15 μm in diameter, respectively), so more nanoparticles were phagositosed than emulsions. Eldridge et al. (1990) mentioned that to have a more effective immunization for nanoparticle formulation, another administration route may be suitable. The multiple emulsion preparation method involves antigen in the inner water phase, so the antigen entrapment efficiency was extremely high (98.73%, 98.27%, 99.44%, respectively). On the other hand the antigen entrapment in nanoparticles (74.21%) was lower than in emulsions. Therefore, initial burst release from nanoparticle suspension was expected by adsorbed and unentrapped antigen, so that nanoparticles give lower immunization than emulsion formulations (Fig.). Chattaraj et al. (1999) pointed out that nanoparticle formulations show a maximum availability of the antigen immunity with subcutaneous injection followed by oral administration. At the end of week 8, F1 and F2 immunization levels were equal to those achieved with nanoparticles (Table), and this may be explained by the mechanism of adjuvant effects. Hydrophobicity increased the adjuvant effect of particulate polymeric devices (Hilbert et al. 1999). Also antigen was released from the nanoparticles as a result of the diffusion through matrix pores, matrix degredation and collapse, succeeding to form continuous release in nanoparticle formulation. Also Chattaraj et al. (1999) indicated that with subcutaneous immunization alone, the polymer degrades for a long time giving better Ig G response. As for the conventional vaccine, there was no statistical difference between pre-immunization – week 4 and pre-immunization – week 8 data ($p > 0.05$) according to the Wilcoxon test. This means, a single administration of conventional vaccine does not lead to complete immunization in rats.

The Kruskal Wallis test was used to determine whether there were any statistically significant differences between the groups in terms of immune responses produced by the formulations on week 4 and week 8 (Table). According to this test, the highest antibody titre on week 4 and week 8 and for all 3 strains was obtained with formulation F3. A decrease was observed in antibody titres of F1 and F2 formulations on week 8. A significant increase was observed in antibody titres of formulation F4 on week 8, because of continuous release from the nanoparticles. Finally, multiple emulsions show a more effective immune response than nanoparticles. Morever, a single administration of entrapped influenza HA in all formulations proved to stimulate a more effective immune response in rats than a conventional vaccine.

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Table: Evaluation of the HAI data with Wilcoxon and Kruskal Wallis tests according to different influenza strains

Time*	Influenza strains	Wilcoxon test datas	Kruskal Wallis test datas
Week 4	Moscow	F2 > F3 > F1 > F4 > CV**	F3 > F2 = F1 > F4 > CV
	Caledonia	F2 > F3 > F1 > F4 = CV	F3 > F1 = F2 > F4 = CV
	Sichuan	F3 = F2 > F4 = F1 > CV	F3 > F1 > F2 > F4 = CV
Week 8	Moscow	F3 > F2 > F1 > F4 > CV	F3 = F2 > F1 > F4 > CV
	Caledonia	F3 = F2 > F1 > CV > F4	F3 > F2 = F1 = F4 > CV
	Sichuan	F3 = F2 > F1 > F4 > CV	F3 > F1 = F2 = F4 > CV

* Correlation with pre-immunization
** Conventional vaccine

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Calcium sulphate dihydrate: an useful excipient for tablets containing labile actives

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Tablets containing an angiotensin-converting enzyme inhibitor and either calcium sulphate dihydrate or lactose monohydrate as main excipients (fillers) exhibited similar technical properties and stability.

Most angiotensin-converting enzyme inhibitors (e.g. enalapril, moexipril, quinapril, ramipril; hereafter called prils) are inherently prone to degradation in solid dosage forms. The main degradates are diketopiperazine derivatives (DKPs) and prilats. The former arise from an intramolecular nucleophilic attack of the secondary amino nitrogen in the aliphatic chain on the carboxylic acid carbon resulting in expulsion of water, formation of a N−C-bond and cyclization whereas the latter (prilats) are hydrolysis products of the carboxylic ethyl ester vector in the drug molecules (Gu and Strickley 1987). Formation of DKPs may be arrested or minimized for example by adding basic reagents, for instance sodium hydrogen carbonate, to the formulation that transform the carboxylic acid moiety into a carboxylate anion (Gu et al. 1990), and degradation to prilats may be reduced by keeping moisture (water) content as low as possible in the tablets. It has been claimed that excipients belonging to the chemical classes of mono- and disaccharides or sugar alcohols like lactose or mannitol have stabilizing effects on prils in solid dosage forms (Harris et al. 1993).

Calcium sulphate dihydrate ($CaSO_4$, $2H_2O$) is available from Penwest Ltd. as a specially-processed direct compression grade named Compactrol. It is a white or off-white, odourless, non-hygroscopic, free flowing powder, slightly soluble (1 : 375) in water, average particle size 120 μm, bulk density not more than 1.10 g/ml, tapped density 0.90–1.35 g/ml (Moreton 2003). We have found pH approx. 6.8 in a 10% aqueous slurry and loss of drying approx. 0.2% (IR moisture balance, 105 °C) in Compactrol samples.

In oder to investigate the technical properties and stability of pril-containing tablets using calcium sulphate dihydrate (Compactrol) as main excipient (filler) a trial batch was prepared (Formulation C) and for comparison another batch (Formulation L) employing lactose monohydrate. The compositions of these two formulations are displayed in Table 1.

Batch sizes were 8.5 kg = 50,000 tablets (formulation C) and 6.5 kg = 50,000 tablets (formulation L). Mixing and granulation was carried out in an intensive mixer. Following drying at 45 °C to a specified loss of drying of not more than 0.80% (IR moisture balance, 100 °C)