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Release kinetics and immunogenicity of parvovirus microencapsulated in PLA/PLGA microspheres

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Abstract

The aim of this work was to examine the immunogenicity of microencapsulated inactivated duck parvovirus in Muscovy duck (*Cairina moschata*) and goose. Inactivated duck parvovirus suspension was microencapsulated into 14–17 kDa poly(lactide) (PLA) and poly(lactide-co-glycolide) (PLGA50:50H) by coacervation. The in vitro antigen release from individual and mixed PLA and PLGA50:50H microspheres (MS) was biphasic with an initial lag-phase of approx. 10 days followed by a relatively constant release over additional 12 days. By varying the composition of PLA + PLGA50:50H MS mixtures from 3 + 1 to 1 + 3, the release kinetics could be altered and controlled efficiently. The antigen-loaded MS were injected subcutaneously into ducks. The immune response, expressed as virus neutralisation (VN) titres, after single administration of MS was modest, i.e. below 200 over the 6 weeks tested, unless the animals were pre-immunised 3 weeks before injecting the MS. The weak immune response was attributed to the low dose injected and inappropriate antigen release kinetics. With pre-immunised animals, however, the results were encouraging and showed that the encapsulated parvovirus was immunogenic.

Keywords: Duck parvovirus; Virus microencapsulation; Biodegradable microspheres; Virus neutralisation; Antigen delivery system

1. Introduction

Biodegradable poly(lactide) (PLA) and poly(-lactide-co-glycolide) (PLGA) microspheres (MS) have been widely studied for delivering and adjuvanting a large number of antigens, including virus antigens (Johansen et al., 2000). However, none of these earlier studies has considered, to our knowledge, duck parvovirus. Parvoviruses are small icosahedral single-stranded viruses (18–26 nm), which replicate in rapidly proliferating cells, causing a variety of severe and often lethal diseases in mammals, including humans (Zadori et al., 1995). Parvovirus replicates in the nucleus of

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erythroid precursor cells and causes cell lysis and transient cessation of red cell production. As a consequence, parvoviruses can cause myocarditis, hepatitis in fetal or neonatal animals, and reduction of circulating lymphocytes in older animals (Derzsy, 1967; Schettler 1971; Kisary, 1993). This so-called Derzsy's disease in geese and Muscovy ducks is prevalent in most large scale breeding and production facilities (Kisary, 1993). Gooslings can be passively immunised with a hyperimmune serum (Derzsy et al., 1970). Alternatively, the offspring can receive yolk-derived antibodies from actively immunised laying geese (Kisary et al., 1978). Serum treatment is expensive and laborious, and passively acquired antibodies are rapidly eliminated (Hoekstra et al., 1973; Kisary, 1993).

In this study, we aimed at enhancing and prolonging the immunogenicity of inactivated parvovirus by microencapsulation technology.

2. Materials and methods

One millilitre of an aqueous suspension of inactivated duck parvovirus (ELISA titre: 447) or its lyophilised form was microencapsulated into end-group capped PLA or uncapped PLGA50:50H (Resomer® 202 and Resomer® RG 502H, respectively, from Boehringer-Ingelheim, Ingelheim, Germany) by coacervation, as described by Thomasin and Gander (1997). The size distribution of the microspheres was determined by laser light scattering (Mastersizer X Malvern, UK).

The parvovirus content in the MS was measured after dissolving approx. 50 mg of MS in dichloromethane (DCM) and collecting the insoluble virus particles on a 0.2 μm regenerated filter. The filters were rinsed thrice with DCM, air-dried, and the virus particles eluted with a pH 7.5 phosphate buffered saline containing polysorbate 20 (PBS-T-N); the PBS-T-N consisted of 29 g/l NaCl, 0.20 g/l KCl, 1.14 g/l $Na_2HPO_4 \cdot 2H_2O$, 0.20 g/l KH_2PO_4, 0.35 g/l polysorbate 20. The aqueous virus suspension was analysed by ELISA.

Parvovirus in vitro release was determined from 200 mg MS incubated in 2.0 ml of 67 mM PBS of pH 7.4, containing 0.2% bovine serum albumin (BSA) and 0.02% sodium azide, in rotating (3 rpm) borosilicate vials at 37°C (Thomasin et al., 1996). At regular intervals, the samples were centrifuged (3500 rpm for 10 min), 200 μl of medium withdrawn and assayed by ELISA. At each sampling, the release medium was replaced by fresh medium.

The antigen was assayed by an enzyme-linked immunosorbent assay (ELISA) using flat-bottom 96-wells microtitre plates (Nunc-Immuno Plate Maxisorb™, Nunc, DK-Roskilde). The wells were filled with 100 μl per well of antiparvo goose IgG (Phylaxia-Ceva, H-Budapest) diluted 1:5000 in 0.05 M carbonate puffer of pH 9.6, and incubated at 37°C for 60 min. After each incubation step, the plates were washed four times with 300 μl of 67 mM PBS of pH 7.4 containing 0.05% polysorbate 20. After 1 h incubation at 37°C with 100 μl serial dilutions of standard and test suspensions of parvovirus in PBS-T-N (see above), the plates were incubated at 37°C for 60 min with 100 μl of Derzsy-conjugatum (Phylaxia-Ceva, H-Budapest) diluted 1:2000 in PBS-T-N containing 0.05% of lactose monohydrate. Extravidin peroxidase conjugate (ExtrAvidin, Sigma, D-Steinheim) diluted 1:2000 in PBS-T-N was added to each well (100 μl), and plates were incubated at 37°C for 30 min. Finally, 200 μl of 0.1 M sodium acetate buffer (pH 5.5) containing 15% of 3,3′,5,5-tetramethylbenzidine dihydrochloride (Sigma, D-Steinheim), 5% of dimethylsulphoxide (Fluka, CH-Buchs) and 0.02% of hydrogenperoxid 30% were added to the plates. After 30 min at room temperature in a dark place, the reaction was stopped by adding 50 μl of 5.0 M sulfuric acid to each well. The optical densities were measured at 450 nm in a plate reader (Thermomax, Molecular Devices, Menlopark, USA).

Muscovy ducks, 7 weeks of age, were immunised with a total amount of 100 μg of MS (equivalent to an ELISA titre of 29) by subcutaneous injection in the neck. For injection, the parvovirus-loaded MS were suspended in 1 ml of a 5% lecithin solution (Phospholipon 90, Rhone-Poulenc Rorer, D-Cologne). One group of ducks was pre-immunised with a life virus vaccine (Deparvac® Phylaxia-Ceva, H-Budapest) at 3 weeks

of age. A third group was treated with the Deparvac® vaccine alone.

The neutralising specific serum antibody titres were measured in flat bottom 96-well microtitre plates (Nunclon™ Delta, MicroWell Plate, Nunc) filled with 100 μl per well of 2.5×10^5 cell per ml of primary goose embryo fibroblasts (Phylaxia-Ceva, H-Budapest) in 10% calf serum (Gibco BRL, A-Wien), containing a nutritional fluid (8.0 g/l NaCl, 0.4 g/l KCl, 0.06 g/l $Na_2HPO_4 \cdot 2H_2O$, 0.06 g/l KH_2PO_4, 0.18 g/l $CaCl_2$, 0.2 g/l $MgSO_4$, 1.10 g/l glucose monohydrate, 0.04 g/l L-tyrosin, 0.02 g/l L-cystin, 0.50 g/l $NaHCO_3$ sodium hydrogen carbonate, and 0.56 ml/l of 1 N HCl). The fibroblasts were incubated with 100 μl per well of 1:1 mixtures of serial dilutions of standard or test immunsera in nutritional fluid with the attenuated parvovirus strain B (titer: 100 tissue culture infectious dose, $TCID_{50}$/0.1 ml); appropriate assay controls were made. After 6 days incubation at 37°C in 3–5% CO_2, the cytopathogenic effect was observed microscopically and evaluated according to an established method (Villegas, 1998). The data were analysed using Wilcoxon test. Data were considered statistically significant if $P < 0.05$.

3. Results and discussion

The inactivated parvovirus particles were microencapsulated into PLA and PLGA50:50H at efficiencies of 43 and 60%, respectively, relative to the nominal loading (447 ELISA titre). The size of the PLGA50:50H MS was smaller ($d_{50\%} = 21.7$ μm; $d_{10\%} = 10.43$ μm; $d_{90\%} = 51.21$ μm) than that of the PLA MS ($d_{50\%} = 43.8$ μm; $d_{10\%} = 12.37$ μm; $d_{90\%} = 82.08$ μm).

The in vitro release of antigenic material showed a biphasic pattern: an initial, almost release-free interval of approx. 8 days, which was independent of the polymer type used, was followed by a second relatively continuous release phase (Fig. 1). This type of release profile differs from the triphasic profiles often reported for peptide and protein release from PLA and PLGA MS (Sah and Chien, 1993; Thomasin et al., 1996). Speculatively, this viral antigen might interact so strongly with the polymer matrix that it is released only after a certain extent of matrix erosion. It is known that PLA and PLGA mass erosion is slow during the initial phase of incubation, and then accelerates upon autocatalytic polymer hydrolysis (Hora et al., 1990). In the

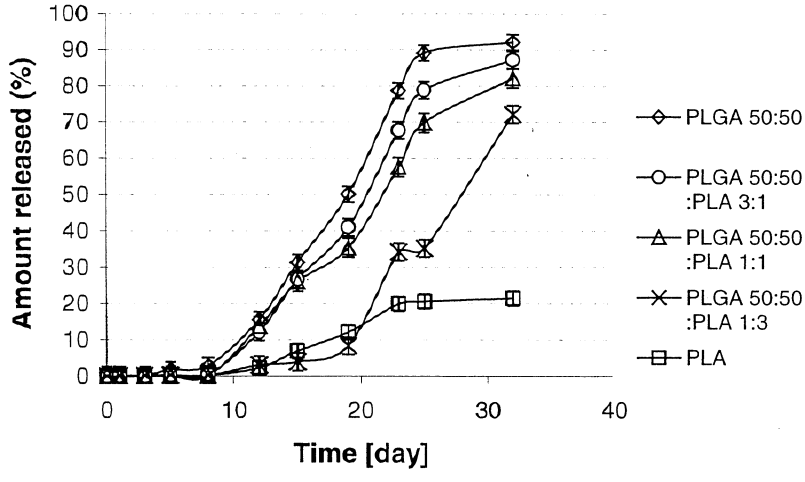

Fig. 1. In vitro release profiles of inactivated parvovirus from coacervated PLA and PLGA50:50H microspheres and from physical mixtures of the microspheres.

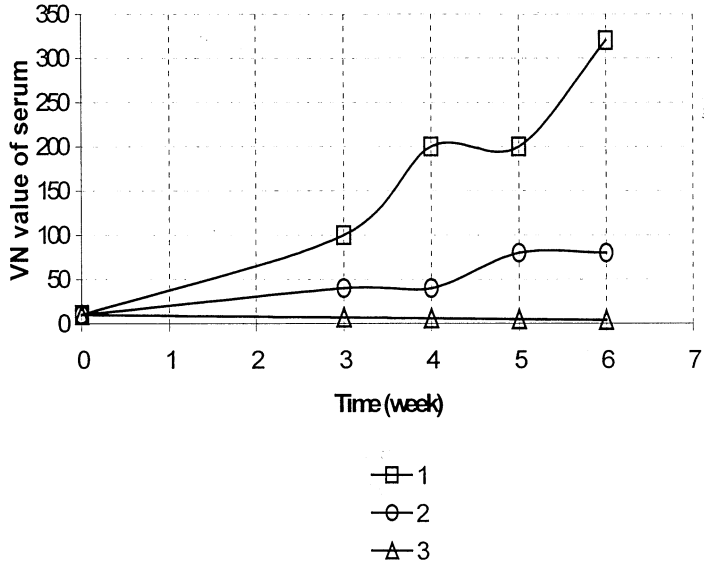

Fig. 2. Virus neutralisation (VN) serum titres of Muscovy ducks after a single immunisation with parvovirus microencapsulated in PLGA50:50H at an age of 7 weeks. At all time points between weeks 3 and 7, the VN-values of the test groups (1) and (2) (see below) were significantly superior ($P < 0.05$) to the VN-values of the control group (3). Key; (1) Ducks pre-immunised with live virus vaccine at 3 weeks of age, and boosted with microencapsulated parvovirus at 7 weeks of age; (2) Ducks immunised with a single injection of microencapsulated parvovirus at 7 weeks of age, without pre-immunisation; (3) Ducks immunised with a single injection of the live virus vaccine (Deparvac®) at 3 weeks of age.

second release phase, i.e. between day 8 and 32, the more hydrophilic PLGA50:50H MS released approx. 90% and the PLA MS 20% of the inactivated parvovirus. The release profiles of the 3 + 1, 1 + 1 and 1 + 3 (PLA + PLGA50:50H) MS mixtures lay in-between those of the single MS types. While the single PLGA 50:50H MS and the 1 + 3 and 1 + 1 (PLA + PLGA50:50H) MS mixtures started to release substantial amounts of antigen on day 8, the 3 + 1 (PLA + PLGA50:50H) MS mixture showed a comparable steady increase in release only after day 18. After this onset, similar release rates were observed. On day 32, the total amount of antigenic material released from the PLGA50:50H MS and from the 1 + 3, 1 + 1 and 3 + 1 (PLA + PLGA50:50H) MS mixtures was 92, 87, 81 and 72%, respectively.

Immunisation of the ducks with antigen-loaded MS produced a steady increase in virus neutralisation (VN) titre over the 6 weeks of study. In the pre-immunised ducks, the 4–6 weeks titres were approx. 4-fold higher than those of the non pre-immunised group (Fig. 2). The lowest VN titres were observed in the ducks treated with a single injection of live virus vaccine. Interestingly, the VN-titres remained rather low for up to 3 weeks after MS-injection, possibly reflecting partly the release-free interval observed in vitro. After 3 weeks the VN titres increased substantially.

Maternal VN-titres of minimum 1:200 or VN-titres of minimum 50 at hatching afford adequate maternal protection to goslings and ducklings up to the end of the laying season (Kelemen et al., 1997). With the MS used in pre-immunised animals, the VN titres achieved this value only after 4 weeks. This is not entirely satisfactory and may be ascribed to, e.g. (i) a reduced immunogenicity of encapsulated antigenic material; (ii) inappropriate release kinetics; (iii) insufficient antigen dose, which was only approx. 1/3 of the dose commonly used in immunisation of ducks. This low dose was a constraint of the moderate antigen content in the MS; the amount of MS injected here was at the upper limit of syringeability. The moderate

antigen loading in turn was due to the relatively low antigen concentration of the virus suspension used, which contained, besides the antigenic material, substantial amounts of non-viral material from the allantois fluid. According to these results, further development is required to optimise this dosage form so that it would be suitable to induce a faster, more elevated and long-lasting immune response.

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References

Derzsy, D., 1967. A viral disease of goslings. I. Epidemiological, clinical, pathological and aetiological studies. Acta Vet. Acad. Sci. Hung. 17, 443–448.

Derzsy, D., Drén, Cs., Szedo, M., Surján, J., Tóth, B., Iró, E., 1970. Viral disease of goslings III. Isolation, properties and antigenic pattern of the virus strains. Acta Vet. Acad. Sci. Hung. 20, 419–428.

Hoekstra, J., Smit, T.H., Van Brakel, C., 1973. Observations on host range and control of goose virus hepatitis. Avian Pathol. 2, 169–178.

Hora, M.S., Rana, R.K., Nunberg, J.H., Tice, T.R., Gilley, R.M., Hudson, M.E., 1990. Release of human serum albumin from poly(lactic acid) and poly(lactic-co-glycolide) microspheres. Pharm. Res. 7, 1190–1194.

Johansen, P., Men, Y., Merkle, H.P., Gander, B., 2000. Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. Eur. J. Pharm. Biopharm. 50, 129–146.

Kelemen, M., Nagy, J., Erdei, J., 1997. Possibilities of prevention of Derzsy's Disease. Magyar Állatorvosok Lapja 12, 732–736 In hungarian, with english abstract.

Kisary, J., 1993. Derzsy's disease of geese. In: McFerran, J.B., McNulty, M.S. (Eds.), Virus Infection of Birds. Elsevier Science Publishers, Amsterdam, pp. 157–162.

Kisary, J., Derzsy, D., Mészáros, J., 1978. Attenuation of the goose parvovirus strain B. Laboratory and field trials of the attenuated mutant for vaccination against Derzsy's disease. Avian Pathol. 7, 397–406.

Sah, H.K., Chien, Y.W., 1993. Evaluation of a microreservoir type biodegradable microcapsule for controlled release of proteins. Drug Dev. Ind. Pharm. 19, 1243–1263.

Schettler, C.H., 1971. Virus hepatitis of geese. II. Host range of goose hepatitis virus. Avian Dis. 15, 323–325.

Thomasin, C., Merkle, H.P., Gander, B., 1996. Physico-chemical parameters governing protein microencapsulation into biodegradable polyesters by coacervation. Int. J. Pharm. 147, 173–186.

Thomasin, C., Merkle, H.P., Gander, B., 1997. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response. J. Controlled Release 41, 131–145.

Villegas, P., 1998. Titration of biological suspension. In: Swayne, D.E., Gilson, J.R., Kackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens. American Association of Avian Pathologists, Kennett Square, PA, pp. 45–253.

Zadori, Z., Stefancsik, R., Rauch, T., Kisary, J., 1995. Analysis of the complete nucleotide sequences of goose and muscovy duck parvoviruses indicates common ancestral origin with adeno-associated virus 2. Virology 212, 562–573.