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Adjuvant activity of linear aliphatic polyester and branched aliphatic oligoester microspheres

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Abstract

Bovine serum albumin (BSA) was entrapped into microspheres prepared of terpolymer of DL-lactic acid, glycolic acid and mannitol (GA-M-DLLA), copolymer of DL-lactic acid and mannitol (M-DLLA) and two lactido-glycolide copolymers (DL-PLGA) by the solvent evaporation emulsion technique ($w_1/o/w_2$). Release of BSA from the microspheres into PBS, pH 7.2, or porcine blood serum was examined for 15 weeks and the effects of blood serum components on the degradation of the microspheres were recorded. Subcutaneous administration of a single dose of the microspheres containing 5 μg BSA to mice elicited biphasic immune responses irrespective of the type of the microspheres. Although the interval between the two phases was shorter in the mice treated with the terpolymer microspheres, adjuvant potencies, assessed in terms of antibody levels, were similar for all the four types of the microspheres.

Keywords: Antibody response; Branched oligoester structures; BSA release; Immunization; Mice; Polyesters

1. Introduction

The basic tools of the specific prophylaxis of infectious diseases of farm animals are live or inactivated vaccines inducing specific protective immunity. The well documented weak immunogenic activity of inactivated or subunit antigens has motivated efforts to enhance the protective effects of such biologicals by completing them with adjuvants. The conventional Freund's adjuvant or aluminium hydroxide are being replaced with new antigen carriers, such as liposomes (Gregoriadis, 1990), microparticles (Kreuter et al., 1986), or ISCOM-type structures (Morein et al., 1984), often combined with immunostimulants

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Table 1
Basic data of synthesized vehicles

Vehicle	Monomer ratio			Tg (°C)	Mn (Kda)	MW (kDa)	MW/Mn (kDa)
	LA (mol)	GA (mol)	MA (mol)				
M-DLLA	99.30	0	0.7	34	2.85	5.30	1.86
GA-M-DLLA	45.05	45.06	0.9	20	2.20	3.95	1.80

based on muramyldipeptides (Ullrich and Fidler, 1992), lipid A (Verma et al., 1992; Alving, 1993), or aviridine (Fatunmbi et al., 1992), or with other immunomodulants.

Recent studies of biologically compatible and degradable microspheres (msp), prepared of synthetic polymers, have revealed them as very good antigen carriers (Chandrasekhar et al., 1994; Cahill et al., 1995). Excellent adjuvant properties, exceeding those of the complete or incomplete Freund's adjuvants or aluminium hydroxide used in commercial vaccines, were demonstrated particularly in polymers and copolymers of lactic and glycolic acids used in the preparation of msp (Eldridge et al., 1991; O'Hagan et al., 1991; Men et al., 1995; Sah et al., 1995).

In this paper, the release and adjuvant activity of msp with incorporated BSA, prepared of new polymer structures-terpolymers-were compared with those of msp prepared of aliphatic copolymers of lactic and glycolic acids (DL-PLGA). A different release mechanism, possibly influencing the course of the immune response, is presumed in msp prepared from terpolymers, in which linear polymers are bridged by mannitol (Kissel et al., 1991). No data on this topic have been found in available literature.

2. Experimental

2.1. Materials

Polyvinylalcohol (PVA) MW 85–146 kDa, 86–89% hydrolyzed, was purchased from Aldrich, USA and dichloromethane (DCM) from Sigma-Aldrich, UK. Bovine serum albumin (BSA) was supplied by USOL, Czech Republic, and DL-PLGA with the copolymer ratio 50/50 (lactide/glycolide%) and MW 50–75 kDa and DL-PLGA with the copolymer ratio 75/25 (lactide/glycolide%), MW 75–120 kDa were purchased from Sigma, USA. GA-M-DLLA and M-DLLA were prepared in our laboratory.

2.2. Preparation of oligoesters

Oligoesters were synthesized of the respective monomers (DL-lactic acid, mannitol, glycolic acid) by the polycondensation method. The reaction ran at 160°C and 550 Pa for 75 h and was catalysed by the strongly acidic cation exchanger Dowex-50 W. The resulting reaction products were purified by double non-isothermal precipitation from methanolic solutions and vacuum-dried at 40°C for 48 h.

Molecular and thermal characteristics of the oligoesters are given in Table 1.

2.3. Preparation of microspheres

Msp of the polymers were prepared using the solvent evaporation emulsion technique ($w_1/o/w_2$) as described by Jeffery et al. (1993). The procedure was as follows: 150 mg polymer were dissolved in 2.5 ml dichloromethane and 15 mg BSA along with the ^{125}I-labelled BSA tracer were dissolved in 0.25 ml distilled water. Both solutions, cooled to 4°C, were dispersed in the homogenizer Ultra-Turrax-T25 at 20 000 rpm for 1 min. The emulsion was immediatelly transferred into 5 ml 2% PVA, cooled to 4°C, and dispersed again at 20 000 rpm for 1 min. The resulting emulsion was stirred at 20°C and 300 rpm for 14 h. Then, the suspension of msp was washed twice with 25 ml distilled water. Each washing step was followed

by centrifugation at 4000 rpm. The rate of entrapment of BSA into msp was calculated from the radioactivity of the non-entrapped ^{125}I BSA in the supernatant. The separated msp were freeze-dried and kept in sealed ampoules at 4°C.

2.4. Morphology and size of microspheres

Surface smoothness and morphology of msp were examined by scanning electron microscopy using the Tesla BS 300 microscope at magnifications 1000 × and 20 000 × .

The size distribution of msp was examined with a light microscope using a pre-calibrated micrometer (Chandrasekhar et al., 1994). Diameters of 550 msp, suspended in 0.2M PBS, pH 7.4, + 0.02% Polysorbate 20, were measured and size distribution was determined by plotting the obtained data into a graphic logarithmic-probability net.

2.5. Release of BSA from microspheres

In the next step 50 mg msp, prepared of individual polymer types and containing BSA + ^{125}I BSA, were mixed in a test tube with 3 ml PBS (pH 7.2), or 3 ml porcine blood serum preserved with 0.02% sodium merthiolate. The test tubes were incubated at 37°C in nonstirred state for 15 weeks and the amount of ^{125}I BSA released into the incubation medium was measured in aliquots collected after 6, 24 and 48 h and subsequently at weekly intervals. The incubation medium was separated by centrifugation, withdrawn with a syringe and replaced with a fresh one. The cumulative growth of released BSA was calculated from the results of the radiometric measurements.

2.6. Biological experiment

Adjuvant potencies of the four types of msp were tested in 60 BALB/c mice divided into four equal groups. The mice of each group received a single subcutaneous dose of msp containing 5 μg BSA suspended in 0.2 ml PBS, pH 7.2. Groups 1, 2, 3, and 4 were treated with msp prepared from DL-PLGA (75/25), DL-PLGA (50/50), M-DLLA, and GA-M-DLLA, respectively. Three mice of each group were exsanguinated after 3, 6, 9, 12 and 15 weeks to determine the levels of antibodies to BSA.

2.7. Serology

Antibodies to BSA were determined by the conventional indirect ELISA using polystyrene microtitre plates, porcine antibodies to murine IgG, purified by affinity chromatography and labelled with horse-radish peroxidase, as the conjugate, and hydrogen peroxide and tetramethyl benzidine as the substrate. All the tests were made in duplicates at the dilution 1:100. The antibody response is expressed in terms of group means of $OD_{450} \pm$ S.E.M.

3. Results and discussion

The rates of protein incorporation and sizes of msp prepared with the four polymer types with different molecular weights using the same procedure are presented in Table 2.

It is evident that the properties of msp prepared from the terpolymer M-DLLA differ considerably from those of the three remaining msp types showing the highest BSA entrapment rate and the smallest size. The merits were apparently due to a higher surface activity of the compound with a shorter oligoester chain (Carrio et al., 1995). On the other hand, the lowest entrapment rate was observed in DL-PLGA (75/25) msp showing,

Table 2
Incorporation of BSA and size of microspheres

Vehicle/MW (kDa)	Entrapment rate (%)	d^a_{50} μm	d^b_{90} μm
M-DLLA (50/50)/5.3	60	2	3
GA-M-DLLA (50/50)/3.95	42	7	20
DL-PLGA (50/50)/50–75	39	7	21
DL-PLGA (75/25)/75–120	31	6	18

[a]50% undersize.
[b]90% undersize.

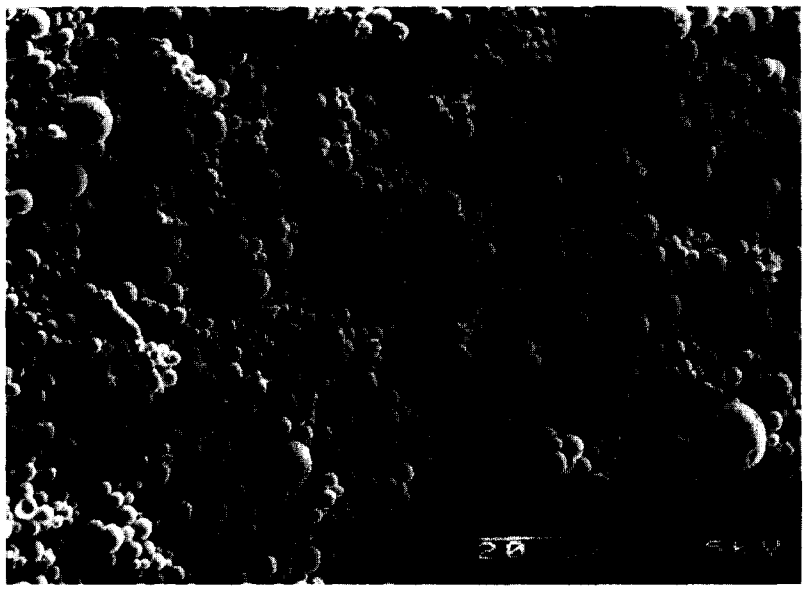

Fig. 1. Scanning electron microscopic photograph of BSA-loaded M-DLLA microspheres. (Magnification × 1000).

among the four msp types, the highest hydrophobicity and molecular weight.

Light microscopic measurements demonstrated almost identical size ranges for msp prepared of GA-M-DLLA, DL-PLGA (75/25) and DL-PLGA (50/50) with 90% of them being smaller than 21 μm.

Surface smoothness was tested by scanning electron microscopy in msp prepared of M-DLLA or DL-PLGA (75/25) showing the highest and the lowest rates of release of BSA into the PBS medium, respectively. It is evident from the micrographs that the occurrence of apparent surface defects and pores was rather rare (Figs. 1–3).

The time-release profile of BSA was investigated not only in PBS, but also in porcine serum simulating a biological environment. Conflicting views of the activity of enzymes in catalytic degradation of polymers have been postulated (Cohen et al., 1991). Prieto et al. (1994) demonstrated the release of entrapped V3 BRU protein from msp prepared of DL-PLGA (75/25) into a simulated intestinal medium containing pancreatin, but not into a simulated gastric medium containing pepsin. It is probable that the difference was due to the activity of esterases contained in pancreatin. Effects of various biological environments on the rate of carrier degradation were described also by Kamei et al. (1992).

The rate of release of BSA during the 15-week experimental period was evidently more rapid in msp incubated in blood serum than in those incubated under the same conditions in PBS, pH 7.2 (Figs. 4 and 5). The fastest release was observed in msp prepared of M-DLLA and DL-PLGA (50/50). Its profile was biphasic, consisting of an initial burst and the second phase of degradation of msp, conditioned the release of BSA. On the other hand, the release from DL-PLGA (75/25) and GA-M-DLLA msp was triphasic, including also a middle, approx. 6-week-long lag phase. The rapid release from M-DLLA msp may have been due to their small size and hence a low barrier efficiency.

The release into PBS, pH 7.2, was triphasic irrespective of the type of msp, the middle lag phase covering the period between Weeks 1 and 7. A higher initial burst was observed in msp prepared of terpolymers. Approximately 80 and 75% of the loaded BSA were released during the 15-week-long period. A similar amount (65–70%) was released from msp prepared of DL-PLGA

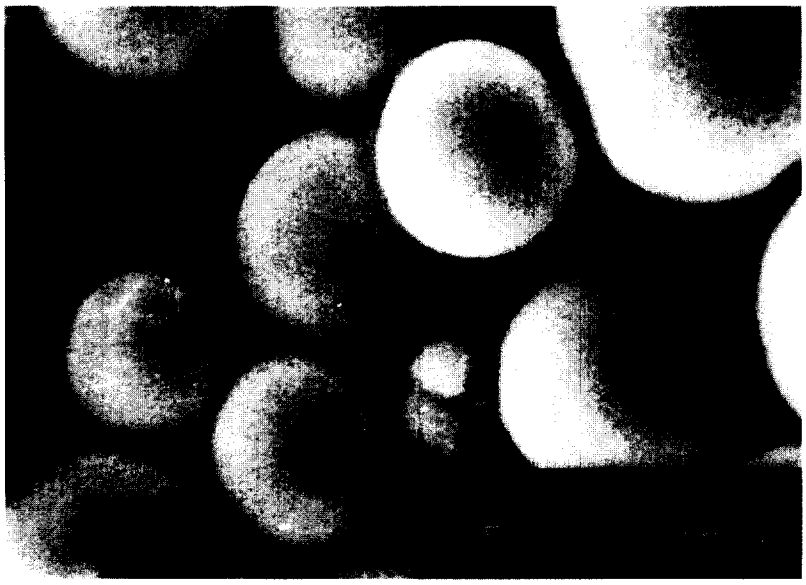

Fig. 2. Scanning electron microscopic photograph of BSA-loaded M-DLLA microspheres. Detail of surface.(Magnification × 20 000).

(75/25) irrespective of the type of the release medium. BSA release during the initial burst (first 48 h of incubation) are shown in Tables 3 and 4.

It is evident from the data presented here that, with the exception of PLGA (75/25) msp, the initial burst for the primary antigenic stimulus was very intensive. The large amount of BSA released during the initial phase of incubation can be attributed to a heterogeneous structure of msp prepared by the emulsion ($w_1/o/w_2$) method and, in the case of M-DLLA msp, also to a higher BSA entrapment rate (Bodmer et al., 1992) and the larger number of cavities associated therewith. On the other hand, the relative stability of PLGA (75/25) msp in any of the incubation media resulted from their high hydrophobicity.

The results of release tests have confirmed that msp prepared of low molecular weight terpolymers are more hydrophilic and degrade more rapidly, and that BSA is released from them more quickly than from msp prepared of high molecular weight linear polymers. This finding is consistent, except for molecular weight, with the view of bulk hydrolysis of ester bonds and a rapid degradation of msp prepared of terpolymers (Kissel et al., 1991).

Immune responses of mice to BSA released from msp prepared of the linear polymers DL-PLGA and the terpolymers M-DLLA and G-M-DLLA were investigated during a 15-week period.

The immune responses in mice receiving a single dose of BSA entrapped in msp prepared of DL-PLGA (75/25) or DL-PLGA (50/50) were biphasic, the first short phase corresponding to the antigenic stimulus of the initial burst. The subsequent gradual decrease in antibody levels was followed by the second phase of the antibody response from Week 9 to 15 (Fig. 6). Although the initial burst release into blood serum was several times lower in msp prepared of DL-PLGA (75/25) than in msp prepared of DL-PLLG (50/50), only a minor difference in immune responses was observed throughout the investigation period. An increase in antibody levels was found in both groups at the end of the experiment, i.e. 15 weeks after a single dose of msp.

The dynamics of immune responses of the mice treated with msp prepared of either of the terpolymers were different (Fig. 7). Although their basic character was biphasic, there was no lag phase between the two peaks. It can be concluded that this course of immune responses resulted from a

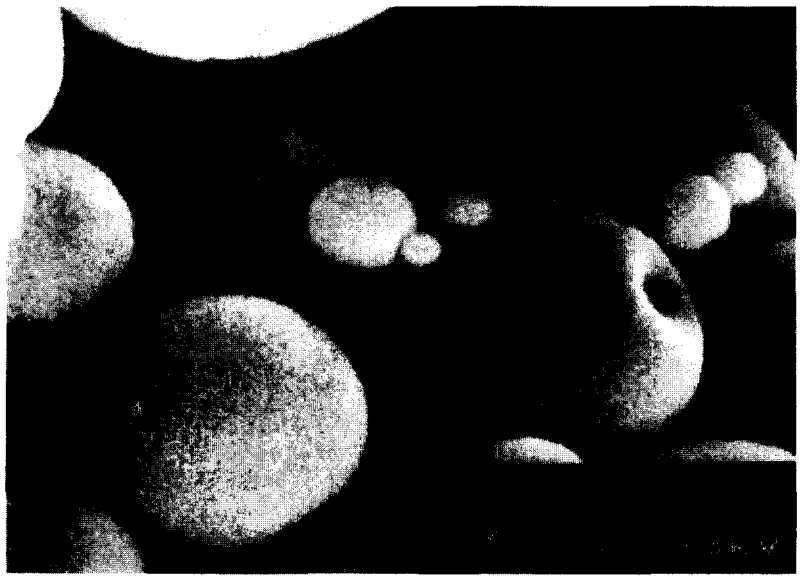

Fig. 3. Scanning electron microscopic photograph of BSA-loaded DL-PLGA (75/25) microspheres. Detail of surface. (Magnification × 20 000).

generally more rapid release of the antigen from msp, as demonstrated also in in vitro release studies. The size of M-DLLA msp is much smaller than that of GA-M-DLLA msp and some authors suggested that this difference was responsible for the stronger adjuvant effect of the former resulting in a higher antibody formation (Eldridge et al., 1993). This effect could not be confirmed in our experiments, however. No significant differences in adjuvant properties were found between linear polymer msp and terpolymer msp when the intensities of antibody responses were expressed in terms of OD_{450}. Both polymer types displayed the expected positive effect of stimulation of antibody responses to highly soluble proteins with a generally low antigenicity (Cohen et al., 1992; Alonso et al., 1994).

This effect is due to biological, physical and chemical properties of msp that are responsible for the basic mechanisms of the adjuvant activity consisting in depot function, protection of antigen

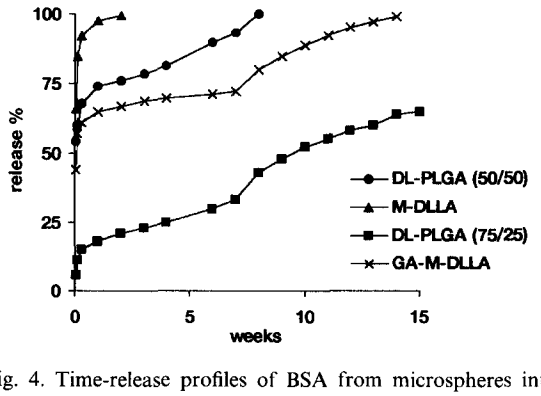

Fig. 4. Time-release profiles of BSA from microspheres into pig blood serum at 37°C.

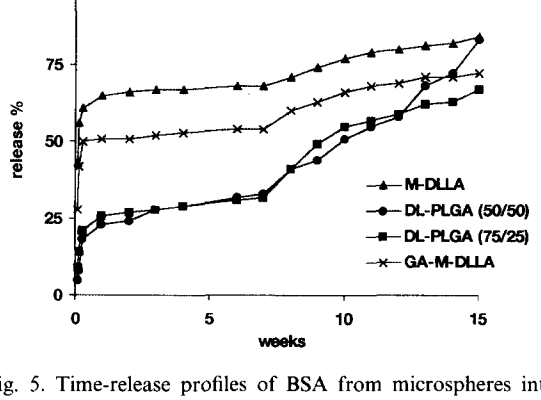

Fig. 5. Time-release profiles of BSA from microspheres into PBS pH 7.2 at 37°C.

Table 3
Dynamics of release of BSA from microspheres during incubation in blood serum (initial burst %)

Vehicle	6 h	24 h	48 h
M-DLLA	66	85	92
GA-M-DLLA	43	56	61
DL-PLGA (50/50)	54	60	67
DL-PLGA (75/25)	6	11	15

Table 4
Dynamics of release of BSA from microspheres during incubation in PBS, pH 7.2 (initial burst %)

Vehicle	6 h	24 h	48 h
M-DLLA	42	55	61
GA-M-DLLA	28	42	50
DL-PLGA (50/50)	4	8	17
DL-PLGA (75/25)	8	13	21

against proteolysis and presentation of antigen to immunocompetent cells (Vogel, 1995). The choice of polymers or a suitable blend thereof allow a controlled release of antigens and thus a prolongation of the antibody response. Similarly as in liposomal and ISCOM adjuvant formulations (Lövgren and Morein, 1991; Antimisiaris et al., 1993; Morein et al., 1994; Kersten and Crommelin, 1995), the effectiveness is considerably enhanced by the fact that weakly immunogenic molecules are presented to the immune system in a corpuscular arrangement resembling natural microorganisms.

Trials of msp prepared from the tested polymers and their blends (Gander et al., 1993) are continued using viral glycoprotein antigens with the intent to develop effective subunit vaccines.

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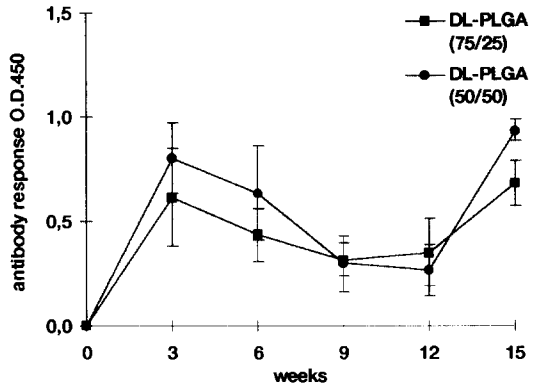

Fig. 6. Antibody response of mice after single immunization with BSA-loaded DL-PLGA microspheres.

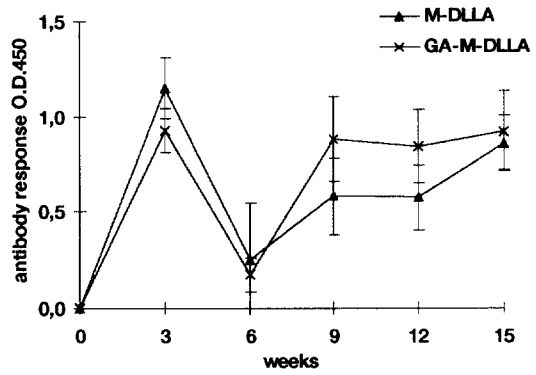

Fig. 7. Antibody response of mice after single immunization with BSA-loaded M-DLLA and GA-M-DLLA microspheres.

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