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Adjuvant efficacy of gelatin particles and microparticles

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Abstract

Gelatin particles and microparticles with mean sizes of 2.0 mm and 20.0 μm, respectively, were prepared using different methods. Bovine serum albumin (BSA) was incorporated into them as a model antigen and the immunomodulators lipopolysaccharide (LPS) or muramyldipeptide (MDP) were incorporated into a part of the particles. In vitro tests showed a complete release of BSA after 18 weeks, but its dynamics were different in the two types. Experiments in rabbits demonstrated that the adjuvant activity of the particles administered subcutaneously was equal to that of aluminium hydroxide. Antibodies were detectable at 3 weeks and low levels were demonstrable at 18 weeks after a single administration. Co-incorporation of the immunomodulators LPS or MDP had a favourable effect on both the onset and the intensity of antibody responses. Gelatin microparticles with incorporated BSA were administered to rabbits subcutaneously or orally. Biphasic antibody responses with maxima in weeks 3–6 and in week 20 were found irrespective of the way of administration. The first and the second peaks were higher in the animals treated subcutaneously and orally, respectively. Both groups showed low and approximately equal antibody levels 38 weeks after the immunization. The adjuvant effect of the orally administered microparticles is evidence of their resistance to the proteolytic environment of the digestive tract of rabbits.

Keywords: Antibodies; Antigen release; Bovine serum albumin; Gelatin microparticles; Immunization; Rabbits

1. Introduction

Adjuvants used to enhance the potency of current vaccines are usually inorganic compounds, mostly aluminium salts, or oil-in-water emulsions. The major drawback of the current adjuvants, resulting from the technology of their production, is poor reproducibility of their properties. The efficacy of vaccines containing such adjuvants is often insufficient, particularly if antigens with a weak immunogenicity (synthetic, soluble) are used

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and, generally, booster immunization is necessary. Attempts to avoid this drawback by the development of new antigen formulations have apparently yielded promising results. The principle of the formulations consists in the incorporation of weak immunogens into corpuscular multimeric structures. Well-known examples are the immunity stimulating complexes (ISCOMs) (Morein et al., 1984) and liposomes (Gregoriadis, 1990).

Another prospective way of enhancing the adjuvant efficacy is the use of biodegradable and biocompatible polymers in the form of microparticles that have been successfully used as carriers of various biologically active substances. Most of such microparticles were prepared of lactic and glycolic acid polymers and co-polymers and proved to be very good antigen carriers (Chandrasekhar et al., 1994, Cahill et al., 1995) with an adjuvant efficacy equal to or higher than the complete or incomplete Freund's adjuvant (CFA, IFA) or aluminium hydroxide (Eldridge et al., 1991, O'Hagan et al., 1991, Men et al., 1995, Sah et al., 1995). According to the recently published classification and on the basis of the mode of action, the efficacy of the microparticles as particulate adjuvants depends on their size and consists in their depot and the targeting components (Cox and Coulter, 1997, Morein et al., 1997).

Materials suitable for the preparation of microparticles also include gelatin as a highly biodegradable polymer prepared by degradation of animal collagen. While experimental use of the synthetic polymers as carriers of various antigens has been described in a number of papers, gelatin particles have been used mostly as matrices for sustained release of drugs and data on their adjuvant activity are rather scarce (Tabata and Ikada, 1989, 1990, Nakaoka et al., 1995).

The conventional procedure for the production of gelatin microparticles consists in the dispersion of heated gelatin solution in liquid paraffin. Cooling of the dispersion results in the formation of gelatin microspheres that are subsequently dried and chemically modified (Tanaka et al., 1963). Many modifications of this basic procedure, allowing the control of various properties of the microparticles such as size and cross-linking, have been developed (Öner and Groves, 1993, Lou and Groves, 1995, Nakaoka et al., 1995, Esposito et al., 1996). The use of the modified microparticles with defined properties and incorporated antigens alone or in combination with immunomodulators may be one of the ways in which to enhance vaccine potency consisting, for example, in the achievement of controlled long-lasting boosting effects and formulation of single-dose vaccines.

The above experience was used in the experiments described here and designed to answer at least partly the question of whether gelatin particles or microparticles with incorporated antigen are prospective vaccine formulations. Two types of gelatin particles differing from each other in their sizes were prepared and bovine serum albumin (BSA) was incorporated into them as a model antigen. The degradation of both the particle types, manifested by antigen release, was tested in vitro and their adjuvant activity in immunization experiments in rabbits. Moreover, the effect of co-incorporation of immunomodulants into and sonication of the larger particles (hereafter referred to only as particles) were tested and the results were compared with the efficacy of aluminium hydroxide as a conventional adjuvant. The objective of the experiments with the smaller particles (hereafter called microparticles) was to find out whether their size range is suitable for a single oral administration and to compare the effects of this inoculation route with those of subcutaneous administration. In all the experiments, the immunization effect was expressed in terms of levels of anti-BSA antibodies.

2. Materials and methods

2.1. Chemicals

The following chemicals were used in our experiments: gelatin type A (300 Bloom), muramyldipeptide (MDP), tetramethylbenzidine (TMB), all supplied by Sigma; lipopolysaccharide (LPS), prepared in our laboratory by phenolic extraction of a culture of *Bordetella bronchiseptica*, as described by Westphal et al. (1952) (for detailed characteristics see Toman et al., 1994); aluminium hydroxide (Aluxid), supplied by

Bioveta Nitra (Slovak Republic); bovine serum albumin (BSA) supplied by USOL (Czech Republic).

2.2. Preparation of gelatin particles and microparticles

Gelatin particles with a size range of 1.0–2.5 mm with incorporated BSA were prepared using the procedure published by Di Silvio et al. (1994). Briefly, 60 mg BSA along with ^{125}I-BSA tracer were dissolved in 3.0 ml of H_2O, and 600 mg of gelatin were dissolved in this solution under intermittent stirring at 45°C. The resulting solution was dropped from a syringe with a G-22 needle into a 30-cm-high column of liquid paraffin cooled to 0°C. The resulting particles were washed twice with chloroform and then treated with vapours of 25% glutaraldehyde in an exsiccator for 48 h. After double washing in phosphate buffered saline (PBS), pH 7.2, the particles were dried with a stream of cold air. The rate of incorporation of BSA and the overall yield were calculated from the weight and radioactivity of the product. Gelatin particles with incorporated MDP or LPS were prepared in a similar way by adding 1 mg of the appropriate immunomodulant to the gelatin + BSA solution. A portion of particles was resuspended in PBS, pH 7.2, and sonicated for 1 min at 100 W for 15 min prior to administration. Only the fraction with the size range 1.6–3.0 mm was used in the immunization experiments.

Gelatin microparticles with the size range of 1–100 μm were prepared using the following procedure: 40 mg BSA along with ^{125}I tracer were dissolved in 0.5 ml of H_2O; the solution was completed with 2.0 ml of 20% gelatin and the mixture was heated under continuous stirring to 45°C. The resulting solution was added dropwise into 50 g of castor oil containing 2.5 g of the surfactant SPAN 20 and heated to 70°C. After 15 min of stirring at 42 kHz, the mixture was cooled under continuous stirring in an ice–water bath for 90 min. After checking the gelification with a light microscope, the microparticles were washed twice with ethanol containing 2% of SPAN 20 and once with ethanol alone.

The resulting volume of microparticles was mixed with 25 ml of ethanol, and glutaraldehyde was added to the final concentration of 4%. After 15 min, the gelatin microparticles were washed three times with ethanol and dried for 18 h. The rate of incorporation of BSA and the overall yield were calculated from the weight and radioactivity of the product.

2.3. Determination of size distribution of gelatin particles and microparticles

The size distribution of the gelatin particles was determined by the sieving technique using the apparatus Analysette 3 (FRITSCH). The size distribution of the gelatin microparticles in ethanol was determined using the laser analyser Analysette 22 (FRITSCH).

2.4. BSA release from gelatin particles and microparticles

Fifty mg of gelatin particles with incorporated BSA + ^{125}I-BSA were mixed in a test tube with 3 ml PBS, pH 7.2, or with 3 ml rabbit serum containing 0.02% sodium merthiolate. The test tubes were incubated in a quiet state at 37°C and the amount of BSA released into the medium was determined at 1-week intervals. For this purpose, a defined volume of the medium was withdrawn with a syringe and replaced with the same volume of fresh medium. The cumulative increase of the released BSA was determined radiometrically.

The same method was used for the determination of BSA release from the gelatin microparticles incubated in PBS, pH 7.2, or rabbit blood serum.

2.5. Immunization experiments

Antibody responses to subcutaneous administration of a single dose of 50 mg of BSA incorporated into the gelatin particles (size range 1.6–2.0 mm) and the effects of sonication or co-incorporation of immunomodulators (1 μg of LPS or MDP per immunization dose) were tested in two 17-week experiments carried out in groups of four rabbits using the scheme given below. A group

Table 1
Size distribution of gelatin particles and preparation yield

Size (mm)				BSA incorporation	Preparation yield
<1.6	1.6–2.0	2.0–2.5	>2.5		
0.0%	70.92%	14.65%[a]	6.81%[b]	92.0%	74.3%

[a] Conglomerate of two particles.
[b] Conglomerate of three particles.

immunized with free BSA in the conventional aluminium hydroxide adjuvant was used as the positive control in the following two experiments.

- Experiment 1
  - Group 1: particles with incorporated BSA
  - Group 2: particles with incorporated BSA, sonicated
  - Group 3: BSA + aluxid 0.1 ml
- Experiment 2
  - Group 1: particles with incorporated BSA
  - Group 2: particles with incorporated BSA + MDP
  - Group 3: particles with incorporated BSA + LPS

Blood serum samples for serological examinations were collected on post-inoculation days 10, 20 and 30, and subsequently at 2-week intervals.

Antibody responses to a single administration of gelatin microparticles with incorporated BSA (size range 1–100 μm) were investigated in a 38-week experiment in two groups of five rabbits:

- Experiment 3
  - Group 1: oral administration of 250 mg BSA incorporated into microparticles
  - Group 2: subcutaneous administration of 50 mg BSA incorporated in microparticles as a comparative experiment

Blood serum samples for serological examinations were collected at 2-week intervals.

The antigenicity of BSA released from the microparticles into PBS, pH 7.2, was checked in mice. Two ml of the eluate collected in week 18 of the in vitro release test were mixed with 1 ml of CFA, and 0.1 ml of the mixture was administered subcutaneously to each of six mice. The treatment was repeated in two of the mice after 3 weeks. The mice were sacrificed 3 or 6 weeks after the last immunization and antibodies to BSA were assayed using enzyme-linked immunosorbent assay (ELISA).

2.6. Serology

Antibodies to BSA were assayed by conventional indirect ELISA using polystyrene microtitre plates, porcine antibodies to rabbit or murine IgG purified by affinity chromatography and labelled with horseradish peroxidase as the conjugate, and hydrogen peroxide and tetramethylbenzidine as the substrate. All the tests were done in duplicates at the dilution 1:100. The antibody responses were expressed in terms of group means of $OD_{450} \pm$ S.E.M.

3. Results and discussion

Rates of incorporation of BSA into the two types of gelatin vehicles prepared by different methods and differing in particle sizes are given in Tables 1 and 2. No considerable differences in the total yield and in BSA incorporation rates were found in spite of the two-log difference in particle size. The integrated size frequency distribution (Fig. 1) shows that approximately 18% of the microparticles were smaller than 10 μm and hence suitable for oral immunization (Eldridge et al., 1989, 1990).

The rate of the release of BSA from the particles and microparticles was tested using PBS, pH 7.2, and rabbit serum simulating the immunized object as the media. As shown in Fig. 2, BSA was released into serum more rapidly than into PBS during the first 9 weeks, apparently due to the

Table 2
Size distribution of gelatin microparticles and preparation yield

Size[a] (μm)			BSA incorporation (%)	Preparation yield (%)
<7.63	<16.77	<30.21		
10%	50%	90%	87.50	70.00

[a] Average size 20.28 μm.

enzymatic activity of the medium. The dynamics of the release of BSA into rabbit blood serum are represented by a triphasic curve with a lag phase between weeks 4 and 12. The release curve for PBS was biphasic with no lag phase. However, the release into PBS and rabbit serum was completed after 14 and 18 weeks, respectively. The marked effect of blood serum enzymes on the release of BSA was confirmed in our earlier experiments using microspheres prepared from oligoesters and polyesters of lactic and glycolic acids (Hampl et al., 1996a). On the other hand, very similar dynamics of release into PBS and rabbit serum were found for the gelatin microparticles (Fig. 3). Their structure seemed to be more consistent and more densely cross-linked and hence more resistant to the effects of the media. The release was biphasic and accelerated markedly after week 15. No burst effect, demonstrated for particles prepared of other polymers in in vitro experiments (Uchida et al., 1994, Hampl et al., 1996a), was observed in the gelatin particles or microparticles. It was evident that the release from substratum parts and desorption from the surface during the first week of incubation in PBS or rabbit serum were very low, but sufficient for antigenic stimulation.

A single immunization dose of the particles or microparticles with incorporated BSA was administered to the rabbits in long-term experiments. Effects of sonication and co-incorporation of immunomodulatory agents were tested along with the effects of gelatin in the rabbits immunized with the larger particles. Since soluble proteins are usually poorly immunogenic (Reid, 1992, Nakaoka et al., 1995) and generally require the use of non-specific potentiators of immune responses (adjuvants), the results were compared with the effects of aluminium hydroxide as a conventional adjuvant.

As can be seen in Fig. 4, the adjuvant effect of the gelatin particles was equal to that of aluminium hydroxide as far as the intensity and length of duration of antibody responses were

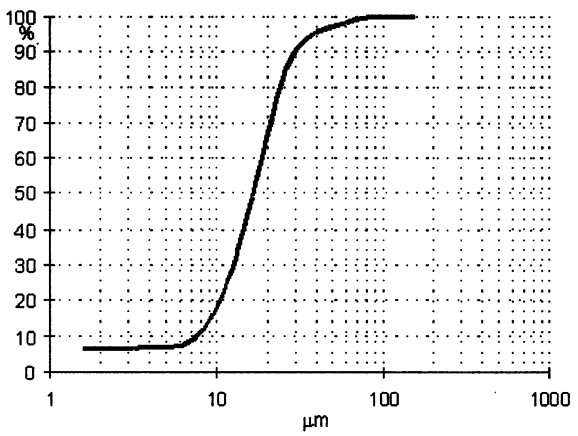

Fig. 1. Integrated frequency distribution of gelatin microparticles.

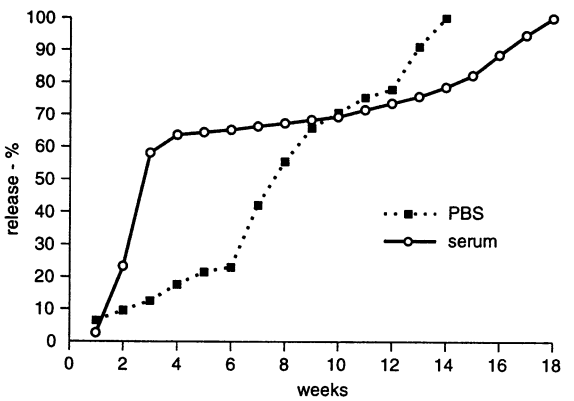

Fig. 2. Release of BSA from gelatin particles into PBS and rabbit blood serum.

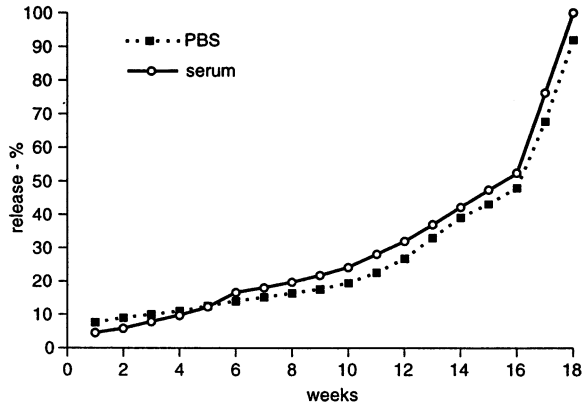

Fig. 3. Release of BSA from gelatin microparticles into PBS and rabbit blood serum.

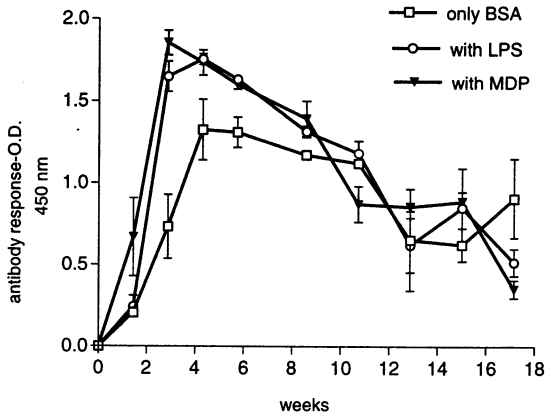

Fig. 5. Antibody responses of rabbits to imunization with gelatin particles with incorporated BSA and LPS or MDP.

concerned. The levels of antibodies to BSA reached their maximal values around the third week and then gradually decreased. Low levels were detectable at the end of the observation period (week 17) in all the rabbits, however. The enhancing effect of sonication was not unambiguous. While the onset of antibody formation was slightly accelerated, apparently due to deaggregation of the particles and partial disintegration of their surface leading to a more rapid release of BSA, the depot effect was suppressed and the decrease of antibody titres began earlier.

The dynamics of antibody titres in the rabbits immunized with the gelatin particles with co-incorporated LPS or MDP are shown in Fig. 5. It is evident that the co-incorporation accelerated the onset and enhanced the intensity of antibody responses. After reaching their maximal values around week 3, the antibody titres gradually decreased and persisted on approximately equal levels in all the three groups from week 9 up to the end of the observation period. A similar result with the same dose of the immunomodulatory agent was obtained in our earlier experiments using bovine herpesvirus (BHV-1) (Hampl et al., 1996b).

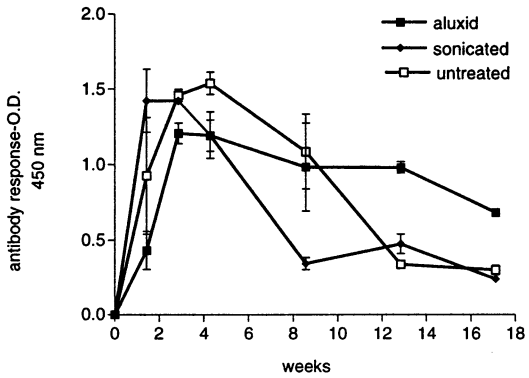

Fig. 4. Antibody responses of rabbits to BSA incorporated into sonicated and untreated gelatin particles.

It can be concluded that the gelatin particles prepared by the method described above are an effective adjuvant formulation enhancing both the intensity and the length of duration of antibody responses to the incorporated antigen. Also the reports of the favourable effects of co-incorporation of immunomodulators (Okada and Toguchi, 1995, Cox and Coulter, 1997) have been confirmed by our results. It should be noted, however, that the administration of the particles is rather difficult owing to their large size.

In another experiment, BSA was incorporated into microparticles with sizes ranging from 1 to 100 μm using an alternative method. Since this range covered also microparticles smaller than 10 μm, they were used for oral immunization and their immunogenic effect after oral administration was compared with that of subcutaneous administration.

The immunization experiments were preceded by a pilot test designed to confirm or eliminate

Table 3
Antigenicity of BSA eluted from gelatin microparticles

Mice nos.	Reimmunization	Antibody titre	
	Week 3	Week 3	Week 6
1, 2	–	0	
3, 4	–		0
5, 6	+		1:50

possible adverse effects of the incorporation procedure, such as those of contact with organic solvents and speed of mechanical stirring, on the antigenicity of BSA. For this purpose, the last eluate collected in week 18 of the release test was administered to six mice. The antigenicity was confirmed by the demonstration of antibodies to BSA at the titre of 1:50 in the mice nos. 5 and 6 in week 6 (Table 3).

BSA incorporated into the microparticles was administered in a single oral dose and the effect was compared with that of subcutaneous administration in groups of five rabbits. Dynamics of antibody responses were monitored for 38 weeks. A difference in the dynamics between the two groups is evident from Fig. 6. The subcutaneous administration induced a steep increase of antibody titres corresponding to the antigenic stimulus of the initial burst as early as in week 2; the titres decreased gradually from week 6 to increase again from week 14 to 24. In the group immunized orally, the antibodies reached their maximum titres in week 6 and, after some decrease, persisted unchanged up to week 20, to increase again and reach maximum values in week 30. The second peak was followed by a decrease and equal antibody titres were found in both the orally and the subcutaneously immunized rabbits in week 38. These dynamics of antibody responses correspond to the results of the previous release test in which the second phase of the release of the remaining approx. 50% BSA was established between weeks 16 and 18 (Fig. 3). A similar relation between the pulsative release and antibody response was described also by other authors (Gander et al., 1993, Cleland et al., 1994).

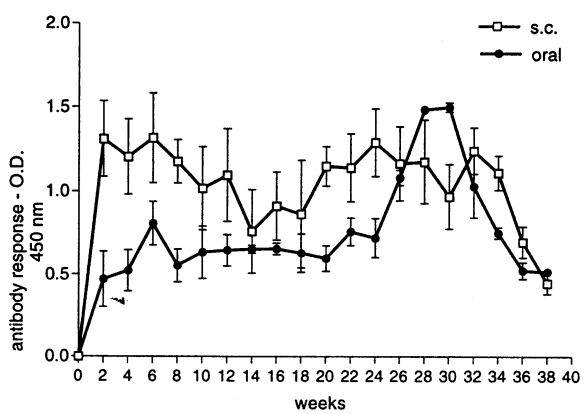

Fig. 6. Antibody responses of rabbits to immunization with gelatin microparticles with incorporated BSA.

The above results of subcutaneous immunization of rabbits with BSA incorporated into gelatin microparticles have confirmed the findings of Nakaoka et al. (1995) who immunized mice with human IgG incorporated into the same vehicle. Their observation period was shorted (50 days), however, and the second increase in antibody titres was recorded 35 days after repeated immunization. The surprising favourable effect of the oral immunization with BSA incorporated into gelatin microparticles is regarded as evidence of the resistance of the microparticles to the proteolytic conditions prevailing in the digestive tract and their absorption into Peyer's plaques. Thus the high resistance of the carrier to proteolysis, demonstrated in experiments in vitro (Fig. 3), has been confirmed.

This effect depends on the biological, physical and chemical properties of the microparticles (Florence, 1997). The most important of these is the particle size and, in the case of gelatin particles, the density of cross-linking controlled by the concentration of glutaraldehyde during the preparation (Nakaoka et al., 1995). The dependence of the effect of oral immunization on particle size is probably primary. The decisive role is attributed only to particles ≤ 10 μm that are able to enter Peyer's plaques; some of them (< 5 μm) are then transported to immunocompetent cells. The stepwise released antigen stimulates the production of both circulating and local antibodies (Eldridge et al., 1989, Challacombe et al., 1992, McGhee et al., 1992, Damgé et al., 1996, Tabata et al., 1996).

The size range of the microparticles used in our experiment was 1–100 μm and approx. 18% of them were < 10 μm. Hence the amount of utilizable microparticles and the incorporated BSA is considerably smaller after oral than after subcutaneous administration and therefore a fivefold dose was used in the orally immunized rabbits to bring the amount of utilizable BSA nearer to the subcutaneous dose. The wider size range is apparently more advantageous, allowing the depot effect and targeting of the subcutaneous and oral administration, respectively, to be achieved. However, the procedure will need to be modified to obtain particles with a size not exceeding 10 μm should oral administration be preferred in the further development of vaccines.

The obtained results are regarded as a contribution to investigations of the usability of gelatin microparticles in immunology, particularly in the development of single-dose immunization.

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