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Evaluation of cholesterol-lecithin implants for sustained delivery of antigen: release in vivo and single-step immunisation of mice

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Summary

Bovine serum albumin (BSA), a model antigen, was delivered from matrix systems (pellets) prepared from cholesterol (C) alone, and from C and hydrogenated egg lecithin (PC) in various C-PC ratios, to mice by implanting the pellets subcutaneously. The in vivo release profile of BSA from these pellets and the antibody responses to BSA were studied at intervals over a period of 10 months following a single-dose administration. An initial burst release of BSA was observed within 2 days of implantation followed by a trickle delivery over 7 months. During the first 5 days, the rate of release of BSA from C pellets (without PC) was significantly slower than from C-PC pellets of 4:1 and 8:1 ratios (w/w). At day 9, the differences became insignificant. The concentration of BSA retained in the pellets increased with an increase in PC content of the pellets. The implants containing BSA induced anti-BSA antibodies at 2 months and maintained the same levels of antibodies for up to 10 months. BSA delivered in the form of three injections produced the same level of antibodies as C-PC pellets of 1:2 ratio (w/w), but a significantly lower level than that produced by the C pellets and C-PC pellets of 1:1, 2:1, 4:1 and 8:1 ratios (w/w) ($P < 0.04$). Bioerosion of the C-PC pellets (at $\leq 4:1$, w/w) was noticeable and an increase in PC content of the pellets increased the erosion rate.

Introduction

Subcutaneous implants prepared from non-biodegradable polymers (Preis and Langer, 1979; Langer, 1981, 1983) and lipidic materials (Opdebeeck and Tucker, 1993) have been demonstrated to be effective for continuous delivery of antigen for single-step immunisation. There have, however, been no reports about their efficacy after 6 months. Furthermore, these implants were not biodegradable, necessitating their surgical removal. The use of biodegradable polymeric implants for sustained delivery of antigen capable of inducing antibodies in mice for about 13 months following a single-dose administration has also been reported, but the preparation techniques of these implants were relatively complex compared

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to lipid implants and required organic solvent for loading the antigen (Kohn et al., 1986, 1988).

Multilamellar and unilamellar phospholipid vesicles (liposomes) were reported to enhance the antibody response in mice and to serve as an effective immunopotentiating carrier for weak antigens (Shek et al., 1983, 1986; Davis and Gregoriadis, 1987). But they do not appear to perform a long-term depot function (Eppstein et al., 1985), and are not considered suitable for single-step immunisation. Delivery systems capable of extending their depot function for long-term antigen delivery were found more appropriate for single-step immunisation (Cohen et al., 1991). Recently, we described subcutaneous implants prepared from cholesterol and lecithin for sustained delivery of antigen, and reported the release profile of bovine serum albumin (BSA), a model antigen, from these pellets and their erosion behaviour in vitro. We also demonstrated that an anti-BSA antibody response was induced by these pellets containing BSA when implanted in mice for 40 days (Khan et al., 1991). Although these pellets were not liposome preparations, we speculated that they might possess an adjuvant effect because of their chemical similarity to liposomes. In this paper, we report the release characteristics of BSA from these pellets in vivo and the anti-BSA antibody responses induced in mice by these pellets over 10 months.

Materials and Methods

Materials

Cholesterol (C) was purchased from Sigma Chemical Co. (St. Louis, U.S.A.) and purified by successive recrystallizations from ethanol and methanol (Opdebeeck and Tucker, 1993). Needle-shaped crystals with m.p. 149°C were obtained. A single spot of pure cholesterol was observed by thin-layer chromatography (Horwitz et al., 1971).

Hydrogenated egg lecithin (PC) (technical grade, iodine value = 0) was supplied by Asahi Chemical Industry Co. Ltd, Tokyo, Japan. Bovine serum albumin (BSA) (catalogue no. A-7030) was purchased from Sigma Chemical Co.

Preparation of pellets

Pellets consisting of five C-PC ratios (1:2, 1:1, 2:1, 4:1 and 8:1, w/w) were prepared from coprecipitates (chloroform) of C and PC as described previously (Khan et al., 1991), except that 40 mg of the total lipid was dissolved in 1 ml of chloroform while preparing the coprecipitates. Pellets from C alone were also prepared as described earlier (Khan et al., 1991). The pellets weighed 28–32 mg (range) and contained 8.7–10.1% (range of average batch content) BSA.

Attempts to prepare pellets from PC alone were not successful because of sticking of the PC to the punches during compression.

Analytical methods

C, PC and BSA contents of the prepared pellets were analysed as described previously (Khan et al., 1991). The BSA content of pellets retrieved after implantation was assayed using high performance size exclusion chromatography (HPSEC). Retrieved pellets were dried to constant weight under vacuum at < 40°C for 24–48 h, homogenised in 5 ml of the mobile phase (0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl) (PBS), centrifuged and the supernatant filtered through 0.45 μm membrane filters (Ministart NML, Gottingen, Germany). Sample solutions (80 μl) were then injected onto a size exclusion column (Waters Protein Pak 300 SW with I-125 guard column), eluted with the mobile phase (1 ml/min) and protein detected by a Waters Model 450 Variable Wavelength Detector at 280 nm. The calibration curves of peak areas vs BSA concentration from triplicate standard solutions of four different concentrations were found to be linear over the ranges of 0.0075–0.150 and 0.032–0.320 mg/ml. The four standard solutions were prepared separately for each range of concentration by dissolving BSA in the mobile phase. C and PC did not interfere with the assay. The BSA contents of pellets not implanted in mice were assayed by both HPSEC and a modified Lowry method (Markwell et al., 1978), and the results obtained from the two methods of analysis were found to be the same. Antibody levels, expressed as absorbance values at 405 nm, were measured in sera diluted (1:200) in PBS with

0.5% Tween 20 (v/v) (PBS-T) by enzyme-linked immunosorbent assay (ELISA) (Opdebeeck and Tucker, 1993).

Release rate of BSA in vivo

Female Quackenbush mice ($n = 16$/group), 9–10 weeks old, were anaesthetised and C-PC and C pellets, containing BSA and prepared as described above, were implanted under the skin behind the neck (Opdebeeck and Tucker, 1993). Four mice from each group were anaesthetised again on days 2, 5, 9 and 15, and the pellets retrieved, washed immediately (briefly) in distilled water and stored at −20°C for analysis of BSA content. The mice were killed after retrieval of the pellets.

Immunisation of mice and study of antibody response

Pellets with the same specifications as described for the previous study on the release rate of BSA in vivo were implanted in the same way in 7–8 weeks old female Quackenbush mice ($n = 24$/group). Another group of mice received the same total dose of BSA in PBS (200 μl containing 1 mg of BSA) divided over three injections administered on days 0, 14 and 28. The control group received blank pellets (without BSA) of C-PC ratio 8:1. Six mice from each group were anaesthetised 2, 4, 7 and 10 months later. Blood was collected by cardiac puncture, sera were separated and stored at −20°C, and the mice were killed. Pellets were also retrieved where possible, washed immediately (briefly) in distilled water and stored at −20°C for analysis of BSA content.

Calculations and statistical tests

The BSA concentrations of pellets retrieved as a whole or partially were calculated by comparing peak areas obtained on HPSEC with those of standards detected at the same elution volume. For pellets retrieved completely, the concentrations were converted into percentages of the initial contents of pellets, the initial contents being determined from the average batch contents. The amount of BSA retained in the implanted pellet was subtracted from the initial content to determine the amount released. BSA contents per mg of the dry weight of retrieved pellets were also calculated. For partially retrieved pellets, only the amount of BSA per mg of the retrieved mass (dry weight) was determined. A peak with a negligible area ($< 0.6\%$ of the initial BSA content of the pellets) was observed in the chromatograms of the control blank pellets retrieved at 2 and 4 months at the same elution volume as for BSA. This peak was not taken into consideration in calculating the amount of BSA in the pellets retrieved at 2 and 4 months. A similar peak was also observed in the blank pellets retrieved at 7 month, but the area of the peak was almost equal for control and treatment pellets retrieved, and therefore, the data were not evaluated.

Analyses of variance and Fisher's protected LSD or calculation of least square means tables (where appropriate) were performed using the software package SuperANOVA (Abacus Concepts, Berkeley, CA, 1989), and $P \leq 0.05$ was considered significant.

Results

An increase in the rate of erosion of the pellets was noticeable with an increase in their PC concentration. Pellets of C-PC ratios 1:2 and 1:1 lost their shape by the second day after implantation and could not be retrieved. These pellets formed a soft diffuse mass and were encapsulated by a thin membrane. Almost all the pellets of 1:2 (C-PC) ratio were eroded away completely by 2 months. Pellets of 1:1 (C-PC) ratio eroded at a slower rate than pellets of 1:2 ratio and bioerosion of these pellets was not complete even after 10 months of implantation. The pellets of 2:1 (C-PC) ratio retained their original shape, but had a layer of a soft mass of lipidic material surrounding the inner hard mass of the pellet, and were also encapsulated by a thin membrane by day 2. Only the inner hard masses of these pellets were retrieved on days 2, 5, 9 and 15, and analysed for BSA content. At 2 and 4 months, the inner hard masses of these pellets had disappeared and the total mass become uniformly diffuse. It could be partially retrieved by squeezing the material out of the encapsulating membranes.

All pellets with a 4:1 (C-PC) ratio were visually intact at 2 months, but thereafter (4, 7 and 10 months) a soft mass was observed surrounding the hard nucleus. The soft layer increased in depth with time. Pellets of 8:1 (C-PC) ratio and C pellets did not lose their shape during the experiment. The thickness and appearance of the encapsulating membrane were different for various formulations. The more rapidly eroding pellets (1:1 and 2:1) were surrounded by thicker membranes than the slower eroding ones (4:1, 8:1 and C pellets), although pellets with very rapid erosion (1:2) were encapsulated by thin membranes. The thick membranes were opaque and glossy, whereas the thin membranes were transparent.

The rate of release of BSA from pellets with C-PC ratios $\geq$ 4:1 (w/w) during the first 2 days increased with the increase in PC content. The rate of release of BSA from C pellets (without PC) was significantly slower over the first 5 days than from the pellets of 4:1 and 8:1 ratios. At day 9 the difference in the cumulative amount of BSA released was not significant (Fig. 1). The concentration of BSA (w/w) retained in the inner hard masses of the pellets of C-PC ratio 2:1 at day 15 was significantly lower than the concentration of BSA retained in the diffuse masses of the pellets of same formulation (2:1) partially retrieved by squeezing the encapsulating membrane at 2 and 4 months (Fig. 2).

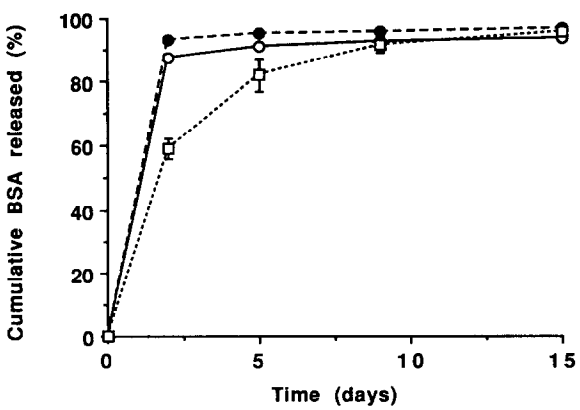

Fig. 1. Profile of release of BSA from pellets implanted in mice up to 15 days. Pellets were prepared from: C (without PC), □; C-PC (8:1), ○; and C-PC (4:1), ●. The pellets contained 2.4–3.2 mg (range) of BSA. Each point represents the mean value for four pellets and vertical bars indicate S.E. of the means.

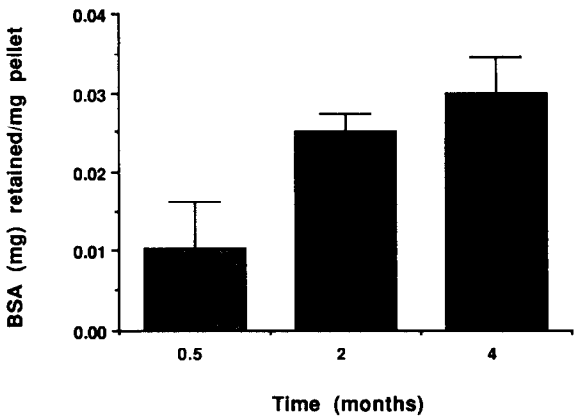

Fig. 2. Distribution of BSA retained in the pellets with C-PC ratio 2:1 at 0.5 months (inner hard mass), 2 months (deformed soft mass) and 4 months (deformed soft mass) after implantation in mice. Vertical bars indicate S.E. of the means ($n = 3$).

In the diffuse masses of the pellets partially retrieved after 2 and 4 months, the concentration of BSA (w/w) retained increased as the PC content of pellets increased in comparison with C. Pellets with C-PC ratios 1:1 and 2:1 had significantly higher concentrations of BSA than the other pellets. Pellets prepared from C alone had the lowest BSA concentration although these levels were not significantly different to the levels in pellets of 4:1 and 8:1 (C-PC) ratios (Fig. 3).

One peak representing a substance with a molecular weight higher than that of BSA (Mol. Wt 66000) and several peaks representing substances with lower molecular weights compared to BSA were present in most chromatograms of the pellets analysed by HPSEC for BSA (data not shown). However, these peaks were also present in chromatograms of the blank pellets (without BSA).

BSA delivered from implants and by injections induced significant ($P \leq 0.0002$) anti-BSA antibody responses in sera of mice by 2 months when compared to the control group of mice (with blank pellets) which had no anti-BSA antibodies. Antibody levels measured in sera of the vaccinated mice at 2, 4, 7 and 10 months did not differ

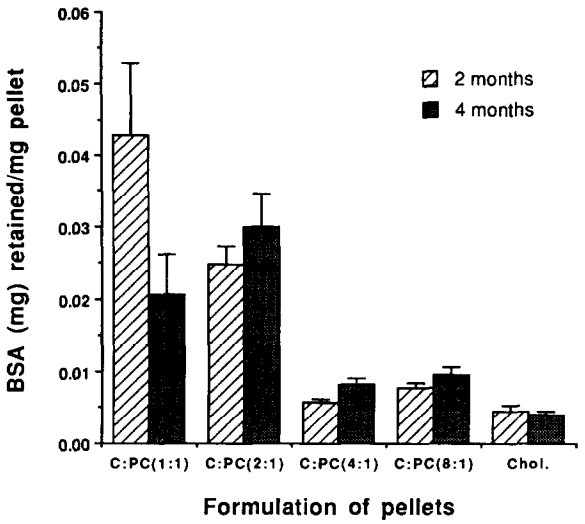

Fig. 3. Variation in the concentrations of BSA retained in C (Chol.) and C-PC (C:PC) pellets following 2 and 4 months after implantation. The C-PC ratios are indicated in parentheses. Vertical bars indicate S.E. of the means ($n = 3$–6).

($P > 0.2$). Three injections of BSA induced the same levels of antibodies as BSA delivered from pellets of 1:2 (C-PC) formulation throughout the experiment ($P > 0.5$). Levels of antibodies induced by BSA delivered from pellets of all other formulations were significantly higher than those induced both by three injections of BSA and by pellets formulated at 1:2 (C-PC) ratio ($P < 0.04$) (Fig. 4).

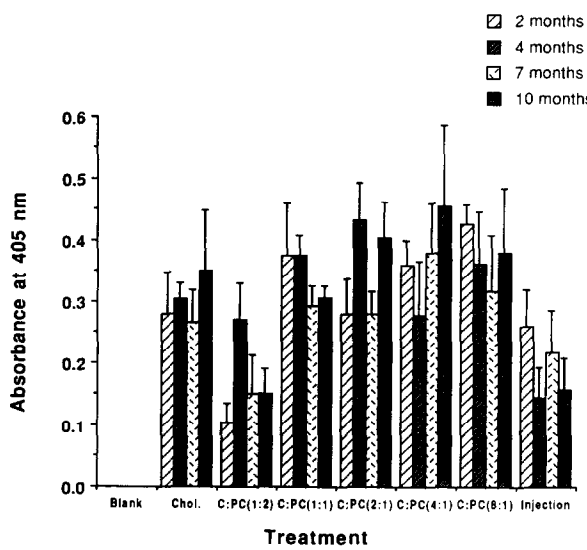

Fig. 4. Anti-BSA antibody levels in sera of mice following administration of BSA from single-dose C (Chol.) and C-PC (C:PC) pellets, and by three injections (Injection). The C-PC ratios are indicated in parentheses. Control mice received blank pellets (without BSA) of C-PC ratio 8:1 (blank). Vertical bars indicate S.E. of the means (n = either 5 or 6).

Discussion

BSA released from implants stimulated the production of anti-BSA antibodies for over a period of at least 10 months, and these antibody levels were significantly higher than those induced by three injections of the same dose of the antigen. Significant antibody responses induced by single-dose administration of an antigen have been reported. Diphtheria toxoid microencapsulated in a biodegradable poly(D,L-lactide) polymer and delivered thus to the immune system of mice induced antibody titres which were comparable until day 75 with those obtained following conventional administration of the toxoid by three injections using calcium phosphate as the adjuvant (Singh et al., 1991). BSA delivered from liposomes encapsulated in Ca-crosslinked alginate microcapsules induced and maintained high levels of antibodies for about 150 days in rats following a single-dose injection (Cohen et al., 1991). BSA loaded in ethylene-vinyl acetate copolymer implants was as or more effective than two injections of BSA emulsified in complete Freund's adjuvant in inducing antibodies in mice for up to 6 months (Preis and Langer, 1979; Langer, 1981, 1983). Kohn et al. (1986, 1988) demonstrated that BSA delivered from biodegradable polymeric implants induced antibodies in mice for up to a period of 56 weeks after a single-dose administration.

An initial release of BSA in a burst from pellets of 4:1, 8:1 (C-PC) ratio (> 87%) and from cholesterol pellets ($\approx$ 60%) within 2 days of implantation and the recovery of pellets intact suggested that release of antigen occurred from these pellets by diffusion. Diffusional release of BSA from cholesterol-lecithin pellets in vitro was reported previously (Khan et al., 1991). Opdebeeck and Tucker (1993) demonstrated similar

profiles for release of BSA from cholesterol pellets in vivo. They also reported the occurrence of high (1 000 000) and low (5000–10 000) molecular weight peaks in the chromatograms of BSA extracted from pellets retrieved from mice in addition to the major peak identified for BSA (Mol. Wt 66 000). The high and low molecular weight peaks were postulated to be either break-down products and aggregates of BSA or murine proteins. The appearance of these peaks in the chromatograms of blank pellets (without BSA) retrieved from mice in the experiment reported here suggests that murine proteins contributed to some extent to the protein profiles obtained in this study. The initial slower release of BSA from C pellets is explained by the inability of the body fluid to penetrate into these pellets as fast as in the presence of PC.

The mechanism for release of BSA from C-PC pellets containing $\geqq 33\%$ PC (compared to C) appears more complex than from pellets with low levels of PC. Significantly higher concentrations of BSA (w/w) were found in the partially retrieved pellets of 1:1 and 2:1 (C-PC) ratios at 2 and 4 months than in 4:1, 8:1 and C pellets suggesting that PC played a role in retarding the diffusional release of BSA. In previous in vitro studies we found that inclusion of PC in pellets enhanced the rate of release of BSA (Khan et al., 1991). The complex nature of release of BSA from the pellets of 1:1 and 2:1 ratios is due either to the faster erosion rate of PC leaving C behind and resulting in higher concentration of BSA or to the formation of PC bilayer structures in the form of myelin bodies, perhaps incorporating a certain amount of C (Bourgès et al., 1967; Phillips and Finer, 1974; Demel and de Kruyff, 1976; Keough et al., 1989). Simultaneous occurrence of both the processes is also a possibility. In the former instance, the release of BSA initially would occur by diffusion and PC would erode at a faster rate than C due to hydration giving rise to a higher concentration of BSA in the pellet residue. Subsequent release of BSA was perhaps delayed by the membrane which encapsulated the pellets since the observed thickness of the membranes encapsulating the pellets of 1:1 and 2:1 (C:PC) was greater than those encapsulating the other pellets. Penetration of body fluids into these pellets might have caused formation of myelin bodies as these were observed to occur in cholesterol-lecithin pellets studied in vitro (Khan and Tucker, 1992). A certain amount of the BSA could have been entrapped in the aqueous compartments of these myelin bodies resulting in a delay in the diffusional release process. The number of myelin bodies would be correlated with the amount of PC present in the pellets suggesting retention of a higher amount of BSA in the pellet residue of higher PC concentration. The highest concentration of BSA was found in the retrieved masses of the pellets of 1:1 and 2:1 ratios at 2 and 4 months. The concentration of BSA in the soft masses of the pellets of 2:1 (C-PC) ratio was higher at 2 and 4 months than was present in the inner hard masses of the same type of pellets retrieved on day 15 supporting that myelin bodies were formed and entrapped a certain amount of BSA in these pellets. The pellets of 4:1 and 8:1 ratios had little chance of forming myelin bodies due to the compactness of the pellets which remained intact for as long as 2 months and for up to 10 months. The comparatively rapid erosion of the pellets of 1:2 (C-PC) ratio made it difficult to ascertain their release characteristics. However, 100% release of BSA occurred within 2 months of implantation since most of these pellets were completely eroded by that time.

The PC content of the pellets influenced the consistency of the mass produced after deformation of the pellets and played a role in the erosion process. The higher the PC content of the pellets the more diffuse were the masses thereby increasing their surface area. The surface area of implanted pellets was shown to be a major factor in controlling the rate of absorption of the pellets (Ballard and Nelson, 1962).

The thickness of the membrane formed in response to implantation of the pellet appeared to be dictated by the degree of erodibility of the pellets. The more erodible pellets were encapsulated by thicker membranes than the slowly eroding pellets, except in the case of very rapidly erodible ones (C:PC, 1:2), where there was perhaps insufficient time for the membranes to develop. Similar observations were reported regard-

ing fibrous capsules formed in response to steroid implants (Foss, 1942); more rapidly absorbable substances resulted in thicker capsules in human than those which were more slowly absorbable. Shimkin et al. (1944) stated that the thickness of the capsule was determined by the rate of absorbability of the implant rather than the slowness of absorption being due to the thicker capsule.

An initial pulse release of the antigen occurred from all pellets, followed by a continued sustained delivery of BSA which was as little as 133 μg of antigen (from C pellets) over the period from 15 days to 7 months. This release profile was capable of inducing antibodies and maintaining the antibody levels for at least up to 10 months. The amount of antigen required for stimulating a secondary response is much lower than that required for the primary response. For many protein antigens a secondary response can be stimulated by as little as 0.001 μg (Mitchison, 1972). More than 93% of BSA was released within 15 days of implantation from most of the pellets studied, and variations in releasing the remaining $< 7\%$ of the antigen from pellets of different formulations did not result in different amounts of antibodies in the sera of the various groups of mice. An increase in antigen delivered to the immune system did not proportionately influence the immune response (Stewart et al., 1983; Hu et al., 1989), and Opdebeeck and Tucker (1993) studying the immune response of mice concluded similarly for a range of 150–3000 μg of BSA. The rapid erosion of pellets with a C-PC ratio 1:2 induced the same level of antibodies as three injections of BSA both of which were significantly less than the levels induced by the pulsatile plus trickle release profile for the other pellets. Higher antibody responses induced by the pellets appear to be due to continuous delivery of the antigen rather than any adjuvant activity of other nature.

The results described in this paper demonstrate that a biocompatible and biodegradable implant prepared from cholesterol and lecithin can deliver antigen, induce antibody formation and maintain these antibody levels for at least 10 months. The antigen was delivered as an initial burst followed by a sustained release of the antigen, perhaps loaded in myelin bodies along with some free antigen. Compared to immunisation by multiple-dose injections, these implants would have the advantage of a single administration. The manufacturing techniques involved in preparing these pellets are simple and do not require heat or any organic solvent treatment of the antigen. The sustained release properties of the pellets, their biodegradable nature and proven efficacy in delivering a model antigen with resultant antibody responses over at least 10 months make them a promising vehicle for delivery of various vaccine antigens.

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