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Cholesterol and lecithin implants for sustained release of antigen: release and erosion in vitro, and antibody response in mice

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Summary

Implantable matrix systems (pellets) were prepared from cholesterol (C), and from C and lecithin (PC), with bovine serum albumin (BSA) as a model antigen. The release profile of BSA from these pellets and their erosion behaviour in borate buffer (pH 7.4) were studied in vitro under different hydrodynamic conditions. The effect of different methods used to mix C and PC on release profile and erosion behaviour was also investigated; pellets were made from physically mixed and coprecipitated C and PC. Ethanol, ethanol-chloroform (1 : 1, v/v) and chloroform were used as coprecipitating solvents. BSA was released by diffusion from all pellets and increasing the concentration of PC enhanced the rate of release. The erosion rate of C and PC depended on the C-PC composition of pellets. Both the release of BSA and erosion of pellets were positively influenced by hydrodynamic activities. At a C-PC ratio of 1 : 2 w/w (approx. 1 : 1 molar), a paracrystalline hydrated phase (gel) was formed which was not a diffusional barrier for the release of BSA. Chloroform used as a coprecipitating solvent reduced the rate of release of BSA significantly at C-PC ratios of 2 : 1 and 1 : 1 (w/w). Erosion of pellets occurred when implanted subcutaneously in mice for 40 days. Implantation of mice with the different formulations containing BSA induced significant ($P \leq 0.0001$) antibodies to BSA in all groups at 40 days, but the levels did not differ significantly between groups ($P \geq 0.088$).

Introduction

The dose, dosage form, route and frequency of administration of antigen are important factors in stimulating an effective immune response (Stewart et al., 1983; Lukes, 1987; Pay and Hingley, 1987); for non-living antigens, small multiple doses have proven effective in achieving protective immunity (Nakashima et al., 1974; Murray et al., 1979; Lau, 1986). Adjuvants with a depot effect are believed to work at least in part because of their role in delivering antigen over a prolonged period of time to the immune system (Vanselow, 1987).

Delivery of BSA from controlled-release pellets of ethylene-vinyl acetate copolymer was as effective or more effective than immunising mice with two injections of BSA emulsified in complete Freund's adjuvant (Langer, 1983). Most at-

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tempts to make sustained release dosage forms for macromolecules have used biocompatible, non-biodegradable (Langer and Folkman, 1976; Preis and Langer, 1979; Creque et al., 1980; Langer, 1981) and biodegradable (Heller, 1984; Hutchinson and Furr, 1987) polymers. These polymers have disadvantages such as the need for their surgical removal after use, denaturation of macromolecules by the organic solvents and the high temperature used in preparation of the dosage form, their cost as well as other practical problems (Kiessel et al., 1988). Thus the use of simple biocompatible systems (e.g. lipids) may be more suitable for protein/antigen delivery.

Cholesterol implants have been used to deliver low molecular weight (< 500) molecules (Kincl et al., 1970; Joseph et al., 1977; Beck and Lowry, 1978) and some macromolecules (MW 1300–400 000) (Kent, 1984; Wang, 1987a,b). Opdebeeck and Tucker (1991) stimulated antibody responses in mice using cholesterol implants to deliver BSA (MW 66 000). A disadvantage of this system is that it is not biodegradable.

Phospholipids have been used alone (Nishihata, 1987; Fujii et al., 1988) and in conjunction with triglycerides (Nishihata et al., 1985, 1986, 1988), benzyl nicotinate (Natsuki and Takabatke, 1987), and cholesterol (Venkataram and Rogers, 1985; Hirotani et al., 1987) as drug carriers. In these studies attempts were made to improve the solubilities and dissolution rates of poorly water-soluble drugs by incorporating phospholipids into the dosage form. Although atherosclerosis research by Adams et al. (1963), Adams and Morgan (1967), and Krut (1982) focused on erosion of cholesterol-lecithin implants in vivo, there have been no reports about their erosion behaviour in vitro.

We prepared pellets containing BSA using cholesterol alone and cholesterol with lecithin as excipients, and studied the effects of cholesterol-lecithin ratio, preparation by physical mixing and coprecipitating from different organic solvents, choice of the organic solvent, and hydrodynamic conditions on the erosion of pellets and the release of BSA in vitro. Antibody responses to BSA by mice implanted with these pellets were also studied.

Materials and Methods

Materials

Cholesterol (C) (Sigma Chemical Co., St. Louis, U.S.A.) was purified by the method of Bourgès et al. (1967). Needle-shaped crystals of anhydrous cholesterol with m.p. 149°C were obtained. The infrared spectrum of cholesterol crystals agreed with the spectrum described in the Pharmaceutical Spectra – infrared (Sadtler Research Laboratories, Inc., 1966). Purity was checked using thin-layer chromatography (Horwitz et al., 1971). Raichem cholesterol enzymatic reagent was supplied by Reagents Applications, Inc. (CA, U.S.A).

Hydrogenated egg lecithin (PC) (technical grade, IV = 0) was a gift from Asahi Chemical Industry Co. Ltd, Tokyo, Japan. BSA (catalogue no.A-7030) was bought from Sigma Chemical Co.

The dissolution medium was 0.185 M borate buffer (pH 7.4) containing 0.1% sodium azide as preservative. All organic solvents used were fractionated.

Preparation of pellets

Physical mixtures of cholesterol and lecithin

Pellets consisting of three C-PC ratios (2 : 1, 1 : 1 and 1 : 2, w/w) and BSA (range 9–10%) were made by crushing and mixing appropriate amounts of C and PC in a mortar and incorporating BSA by geometric progression. The powder was sieved (420 μm) and then compressed into pellets (range 27–31 mg) using 5.5 mm concave punches in a single punch tablet machine (Manesty, Liverpool, U.K.). Blank pellets containing the same ratios of C and PC but no BSA were prepared in the same way.

Cholesterol pellets

Pulverised cholesterol was mixed by geometric progression with BSA and compressed into 60 mg slugs. These were granulated through a 420 μm sieve and compressed as described above.

Coprecipitates of cholesterol and lecithin

C and PC were dissolved in chloroform, chloroform-ethanol (1 : 1, v/v) or ethanol (10 mg of lipid per ml) and the solvent evaporated in a

Buchi (RE 120) rotavapor (Flawil, Switzerland) under reduced pressure for 4 h at 40–42°C. The mass was dried to constant weight under reduced pressure at 37–40°C for 24–72 h. Coprecipitates were pulverised, mixed with BSA, sieved (420 μm) and pelleted as described above. At C-PC ratios of 1:1 and 1:2 granules were redried for 2–3 h before punching when humid conditions caused pellets to stick to the punches during compression.

Pellets were stored at below 4°C in light-proof containers over silica gel, and were analysed for C, PC and BSA contents.

BSA release rate and erosion of pellets in vitro

(a) The effect of the C-PC composition of pellets on the release of BSA and the erosion of pellets was studied by exposing physically mixed pellets of three C-PC ratios (2:1, 1:1 and 1:2, w/w) to active hydrodynamic conditions. Pellets (12/group) were weighed individually and placed into 12.5 ml plastic tubes containing 12.5 ml of borate buffer. The tubes were tumbled (end over end) at 27 rpm in a water bath at 37°C. Two pellets from each group were retrieved at designated times and dried under reduced pressure at less than 40°C to constant weight. Borate buffer separated from pellets was stored below 4°C and quantitatively assayed for BSA within 7 days of collection.

(b) The release of BSA and erosion of pellets made from physical mixtures and coprecipitates of C and PC (1:1, w/w) were compared under active hydrodynamic conditions as described above except that pellets were placed in 12 ml of borate buffer and tubes were tumbled at 25 rpm. Ethanol, chloroform and ethanol-chloroform (1:1, v/v) were used as coprecipitating solvents in preparing pellets. Reproducibility of the release and erosion rates among different batches of pellets was also checked.

(c) The influence of low hydrodynamic activity on release of BSA and erosion of pellets was also investigated. Pellets of C-PC 1:1 (w/w) made from physical mixtures, and coprecipitated from chloroform were weighed individually and placed into 12.5 ml plastic tubes containing 7.5 ml of borate buffer. Tubes were mounted horizontally in an Orbital Shaking water bath (model OW1412, Paton Industries Pty Ltd, Stephany, Australia) at 37°C and oscillated at 70 times/min. Pellets were retrieved and dried to constant weight. Borate buffer was stored as described and quantitatively assayed for BSA within 7 days of collection. Two batches of each pellet type were studied.

(d) The influence of low hydrodynamic activity on release of BSA and erosion of pellets was also studied in pellets of C-PC ratios of 2:1 and 1:2 (w/w), and made from physical mixtures and coprecipitates (chloroform) in a similar way to that described in (c).

Immunisation of mice and study of antibody responses

Pellets made from physical mixtures and coprecipitates (chloroform) of C-PC ratios of 2:1, 1:1 and 1:2 (w/w), and from cholesterol alone were implanted in female Quackenbush mice (5/group) weighing 24–30 g. The pellets weighed 29–31 mg and contained 8–10% BSA (range of average batch contents). Two control groups were implanted either with pellets of physically mixed C-PC or with coprecipitated C-PC (both 2:1 w/w) without BSA. The mice were anaesthetised by injecting ketamine hydrochloride and xylazine, and pellets were implanted subcutaneously behind the neck (Opdebeeck and Tucker, 1991). Mice were again anaesthetised 40 days later and blood was collected by cardiac puncture; sera were separated and stored at −20°C. Antibody levels to BSA were measured in serially diluted sera by enzyme-linked immunosorbent assay (ELISA) (Opdebeeck and Tucker, 1991).

Analytical methods

Pellets were dissolved in ethanol (10 ml) and an aliquot of supernatant taken to quantify PC (Bartlet, 1959) and C. BSA, borate buffer and C did not interfere with the PC assay. C content was analysed using a Raichem cholesterol reagent kit which uses a modification of the enzymatic method of Allian et al. (1974). An adjustment (< 15%) to this method was made due to interference by PC in the assay. The changes in slopes and intercepts of the standard curve at various PC concentrations were examined and used to

make the adjustment. Neither BSA nor borate buffer interfered with the assay. A modified Lowry procedure (Markwell et al., 1978) was used to quantify BSA in retrieved borate buffer and in pellets. Pellets were sonicated in 10 ml of dissolution medium until disintegrated and an aliquot analysed for BSA content. C, PC and borate buffer did not interfere with the assay.

Calculations and statistical tests

Erosion of C and PC was calculated by deducting the contents assayed in the retrieved pellets from their initial values determined from the average batch content. The amounts of BSA released were quantified in the dissolution media and converted into percentages of the initial contents of pellets determined from the average batch content. Antibody levels were expressed as the logarithms of dilutions of sera at an absorbance of 0.4. These were obtained by inverse prediction from the least-squares regression lines of absorbance versus the logarithm of dilution of sera.

Analyses of Variance, *t*-tests and Fisher's protected LSD tests were performed where appropriate. Correlation analysis was applied to compare the relationship between C-PC erosion and BSA release. A significance level of $P \leqq 0.05$ was used in all tests.

Results

Under active hydrodynamic conditions the release rate of BSA from pellets (Fig. 1A) and erosion of pellets (Fig. 1B) was higher for pellets containing more PC. The relative erosion rates of C and PC differed for the different ratios (Table 1). At a C-PC ratio of 2:1, PC eroded at a faster rate than C for the first 5 h, followed by a gradual slowing of the erosion rate of PC. At a 1:1 C-PC ratio, C and PC eroded at approximately the same rate throughout, whereas at a 1:2 C-PC ratio, there was minimal erosion of PC and almost no erosion of C before these pellets disintegrated after 1.5–2 h.

Pellets made from ethanol coprecipitates and ethanol-chloroform (1:1, v/v) coprecipitates differed neither in their BSA release rates nor in

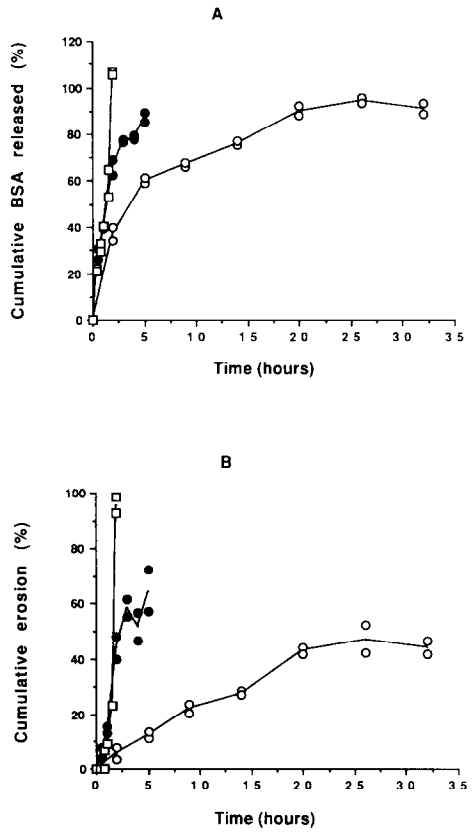

Fig. 1. Effect of C-PC composition of pellets on release of BSA (A) and erosion of C-PC (B) under active hydrodynamic conditions from pellets made of physical mixtures of C and PC: ○, C:PC (2:1); ●, C:PC (1:1); □, C:PC (1:2). Each point represents the result from an individual pellet.

their erosion rates. However, they showed significantly higher release and erosion rates than pellets made from physical mixture of C and PC (Fig. 2A and B). Chloroform as coprecipitating solvent significantly reduced the release rate of BSA from pellets with C-PC ratios of 2:1 and 1:1 in all hydrodynamic conditions tested (Figs 2A, 3A and 4A), but under conditions of low hydrodynamic activity at a C-PC ratio of 1:2 the release rates of BSA from pellets of physical mixtures and chloroform coprecipitates did not differ significantly (Fig. 4A). Under active hydrodynamic conditions at a C-PC ratio of 1:1, the pellets made from chloroform coprecipitates eroded at a slower rate than the pellets made from physical mixture of C and PC (Fig. 2B). Under conditions with low hydrodynamic activity

TABLE 1

C and PC erosion under active hydrodynamic conditions from physically mixed type pellets (values are means for two pellets; [a] one pellet only)

Theoretical C:PC (w/w)	Time (h)	Erosion (mg)		Erosion (%)	
		C	PC	C	PC
2:1	2	0.55	0.97	3.10	11.18
	5	0.70	2.33	4.29	29.32
	9	2.79	2.79	16.48	33.78
	14	3.66	3.37	21.46	40.57
	20	6.38	4.90	36.27	57.22
	26	7.15	4.79	42.03	57.68
	32	6.54	4.85	37.72	57.41
1:1	0.5	1.41	0.0	10.68	0.00
	1	2.29	1.85	16.82	14.03
	2	5.66	5.70	43.08	44.84
	3	7.81	7.78	57.75	59.43
	4	7.24	6.68	53.05	50.60
	5	8.53	8.43	64.00	65.37
1:2	0.4	0.00	0.00	0.00	0.00
	0.75	0.00	0.33	0.00	2.03
	1 [a]	0.00	2.11	0.00	13.18
	1.5 [a]	0.78	4.66	11.41	28.37
	2	6.71	15.10	100.00	93.74

at C-PC ratios of 1:1 and 2:1, pellets made from physical mixtures and chloroform coprecipitates did not differ significantly in their erosion behaviour; at a C-PC ratio of 1:2, pellets made from chloroform coprecipitates eroded rapidly within 6 days, whilst erosion of pellets made from physical mixtures was less than 5% (Figs 3B and 4B).

The variations in the release of BSA from pellets and in erosion of pellets of different batches of the same formulation were not significant.

The rates of release of BSA from pellets (Figs 2A and 3A) and erosion of pellets (Figs 2B and 3B) were greatly increased with an increase in hydrodynamic activity. Pellets made from cholesterol alone did not erode over 15 days under low hydrodynamic conditions (Fig. 4B) and about 20% of BSA was released within 8 h with almost no further release over 15 days (Fig. 4A).

All pellets implanted in mice for 40 days were enveloped by a membrane; no inflammatory reaction was seen at the implantation site although vascularisation of the fibrous tissue which encapsulated some pellets of C-PC ratios 1:1 and 2:1, and of the underlying tissue was evident in some cases. The pellets lost their shape to various degrees, depending on their C-PC compositions; pellets made with the highest PC concentration were more likely to lose their shapes and some of these pellets eroded completely.

The various formulations of the pellets containing BSA induced significant levels of anti-BSA antibodies in all groups of mice implanted with these pellets for 40 days, but the antibody levels

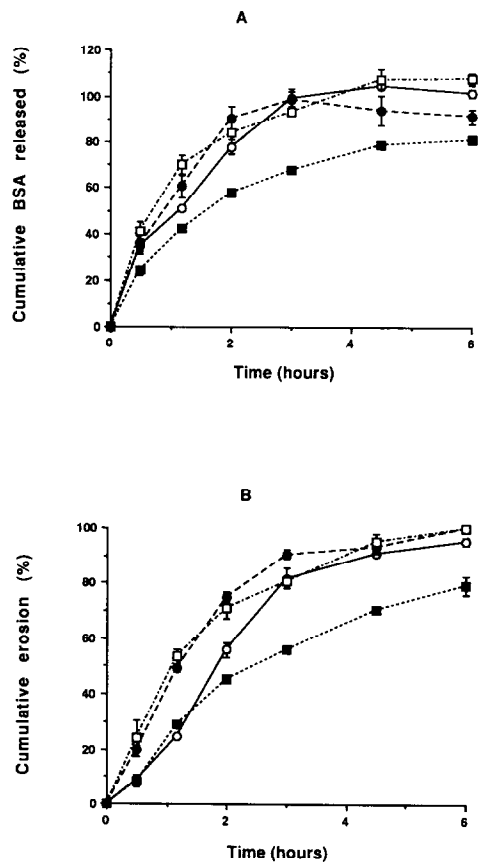

Fig. 2. Release of BSA (A) and erosion of C-PC (B) under active hydrodynamic conditions from pellets (C:PC, 1:1) made of physical mixtures and coprecipitates of C and PC: ○, physical mixture; ●, ethanol coprecipitates; □, ethanol-chloroform (1:1, v/v) coprecipitates; ■, chloroform coprecipitates. Each point is the mean of the results from four pellets of two different batches and vertical bars represent standard errors of the means.

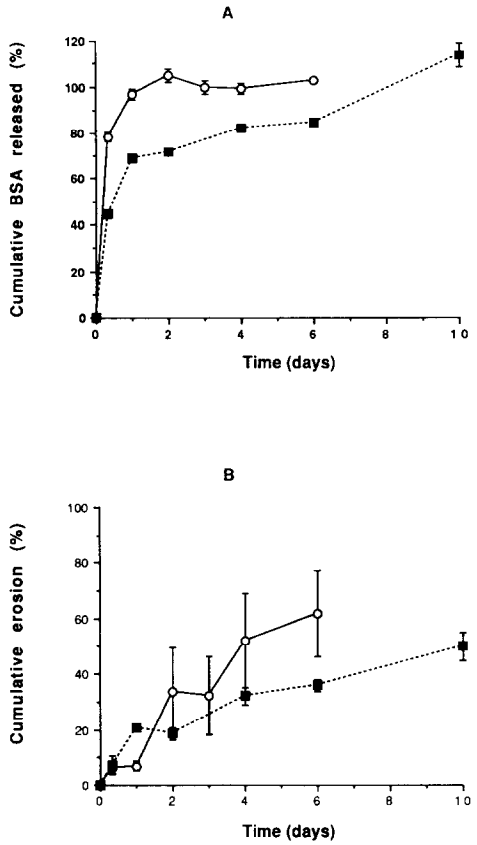

Fig. 3. Release of BSA (A) and erosion of C-PC (B) under gentle hydrodynamic conditions from pellets (C:PC, 1:1) made of physical mixtures and chloroform coprecipitates: ○, physical mixture; ■, coprecipitates. Each point is the mean of the results from four pellets of two different batches and vertical bars represent standard errors of the means.

were not significantly different among groups. No antibodies against BSA were detected in the sera of the control groups of mice which were implanted with blank pellets without BSA (Fig. 5).

Discussion

The rate of release of BSA and erosion of pellets could be manipulated by varying the C-PC compositions of pellets, altering the hydrodynamic conditions and by coprecipitating C and PC from various organic solvents. Significant levels of anti-BSA antibodies detected in the sera of mice implanted with these pellets for 40 days suggest that this type of implant has potential as a bioerodible carrier for delivery of antigen.

The increased rate of release of BSA from pellets with higher PC content was anticipated because of the ability of PC to hydrate in the presence of water. At C-PC ratios of 2:1 and 1:1, 34–40% and 62–70% release of BSA occurred within 2 h, respectively. When the PC content was increased to a 1:2 C-PC ratio, 100% release of BSA occurred in less than 2 h. Addition of lecithin to a triglyceride base increased the release of sodium diclofenac from suppositories (Nishihata et al., 1985, 1986) and addition of cholesterol to sodium diclofenac granules pre-

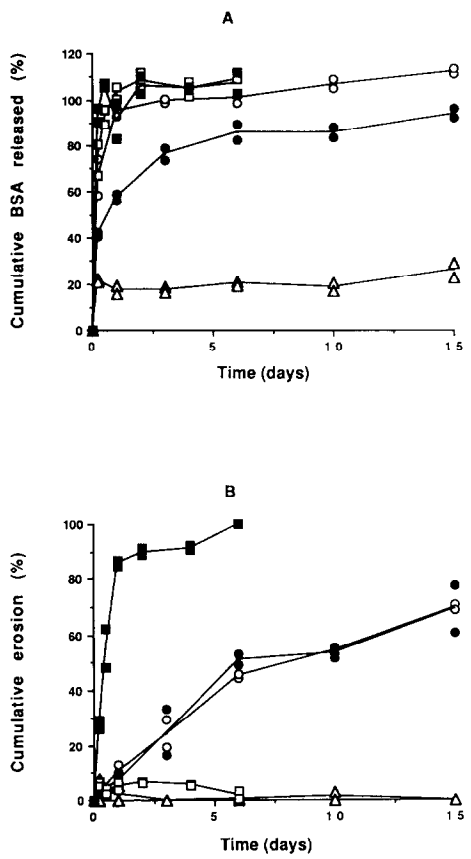

Fig. 4. Effect of C-PC composition on the release of BSA (A) and erosion of C-PC (B) under gentle hydrodynamic conditions. Physical mixtures: ○, C:PC (2:1); □, C:PC (1:2); chloroform coprecipitates: ●, C:PC (2:1); ■, C:PC (1:2); △, cholesterol pellets. Each point represents the result from an individual pellet.

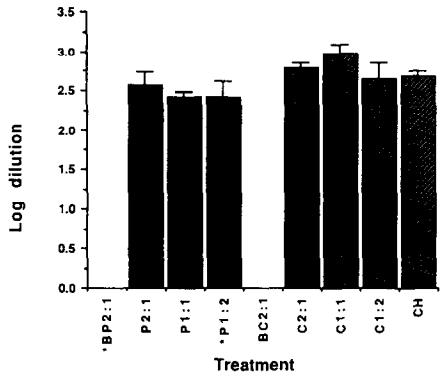

Fig. 5. Antibody response of mice implanted for 40 days with cholesterol pellets and pellets made from physical mixtures and chloroform coprecipitates of C and PC containing 2.32–3.10 mg (range) of BSA. Physical mixtures: BP2:1, C:PC (2:1) without antigen; P2:1, C:PC (2:1); P1:1, C:PC (1:1); P1:2, C:PC (1:2); coprecipitates: BC2:1, C:PC (2:1) without antigen; C2:1, C:PC (2:1); C1:1, C:PC (1:1); C1:2, C:PC (1:2); cholesterol pellets: CH. Vertical bars represent standard errors of means ($n = 5$, $*n = 4$).

pared with hydrogenated soya lecithin caused a significantly slower release of sodium diclofenac (Hirotani et al., 1987). These workers suggested that the mechanism of drug release was dependent on the infiltration of water into the matrix and the amount of infiltrated water was regulated by the content of lecithin. Venkataram and Rogers (1985) also showed that the addition of cholesterol to griseofulvin-phospholipid coprecipitates reduced the release rate of griseofulvin.

Under active hydrodynamic conditions a significant correlation ($r > 0.96$) existed between the release of BSA and erosion of pellets, which suggests the release of BSA was substantially influenced by the erosion process. However, erosion was not the controlling factor for the release of BSA since at a 2:1 ratio of C-PC, 90% of BSA was released at 20 h, whereas only 45% erosion had occurred. Pellets with other C-PC ratios behaved similarly suggesting that diffusional release was operative.

Differences in relative erosion rates of C and PC at different C-PC compositions exposed to active hydrodynamic conditions can be explained by the ability of PC to associate with C under certain conditions. In the presence of excess water, cholesterol and lecithin form a paracrystalline hydrated phase with an equimolar C-PC (approx. 1:2, w/w) ratio (Bourgès et al., 1967). Although these workers suggested that chemically homogeneous lecithins with saturated long chains do not swell in water at room temperature (or even at 40°C) and therefore, do not form the paracrystalline hydrated phase, in our studies (lecithin with iodine value = 0) the dissolution media examined under a light microscope showed the presence of myelin structures as a result of erosion. Thus, it is proposed that erosion of PC occurred in association with C in the form of a paracrystalline hydrated phase (gel) with equimolar C-PC composition (C:PC, 1:2 w/w); and as a result at a 2:1 C-PC ratio PC eroded initially at a faster rate followed by a gradual decrease due to the low content of PC remaining and its strong association with C. At a 1:1 ratio of C-PC, erosion also occurred in a similar way in the form of a gel. In both cases, escape of PC with equimolar quantities of C left excess cholesterol in the pellets in a fragile condition to be broken away by active hydrodynamic activity. Erosion of C and PC at the same rate throughout for pellets of C-PC ratio 1:1 can be explained by simultaneous breaking of excess C (above equimolar quantities) and erosion of C-PC in the form of a gel (in equimolar quantities). At a 1:2 ratio of C-PC, the pellets remained intact for 1.5–2 h due to the formation of the paracrystalline hydrated phase of the same C-PC composition as in the pellet. After this time they underwent rapid disintegration due to the hydrodynamic activity.

The various erosion rates of pellets made from different C-PC compositions and exposed to gentle hydrodynamic conditions were also associated with the formation of the paracrystalline hydrated phase. Pellets made from physical mixtures (C:PC, 1:2) eroded by less than 5% over 6 days because they formed the paracrystalline hydrated phase which kept them intact. These hydrated intact pellets had a gel-like appearance and broke easily on handling. The rapid erosion of pellets made from chloroform coprecipitates suggests that these pellets formed a less stable hydrated phase. However, almost 100% of BSA was released within 1 day from both types of

pellets, indicating the gel was not an effective diffusional barrier.

The role of chloroform as a coprecipitating solvent in reducing the release rate of BSA at C-PC ratios of 2:1 and 1:1 is not clear. The effect of chloroform as a coprecipitating solvent on the erosion of pellets was not consistent and varied in different conditions. It has been suggested that the orientation of phospholipid molecules in coprecipitates is affected by the solvent used (Venkataram and Rogers, 1984, 1985), and cholesterol as an additive influences the phase transitions of phospholipids to some extent depending on the amounts (Ladbrooke et al., 1967; Keough et al., 1989). This may be responsible for the observed behaviour but it requires further investigation.

Hydrodynamic conditions are important parameters in the release of BSA and erosion of pellets. Under the different hydrodynamic conditions, the release of 80–85% of BSA from pellets made from chloroform coprecipitates with a C-PC ratio of 1:1 varied from 6 days to 6 h. These pellets were about 30% eroded over 4 days under gentle hydrodynamic conditions, and within 1.2 h under active conditions. The initial burst release of BSA from both physical mixtures and coprecipitated types of pellets, when only minor erosion had occurred, again indicated that diffusional release of BSA had occurred and that erosion was not the major process controlling the release.

The in vitro release of BSA increased with an increase in PC content of the pellets; however, altering hydrodynamic conditions affected the release rate and erosion significantly making prediction of in vivo behaviour difficult. The rate of release of BSA and erosion of pellets were not studied quantitatively in vivo, although it was clear that retrieved pellets of C-PC ratio 1:2 eroded more than the pellets of other C-PC ratios. Stewart et al. (1983) reported an increase in the protective capacity of *Bacteroides nodosus* vaccines which was not directly proportional to the increase in protein concentration of pili delivered to the immune system. They found that a 16-fold difference in the concentrations of antigen delivered resulted in a 1.5–2-fold difference in the mean agglutinin titres. Thus, although various amounts of BSA may have been released from pellets formulated differently, the variation was not enough to produce significantly different levels of anti-BSA antibodies in the sera of mice. This is supported by earlier studies of Opdebeeck and Tucker (1991) who reported that decreasing the dose of BSA from 3 to 0.5 mg delivered from cholesterol pellets did not produce significantly different levels of anti-BSA antibodies in mice despite significant variations of in vivo release profiles of antigen. However, decreasing the antigen load further to 0.15 mg significantly reduced the level of antibodies.

Small et al. (1966) and Bourgès et al. (1967) found that on dissolution, cholesterol-lecithin mixtures form cylindrical myelin figures comprising concentric lecithin bilayers with cholesterol intercalating the lecithin. The presence of myelinic bodies with elongated, tube-like structures were observed by light microscopy in the dissolution media taken from all the samples of C-PC pellets. Water-soluble antigens might be entrapped in the aqueous compartments of the myelinic bodies which would be released slowly from the encapsulated pellet in vivo. This system for delivering antigens may thus be expected to have the added advantage of acting as an adjuvant, since antigens loaded in liposomes induce a better primary antibody response than free antigen (Warren et al., 1986; Alving, 1987), and multilamellar and unilamellar liposomes have been used to induce responses to BSA (Shek et al., 1986).

The simplicity of manufacture of these pellets without the use of organic solvents and heat, their biocompatibility and bioerodible nature make this system promising for the delivery of antigens and other macromolecules.

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